ARABELA GUEDES DE AZEVEDO VIANA

PROTEOMIC APPLICATIONS FOR STUDIES OF BULL FERTILITY

Thesis submitted to the Veterinary Medicine Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Mariana Machado Neves

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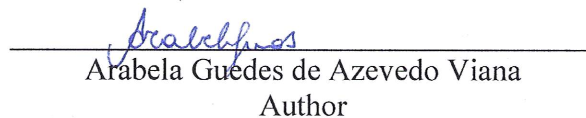

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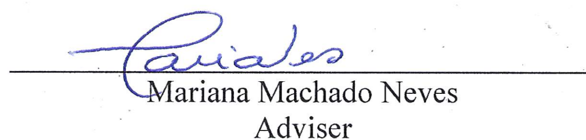

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Adviser

To God and to my family.

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ABSTRACT

VIANA, Arabela Guedes de Azevedo, D.Sc., Universidade Federal de Viçosa, October, 2022. **Proteomic applications for studies of bull fertility.** Adviser: Mariana Machado Neves. Co-adviser: Arlindo de Alencar Araripe Noronha Moura.

During spermatogenesis, the immature spermatozoon undergoes a series of morphological changes, and the nucleus is highly condensed to protect the DNA. Thus, the apparently transcriptionally silent sperm exclusively depends on the extracellular factors for survival and functionality. The seminal plasma (SP) is a fluid produced by accessory sexual glands, epididymides, and testes, and plays crucial roles in the ejaculated sperm. Understanding the complexity of the SP proteins is still a challenge and proteomic approaches have been widely used to uncover SP proteins and, consequently, potential protein biomarkers of bull fertility. To verify the state of the art of the SP proteome and its relationship with sperm fertility in bulls, we applied an extensive and systematic literature search to select 71 and 29 studies for systematic review and meta-analysis research, respectively. Qualitatively, the exposure to a single protein, such as osteopontin (OPN), binder of sperm proteins (BSPs), and heparin-binding proteins (HBPs), positively associates with sperm motility, capacitation, and fertilizing ability. Quantitatively, our meta-analysis revealed that seminal proteins ameliorate sperm parameters. BSPs and beta-defensin 126 highly favored sperm protection when cells were collected from the epididymis by retrograde flux and analyzed under room temperature conditions. Total SP proteins improved the motility and quality of *Bos taurus* sperm collected by artificial vagina, mainly in the presence of HBPs. Due to the diversity of methodologies applied in the eligible studies, the translational aspects of SP research should be taken into consideration to precisely define how seminal proteins can be harnessed to advance reproductive biotechnology. Then, we suggested that future studies should evaluate intact proteins to ascertain their biological functions. To address this question, we evaluated the associations between seminal proteoforms in bulls with contrasting freezability statuses. We employed top-down mass spectrometry to generate sperm and seminal plasma proteoform atlas of bulls with low (LF; n = 6) and high (HF; n = 9) semen freezability. Sperm and seminal plasma proteins were fractionated by tandem size exclusion chromatography (< 30 kDa) and analyzed by mass spectrometry (operated in protein intact mode). We characterized 299 and 267 bull SP and sperm proteoforms, respectively; most of them, truncated. Interestingly, the two proteins that presented the greatest number of proteoforms play opposite roles in the regulation of calcium intake, the c-type natriuretic peptide and the caltrin. A truncated

proteoform of beta-defensin 10 with pyrrolidone carboxylic acid in the N-terminal was exclusively identified in the SP of HF bulls and may be further investigated to be pointed out as a potential biomarker. Eight truncations of OPN, including one with a serine phosphorylation site, were uniquely expressed in the SP of LF bulls. Regarding sperm proteoforms, we described the acetylation of histone H2A type 1 and c-Myc protein binding proteoform in LF sires, which may indicate a deficiency in DNA packing and/or nuclear activity. This first sperm and seminal plasma proteoform atlas of any species, showed that post-translational processing appears to define bio-attributes of proteins and their empirical associations with sperm cryoresistance.

Keywords: Bull. Fertility. Seminal plasma. Proteins. Proteoforms. Systematic review. Meta-analysis.

RESUMO

VIANA, Arabela Guedes de Azevedo, D.Sc., Universidade Federal de Viçosa, outubro de 2022. **Aplicações proteômicas no estudo da fertilidade de touros.** Orientadora: Mariana Machado Neves. Coorientador: Arlindo de Alencar Araripe Noronha Moura.

Durante a espermatogênese, o espermatozoide imaturo sofre uma série de mudanças morfológicas, e o núcleo é condensado para proteger o DNA. Assim, o espermatozoide aparentemente transcricionalmente silenciado depende exclusivamente dos fatores extracelulares para sua sobrevivência e funcionalidade. O plasma seminal (PS) é um fluido produzido pelas glândulas sexuais acessórias, epidídimos e testículos, e desempenha papéis cruciais no espermatozoide. Compreender a complexidade das proteínas seminais ainda é um desafio e abordagens proteômicas têm sido amplamente utilizadas para descrevê-las e indicar potenciais biomarcadores de fertilidade. Para investigar o proteoma seminal e sua relação com a fertilidade espermática em touros, aplicamos uma extensa e sistemática pesquisa bibliográfica, selecionando 71 e 29 estudos para revisão sistemática e meta-análise, respectivamente. Qualitativamente, a exposição a uma única proteína, como a osteopontina (OPN), proteína ligadora do espermatozoide (BSPs) e proteínas de ligação à heparina (HBPs), se associa positivamente com motilidade, capacitação e fertilização dos espermatozoides. Quantitativamente, nossa meta-análise revelou que as proteínas seminais melhoram os parâmetros espermáticos. BSPs e beta-defensina 126 atuam positivamente na proteção espermática quando as células foram coletadas do epidídimo por fluxo retrógrado e analisadas em condições de temperatura ambiente. As proteínas seminais totais melhoraram a motilidade e a qualidade dos espermatozoides *Bos taurus* coletados por vagina artificial, principalmente na presença de HBPs. Devido à diversidade de metodologias aplicadas nos estudos, os aspectos translacionais da pesquisa em PS devem ser levados em consideração para definir com precisão como as proteínas seminais podem ser aproveitadas para o avanço da biotecnologia reprodutiva. Então, sugerimos que estudos futuros avaliem proteínas intactas para averiguar suas funções biológicas. Para abordar esta questão, avaliamos as associações entre proteoformas seminais e o status de congelabilidade de sêmen. Empregamos a espectrometria de massa *top-down* para gerar o atlas de proteoformas de espermatozóides e PS de touros com baixa (BC; n = 6) e alta (AC; n = 9) congelabilidade do sêmen. As proteínas espermáticas e seminais foram fracionadas por cromatografia de exclusão de molecular (< 30 kDa), e analisadas por espectrometria de massa (operado no modo proteína intacta). Caracterizamos 299 e 267 proteoformas de espermatozoide e PS de touro, respectivamente; a maioria delas, truncado. Curiosamente, as

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Palavras-chave: Touro. Fertilidade. Plasma seminal. Proteínas. Proteoformas. Revisão sistemática. Meta-análise.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
SP	Seminal plasma
OPN	Osteopontin
BSP	Binder of sperm protein
HBP	Heparin-binding protein
DEFB	Beta-defensin
HF	High fertility
LF	Low fertility
kDa	kilodaltons
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
CEF	Caudal epididymal fluid
IGF	Insulin-like growth fator
IL	Interleukin
aSFP	Acidic seminal plasma protein
LPGDS	Lipocalin-type prostaglandin D synthase
FAA	Fertility-associated antigen
TIMP	Metalloproteinase inhibitor
GGT	Gamma-glutamyl transferase
AV	Aritificial vagina
EJ	Electro-ejaculation
RF	Retrograde flushing
F	Fresh
FT	Frozen-thawed
PSAP	Prosaposin
RLX/ RLN	Relaxin
WSP	Whole seminal plasma proteins
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
HCD	High energy collision dissociation
EthCD	Electron-transfer/higher-energy collision dissociation
NPPC	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
PPI	Protein-protein interaction

FDR	False Discovery rate
USDA	United States Department of Agriculture
AI	Aritificial Insemination
BSE	Breeding soundness examination
RNA	Ribonucleic acid
MS	Mass spectrometry
PTM	Post-translational modification
ART	Assisted reproductive technology
SYRCLE	Systematic Review Centre for Laboratory Animal Experimentation
USA	United States of America
FUCA	Fucosidase
ALB	Albumin
NUCB	Nucleobindin 2
NT5E	5′-nucleotidase
CLU	Clusterin
GH	Growth hormone
QT	Total heterogeneity
QW	Heterogeneity within
QB	Heterogeneity between
ARRIVE	Animal Research: Reporting In Vivo Experiments
AGC	Automatic gain control
DDA	Data-dependent acquisition
TPP2	Tubulin 2446 polymerization-promoting protein family member 2
TB4	Tymosin beta 4
Pro-ADM	Pro-adrenomedulin
ACBP	Acyl-CoA binding protein
PolyUb-B	Polyubiquitin B
SERPINA5	Serine protease inhibitor
RPABC2	DNA-directed RNA polymerases I, II, and III subunit RPABC2
UBB	Ubiquitin
CALM	Calmodulin
ATP5F1E	ATP synthase subunit epsilon
GAPDHS	Glyceraldehyde-3-Phosphate Dehydrogenase

CENPX	Centromere protein x
UQCR10	Cytochrome b-c1 complex subunit 9
HSPD1	Heat shock factor protein 1
NDUFS6	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial
ATP5F1B	ATP synthase subunit beta, mitochondrial
TUBA1B	Tubulin alpha-1B chain
BRF2	Transcription factor IIIB 50 kDa subunit
SRN	Seminal ribonuclease
pGlu	Pyrrolidone carboxylic acid
SPINK6	Serine Peptidase Inhibitor Kazal Type 6
NGF	Nerve growth factor

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1. GENERAL INTRODUCTION

Currently, Brazil has one of the largest herds of cattle in the world and stands out among the leading exporters of beef and animal products (USDA, 2020). Although these data are extremely important for the country, leading to employment and incomes generation, maintaining productivity indices and the attention to market demands are major challenges for the future. Given the complexity of Brazilian ecosystems, there is an urgent need to preserve natural resources and biodiversity (LIBERA et al., 2020). Significant efforts have been dedicated to animal biotechnology research, in the incessant search for efficient, economically and environmentally sustainable production systems (LIBERA et al., 2020). The fertility index is determinant for profitability in all animal production models (NAYERI et al., 2017). It is influenced by numerous factors and variables (KENNY; BYRNE, 2018; LLAMAS-LUCENO et al., 2020; MORRELL, 2020; COPLEY et al., 2022), and should, therefore, be a permanent object of studies and research.

Research dedicated to gamete biotechnology and reproductive physiology has shown remarkable growth and a wide range of approaches and areas. It includes protocols and methods for estrus manipulation and synchronization, embryo production and transfer, cloning, cryopreservation of tissues, gametes and embryos, sperm sexing and stem cell transfer (BARNES et al., 1993; CAVALIERI et al., 2006; VIANA, 2017; CAVALIERi et al., 2018; VALENTE et al., 2022; YANEZ-ORTIZ et al., 2021; UPDHYAY et al., 2021; QUELHAS et al., 2021; XU et al., 2021). Reproductive biotechnologies, such as those described above, have also provided incalculable benefits for society and the individuals' welfare. It also includes the scope of environmental preservation programs and assisted reproduction techniques, contributing to the reproduction of wild species.

With the worldwide application of artificial insemination (AI) in the cattle industry, the use of selected superior males became crucial to improve the genetic quality of livestock and ensure the profitability (BUTTLER et al., 2020). The bull selection based on scrotal circumference size showed the decreased calving interval, improved daughter pregnancy rate and increased average daily gain (MEYER et al., 1991; VAN MELIS et al., 2010; RAIDAN et al., 2016). Bull fertility is currently evaluated by breeding soundness examinations (BSEs), that includes seminal parameters. At AI centers, besides the BSE, they also use the non-returning rate or conception rates to evaluate bull fertility. However, bulls with similar seminal parameters (sperm motility, concentration, and morphology) may have different fertility indexes (NONGBUA et al., 2014). Studies indicate that molecular components present in

gametes or in the fluids that surround them (seminal plasma - SP) also contribute to the definition of an individual's real fertility (KILLIAN et al., 1993; MOURA et al., 2006; PEDDINTI et al., 2008; REGO et al., 2016; VIANA et al., 2018; GOMES et al., 2020). Sperm factors are defined as the chromatin integrity of these cells, RNA species and proteins. A range of research also shows that SP proteins influence various aspects of sperm function before and after ejaculation, with relationships with seminal parameters and fertility indices assessed *in vitro* and *in vivo* (VIANA et al., 2021).

In this context, proteomics approaches complement and expand, in terms of qualitative and quantitative data, the information obtained through cytological, histological or phenotypical exams. Thus, studies on the proteomics of sperm and SP are essential for the identification of specific changes, at a given time and environment, in a precise and minimally invasive way. Its use may be more accurately indicated in the diagnosis and treatment of other mammalian male infertility factors, increasing a man's potential for fatherhood (KOVAC et al., 2013). Recent discoveries of male fertility biomarkers may be helpful in eliminating the need for invasive and costly tests such as karyotyping and testicular biopsy, allowing for early classification and diagnosis of male infertility factor.

Proteomic approaches

Proteomics is the study of a set of proteins related to a cellular or organismic function, under certain conditions of environment and time (ZIMMER et al., 2006). Proteins are made up of amino acid residues encoded from genetic material. Although it is possible to deduce the sequence of their residues based on genes, it is not always feasible to determine when these proteins will be produced, nor their quantity or modifications. This production is controlled by the local and temporal conditions in which the organism finds itself. Thus, methods in molecular biology allow the quantification of proteins and, consequently, the interpretation of the influence of stimuli on the phenotype of organisms.

Mass spectrometry (MS) has become one of the most used methods in proteomic studies and currently there are two main approaches, one called bottom-up and the other, top-down (AEBERSOLD; MANN, 2003). The standard flowchart for bottom-up proteomics analysis begins with preparing the organism, tissue, cell or fluid under the desired conditions and extracting the proteins. After extraction, the proteins are cleaved in a protease digestion process and their peptides are ionized and fragmented in MS. In the top-down approach, the steps are similar, however the proteins are ionized intact and do not undergo the digestion process, being

fragmented into MS (KELLIE et al., 2012). This approach is favored when it comes to the analysis of post-translational modifications (PTMs), because during the digestion step some PTMs can interfere with the cleavage process, affecting the identification of peptides. Furthermore, the peptides responsible for identifying proteins may not contain PTMs or this information may be present in other sequences of unidentified residues (CATHERMAN et al., 2014).

The bottom-up approach is currently better consolidated and has more instrumental, analytical and bioinformatics tools than the top-down one. However, the analysis of intact proteins has become increasingly efficient with technological advances, increased accuracy and resolution of mass spectrometers, in addition to improvements in techniques for sample preparation (CATHERMAN et al., 2014). Sample fractionation may be one of the key steps to reduce its complexity, improving the signal/noise ratio and coverage of identification and quantification, mainly of peptides and proteins in lower abundance (MOSTOVENKO et al., 2013).

The top-down approach is a recent and very effective technology for proteoform characterization. “Proteoforms” encompass all forms of genetic variation, alternative splicing of RNA transcripts and PTMs expressed from a single gene (SMITH; KELLEHER, 2013). The characterization and quantification of proteoforms contribute to clinical and translational research in a way that goes beyond the limitations of the bottom-up approach. The pathogeny of many diseases, physiological processes and the fertilizing capacity of gametes are based on the operation of functional proteoforms modified in their natural context. The study of intact proteins and their isoforms (proteoforms) has the potential to provide accurate information for phenotypic understanding and early detection of diseases and traits of interest in farm animals.

Phosphorylation, glycosylation, ubiquitination, acetylation and methylation are some of the most studied PTMs. Phosphorylation is widely investigated in sperm and during sperm maturation, phosphorylation of histones and protamines is crucial for the compaction of genetic material (MORIN et al., 1999; Brunner et al., 2014). It also regulates processes related to sperm motility and capacitation (SIGNORELLI et al., 2012), as well as interaction with the zona pellucida (LIU et al., 2006). Glycosylation of sperm proteins occurs mainly during the epididymal tract, where many glycoproteins secreted by epididymal cells are incorporated into the sperm membrane (KIRCHHOF et al., 1996). These glycoproteins are essential for the recognition and interaction between spermatozoa and zona pellucida (YEUNG et al., 2001). Ubiquitination is crucial to prevent the accumulation of defective proteins and cells, through

the mediation of protein degradation by the ubiquitin-proteasome system. Histone acetylation and ubiquitination during spermatogenesis is pivotal for histones degradation and replacement by protamines (Gaucher et al., 2010). Failures in the histone acetylation process can lead to decreased fertility or infertility (Kim et al., 2014). In sperm, methylation is associated with histone retention, which play a role in epigenetic signaling.

Seminal proteins are also commonly expressed as numerous isoforms, such as clusterin, binder of sperm proteins BSPs, spermadesins, metalloproteinase inhibitors (TIMPs) and osteopontin (OPN). Studies clearly show that the SP of bulls (MOURA et al., 2007, REGO et al., 2016; VIANA et al., 2018; GOMES et al., 2020); sheep (VAN TILBURG et al., 2021), buffaloes (BRITO, 2018), goats (VAN TILBURG et al., 2015), dogs (AQUINO-CORTEZ et al., 2017), rabbits (BEZERRA et al., 2019) and swine (GONZALEZ-CADAVID et a., 2014) contains several isoforms of conserved proteins. The amount and distribution of these isoforms shows considerable variation between species, but their biochemical and functional details are still unknown in most cases. However, studies suggest that the proteoform of BSPs (SINGH et al., 2019) and clusterin (JANISZEWSKA et al., 2020) are determinant for their specific roles on sperm physiology.

Systematic reviews and meta-analysis research

In the last decades, there is a large volume of papers published and it points to the necessity for accurate synthesis, based on scientific evidence. Narrative reviews are broad publications, suitable for describing and discussing the development or "state of the art" of a given subject, from a theoretical or contextual point of view (CORDEIRO et al., 2007). However, it may not use explicit and systematic criteria for the search and critical analysis of the literature, and it may be loaded with the authors' personal opinion and biases. A review with a systematic approach impartially evaluates the results of multiple primary studies, critically selected (GALVÃO; PEREIRA, 2014).

Systematic reviews aim to answer a clear question, using systematic and transparent methods, therefore, reproducible. To formulate the research question, the commonly used structure is called PICO, which specifies the population (P), the intervention (I) and its comparison (C), and the outcome (O; GALVÃO; PEREIRA, 2014). Statistical methods, such as meta-analysis, may or may not be used to analyze and summarize the results of the included studies. In the present study, the target population was the bull, the intervention was the seminal

protein, the comparator was the controls evaluated without the protein, the outcome, in general, was bull fertility and sperm freezeability.

Therefore, the objective of this thesis was to evaluate the effect of SP proteins on sperm freezeability and bull fertility. It also aims to describe and compare seminal and sperm proteoforms from bulls with different freezeability phenotypes, using top-down approach. In the chapter 1, we carried out a systematic review, in which we included 71 papers published in the last almost 30 years, to understand the functions of SP proteins and their potential associations with sperm parameters and fertility in bulls. In the chapter 2, in which we included 29 articles published in the last 30 years, the meta-analytical approach was applied to find out the magnitude of the effects of SP proteins on parameters associated with bull fertility. There was also evaluated the impact of different methodological moderators, such as bovine breeds, semen collection, sperm source, sample conditioning, and seminal proteins on such effects. The reveal the potential contributions of seminal proteins on bull sperm fertility outcomes. Finally, in chapter 3, we aimed to evaluate the associations of sperm and SP proteoforms in bulls with contrasting freezeability status, applying a top-down proteomics strategy that combines tandem size-exclusion chromatography, reversed-phase liquid chromatography and tandem mass spectrometry.

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2. CHAPTER 1

Functional attributes of seminal proteins in bull fertility: A systematic review

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Functional attributes of seminal proteins in bull fertility: A systematic review

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Key words: Bovine, seminal plasma, semen, spermatozoa, proteome.

ABSTRACT

Proteomic approaches have been widely used in reproductive studies to uncover protein biomarkers of bull fertility. Seminal plasma is one of the most relevant sources of these proteins that may influence sperm physiology. Nonetheless, there are still gaps in existing knowledge in the functional attributes of seminal proteins. Thus, we reviewed the relationships between seminal plasma proteins and bull fertility by conducting a systematic review with data obtained from 71 studies. This review showed that the associations between fertility improvement with the use of total seminal plasma proteins are still controversial. None of the studies explored the sperm fertilizing ability following these interactions. By contrast, the exposure to a single protein, such as osteopontin, binder of sperm proteins, and heparin binding proteins, can increment sperm motility, capacitation, and fertilizing ability by modulating intracellular calcium concentrations, removing lipids from sperm membranes, and regulating the acrosome reaction. Variations in protein analyses and the protein contents and their abundances between animals contributed to the difficulty of establishing protein biomarkers of fertilizing potential of the bull sperm. Indeed, the heterogenicity of methodologies was a limitation of this review. Standardized methods of seminal protein analyses, as well as sperm endpoints, may minimize such discrepancies. In conclusion, potential biomarkers of sperm parameters are still to be established. Future studies should evaluate protein isoforms and how they interact with sperm to ascertain their biological functions.

INTRODUCTION

Historically, the cattle industry has been investing efforts in strategies for accurate evaluation and prediction of male fertility (Butler *et al.* 2020). This knowledge has the potential to significantly increase the conception rates of herds and contribute to the early selection of sires for field reproduction and assisted reproductive technologies (ARTs; Park *et al.* 2019). For decades, sperm parameters have been used to ascertain male fertility (Blom 1950; Mocé & Graham 2008). Alternatively, recent "OMICs" approaches started to add more information about the physiology of male gamete and the non-cellular microenvironment of semen, emerging the concept that bull fertility is also determined by molecular components of that medium (Killian *et al.* 1993; Moura *et al.* 2006a; Harayama *et al.* 2017; Velho *et al.* 2018).

Mammalian seminal plasma (SP) is a complex medium (for review, see Juyena & Stelletta 2012) that supports the functions and survival of the transcriptionally inactive spermatozoa. Proteins of seminal medium have been studied in several species, evidencing their roles in events associated with sperm physiology and fertility (Camargo *et al.* 2018), as well as fetal developmental programming (Ortiz *et al.* 2019). Recently, major studies have made significant contributions to decipher the complexity of bovine seminal fluid proteome and its functional attributes in male reproduction (Rego *et al.* 2014; Westfalewicz *et al.* 2016; Viana *et al.* 2018). Nevertheless, seminal proteins vary in quantity and quality according to the age, breed, management, and stress conditions (Rego *et al.* 2015; Morrell *et al.* 2017; Boe-Hansen *et al.* 2020). Bulls with similar semen parameters present differences in fertility rates and studies indicate that such differences relate to specific proteins of the SP (Moura *et al.* 2006a; Viana *et al.* 2018).

Despite the evidence brought by these empirical data, interventional studies focused on seminal proteins and bull fertility are limited and, in some cases, with inconclusive results. For instance, osteopontin (OPN) concentration in reproductive fluids are associated with the fertility scores of dairy bulls (Cancel *et al.* 1995; Moura *et al.* 2006a). There are also controversies regarding the role(s) of heparin binder proteins (HBPs) on the improvement of sperm fertility in *in vivo* studies (Bellin *et al.* 1996; Dalton *et al.* 2012; Alvarez-Gallardo *et al.* 2013). Other studies have described associations between seminal fluid proteins and parameters of the bovine sperm, such as motility, capacitation, and post-thaw viability (Lackey *et al.* 1998; Thérien *et al.* 2001; Karunakaran & Devanathan 2015; Stewart *et al.* 2018; Gomes *et al.* 2020). The list of proteins mentioned by these authors is vast but confirmation of functional attributes of fertility-related proteins by interventional assays is still missing in most cases.

Overwhelming amounts of data about SP proteomes and empirical associations between seminal proteins and sperm parameters and fertility emphasize the importance of such components for male fertility. However, evaluation of those data poses a challenge for researchers dedicated to study male fertility. Therefore, this systematic review is aimed at analyzing the functions of seminal fluid proteins and their potential associations with sperm parameters and fertility in bulls.

METHODS

Strategy for literature search

In the current work we followed the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA; Liberati *et al.* 2009). The review included studies published from 1990 until D. This period has been chosen based on two main events: 1) the development of mass spectrometry-based techniques that led to the emerging term "proteomics" first mentioned (Wasinger *et al.* 1995); 2) the publication of a cutting-edge study by Killian and collaborators in 1993, which argued that the bull SP has fertility-associated proteins. Web of Science, Scopus, and PubMed databases were used for a comprehensive search of papers, using 'bull' or 'bovine' or '*Bos taurus*' and 'seminal plasma' or 'seminal fluid' and 'fertility' or 'cryopreservation' as search terms. The wildcard symbol "*" was used in the terms 'fertility' and 'cryopreservation' to expand the search. Reference lists of the studies included were hand searched to identify other relevant trials.

Studies reviewed

For a study to be included in this review, some criteria had to be met, as follows: (1) journals in which articles were evaluated by ad hoc reviewers; (2) studies that used cattle as the animal model. In addition, other reasons for study exclusion were considered: (1) the study evaluated biochemistry and functions of seminal proteins with no association with fertility; (2) there was no analysis of SP and its proteins; (3) the study was a review article.

Data extraction and synthesis

The following variables of interest were considered for studies evaluating bovine SP: title, authors, year, and country. Additionally, included studies were categorized into 1) observational studies, when authors described seminal proteins in animals with varied fertility; 2) interventional studies, when authors incubated sperm with seminal protein(s) and evaluated

the outcome. Moreover, we extracted experimental model features, research methods, and the main findings. Additionally, reporting quality of all eligible studies was evaluated using the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE) Risk of Bias tool (Hooijmans *et al.* 2014).

RESULTS

Selected studies and qualitative data

The initial search resulted in 880 studies. Following the removal of duplicates and exclusions, 71 studies were eligible for the present systematic review (Fig. 1). Most of the eligible studies were from USA (n = 24), followed by India (n = 13), Brazil (n = 12 Canada and Germany (n =5, each), Japan (n = 3), Colombia and Ireland (n = 2, each). China, France, Italy, Mexico, and Venezuela contributed to one study, each. Moreover, nine papers were published from 1990 to 1999, 23 from 2000 to 2009, and 39 from 2010-2020. Thirty-six studies were observational, while 35 were interventional studies (Supplementary Table 1).

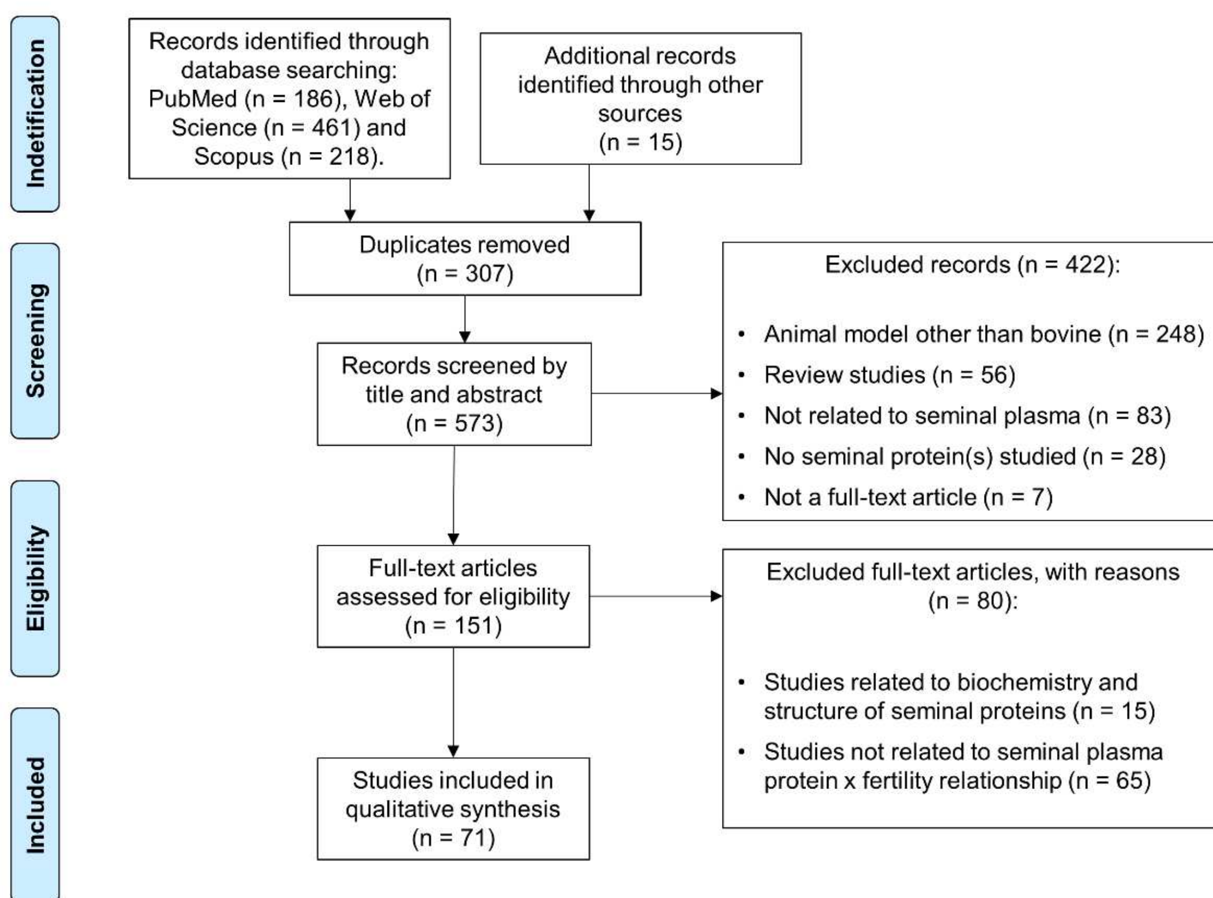

Figure 1. Flow diagram of search results to define the articles to be included in systematic review and meta-analysis, according to PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analysis)

Bos taurus taurus was the animal model used in 36 of the eligible studies, whereas other studies used *Bos taurus indicus* (n = 8), both species (n = 6), and crossbred bulls (n = 12). Nine studies did not mention the species used (Supplementary Table 1). Furthermore, 56 articles used ≤ 20 bulls, while 12 used > 21 bulls to conduct their experiments. By contrast, three papers did not inform the number of animals (Supplementary Table 1).

Most researchers harvested semen samples using artificial vagina (AV; n = 30), followed by electro ejaculator (n = 17). In other studies, seminal fluids were collected by retrograde flushing (RF) of the epididymis (n = 1), mincing epididymis (n = 1), and epididymal fluid via cannulation of vas deferens (n = 1). In four studies, fluids were collected using two methods, electro ejaculator and RF (n = 1), AV and RF (n = 2), and mincing epididymis and RF (n = 1). Finally, the methods used to collect the sample were not described in 17 studies (Supplementary Table 1).

Researchers of most of the studies used total SP proteins (n = 30), followed by fractioned (n = 15), and purified seminal proteins (n = 14). From the experiments where total seminal proteins were used, seven performed protein separation, whereas in two studies known proteins were used (Supplementary Table 1). While in ten studies both protein separation and identification were performed, in eleven studies the protein content of SP used was not evaluated. Regarding the fractioned SP, in nine studies only protein separation was performed, in contrast to six studies where seminal proteins were separated and identified. Studies working with purified proteins performed protein separation and identification (Supplementary Table 1). Moreover, recombinant proteins were used in eight studies, and proteins were purchased in five of them. Ultimately, in four studies antibodies against the target proteins were used, and in only two of these some methods of protein separation and identification were performed (Supplementary Table 1).

The most used techniques to separate SP proteins were chromatography and gel electrophoresis. A single approach to separate seminal proteins was preferentially used in thirty-seven studies. By contrast, in other studies, two approaches (n = 15) and three approaches (n = 1) were used, respectively, and the separation technique(s) used were not mentioned in 18 (Supplementary Table 1). Protein identification was assessed using either one method (n = 30) or more than one (n = 9). In most of the studies, protein identification using mass spectrometry, followed by Western Blotting, ELISA, N-terminal sequence, and radioimmunoassay was performed. In the remaining 32 articles, approaches for SP protein identification were not used (Supplementary Table 1).

From the 44 studies that evaluated the interaction between spermatozoa and SP proteins, 33 used ejaculated sperm, while one used epididymal sperm (Tables 1-4). The other ten studies used both epididymal and ejaculated sperm in their experiments. The incubation with SP proteins used either fresh (n = 32), frozen-thawed sperm (n = 9), or both (n = 3). The analyses of sperm parameters after incubation with seminal proteins were mainly conducted with frozen-thawed sperm (n = 19), followed by fresh gametes (n = 14), frozen-thawed and fresh sperm (n = 6), cooled and thawed sperm (n = 2), fresh and cooled sperm (n = 1), and the three forms of sperm storage (n = 1; Tables 1-4). The seminal proteins studied were the total SP proteins (n = 6) and epididymal fluid proteins (n = 1; Table 1); HBPs (n =12; Table 2); binder of sperm proteins (BSPs; n = 8; Table 3); OPN (n = 4; Table 3); acid seminal fluid protein (aSFP), insulin-like growth factors (IGFs), relaxin (RLN), and beta defensin 126 (DEFB126; n = 2 studies each; Table 4); nerve growth factor (NGF), beta nerve growth factor (NGFB), lipocalin-type prostaglandin-D synthase (PTGDS), growth hormone (GH), and prosaposin (PSAP; n = 1 study each; Table 4).

Main findings

From the 71 studies used in the present review, 44 described the cause-and-effect relationship between a SP protein and sperm fertilizing capacity (Tables 1-4). According to three studies, the addition of total SP proteins to semen may decrease sperm fertilizing capacity due to the masking of deoxyribonuclease activity, which leads to an increase in sperm-neutrophil binding (Alghamdi *et al.* 2009; Alghamdi *et al.* 2010), and a delay of capacitation of frozen-thawed sperm (Almadaly *et al.* 2015). In one study no effect of seminal proteins treatment of sex-sorted semen on embryo development was found (Stinshoff *et al.* 2012), whereas in another study an increase in frozen-thawed sperm viability after incubation with SP proteins was shown (Rueda *et al.* 2013a). In one study positive roles of epididymal fluid proteins on sperm motility and protection against oxidative stress were reported (Reyes-Moreno *et al.* 2002; Table 1).

Table 1. Characterization and main findings of studies (*n*=6) evaluating the functions of bull seminal plasma (SP) proteins and epididymal fluid proteins.

Reference	Sperm source	Sperm conditions		Protein	Main findings
		At proteins incubation	At sperm analysis		
Alghamdi *et al.* (2009)	Ej	Fresh	Fresh	SP proteins	SP proteins introduced into the uterus at AI may increase sperm-

					neutrophil binding, contributing to reduce fertility.
Alghamdi *et al.* (2010)	Ej	Thawed	Thawed	SP proteins	SP proteins introduced into the uterus at AI may mask DNase activity, responsible to digest DNA-based neutrophil extracellular traps and frees spermatozoa.
Almadaly *et al.* (2015)	Ej	Fresh	Thawed	SP proteins	SP proteins delayed sperm capacitation.
Stinshoff *et al.* (2012)	Ej	Fresh	Thawed	SP proteins	SP proteins supplementation in sorted semen had no effect in cleavage and development of embryo in vitro.
Rueda *et al.* (2013a)	Ej	Fresh	Thawed	SP proteins	Incubation of semen with SP proteins increased the thawed sperm viability 20% when used 1 and 1,5mg in CFY media, and increased 25% when used 0.5 mg, using Bioxcell media.
Reyes-Moreno *et al.* (2002)	Ej	Thawed	Thawed	Epididymal fluid proteins	Proteins of epididymal fluid kept sperm motility and protect sperm against oxidative stress caused by hydrogen peroxide.

AI, artificial insemination; CFY, citrate-fructose with egg yolk; Ej, ejaculate.

From the 12 studies where the effect of HBPs on bull fertility was evaluated, eleven described positive associations between HBPs and sperm parameters (Karunakaran & Devanathan 2015; Krishnan *et al.* 2015; Patel *et al.* 2015; Krishnan *et al.* 2016a; Krishnan *et al.* 2016b; Patel *et al.* 2016; Pande *et al.* 2018; Table 2), and HBPs associated with *in vivo* bull fertility (Bellin *et al.* 1994; Bellin *et al.* 1996; Sprott *et al.* 2000; Alvarez-Gallardo *et al.* 2013). A negative association between a 31-kDa sperm membrane HBP and Nelore fertility rates was described in one study (Dalton *et al.* 2012; Table 2).

Table 2. Characterization and main findings of studies (*n*=12) evaluating the functions of bull heparin binding proteins (HBPs).

Reference	Sperm source	Sperm conditions		Protein	Main findings
		At proteins incubation	At sperm analysis		
Karunakaran & Devanathan (2015)	Ej	Fresh	Thawed	HBPs	Bulls positive for 28-30 kDa HBPs in sperm had greater sperm motility, quality, capacitation, protection against oxidative stress and higher conception rates.
Krishnan *et al.* (2015)	Ej	Fresh	Thawed	HBPs	The absence of HBPs in sperm membrane led to decreasing in sperm motility. The cryopreservation increased MDA level among bulls' positive for FAA proteins and 2-fold in negative bulls.

Patel *et al.* (2015)	Ej	Fresh	Cooled and thawed	HBPs	HBPs enhanced sperm motility, viability and membrane integrity in pre-frozen and frozen-thawed semen and increased capacitation and AR of frozen-thawed semen. Binding of HBPs to sperm declined acrosome damage and lipid peroxidation.
Krishnan *et al.* (2016a)	Ej	Fresh	Thawed	HBPs	Bulls positive for 30 kDa HBP had lowest sperm abnormalities and DNA fragmentation. Fresh semen positive for 30 kDa showed higher intact acrosome. Bulls positive for 24 and 30 kDa HBPs were able to sustain lower percentage of post-thaw sperm abnormalities with higher chromatin integrity.
Krishnan *et al.* (2016b)	Ej	Fresh	Thawed	HBPs	Bulls positive for 30 kDa had higher total motile sperm, plasma membrane integrity, mitochondrial membrane potential and traveled longer distance though cervical mucus. 24 and 30 kDa presence associated with had higher sperm progressive motility.
Patel *et al.* (2016)	Ej	Fresh	Cooled and thawed	HBPs	Incubation of semen with 31 kDa protein (DNASE1L1) increased sperm motility, viability, and hypo-osmotic swelling responsive in spermatozoa.
Pande *et al.* (2018)	Ej	Fresh	Fresh and thawed	HBP	FAA in sperm membrane led to enhance the motility. Followed by FAA presence in SP only and by FAA presence both in SP and sperm membrane.
Bellin *et al.* (1994)	Ej	Fresh	Fresh	HBPs	HBP-B5 (group of greatest heparin affinity) in sperm membrane had greater fertility in vivo.
Bellin *et al.* (1996)	Ej	Fresh	Fresh	HBPs	HBPs 21.5, 24 and 30 kDa and with HBP 30kDa only in sperm membrane had greater fertility in vivo.
Sprott *et al.* (2000)	Ej	Fresh	Fresh	HBPs	FAA in sperm membrane had greater fertility rates in vivo.
Alvarez-Gallardo *et al.* (2013)	Ej	Fresh	Thawed	HBPs	FAA and TIMP2 added to semen before cryopreservation increased fertility in vivo.
Dalton *et al.* (2012)	Ej	Fresh	Thawed	HBPs	FAA in sperm membrane did not show difference in bull fertility when cows were inseminated. But heifers inseminated with semen from FAA-negative bulls showed higher fertility rates.

AR, acrosome reaction; Ej, ejaculate; DNA, deoxyribonuclease; DNASE1L1, deoxyribonuclease 1 like 1; FAA, fertility-associated antigen; kDa, kilodalton; MDA, malonaldehyde; TIMP2, metalloproteinase inhibitor 2.

Regarding the role of BSPs on sperm functionality, two studies reported an increase in sperm capacitation, sperm-oviduct binding, and motility of fresh sperm after incubation with

these proteins (Thérien *et al.* 2001; Gwathmey *et al.* 2006; Table 3). Other authors described that low density fraction of egg yolk or anti-BSP antibodies prevented sperm-BSP binding to cryopreserved sperm, leading to the prevention of early capacitation and increasing sperm longevity (Bergeron *et al.* 2004; Srivastava *et al.* 2012; Srivastava *et al.* 2013). An increase in acrosome reaction and early embryo development with the BSP1 added into the fertilization medium *in vitro* was reported in two studies (Rodriguez-Villamil *et al.* 2016; Rodriguez-Villamil *et al.* 2020). Also, bull sperm survival to cryopreservation was negatively associated with the presence of BSP1 in the acrosome region and the epididymal fluid (D'Amours *et al.* 2012; Table 3). The positive effects of OPN on *in vitro* sperm capacitation, sperm-egg binding, and early embryo development were confirmed in four studies (Erikson *et al.* 2007; Gonçalves *et al.* 2007; Gonçalves *et al.* 2008a; Monaco *et al.*2009; Table 3).

Table 3. Characterization and main findings of studies (*n*=12) evaluating the functions of bull binder of sperm proteins (BSPs) and osteopontin (OPN).

Reference	Sperm source	Sperm conditions		Protein	Main findings
		At proteins incubation	At sperm analysis		
Thérien *et al.* (2001)	Ep	Fresh	Fresh	BSPs	BSPs and follicular fluid high-density lipoprotein increase sperm capacitation.
Gwathmey *et al.* (2006)	Ep and Ej	Fresh	Fresh	BSPs	BSPs increase sperm-binding to oviductal epithelium and maintain sperm motility.
Bergeron *et al.* (2004)	Ej	Fresh	Fresh and cooled	BSPs	The low-density fraction of egg yolk in semen extender prevents BSPs to bind spermatozoa, which is beneficial to sperm cryopreservation.
Srivastava *et al.* (2012)	Ej	Fresh	Fresh, cooled and thawed	BSP1	Sequestration of BSP1 by synergistic action of egg yolk and specific antibody, compared to their use alone (egg yolk or antibody), improved sperm motility, quality and capacitation in pre-freeze and post-thawed values.
Srivastava *et al.* (2013)	Ej	Fresh	Fresh, cooled and thawed	BSP1	Sequestration of BSP1 by specific antibodies in EYTG media improve sperm motility, quality and capacitation in pre-freeze and post-thawed sperm.
Rodríguez-Villamil *et al.* (2016)	Ep and Ej	Thawed	Thawed	BSP1	BSP1 showed a dose-dependent effect in epididymal and ejaculated sperm acrosome reaction. BSP1 increased early embryo development in vitro of epididymal sperm, as well as ejaculated sperm in higher doses above 20 µg/ml.
Rodríguez-Villamil *et al.* (2020)	Ep and Ej	Fresh	Thawed	BSP1	Epididymal sperm: BSP increased capacitation and fertilization rates in vitro. Ejaculated sperm: heparin treatment accelerated sperm capacitation.
D'Amours *et al.* (2012)	Ep and Ej	Fresh and thawed	Fresh and thawed	BSPs and ELSPbP1	The presence of BSP1 in the sperm acrosome is associated with sensitivity

					to cryopreservation. The presence of ELSPbP1 relates to dead spermatozoa.
Erikson *et al.* (2007)	Ep and Ej	Fresh	Fresh	OPN	OPN increases fertilization to oocyte and play role in blocking polyspermy.
Gonçalves *et al.* (2007)	Ej	Fresh	Fresh	OPN	Sperm treated with peptides from OPN, anti-OPN and anti-Integrin subunits evidenced the crucial interaction between OPN-Integrins for sperm-egg binding.
Gonçalves *et al.* (2008a)	Ej	Thawed	Thawed	OPN	OPN increases fertilization and embryo development in vitro.
Monaco *et al.* (2009)	Ej	Thawed	Thawed	OPN	OPN improved sperm capacitation and embryo development in vitro.

BSP, binder of sperm protein; Ej, ejaculate; ELSPbP1, Epididymal Sperm Binding Protein 1; Ep, epididymis; EYTG, Egg yolk-tris glycerol; OPN, osteopontin.

The IGFs were positively related to sperm motility (Henricks *et al.* 1998; Lackey *et al.* 1998, Table 4). NGF decreased sperm motility and did not influence sperm viability (Li *et al.* 2010), though NGFB played positive roles in sperm viability and did not affect sperm acrosome reaction (Stewart *et al.* 2019; Table 4). Recombinant bovine GH increased both sperm motility and *in vivo* bull fertility (Sauerwein *et al.* 2000; Table 4). Furthermore, DEFB126 played a positive role in sperm motility, enhancing the ability of sperm to bind oviductal cells and reducing agglutination of sperm obtained from corpus epididymis (Fernandez-Fuertes *et al.* 2016; Lyons *et al.* 2018; Table 4). RLN, in turn, induced sperm motility, capacitation, and acrosome reaction with no impact on post-thaw sperm viability (Miah *et al.* 2007; Miah *et al.* 2011; Table 4). It was reported that aSFP enhanced mitochondrial activity and protected sperm against lipid peroxidation, possibly acting as a decapacitating factor (Dostàlova *et al.* 1994; Schöneck *et al.* 1996; Table 4). PSAP added into frozen-thawed semen before artificial insemination (AI) increased *in vivo* fertility of bulls (Amann *et al.* 1999; Table 4). Sperm incubated with anti-lipocalin PTGDS antibodies had better oocyte binding but lower *in vitro* fertilization rates (Gonçalves *et al.* 2008b; Table 4). It was reported that bulls had more abnormal sperm when a 15-kDa protein was absent, as well as in the presence of a low abundance of a 25-kDa protein in SP (Sood *et al.* 2018; Table 4).

Table 4. Characterization and main findings of studies (*n*=14) evaluating the functions of insulin-like growth factors (IGFs), nerve growth factor (NGF), growth hormone (GH), beta defensin 126 (DEFB126), relaxin (RLN), acid seminal fluid protein (aSFP), prosaposin (PSAP), and lipocalin-type prostaglandin D-synthase (PGTDS).

Reference	Sperm source	Sperm conditions		Protein	Main findings
		At proteins incubation	At sperm analysis		
Henricks *et al.* (1998)	Ej	Fresh	Fresh	IGF1	IGF1 stimulates sperm motility.

Lackey *et al.* (1998)	Ej	Fresh	Fresh	IGFs	IGF1 and 2 are involved in initiation and maintenance of sperm motility.
Li *et al.* (2010)	Ej	Thawed	Thawed	NGF	NGF had no effect in acrosome reaction, but had significant effects on leptin secretion, sperm viability, and apoptosis.
Stewart *et al.* (2019)	Ep and Ej	Thawed	Thawed	NGFB	Semen incubated with NGFB prior to cryopreservation showed no difference in sperm quality and a decrease in sperm motility.
Sauerwein *et al.* (2000)	Ej	Fresh	Fresh and Thawed	GH	Increased fresh sperm motility and enhanced in vivo fertility of cryopreserved sperm.
Fernandez-Fuertez *et al.* (2016)	Ep and Ej	Fresh and thawed	Fresh and thawed	DEFB126	DEFB126 plays role in the acquisition of sperm motility.
Lyons *et al.* (2018)	Ep and Ej	Fresh	Fresh	DEFB126	DEFB126 enhanced the ability of epididymal and ejaculated sperm to bind oviductal cells. The protein also prevented epididymal corpus sperm agglutination
Miah *et al.* (2007)	Ej	Fresh and thawed	Fresh and thawed	RLN	Sperm motility and acrosome reaction was improved in both fresh and frozen-thawed spermatozoa incubated with RLN. But RLN showed no significant effect on viability in either fresh or frozen-thawed spermatozoa.
Miah *et al.* (2011)	Ej	Fresh	Fresh and thawed	RLN	RLN induces capacitation and acrosome reaction.
Dostàlova *et al.* (1994)	Ep and Ej	Fresh	Fresh	aSFP	aSFP sperm-coating decreased in capacitated gamete (1.5 h) and it was indetectable after 18h in capacitation media. aSFP may act as decapacitating factor.
Schöneck *et al.* (1996)	Ej and Ep	Fresh	Fresh	aSFP	aSFP stimulates sperm mitochondrial activity and inhibits lipid peroxidation in a dose-dependent manner.
Amann *et al.* (1999)	Ej	Thawed	Thawed	PSAP	Thawed semen treated with PSAP before AI increases fertility in vivo.
Gonçalves *et al.* (2008b)	Ej	Thawed	Thawed	Lipocalin-type PGTDS	Sperm treated with antibody against lipocalin-type PGTDS increased sperm-oocyte binding and inhibited fertilization in vitro.
Sood *et al.* (2018)	Ej	Fresh	Fresh	15 and 25 kDa SP protein	Bulls showed abnormal sperm in absence of 15kDa protein and weak expression of 25kDa protein.

AI, artificial insemination; aSFP, acid seminal fluid protein; DEFB126, beta defensin 126; ELSPB1, epididymal sperm binding protein 1; Ej, ejaculate; Ep, epididymis; GH, growth hormone; IGF, insulin-like growth factor; kDa, kilodalton; LPGDS, lipocalin-type prostaglandin D-synthase; NGF, nerve growth factor; NGFB, beta nerve growth factor; PGDS, prostaglandin D-synthase; PSAP, prosaposin; RLN, relaxin.

Sixteen studies related the proteome of bull SP with pre-established fertility and sperm freezability phenotypes (Table 5). Overall, those studies used different techniques for searching potential protein biomarkers, with different outcomes and results. Among them, seven studies considered cows' nonreturn rates as the endpoint to select bulls with low and high fertility (Killian *et al.* 1993; Hoelfich *et al.* 1999; Fouchécourt *et al.* 2002; Moura *et al.* 2006a, Moura *et al.* 2006b; Moura *et al.* 2007; Viana *et al.* 2018). Likewise, three studies used conception

rates to categorize fertile bulls (Aslam *et al.* 2014; Stewart *et al.* 2018; Kasimanickam *et al.* 2019). In other studies, however, bulls were selected with low and high sperm freezability according to characteristics of frozen-thawed ejaculates (Jobim *et al.* 2004; Roncoletta *et al.* 2013; Magalhães *et al.* 2016; Rego *et al.* 2016; Gomes *et al.* 2020). The endpoint used to classify low and high fertility bulls was not mentioned only in one study (Vickram *et al.* 2016). Results from the eligible studies indicated that IGF2, BSP3, BSP5, cathepsin-D (CTSD), α-L-fucosidase (FUCA), OPN, phospholipase A2 (PLA2), spermadhesin 1, and albumin (ALB) were detected in greater abundances in the SP of high fertility bulls, whereas IGF1, spermadhesins 1 and Z13, nucleobindin (NUCB), acrosin inhibitor, and 5'nucleotidase (NT5E) were expressed at higher levels in the SP of low fertility sires (Table 5). Depending on the study, clusterin (CLU), BSP1, and metalloproteinase inhibitor (TIMP) were predominant in the semen of either low or high fertility, while lipocalin-type PTGDS and NGFB could not be associated with any specific fertility phenotype (Table 5). Other differentially abundant SP proteins according to sire fertility phenotype are listed in Table 5.

Table 5 Main outcomes of studies (*n*=16) included in the systematic review that described significant associations between seminal plasma (SP) proteins and fertility or sperm freezability (SF) status of bulls.

Reference/ Phenotype	Proteins expressed	Endpoints	Sample evaluated
Killian *et al.* (1993)		NRR	SP
H Fert	Spot 26 kDa/pI 6.2 and spot 55 kDa/pI 4.5		
L Fert	Spot 16 kDa/pI 4.1 and spot 16 kDa/pI 6.7		
Hoelfich *et al.* (1999)		NRR	SP
H Fert	IGF2		
L Fert	IGF1		
Fouchécourt *et al.* (2002)		NRR	SP
H Fert	PTGDS - higher and lower abundance		
L Fert	PTGDS - lower abundance		
Moura *et al.* (2006a)		NRR	AGF
H Fert	OPN, PLA2		
L Fert	Spermadhesin Z13		
Moura *et al.* (2006b)		NRR	CEF
H Fert	CTSD, FUCA		
L Fert	PTGDS		
Moura *et al.* (2007)		NRR	AGF
H Fert	BSP1, BSP3, BSP5, ALB, CLU, PLA2, OPN		
L Fert	NUCB		
Viana *et al.* (2018)		NRR	SP
H Fert	NPPC, TIMP2, BSP5, sulfhydryl oxidase		
L Fert	CLU, TFPI2, LGALS3BP, NT5E		
Aslam *et al.* (2014)		ACR	SP
H Fert	BDNF, COMMD6, Uncharacterized protein C11orf70 homolog, POT1, ALB, GSTM1, CPXM2, NDUFAF2, PDE6B		
L Fert	SORCS1, TANGO2, CYP11B2, CASP8, PTGER3, ZNF696, P2RX5, DST, GRK2		
Stewart *et al.* (2018)		SCR	SP

H Fert	NGF- higher abundance		
L Fert	NGF- lower abundance		
Kasimanickam *et al.* (2019)		DPR	SP
H Fert	BSP1, BSP3, BSP5, Spermadhesin-1, ALB, TIMP, AK1, PEBP1		
L Fert	CCT5, CCT8, ELSPbP1, PSMA6, CLU		
Jobim *et al.* (2004)		Viability of EJ*	SP
H Freeze	CLU; aSFP; spot 15–16 kDa/pI 4.7–5.2; spot 13–14 kDa/pI 4–4.5		
L Freeze	Spot 25-26 kDa/pI 6-6.5		
Roncoletta *et al*. (2013)		Thawed EJ†	SP
H Freeze	Spot 18.20 kDa/pI 5.3 and spot 17.90 kDa/pI 4.8		
Magalhães *et al*. (2016)		SF	SP
H Freeze	BSP1 isoform15.52 kDa/pI 5.78 absent		
L Freeze	BSP1 15.52 kDa/pI 5.78 present		
Rego *et al.* (2016)		SF	SP
H Freeze	OPN-K, DNASE1L3		
L Freeze	SPINK2, GPX 3, TIMP2, EFNA1, ANXA1, PAFA		
Gomes *et al*. (2020)		SF	SP
H Freeze	BSP5, seminal ribonuclease		
L Freeze	Spermadhesin-1, GSN, TUB, GAPHD, CALM, ATP synthase, SPESP1, PRDX5, SCGB1D members, GPI		
Vickram *et al*. (2016)		Not mentioned	SP
H Fert	Band 250 kDa		
L Fert	Band 160 kDa		

ACR, absolute conception rate; AGF, accessory gland fluid; AK1, adenylate kinase isoenzyme 1; ALB, albumin; ANXA1, Anexin A1; aSFP, acid seminal fluid protein; BDNF, brain-derived neurotrophic factor; BSP, binder of sperm protein; CALM, calmodulin; CASP8, Caspase-8 isoform 1; CCT5, T-complex protein 1 subunit epsilon; CCT8, T-complex protein 1 subunit theta; CEF, cauda epididymal fluid; CLU, clusterin; COMMD6, COMM domain-containing protein 6-like; CPXM3, Cytosolic carboxypeptidase 3; CTSD, cathepsin D; CYP11B2, Cytochrome P450 11B2 mitochondrial-like isoform1; DNASE1L3, Deoxyribonuclease 1 Like 3; DPR, daughter pregnance rate; DST, Dystonin isoform 1eA precursor isoform 150; EFNA1, ephrin-A1; ELSPbP1, Epididymal Sperm Binding Protein 1; FUCA, alfa L fucosidase; GAPHD, glyceraldehyde-3-phosphate dehydrogenase; GPI, glucose-6-phosphate isomerase; GPX3, Glutathione peroxidase 3; GRK2, Beta-adrenergic receptor kinase 2; GSN, gelsolin; GSTM1, Glutathione S-transferase Mu1; H Freeze, high freezability; H Fert, high fertility; IGF, insulin-like growth factor; kDa, kilodalton; L Freeze, low freezability; L Fert, low fertility; LGALS3BP, galectin-3-binding protein; NDUFAF2, Mimitin mitochondrial; NGF, nerve growth factor; NT5E, 5'-nucleotidase; NPPC, C-type natriuretic peptide; NRR, nonreturn rates; NUCB, nucleobindin; OPN, osteopontin; PAFA, platelet-activating factor acetyl hydrolase; PDE6B, cGMP-dependent 3',5'-cyclic phosphodiesterase; PEBP1, phosphatidylethanolamine-binding protein 1; pI, isoelectric point; PLA2, phospholipase A2; POT1, Protection of telomers 1; PRDX5, peroxiredoxin-5; PSMA6, proteasome subunit alpha type-6; PTGDS, prostaglandin D synthase; PTGER3, Prostaglandin E2 receptor EP3; P2RX5, P2X purinoceptor 5-like; SCGB1D, secretoglobin family 1D; SCR, sire conception rate; SORCS1, VPS10 domain-containing receptor SorCS1 isoform X4; SP, seminal plasma; SPESP1, sperm equatorial segment protein 1; SPINK2, Acrosin inhibitor; TANGO2, Transport and Golgi organization protein 2 homolog; TFPI2, tissue factor pathway inhibitor 2; TIMP, metalloproteinase inhibitor; TUB, tubulin; ZNF696, Zinc finger protein 696;

* Viability of thawed ejaculate (> 90%); †Thawed EJ and approved ejaculates.

In eleven studies the correlations involving bovine SP proteins, sperm parameters, and fertility were evaluated (Table 6). The concentration of total SP proteins was positively correlated with sperm freezability (Cheema *et al.* 2008), while interleukin 10 (IL10) and

spermadhesin Z13, among other proteins, had positive correlations with sperm motility (Vera *et al.* 2003; Menezes *et al.* 2017). Spermadhesin 1 showed a negative correlation with the percentage of abnormal frozen-thawed and fresh sperm (Montanholi *et al.* 2016; Menezes *et al.* 2017). BSP5 presented a positive correlation with abnormal sperm in fresh semen, but negative with abnormal sperm in frozen-thawed semen (Menezes *et al.* 2017). Spermadhesin 1 and paraoxonase (PON1) were negatively correlated with sperm vigor, whereas CLU exhibited a positive association with this parameter (Menezes *et al.* 2017; Ferreira *et al.* 2017). BSP5 and CLU had positive correlations with the viability of bovine sperm (Menezes *et al.* 2017), and seminal gamma glutamyl transferase (GGT) activity showed positive associations with *in vitro* embryo development (Pero *et al.* 2017). In the remaining studies significant correlations between the amount of certain SP proteins and sperm parameters or fertility were uncovered, with r-values under 0.8 (Marques *et al.* 2000; Kohsaka *et al.* 2003; Assumpção *et al.* 2005; Assumpção *et al.* 2013; Rueda *et al.* 2013b; Table 6).

Table 6. Main findings of the studies (*n*=11) included in the systematic review that evaluated the correlations of seminal plasma proteins with sperm parameters and bull fertility.

Reference	Protein	*r*	Parameter description
Cheema *et al.* (2008)	SP proteins	0.86	sperm freezability
Vera *et al.* (2003)	IL10	0.84	sperm motility
Menezes *et al.* (2017)	Acrosin inhibitor 1 and CLU	-0.68	frozen-thawed sperm total defects
	Spermadhesin 1	-0.64	frozen-thawed sperm total defects
		-0.81	sperm total defects
	CLU and BSP5	0.8	fresh sperm viability
		0.81	frozen-thawed sperm viability
	BSP5	0.8	sperm total defects
		0.76	sperm viability
		-0.81	frozen-thawed sperm total defects
	Spermadhesin 1	-0.89	sperm vigor
		-0.64*	frozen-thawed sperm motility
		0.64*	frozen-thawed sperm motility
	BSP1	0.66	sperm motility
	ENO1, CK M-type, BSP3, GAPDH	0.72	sperm viability
	CLU	0.67	sperm motility
		0.78	frozen-thawed sperm motility
		0.82	frozen-thawed sperm vigor
		0.71	sperm vigor
	ALB	0.64	sperm viability
	Spermadhesin Z13	0.85	frozen-thawed sperm motility
		0.79	frozen-thawed sperm vigor
		-0.73	frozen-thawed sperm total defects
	TIMP2	0.72	sperm vigor
	CATSS	0.64	sperm vigor
	CTSLP1	-0.68	frozen-thawed sperm total defects

	NT5E and LGMN	-0.73	frozen-thawed sperm total defects
	Inhibitor of carbonic anhydrase	0.78	sperm total defects
		0.79	frozen-thawed sperm total defects
Montanholi *et al.* (2016)	aSFP	0.39	sperm motility
	ACR	0.49	sperm concentration
	Niemman-Pick type 2	0.37	sperm concentration
	PGK2	0.35	sperm pathology (loose head)
	BSP1	0.45	sperm motility
	Spermadhesin 1	-0.81	sperm total defects
Ferreira *et al.* (2017)	PON1	-0.89	sperm vigor
Pero *et al.* (2017)	GGT activity	0.41	frozen-thawed sperm motility
		0.80	embryo cleavage *in vitro*
		0.87	blastocyst formation *in vitro*
	ALP activity	0.51	frozen-thawed sperm viability
Marques *et al.* (2000)	15.7 kDa in sperm membrane	0.71	acrosome reaction
	31.1 kDa in sperm membrane	-0.61	acrosome reaction
Kohsaka *et al.* (2003)	RLN	0.64	sperm motility
Assumpção *et al.* (2005)	SP proteins	-0.07	bull fertility
Assumpção *et al.* (2013)	SP proteins	0.43	sperm concentration
		-0.43	sperm total defects
Rueda *et al.* (2013b)	16.20 kDa, pI 5.5	0.64	sperm viability

ACR, acrosin; ALB, albumin; ALP, alkaline phosphatase; aSFP, acid seminal fluid protein; BSP, binder of sperm protein; CATSS, cathepsin S; CK, creatine kinase; CLU, clusterin; CTSLP1, cathepsin L1; kDa, kilodalton; ENO1, alfha-enolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGT, γ-glutamyltransferase; IL, interleukin; LGMN, legumain; NT5E, 5'-nucleotidase; pI, isoeletric point; SP, seminal plasma; TIMP2, tissue inhibitor of metalloproteinase 2; PGK2, Phosphoglycerate kinase 2; PON1, Paraoxonase 1; RLN, relaxin; *r*, Pearson's correlation.

[*]Menezes et al. (2017) correlated the optical density of bull seminal plasma protein spots from two-dimensional gels electrophoresis with sperm parameters. Some proteins were identified in more than one spot and some spots had more than one protein. This is why the same protein may present different correlations with the same sperm parameter.

Risk of bias

The methods applied to set up a random sequence generation were not mentioned in any of the papers (Fig. 2). Baseline similarities were reported in 63% of the studies, whereas the methods used to conceal the allocation were not reported and often defined as "unclear" (97%; Fig. 2). Random housing and blindness of caregivers were reported in only three and two studies, respectively. None of the studies reported whether the selection of animals to assess at each outcome point was determined at random, whereas only two studies reported blindness of the outcome assessment (Fig. 2). Data outcome was adequately reported in 96% of the studies and three studies presented incomplete outcomes. Eight studies were defined as "high risk" of bias due to the selective reporting data. Another potential source of bias was scored as "high risk" in three studies, due to the use of different treatments in different animals, the use of a single sire as the animal sample, and the correlation of densities of protein spots (which may contain more than one protein) with sperm parameters (Fig. 2).

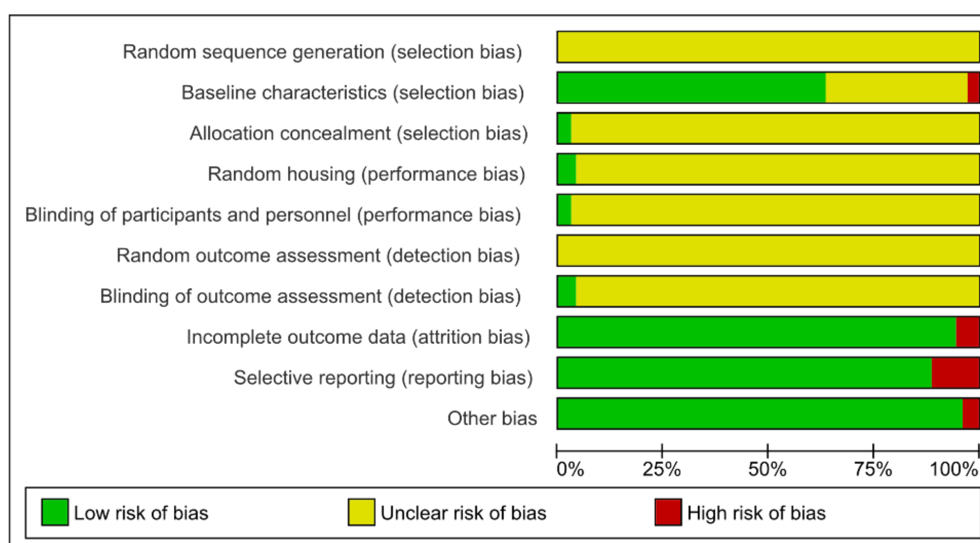

Figure 2. Results of the risk of bias to evaluate the methodological quality of the 71 papers included in the systematic review.

DISCUSSION

The present review provides evidence for the potential roles of SP proteins in sperm motility, quality, and capacitation, contributing to the establishment of sperm reservoir, sperm protection, and fusion of the gametes. These proteins, in the end, increment bull fertility scores and improve early embryo development (Fig. 3). In addition, we also described discrepancies among studies regarding potential functions of seminal proteins in male reproduction. Indeed, the study of bull fertility is challenging from numerous aspects. Sperm physiology and fertilizing capacity are determined by dynamic and complex processes that, in turn, rely on intrinsic and extrinsic factors. While intrinsic factors involve features of the spermatozoon itself, such as metabolism, morphology, membrane structure, acrosome and DNA integrity, extrinsic factors comprise those in the milieu surrounding the male gamete, from the epididymis to ejaculation. Thus, this systematic review specifically focused on the protein segment of that milieu, analyzing studies conducted during the last three decades.

In this light, most of the eligible studies were performed by scientists from the USA, India, and Brazil. It was an expected result given the importance of the USA and Brazil as the world's top beef producers, while India has the largest cattle livestock population in the world (USDA 2020). *Bos taurus* was the animal model mostly used due to its importance for AI programs in dairy farms (Kabuga & Appiah 1992). Dairy bulls have been used in commercial ARTs for several decades. These animals are subjected to routine evaluation, allowing researchers to compile detailed information about their semen parameters and fertility scores

based on thousands of semen samples and inseminations. This fact makes *Bos taurus* a suitable model to study mammalian reproduction. Moreover, AV was the method of semen collection mainly used since it mimics natural mating (Rego *et al.* 2015). Electroejaculation was the second method chosen by authors, which increases the volume of SP because of the application of short and low-voltage pulses of electrical current to the pelvic nerves that stimulate the contraction of smooth muscles of the pelvic urethra and secretion of seminal plasma by accessory sex glands (Seidel & Foote 1969). This approach dilutes the ejaculated sperm and affects their interaction with seminal proteins.

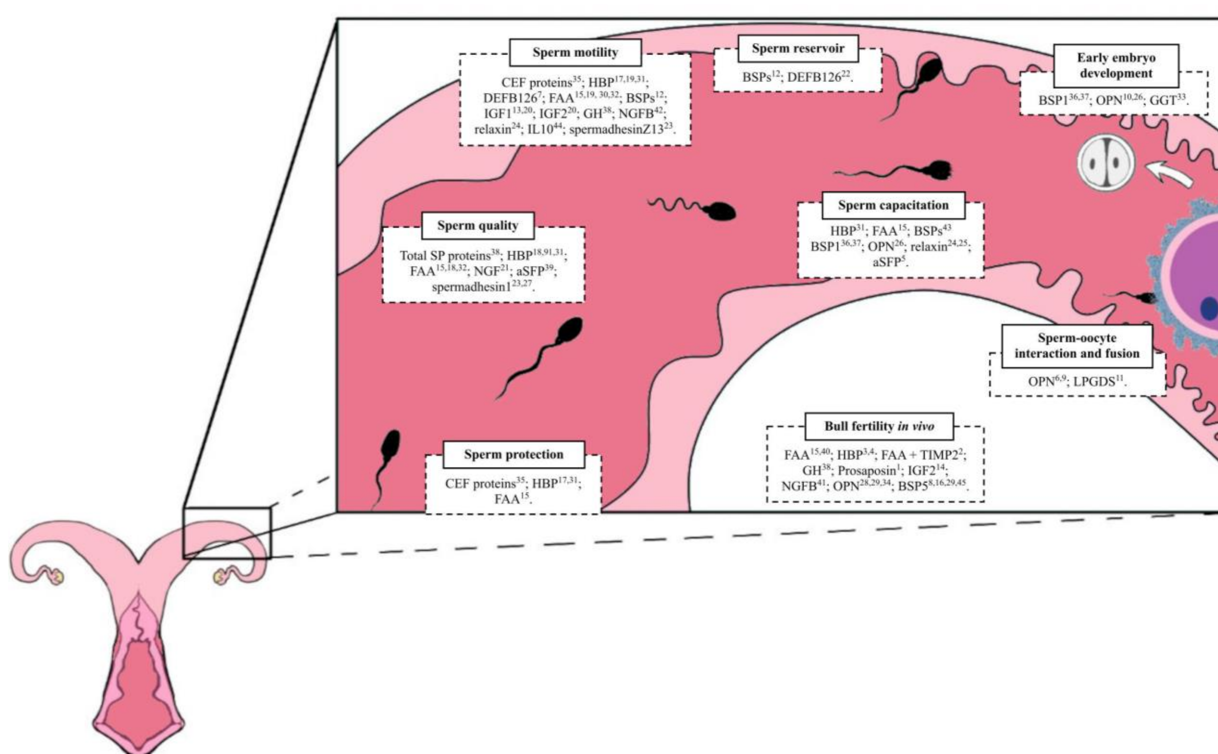

Figure 3. Schematic representation of the potential roles of seminal plasma (SP) proteins on sperm parameters and bull fertility *in vivo*. Sperm motility, quality, and capacitation may be positively influenced by seminal proteins, such as cauda epididymal fluid (CEF), beta defensin 126 (DEFB126), heparin binding proteins (HBPs), fertility-associated antigen (FAA), binder of sperm protein (BSP), insulin-like growth factors (IGF1 and 2), growth hormone (GH), nerve growth factor (NGF), nerve growth factor beta (NGFB), relaxin, interleukin (IL) 10, spermadhesins (including Z13), acid seminal fluid protein (aSFP), osteopontin (OPN), as well as total SP proteins. Regarding sperm capacitation, however, aSFP may act as decapacitating factor. Proteins can also contribute to the establishment of sperm reservoir (BSPs and DEFB126) and the sperm protection against oxidative stress (CEF proteins and HBPs). Moreover, OPN may improve the sperm-oocyte interaction, whereas lipocalin-type prostaglandin D synthase (LPGDS) plays negative roles in gametes interaction and fusion. Several proteins increment bull fertility scores (FAA, FAA + metalloproteinase inhibitor 2 [TIMP2], HBP, GH, prosaposin, IGF2, NGFB, OPN, and BSP5) and early embryo development (BSP1, OPN, and gamma glutamyl transferase [GGT]). [1] Amman *et al.* 1999; [2] Alvarez-Gallardo *et al.* 2013; [3] Bellin *et al.* 1994; [4] Bellin *et al.* 1996; [5] Dostàlova *et al.* 1994; [6] Erikson *et al.* 2007; [7] Fernandez-Fuertez *et al.* 2002; [8] Gomes *et al.* 2020; [9] Gonçalves *et al.* 2007; [10] Gonçalves *et al.* 2008a; [11] Gonçalves *et al.* 2008b; [12] Gwathmey *et al.* 2006; [13] Henricks *et al.* 1998; [14] Hoeflich *et al.* 1998; [15] Karunakaran & Devanathan 2015; [16] Kasimanickam *et al.* 2019; [17] Krishnan *et al.* 2015; [18] Krishnan *et al.* 2016a; [19] Krishnan *et al.* 2016b; [20] Lackey *et al.* 1998; [21] Li *et*

al. 2010; [22] Lyons *et al.* 2018; [23] Menezes *et al.* 2017; [24] Miah *et al.* 2007; [25] Miah *et al.* 2011; [26] Monaco *et al.* 2009; [27] Montanholi *et al.* 2016; [28] Moura *et al.* 2006a; [29] Moura *et al.* 2006b; [30] Pande *et al.* 2018; [31] Patel *et al.* 2015; [32] Patel *et al.* 2016; [33] Pero *et al.* 2017; [34] Rego *et al.* 2016; [35] Reyes-Moreno *et al.* 2002; [36] Rodriguez-Villamil *et al.* 2016; [37] Rodriguez-Villamil *et al.* 2020; [38] Sauerwein *et al.* 2000; [39] Schöneck *et al.* 1996; [40] Sprott *et al.* 2000; [41] Stewart *et al.* 2018; [42] Stewart *et al.* 2019; [43] Thérien *et al.* 2001; [44] Vera *et al.* 2003; [45] Viana *et al.* 2018.

This review detected controversies on the use of the whole SP proteins in AI. Discrepancies among studies and their results probably relate to the fact that proteins of SP play a wide range of roles (Rodríguez-Martínez *et al.* 2011). While some authors reported positive correlations between seminal fluid proteins and sperm parameters (Cheema *et al.* 2008; Assumpção *et al.* 2013), others reported a negative correlation with bull fertility (Assumpção *et al.* 2005). However, none of the selected studies evaluated the effects of seminal proteins on the sperm fertilizing ability, as well as the functional mechanism of the complex mixture of proteins in this outcome.

Comparison of seminal protein abundances from bulls with contrasting sperm freezability and fertility scores was another common approach used by researchers. Based on such studies, there is a large variability in protein abundances in bulls of high and low fertility and sperm freezability. Experiments listed in our analytical review identified several types of SP proteins and investigate how they affected sperm functions and parameters. Below, we discuss the biochemical characteristics and roles of some of these proteins.

HBPs

According to our review, HBPs favor both capacitation and acrosome reaction when present in the sperm membrane or SP of bulls (Bellin *et al.* 1996; Karunakaran & Devanathan 2015; Fig. 3). HBPs also exert positive impacts on sperm cryopreservation through stabilization of acrosome membrane (Alvarez-Gallardo *et al.* 2013), as well as protection of bovine sperm against oxidative stress (Karunakaran & Devanathan 2015) and DNA fragmentation (Krishnan *et al.* 2016a). Once in the female reproductive tract, sperm bind to the glycosaminoglycan heparin through HBPs that, in turn, regulates sperm capacitation (Miller *et al.* 1990; Alvarez-Gallardo *et al.* 2013). Known HBPs of the bull SP include a 24-kDa TIMP2 (McCauley *et al.* 2001), 55-kDa OPN (Cancel *et al.* 1995), and a 30-kDa DNAse I-like (DNASE1L1) molecule described as fertility-associated antigen (FAA; McCauley *et al.* 1999). Controversy, an increase in *in vivo* fertility of Nelore heifers inseminated with FAA-negative bulls was described in one study. The authors reported that either the presence or absence of FAA on sperm was not a single valid criterion for bull fertility prediction (Dalton *et al.* 2012).

BSPs

In this review, we provide evidence that BSP1 and BSP5 act positively on sperm physiology (Fig. 3). For instance, interventional studies confirmed the positive effects of BSP1 on sperm capacitation and early embryo development (Thérien *et al.* 2001; Rodríguez-Villamil *et al.* 2016), even with variations in its abundance among sires with different fertility scores (Magalhães *et al.* 2016). Moreover, enhancement in sperm freezability and fertility scores was associated with high abundance of BSP5 in bulls (Moura *et al.* 2007; Viana *et al.* 2018; Kasimanickam *et al.* 2019; Gomes *et al.* 2020). Both BSP1 (also named as PDC-109) and BSP5 (BSP 30 kDa) are glycosylated proteins with several isoforms (Calvete *et al.* 1996; van Tilburg *et al.* 2021). The levels of glycosylation influence the ability of BSPs to remove lipids from the cell membrane, which occurs when sperm undergo BSP-mediated capacitation (Plante *et al.* 2016; Singh *et al.* 2019). Those proteins belong to the BSP family, the main component of bovine SP (Manjunath & Thérien 2002; Gomes *et al.* 2020). Several authors have demonstrated the beneficial uses of BSP's as sequestering agents to increase sperm longevity (Bergeron *et al.* 2004; Srivastava *et al*. 2013). The continuous exposure of sperm to BSPs causes phospholipid and cholesterol efflux that promotes membrane reorganization, leading to early capacitation and damage to membranes during cryopreservation procedures (Manjunath & Thérien 2002). BSPs also mediate the interactions between sperm and oviductal epithelial cells to form the sperm reservoir, prolonging sperm survival (Gwathmey *et al.* 2006).

OPN

Positive role of OPN enhancing the capacitation process and early embryo development in *in vitro* studies was reported after its incubation with frozen-thawed bovine sperm (Gonçalves *et al.* 2008a; Monaco *et al.* 2009; Fig. 3). The case of OPN is one of the few empirical associations between an SP protein and bull fertility that has been confirmed by results of interventional studies. This finding may be related to the amounts of OPN in reproductive fluids of bulls (Killian *et al.* 1993; Cancel *et al.* 1995; Moura *et al.* 2006a; Rego *et al.* 2016). Seminal OPN is a 55-kDa acidic, calcium-binding, phosphorylated, and glycosylated protein (Cancel *et al.* 1995). However, the exact mechanism(s) by which OPN affects sperm function is still unclear. OPN may influence intracellular calcium concentrations and stimulates exocytosis of acrosomal vesicle and gamete fusion, which are crucial steps for sperm capacitation and fertilizing ability (Johnson *et al.* 2003). Moreover, OPN interacts with

sperm, zona pellucida, and oolemma through integrins and CD44 receptors, triggering intracellular mechanisms in the oocyte and affecting the early stages of embryo growth (Erikson *et al.* 2007). The latter is a plausible hypothesis to explain OPN's effects on early embryo development, but further studies are needed for its confirmation.

GH and the growth factors: IGFs and NGF

According to the studies analyzed here, GH, IGF1, and IGF2 maintained sperm motility through energy metabolism and glucose uptake (Lackey *et al.* 1998; Sauerwein *et al.* 2000; Fig. 3). The GH plays a central role in tissue development, including in male reproductive organs. Additionally, GH levels modulate IGF1 production that, in turn, play roles in Leydig cell differentiation and function, steroidogenesis, and spermatogenesis (Tsuruta & O´Brien 1995; Baker *et al.* 1996).

This review showed that NGFB is more abundant in the SP of high fertility sires and that exogenous NGF added to frozen-thawed bull spermatozoa improved sperm quality (Li *et al.* 2010; Stewart *et al.* 2018; Fig. 3) through the increase of leptin secretion from sperm. Leptin is a cytokine-like hormone that regulates energy balance and promotes sperm capacitation and survival in pigs and acrosome reaction and motility in men (Aquila *et al.* 2008; Lampiao & du Plessis 2008). Moreover, NGF plays roles in spermatogenesis and stimulates tissue differentiation, development, and survival (Mutter *et al.* 1999). Authors speculated that NGF presence in the SP of species with spontaneous ovulation has a function as ovulation-inducing factor in llamas and alpacas (Ratto *et al.* 2012). However, the actual mechanisms by which this protein modulates sperm function and whether or not seminal NGFB contributes to ovulation in these species remain to be elucidated.

Peptides: DEFB126, prosaposin, and relaxin

Roles of DEFB126 in sperm motility, prevention of sperm agglutination, and sperm-oviduct binding were confirmed here (Fernandez-Fuertes *et al.* 2016; Lyons *et al.* 2018; Fig. 3). Indeed, DEFB126 coating increases the negative charge of sperm due to multiple sialylated oligosaccharides of the protein, allowing easy migration of sperm through electronegative cervical mucous (Tollner *et al.* 2008). The epididymis is the main organ to secrete DEFB126, which binds to sperm and modulates sperm functions in the female tract (Lyons *et al.* 2018).

This review showed that PSAP may improve the fertility of bull frozen-thawed sperm. Its role is probably related to promoting gamete interaction (Amann *et al.* 1999; Hammerstedt

et al. 1997). PSAP is a 60-aminoacid precursor of saposins that plays a role in the hydrolysis of sphingolipids present in cell membranes (Kishimoto *et al.* 1992). In humans, sphingolipids in the sperm membrane slow the capacitation rate, by delaying cholesterol efflux (Cross *et al.* 2000). Finally, seminal RLN induces sperm motility and capacitation by accelerating intracellular signaling cascades, acting on cholesterol efflux, increasing calcium influx and intracellular cAMP, and inducing protein tyrosine phosphorylation (Miah *et al.* 2011).

Spermadhesins and aSFP

Our findings reveal controversies among studies regarding the impact of spermadhesins on male fertility parameters. Both spermadhesin Z13 and spermadhesin 1 were associated with low fertility (Moura *et al.* 2006a) and low sperm freezability (Gomes *et al.* 2020), even being more abundant in high fertility bulls (Kasimanickam *et al.* 2019). Differences in their effects may be either beneficial or detrimental, depending on their concentrations. Further, spermadhesin Z13 was positively correlated with sperm motility and vigor (Menezes *et al.* 2017; Fig. 3), whereas spermadhesin 1 was negatively correlated with total sperm defects (Montanholi *et al.* 2016; Menezes *et al.* 2017). These proteins have antioxidative effects, mediate sperm interaction with the oviduct epithelium, and participate in sperm-egg binding, sperm membrane stability, and sperm motility (Töpfer-Petersen *et al.* 1998).

Acid seminal plasma protein (aSFP) shares identity with spermadhesins (Romão *et al.* 1997). Although aSFP coats sperm during ejaculation, it is released from sperm during capacitation in the female tract, suggesting its role in sperm decapacitation (Fig. 3). Up to date, there is no evidence of its participation in sperm-oocyte interaction (Dostàlova *et al.* 1994). Moreover, aSFP abundance in SP is associated with bull sperm freezability (Jobim *et al.* 2004). Improvement in sperm motility, metabolism, and protection against oxidative stress are other potential attributes of aSFP (Schöneck *et al.* 1996; Montanholi *et al.* 2016).

Lipocalin-type PTGDS

PTGDS expression in the epididymal fluid had an inverse link with fertility of dairy sires (Moura *et al.* 2006b). Conversely, reports released by Killian *et al* (1993) and Gerena *et al* (1998) described positive associations between seminal PTGDS and fertility phenotypes of bulls, but Fouchécourt *et al* (2002) concluded that PTGDS does not have meaningful correlations with fertility indexes. Later on, Gonçalves *et al* (2008b) verified that incubation of bovine oocyte and spermatozoa in the presence of anti-LTPGDS antibodies increased sperm-

oocyte binding and decreased fertilization rate (Fig. 3), suggesting LTPGDS's role in blocking polyspermy and mediating fertility. PTGDS converts prostaglandin (PG)H_2 to $PTGD_2$ and is the major protein secreted by the epididymis in several species, including bulls (Fouchécourt *et al.* 2002; Moura *et al.* 2010).

Clusterin

This systematic review uncovers that CLU abundance in bull SP can be either positively (Jobim *et al.* 2004; Moura *et al.* 2007; Menezes *et al.* 2017) or negatively (Viana *et al.* 2018; Kasimanickam *et al.* 2019; Boe-Hansen *et al.* 2015) associated with sperm freezability and bull fertility. CLU apparently interacts with and becomes a selective marker of morphologically abnormal sperm (Bailey & Griswold 1999). This protein acts as a chaperone, participates in sperm maturation (Belleannée *et al.* 2011), protects spermatozoa against reactive oxygen species (Reyes-Moreno *et al.* 2002), halts cell lysis by complement-mediated processes, and contributes to lipid transport and membrane remodeling (Humphreys *et al.* 1999). The glycosylation pattern of secreted–CLU determines several of its functional characteristics (Janiszewska & Kratz 2019), which may imply in specific functions for each seminal CLU isoform (or groups) with a unique relationship with male fertility.

Review limitations

The SYRCLE's toll revealed specific limitations in the research reports analyzed here. The risk of bias showed a high number of unclear answers for the individual studies. This result is mainly associated with the heterogenicity in methodologies evaluating SP effects in sperm physiology and the low quality of methodological description. It may explain why studies focused on similar objectives report different results that, in some cases, are conflicting. Methodologies related to protein analysis also influenced our findings. Mass spectrometry-based methods were used to identify and quantify proteins in SP from bulls with contrasting fertility scores and sperm parameters. While some authors used gel-based, others evaluated SP samples using LC-MS/MS. These approaches considerably differ from each other concerning the technical details. Moreover, mass spectrometry methods and bioinformatics tools have changed from 1990 to 2020, affecting data analyses and interpretation.

Final remarks and future directions

The present review focused on a comprehensive and systematic analysis of SP proteins, their functions, and potential associations with sperm parameters and fertility phenotypes of bovine sires. Although we do not have reliable biomarkers of bull fertility (yet), lessons and future directions can be taken from this review. One of the key steps for building up a baseline for future studies regarding SP and bull fertility is to standardize as much as possible the methods for quantifying seminal proteins and the definition of endpoints that are of actual importance for bulls used in commercial AI and sires kept in free-range production systems. These methodological steps and standardizations will minimize the sources of biases. The other point we must emphasize is that statistical associations detected between seminal proteins and fertility parameters should be validated in additional studies with larger groups of animals with the same phenotypes. Next, fertility-related proteins must be tested using *in vitro* and/or *in vivo* assays for understanding how these proteins work. Future experiments focused on SP should evaluate protein isoforms, roles of post-translational modifications, and protein complexes in SP. Several proteins appear in SP as numerous isoforms, and their functions may also be determined by how they interact in the seminal milieu (Gomes *et al.* 2020; van Tilburg *et al.* 2021).

Studies on SP proteins, their empirical relation to fertility scores of bulls, and further experimental tests may become the basis for two types of applications. First, specific seminal proteins could be used as biomarkers of fertility, being an additional parameter employed to select superior sires. Although possible, this is a demanding task because of many required processes for biomarker validation and qualification. Alternatively, selected seminal proteins could be used as fertility enhancers, being added into extenders to improve sperm cryotolerance and fertilizing capacity. These candidates as fertility enhancers could also be added into *in vitro* fertilization media to boost embryo production. Therefore, SP protein-based technologies may contribute to bovine reproduction, making herd production systems more profitable and sustainable.

Declaration of interest

The authors declare that they have no conflict of interest.

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Author contribution statement

G. Viana, A. Moura, and M. Machado-Neves conceived the study design. A. G. Viana, I. M. Ribeiro, and R. P. R. Carvalho performed the literature search and data extraction. All authors contributed to the data interpretation and article preparation. A. Moura, E. Memili, and M. Machado-Neves reviewed the intellectual content before submission. Machado-Neves supervised the project.

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Supplementary Table 1. Characteristics of studies (*n*=71) evaluating the associations and effects of seminal plasma proteins on bull fertility.

Reference	Country	Type of study	Species	Bulls (*n*)	Sample collection	Sample type	Method of protein:	
							Separation	Identification
Killian *et al.* (1993)	USA	Observational	*Bos taurus*	35	Unk	Whole SP	1D and 2D	NA
Bellin *et al.* (1994)	USA	Observational	*Bos taurus*	300	EJ	Fractioned SP	C	NA
Dostàlova *et al.* (1994)	Germany	Interventional	Unk	Unk	Unk	Purified aSFP	C	ELISA
Bellin *et al.* (1996)	USA	Observational	*Bos taurus*	53	EJ	Fractioned SP	C	ELISA and WB
Schöneck *et al.* (1996)	Germany	Interventional	*Bos taurus*	Unk	Unk	Purified aSFP	C	Analytical isoelectric focusing
Henricks *et al.* (1998)	USA	Interventional	*Bos indicus*	7	EJ	Purified IGF1	C	RIA
Lackey *et al.* (1998)	USA	Interventional	*Bos taurus*	2	EJ	Purchased IGFs	NA	NA
Amann *et al.* (1999)	USA	Interventional	*Bos taurus*	3	Unk	Synthetic PSAP	NA	NA
Hoeflich *et al.* (1999)	USA	Observational	*Bos taurus*	19	Unk	Whole SP	1D	WB
Alonso Marques *et al.* (2000)	Brazil	Observational	*Bos taurus* and *Bos indicus*	37	Unk	Whole SP	1D	NA
Sauerwein *et al.* (2000)	Germany	Interventional	*Bos taurus*	10	AV	Recombinant GH	NA	NA
Sprott *et al.* (2000)	USA	Observational	Crossbred	25	EJ	Fractioned SP	1D	WB
Thérien *et al.* (2001)	Canada	Interventional	Unk	Unk	Unk	Purified BSP	1D and C	N-terminal
Fouchécourt *et al.* (2002)	France	Observational	*Bos taurus*	133	AV	Whole SP	1D	WB
Reyes-Moreno *et al.* (2002)	Canada	Interventional	Unk	5	RF	Fractioned SP	Filtration and 1D	WB and MS (MALDI-TOF)
Kohsaka *et al.* (2003)	Japan	Observational	*Bos taurus*	13	Unk	Whole SP	NA	RIA
Vera *et al.* (2003)	Venezuela	Observational	Crossbred	7	EJ	Whole SP	NA	NA
Bergeron *et al.* (2004)	Canada	Interventional	Unk	4	Unk	Whole SP	1D	WB and RIA

Jobim *et al.* (2004	Brazil	Observational	*Bos taurus* and *Bos indicus*	16	AV	Whole SP	2D	N-terminal
Assumpção *et al.* (2005)	Brazil	Observational	*Bos indicus*	19	EJ	Whole SP	NA	NA
Gwathmey *et al.* (2006)	USA	Interventional	*Bos taurus*	11	Unk	Purified BSPs	C	1D and amino acid sequence
Moura *et al.* (2006a)	USA	Observational	*Bos taurus*	37	AV	Fractioned SP (AGF)	2D	CapLC-MS/MS q-TOF
Moura *et al.* (2006b)	USA	Observational	*Bos taurus*	20	Cannulation vas deferens	Fractioned SP (CEF)	2D	CapLC-MS/MS q-TOF
Erikson *et al.* (2007)	USA	Interventional	*Bos taurus*	13	AV and RF	Anti-OPN	1D	WB
Gonçalves *et al.* (2007)	USA	Interventional	*Bos taurus*	2	AV	Anti-OPN, OPN, and ITG	NA	NA
Miah *et al*. (2007)	Japan	Interventional	*Bos taurus*	4	AV	Purified RLN	1D	Amino acid sequence
Moura *et al.* (2007)	USA	Observational	*Bos taurus*	12	AV and RF	Fractioned SP (AGF)	2D	MS (ESI-qTOF)
Gonçalves *et al*. (2008a)	USA	Interventional	*Bos taurus*	6	AV	Purified OPN	1D and C	WB
Gonçalves *et al.* (2008b)	USA	Interventional	*Bos indicus*	1	Unk	Anti-PTGDS	NA	NA
Cheema *et al.* (2008)	India	Observational	Crossbred	14	AV	Whole SP	1D	NA
Alghamdi *et al.* (2009)	USA	Interventional	*Bos taurus*	5	EJ	Whole SP	NA	NA
Monaco *et al.* (2009)	USA	Interventional	*Bos taurus*	3	Unk	Purified OPN	1D and C	WB
Alghamdi *et al.* (2010)	USA	Interventional	*Bos taurus*	5	EJ	Whole SP	NA	NA
Li *et al*. (2010)	China	Interventional	*Bos taurus*	3	Unk	Recombinant NGF	1D	WB and ELISA
Miah *et al*. (2011)	Germany	Interventional	*Bos taurus*	4	AV	Recombinant RLN	1D	WB
D'Amours *et al.* (2012)	Canada	Observational	*Bos taurus*	9	Unk	Recombinant BSP1 and ELSPbP1	C	WB
Dalton *et al.* (2012)	Brazil	Observational	*Bos indicus*	6	AV	Whole SP	NA	NA

Srivastava *et al.* (2012)	India	Interventional	Crossbred	6	AV	Purified BSP1	1D and C	WB and ELISA
Stinshoff *et al.* (2012)	Germany	Interventional	Unk	1	Unk	Whole SP	NA	NA
Alvarez-Gallardo *et al.* (2013)	Mexico	Interventional	*Bos indicus*	1	AV	Recombinant TIMP2 and FAA	NA	NA
Assumpção *et al.* (2013)	Brazil	Observational	*Bos indicus*	20	EJ	Whole SP	NA	NA
Roncoletta *et al.* (2013)	Brazil	Observational	*Bos taurus* and *Bos indicus*	20	AV	Whole SP	2D	NA
Rueda *et al.* (2013a)	Colombia	Interventional	*Bos taurus* and *Bos indicus*	20	EJ	Fractioned SP	C and 2D	NA
Rueda *et al.* (2013b)	Colombia	Interventional	Crossbred	20	EJ	Fractioned SP	C, 1D, and 2D	NA
Srivastava *et al.* (2013)	India	Interventional	Crossbred	6	AV	Purified BSP1	C	WB and ELISA
Aslam *et al.* (2014)	India	Observational	Crossbred	6	AV	Whole SP	2D	MS (MALDI-TOF)
Almadaly *et al.* (2015)	Japan	Interventional	*Bos taurus*	9	AV	Whole SP	C and 2D	NA
Karunakaran & Devanathan (2015)	India	Interventional	Unk	22	AV	Fractioned HBP 28-30 kDa	1D	NA
Krishnan *et al.* (2015)	India	Observational	*Bos taurus*	17	AV	Fractioned SP	C and 1D	NA
Patel *et al.* (2015)	India	Interventional	Crossbred	6	AV	Fractioned HBPs	C	NA
Fernandez-Fuertes *et al*. (2016)	Ireland	Interventional	Unk	3	Minced epididymis	Anti-DEFB126	1D	WB
Krishnan *et al.* (2016a)	India	Observational	*Bos taurus*	17	AV	Fractioned SP	C and 1D	NA
Krishnan *et al.* (2016b)	India	Observational	*Bos taurus*	17	AV	Fractioned SP	C and 1D	NA
Magalhães *et al.* (2016)	Brazil	Observational	*Bos indicus*	9	AV	Whole SP	1D and 2D	MS (MALDI-TOF/TOF)
Montanholi *et al.* (2016)	Canada	Observational	*Bos taurus*	31	EJ	Whole SP	C	MS (LTQ-Orbitrap Hibrid)

Patel *et al.* (2016)	India	Interventional	Crossbred	6	AV	Purified 31kDa HBP	C and 1D	MS (MALDI-TOF)
Rego *et al*. (2016)	Brazil	Observational	*Bos indicus*	13	EJ	Whole SP	2D	MS (ESI-qTOF)
Rodríguez-Villamil et al. (2016)	Brazil	Interventional	*Bos taurus* and *Bos indicus*	7	EJ	Purified BSPs	C and 1D	LC-MS/MS, N-terminal, and WB
Vickram *et al.* (2016)	India	Observational	Crossbred	20	AV	Whole SP	1D	NA
Ferreira *et al.* (2017)	Brazil	Observational	*Bos taurus*	110	EJ	Whole SP	NA	NA
Menezes *et al.* (2017)	USA	Observational	*Bos taurus*	10	EJ	Whole SP	2D	LC-MS/MS
Pero *et al.* (2017)	Italy	Observational	Unk	8	Unk	Whole SP	NA	NA
Lyons *et al.* (2018)	Ireland	Interventional	*Bos taurus*	6	Minced epididymis and RF	Recombinant DEFB126	NA	NA
Pande *et al.* (2018)	India	Observational	Crossbred	36	AV	Fractioned SP	1D	NA
Sood *et al.* (2018)	India	Observational	*Bos taurus*	5	AV	Whole SP	1D	NA
Stewart *et al.* (2018)	USA	Observational	*Bos taurus* and *Bos indicus*	145	AV	Whole SP	NA	ELISA
Viana *et al*. (2018)	Brazil	Observational	*Bos taurus*	10	Unk	Whole SP	C	MS (Orbitrap Elite)
Kasimanickam *et al.* (2019)	USA	Observational	*Bos taurus*	8	AV	Whole SP	2D	MS (ESI-qTOF)
Stewart *et al.* (2019)	USA	Interventional	Crossbred	10	EJ and RF	Purified NGFB	C	LC-MS/MS
Gomes *et al*. (2020)	USA	Observational	*Bos taurus*	14	AV	Whole SP	C	LC-MS (MudPit)
Rodríguez-Villamil et al. (2020)	Brazil	Interventional	Unk	10	AV	Purified BSP1	C and 1D	LC-MS/MS, N-terminal, and WB

1D, one-dimensional electrophoresis; 2D, two-dimensional electrophoresis; AGF, accessory gland fluid; aSFP, acid seminal fluid protein; AV, artificial vagina; BSP, binder of sperm protein; C, chromatography; CapLC, Capillary liquid chromatography; CEF, caudal epididymal fluid; DEFB126, beta defensin 126; EJ, electroejaculation; ELISA, Enzyme-Linked Immunosorbent Assay; ELSPbP1, epididymal sperm binding protein 1; ESI, electrospray ionization; FAA, fertility-associated antigen; HBP, heparin binding protein; IGF, insulin-like growth factor; ITG, integrin; kDa, kilodalton; LC, liquid chromatography; MALDI, Matrix-assisted laser desorption / ionization; MS, mass spectrometry; MS/MS, tandem liquid chromatography; NA, Not applied; NGF, nerve growth factor; OPN, osteopontin; PSAP, prosaposin; qTOF, quadrupole Time of Flight; RF, retrograde flushing; RIA, radioimmunoassay; RLN, relaxin; SP, seminal plasma; TIMP2, metalloproteinase inhibitor 2; TOF, Time of Flight; Unk, unknown; WB, western blotting.

3. CHAPTER 2

Contributions of seminal plasma proteins to fertilizing ability of bull sperm: A meta-analytical review

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Contributions of seminal plasma proteins to fertilizing ability of bull sperm: A meta-analytical review

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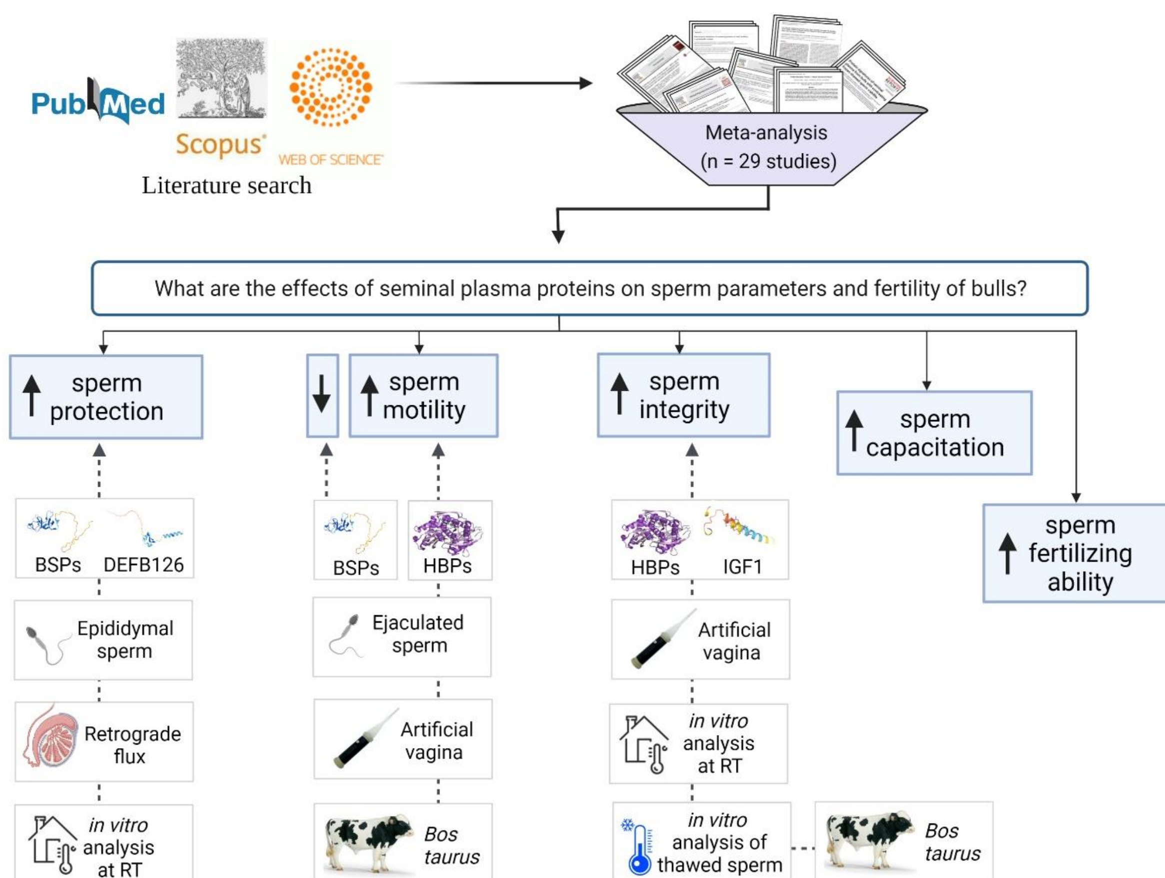
Short title: The effects of seminal proteins on bull spermatozoa



ABSTRACT

Seminal plasma is a dynamic, intricate combination of fluids from the testicles, epididymides, seminal vesicles, bulbourethral glands, and prostate, containing molecules that modulate sperm functions, post-fertilization events, and the female reproductive tract physiology. Significant variations in sperm parameters and fertility status of bulls relate to differences in the seminal plasma proteome. In this framework, a meta-analytical study was conducted examining 29 studies (published between 1990 and 2021) to ascertain the impact of seminal proteins on bull fertility and the influence of distinct methodologies on such effects. Our results revealed that seminal proteins ameliorate sperm parameters, such as motility, integrity, capacitation, and fertilizing ability, and favors sperm protection. Seminal binder of sperm proteins and beta-defensin 126 highly favored sperm protection when cells were collected from the epididymis by retrograde flux and analyzed under room temperature conditions. Furthermore, seminal proteins improved the motility and quality of *Bos taurus* sperm collected by artificial vagina, mainly in the presence of heparin-binding proteins. The key limitations faced by this study were the paucity of studies evaluating the effects of whole seminal fluid proteins and the limited number of studies conducted *in vivo*. In conclusion, the present meta-analytical study confirms that seminal proteins improve fertility-related parameters in the bovine species. However, methodological strategies used by authors are diverse, with distinct endpoints and methods. Thus, the translational aspects of seminal plasma research should be taken into consideration to precisely define how seminal proteins can be harnessed to advance reproductive biotechnology.

Keywords: Bovine, semen, proteome, male reproduction, meta-analysis.

INTRODUCTION

Significant efforts have been made to uncover the relationships between semen analysis and bull fertility. The fertilizing capacity of a male is influenced by sexual behavior, endocrinology, genetics, and sperm intrinsic and extrinsic factors (Kastelic & Thundathil, 2008; Butler et al., 2019; Rodriguez-Martinez, 2021). While intrinsic factors include sperm features, such as morphology, motility, concentration, and membrane integrity, extrinsic factors are related to non-cellular components of semen capable of modulating sperm functional attributes (Evans et al., 2020; Özbek et al., 2021; Viana et al., 2021). Seminal plasma is one of these extrinsic factors. It consists of combination of fluids from male reproductive organs containing proteins, amino acids, lipids, carbohydrates, organic acids, hormones, and ions (Juyena & Stelletta, 2012). Proteins from seminal fluid regulate vital biological events during the male gamete's journey throughout uterus and oviduct, including sperm protection, metabolism, capacitation, acrosome reaction, endometrial gene expression, sperm-oviduct and sperm-oocyte interactions, and fertilization (Gonçalves et al., 2008; Plante et al., 2016; Rodriguez-Villamil *et al.*, 2016; Suarez, 2016; Camargo et al., 2018; Moura et al., 2018). Collectively, those events are essential for male reproductive efficiency, early embryo development, and beyond.

Significant variations in fertility scores, sperm parameters, and sperm freezability of bulls relate to dissimilarities in the proteome of seminal fluid (Killian et al., 1993; Fouchercourt et al., 2002; Jobim et al., 2004; Moura et al., 2006; 2007; Rego et al., 2016; Viana et al., 2018; Kasimanickan et al., 2019; Gomes et al., 2020; Willforss et al., 2021). However, our recent systematic review found substantial asymmetry among the results published in those studies due to variations in methods of protein analysis, bovine breeds, semen collection, sperm endpoints, and sample storage conditions (Viana et al., 2021). Moreover, proteins associated with fertility parameters were tested in cause-and-effect experiments, which helped to confirm (or not) the empirical link detected between seminal protein and bull fertility (Viana et al., 2021).

The association between fertility performance and seminal proteins is still controversial in the bovine species. Several studies have clearly shown that incubation of sperm with a specific seminal protein, a group of proteins (e.g., heparin binding proteins [HBPs]), or even with the whole seminal plasma protein (WSP) content affects sperm physiology (for review, see Viana et al., 2021). However, the molecular and cellular underpinnings of the specific contributions of seminal proteins to fertility are still unclear. For example, binder of sperm

proteins (BSPs), secreted by the accessory sex glands in the bovine species, act as chaperones by binding to the spermatozoa membrane, favoring the maintenance of its integrity, the sperm-oviduct interaction, and capacitation (Thérien et al., 2001; Gwathmey et al., 2006; Rodriguez-Villamil et al., 2016; Suarez, 2016; Singh et al., 2019). Other proteins, such as osteopontin (OPN) and beta-defensin 126 (DEFB126), play roles in fertilization as well as in early embryogenesis (Moura, 2005; Gonçalves et al., 2008; Lyons et al., 2018; Bustamante-Filho et al., 2021).

Considering the complexity of protein composition of seminal plasma and the diverse methodological strategies used by authors to ascertain this subject, it remains a challenge to explain and precisely quantify the effects of each seminal protein on sperm parameters and male fertility. Thus, here, a meta-analytical approach was applied to find out the magnitude of the effects of seminal fluid proteins on parameters associated with bull fertility. Also, we evaluated the impact of different methodological moderators, such as bovine breeds, semen collection, sperm source, sample conditioning, and seminal proteins on such effects. The outcomes reveal the potential contributions of seminal proteins on bull sperm fertility.

METHODS

Search strategy

This study was designed according to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA; Moher et al., 2015). The central question we pursued was: What are the effects of seminal plasma proteins on sperm parameters and fertility of bulls? The search and selection of the eligible studies were performed according to Viana et al. (2021). Briefly, three databases (Web of Science, Scopus, and PubMed) were chosen for an electronic search, using the words “bull” or “bovine” or “Bos taurus” and “seminal plasma” or “seminal fluid” and “fertility” or “cryopreservation”. The review included studies published between 1990 and September 28th, 2021, a period coincident with the significant development of mass spectrometry-based techniques, as explained in Viana et al. (2021). The search also included the reference lists cited in review studies.

Studies reviewed

Studies had to meet some criteria for inclusion in the current meta-analytical review: (1) used bovine as the animal model; (2) were full-text articles and not review papers; (3) used

seminal plasma; (4) evaluated seminal plasma protein(s); (5) be related to bull fertility; and (6) stated means, sample sizes, and variance measures for both the control and treatment groups. Finally, we excluded studies in which the function of seminal plasma proteins was evaluated indirectly by using antibodies or other sequestrating agents. For the meta-analysis, mean values (Xcontrol and Xtreatment), standard deviations (Scontrol and Streatment), and sample sizes (Ncontrol and Ntreatment) were obtained from each study. ImageJ software was used to assess the averages and statistical dispersion of the data available in figures.

An independent random-effects model of the meta-analysis was performed for each of the six distinct variables: (1) sperm protection; (2) sperm motility; (3) sperm integrity; (4) sperm damage; (5) sperm capacitation; and (6) sperm fertilizing ability (Table 1). We settled these variables regarding the data obtained from all studies, and, frequently, the same study contributed to data from more than one variable. However, to be included, it must contain at least five independent comparisons of each variable. Moreover, the mixed-effects model of the meta-analysis categorized the studies in accordance with the five methodological moderators: bovine breeds (Bos taurus taurus, Bos taurus indicus, and crossbred), sample collection (artificial vagina, electroejaculation, and retrograde flushing), sample source (epididymal and ejaculated sperm), sperm sample conditioning (fresh and frozen-thawed sperm) during protein incubation and sperm analysis, and the proteins studied (WSP, HBPs, BSPs, OPN, relaxin [RLX], insulin-like growth factor [IGF], nerve growth factor [NGF], DEFB126, and prosaposin [PSAP]).

Table 1. Analyzed variables based on outcomes related to bovine sperm fertility.

Variables	Parameters
Sperm protection	Sperm-neutrophil binding (%); sperm binding to oviductal epithelial cells (sperm/1 mm^2); sperm agglutination (%).
Sperm motility	Motile sperm (%); progressive/general/total motility (%); straight-line velocity (μm/s); number of sperm penetrated in the mucosa; sperm penetration distance (mm).
Sperm integrity	Sperm concentration (10^8/ml); intact acrosome (%); normal morphology (%); membrane integrity (%); intact DNA (%); viable sperm cells (%); insulin secretion (μU/ml); leptin secretion (ng/ml); mitochondrial activity/potential (%); hypo-osmotic swelling test (%); SOD activity (%); F-actin (%).
Sperm damage	Sperm abnormality (%); loss of mitochondrial membrane potential (%); malondialdehyde level (μmol/ml and μM/10^9 sperm); apoptotic sperm cells (%); DNA fragmentation (%); lipid peroxidation (%);

Sperm capacitation	Acrosome reaction (%); acrosome damage (%); capacitation (%); calcium level (fluorescence intensity); cholesterol (ng/10^6 sperm); cyclic adenosine monophosphate (fmol/10^6 sperm).
Sperm fertilizing ability	Total/normal/polyspermy fertility (%); sperm bind to zona pellucida (sperm/ZP); cleavage (%); blastocyst (%); Day 8 embryo (%); pregnancy (%).

Meta-analysis

The magnitude of the effects (d) of seminal plasma proteins on bull sperm parameters and fertility was calculated using the standardized difference between control and treatment groups. For each study, the equation $d = (Xt - Xc / SD) * J$ was applied, where Xt is the treatment group response, Xc is the control group response, SD is the pooled standard deviation, and J is an adjustment term to delete the bias of the small sample sizes (Rosenberg et al., 2000). For sample sizes equal to or larger than 25, J reaches 1.0. Then, the cumulative effect (d++) for each of the variables explored was assessed applying a mixed-effects model to analyze the methodological moderators. This model assumes that studies within a class share a common mean effect, but there are also random variations beyond the sampling variation (Carvalho et al., 2020; Machado-Neves, 2021). Inferior and superior CIs were calculated according to the average cumulative effects, and intervals that did not overlap with zero, with n-1 degrees of freedom (df), were considered significant. According to Cohen et al. (1992), d++ is classified as weak, moderate, and strong when its values are around 0.2, 0.5, and 0.8, respectively. In addition, positive d++ values indicate that seminal proteins have positive effects on the variables measured. Negative values, in turn, indicate a negative effect on variables.

Finally, heterogeneity analyses (Q statistic) were employed to test whether categorical groups in mixed models were homogeneous to calculate the effect sizes. The total heterogeneity (QT) was calculated for all variables tested, as well as heterogeneity within (QW) and between groups (QB). The significance of these statistical methods was evaluated using Chi-square distribution with n-1 df. Rosenthal's fail-safe number was calculated for each effect tested to avoid publication bias and file-drawer problem (Rosenberg et al., 2000). Rosenthal's fail-safe number indicates the number of missing studies to change the results from significant to non-significant. We assessed a robust outcome when the fail-safe number exceeded 5k + 10 (k is the number of comparisons in the analysis (Møller & Jennions, 2001). MetaWin 2.1 and GraphPad Prism 9.0.2 software were used to analyze and build the graphics, respectively (Rosenberg et al., 2000).

Risk of bias

The risk of bias analysis was performed to assess the transparency and accuracy of the included studies to report methodologies and results. To this end, we followed the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines 2.0 with modifications (du Sert et al., 2020). The ARRIVE 2.0 includes 21 items in 38 questions about the reported abstract, objectives, study design, statistical methods, ethical statement, data access, and declaration of interest. Also, we included questions about sample size, animals' inclusion criteria, randomization, management, and experimental procedures, and evaluated the quality report on outcome measures, results' presentation, interpretation, and scientific implications. We did not include questions related to primary outcome measurements, effect size, confidence interval, animal model, animal interventions, human health relationship, protocol registration, and adverse event reporting.

RESULTS

Selected studies and qualitative data

The primary literature search resulted in 1,179 studies through database searches and reference lists of eligible studies (Figure 1; Supplementary Table 1 [ST1]). After removing duplicates and exclusions, 29 studies were eligible for this meta-analytical review, which generated 176 independent comparisons for the effects of seminal plasma proteins on sperm parameters and bull fertility (Figure 1). Qualitative aspects of the methodological strategies used in the eligible studies are shown in Figure 2 and ST1. Briefly, Bos taurus was the main animal model used to assess the effects of seminal proteins on fertility parameters (Figure 2a), and artificial vagina was the preferred method of sample collection (Figure 2b). Ejaculated sperm were used in most of the selected studies in contrast to those studies that used only epididymal sperm or both ejaculated and epididymal sperm (Figure 2c). Sperm storage conditions, including fresh sperm, frozen-thawed sperm, and any combination of these protocols, differed among studies (Figure 2d). HBPs were the most common type of seminal proteins used in the studies evaluated in the present meta-analysis, followed by WSP and BSPs. IGF and RLX were analyzed in three studies each, whereas OPN, DEFB126, and NGF were analyzed in two studies. One study evaluated the effect of PSAP on bull fertility (Figure 2e).

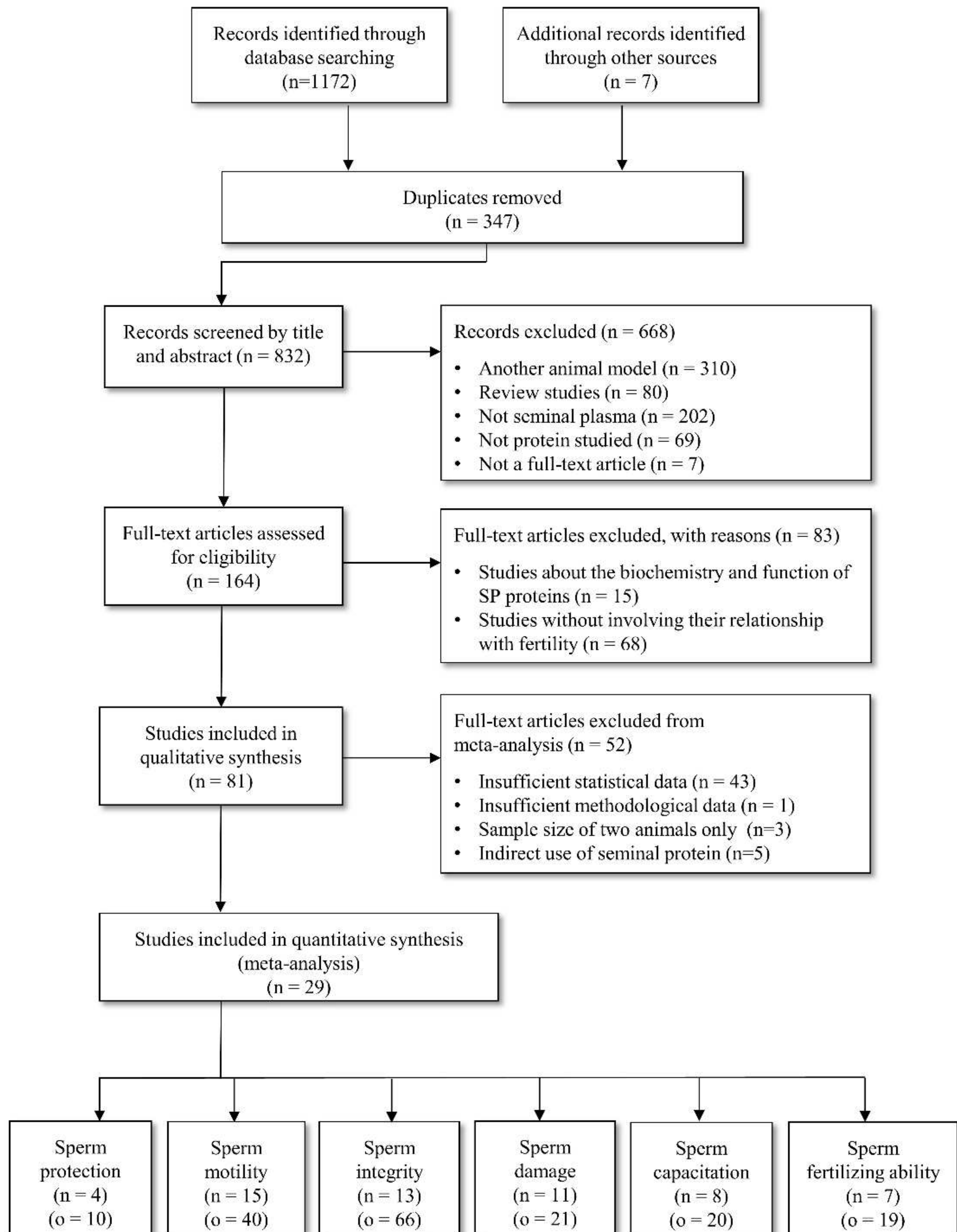


Figure 1. Flow chart of search results according to PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analysis). SP = seminal plasma; n = number of studies; o = number of independent comparisons extracted from the studies.

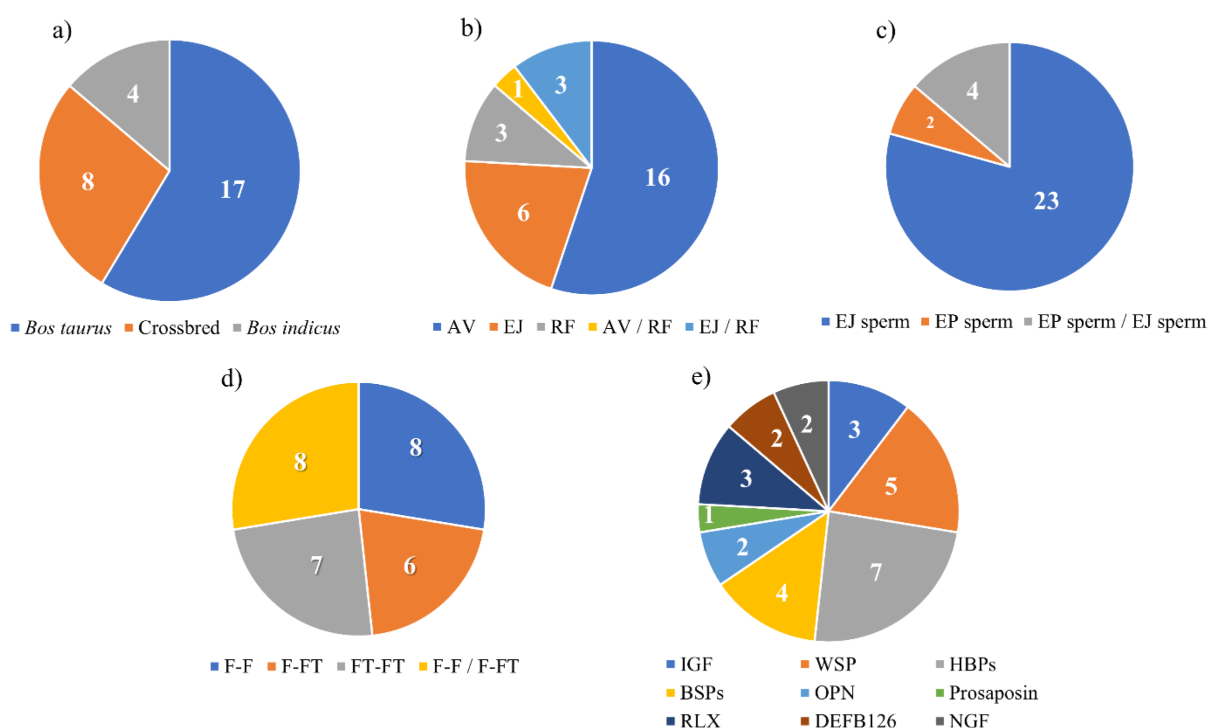


Figure 2. Methodological strategies related to the 29 eligible studies used in this meta-analysis. a) bovine breeds: *Bos indicus*, *Bos taurus*, and crossbred sires; b) sample collection: artificial vagina (AV), electroejaculation (EJ), and retrograde flushing (RF); c) sample type: epididymal (EP sperm) and ejaculated sperm (EJ sperm); d) sperm condition at protein incubation and at analysis: fresh and fresh (F-F), frozen-thawed and frozen-thawed (FT-FT), fresh and frozen-thawed (F-FT), and those studies evaluating both F-F and F-FT; e) seminal protein studied: insulin-like growth factor (IGF), whole seminal plasma proteins (WSP), heparin-binding proteins (HBPs), binder of sperm proteins (BSPs), osteopontin (OPN), prosaposin (PSAP), relaxin (RLX), beta defensin 126 (DEFB126), and nerve growth factor (NGF). White numbers indicate the number of studies for each methodological parameter analyzed.

Meta-analysis

Our results revealed that seminal plasma proteins elicited strong positive effects on sperm protection, motility, integrity, capacitation, and fertilizing ability (Figure 3). Moreover, seminal plasma proteins showed a strong effect on sperm damage, diminishing the occurrence of sperm injuries (Figure 3). Rosenthal's fail-safe numbers for the effects of seminal proteins on sperm motility (684.5), sperm integrity (2,227), sperm damage (227.7), and sperm capacitation (116.6) were remarkably high, indicating the robustness of the analysis. On the other hand, Rosenthal's fail-safe numbers for the effects of seminal plasma proteins on sperm protection (55.8) and sperm fertilizing ability (86.4) were low, indicating that there is still a demand for additional studies to ensure the accuracy of the effects without bias.

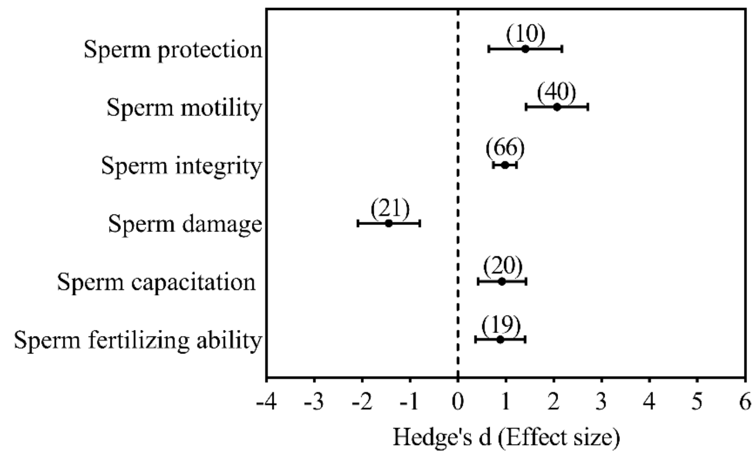


Figure 3. Effects of seminal plasma proteins on variables associated with bull fertility. The cumulative effect sizes (95% confidence interval) are significant when the confidence intervals do not overlap with zero. (n) = number of independent comparisons.

Based on the mixed-effects model, the positive effects of seminal proteins on sperm protection were significant in the case of epididymal sperm collected by retrograde flux and analyzed under fresh conditions. In vitro incubation with BSPs and DEFB126 influenced the protective effects of seminal plasma on sperm rather than incubation with WSP (Figure 4a). Bull seminal proteins had a positive effect on motility when spermatozoa were collected using artificial vagina from Bos taurus sires and incubated with HBPs (Figure 4b). Proteins of seminal plasma contributed to the integrity of sperm membrane in Bos taurus and when semen was collected using artificial vagina. The benefit of interactions between sperm and seminal proteins was reached when fresh-fresh and fresh-frozen sperm were incubated with HBPs and IGF1 in vitro (Figure 4c). Finally, seminal proteins reduced sperm damage when Bos taurus semen was incubated with HBPs (Figure 4d). Variables defined as sperm capacitation and sperm fertilizing ability were not analyzed using a mixed-effects model due to an insufficient number of independent comparisons in each category. The detailed result of the mixed-effects model can be found in (Supplementary Table 2 [ST2]).

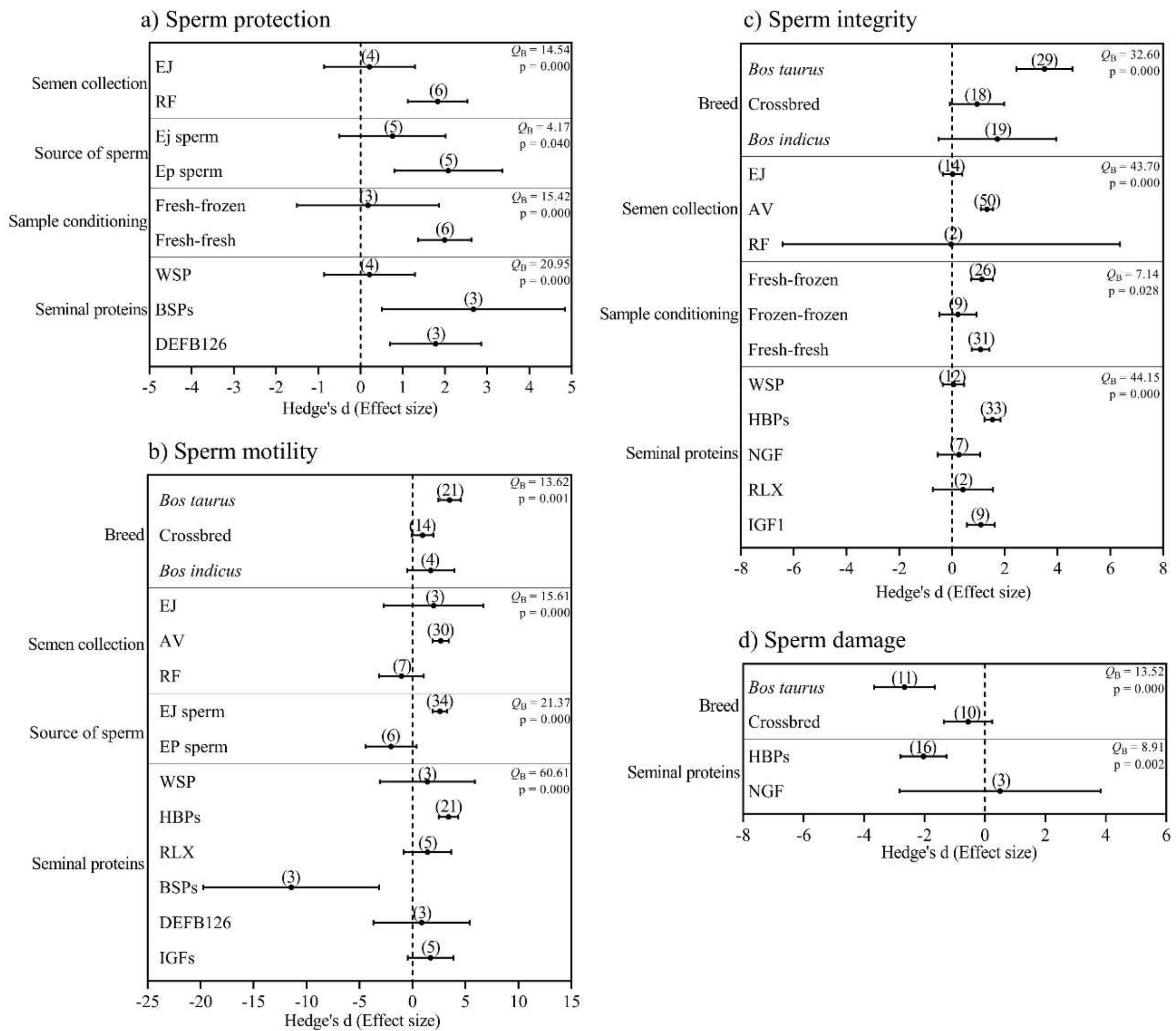


Figure 4. Effects of bovine breeds, semen collection, sperm source, sample conditioning, and seminal proteins on a) sperm protection; b) sperm motility; c) sperm integrity; and d) sperm damage. The cumulative effect size is reported for each effect (95% confidence intervals), and effects are significant when confidence intervals do not overlap with zero. (n) = number of independent comparisons for each category. Q_B indicates heterogeneity between groups. AV: artificial vagina; BSPs: binder of sperm proteins; DEFB126: beta-defensin 126; EJ: electroejaculation: EJ sperm: ejaculated sperm; EP sperm: epididymal sperm; HBPs: heparin binding proteins; IGF: insulin-like growth factor; NGF: nerve growth factor; RF: retrograde flushing; RLX: relaxin; WSP: whole seminal proteins.

Risk of bias

The 29 studies included in this meta-analysis reported their study design, experimental unit, sample size, outcome measurements, descriptive results, and abstract (Fig. 5; Supplementary Table 3 [ST3]). None of the authors reported how the sample size was determined and if there was an exclusion of animals or experimental units and strategies to minimize confounders. Moreover, it is not clear whether the authors used blind experiments on the researchers and the available data. Only 60% (n = 17) of the studies used semen parameters or bull fertility status as criteria to include the animal in the study and only one study reported

the randomization to allocate the units (Fig. 5; Supplementary Table 3 [ST3]). Most studies provided information about the statistical software used, and the reasons for choosing the experimental procedures, except for reporting time, frequency, and local. Only around half of the studies reported species-appropriate details of the animals used, housing, and husbandry conditions. However, few studies (n = 3) reported the animals' health status. Ethical statements and declarations of conflict of interest were reported by nine studies and funding sources, by 12. Although the interpretation and scientific implications were properly reported by the majority of the studies, the review limitations and potential sources of bias were reported only in three studies.

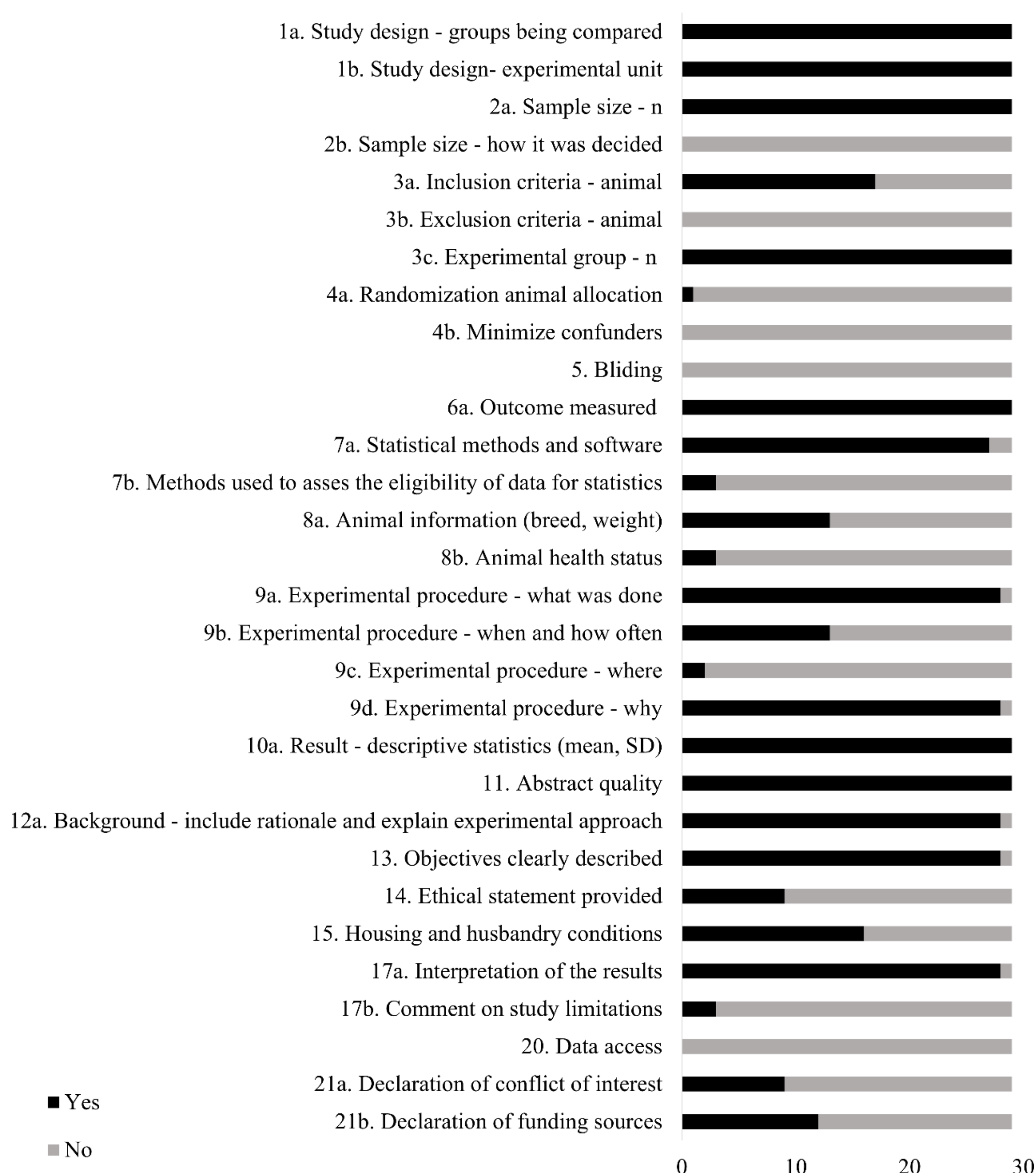


Figure 5. Bar chart displaying the frequencies of the options ‘Yes’ and ‘No’ among the 29 eligible studies included in the meta-analysis.

DISCUSSION

This meta-analysis evidenced that seminal plasma proteins have meaningful effects on fertility parameters in the bovine species. The seminal proteins mediated strong positive responses on sperm protection, motility, quality, capacitation, and fertilizing ability. Moreover, we demonstrate that methodological strategies influenced the outcomes of studies proposed to measure seminal protein effects on sperm and fertility parameters. Our findings quantified the effects of seminal proteins on bull sperm and fertility, complementing the qualitative information previously reunited in our systematic review describing the function of seminal fluid proteins and their potential association with sperm parameters and fertility in bulls (Viana et al., 2021). In this sense, we expect that our findings and inference will contribute to the perception of seminal plasma proteins as players in physiological processes defining sperm fecundity. Over the last decades, scientists have made remarkable contributions to the understanding of seminal plasma protein functions and their empirical associations with bull fertility (Killian et al., 1993; Moura et al., 2006; 2007; Gonçalves et al., 2008; Alvarez-Gallardo et al., 2013; Plante et al., 2016; Rodriguez-Villamil et al., 2016; 2020; Viana et al., 2018). However, the major challenge faced by researchers is the precise identification of seminal plasma-based markers of fertility, which may improve the outcomes of assisted reproductive technologies.

The effects of seminal plasma proteins on sperm protection

This review reinforced the role of seminal proteins to elicit a positive response in sperm protection. This effect depends on the methodological strategies used by the authors. Results from the mixed-effects model showed that only epididymal sperm collected via retrograde flushing were positively affected by seminal proteins. The explanation for this finding may be related to the lack of interaction(s) between epididymal sperm and seminal proteins secreted only by the accessory sex glands. This interaction will occur during ejaculation when epididymal sperm come in contact for the first time with proteins exclusively secreted by accessory sex glands. Thus, epididymal sperm will be more responsive to the action of proteins from these glands and, in fact, Rodriguez-Villamil et al. (2016; 2020) described the low responsiveness of ejaculated spermatozoa incubated with BSP proteins when compared with

epididymal sperm. According to our concept, ejaculated sperm already in contact with accessory sex glands proteins may become non-responsive to new stimuli by the same proteins when incubated with the seminal components in vitro.

Seminal proteins provided better protection for fresh sperm incubated in vitro at room temperature than for frozen-thawed cells. This outcome may be attributed to the maintenance of membrane integrity in sperm manipulated at room temperature in contrast to frozen cells. In addition to the formation of ice crystals during cryopreservation, cold shock and osmotic stress also cause injuries to the sperm membranes (Upadhyay et al., 2021). Accordingly, alterations in lipid membrane organization, fluidity, permeability, and composition during cryopreservation lead to the failure of kinetic parameters, as well as sperm mitochondrial and acrosomal functions (Bailey et al., 2000). The misconfiguration of lipids disrupts the interaction between sperm membrane and proteins, which interfere with the action(s) of seminal proteins on cryopreserved sperm cells (Leahy & de Graaf, 2012). Cryoinjuries in sperm membranes may be reduced by adding cholesterol, low-density lipoproteins, and alternative sources of phospholipids to extenders rather than proteins (Neves et al., 2014; Silva et al., 2014; Ondřej et al., 2019). Authors postulated that the seminal plasma contains potential biomarkers of sperm freezability, such as clusterin, acidic seminal fluid protein, OPN, BSP1, BSP5, deoxyribonuclease 1 like 3, and seminal RNase (Jobim et al., 2004; Magalhães et al., 2016; Rego et al., 2016; Gomes et al., 2020). Despite this empirical evidence, the results of our meta-analysis failed to confirm that seminal proteins have beneficial effects on frozen-thawed sperm in bulls.

This review evidenced that incubation of spermatozoa with BSP1, BSP3, and BSP5 improved sperm protection. Overall, BSPs are the most abundant protein group of the bovine seminal fluid, comprising approximately 23% of the entire seminal proteome (Gomes et al., 2020). BSP1 is the major BSP in the seminal plasma of bulls, whereas BSP3 and BSP5 are present at low abundance. BSPs exhibit chaperone-like activity, being capable of interacting with and stabilizing another protein to either acquire its functionally active conformation or neutralize its activity. In this case, seminal proteins exerting sperm-protecting roles, such as BSPs, minimize oxidative stress and immune attacks of proteins from the uterus and oviduct (Calvete et al., 2010; Min et al., 2013; Singh et al., 2019). BSP1 binds to sperm at ejaculation (Souza et al., 2008) and promotes cholesterol efflux from the membrane, acting on sperm capacitation and fertilization (Thérien et al., 1998; Rodriguez-Villamil et al., 2016; 2020). BSPs

also intervene the sperm-oviduct interactions, contribute to the formation of sperm reservoir, and, thus, increase the life span of gametes in the oviduct (Suarez, 2016).

Unfortunately, none of the studies eligible for the current meta-analysis has evaluated the effects of seminal proteins with specific antioxidant properties. Mammalian seminal plasma has a vast number of components that act specifically for the protection of sperm from oxidative stress (Alkan et al., 1997; Tavilani et al., 2008; Barranco et al., 2015). These enzymes are crucial to protect sperm against free radicals and oxidants known as reactive oxygen species (ROS), which disturb the structures of lipids, proteins, and DNA (Aitken and Drevet, 2020; Tiwari et al., 2022). On the other hand, authors have incubated spermatozoa with DEFB126, which is mainly found in the cauda epididymis and prevents sperm agglutination (Lyons et al., 2018). Like BSPs, DEFB126 mediates the formation of the sperm reservoir by promoting the interactions between sperm and the oviduct epithelia (Gwathmey et al., 2006; Lyons et al., 2018). Assessment of sperm binding to oviduct epithelium is essential because only fertile sperm can form a sperm reservoir (Coy et al., 2012; Holt & Frazeli, 2015).

The effects of seminal plasma proteins on sperm motility

Our findings indicated that seminal plasma proteins ameliorate the motility of bull sperm. This effect is relevant since motility is required for the gamete to complete its journey to the fertilization site in vivo (Suarez, 1996). The current study also showed that motility is ameliorated by seminal plasma proteins only in studies using Bos taurus sperm. The paucity of studies evaluating the effects of seminal proteins on Bos indicus sperm motility may have influenced this result. Nevertheless, it remains to be determined whether the motility and physiology of Bos taurus and Bos indicus sperm are meaningfully different. One study has documented differences in the structure of flagella and energy supply after comparing sperm proteomes of Bos indicus and crossbred bulls, which may contribute to differences in sperm motility (Ashrafzadeh et al., 2013).

Herein, sperm samples collected via artificial vagina had better motility after interaction with seminal proteins. Studies report that semen collection by electroejaculation modifies the proteome of bovine seminal plasma (Rego et al., 2015), which probably explains why studies using artificial vagina and other methods of semen collection elicit different outcomes. At ejaculation, seminal proteins bind to sperm and activate signaling pathways for sperm motility and capacitation (Miah et al., 2011). Additionally, we observed that in vitro incubation of ejaculated sperm with seminal proteins improved motility when compared to epididymal sperm,

an effect intensified in the presence of HBPs (Karunakaran & Devanathan, 2015; Krishnan et al., 2015; Patel et al., 2015; Patel et al., 2016; Krishnan et al., 2016; Pande et al., 2018). Heparin-binding proteins are present in the seminal plasma of many species and are multifunctional, acting in acrosome reaction (Singh et al., 2016) and as chaperones (Sankhala et al., 2012). From the seven studies included in our meta-analysis that evaluated seminal HBPs, four analyzed the effect a 28-31 kDa HBP identified as either DNAse-I-like protein or fertility-associated antigen (FAA) (McCauley et al., 1999). Sperm membrane FAA has been related to bull fertility owing to its role in sperm motility, capacitation, and protection against oxidative stress (Ax, 2004; Karunakaran & Devanathan, 2015).

The effects of seminal plasma proteins on sperm integrity and damage

The current meta-analysis indicated that seminal proteins mitigate sperm damage and preserve sperm integrity. This finding may be explained by the protective effects of seminal proteins on the integrity and morphology of sperm organelles (mitochondria and acrosome), DNA, and membranes, which contribute to the inactivation of the apoptotic cascade (Karunakaran & Devanathan, 2015; Robaire & Hinton, 2015; Robles et al., 2017). Typically, seminal proteins directly bind to the sperm membrane, preserving plasma and acrosome membranes and promoting cell stability (Gwathmey et al., 2006; Singh et al., 2019; Zoca et al., 2021). According to the eligible studies included in this review, HBPs and IGF1 exhibited the most pronounced effects on sperm integrity in contrast to the other seminal proteins. During ejaculation, sperm interact with seminal HBPs, as well as with heparin and glycosaminoglycans present in the uterus. These molecules modulate sperm capacitation (Karunakaran & Devanathan, 2015), protect sperm against lipid peroxidation (Patel et al., 2015), and stabilize the acrosome membrane (Alvarez-Gallardo et al., 2013; Pande et al., 2018), maintaining the integrity of sperm structure. In addition, IGFs improve metabolic conditions and changes in fresh and thawed sperm (Costa et al., 2020; Singh et al., 2020; Susilowat et al., 2021). The IGF-I binds to its receptors on the bovine spermatozoa membrane and maintains its motility through energy metabolism (Susilowat et al., 2021). IGF1 increases glucose uptake (Stewart & Rotwein, 1996), maintaining insulin levels, preventing the sperm from mobilizing their reserves, and maintaining membrane stability. Our meta-analysis also pointed out that the breed of bulls influenced the outcomes of studies that evaluated the effects of seminal plasma proteins on sperm integrity. Thus, further investigation is needed to precisely assess the biotasks of seminal plasma components from Bos indicus bulls and their crosses.

The effects of seminal plasma proteins on sperm capacitation

This meta-analytical study evidenced the significance of the seminal plasma molecules in promoting sperm capacitation. BSPs are the leading seminal proteins coating bovine sperm at ejaculation, causing cholesterol efflux and membrane reorganization. This mechanism avoids the free movement of phospholipids and stabilizes the membranes of sperm while the cells travel through the uterus and oviduct (Greube et al., 2001; Thérien et al., 2001). In the oviduct, BSP-bound sperm interact with oviductal fluid components, such as lipoproteins and glycosaminoglycans, and lose more cholesterol from their surface membranes as part of the capacitation process (Cross, 1998; Souza et al., 2008). The continuous sperm exposure to BSPs during cryopreservation may cause early capacitation and damage sperm (Manjunath & Thérien, 2002). To overcome this issue, authors have suggested that BSP sequestering agents, such as milk proteins, delay sperm capacitation in vitro, increasing sperm longevity (Bergeron et al., 2004; Menezes et al., 2016). Thus, when ascertaining the positive effects of seminal proteins on sperm capacitation, authors should consider that BSPs have either beneficial or detrimental effects on the timing of capacitating sperm.

The effects of seminal plasma proteins on sperm fertilizing ability

This meta-analytical study evidenced that seminal proteins exert a positive effect on bull fertility, even with the pronounced variations in methodologies observed in the selected studies. In fact, better sperm motility, quality, capacitation, protection, and sperm-oocyte interaction tends to improve fertility status of the sires. Herein, the eligible studies evaluated the effect of BSPs (Rodriguez-Villamil et al., 2016, 2020), OPN (Gonçalves et al., 2008; Erikson et al., 2007), and DEFB126 (Sprott et al., 2000; Fernandez-Fuertes et al., 2016). All these studies confirmed the association between BSPs, OPN, and DEFB126 and bull fertility using in vitro assays. Only Amann et al. (1999) reported a positive effect of PSAP on bull fertility under in vivo conditions. The use of in vitro assays is a double-edged sword. Undoubtedly, in vitro studies contribute to elucidation of molecular mechanisms and are fundamental to guide studies in vivo, mainly because in vivo experiments take longer to be completed and are high-costly. However, none of the existing in vitro assays fully mimic in vivo fertilization because of the absence of factors such as natural sperm selection within the female organs, endocrine environment, and female reproductive fluids (Talevi & Gualtieri, 2004).

REVIEW LIMITATIONS

This meta-analysis faced some limitations regarding data collection. Forty-three articles provided insufficient statistical data, whereas other studies did not inform methodological data (n = 4; Fig. 1). Other five studies analyzed seminal proteins indirectly, applying agents (e.g., egg yolk and antibodies) for protein sequestration. Another limitation was the methodological variation between studies by authors from different research groups over more than 30 years. Indeed, the development of methods and techniques of detection improved the quality of sperm analysis, sperm cryopreservation procedures, and protein separation, detection, and identification. This issue was circumvented here using the mixed-effects model of meta-analysis. This model evaluates study heterogeneity and its influence on the magnitude of the effects. Finally, the elegant ARRIVE guidelines 2.0 tool revealed punctual limitations in the research reporting of the 29 eligible studies. The lack of correct and complete reporting of methodologies impairs the repeatability of experimental design. Also, the gap in information about the randomization and blinding strategies supports the existence of biases because the researchers may expect a specific outcome and it may cause unintentionally influence the data collection and interpretation. Only almost 10% of the authors discussed the limitations of their studies. In scientific research, limitations are unavoidable, and scoring them helps the planning and performance of future studies. Shedding light on the risks of bias is relevant for the scientific community and the direction of efforts to increase knowledge in the area. It is worth mentioning that, up to date, there is no database to register the protocol of reviews performed on domestic animals. Protocol registration is recommended to publish the review protocol before the article submission. Due to the exponential growth of systematic reviews and meta-analyses' publications, the protocol registration protects the authors from duplicates and overlapping.

CONCLUSIONS AND FUTURE DIRECTIONS

The concept today is that seminal plasma is a dynamic and complex secretome from the testicles, epididymis, seminal vesicles, bulbourethral glands, and prostate, with molecules that regulate not only sperm functions but also post-fertilization events and uterine physiology. In fact, a systematic analysis of 71 articles published over the last three decades showed that research devoted to seminal plasma proteins has generated unprecedented information about

the biochemistry and functions of these molecules (Viana et al., 2021). Accordingly, the present meta-analytical investigation brings evidence that seminal plasma proteins improve sperm motility, quality, capacitation, and stimulate sperm protection and fertilizing ability in the bovine species. However, our findings show that strategies used by authors to study seminal proteins and bull fertility-related parameters are diverse, with distinct endpoints and methods. The associations between seminal proteins and sperm and fertility parameters of bulls are influenced by animal breed, method of sample collection, type and condition of semen samples (epididymal vs. ejaculated sperm; fresh vs. frozen-thawed sperm), and type of proteins tested in the experiments. This lack of uniformity in the studies is somehow expected in the first phase of the research about seminal proteins. At the present scenario, however, we suggest that methods and endpoints need to become more standardized to define which (and how) seminal proteins could be used to select superior sires and/or to enhance the outcomes of reproductive biotechnology. According to our view, scientists ought to focus on the translational aspects of the research about seminal plasma proteins as well. The knowledge of seminal molecular configuration will contribute to the efficiency of assisted reproductive technologies in the bovine species and support reproductive biology science in other species.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION STATEMENT

A.G. Viana, A. Moura, and M. Machado-Neves conceived the study design. A. G. Viana, I. M. Ribeiro, and R. P. R. Carvalho performed the literature search and data extraction. All authors contributed to the data interpretation and article preparation. A. Moura, E. Memili, and M. Machado-Neves reviewed the intellectual content before submission. Machado-Neves supervised the project.

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Supplementary Table 1. Primary studies used in the meta-analysis about the effects of seminal plasma proteins on sperm fertility of bulls. Information about the authors, year of publication, bovine breed, sample collection, sample type, sample condition at protein incubation and at analysis, seminal protein studied, and the DOI.

Author/year	Bovine breed	Sample collection	Sample type	Sample condition*	Seminal protein studied	DOI
Henricks *et al*., 1998	*Bos indicus*	electro-ejaculator	ejaculated sperm	fresh-fresh	IGF	Bio Reprod 59(2):330-337
Amann *et al*., 1999	*Bos taurus*	artificial vagina	ejaculated sperm	frozen-frozen	PSAP	10.1002/j.1939-4640.1999.tb02493.x
Sprott *et al*., 2000	crossbred	electro-ejaculator	ejaculated sperm	frozen-frozen	BSPs	10.2527/2000.784795x
Reyes-Moreno *et al*., 2002	crossbred	retrograde flushing	ejaculated sperm	frozen-frozen	WSP	10.1095/biolreprod66.1.159
Gwathmey *et al*., 2006	*Bos taurus*	retrograde flushing	epididymal sperm	fresh-fresh	BSPs	10.1095/biolreprod.106.053306
Erikson *et al*., 2007	*Bos taurus*	artificial vagina	epididymal sperm	fresh-fresh	OPN	10.1530/REP-06-0228
Miah *et al*., 2007	*Bos taurus*	artificial vagina	ejaculated sperm	fresh-fresh and fresh-frozen	OPN	10.1111/j.1740-0929.2007.00468.x
Gonçalves *et al*., 2008	*Bos taurus*	artificial vagina	ejaculated sperm	frozen-frozen	WSP	10.1016/j.anireprosci.2007.09.006
Alghamadi *et al*., 2009	*Bos taurus*	electro-ejaculator	ejaculated sperm	fresh-frozen	WSP	10.1016/j.anireprosci.2008.10.015
Alghamadi *et al*., 2010	*Bos taurus*	electro-ejaculator	ejaculated sperm	frozen-frozen	NGF	10.1016/j.anireprosci.2010.06.003
Li *et al*., 2010	*Bos taurus*	artificial vagina	ejaculated sperm	frozen-frozen	RLX	10.1016/j.theriogenology.2010.06.033
Miah *et al*., 2011	*Bos taurus*	artificial vagina	ejaculated sperm	fresh-fresh and fresh-frozen	RLX	10.1016/j.anireprosci.2011.03.010.
Almadaly *et al.,* 2015	*Bos taurus*	artificial vagina	ejaculated sperm	fresh-frozen	WSP	10.1016/j.theriogenology.2014.09.004
Karunakaran & Devanathan, 2015	crossbred	artificial vagina	ejaculated sperm	fresh-fresh	HBPs	10.1080/09712119.2015.1129343
Krishnan *et al.,* 2015	*Bos taurus*	artificial vagina	ejaculated sperm	fresh-fresh	HBPs	Indian J Anim Sci 85(11):1176 - 1180
Patel *et al*., 2015	crossbred	artificial vagina	ejaculated sperm	fresh-fresh and fresh-frozen	HBPs	10.5713/ajas.15.0586
Krishnan *et al*., 2016	*Bos taurus*	artificial vagina	ejaculated sperm	fresh-fresh and fresh-frozen	HBPs	Indian J Anim Sci 86(5):528-534
Krishnan *et al*., 2016	*Bos taurus*	artificial vagina	ejaculated sperm	fresh-fresh and fresh-frozen	HBPs	Indian J Anim Sci 86 (4): 392–396

Fernandez-Fuertes *et al*., 2016	crossbred	retrograde flushing	epididymal sperm	fresh-fresh	DEFB126	10.1095/biolreprod.116.138792
Rodriguez-Villamil *et al*., 2016	*Bos taurus*	electro-ejaculator and retrograde flushing	ejaculated sperm and epididymal sperm	fresh-frozen	BSPs	10.1016/j.theriogenology.2015.09.044
Patel *et al*., 2016	crossbred	artificial vagina	ejaculated sperm	fresh-fresh and fresh-frozen	HBPs	10.1016/j.theriogenology.2016.05.020
Lyons *et al.,* 2018	*Bos taurus*	artificial vagina and retrograde flushing	ejaculated sperm and epididymal	fresh-fresh	DEFB126	10.1071/RD17415
Pande *et al*., 2018	*Bos taurus*	artificial vagina	ejaculated sperm	fresh-fresh and fresh-frozen	HBPs	Indian J Anim Sci 88 (1): 39–45
Stewart *et al*., 2019	crossbred	electro-ejaculator and retrograde flushing	ejaculated sperm and epididymal	fresh-frozen	NGF	10.1016/j.anireprosci.2019.06.010
Costa *et al*., 2020	*Bos indicus*	electro-ejaculator	ejaculated sperm	fresh-frozen	IGF	10.1590/1678-4162-11291
Elkhawagah *et al.,* 2020	crossbred	artificial vagina	ejaculated sperm	fresh-frozen	RLX	10.1016/j.cryobiol.2020.06.006
Singh *et al*., 2020	*Bos indicus*	artificial vagina	ejaculated sperm	fresh-fresh	IGF	10.1016/j.bbrc.2019.02.002
Rodriguez-Villamil *et al.,* 2020	*Bos taurus*	electro-ejaculator and retrograde flushing	ejaculated sperm and epididymal	frozen-frozen	BSPs	10.1017/S0967199420000374
Zoca et al., 2021	*Bos indicus*	electro-ejaculator	Eeaculated sperm	fresh-fresh and frozen-frozen	WSP	10.1111/rda.13928.

*Sample condition at protein incubation and at analysis. BSPs: binder of sperm protein; DEFB126: beta-defensin 126; HBPs: heparin binding protein; IGF: insulin-like growth factor; NGF: nerve growth factor; OPN: osteopontin; PSAP: prosaposin; RLX: relaxin; WSP: whole seminal proteins.

Supplementary Table 2. Effect sizes, heterogeneity and Rosenthal's fail-safe number for the analyzed variables according to methodological variations of the effect of seminal plasma proteins in bull fertility.

Variables	Q_B	*p*-value	d++	Df	CI	Rosenthal's fail-safe number	5*k* + 10
			BOVINE BREEDS: ***Bos taurus vs. Bos indicus vs.*** **Crossbred**				
SPERM MOTILITY	13.62	0.0011†				680.4§	210
Bos taurus			3.4997‡	20	2.4376 to 4.5618		
Crossbred			0.9456	13	-0.0861 to 1.9772		
Bos indicus			1.7178	4	-0.5054 to 3.9411		
SPERM INTEGRITY*	32.60	0.0000†				1201.6§	340
Bos taurus			1.9689	28	1.5348 to 2.4031		
Crossbred			0.6503	17	0.2328 to 1.0678		
Bos indicus			0.4708	18	0.0955 to 0.8460		
SPERM DAMAGE	13.52	0.0002†				2478.5§	115
Bos taurus			-2.6604‡	10	-3.6579 to -1.6629		
Crossbred			-0.5428	9	-1.3623 to 0.2367		
SPERM CAPACITATION*	0.00	0.9738				116.6	110
Bos taurus			0.9325	14	0.2841 to 1.5809		
Crossbred			0.9155	4	-0.24301 to 2.0740		
SPERM FERTILIZING ABILITY	2.22	0.1357	0.02742			81.2	105
Bos taurus			1.0574	15	0.4761 to 1.6388		
Crossbred			0.0959	2	-2.4169 to 2.6088		
SPERM PROTECTION** Turned off							
			SEMEN COLLECTION: Electroejaculation (EJ) ***vs.*** **artificial vagina (AV)** ***vs.*** **retrograde fluxing (RF)**				
SPERM PROTECTION*	14.54	0.0001†				110.4§	60
EJ			0.2126	3	-0.8630 to 1.2881		
RF			1.8263‡	5	1.1191 to 2.5334		

SPERM MOTILITY	15.61	0.0004†				668§	210
EJ			1.9889	2	-2.7033 to 6.6812		
AV			2.6630‡	29	1.9108 to 3.4152		
RF			-1.0410	6	-3.1514 to 1.0694		
SPERM INTEGRITY	43.70	0.0000†				3118.5§	340
EJ			0.0201	13	-0.3449 to 0.3851		
AV			1.3233‡	49	1.0897 to 1.5570		
RF			-0.0295	1	-6.4252 to 6.3663		
SPERM FERTILIZING ABILITY	2.11	0.3470				81.2	125
EJ			0.7905	4	-0.5771 to 2.1581		
AV			0.6064	11	-0.2728 to 1.4856		
RF			1.4892	5	0.2291 to 2.7494		
SPERM DAMAGE** Turned off							
SPERM PROTECTION** Turned off							
SAMPLE SOURCE: Ejaculated (Ej) sperm *vs.* Epididymal (Ep) sperm							
SPERM PROTECTION	4.18	0.0409†				61.5§	60
Ej sperm			0.7579	4	-0.4994 to 2.0152		
Ep sperm			2.0788‡	4	0.7991 to 3.3585		
SPERM MOTILITY	21.37	0.0000†				694.7§	210
Ej sperm			2.5809‡	33	1.9000 to 3.2617		
Ep sperm			-2.0321	5	-4.4481 to 0.3839		
SPERM INTEGRITY	2.53	0.0643				2224.9	345
Ej sperm			1.0227	63	0.7752 to 1.2703		
Ep sperm			-0.0296	1	-8.2859 to 8.2268		
SPERM FERTILIZING ABILITY	2.11	0.1469				87.5	105
Ej sperm			0.6699	12	0.0501 to 1.2897		
Ep sperm			1.4752	5	0.2531 to 2.6974		
SPERM DAMAGE** Turned off							

SPERM CAPACITATION** Turned off							
			SAMPLE CONDITIONING: fresh-fresh *vs.* fresh-frozen *vs.* frozen-frozen thawed sperm				
SPERM PROTECTION*	15.42	0.0000†				153.6§	55
Fresh-frozen			0.1760	2	-1.5041 to 1.8562		
Fresh-fresh			1.9902‡	5	1.3560 to 2.6244		
SPERM INTEGRITY	7.14	0.0281†				2328.4§	340
Fresh-frozen			1.1333‡	25	0.7227 to 1.5439		
Frozen-frozen			0.2241	8	-0.4769 to 0.9251		
Fresh-fresh			1.0814‡	30	0.7418 to 1.4210		
SPERM MOTILITY*	0.93	0.3338				606.5	205
Fresh-frozen			5.687	13	1.2899 to 3.6776		
Fresh-fresh			3.2575	24	0.9673 to 2.6678		
SPERM DAMAGE*	1.73	0.1874				253.3	110
Fresh-frozen			-1.1635	8	-2.2134 to -0.1135		
Fresh-fresh			-1.9999	10	-2.9846 to -1.0152		
SPERM CAPACITATION	4.05	0.1319				119.5	110
Fresh-frozen			0.9617	9	0.2369 to 1.6865		
Frozen-frozen			2.7275	1	-9.8609 to 15.3159		
Fresh-fresh			0.6115	7	-0.2574 to 1.4803		
SPERM FERTILIZING ABILITY	3.91	0.1411				86	105
Fresh-frozen			1.2037	3	-0.8120 to 3.2195		
Frozen-frozen			1.1526	8	0.4206 to 1.8846		
Fresh-fresh			0.0318	5	-1.2449 to 1.3084		
			SEMINAL PROTEINS: WSP *vs.* HBP *vs.* BSPs *vs.* DEFB126 *vs.* IGF *vs.* RLX *vs.* NGF				
SPERM PROTECTION*	20.95	0.0000†				185.5§	60
WSP			0.2126	3	-0.8630 to 1.2881		
BSPs			2.6719‡	2	0.5058 to 4.8379		
DEFB126			1.7762‡	2	0.6942 to 2.8583		

SPERM MOTILITY*	60.61	0.0000†				711.5§	210
WSP			1.4002	2	-3.0768 to 5.8771		
HBPs			3.3911‡	20	2.4663 to 4.3159		
RLX			1.4194	4	-0.8221 to 3.6609		
BSPs			-11.4466‡	2	-19.7432 to -3.1500		
DEFB126			0.8569	2	-0.4507 to 3.8565		
IGFs			1.7029	4	-3.6953 to 5.4091		
SPERM INTEGRITY*	44.15	0.0000†				3022.8§	300
WSP			0.0529	11	-0.3565 to 0.4624		
HBPs			1.5306‡	32	1.2240 to 1.8372		
NGF			0.2591	6	-0.5434 to 1.0615		
RLX			0.4132	1	-0.7291 to 1.5554		
IGF1			1.0882‡	8	0.5623 to 1.6141		
SPERM DAMAGE*	8.91	0.0028†				231.1§	105
HBPs			-2.0330‡	15	-2.7895 to -1.2765		
NGF			0.5032	2	-2.8174 to 3.8238		
SPERM CAPACITATION*	0.04	0.825				87.2	95
HBPs			0.9204	4	0.1930 to 1.4542		
RLX			0.8236	11	0.1930 to 1.4542		
SPERM FERTILIZING ABILITY*	4.39	0.11				65.2	100
DEFB126			-0.0398	1	-10.1119 to 10.0322		
BSPs			1.4032	8	0.5293 to 2.2770		
OPN			0.4693	6	-0.4585 to 1.3970		

BSPs = binder of sperm proteins; CI = confidence interval; *d*++ = cumulative effect; DEFB126 = beta-defensin 126; Df = degrees of freedom; HBP = heparin binding protein; IGF = insulin-like growth factor; *k* = number of comparisons in the analysis; NGF = nerve growth factor; OPN = osteopontin; PSAP = prosaposin; RLX = relaxin; Q_B = heterogeneity between groups; vs. = versus; WSP = whole seminal plasma.

*Groups with fewer than two valid studies were eliminated from the analysis.

** Variables not analyzed using a mixed-effects model due to an insufficient number of independent comparisons in each category.

† Significant p-value ($p \leq 0.05$).

‡ Significative effect ($p \leq 0.5$) and a confidence interval (CI) that does not include zero.

§ Significative effect ($p \leq 0.5$), with a confidence interval (CI) that does not include zero and Rosenthal's fail-safe number indicating the robustness of the outcome (Rosenthal's fail-safe number > $5k+10$).

Supplementary Table 3. Risk of bias - ARRIVE Guideliness 2.0 items

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM	AN
1	**Author (year)**	**1a**	**1b**	**2a**	**2b**	**3a**	**3b**	**3c**	**4a**	**4b**	**5**	**6a**	**6b**	**7a**	**7b**	**8a**	**8b**	**9a**	**9b**	**9c**	**9d**	**10a**	**10b**	**11**	**12a**	**12b**	**13**	**14**	**15**	**16a**	**16b**	**16c**	**17a**	**17b**	**18**	**19**	**20**	**21a**	**21b**	**Quality score**
2	**Henricks *et al*., 1998**	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	N	Y	N	Y	Y	N	Y	Y	NA	Y	Y	NA	Y	Y	N	NA	NA	N	Y	N	NA	NA	N	N	N	16
3	**Amann *et al*., 1999**	Y	Y	Y	N	Y	N	Y	Y	N	N	Y	NA	Y	N	Y	N	Y	Y	Y	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	Y	NA	NA	N	N	N	20
4	**Sprott *et al.* , 2000**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	N	N	N	N	N	Y	N	N	Y	NA	Y	N	NA	Y	N	N	NA	NA	Y	N	N	NA	NA	N	N	N	11
5	**Reyes-Moreno *et al*., 20**	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	Y	N	Y	Y	NA	Y	Y	NA	N	N	N	NA	NA	N	Y	N	NA	NA	N	N	N	13
6	**Gwathmey *et al*., 2006**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	Y	N	Y	Y	NA	Y	Y	NA	Y	N	N	NA	NA	N	Y	N	NA	NA	N	N	N	15
7	**Erikson *et al*., 2007**	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	N	Y	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	N	NA	NA	N	Y	Y	NA	NA	N	N	Y	16
8	**Miah *et al*., 2007**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	Y	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	N	N	16
9	**Gonçalves *et al*., 2008**	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	N	NA	NA	N	Y	N	NA	NA	N	N	Y	14
10	**Alghamdi *et al.* , 2009**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	Y	N	Y	Y	N	Y	Y	NA	Y	Y	NA	Y	Y	Y	NA	NA	N	Y	N	NA	NA	N	N	Y	19
11	**Alghamdi *et al*., 2010**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	Y	N	Y	Y	N	Y	Y	NA	Y	Y	NA	Y	Y	Y	NA	NA	N	Y	N	NA	NA	N	N	Y	19
12	**Li *et al*., 2010**	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	N	NA	NA	N	Y	N	NA	NA	N	N	Y	14
13	**Miah *et al*., 2011**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	Y	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	N	N	16
14	**Almadaly *et al*., 2015**	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	N	Y	15
15	**Krishnan *et al.* , 2015**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	N	N	15
16	**Patel *et al*., 2015**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	Y	Y	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	Y	N	18
17	**Krishnan *et al.* , 2016**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	N	N	15
18	**Krishnan *et al.* , 2016**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	N	N	15
19	**Fernandez-Fuertes *et al.***	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	Y	N	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	N	NA	NA	N	Y	Y	NA	NA	N	N	N	15
20	**Rodriguez-Villamil *et al.***	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	Y	N	Y	Y	NA	Y	Y	NA	Y	Y	N	NA	NA	N	Y	N	NA	NA	N	Y	Y	17
21	**Patel *et al*., 2016**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	Y	Y	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	Y	Y	19
22	**Karunakaran & Devana**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	Y	Y	NA	NA	N	Y	N	NA	NA	N	Y	N	17
23	**Lyons *et al*., 2018**	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	Y	N	Y	Y	NA	Y	Y	NA	Y	N	N	NA	NA	N	Y	N	NA	NA	N	Y	Y	16
24	**Pande *et al*., 2018**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	Y	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	N	N	16
25	**Stewart *et al*., 2019**	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	Y	N	N	Y	Y	N	Y	Y	NA	Y	Y	NA	Y	Y	N	NA	NA	N	Y	N	NA	NA	N	Y	N	17
26	**Costa *et al*., 2020**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	Y	Y	NA	NA	N	Y	N	NA	NA	N	N	N	16
27	**Elkawagah *et al*., 2020**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	Y	N	Y	N	Y	N	N	Y	Y	NA	Y	Y	NA	Y	N	Y	NA	NA	N	Y	N	NA	NA	N	Y	Y	18
28	**Singh *et al*., 2020**	Y	Y	Y	N	Y	N	Y	N	N	N	Y	NA	N	N	Y	N	Y	Y	Y	Y	Y	NA	Y	Y	NA	Y	N	N	NA	NA	N	Y	N	NA	NA	N	N	N	16
29	**Rodriguez-Villamil *et al***	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	N	N	N	Y	Y	N	Y	Y	NA	Y	Y	NA	Y	Y	N	NA	NA	N	Y	N	NA	NA	N	Y	Y	17
30	**Zoca *et al.* , 2021**	Y	Y	Y	N	N	N	Y	N	N	N	Y	NA	Y	Y	Y	Y	Y	Y	N	Y	Y	NA	Y	Y	NA	Y	Y	Y	NA	NA	N	Y	N	NA	NA	N	Y	Y	21
31	N = No; Y = Yes; NA = not applied																																							

Content:

1a. Study design - The groups being compared, including control groups. If no control group has been used, the rationale should be stated.

1b. Study design - The experimental unit (e.g., a single animal, litter, or cage of animals).

2a. Sample size - Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.

2b. Sample size - Explain how the sample size was decided. Provide details of any a priori sample size calculation, if done.

3a. Inclusion and exclusion criteria - Describe any criteria used for including or excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established a priori. If no criteria were set, state this explicitly.
3b. Inclusion and exclusion criteria - For each experimental group, report any animals, experimental units, or data points not included in the analysis and explain why. If there were no exclusions, state so.
3c. Inclusion and exclusion criteria - For each analysis, report the exact value of n in each experimental group.
4a. Randomization - State whether randomization was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomization sequence.
4b. Randomization - Describe the strategy used to minimize potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.
5. Blinding- Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).
6a. Outcome measures - Clearly define all outcome measures assessed (e.g., cell death, molecular markers, or behavioral changes).
6b. Outcome measures - For hypothesis-testing studies, specify the primary outcome measure, i.e., the outcome measure that was used to determine the sample size.
7a. Statistical methods - Provide details of the statistical methods used for each analysis, including the software used.
7b. Statistical methods - Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.
8a. Experimental animals - Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.
8b. Experimental animals - Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.
9a. Experimental procedures - What was done, how it was done, and what was used.
9b. Experimental procedures - When and how often.
9c. Experimental procedures - Where (including detail of any acclimatization periods).
9d. Experimental procedure - Why (provide a rationale for procedures).
10a. Results - Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g., mean and SD, or median and range).
10b. Results - If applicable, the effect size with a confidence interval.
11. Abstract - Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.
12a. Background - Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.
12b. Background - Explain how the animal species and model used address the scientific objectives and, where appropriate, the relevance to human biology.
13. Objectives - Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.
14. Ethical statement - Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study and any relevant license or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.
15. Housing and husbandry - Provide details of housing and husbandry conditions, including any environmental enrichment.
16a. Animal care and monitoring - Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering, and distress.
16b. Animal care and monitoring - Report any expected or unexpected adverse events.
16c. Animal care and monitoring - Describe the humane endpoints established for the study, the signs that were monitored, and the frequency of monitoring. If the study did not set humane endpoints, state this.
17a. Interpretation/scientific implications - Interpret the results, taking into account the study objectives and hypotheses, current theory, and other relevant studies in the literature.

17b. Interpretation/scientific implications - Comment on the study limitations, including potential sources of bias, limitations of the animal model, and imprecision associated with the results.
18. Generalisability/translation - Comment on whether, and how, the findings of this study are likely to generalize to other species or experimental conditions, including any relevance to human biology (where appropriate).
19. Protocol registration - Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.
20. Data access - Provide a statement describing if and where study data are available.
21a. Declaration of interests - Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated.
21b. Declaration of interests - List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis, and reporting of the study.

4. CHAPTER 3

Sperm and seminal plasma proteoform atlas in bulls with contrasting semen freezability: a story deciphered by top-down mass spectrometry

In preparation to be submitted to *Biology of Reproduction*

Sperm and seminal plasma proteoform atlas in bulls with contrasting semen freezability: a story deciphered by top-down mass spectrometry

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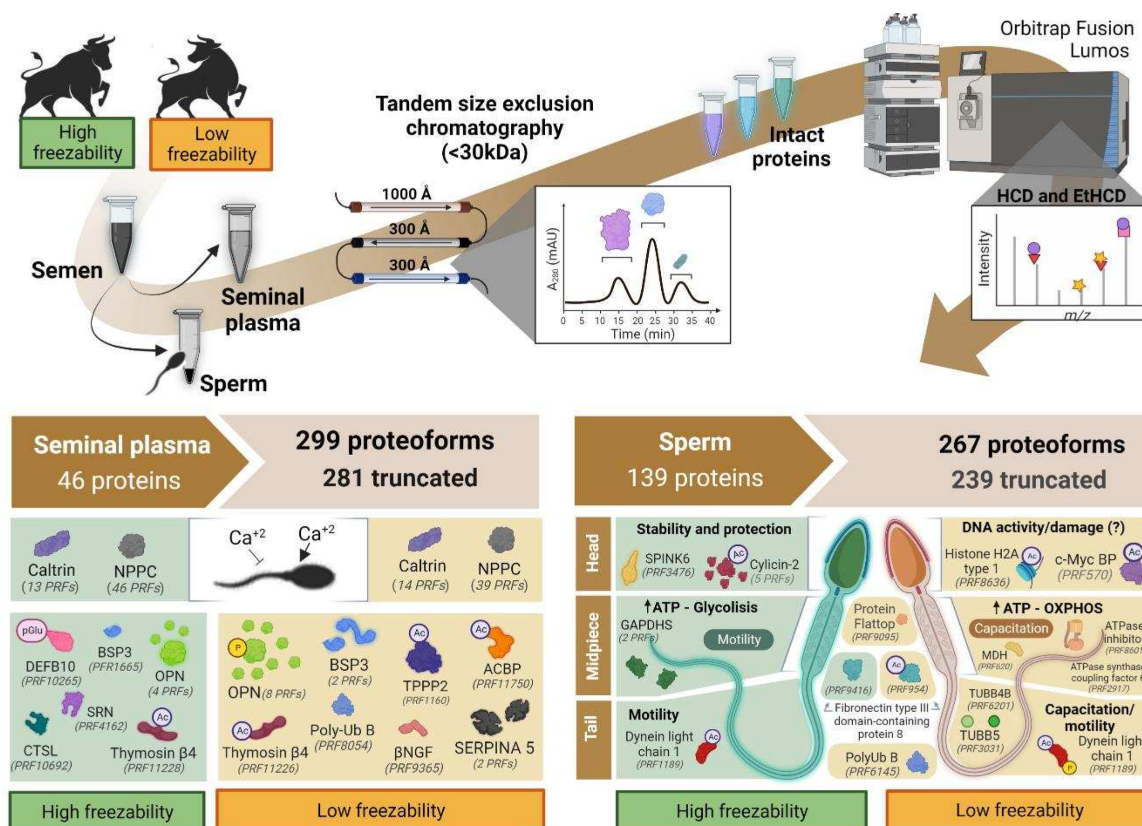


ABSTRACT

The present research employs top-down mass spectrometry to generate sperm and seminal plasma proteoform atlas of bulls with low (LF; n = 6) and high (HF; n = 9) semen freezability. Sperm and seminal proteins were fractionated by tandem size exclusion chromatography (< 30 kDa) and analyzed by mass spectrometry (operated in protein intact mode). This approach allowed the identification of 299 seminal plasma (from 46 proteoform families) and 267 sperm proteoforms (from 139 proteoform families). Bovine seminal plasma contained 145 c-type natriuretic peptide (NPPC), 46 caltrin, 27 osteopontin, five BSP1 and six BPS3 proteoforms. Seventy proteoforms defined the seminal plasma signature of HF bulls, belonging to beta-defensin 10, NPPC, caltrin, seminal ribonuclease, osteopontin and BSP3 proteoform families. Seminal proteoforms (77) upregulated in LF bulls belonged to caltrin, NPPC, osteopontin, BSP3, serpina5, βNGF families, among others. Different NPPC, caltrin, osteopontin and BSP3 proteoforms existed in HF and LF bull seminal plasma. Sperm proteoforms (33) upregulated in HF bulls belonged to NPPC, caltrin and cylicin-2 families, while sperm proteoforms (22) of LF bulls related to caltrin, cylicin-2, ATP synthases, malate dehydrogenase families, among others. A truncated and acetylated histone H2A and a non-truncated and acetylated c-Myc protein binding proteoforms were prevalent in LF sperm. There were cylicin-2 proteoforms in HF and LF sperm and truncated glyceraldehyde-3-phosphate dehydrogenases in HF sperm. In conclusion, this is the first sperm and seminal plasma proteoform atlas of any species. Post-translational processing appears to define bio-attributes of proteins and their empirical associations with sperm cryoresistance.

Keywords: Bovine, semen, top-down proteomics, proteoform, freezability.

INTRODUCTION

Semen cryopreservation has made possible the massive use of artificial insemination in livestock, for wildlife conservation and human medicine [1-4]. Methods for sperm cryopreservation have improved in recent decades and become specific for every species but, despite this remarkable accomplishment, sperm damages inflicted by cold shock are still significant. Freezing and thawing cause intracellular dehydration and formation of ice crystal, organelle and membrane damage, and sperm are particularly susceptible to temperature changes because of their low fluid content and high membrane fluidity [5]. These events inevitably affect male fertility when cryopreserved gametes are used [5, 6].

A single ejaculate contains sperm with distinct morphology and biochemical composition, and individuals with comparable parameters of fresh sperm may have different sperm kinetics after semen cryopreservation [5]. The reasons for such differences are not completely understood but the proteomic synopsis of sperm and the seminal milieu seems to determine, at least partially, the sperm fertilizing capacity and its resilience to cold shock.

Sperm proteins function as contractile components and regulate motility, energy metabolism, chromatin organization, ion channels, sperm interaction with the female reproductive tract, acrosome reaction and fertilization [7, 8]. Empirical relationships exist among sperm proteins, parameters of both fresh and frozen-thawed sperm and fertility of bulls [9-15]. During spermatogenesis, most sperm histones are replaced by protamines, which condense sperm DNA into a transcriptionally inactive state [16]. However, although unable to transcribe genes, sperm may synthesize new proteins from pre-existing mRNAs and alter proteins through post-translational modifications (PTMs) [17-19]. Seminal plasma proteins also exert major functions, influencing sperm metabolism, motility, capacitation and acrosome reaction, and fertilization, among other processes [20, 21]. Associations have been reported between seminal proteins, semen freezability and fertility of bulls [11, 22-27] and certain seminal proteins potentiate the fertilizing capacity of sperm in vitro as well [28-33]. All these findings confirm the relevance of sperm and seminal plasma molecular signatures for sperm´s fate.

Early studies based on 2-D gel electrophoresis suggested that seminal plasma of species such as bovine [34, 35], rams [36] and humans [37] contain diverse proteoforms. This is the case of the binder of sperm proteins (BSPs), the most abundant protein in the seminal plasma of bulls that bind to sperm at ejaculation [38] and act in sperm capacitation [39], sperm-oviduct

interaction [40] and fertilization [32, 33, 41]. The biological significance of BSP proteoforms is intriguing as glycosylated BSP1 has chaperone-like activity, while non-glycosylated BSP1 is responsible for membrane cholesterol efflux and sperm capacitation [42]. Sperm PTMs such as phosphorylation and acetylation define important pathways related to sperm physiology as well [43, 44]. Tyrosine phosphorylation of several sperm proteins are crucial to sperm motility, capacitation and acrosomal reaction [45, 46] and global histone acetylation is necessary for the relaxation of chromatin for protamine replacement [47]. Thus, the existence of proteoforms is a common feature of seminal plasma and sperm, but their characteristics and significance for male fertility are still largely unknown.

Bottom-up mass spectrometry has brought exceptional knowledge about the proteome of sperm cells and reproductive secretions. However, methods of bottom-up proteomics, where all proteins are digested prior to mass spectrometry, interfere with the precise mapping of proteoforms. Top-down mass spectrometry, instead, analyzes intact proteins and has become a powerful tool for the characterization of proteoforms resulting from post-transcription and post-translation alterations [48-50]. Top-down proteomics approaches have recently described different patterns of phosphorylation in osteopontin (OPN) from bovine seminal fluid [51], suggesting that PTMs define specific OPN functions in sperm physiology. OPN is involved in cell adhesion, tissue and extracellular remodeling, affects sperm capacitation and fertilization and it is a promising seminal protein biomarker of bull fertility [23, 28, 29, 52, 53].

The definition of structures and roles of proteoforms represent major challenges for both clinical and fundamental research [54, 55], and proteoform mapping is a necessary step for the full comprehension of protein attributes. Thus, the present study was conducted to evaluate the associations of sperm and seminal plasma proteoforms in bulls with contrasting freezability status, applying a top-down proteomics strategy that combines tandem size-exclusion and reversed-phase liquid chromatography, mass spectrometry and label-free quantitation.

MATERIAL AND METHODS

Sample collections and determination of semen freezability

Semen samples from Holstein sires were provided by Alta Genetics Inc. (Watertown, WI, USA), as reported before [27]. Briefly, semen was frozen according to Alta Genetics standard protocols and post-thaw sperm viability was assessed by evaluating the membrane integrity of ten thousand cells per sample, using flow cytometry (SYBR-14/PI, Live/Dead

Sperm Viability Kit L-7011; Thermo Fisher Scientific, USA). Sires' freezability status were ranked based on the percentage of viable sperm (%ViableSperm) deviation from the population average (54.7 ± 5.4%). Bulls with low semen freezability (LF; n = 6) had -5.8 to +0.3 %ViableSperm and bulls with high semen freezability (HF; n = 9) had +4.6 to +11.5 %ViableSperm relative to the average %ViableSperm of the population (Table 1).

Table 1. Semen freezability phenotypes of Holstein bulls. Bulls 1-6 are defined as low-semen freezability (LF) and bulls 7-16, as high-freezability (HF). This set of samples is the same used in a previous study conducted by Gomes et al [27].

Bull#	Freezablity status	Number of frozen-thawed samples	% Average of post-thaw viability (flow cytometry)	% Difference from the population average
1	LF	79	48.9	-5.8
2	LF	107	49.2	-5.5
3	LF	194	52.7	-2.0
4	LF	264	54.8	-0.1
5	LF	71	54.9	0.2
6	LF	229	55.0	0.3
7	HF	138	59.3	4.6
8	HF	81	59.9	5.2
9	HF	113	61.9	7.3
10	HF	153	62.3	7.7
11	HF	207	62.8	8.1
12	HF	266	63.0	8.3
13	HF	205	64.3	9.6
14	HF	50	64.4	9.714
15	HF	116	66.2	11.5

One semen sample was collected by artificial vagina from the HF and LF bulls and centrifuged at 800 × g (15 min., 4 °C). The supernatant (seminal plasma) was centrifuged again at 10,000 x g (30 min., 4 °C), transferred to new vials, and treated with protease (product # 8830; Sigma-Aldrich, USA) and RNAse inhibitors (product # 1158; Sigma-Aldrich, USA) and stored (– 80 ºC). The sperm pellets left after the first centrifugation of semen samples were washed three times in phosphate-buffered saline (800 × g, 15 min., 4 °C), mixed with protease and RNAse inhibitors (same as above), aliquoted and stored (− 80 °C).

Protein extraction

Sperm from bulls with high (n=9) and low semen freezability (n=6) were used to form two pools, using 40 x 10^6 sperm per animal. Protein was extracted by treating sperm pools (1:5, v/v) with lysis buffer (8 M urea, 0.1 M ammonium bicarbonate), and with protease and phosphatase inhibitors (cOmplete™, EDTA-free Protease Inhibitor Cocktail and PhosSTOP™, Roche, USA, respectively) followed by 20 homogenization cycles (Dounce homogenizer) and centrifugation (17,000 × g, 15 min., 4 °C). Extracted proteins were precipitated with cold acetone (1:4) overnight (– 20 °C), followed by three sequential centrifugations and washings in cold acetone (14,000 × g, 10 min., 4 °C) [25]. The dried pellet was resuspended in formic acid (50 mM) and an aliquot was used to determine protein concentration (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific, USA). Seminal plasma samples were also pooled for HF bulls (n = 9) and LF bulls (n = 6), using 60 µl of fluid from each animal, and subjected to protein precipitation with cold acetone as reported above.

Tandem size exclusion chromatography (tSEC)

The pools of sperm (300 µg) and seminal plasma (300 µg) proteins from HF and LF bulls were fractionated by tSEC using an Agilent 1200 HPLC system consisting of an Agilent 1260 quaternary pump (G1311B), a G-1315D diode array detector, and G1364C fraction collector (Agilent, USA). Separation was achieved with a series of three PolyHYDROXYETHYL A (PolyHEA) columns (9.4 × 200 mm, 3 µm, 1000 Å−300 Å− 300 Å pores) at room temperature [49, 56]. The mobile phase contained 50 mM formic acid, 0.1% difluoroacetic acid, and the fractionation occurred at 0.45 mL/min. The elution of protein was measured by detecting absorption at 280 nm using Agilent ChemStation Software and one-minute fractions were collected for 13 minutes (24 – 37 min.). Afterward, samples were concentrated by using 3-kDa molecular weight cutoff filter units (Amicon Ultra 3 kDa centrifugal filter devices; Millipore, USA).

One-dimensional gel electrophoresis

Ten microliter aliquots of proteins extracted from sperm and seminal plasma pools were reduced using the same volume of Novex™ Sample Reducing Agent (Thermo Fischer Scientific, USA), followed by heating at 70 °C for 10 min. Then, samples were added to each lane of a 4−20% tris-glycine polyacrylamide gel and 10 µl of SeeBlue™ Plus2 Prestained Standard (Thermo Fisher Scientific, USA) were used as molecular weight standards. The gel was run at 150 V for 60 min., stained with Coomassie blue (Bio Rad, USA), unstained with

40% methanol and 7% acetic acid (Sigma-Aldrich, USA), and scanned at 300 dpi (c600; Azure Biosystems, USA).

LC-MS/MS

Sperm and seminal plasma protein fractions obtained were analyzed using a nanocapillary liquid chromatography (Easy-nLC 1200) system coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, USA), operated in protein intact mode. For protein fraction from each pool (∼1000 ng) we used a fused silica capillary column (∼40 cm × 75 µm i.d.) packed in-house with C4 reversed-phase resin (Jupiter, 3 µm, 300 Å, Phenomenex, USA) under mobile phases A (95% H2O, 5% ACN in 0.1% formic acid) and B (80% ACN, 20% H2O in 0.1% formic acid). The gradient started at 5 % B, kept constant for 3 min, followed by an increase to 60 % within 30 min and was held for the next 10 min. Next, the percentage of B was returned to 10% and held constant for 2 min at a constant flowrate of 200 nL/min. We set the temperatures of autosampler to 7 °C and column oven to 60 °C. Precursor and fragment ions were captured with a resolving power of 120,000 and we set the automatic gain control (AGC) of 1E6 ions in 200 ms for MS and in 250 ms for MS/MS scans. MS/MS spectra were acquired in data-dependent acquisition (DDA) mode, by isolating the most abundant precursors ions at 3 m/z isolation window for 20 s, followed by its exclusion after 60 s of the previous selection. Two activation techniques were used in consecutive scans: high energy collision dissociation (HCD) and electron-transfer/higher-energy collision dissociation (EthCD) with 12 % normalized collision energy [49].

Data analysis

Mass spectrometer raw files were processed using ProSight PD 4.1 (Thermo Fisher Scientific, USA) embedded in Proteome Discoverer Software 2.5. Precursor and fragment ions were deconvoluted using Xtract algorithm and then searched against a database generated via shotgun annotation for *Bos taurus* (Uniprot release 2022_March). *Bos taurus* database consisted of 6,133 entries corresponding to 5,817 isoforms. All searches were performed using a three-tiered approach [57, 58]. The first tier consisted of a tight absolute mass search with MS^1 and MS^2 tolerances of 2.2 Da and 10 ppm, respectively. This first tier was used to identify proteins with complete sequences while in the second search, we used a biomarker search with both MS^1 and MS^2 tolerance of 10 ppm. Then, in the third tier, we used 200 Da as MS^1 tolerance and we keep 10 ppm for MS^2 tolerance to detect proteoforms with unknown modifications. For

all searches, we used a global false discovery rate (FDR) of 1%, and [-log(P-value)] ≤ 4 threshold was defined for reliable identification of proteoforms [49]. Proteoforms were filtered by the following characterization scores: 3-40, confidently identified, but not fully characterized; > 40, confidently identified and extensively characterized [57]. Only those proteoforms detected in at least two technical replicates were used in the comparative analysis between HF and LF bulls. Only those molecules present in all three technical replicates were used for the detection of uniquely expressed proteoforms in the seminal plasma and sperm of HF and LF bulls. We used Prosight Lite to match and get a graphic interpretation of the candidate sequences and their modifications against mass spectrometry observations [59].

Protein–protein interaction (PPI) network models

Network models were reconstructed by STRING Cytoscape's App [60] using uniquely expressed proteoforms in HF and LF bulls as seed nodes (*Bos taurus* species). Only physical and/or functional interactions were retained by filtering those "experiments" or/and "databases" annotated, with a STRING Score ≥ 0.5, considering only edges between seed nodes and their neighbors. Reconstructed networks were analyzed by Centiscape Cytoscape's App to calculate Degree centrality [61], while STRING Cytoscape's App was used to extract enriched functional modules by KEGG, Reactome and WikiPathways databases (FDR < 0.01) [60]. Only those pathways with at least 5 genes contributing to their enrichment were considered.

RESULTS

Relative abundances of bull seminal plasma and sperm proteins were predominant in the low molecular weight ranges, as determined by tandem size-exclusion chromatography (Figure 1). The association of higher-energy collisional dissociation (HCD) and electron-transfer/higher-energy collisional dissociation (EthCD) activation allowed the identification of 299 seminal plasma proteoforms, belonging to 46 proteoform families. The same approach identified 267 proteoforms in the bovine sperm (from 139 proteoform families), considering 1% FDR and molecules with less than 30 kDa (Supplementary table 1: ST1 and ST3). Although intact, the majority of proteoforms were detected in their truncated form. There were only 19 and 29 non-truncated proteoforms identified by the tight absolute mass search in seminal plasma and sperm, respectively.

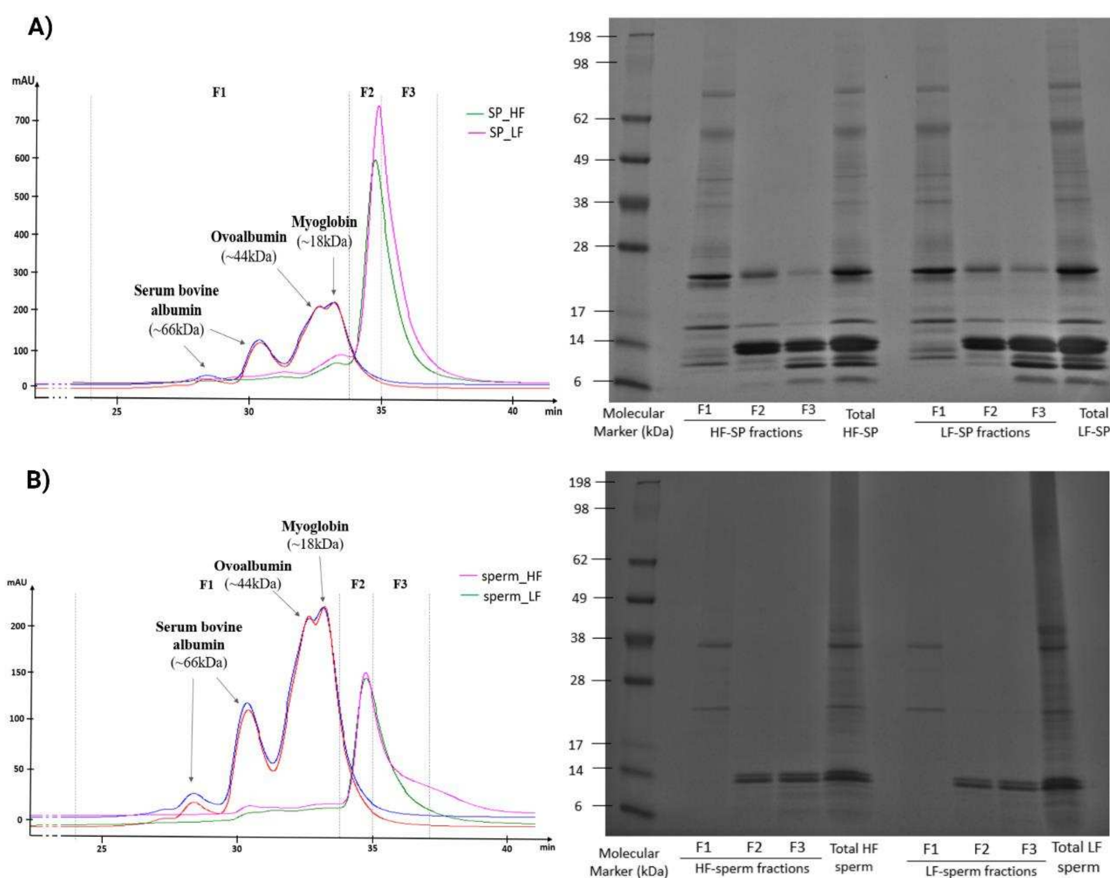


Figure 1. Tandem size exclusion chromatography and SDS-PAGE (4-20% tris-glycine) profile of proteins extracted from (A) seminal plasma and (B) sperm of bulls with low (LF) and high semen freezability (HF) phenotypes.

Employing EThcD fragmentation allowed the identification of 92 seminal plasma proteoforms, while the application of HCD fragmentation detected more sperm proteoforms (n = 89) when compared to the sole use of EThcD (n = 74; Figures 2A and 2E). However, fragmentation methods based on the combined use of HCD and EthCD allowed identification of greater number of proteoforms in seminal plasma (153) and sperm (104).

The number of seminal plasma and spermatozoa proteoforms identified by HCD and EThcD are listed according to molecular weight, *q* value and C-score (Figures 2B – 2H). Most of the proteoforms had less than 5 kDa, except for sperm proteoforms detected by EThcD, which had 5-10kDa. HCD fragmentation, alone or in association with ETD, detected seminal plasma and sperm proteoforms with the highest molecular weights (Figures 2B and 2F). In this study, the *q*-value represents a measure of significance, ranging from 8.2×10^{-2} to 4.8×10^{-142} for seminal plasma and from 3.7×10^{-2} to 3.4×10^{-203} for sperm proteoforms. Proteoforms with the highest *q* values were detected using both HCD and EThcD fragmentations (Figures 2C and 2G). Characterization scores (C-score) project the confidence of proteoform differential

abundances and were above 100 and 50 for seminal plasma and sperm, respectively. C-scores of proteoforms detected using only HCD as a fragmentation method were below 20 in almost 50% of the features (Figures 2D and 2H).

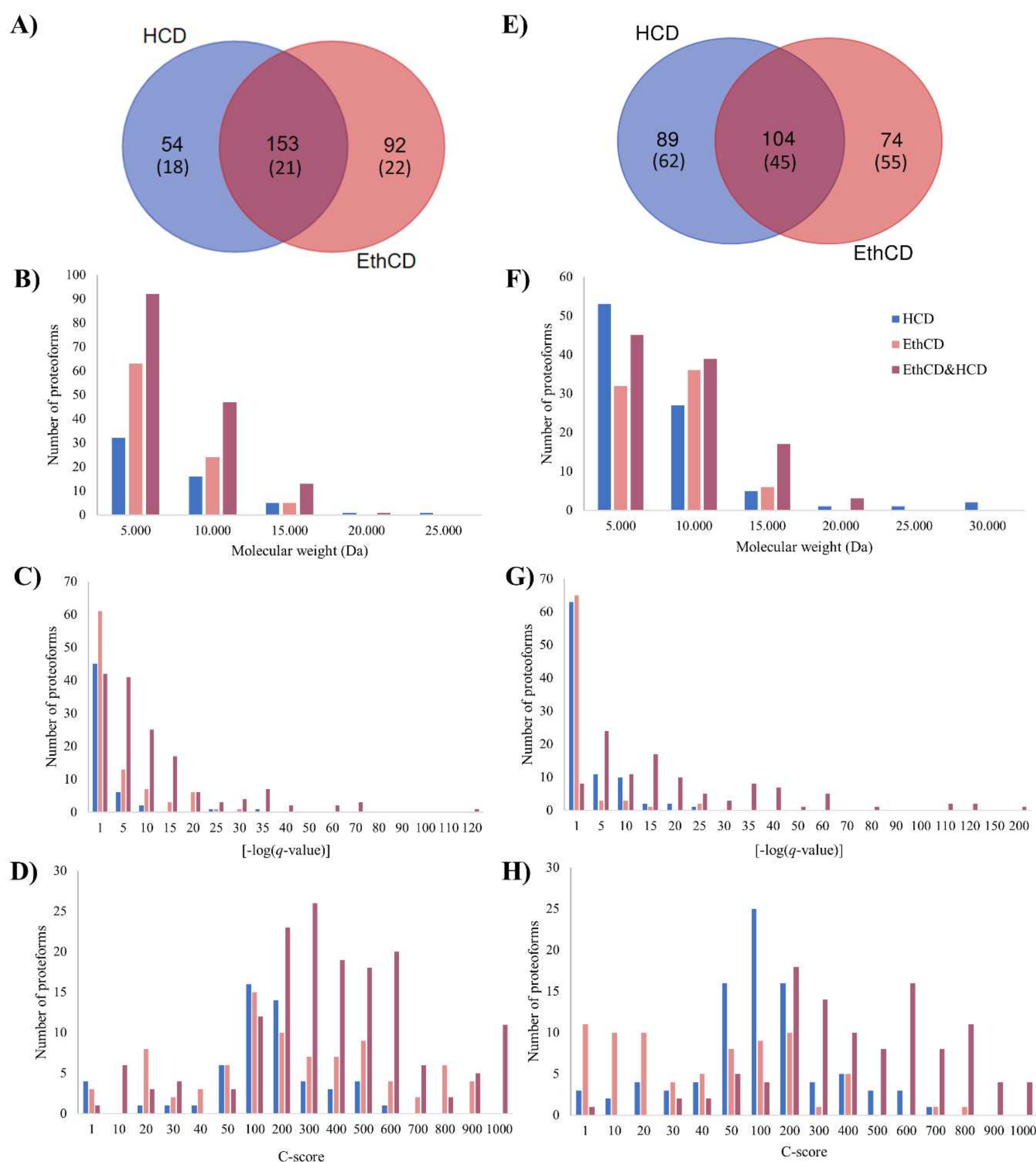


Figure 2. Venn diagram showing the number of proteoforms detected in bovine seminal plasma (A) and sperm (E). Three fractions from seminal plasma and sperm proteins were obtained by high energy collision dissociation (HCD) and electron-transfer/higher-energy collision dissociation (EthCD) and analyzed using top-down mass spectrometry. Numbers in parentheses correspond to the overlapped and unique proteins detected by the fragmentation methods. Molecular weight (Da), [-log(Q-value)] and C-score for seminal plasma (B, C and D) and sperm (F, G, H) proteoforms detected by HCD, EthCD and both HCD and EthCD.

Seminal plasma proteoforms

A total of 222 and 247 proteoforms were identified in the seminal plasma of HF and LF bulls, respectively, and 170 proteoforms were common to both groups (Supplementary table 1: ST1). The majority of all seminal proteoforms (87%) belong to nine proteoform families, being almost 50% (n = 145) from the c-type natriuretic peptide (NPPC) proteoform family (Figure 3) and nearly 15% (n = 46) of seminal plasma proteoforms belong to the caltrin family (Figure 4). Osteopontin, in turn, appeared in the bovine seminal plasma as 27 truncated proteoforms containing 12 to 109 amino acid residues, with one OPN proteoform with phosphorylation on serine 106 (Figure 5).

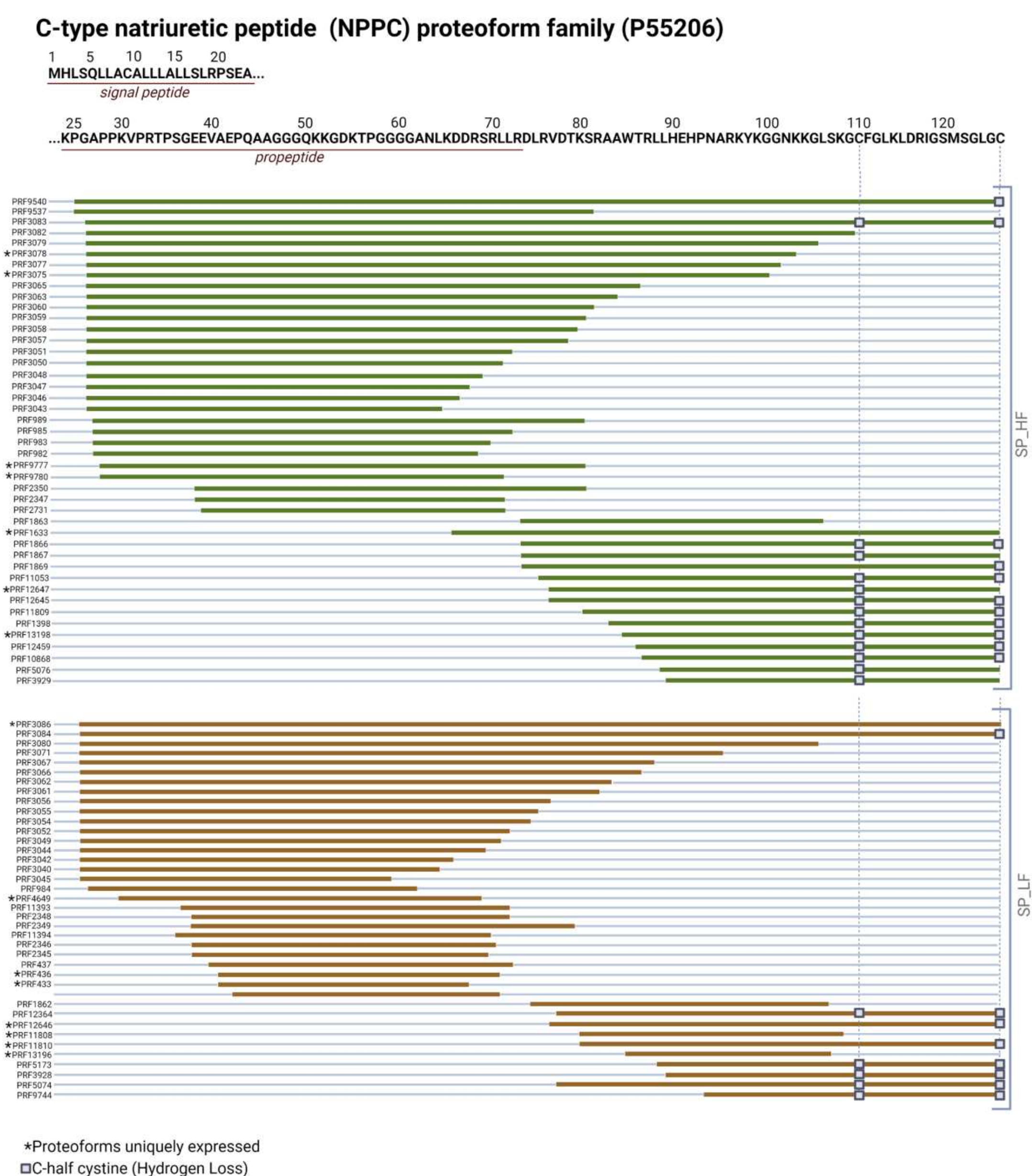


Figure 3. C-type natriuretic (NPPC) truncated proteoform profile of uniquely (*) and differentially abundant in the seminal plasma (SP) of bulls with high (HF; green) and low (LF; orange) freezability status. The detailed list of proteoforms can is presented in the Supplementary Data ST1.

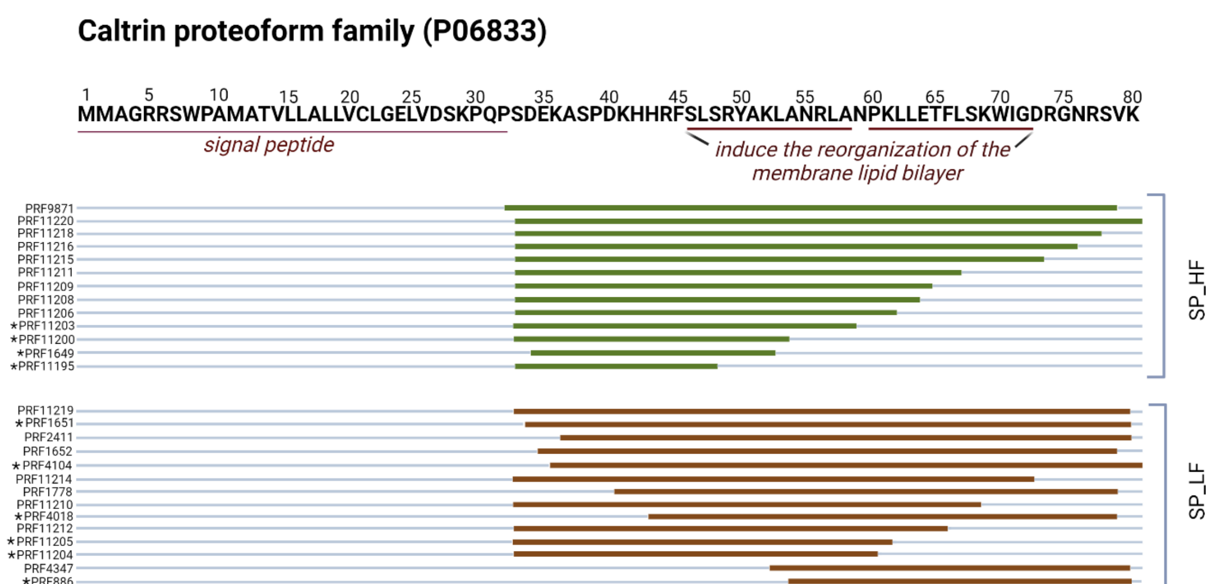


Figure 4. Caltrin truncated proteoform profile of uniquely (*) and differentially abundant in the seminal plasma (SP) of bulls with high (HF; green) and low (LF; orange) freezability status. The detailed list of proteoforms can is presented in the Supplementary Data ST1.

Osteopontin (OPN) proteoform family (P31096)

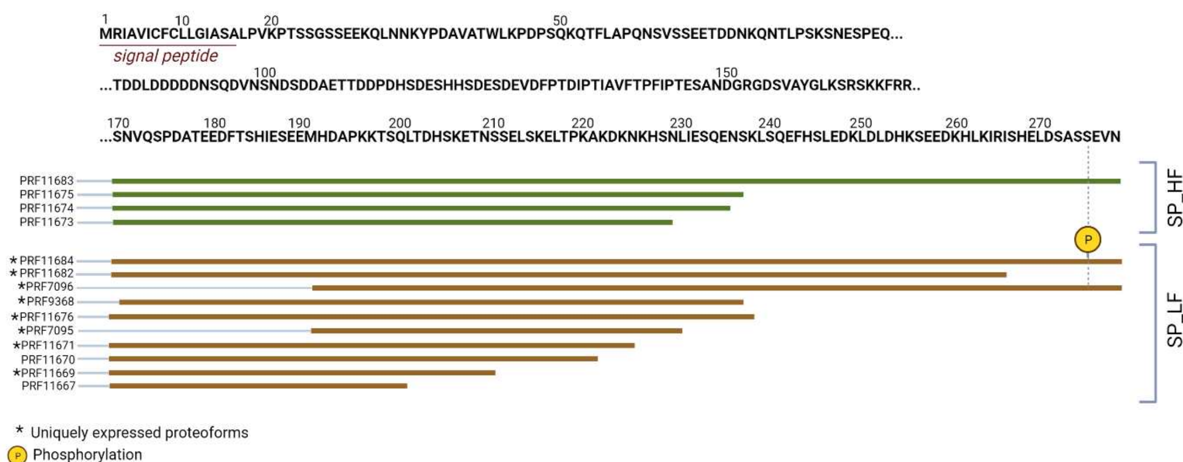


Figure 5. Osteopontin (OPN) truncated proteoform profile of uniquely (*) and differentially abundant in the seminal plasma (SP) of bulls with high (HF; green) and low (LF; orange) freezability status. The detailed list of proteoforms can is presented in the Supplementary Data ST1.

We describe two proteoforms of intact BSP1 (PDC-109), one of them with half cystine residues (C69 and C94), and three BSP1 proteoforms with truncations from the N-terminus containing the signal sequence. There were six BPS3 proteoforms identified in the bull seminal secretion, including one intact proteoform with eight half cystine residues (C29, C43, C53, C66,

C74, C88, C100 and C115) and five truncated proteoforms. One of these truncated BSP3 proteoforms came without any of the two fibronectin II domains. The remaining proteoforms in the bovine seminal plasma are related to pro adrenomedullin, procathepsin L, clusterin and beta-2 microglobulin proteoform families, among others (Supplementary table 1: ST1).

Fifteen proteoforms were uniquely expressed in the seminal plasma of HF bulls, considering the molecules detected in all three technical replicates obtained from HCD and EThcD fragmentations. Among these 15, eight proteoforms related to the NPPC family, four to the caltrin family and one proteoform of beta-defensin 10, procathepsin L and seminal ribonuclease family (Table 2). There were 25 proteoforms uniquely detected in the seminal plasma of LF bulls, considering all three technical replicates. Of these 25, nine protoforms belonged to the NPPC family and six, to the caltrin family. Additionally, eight OPN proteoforms, one pro adrenomedullin proteoform and one proteoform of tubulin polymerization-promoting protein family member 2 (TPP2) were unique to the seminal plasma of LF bulls (Table 2).

There were 107 seminal plasma proteoforms common to HF and LF bulls but with different abundances [Log2(FC) > 1.5] between these two groups, considering the molecules detected in at least two technical replicates obtained from HCD and EThcD fragmentations. Fifty-five proteoforms had greater expression in the seminal plasma of HF bulls, all belonging to OPN, BSP3, B2M, NPPC, caltrin and pro-ADM proteoform families. Among those 55, there were 45 proteoforms with 100-fold increase [Log2(FC) = 6.64] in HF bulls in comparison with LF bulls. Fifty-two proteoforms were less prevalent in the seminal plasma of HF bulls, which belong to BSP3, TPPP2, TB4, pro-ADM, ACBP, polyUb-B, OPN, SERPINA5, NPPC and caltrin proteoform families. Among those 52, there were 46 molecules showing 100-fold [Log2(FC) = -6.64] decreases in the seminal plasma of HF in comparison with LF bulls (Figure 6; Supplementary table 1:ST1).

Uniquely expressed proteoform families in the seminal plasma of HF and LF bulls were used as seed proteins to reconstruct networks of high-confident protein interactors, biological pathways, molecular functions and biological processes. DNA-directed RNA polymerases I, II, and III subunit RPABC2 (POLR2F) and NEDD8 were the most connected proteins in the seminal plasma of HF bulls, while polyubiquitin (UBB), calmodulin (CALM) and ATP synthase subunit epsilon, mitochondrial (ATP5F1E) were those most connected in LF ones (Figure 7A).

Table 2. Seminal plasma proteoforms uniquely identified in Holstein bulls with high (HF) or low freezability (LF) status.

PRF	Protein Accession	Description	Sequence	Modification	Theo. Mass [Da]	C-Score	[-Log(q-value)]
Proteoforms expressed uniquely in seminal plasma of bulls with high freezability (HF) status							
10265	P46168	Beta-defensin 10	QGVRSYLSCWGNRGICLLNRCPGRMRQIGTCL APRVKCCR	Q1(2-pyrrolidone-5-carboxylic acid (Gln)); C9(half cystine); C16(half cystine); C21(half cystine); C31(half cystine); C38(half cystine); C39(half cystine)	4500.1840	115.72	7.86
11200	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLA		2542.3041	226.97	4.17
11195	P06833	Caltrin	SDEKASPDKHHRFSLS		1839.8864	174.22	3.14
11203	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLAN		3110.6122	150.48	2.68
1649	P06833	Caltrin	DEKASPDKHHRFSLSRYAKL		2384.2349	123.63	2.37
9777	P55206	C-type natriuretic peptide	PPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGG GGANLKDDRSRL		4409.2800	449.48	9.73
9780	P55206	C-type natriuretic peptide	PPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLRVDTKS		5592.9473	218.04	5.02
3075	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTP GGGGANLKDDRSRLLRDLRVDTKSRAAWTRL LHEHPNARKYKG		8006.2465	548.53	4.52
3078	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTP GGGGANLKDDRSRLLRDLRVDTKSRAAWTRL LHEHPNARKYKGGNK		8305.4059	378.34	4.42
13198	P55206	C-type natriuretic peptide	WTRLLHEHPNARKYKGGNKKGLSKGCFGLKL DRIGSMSGLGC	C26(half cystine); C42(half cystine)	4610.4050	254.06	4.26
1633	P55206	C-type natriuretic peptide	DDRSRLLRDLRVDTKSRAAWTRLLHEHPNARK YKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C45(half cystine); C61(half cystine)	6834.6198	246.75	3.31
12647	P55206	C-type natriuretic peptide	VDTKSRAAWTRLLHEHPNARKYKGGNKKGLS KGCFGLKLDRIGSMSGLGC	C34(half cystine)	5439.8581	29.74	2.49
3929	P55206	C-type natriuretic peptide	HEHPNARKYKGGNKKGLSKGCFGLKLDRIGSM SGLGC	C21(half cystine)	3942.0165	40.29	1.66
10692	P25975	Procathepsin L	RAVWEKNKKIIDLHNQEYSEGKHGFR		3181.6534	273.64	2.57
4162	P00669	Seminal ribonuclease	KESAAAKFERQHMD		1646.7835	426.28	2.68
Proteoforms expressed uniquely inthe seminal plasma of bulls with low freezability (LF) status							
886	P06833	Caltrin	ANRLANPKLLETFLSKWIGDRGNRSV		2954.6203	546.84	12.91
11205	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPK		3335.7599	342.20	6.84
4104	P06833	Caltrin	KASPDKHHRFSLSRYAKLANRLANPKLLETFLS KWIGDRGNRSV		5076.7751	138.28	4.21
1651	P06833	Caltrin	DEKASPDKHHRFSLSRYAKLANRLANPKLLETF LSKWIGDRGNRS		5221.7762	42.30	3.97

11204	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANP		3207.6650	259.98	3.62
4018	P06833	Caltrin	HRFSLSRYAKLANRLANPKLLETFLSKWIGDRG NRSV		4313.3774	133.44	3.14
433	P55206	C-type natriuretic peptide	AEPQAAGGGQKKGDKTPGGGGANLKDDR		2679.3325	824.66	8.99
436	P55206	C-type natriuretic peptide	AEPQAAGGGQKKGDKTPGGGGANLKDDRSRL		3035.5497	354.61	5.87
13196	P55206	C-type natriuretic peptide	WTRLLHEHPNARKYKGGNKKGL		2602.4357	197.45	3.45
3086	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTP GGGGANLKDDRSRLLRDLRVDTKSRAAWTRL LHEHPNARKYKGGNKKGLSKGCFGLKLDRIGS MSGLGC		10613.5913	273.15	3.33
4649	P55206	C-type natriuretic peptide	KVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGG ANLKDDRS		3945.9893	197.50	2.68
12646	P55206	C-type natriuretic peptide	VDTKSRAAWTRLLHEHPNARKYKGGNKKGLS KGCFGLKLDRIGSMSGLGC	C50(half cystine)	5439.8581	24.24	2.57
9806	P55206	C-type natriuretic peptide	PQAAGGGQKKGDKTPGGGGANLKDDRSRL		2835.4700	289.50	2.49
11808	P55206	C-type natriuretic peptide	SRAAWTRLLHEHPNARKYKGGNKKGL		2987.6431	132.52	2.49
11810	P55206	C-type natriuretic peptide	SRAAWTRLLHEHPNARKYKGGNKKGLSKGCF GLKLDRIGSMSGLGC	C46(half cystine)	4996.6201	21.35	1.71
11667	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQ		3473.5165	716.63	11.28
7095	P31096	Osteopontin	MHDAPKKTSQLTDHSKETNSSELSKELTPKAK DKNKHSN		4389.1983	623.88	8.60
11670	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLT DHSKETNSSELSKELTPK		5698.6341	381.75	8.17
11676	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLT DHSKETNSSELSKELTPKAKDKNKHSNLIESQE NSK		7749.6736	394.63	7.18
9368	P31096	Osteopontin	NVQSPDATEEDFTSHIESEEMHDAPKKTSQLTD HSKETNSSELSKELTPKAKDKNKHSNLIESQEN S		7534.5466	84.25	3.75
11682	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLT DHSKETNSSELSKELTPKAKDKNKHSNLIESQE NSKLSQEFHSLEDKLDLDHKSEEDKHLKIR		11020.3369	216.10	2.68
11684	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLT DHSKETNSSELSKELTPKAKDKNKHSNLIESQE NSKLSQEFHSLEDKLDLDHKSEEDKHLKIRISHE LDSASSEVN	S106(O-phospho-L-serine)	12468.9190	4.01	2.42
7096	P31096	Osteopontin	MHDAPKKTSQLTDHSKETNSSELSKELTPKAK DKNKHSNLIESQENSKLSQEFHSLEDKLDLDHK SEEDKHLKIRISHELDSASSEVN		10056.9912	191.35	2.37
12377	O62827	Pro-adrenomedullin	TLIRPQDVKGASRSPQASSPDAARIRV		2875.5740	227.57	3.14
9449	Q3T077	Tubulin polymerization-promoting protein family member 2	PATTGVTKATTVGGVSRLTDTSKYTGTHKERF DESGKGKGIAGREDVTDNSGYVSGYKGAGTY DKKGSN		7115.5097	493.813 2607	8.63

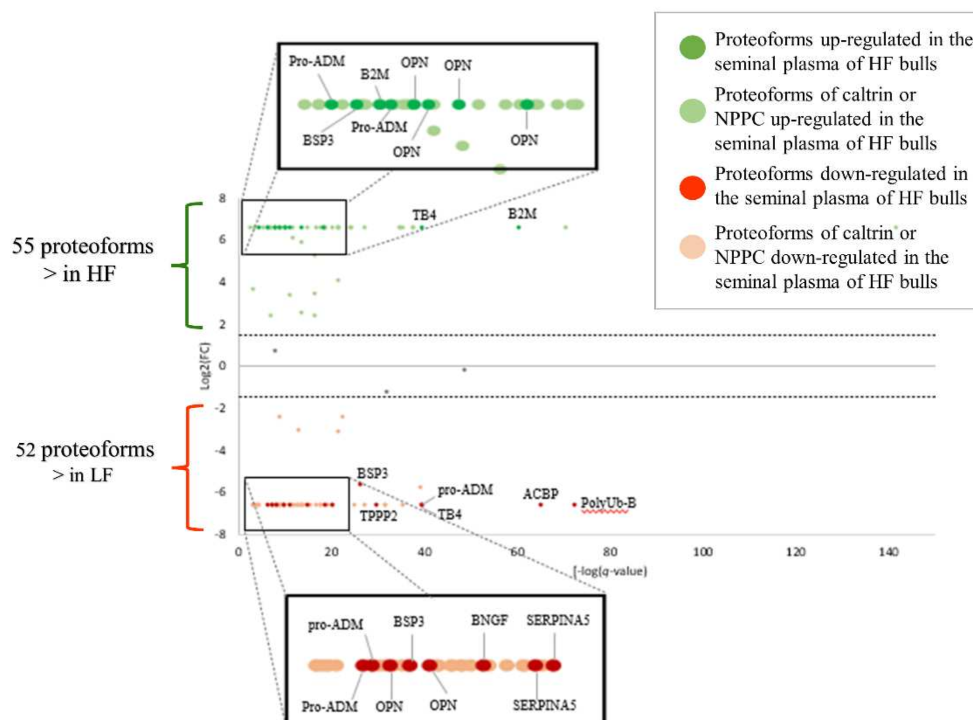
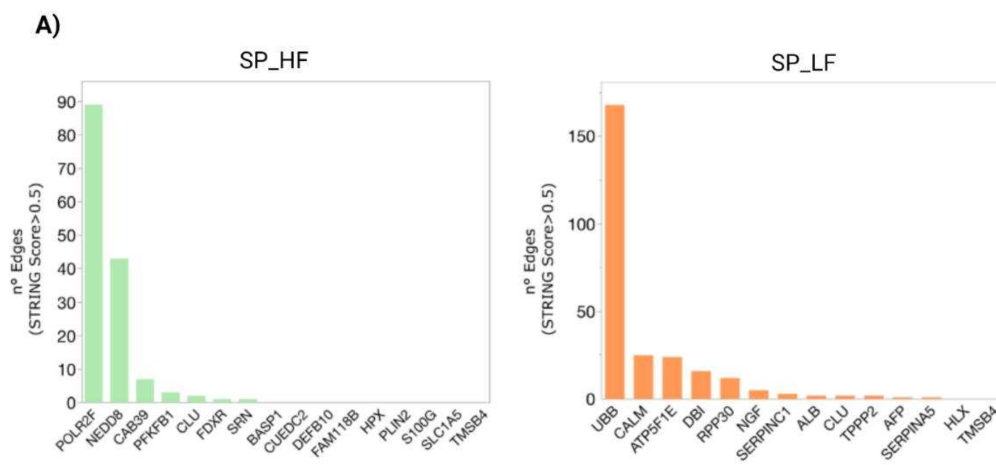


Figure 6. Proteoforms differentially abundant in the seminal plasma of bulls with low (LF) and high (HF) semen freezability phenotypes, as determined by top-down mass spectrometry. Proteoforms were present in at least two technical replicates obtained by HCD and EThcD fragmentations. The x-axis represents the significance calculated by the negative logarithmic function of *q*-values. The y-axis shows the fold-change based on proteoforms of HF and LF bulls (HF/LF ratio). The list of all seminal plasma proteoforms is shown in Supplementary table 1:ST1.

Enriched protein interactors of uniquely expressed seminal proteoforms in HF bulls were involved in transcription, cytosolic DNA-sensing and proteolysis with a specific reference to neddylation. Ubiquitin-mediated proteolysis was more enriched in the seminal plasma of LF bulls, along with electron transport chain/thermogenesis, ribosome/RNA transport and calcium and BDNF signaling pathways (Figure 7B; Supplementary table 1:ST2).



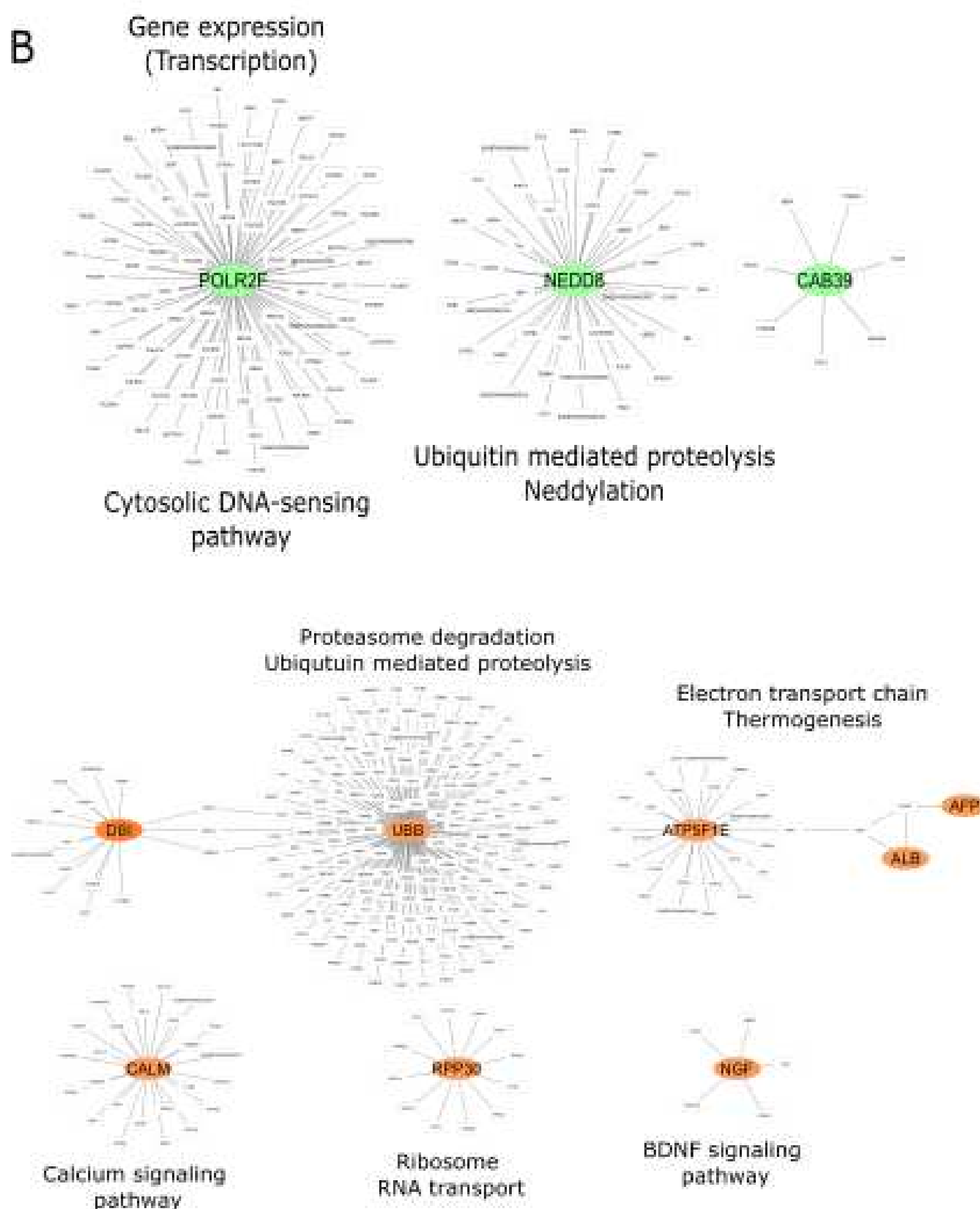


Figure 7. *In silico* reconstruction of PPI networks using the uniquely expressed proteoforms as seed proteins/genes. A) Number of edges/interactions (degree centrality) for each seminal plasma protein (proteoform) in high (HF; green) and low (LF; orange) bulls. B) PPI network reconstructed considering uniquely expressed seminal plasma proteoforms in HF (green) and LF (orange) bulls. The most enriched pathways (from KEGG, Reactome and WikiPathways) for each functional module and the complete list of proteins are shown in Supplementary table 1:ST2.

Sperm proteoforms

A total of 176 and 202 proteoforms were identified in the sperm of HF and LF bulls, respectively, with 111 proteoforms common to both HF and LF phenotypes. The most common proteoforms detected in the sperm belonged to the NPPC (n = 21), caltrin (n = 21) and cylicin

(n = 12) family. All NPPC proteoforms were truncated, with four of them with half-cystine modifications, and all caltrin proteoforms were truncated as well. Cylicin proteoforms were truncated and two of them had N-terminal alpha-amino acetylated residues. Four BSP3 proteoforms were found in the sperm, all truncated and one with a half cystine modification. One intact BSP1 proteoform with eight half cystine modifications and one truncated BSP1 were detected in the bovine sperm. Other predominant proteoforms belonged to the calmodulin, dynein, glyceraldehyde-3-phosphate dehydrogenase, among others (Supplementary table 1: ST3).

Considering the molecules detected in three technical replicates, 15 sperm proteoforms were unique in HF bulls and other 15 were uniquely expressed in LF bulls (Table 3). The specific HF sperm proteoforms belong to 10 proteoforms families, four of the NPPC family, three of the cylicin-2 family and one proteoform of glyceraldehyde-3-phosphate dehydrogenase, testis-specific, cytochrome b-c1 complex subunit 9, presenilins-associated rhomboid-like protein, mitochondrial, ubiquinone biosynthesis O-methyltransferase, mitochondrial, collagen alpha-1(X) chain, fibronectin type III domain-containing protein 8, zinc finger protein 474 and IQ domain-containing protein F1 family. The 15 proteoforms unique to sperm cells of LF bulls relate to 14 proteoform families: NPPC, caltrin, cylicin-2, tubulin beta 4B and 5 chains, ATP synthase-coupling factor 6, mitochondrial, cytochrome b-c1 complex subunit 2, mitochondrial, cytochrome c oxidase subunit 5B, mitochondrial and malate dehydrogenase, mitochondrial, actin-related protein T2, dynein light chain 1, cytoplasmic, fibronectin type III domain-containing protein 8, peripherin and histone H2A type 1 (Table 3).

There were 111 proteoforms commonly identified in the sperm of HF and LF bulls, with 15 appearing in different abundances [Log2(FC) > 1.5] in the two groups and present in at least two technical replicates. Eight proteoforms were up-regulated in the sperm of HF bulls, including one proteoform of GAPDHS, testis-specific was [Log2(FC) = 6.64] and seven proteoforms with Log2(FC) values from 1.53 to 3.76. Seven proteoforms were down-regulated in the sperm of HF bulls, including one truncated proteoform of testis-expressed protein 43 [Log2(FC) = 6.64)] and other six proteoforms with abundance fold-changes varying from 1.52 to 2.39 (Figure 8; Supplementary table 1: ST3).

Table 3. Sperm proteoforms uniquely identified in Holstein bulls with high (HF) or low freezability (LF) status.

PRF	Protein Accession	Description	Sequence	Modification	Theo. Mass [Da]	C-Score	[-Log(*q*-value)]
Proteoforms expressed uniquely in sperm of bulls with high freezability (HF) status							
2370	P23206	Collagen alpha-1(X) chain	GEMGHCTPCRPGER-GLPGPQGPTGPPGPPGVGKRGENGLPGQPGLKGDQGVPGERGAAGPSGPQGPPGEQGPEGIGKP-GAPGIPGQPGIPGMKGQPGAPGTAG	C6(half cystine)	9548.6787	13.52	1.79
3633	P55206	C-type natriuretic peptide	LLHEHPNARKYKGG-NKKGLSKGCFGLKLDRIGSMSGLGC	C23(half cystine); C39(half cystine)	4167.1768	139.91	3.80
9250	P55206	C-type natriuretic peptide	TPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSR		3621.7731	219.99	3.42
201	P55206	C-type natriuretic peptide	AAWTRLLHEHPNARKYKGG-NKKGLSKGCFGLKLDRIGSMSGLGC	C28(half cystine); C44(half cystine)	4752.4792	98.58	2.31
1502	P55206	C-type natriuretic peptide	DLRVDTKSRAAW-TRLLHEHPNARKYKGG-NKKGLSKGCFGLKLDRIGSMSGLGC	C37(half cystine); C53(half cystine)	5823.06243	205.37	23.52
2228	Q28092	Cylicin-2	FQKINFGAYDNYVPVSELSKKSWNQQHFALVFPKPPRPGKRRR		5129.7369	457.30	11.59
2227	Q28092	Cylicin-2	FQKINFGAYDNYVPVSELSKKSWNQQHFALVFPKPPRPGKRR		4973.6358	283.85	4.30
9073	Q28092	Cylicin-2	SWNQQHFALVFPKP-PRPGKRRRSKPSLLQENTSPKYDAEKLRGDRKQPLWMHR		6391.4155	152.29	1.99
9349	P00130	Cytochrome b-c1 complex subunit 9	VAPTLTARLYSLLFRRT-STFALTIVVGALFFERAFDQGADAIYE-HINEGKLWKHIKHKYENKE		7321.8986	756.44	60.54
9416	Q2YDH8	Fibronectin type III domain-containing protein 8	VGDGEEAM-LKKETLNVLNALDQMSKPFPNPKSMNR		3900.9536	319.28	8.63
9400	Q2KJE5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	VEAEPEPPAQPQPQPEP-IKEEVPPPPPPPPAP-KKVRELIVGINGFGR		5048.7128	591.57	11.07
2539	Q3SYS7	IQ domain-containing protein F1	GLVENSLPSEPSPT-LEKAEAQQENKAATKGADDADKR		3893.9243	216.24	5.41
2237	Q2KHV4	Presenilins-associated rhomboid-like protein, mitochondrial	FRKAPRKVEPRRSDTSSEAYKR		2663.4368	249.66	4.00
7171	Q3T131	Ubiquinone biosynthesis O-methyltransferase, mitochondrial	NRIMWFKSYSITFACLNWMKSYRLP		3154.5707	60.66	1.86

8996	A6QQM4-1	Zinc finger protein 474	STLQQSFHHSKEPTFLINQAVLFGE-SHSSFLPEIERD		4255.0974	22.43	1.57
Proteoforms expressed uniquely in sperm of bulls with low freezability (LF) status							
9245	Q2TA43	Actin-related protein T2	TPLTGANQK-KYFVGEEALHRHEVLQLHYPIER		3772.9802	213.18	3.48
2917	P02721	ATP synthase-coupling factor 6, mitochondrial	KELDPVQKLFVDKIREYRTKRQTSGG-PVDAGPEYQQDLDRELF-KLKQMYGKADMNTFPNFT-FEDPKFEVVEKPQS		8838.4531	323.71	13.98
9748	P06833	Caltrin	YAKLANRLANPKLLETFLSKWIG-DRGNRSV		3429.8997	465.98	14.13
8467	P06833	Caltrin	SDEKASPDKHHRFSLSR		1995.9875	465.49	10.36
2312	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLR		4806.5237	165.38	4.55
8685	Q28092	Cylicin-2	SKPSLLQENTSPKYDAEKLRG-DRKQPLWMHR		3707.9318	229.22	6.43
8705	P23004	Cytochrome b-c1 complex subunit 2, mitochondrial	SLKVAPKVKATEAPAGVP-PHPQDLEFTRLPNGLVIASLEN-YAPASR		4848.6290	291.00	6.35
8606	P00428	Cytochrome c oxidase subunit 5B, mitochondrial	SGGGVPTDEEQATGLEREVMLAARKGQDPYNILAPKATSGTKED		4586.2559	286.38	3.23
1189	P61285	Dynein light chain 1, cytoplasmic	CDRKA-VIKNADMSEEMQQDSVECATQAL-EKYNIEKDIAAHIKKEFDKKYNPT-WHCIVGRN-FGSYVTHETKHFIYFYLGQVAILLFKSG	N-term(alpha-amino acetylated residue); S87(O-phospho-L-serine)	10350.0444	29.04	7.42
954	Q2YDH8	Fibronectin type III domain-containing protein 8	ASETFYKVGDGEEAM-LKKETLNVLNALDQMSKPFPNPKSMNRTVTTK	N-term(alpha-amino acetylated residue)	5299.6567	483.18	12.86
8636	P0C0S9	Histone H2A type 1	SGRGKQGGKARAKAKTRSSR	N-term(alpha-amino acetylated residue)	2128.2050	181.67	3.23
620	Q32LG3	Malate dehydrogenase, mitochondrial	AKVAVLGASGGIGQPLSLLLKNS-PLVSRLTLYDIAHTPGVAADLSHIETR		5118.8557	780.54	28.43
2520	A6QQJ3	Peripherin	GLRSSVSSTSYRRTFGPPPS-LSPGAFSYSSSSRFSSSRLL		4255.1410	16.81	3.23
6201	Q3MHM5	Tubulin beta-4B chain	MREIVHLQAGQCGNQIGAKFWEV-ISDEH-GIDPTGTYHGDSDLQLERINVYYNEATGGKYVPR		6974.3569	456.41	16.21
3031	Q2KJD0	Tubulin beta-5 chain	KLAVNMVPFPRLHFFMPG-FAPLTSRGSQQYR		3591.8748	321.86	6.80

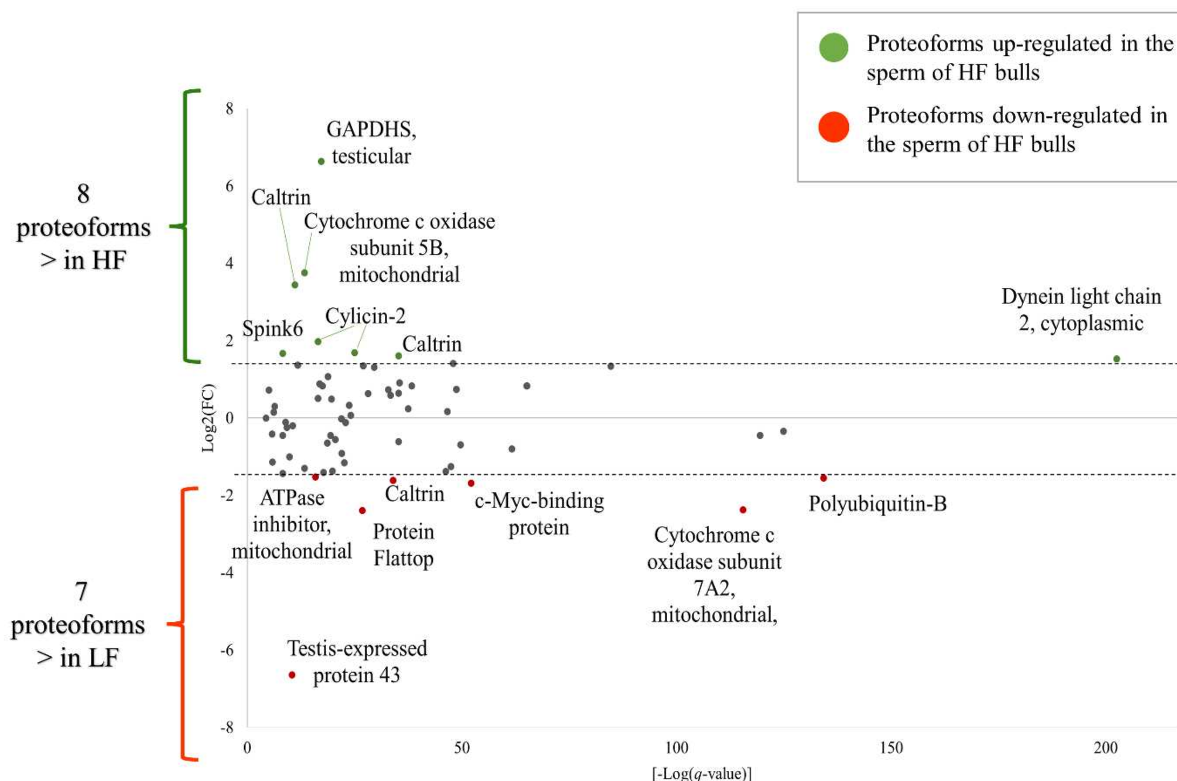


Figure 8. Proteoforms differentially abundant in the sperm of bulls with low (LF) and high (HF) semen freezability phenotypes, as determined by top-down mass spectrometry. Proteoforms were present in at least two technical replicates obtained by HCD and EThcD fragmentations. The x-axis represents the significance calculated by the negative logarithmic function of *q*-values. The y-axis shows the fold-change based on protoforms of HF and LF bulls (HF/LF ratio). The list of all sperm proteoforms is shown in Supplementary table 1:ST3.

Using uniquely expressed sperm proteoform families of HF and LF bulls as seed proteins to reconstruct networks of high-confident protein interactors, we detected cytochrome b-c1 complex subunit 9 (UQCR10), centromere protein x (CENPX), heat shock factor protein 1 (HSPD1) and interleukin-7 (IL7) as the most connected proteins in the HF group. On the other hand, NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial (NDUFS6), ATP synthase subunit beta, mitochondrial (ATP5F1B), TUBA1B and BRF2 were those most connected molecules of the LF phenotype (Figure 9A).

The enrichment of mitochondrial metabolic pathways was pronounced in HF sperm, including such as fatty acid metabolism and TCA cycle coupled to respiration electron chain. In this context, we also found proteoforms involved in ribosome, spliceosome, cytokine-cytokine receptor interaction, and a module enriched in cell cycle/Fanconi anemia pathway/deubiquitination (Figure 9B, Supplementary table 1: ST4). Differently, at metabolic level, LF sperm was characterized by the enrichment of proteins involved in cysteine and methionine metabolism, as well as in ATP synthesis by chemiosmotic coupling and heat

production by uncoupling proteins. In addition, a series of pathways, such as neutrophil degranulation, phagosome and cytosolic DNA-sensing pathway, involved in the immune response, were enriched in the sperm of HF bulls.

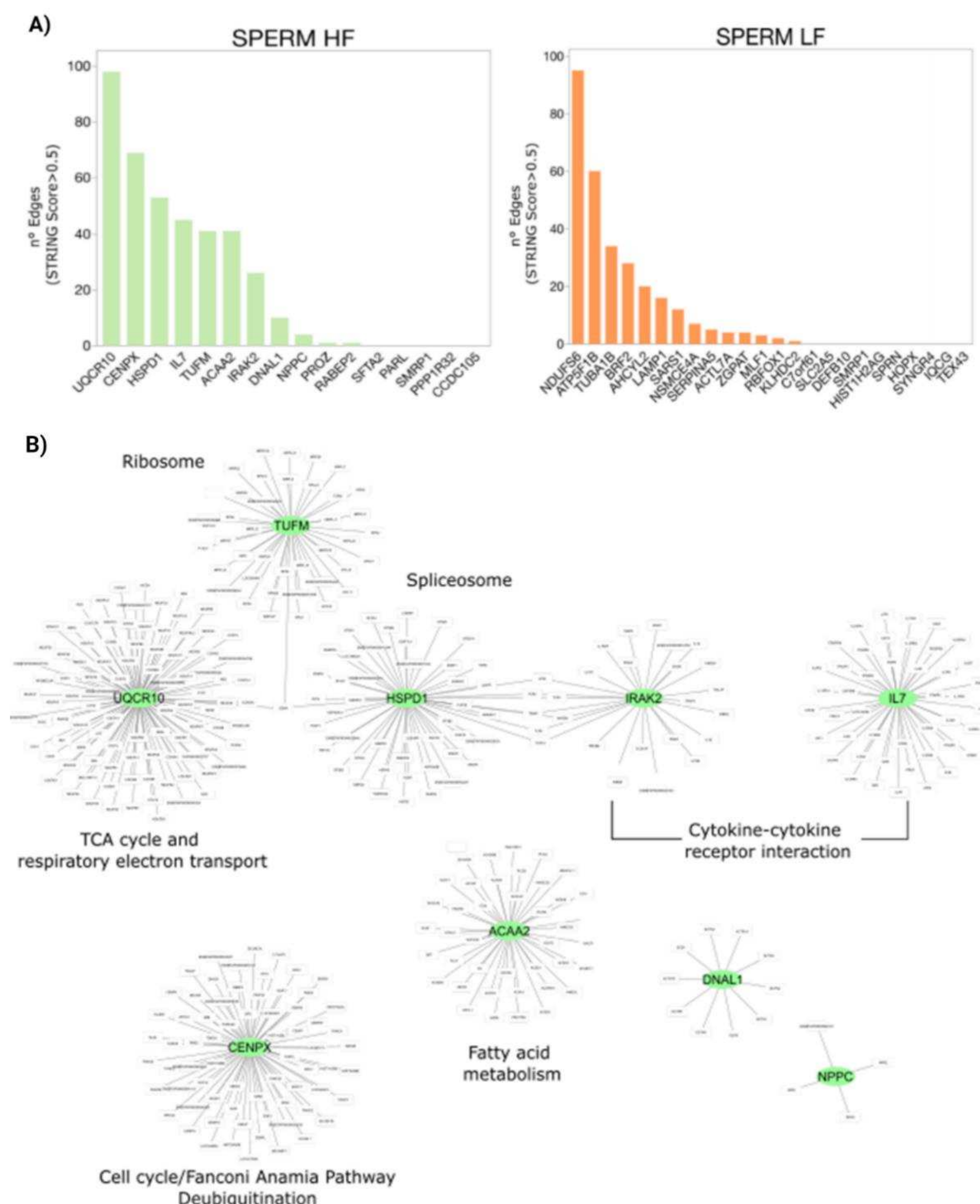


Figure 9. *In silico* reconstruction of PPI networks using the uniquely expressed proteoforms as seed proteins/genes. A) Number of edges/interactions (degree centrality) for each sperm protein (proteoform) in high (HF; green) and low (LF; orange). B) PPI network reconstructed by considering uniquely expressed sperm proteoforms in HF (green) and LF (orange) bulls. The most enriched pathways (from KEGG, Reactome and WikiPathways) for each functional module and the complete list of proteins are shown in Supplementary table 1:ST4.

DISCUSSION

The present study brings novel information about the proteoform atlas of bovine seminal plasma and spermatozoa, using methods of protein separation and label-free top-down mass spectrometry. Furthermore, we established that proteoform landscapes of both seminal plasma and sperm cells have significant differences in bulls with contrasting semen freezability phenotypes. This is the first characterization of seminal plasma and sperm proteoform atlas of any species.

Separation of seminal plasma and sperm proteins by tandem size exclusion chromatography allowed the characterization of proteoforms with up to 30 kDa, combining electron transfer and HCD generated both b/y and c/z ions, greater ion fragmentation and enriched MS/MS data. EThcD is a suitable fragmentation method to characterize proteoforms with ambiguous phosphorylation sites and improves the localization of other PTMs such as ubiquitination, glycosylation and acetylation [62]. Here we show that application of HCD and EThcD was more reliable for the identification of seminal plasma and sperm proteoforms than the use of a single fragmentation method, based on *q*-values and C-scores.

The majority of proteoforms identified in the bovine seminal plasma and sperm appeared as truncated molecules. Protein truncations are irreversible modifications caused by cleavage, alternative translation, or alternative splicing, generating diversity to the repertoire of protein functions and structures [63]. Similar to these results, our previous investigation [27] reported that more than 70% of proteoforms found in the seminal plasma of Angus bulls were truncated. As well known, reproductive fluids of bulls express a diverse range of proteases [12, 25-27, 35, 64] and we assume that such proteases cleave newly secreted proteins as part of their activation or inactivation.

As summarized in Figure 10, males with contrasting semen freezability have distinct seminal plasma and sperm proteoform landscapes. The HF profile consists of nine seminal plasma and six sperm proteoform families. On the other hand, the profile of LF bulls was characterized by 10 seminal plasma and 12 sperm proteoform families. In some cases, HF and LF seminal plasma and sperm profiles contained different proteoforms of the same protein, suggesting that truncations and PTMs have actual importance for sperm resistance to cold shock.

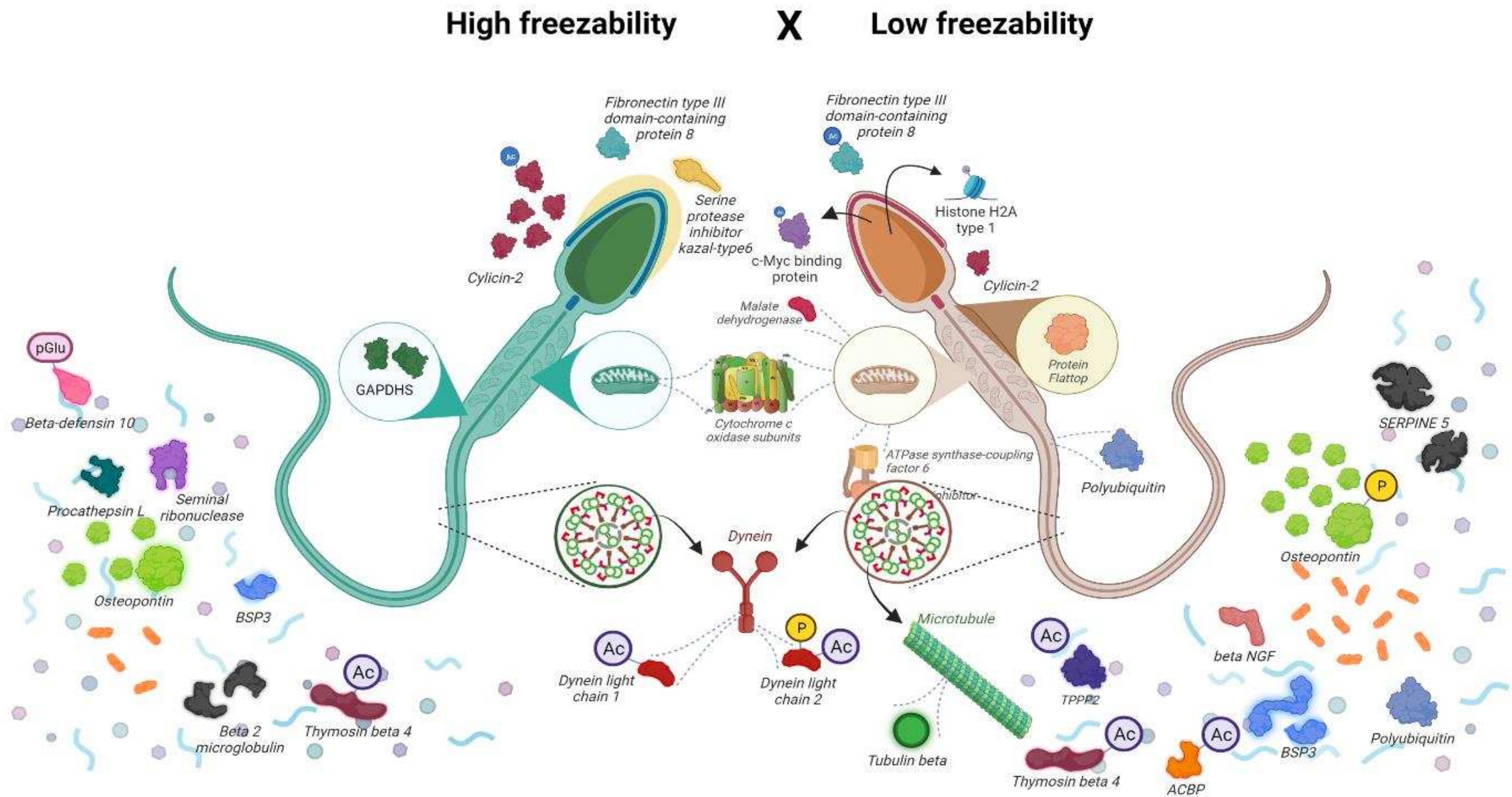


Figure 10. Major proteoforms characterizing the seminal plasma and sperm of bulls with low (LF) and high semen freezability (HF) phenotypes. Proteoforms were identified by high energy collision dissociation (HCD) and electron-transfer/higher-energy collision dissociation (EthCD), followed by top-down mass spectrometry. Only proteoforms detected in at least two fractions of the fragmentation methods are shown. Methods allowed the identification of proteins and proteoforms under 30 kDa

In our study, in silico analyses indicated that seminal and sperm proteoforms of HF and LF bulls belonged to specific protein hubs. Proteoform families of HF seminal fluid were linked to transcription, cytosolic DNA-sensing and proteolysis, while ubiquitin-mediated proteolysis, electron transport chain/thermogenesis, ribosome/RNA transport and calcium and BDNF signaling pathways were enriched in the seminal plasma of LF bulls. Enrichment of mitochondrial metabolic pathways was pronounced in HF sperm, as well as ribosome, spliceosome, cytokine-cytokine receptor interaction, and cell cycle/Fanconi anemia pathway/deubiquitination. Sperm of LF bulls related to cysteine and methionine metabolism, ATP synthesis and heat production. Conversely, pathways of neutrophil degranulation, phagosome and cytosolic DNA-sensing pathway were enriched in HF bulls. Below, we discuss how the identified seminal and sperm proteoforms relate to these hubs and gamete function.

The majority of seminal plasma proteoforms belonged to NPPC and caltrin families. Our methods detected 145 NPPC truncations, being 41 and 39 characteristics of HF and LF bulls, respectively. This obviously indicates that cleavage of native NPPC is an important process in the seminal milieu, but how and when truncated NPPC forms are made, and what roles they play in sperm physiology are still unexplored topics. NPPC is found in the seminal plasma of boars and men [65, 66], and has positive association with bull fertility, as shown in one of our previous studies that used bottom-up proteomics [25]. Rat and human sperm have NPPC receptors and NPPC from seminal plasma and uterus regulates sperm capacitation by stimulating cGMP/PKG signaling, Ca2+ uptake and tyrosine-phosphorylation [67].

Caltrin refers to a family of basic proteins with 80 amino acid residues, secreted by seminal vesicles. Caltrin binds to sperm and has antagonistic activities as regard to extracellular Ca2+ uptake [68-70]. According to this concept, caltrin inhibits Ca2+ uptake with the aid of seminal plasma anionic cofactors, such as citric and L-lactic acids, phosphatidylserine, phosphatidylglycerol and cardiolipin. Once the cofactors dissociate, caltrin assumes a new conformation and stimulates Ca2+ uptake [70].

Caltrin is also a bactericide because of the presence of SLSRYAKLANRLA and PKLLETFLSKWIG segments that induce membrane reorganization [70, 71]. Here, we described 45 seminal plasma caltrin truncations ranging from 24 to 54 amino acid residues. Three caltrin proteoforms belonged to the seminal plasma landscape of HF bulls, but only one of these contained

the SLSRYAKLANRLA peptide. The seminal fluid of LF bulls had other six caltrin proteoforms, all with at least one of those two peptides and three proteoforms with both SLSRYAKLANRLA and PKLLETFLSKWIG. Interestingly, both NPPC and caltrin functions are linked to Ca2+ uptake, a crucial event for sperm capacitation, hyperactivation and acrosome reaction. So, as multifunctional and versatile molecules are NPPC and caltrin, it is plausible to assume that truncation determines how they work, either as sperm capacitating or decapacitating factor, or as a protector against pathogens (caltrin).

Binder of sperm proteins are the most abundant protein family of the bull seminal plasma [27], coating sperm cells at ejaculation [38] to promote capacitation [72], sperm-oviduct interaction [73] and fertilization as well [32, 33]. BSPs can even damage sperm if in contact with these cells for too long or when in excessive amounts [23, 74]. Our initial studies using 2-D electrophoresis coupled with mass spectrometry clearly suggested the existence of BSP proteoforms [12, 23, 26, 35, 64] and understanding the versatility of BSPs is now coming to light because of advances in the analyses of intact proteins. In fact, we presently show, for the first time, the existence of several truncations of both BSP1 and BSP3. Proteoforms of BSP1 were not associated with semen freezability but two truncated BSP3 proteoforms were characteristic of HF bulls and the other two, prevalent in LF bulls. As we have pointed out in the case of NPPC and caltrin, it appears that post-translation processing of BSPs guides their functional attributes in bovine seminal plasma.

Proteases and protease inhibitors are among the most conserved genes in mammalian species [75]. Proteolysis is crucial for biological entities, such as semen, and activation or inhibition of proteases amplifies signaling pathways [75]. We presently identified truncated proteoforms of proteases distinctive of seminal plasma of HF (seminal ribonuclease (SRN) and procathepsin L) and LF bulls (two proteoforms from C- terminus of SERPINA5). Seminal ribonuclease has catalytic, antioxidant and immunosuppressive functions [76, 77]. Using bottom-up proteomics, we previously detected SRN as one of the seminal plasma signatures of bull high semen freezability [27], but our present study is the first to describe SRN-specific proteoform structures and their association with frozen-thawed sperm membrane integrity. In our previous studies, SERPINA5 was not differentially abundant in bulls with contrasting fertility and semen freezability [25, 27], but here two truncated SERPINA5 proteoforms were more prevalent in LF bulls. We describe, for

the first time, that proteoforms of proteolytic regulators are part of the seminal molecular atlas of bulls with proper frozen-thawed sperm viability.

A truncated beta-defensin 10 (DEFB10) with pyrrolidone carboxylic acid (pGlu) in the N-terminal and six half cystines contributed to the HF bull seminal plasma profile. Beta-defensins are immune modulators in several tissues, and the epididymis is the dominant site of their expression in male reproductive organs [78]. Beta-defensin (including DEFB10) knocked out rats showed high intracellular Ca2+ and damaged microtubule structures. In bulls, DEFB126 plays a key role in sperm motility, binds to sperm membrane and participates in sperm-zona interaction [79]. The N-terminal pGlu modification of DEFB10 proteoform was probably formed in the acidic environment of the epididymis. pGlu minimizes protein susceptibility to degradation and it is possible that this single PTM contributes to specific DEFB10 roles in the seminal milieu. Interesting that we have described DEFB10 before in seminal plasma of bulls using bottom-up approaches, but with no significant links with fertility-related phenotypes [25, 27].

Our previous gel-based proteomic studies indicated the existence of multiple OPN proteoforms in the seminal plasma of bulls [23, 35, 12, 26]. Now, we report the identification of 27 OPN truncations and eight of them, including a phosphorylated one, defined the molecular signature of LF bull seminal plasma. On the other hand, an OPN proteoform without PTMs was characteristic of the seminal plasma of HF bulls. Seminal plasma OPN is associated with bull fertility [22, 52, 24, 26] and membrane stabilization during cryopreservation [80], affects acrosome reaction, fertilization and early development of bovine embryos [28, 29]. The diversity of OPN proteoforms found in the seminal plasma obviously indicates that truncations and amino acid modifications dictate OPN´s role in sperm physiology. Osteopontin is secreted by several tissue types in addition to the male reproductive organs, being involved in cell adhesion, tissue and extracellular remodeling, immune-related events, tumor angiogenesis, metastasis and cell proliferation pathways [81-84]. All these bio-attributes of OPN and the existence of so many OPN proteoforms reported in our study raise the possibility that functions of OPN in semen go far beyond what is known today.

Thymosin beta 4 inhibits actin monomers polymerization [85] and N-terminal acetylation of thymosin beta 4 was predominant in the seminal plasma of LF bulls, while N6-acetyl-L-lysine acetylation was peculiar to HF bulls. A truncated proteoform of TPPP2 and a non-truncated proteoform (N-terminus acetylated) were part of the LF bull seminal plasma proteoform profile.

N-terminal acetylation is an irreversible PTM that regulates protein half-life, folding and interactions with other proteins or sperm membrane [86] while Lysine acetylation is a reversible PTM [87]. TPPP2 is found in the cytosol or flagellum of sperm and regulates the microtubule dynamics required for motility [88]. A non-truncated proteoform of acyl-CoA-binding protein (ACBP) without methionine and N-terminal acetylation was differentially abundant in the seminal plasma of bulls with LF phenotype. ACBP are cytosolic proteins found in different stages of spermatogenic cells and in the midpiece of ejaculated rat sperm [89] that binds to long-chain fatty acyl CoA and is a lipid transporter [90]. ACBP abundance in seminal plasma may be an indicator of low sperm quality due to membrane disruption, or that LF spermatozoa have increased fat mobilization as an energy source to ensure sperm motility and hyperactivation, caused by early sperm capacitation. Nevertheless, here we have proteoforms of cytoskeleton (TPP2 and thymosin beta 4) and cytosol components (ACBP) of sperm related to low semen freezability. Detachment of sperm proteins into the seminal fluid is a natural process, and it may also be the result of the centrifugation used to separate sperm from seminal plasma.

The molecular signature of sperm from HF and LF bulls includes proteoforms from 6 and 12 proteoform families, respectively (Figure 7). A truncation and acetylation in the N-terminus of histone H2A type 1 was detected only in the sperm of LF sires. When non-acetylated, histones compact nucleosomes due to attraction from the lysine positive charge and DNA negative charges. However, acetylation facilitates the opening of chromatin and gene expression but allows damage caused by oxidative stress [91]. This vulnerability to oxidative stress is harmful to sperm viability and would explain the presence of acetylated histone in sperm of LF animals.

There is a non-truncated c-Myc protein binding proteoform with N-terminal acetylation in LF bulls, possibly regulating apoptosis of damaged sperm. c-Myc binding protein is a transcriptional factor that regulates the activity of MYC, a family of proto-oncogenes genes. c-myc gene acts is a growth promoter [92] and overexpression of c-myc induces apoptosis of spermatocytes, causing sterility [93]. A proteoform of transcription factor IIIB (BRF2) was detected in the sperm of LF bulls. BRF2 is linked to the enrichment of the cytosolic DNA-sensing pathway, which supports the idea of some nuclear activity in sperm with lower cryoresistance. On the other hand, the proteoform of centromere protein x (CENPX) present in HF sperm contributed to the enrichment of the cell cycle/Fanconi anemia pathway/deubiquitination pathway. In fact,

FANCI, a component of the Fanconi anemia pathway and responsible for the repair of DNA cross-links, allegedly plays a role in spermatogenesis [94].

Proteoforms of cylicin-2, SPINK6 and fibronectin type III domain-containing protein 8 were part of the sperm profiles from bulls with contrasting semen freezability. A greater abundance of cylicin-2 proteoforms would be expected in HF sperm due to its contribution to the stability and protection of the genetic material and N-terminus acetylation may enhance such cylicin-2 functions. One truncated cylicin-2 proteoform was uniquely detected in LF sperm but its effect on gamete physiology is still uncertain. Cylicin-2 has actin-binding activity and plays a role in the morphogenesis of perinuclear structure during spermatogenesis [95, 96]. A SPINK6 proteoform without the signal peptide and three disulfide bonds was characteristic of sperm from HF bulls. SPINK6 is secreted by accessory sex glands, binds to the sperm, inhibits the activity of proteases such as kallikreins, trypsin and acrosin, and delays acrosome reaction in mouse sperm [97]. One truncated proteoform of fibronectin type III domain-containing protein 8 was uniquely expressed in sperm cells of HF bulls and the other, with N-terminal acetylation, in LF sperm. Fibronectin type III domain-containing proteins may participate in fertilization through the interaction of its RGD sequence with the extracellular domain of beta 1 integrin [98]. However, both HF and LF sperm proteoforms showed truncations in the portion of the protein without the RGD sequence. In this case, N-terminus acetylation may be a key factor for protective or detrimental roles of fibronectin type III domain-containing protein 8 on sperm membrane after cryopreservation.

The sperm midpiece is composed of a packed mitochondrial helical sheath that surrounds outer longitudinal fibers and axoneme [99]. We identified several truncated proteoforms of subunits of cytochrome c and b-c complex and NADH dehydrogenases in both HF and LF sperm and some of them contributed to the enrichment of fatty acid metabolism, TCA cycle and respiration electron chain pathways in HF sperm. However, proteoforms of ATPase inhibitor, ATP synthase-coupling factor 6 and malate dehydrogenase (MDH) were characteristic of LF sperm. Bull spermatozoa depend on oxidative phosphorylation for capacitation [100] and ATP yield but glycolysis is another source of energy for sperm [101]. In fact, sperm motility is arrested in knock-out mice for enzymes involved in the glycolytic pathway, even with normal mitochondrial activity [103, 104].

However, what actually fuels the sperm for both capacitation and motility it is not completely understood because ATP produced in the midpiece is not sufficient to feed the entire

sperm tail and hyperactivation [102]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDHS) catalyzes glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate during the glycolytic pathway to produce ATP. Two truncations of GAPDHS were characteristic of HF bulls indicating that these cells may have a higher ATP producing capacity as compared to LF sires.

Sperm dyneins are ATPase complexes present in the axoneme and convert ATP to mechanical force [105]. A proteoform of dynein light chain 1 was one of the LF sperm markers and a proteoform of dynein light chain 2 was more abundant in HF sperm. Both dyneins 1 and 2 had acetylation at N-terminus and serine phosphorylation. We also identified microtubular proteoforms uniquely expressed in LF sperm. Dynein phosphorylation is caused by kinase activation during sperm capacitation, which also includes alteration in the sperm membrane fluidity, negatively related to sperm cryoresistance. Protein flattop regulated basal body docking and position in ciliated cells [106]. Here we showed that a proteoform of protein flattop is more expressed in LF sperm. As far as we know, this is the first-ever association between the protein flattop and sperm membrane viability, and more studies are needed to understand the mechanisms involved in its association.

Different proteoforms of polyubiquitin-B were identified in the proteome atlas of HF and LF bovine sperm. Polyubiquitin-B is secreted from the epididymis and accessory sex glands and the reason why they appear in both seminal fluid and sperm is because, like many other seminal proteins, they strongly bind to the sperm membrane before and/or after ejaculation. Based on our personal experience, many seminal plasma proteins remain bound to ejaculated sperm even after sequential washings. Our in-silico analyses indicate that ubiquitination was enriched in the seminal fluid of LF bulls, while neddylation was enriched in the seminal plasma of HF bulls. Both signaling pathways can be activated in response to DNA double-strand breaks [107], but there is evidence of neddylation as a mitochondria structure and function regulator [108]. Sperm ubiquitination, in turn, relates to chromatin damages [109] and tags epididymal defective sperm for further proteasome degradation and phagocytosis [110], a mechanism of sperm quality control.

A truncation of βNGF, corresponding to its propeptide sequence, was prevalent in the seminal plasma of LF bulls. Cleavage of the main βNGF during its maturation may act during the folding or transport of other proteins, reports a study conducted by Eder and Fersht [112]. The truncation containing the mature βNGF was not detected in our study but βNGF contributed to the enrichment of BDNF signaling pathway. BDNF (brain-derived neurotrophic factor) affects sperm

mitochondrial activity and apoptosis, as well as sperm viability [113, 114]. Enrichment of the calcium signaling pathway, electron transport chain and thermogenesis linked to the seminal plasma profile of LF bulls supports the evidence that sperm membrane from these individuals have lower resistance to cryoinjuries due to premature activation of capacitation and metabolic processes.

The current research has granted us unprecedented information about the co-existence of proteoforms in the bovine semen, bringing additional levels of complexity to the roles of seminal fluid and sperm components. Novel insight is also provided about the diversity of PTMs and truncated seminal and sperm proteoforms and the how proteoform atlas relate to semen freezability phenotypes of bulls. These results reflect the intricacy of the semen proteome, which tends to increase exponentially as other fractions (> 30 kDa) of seminal and sperm proteoforms are scrutinized with new separation and mass spectrometry methods. The present strategies carried out with the bovine model ought to guide similar studies in other species, as proteoform signatures emerge as vital for clinical investigation, drug screening, as therapeutic targets and fertility markers.

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Conflict of interests

The authors declare no conflict of interest.

Author's contribution

A. Viana, F. Gomes, E. Memili, A. Kaya, A. Moura and J. Yates conceived the design of the study. A. Viana and F. Gomes prepared the samples for chromatography and mass spectrometry and analyzed the raw data. A. Viana, D. Silvestre and A. Moura worked on data interpretation. A. Viana and A. Moura worked on the writing of the original article. E. Topper, A. Kaya, J. Diedrich, M. Machado-Neves, E. Memili and Yates JR III provided critical revision of the article. Moura AA and Yates JR III supervised the work. All authors read and approved the final manuscript.

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ST1 _SP proteoforms: Seminal plasma (SP) proteoforms of high (HF) and low freezability (LF) bulls .

PRF ID	Protein Accession	Description	Sequence	Modification	Type	Activation type	Group	Log2 FC	C-Score	Q-value	% Residue Cleavages
592	P61603	10 kDa heat shock protein, mitochondrial	AGQAFRKFLPLFDRVLVE RSAAETVTKGGIMLPEKS QGKVLQATVVAVGSGSK GKGGEIQPVSVKVGDKV LLPEYGGTKVVLDDKDY FLFRDGDILGKYVD	K7(N6-acetyl-L-lysine);	Non-truncated	HCD/EthCD	Both	-0,22	4,0103	5,09E-49	28,0
593	P61603	10 kDa heat shock protein, mitochondrial	AGQAFRKFLPLFDRVLVE RSAAETVTKGGIMLPEKS QGKVLQATVVAVGSGSK GKGGEIQPVSVKVGDKV LLPEYGGTKVVLDDKDY FLFRDGDILGKYVD	N-Term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	Both	-0,22	37,5281	5,66E-49	28,0
5406	P49872	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1	LQKIWIPHNNGNSRLQRR RGSSIPQFTNSPTMVIMV GLPAR	S21(O-phospho-L-serine)	Truncated	EthCD	HF	6,64	45,17409	0,0149937	22,5
11750	P07107	Acyl-CoA-binding protein	SQAEFDKAAEEVKHLKT KPADEEMLFIYSHYKQAT VGDINTERPGMLDFKGK AKWDAWNELKGTSKED AMKAYIDKVEELKKKYG	N-Term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	LF	-6,64	63,73948	1,25E-65	44,7
2084	P02769	Albumin	DTHKSEIAHRFKDLGEEH FKGLVL		Truncated	HCD	LF	NA	205,9593	0,0002263	65,2
10094	Q3SZ57	Alpha-fetoprotein	QESQALAKRSCGLFQKLG EYYLQNAFLVAYTKKAP QLTSPELMALTRKMANA		Truncated	EthCD	LF	-6,64	14,72805	0,0468446	20,8
10774	P41361	Antithrombin-III	RKANKSSELVSANRLFGG KSITFNETYQDISEVVYG AKLQPLDFKGNAEQSRLT		Truncated	EthCD	LF	-6,64	16,69387	0,0713202	20,4
13183	P05632	ATP synthase subunit epsilon, mitochondrial	WRQAGLSYIRYSQICAKA VRDALKTEFKANAMKTS GSTIKIVKVKKE	N-Term(alpha-amino acetylated residue); K21(N6-acetyl-L-lysine); K37(N6-acetyl-L-lysine); K44(N6-acetyl-L-lysine)	Truncated	HCD	LF	-6,64	353,3674	4,27E-11	56,5
5429	P01888	Beta-2-microglobulin	IQRPPKIQVYSRHPPEDG KPNYLNCYVYGFHPPQIE IDLLKNGEKIKSEQSDLSF SKDWSFYLLSHAEFTPNS KDQYSCRVKHVTLEQPRI	C25(half cystine); C79(half cystine)	Non-truncated	HCD/EthCD	HF	6,64	608,9121	3,73E-61	29,9
5430	P01888	Beta-2-microglobulin	IQRPPKIQVYSRHPPEDG KPNYLNCYVYGFHPPQIE IDLLKNGEKIKSEQSDLSF SKDWSFYLLSHAEFTPNS KDQYSCRVKHVTLEQPRI	C79(half cystine)	Truncated	HCD	Both	-1,01	4,0103	0,0043	11,3

5431	P01888	Beta-2-microglobulin	IQRPPKIQVYSRHPPEDGKPNYLNCYVYGFHPPQIEIDLLKNGEKIKSEQSDLSFSKDWSFYLLSHAEFTPNSKDQYSCRVKHVTLEQPRI		Truncated	HCD/EthCD	HF	6,64	225,0526	2,86E-08	9,3
5428	P01888	Beta-2-microglobulin	IQRPPKIQVYSRHPPEDGKPNYLNCYVYGFHPPQIEIDLLKNGEKIKSEQSDLSFSKDWSFYLLSHAEFTPNSKDQYSCRVKHVTLEQPRI	C79(half cystine)	Truncated	HCD	Both	5,55	4,0103	0,025746	11,6
5427	P01888	Beta-2-microglobulin	IQRPPKIQVYSRHPPEDGKPNYLNCYVYGFHPPQIEIDLLKNGEKIKSEQSDLSFSKDWSFYLLSHAEFTPNSKDQYSCRVKHVTLEQPRI	C25(half cystine); C79(half cystine)	Truncated	EthCD	LF	-6,64	143,4677	0,0710813	12,6
10265	P46168	Beta-defensin 10	QGVRSYLSCWGNRGICLLNRCPGRMRQIGTCLAPRVKCCR	Q1(2-pyrrolidone-5-carboxylic acid (Gln)); C9(half cystine); C16(half cystine); C21(half cystine); C31(half cystine); C38(half cystine); C39(half cystine)	Non-truncated	HCD/EthCD	HF	6,64	115,7231	1,38E-08	15,4
9365	P13600	Beta-nerve growth factor	NVPAGHAIPQAHWIKLQHSLDTVLRRAHSAPAGP		Truncated	HCD/EthCD	LF	-6,64	792,9651	1,05E-15	78,8
3308	P80724	Brain acid soluble protein 1	GGKLSKKKKGYNVNDEKAKDKDKKAEGAGTEEEGTPKENEAQAAAETPEVK	G1(N-myristoylglycine)	Truncated	HCD	HF	NA	326,1342	0,0020766	22,0
7828	Q29RI6	Calcium-binding protein 39	MPFPFGKSHKSPADIVKNLKESMAVLEKQDISDKKAEKATEEVSKNLVA	N-Term(alpha-amino acetylated residue); K21(N6-acetyl-L-lysine)	Truncated	HCD	HF	6,64	59,99977	0,0518417	12,5
352	P62157	Calmodulin	ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEF		Non-truncated	HCD	LF	-6,64	664,1582	0,0042964	6,1
11220	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSVK		Non-truncated	HCD/EthCD	HF	6,64	1119,114	4,79E-142	91,5
12642	P06833	Caltrin	VDSKPQPSDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV		Truncated	HCD	HF	6,64	299,0676	2,51E-10	45,3
9871	P06833	Caltrin	PSDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV		Truncated	HCD/EthCD	HF	6,64	417,4682	5,71E-10	59,6
11219	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV		Truncated	HCD/EthCD	LF	-6,64	1051,901	4,08E-32	91,3

11218	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRS	Truncated	HCD	HF	6,64	251,2322	2,28E-08	46,7
1652	P06833	Caltrin	DEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV	Truncated	HCD/EthCD	LF	-6,64	404,4054	5,86E-10	60,0
11217	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNR	Truncated	HCD/EthCD	HF	6,64	375,4646	4,44E-11	59,1
1651	P06833	Caltrin	DEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRS	Truncated	HCD	LF	-6,64	42,30077	0,0001084	45,5
2411	P06833	Caltrin	EKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV	Truncated	HCD/EthCD	LF	-6,64	560,6221	2,25E-09	47,7
9870	P06833	Caltrin	PSDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGN	Truncated	HCD	LF	-6,64	120,5027	0,0388626	25,0
11216	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGN	Truncated	HCD/EthCD	HF	6,64	1013,42	1,36E-27	90,7
4104	P06833	Caltrin	KASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV	Truncated	HCD	LF	-6,64	138,2833	6,22E-05	46,5
11215	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDR	Truncated	HCD/EthCD	HF	6,64	564,3315	2,31E-09	53,7
11214	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGD	Truncated	HCD/EthCD	LF	-6,64	595,5165	2,03E-18	85,0
1778	P06833	Caltrin	DKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV	Truncated	HCD/EthCD	LF	-6,64	268,9063	1,05E-07	46,2
1777	P06833	Caltrin	DKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDR	Truncated	HCD	LF	-6,64	99,41704	0,0376783	33,3
4018	P06833	Caltrin	HRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRS	Truncated	HCD	LF	-6,64	133,4403	0,0007314	36,1
11213	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLS	Truncated	EthCD	LF	-6,64	165,7324	0,0278621	27,8
11212	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLS	Truncated	HCD/EthCD	LF	-6,64	607,6174	2,36E-13	80,0
11211	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFL	Truncated	HCD/EthCD	HF	6,64	667,4994	3,64E-17	82,4
11210	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETF	Truncated	HCD/EthCD	Both	-2,4	987,0926	3,55E-23	87,9
11627	P06833	Caltrin	SLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV	Truncated	HCD/EthCD	LF	-6,64	546,0679	1,05E-12	66,7
11209	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLET	Truncated	HCD/EthCD	Both	6,13	589,3744	2,61E-12	81,3
11208	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLE	Truncated	HCD/EthCD	HF	6,64	416,3688	1,32E-10	77,4
11207	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLL	Truncated	EthCD	LF	NA	269,2216	0,0007322	56,7
11206	P06833	Caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKL	Truncated	HCD/EthCD	HF	6,64	567,9733	3,92E-12	82,8
13228	P06833	Caltrin	YAKLANRLANPKLLETFLSKWIGDRGNRSV	Truncated	HCD	LF	NA	145,0484	0,0042929	37,9

685	P06833	Caltrin	AKLANRLANPKLLETFLS KWIGDRGNRSV		Truncated	HCD	HF	NA	88,47547	0,0423872	39,3
11205	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANRLANPK		Truncated	HCD/EthCD	LF	-6,64	342,1964	1,46E-07	71,4
4347	P06833	Caltrin	KLANRLANPKLLETFLSK WIGDRGNRSV		Truncated	HCD/EthCD	LF	-6,64	492,6614	3,51E-08	85,2
11204	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANRLANP		Truncated	HCD/EthCD	LF	-6,64	259,9847	0,0002404	70,4
11203	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANRLAN		Truncated	HCD	HF	6,64	150,4819	0,0020744	38,5
1776	P06833	Caltrin	DKHHRFSLSRYAKLANR LANPKLLETF		Truncated	HCD/EthCD	LF	-6,64	164,0658	0,0081152	50,0
886	P06833	Caltrin	ANRLANPKLLETFLSKWI GDRGNRSV		Truncated	HCD	LF	NA	546,8426	1,24E-13	80,0
9321	P06833	Caltrin	NRLANPKLLETFLSKWIG DRGNRSV		Truncated	HCD	Both	NA	200,1134	0,0004121	62,5
11202	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANRL		Truncated	HCD/EthCD	Both	NA	429,5439	1,73E-08	83,3
11201	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANR		Truncated	HCD/EthCD	Both	NA	224,4362	9,91E-05	56,5
11200	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLA		Truncated	HCD/EthCD	HF	NA	226,9715	6,74E-05	81,0
11199	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKL		Truncated	EthCD	HF	NA	111,5731	0,0140299	55,0
1649	P06833	Caltrin	DEKASPDKHHRFSLSRYA KL		Truncated	HCD/EthCD	HF	NA	123,6294	0,0042893	42,1
11198	P06833	Caltrin	SDEKASPDKHHRFSLSRY AK		Truncated	HCD	LF	NA	267,9611	3,32E-06	68,4
11197	P06833	Caltrin	SDEKASPDKHHRFSLSRY A		Truncated	HCD/EthCD	Both	NA	258,2474	1,98E-05	66,7
11196	P06833	Caltrin	SDEKASPDKHHRFSLSRY		Truncated	EthCD	LF	NA	112,5742	0,0207519	70,6
11195	P06833	Caltrin	SDEKASPDKHHRFSLS		Truncated	HCD	HF	NA	174,2249	0,0007297	66,7
12321	P06833	Caltrin	TFLSKWIGDRGNRSV		Truncated	HCD	HF	NA	141,3224	0,0496566	50,0
5494	P17697	Clusterin	ISDKELQEMSTEGSKYVN KEIKNALKEVKQIKTQIE QTNEERKLLLSSLEEAKK KKEDALNDTRDSENKLK ASQGVCNETMTALWEEC KPCLKQTCMKFYARVCR SGSGLVGHQLEEFLNQSS PFYFWINGDRIDSLMEND REQSHVMDVMEDSFTRA SSIMDELFQDRFFLRRPQ DTQYYSPFSSFPRGSLFFN	C77(half cystine); C88(half cystine)	Truncated	HCD	HF	NA	345,2353	0,0020763	6,5
1995	P17697	Clusterin	DREQSHVMDVMEDSFTR ASSIMDELFQDRFFLRRP QDTQYYSPFSSFPRGSLFF NPKSRFA		Truncated	HCD	LF	-6,64	451,3103	0,0207436	13,3
11378	P17697	Clusterin	SFTRASSIMDELFQDRFFL RRPQDTQYYSPFSSFPRG SLFFNPKSRFA		Truncated	HCD	LF	-6,64	400,9249	2,75E-05	23,4
2861	P17697	Clusterin	FLRRPQDTQYYSPFSSFPR GSLFFNPKSRFA		Truncated	HCD/EthCD	LF	-6,64	745,1355	2,35E-13	76,7
12454	P17697	Clusterin	TQYYSPFSSFPRGSLFFNP KSRFA		Truncated	HCD	LF	NA	254,4573	0,0007318	60,9
1866	P55206	C-type natriuretic peptide	DLRVDTKSRAAWTRLLH EHPNARKYKGGNKKGLS KGCFGLKLDRIGSMSGLG	C37(half cystine); C53(half cystine)	Non-truncated	HCD/EthCD	HF	6,64	693,5884	7,32E-71	57,7

9539	P55206	C-type natriuretic peptide	PGAPPKVPRTPSGEEVAE PQAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKGLSKGC FGLKLDRIGSMSGLGC	C86(half cystine); C102(half cystine)	Truncated	HCD/EthCD	Both	3,72	223,6032	0,0002928	19,8
9540	P55206	C-type natriuretic peptide	PGAPPKVPRTPSGEEVAE PQAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKGLSKGC FGLKLDRIGSMSGLGC	C102(half cystine)	Truncated	HCD/EthCD	Both	3,72	24,90791	0,0020716	25,7
3083	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKGLSKGC FGLKLDRIGSMSGLGC	C85(half cystine); C101(half cystine)	Truncated	HCD/EthCD	Both	2,38	615,0055	1,14E-16	40,0
3085	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKGLSKGC FGLKLDRIGSMSGLGC	C85(half cystine)	Truncated	EthCD	Both	2,38	14,35068	1,23E-06	31,0
3084	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKGLSKGC FGLKLDRIGSMSGLGC	C101(half cystine)	Truncated	HCD/EthCD	LF	-6,64	27,38232	3,08E-09	39,0
3086	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKGLSKGC FGLKLDRIGSMSGLGC		Truncated	EthCD	LF	-6,64	273,1506	0,0004729	24,0
3082	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKGLSKG		Truncated	EthCD	HF	6,64	484,829	3,38E-07	39,8
3081	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKGLS		Truncated	EthCD	Both	4,17	245,4185	0,0004239	33,3
3080	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKGL		Truncated	HCD/EthCD	LF	-6,64	340,9376	3,05E-10	50,0
3079	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAWTRLLHEHP NARKYKGGNKKG		Truncated	HCD/EthCD	Both	3,49	603,2038	5,13E-17	55,7

3078	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLLHEHPNARKYKGGNK		Truncated	EthCD	HF	6,64	378,3446	3,82E-05	28,6
3077	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLLHEHPNARKYKGGN		Truncated	HCD/EthCD	Both	2,56	620,6995	5,21E-14	46,1
3075	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLLHEHP		Truncated	EthCD	HF	6,64	548,5346	3,01E-05	28,4
3074	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLLHEHP		Truncated	HCD/EthCD	Both	0,78	348,1281	2,27E-08	41,7
3073	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLLHEHP		Truncated	EthCD	LF	-6,64	503,6441	0,0001425	47,9
4550	P55206	C-type natriuretic peptide	KTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSG	C56(half cystine); C72(half cystine)	Truncated	HCD	LF	-6,64	106,87	0,0043018	19,7
3072	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLLHEHP		Truncated	EthCD	LF	-6,64	189,6815	0,0166323	18,6
3071	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLLHEHP		Truncated	HCD/EthCD	LF	-6,64	656,5235	9,61E-20	60,9
3070	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLLHEHP		Truncated	EthCD	HF	6,64	359,4832	1,26E-08	55,9
3068	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRLL		Truncated	HCD/EthCD	LF	-6,64	384,7562	4,81E-05	38,1
3067	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTRL		Truncated	EthCD	LF	-6,64	503,1812	2,15E-10	50,0
3066	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWTR		Truncated	HCD/EthCD	LF	-6,64	339,9278	9,51E-05	44,3
1633	P55206	C-type natriuretic peptide	DDRSRLLRDLRVDTKSRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C45(half cystine); C61(half cystine)	Truncated	HCD	HF	6,64	246,7459	0,0004939	23,3
3065	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAAWT		Truncated	HCD/EthCD	HF	6,64	825,0044	1,12E-24	76,7

3064	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAAW		Truncated	EthCD	Both	-2,35	128,9278	0,0038354	25,4
9538	P55206	C-type natriuretic peptide	PGAPPKVPRTPSGEEVAE PQAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAA		Truncated	EthCD	LF	-6,64	335,3091	1,51E-09	50,8
3063	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRAA		Truncated	HCD/EthCD	HF	6,64	980,2595	3,41E-35	84,5
3062	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSRA		Truncated	HCD/EthCD	LF	-6,64	630,738	2,11E-18	73,7
9537	P55206	C-type natriuretic peptide	PGAPPKVPRTPSGEEVAE PQAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKS		Truncated	HCD/EthCD	HF	6,64	353,5654	7,41E-11	57,1
3061	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR VDTKSR		Truncated	HCD/EthCD	LF	-6,64	547,6303	6,46E-16	69,6
3060	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR		Truncated	HCD/EthCD	HF	6,64	1048,958	3,68E-38	87,3
989	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQ AAGGGQKKGDKTPGGG GANLKDDRSRLLRDLRV		Truncated	HCD/EthCD	Both	5,29	516,0321	1,06E-16	66,7
3059	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR		Truncated	HCD/EthCD	Both	5,82	491,7729	5,20E-14	63,0
3058	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR		Truncated	HCD/EthCD	HF	6,64	1122,652	2,62E-35	90,6
9780	P55206	C-type natriuretic peptide	PPKVPRTPSGEEVAEPQA AGGGQKKGDKTPGGGG ANLKDDRSRLLRDLRVD		Truncated	HCD	HF	NA	218,037	9,62E-06	34,0
3057	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR		Truncated	EthCD	Both	4,14	860,3456	2,49E-22	78,8
1867	P55206	C-type natriuretic peptide	DLRVDTKSRAAWTRLLH EHPNARKYKGGNKKGLS KGCFGLKLDRIGSMSGLG	C53(half cystine)	Truncated	HCD/EthCD	HF	6,64	34,29168	0,0004624	23,1
1868	P55206	C-type natriuretic peptide	DLRVDTKSRAAWTRLLH EHPNARKYKGGNKKGLS KGCFGLKLDRIGSMSGLG	C37(half cystine)	Truncated	HCD/EthCD	HF	6,64	24,63128	8,86E-06	36,5
988	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQ AAGGGQKKGDKTPGGG GANLKDDRSRLLRDLRV		Truncated	HCD/EthCD	LF	-6,64	502,9376	3,86E-12	67,3
3056	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEP QAAGGGQKKGDKTPGG GGANLKDDRSRLLRDLR		Truncated	HCD/EthCD	Both	-3,1	753,5526	6,26E-22	74,0
11053	P55206	C-type natriuretic peptide	RVDTKSRAAWTRLLHEH PNARKYKGGNKKGLSKG CFGLKLDRIGSMSGLGC	C35(half cystine); C51(half cystine)	Truncated	EthCD	Both	3,32	499,4601	7,10E-12	48,0

11054	P55206	C-type natriuretic peptide	RVDTKSRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C35(half cystine)	Truncated	EthCD	Both	3,32	19,20414	0,0203489	26,0
12645	P55206	C-type natriuretic peptide	VDTKSRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C34(half cystine); C50(half cystine)	Truncated	HCD/EthCD	HF	6,64	615,0898	2,15E-10	44,9
12647	P55206	C-type natriuretic peptide	VDTKSRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C34(half cystine)	Truncated	EthCD	HF	6,64	29,74234	0,0032288	24,5
3055	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDL		Truncated	HCD/EthCD	LF	-6,64	607,8956	1,49E-20	75,5
12646	P55206	C-type natriuretic peptide	VDTKSRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C50(half cystine)	Truncated	EthCD	LF	-6,64	24,2428	0,00272	34,7
2086	P55206	C-type natriuretic peptide	DTKSRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C33(half cystine); C49(half cystine)	Truncated	HCD/EthCD	LF	-6,64	230,5587	0,000692	27,1
3054	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRD		Truncated	EthCD	LF	-6,64	942,2772	6,16E-32	85,4
3053	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLR		Truncated	HCD/EthCD	Both	-1,22	936,8394	1,28E-32	85,1
987	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRD		Truncated	HCD/EthCD	LF	-6,64	470,0658	3,51E-12	59,6
12364	P55206	C-type natriuretic peptide	TKSRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C32(half cystine); C48(half cystine)	Truncated	HCD/EthCD	LF	-6,64	255,4397	3,38E-05	31,9
3052	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	HCD/EthCD	Both	-5,71	1111,851	7,80E-40	89,1
986	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLR		Truncated	HCD	LF	-6,64	274,4132	0,0003399	28,3
2351	P55206	C-type natriuretic peptide	EEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKSRAA		Truncated	HCD	LF	-6,64	97,52481	0,076027	19,6
985	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	HCD/EthCD	HF	6,64	618,8847	7,78E-21	80,0
3051	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRL		Truncated	HCD/EthCD	HF	6,64	1017,446	4,98E-36	91,1
11809	P55206	C-type natriuretic peptide	SRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C30(half cystine); C46(half cystine)	Truncated	HCD/EthCD	HF	6,64	386,5751	2,00E-07	37,8
11810	P55206	C-type natriuretic peptide	SRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C46(half cystine)	Truncated	EthCD	LF	-6,64	21,34693	0,0194364	26,7
11811	P55206	C-type natriuretic peptide	SRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C30(half cystine)	Truncated	EthCD	LF	-6,64	17,12559	0,0673969	22,2
9778	P55206	C-type natriuretic peptide	PPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	HCD/EthCD	Both	2,44	498,9484	6,18E-15	65,9

3050	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSR		Truncated	HCD/EthCD	HF	6,64	1007,464	2,20E-38	100,0
984	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRL		Truncated	HCD/EthCD	LF	-6,64	513,2196	2,54E-13	70,5
983	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSR		Truncated	EthCD	HF	6,64	861,1965	6,43E-22	81,4
2350	P55206	C-type natriuretic peptide	EEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKS		Truncated	EthCD	HF	6,64	775,9565	2,60E-22	83,7
9777	P55206	C-type natriuretic peptide	PPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRL		Truncated	EthCD	HF	NA	449,4763	1,88E-10	65,1
3049	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	HCD/EthCD	LF	-6,64	1021,706	4,20E-36	86,0
9688	P55206	C-type natriuretic peptide	PKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	HCD	LF	-6,64	289,6767	2,34E-05	39,5
982	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	HCD/EthCD	HF	6,64	560,3447	3,33E-19	78,6
1398	P55206	C-type natriuretic peptide	AWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C27(half cystine); C43(half cystine)	Truncated	EthCD	HF	6,64	233,1026	0,0003999	33,3
3048	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDR		Truncated	EthCD	HF	6,64	594,0778	2,13E-19	76,2
3047	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDD		Truncated	EthCD	HF	6,64	768,3052	3,53E-25	78,0
13198	P55206	C-type natriuretic peptide	WTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C26(half cystine); C42(half cystine)	Truncated	HCD/EthCD	HF	6,64	254,0551	5,51E-05	36,6
2349	P55206	C-type natriuretic peptide	EEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDT		Truncated	EthCD	LF	-6,64	254,7496	0,0007315	39,0
3046	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKD		Truncated	HCD/EthCD	HF	6,64	652,8041	8,52E-19	85,0
12459	P55206	C-type natriuretic peptide	TRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C25(half cystine); C41(half cystine)	Truncated	EthCD	HF	6,64	223,772	4,29E-07	42,5
438	P55206	C-type natriuretic peptide	AEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKS		Truncated	HCD	LF	-6,64	87,44746	0,0038371	27,5
10868	P55206	C-type natriuretic peptide	RLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C24(half cystine); C40(half cystine)	Truncated	EthCD	HF	6,64	402,3038	1,92E-08	51,3
3045	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLK		Truncated	EthCD	LF	-6,64	380,561	6,55E-08	64,1
4649	P55206	C-type natriuretic peptide	KVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	HCD/EthCD	LF	-6,64	197,4984	0,0020785	41,0

9808	P55206	C-type natriuretic peptide	PQAAGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKS		Truncated	EthCD	Both	4,19	162,0409	0,0032258	42,1
3044	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANL		Truncated	HCD/EthCD	LF	-6,64	793,9479	2,02E-25	89,5
5173	P55206	C-type natriuretic peptide	LLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C23(half cystine); C39(half cystine)	Truncated	HCD/EthCD	LF	-6,64	262,8823	0,0002146	39,5
3043	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGAN		Truncated	EthCD	HF	6,64	626,2498	2,92E-18	86,5
5075	P55206	C-type natriuretic peptide	LHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSG	C38(half cystine)	Truncated	EthCD	HF	6,64	21,5029	0,049989	27,0
5076	P55206	C-type natriuretic peptide	LHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSG	C22(half cystine)	Truncated	HCD/EthCD	HF	6,64	61,72868	0,0042911	32,4
5074	P55206	C-type natriuretic peptide	LHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C22(half cystine); C38(half cystine)	Truncated	HCD/EthCD	LF	-6,64	551,6602	5,04E-14	62,2
3928	P55206	C-type natriuretic peptide	HEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C21(half cystine); C37(half cystine)	Truncated	HCD/EthCD	Both	-2,4	528,8869	2,08E-09	55,6
11394	P55206	C-type natriuretic peptide	SGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	EthCD	Both	-3,07	527,4188	1,55E-13	75,0
3929	P55206	C-type natriuretic peptide	HEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGL	C21(half cystine)	Truncated	EthCD	HF	6,64	40,29072	0,0218031	30,6
3042	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGG		Truncated	EthCD	LF	-6,64	535,776	1,18E-14	83,3
518	P55206	C-type natriuretic peptide	AGGGQKKGDKTPGGGGANLKDDRSRLLRDLRVD		Truncated	HCD/EthCD	HF	6,64	273,2235	4,18E-06	57,1
1865	P55206	C-type natriuretic peptide	DLRVDTKSRAAWTRLLHEHPNARKYKGGNKKGLS		Truncated	HCD/EthCD	LF	-6,64	177,2282	0,0007304	37,1
2399	P55206	C-type natriuretic peptide	EHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C20(half cystine); C36(half cystine)	Truncated	HCD/EthCD	LF	-6,64	269,3348	0,0020734	34,3
1864	P55206	C-type natriuretic peptide	DLRVDTKSRAAWTRLLHEHPNARKYKGGNKKGLS		Truncated	EthCD	LF	-6,64	96,47563	0,0086517	35,3
2348	P55206	C-type natriuretic peptide	EEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRL		Truncated	EthCD	LF	-6,64	962,5152	5,02E-28	97,1
11393	P55206	C-type natriuretic peptide	SGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	HCD/EthCD	LF	-6,64	607,5883	1,91E-12	70,6
2732	P55206	C-type natriuretic peptide	EVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	HCD/EthCD	Both	NA	382,27	1,50E-09	69,7
3040	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGG		Truncated	HCD/EthCD	LF	-6,64	499,5759	5,24E-14	87,9
3329	P55206	C-type natriuretic peptide	GGQKKGDKTPGGGGANLKDDRSRLLRDLRVDTKS		Truncated	EthCD	LF	-6,64	175,703	0,00323	51,5
9744	P55206	C-type natriuretic peptide	PNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C18(half cystine); C34(half cystine)	Truncated	HCD/EthCD	LF	-6,64	236,9943	0,0002879	39,4
11392	P55206	C-type natriuretic peptide	SGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	HCD/EthCD	LF	-6,64	380,8207	3,32E-06	54,5
1863	P55206	C-type natriuretic peptide	DLRVDTKSRAAWTRLLHEHPNARKYKGGNKKGL		Truncated	HCD/EthCD	HF	6,64	648,4351	2,09E-15	90,6
2347	P55206	C-type natriuretic peptide	EEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSR		Truncated	EthCD	HF	6,64	855,635	4,28E-22	90,6
2731	P55206	C-type natriuretic peptide	EVAEPQAAGGGQKKGDKTPGGGGANLKDDRSR		Truncated	HCD/EthCD	Both	2,39	534,984	2,21E-07	54,8
437	P55206	C-type natriuretic peptide	AEPQAAGGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	HCD/EthCD	LF	-6,64	409,8108	1,28E-08	77,4

1112	P55206	C-type natriuretic peptide	ARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C16(half cystine); C32(half cystine)	Truncated	HCD/EthCD	LF	NA	135,0649	0,0098522	29,0
1862	P55206	C-type natriuretic peptide	DLRVDTKSRAAWTRLLHEHPNARKYKGGNKKG		Truncated	HCD/EthCD	LF	-6,64	195,7492	0,0007311	71,0
2346	P55206	C-type natriuretic peptide	EEVAEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	HCD/EthCD	LF	-6,64	811,132	3,06E-17	87,1
2730	P55206	C-type natriuretic peptide	EVAEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	HCD	Both	NA	317,6504	1,11E-08	76,7
436	P55206	C-type natriuretic peptide	AEPQAAGGGQKKGDKTPGGGGANLKDDRSRL		Truncated	HCD/EthCD	LF	-6,64	354,6126	1,34E-06	63,3
2345	P55206	C-type natriuretic peptide	EEVAEPQAAGGGQKKGDKTPGGGGANLKDDR		Truncated	EthCD	LF	-6,64	624,6308	2,45E-12	73,3
10782	P55206	C-type natriuretic peptide	RKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C15(half cystine); C31(half cystine)	Truncated	HCD	LF	-6,64	176,7202	0,0032272	26,7
435	P55206	C-type natriuretic peptide	AEPQAAGGGQKKGDKTPGGGGANLKDDRSR		Truncated	HCD/EthCD	Both	NA	1005,094	5,75E-15	72,4
2344	P55206	C-type natriuretic peptide	EEVAEPQAAGGGQKKGDKTPGGGGANLKDD		Truncated	HCD/EthCD	Both	NA	128,4486	0,0203407	44,8
12644	P55206	C-type natriuretic peptide	VDTKSRAAWTRLLHEHPNARKYKGGNKKGL		Truncated	EthCD	LF	NA	96,25333	0,0086555	44,8
434	P55206	C-type natriuretic peptide	AEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	EthCD	Both	NA	887,3044	9,72E-13	92,9
3039	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGD		Truncated	EthCD	Both	NA	898,7748	3,31E-21	96,4
13277	P55206	C-type natriuretic peptide	YKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C13(half cystine); C29(half cystine)	Truncated	HCD/EthCD	Both	NA	354,1546	4,67E-23	39,3
9806	P55206	C-type natriuretic peptide	PQAAGGGQKKGDKTPGGGGANLKDDRSRL		Truncated	HCD/EthCD	LF	NA	289,498	0,0032247	57,1
4279	P55206	C-type natriuretic peptide	KGGNKKGLSKGCFGLKLDRIGSMSGLGC	C12(half cystine); C28(half cystine)	Truncated	HCD/EthCD	Both	NA	262,9915	0,0007308	37,0
9805	P55206	C-type natriuretic peptide	PQAAGGGQKKGDKTPGGGGANLKDDRSR		Truncated	HCD/EthCD	Both	NA	579,0922	1,53E-06	40,7
433	P55206	C-type natriuretic peptide	AEPQAAGGGQKKGDKTPGGGGANLKDDR		Truncated	EthCD	LF	NA	824,6573	1,03E-09	70,4
981	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQAAGGGQKKGD		Truncated	HCD	LF	NA	177,3145	0,0042982	22,2
517	P55206	C-type natriuretic peptide	AGGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	EthCD	Both	NA	436,5497	2,48E-11	88,5
432	P55206	C-type natriuretic peptide	AEPQAAGGGQKKGDKTPGGGGANLKDD		Truncated	EthCD	HF	NA	420,4891	0,0020747	38,5
9804	P55206	C-type natriuretic peptide	PQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	EthCD	HF	NA	938,8614	5,33E-14	96,2
4549	P55206	C-type natriuretic peptide	KTPGGGGANLKDDRSRLLRDLRVDTKS		Truncated	EthCD	LF	NA	214,3554	0,0020756	50,0
516	P55206	C-type natriuretic peptide	AGGGQKKGDKTPGGGGANLKDDRSRL		Truncated	HCD/EthCD	Both	NA	156,1147	0,0032316	44,0
3303	P55206	C-type natriuretic peptide	GGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	HCD	HF	NA	217,603	0,0007294	56,0
11808	P55206	C-type natriuretic peptide	SRAAWTRLLHEHPNARKYKGGNKKGL		Truncated	HCD/EthCD	LF	NA	132,5174	0,0032302	40,0
1861	P55206	C-type natriuretic peptide	DLRVDTKSRAAWTRLLHEHPNARKY		Truncated	HCD/EthCD	Both	NA	375,563	3,57E-07	75,0
3328	P55206	C-type natriuretic peptide	GGQKKGDKTPGGGGANLKDDRSRLL		Truncated	EthCD	LF	-6,64	383,86	7,49E-10	83,3

13197	P55206	C-type natriuretic peptide	WTRLLHEHPNARKYKGGNKKGLSKG		Truncated	EthCD	LF	NA	129,0839	0,0032286	54,2
515	P55206	C-type natriuretic peptide	AGGGQKKGDKTPGGGGANLKDDRS		Truncated	EthCD	Both	NA	928,8328	5,22E-18	95,7
3302	P55206	C-type natriuretic peptide	GGGQKKGDKTPGGGGANLKDDRSR		Truncated	EthCD	HF	NA	376,3964	0,0027188	56,5
514	P55206	C-type natriuretic peptide	AGGGQKKGDKTPGGGGANLKDDR		Truncated	EthCD	HF	NA	258,9086	0,0199306	59,1
3327	P55206	C-type natriuretic peptide	GGQKKGDKTPGGGGANLKDDRSR		Truncated	EthCD	HF	NA	567,4478	0,0007329	72,7
3301	P55206	C-type natriuretic peptide	GGGQKKGDKTPGGGGANLKDDRS		Truncated	EthCD	LF	NA	608,6771	2,88E-06	77,3
12643	P55206	C-type natriuretic peptide	VDTKSRAAWTRLLHEHPNARKY		Truncated	HCD	Both	NA	87,05697	0,0128847	47,6
3038	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAG		Truncated	HCD	HF	NA	205,3292	0,0288229	33,3
3326	P55206	C-type natriuretic peptide	GGQKKGDKTPGGGGANLKDDRS		Truncated	EthCD	LF	NA	589,1577	2,04E-05	90,5
13196	P55206	C-type natriuretic peptide	WTRLLHEHPNARKYKGGNKKGL		Truncated	HCD	LF	NA	197,4457	0,0003545	47,6
5073	P55206	C-type natriuretic peptide	LHEHPNARKYKGGNKKGLSKG		Truncated	EthCD	HF	NA	284,5101	0,0189297	60,0
5072	P55206	C-type natriuretic peptide	LHEHPNARKYKGGNKKGL		Truncated	EthCD	HF	NA	412,2131	0,0007301	94,1
1098	Q3ZBN4	CUE domain-containing protein 2	AQWVLAKARGDLEEAVQMLVEGKQGPPAWDGPNQDLPRRLRGP		Truncated	EthCD	HF	6,64	35,35428	0,0795744	14,3
10143	P01035	Cystatin-C	QGPRKGRLLGGLMEADVNEEGVQEALSFAVSEFNKRSNDAYQSRVVRVVRARKQVVSGMNYFLDVELGRTTCTKSQANLDSCPFHNQPHLKREKLCSFQVYVVPWMNTINLVKFSCQD	Q1(2-pyrrolidone-5-carboxylic acid (Gln)); C72(half cystine); C82(half cystine); C96(half cystine); C116(half cystine)	Non-truncated	HCD/EthCD	Both	-3,49	392,0395	6,08E-20	12,8
10898	Q32PE0	DNA-directed RNA polymerases I, II, and III subunit RPABC2	RITTPYMTKYERARVLGTRALQIAMCAPVMVELEGETDPLLI		Truncated	EthCD	HF	6,64	23,89604	0,0773304	24,4
9227	Q05B84	Fibronectin type III and SPRY domain-containing protein 1	NLRVDDLSVEWDAMGGKVQDIKAREKDGKGRTASPVNSPARG		Truncated	EthCD	Both	-2,73	85,02645	0,047746	22,0
12557	Q9NZ50	Gamma-synuclein	VAEKTKEQANAVSEAVVSSVNTVATKTVEEVENIAVTSGVVHKEA		Truncated	EthCD	Both	-5,71	7,205703	0,0468599	22,7
4729	A7MB54	H2.0-like homeobox protein	IADILHAGVGEPGATPEGLAGASAAALTAHLGSAHPHASFQAAARSPL		Truncated	EthCD	LF	-6,64	19,56415	0,0556398	17,0
9134	Q3SZV7	Hemopexin	NGLGLYLVQGPNLYCYKDVEELSKTKDLPQAQRM	C15(half cystine)	Truncated	EthCD	HF	6,64	22,51674	0,0771188	23,7
1478	Q3SZ71	Mitochondrial-processing peptidase subunit beta	CGFTERLLIGGAAGRSLYFGGNRLRSTQAAAQVVLNVPETRVTC		Truncated	EthCD	Both	-5,71	6,909694	0,0376916	14,0

12728	P03972	Muellerian-inhibiting factor	VGVLSSYEQAFLEAVRRTHWGLSDLTTFAVCPAGNGQPVLPHL		Truncated	EthCD	Both	-2,85	30,86035	0,0760473	21,4
10042	P08165	NADPH:adrenodoxin oxidoreductase, mitochondrial	QDLKAGHLPSGPRPGSAFIKALLDSRGVWPVSFSDWEKLDAEEVSRGQASGKPREK		Truncated	EthCD	HF	6,64	40,44311	0,0595278	20,0
7527	P61282	NEDD8	MLIKVKTLTGKEIEIDIEPTDKVERIKERVEEKEGIPPQQQRLIYSGKQMNDEKTAADYKILGGSVLHLVLAL		Non-truncated	HCD	HF	NA	447,6197	1,96E-29	22,7
1555	Q95JC7	Neutral amino acid transporter B(0)	CVEEKNGVARHISRFILPIGATVNMDGAALFQCVAAVFIAQLNHRSLDFV		Truncated	EthCD	HF	6,64	86,60374	0,081711	14,3
3023	P02316	Non-histone chromosomal protein HMG-14	GAKGKQAEVANQETKEDLPAENGETKNEESPASDEAEEKEAK		Truncated	EthCD	Both	NA	73,27666	0,0784472	17,1
11683	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLSQEFHSLEDKLDLDHKSEEDKHLKIRISHELDSASSEVN		Truncated	HCD/EthCD	HF	6,64	669,3701	8,01E-19	50,0
11684	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLSQEFHSLEDKLDLDHKSEEDKHLKIRISHELDSASSEVN	S106(O-phospho-L-serine)	Truncated	EthCD	LF	-6,64	4,0103	0,0038387	13,9
11682	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLSQEFHSLEDKLDLDHKSEED		Truncated	HCD/EthCD	LF	-6,64	216,1009	0,0020728	16,8
11681	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLSQEFHSLEDKLDLDHKSEED		Truncated	HCD	LF	-6,64	144,6568	0,0542778	10,8
7096	P31096	Osteopontin	MHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLSQEFHSLEDKLDLDHKSEEDKHLKIRISHELDSASSEVN		Truncated	EthCD	LF	-6,64	191,3508	0,0042946	20,7
947	P31096	Osteopontin	APKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLSQEFHSLEDKLDLDHKSEEDKHLKIRISHELDSASSEVN		Truncated	EthCD	LF	-6,64	165,3108	0,0760068	14,3
11679	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLSQ		Truncated	EthCD	Both	5,12	361,0089	0,0007318	22,7
11678	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLSQ		Truncated	EthCD	LF	-6,64	143,1692	0,0007311	28,4

11677	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSKLS	Truncated	EthCD	Both	-1,09	516,7293	1,11E-07	34,3
11676	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENSK	Truncated	HCD/EthCD	LF	-6,64	394,6268	6,55E-08	42,6
11675	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENS	Truncated	HCD/EthCD	HF	6,64	528,3052	1,10E-11	44,8
11674	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQEN	Truncated	HCD/EthCD	HF	6,64	391,0571	9,82E-11	47,0
9368	P31096	Osteopontin	NVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSNLIESQENS	Truncated	HCD/EthCD	LF	-6,64	84,24617	0,0001772	30,3
11673	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKNKHSN	Truncated	HCD/EthCD	HF	6,64	711,8256	5,34E-14	50,8
12945	P31096	Osteopontin	VQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKN	Truncated	HCD/EthCD	HF	6,64	193,8999	8,44E-05	42,1
11672	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKD	Truncated	EthCD	LF	-6,64	26,59553	0,0001766	40,4
11671	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPKAKD	Truncated	HCD/EthCD	LF	-6,64	387,3873	5,54E-09	50,9
11670	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSELSKELTPK	Truncated	HCD/EthCD	LF	-6,64	381,7463	6,81E-09	64,0
11669	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETNSSE	Truncated	EthCD	LF	NA	182,6522	0,0140237	21,4
11668	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKETN	Truncated	HCD/EthCD	LF	-6,64	464,9275	8,90E-12	69,2
7095	P31096	Osteopontin	MHDAPKKTSQLTDHSKETNSSELSKELTPKAKDKN	Truncated	HCD/EthCD	LF	-6,64	623,8752	2,50E-09	52,6
12944	P31096	Osteopontin	VQSPDATEEDFTSHIESEEMHDAPKKTSQLTDHSKE	Truncated	HCD/EthCD	LF	-6,64	328,1856	3,24E-08	70,3
11667	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHDAPKKTSQ	Truncated	HCD/EthCD	LF	NA	716,6309	5,25E-12	80,0
4351	P31096	Osteopontin	KLDLDHKSEEDKHLKIRISHELDSASSEVN	Truncated	HCD/EthCD	Both	0,58	373,2105	0,0020753	34,5
11666	P31096	Osteopontin	SNVQSPDATEEDFTSHIESEEMHD	Truncated	HCD	HF	NA	151,1315	0,0150002	52,2
4306	P31096	Osteopontin	KHLKIRISHELDSASSEVN	Truncated	HCD/EthCD	Both	NA	424,4409	2,34E-06	100,0
11664	P31096	Osteopontin	SNVQSPDATEED	Truncated	EthCD	HF	NA	605,0375	9,97E-05	81,8
12515	Q9TUM6-1	Perilipin-2	TTVTGAKDSVASTITGVVDRTKGAVTGSVEKTKSVVSGSINTVLRSRVMQLMS	Truncated	HCD	HF	6,64	25,58309	0,0673766	17,3

11149	Q9N2I2	Plasma serine protease inhibitor	SARLGSQRIVFNRPFLVLI VKNSKHILFLGKVTRP	Truncated	HCD/EthCD	LF	-6,64	998,3683	1,45E-20	91,2
5051	Q9N2I2	Plasma serine protease inhibitor	LGSQRIVFNRPFLVLIVKN SKHILFLGKVTRP	Truncated	HCD/EthCD	LF	-6,64	951,0697	2,87E-19	83,9
1946	Q28165	Polyadenylate-binding protein 2	DQALKPTKPMRFLGDEE TVRKAMEAVAAQGKAK	Truncated	HCD/EthCD	Both	-0,31	414,9417	1,11E-08	68,8
8054	P0CG53	Polyubiquitin-B	MQIFVKTLTGKTITLEVE PSDTIENVKAKIQDKEGIP PDQQRLIFAGKQLEDGRT LSDYNIQKESTLHLVLRL	Truncated	HCD/EthCD	LF	-6,64	615,1812	6,35E-73	42,7
2496	O62827	Pro-adrenomedullin	ELRESSSYPTGLADVKAG PVQTLIRPQDVKGASRSP QASSPDAARIRV	Non-truncated	HCD/EthCD	LF	-6,64	386,6064	3,40E-40	46,8
11607	O62827	Pro-adrenomedullin	SLPEAGLGRTLLQPPEPK LRGAPDSRVHQVLATLRI	Non-truncated	HCD/EthCD	LF	-6,64	483,3547	1,94E-19	31,4
2498	O62827	Pro-adrenomedullin	ELRESSSYPTGLADVKAG PVQTLIRPQDVKGASRSP QASSPDAARIRVK	Truncated	HCD/EthCD	HF	6,64	283,5373	6,33E-05	43,8
5451	O62827	Pro-adrenomedullin	LRESSSYPTGLADVKAGP VQTLIRPQDVKGASRSPQ ASSPDAARIRV	Truncated	HCD	HF	6,64	180,961	0,0020725	28,3
10730	O62827	Pro-adrenomedullin	RESSSYPTGLADVKAGPV QTLIRPQDVKGASRSPQA SSPDAARIRVK	Truncated	HCD/EthCD	HF	6,64	459,6136	3,70E-09	50,0
10729	O62827	Pro-adrenomedullin	RESSSYPTGLADVKAGPV QTLIRPQDVKGASRSPQA SSPDAARIRV	Truncated	HCD/EthCD	HF	6,64	435,0114	1,29E-09	48,9
10728	O62827	Pro-adrenomedullin	RESSSYPTGLADVKAGPV QTLIRPQDVKGASRSPQA SSPDAARIR	Truncated	HCD/EthCD	LF	-6,64	333,477	4,27E-07	38,6
11606	O62827	Pro-adrenomedullin	SLPEAGLGRTLLQPPEPK LRGAPDSRVHQVLATLR	Truncated	HCD	HF	NA	564,2967	0,0007307	32,4
11605	O62827	Pro-adrenomedullin	SLPEAGLGRTLLQPPEPK LRGAPDSRVHQVLATL	Truncated	HCD/EthCD	LF	-6,64	267,3512	0,0007044	42,4
11604	O62827	Pro-adrenomedullin	SLPEAGLGRTLLQPPEPK LRGAPDSRVHQVLAT	Truncated	HCD/EthCD	LF	-6,64	239,7114	0,0043036	34,4
11603	O62827	Pro-adrenomedullin	SLPEAGLGRTLLQPPEPK LRGAPDSRVHQVLA	Truncated	EthCD	LF	-6,64	132,3414	0,0379215	32,3
12946	O62827	Pro-adrenomedullin	VQTLIRPQDVKGASRSPQ ASSPDAARIRV	Truncated	HCD/EthCD	LF	-6,64	375,2247	8,34E-08	71,4
12377	O62827	Pro-adrenomedullin	TLIRPQDVKGASRSPQAS SPDAARIRV	Truncated	EthCD	LF	NA	227,5683	0,000729	50,0
5230	O62827	Pro-adrenomedullin	LIRPQDVKGASRSPQASS PDAARIRV	Truncated	HCD/EthCD	Both	NA	195,6768	0,0032261	44,0
949	P25975	Procathepsin L	APKLDPNLDAHWHQWK ATHRRLYGMNEEEWRRA VWEKNKKIIDLHNQEYSE	Truncated	HCD/EthCD	LF	-6,64	247,6155	2,91E-05	39,3
7715	P25975	Procathepsin L	MNEEEWRRAVWEKNKKI IDLHNQEYSEGKHGFR	Truncated	HCD/EthCD	Both	1,64	339,3866	4,73E-05	40,6
10692	P25975	Procathepsin L	RAVWEKNKKIIDLHNQE YSEGKHGFR	Truncated	HCD/EthCD	HF	NA	273,6359	0,0027212	28,0
1378	P25975	Procathepsin L	AVWEKNKKIIDLHNQEY SEGKHGFR	Truncated	EthCD	HF	NA	137,2822	0,0166393	37,5
948	P25975	Procathepsin L	APKLDPNLDAHWHQWK ATHRRLYG	Truncated	HCD/EthCD	Both	NA	351,0722	1,28E-06	60,9

4352	P25975	Procathepsin L	KLDPNLDAHWHQWKATHRRLYG		Truncated	HCD	LF	NA	111,0953	0,0335632	47,6
6302	P25975	Procathepsin L	MAMNAFGDMTNEEFR		Truncated	HCD	HF	NA	545,2689	0,0007325	71,4
3028	Q5E977	Protein FAM118B	GALVLTTNFDNLLELYAADQGKQLESLDLTDEKKVLEWAQEK		Truncated	EthCD	HF	6,64	27,93183	0,0314975	26,8
11134	P02633	Protein S100-G	SAKKSPEELKGIFEKYAAKEGDPNQLSKEELKLLLQTEFPSLLKGPSTLDELFEELDKNGDGEVSFEEFQVL	N-Term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	HF	6,64	1011,517	5,41E-79	46,8
11091	Q3SZ21	Ribonuclease P protein subunit p30	RYTISNALNLMQVCKGKNVIISSAAERPLEIRGPYDVANLGLLFGLSESDAK		Truncated	HCD	LF	-6,64	39,25551	0,0468752	15,7
1667	P04557	Seminal plasma protein A3	DEQLSEDNVILPKEKKDPASGAETKDNKCVFPFIYGNKKYFDCTLHGSLFLWCSLDADYTGRWKYCTKNDYAKCVFPFIYEGKSYDTCIIIGSTFMNYWCSLSSNYDEDGVWKYC	C29(half cystine); C43(half cystine); C53(half cystine); C66(half cystine); C74(half cystine); C88(half cystine); C100(half cystine); C115(half cystine)	Non-truncated	HCD/EthCD	Both	-5,69	222,0491	5,57E-27	15,8
1666	P04557	Seminal plasma protein A3	DEQLSEDNVILPKEKKDPASGAETKD		Truncated	HCD/EthCD	LF	-6,64	505,4867	2,11E-10	84,0
5499	P04557	Seminal plasma protein A3	LSEDNVILPKEKKDPASGAETKD		Truncated	HCD	LF	NA	122,2426	0,0397961	31,8
1665	P04557	Seminal plasma protein A3	DEQLSEDNVILPKEKKD		Truncated	HCD/EthCD	HF	6,64	596,1237	1,02E-06	87,5
2605	P04557	Seminal plasma protein A3	EQLSEDNVILPKEKKD		Truncated	HCD	HF	NA	223,3614	0,0145814	53,3
10356	P04557	Seminal plasma protein A3	QLSEDNVILPKEKKD		Truncated	HCD/EthCD	Both	NA	310,5937	0,0020707	85,7
1967	P02784	Seminal plasma protein PDC-109	DQDEGVSTEPTQDGPAELPEDEECVFPFVYRNRKHFDCTVHGSLFPWCSLDADYVGRWKYCAQRDYAKCVFPFIYGGKKYETCTKIGSMWMSWCSLSPNYDK		Non-truncated	HCD/EthCD	Both	NA	38,22138	3,83E-13	9,3
1965	P02784	Seminal plasma protein PDC-109	DQDEGVSTEPTQDGPAELPEDEECVFPFVYRNRKHFDCTVHGSLFPWCSLDADYVGRWKYCAQRDYAKCVFPFIYGGKKYETCTKIGSMWMSWCSLSPNYDK	C69(half cystine); C94(half cystine)	Non-truncated	HCD	Both	NA	231,1954	0,0379348	9,3
1949	P02784	Seminal plasma protein PDC-109	DQDEGVSTEPTQDGPAELPED		Truncated	HCD/EthCD	Both	NA	406,6447	0,0007321	55,0
1948	P02784	Seminal plasma protein PDC-109	DQDEGVSTEPTQDG		Truncated	HCD	LF	NA	522,7084	0,0032275	38,5
1947	P02784	Seminal plasma protein PDC-109	DQDEGVSTEPTQD		Truncated	HCD/EthCD	Both	NA	659,6604	0,0020776	58,3
4162	P00669	Seminal ribonuclease	KESAAAKFERQHMD		Truncated	HCD/EthCD	HF	NA	426,2824	0,0020737	92,3

11228	P62326	Thymosin beta-4	SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	K3(N6-acetyl-L-lysine);	Non-truncated	HCD	HF	6,64	4,0103	5,24E-40	50,0
11226	P62326	Thymosin beta-4	SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES	N-Term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	LF	-6,64	33,03005	5,67E-40	50,0
5083	Q5E971	Transmembrane emp24 domain-containing protein 10	IHKDLLVTGAYEITDQSGGAGGLRTHLKITDSAGHILYSKEDAT		Truncated	EthCD	Both	-5,71	67,73409	0,0081115	20,9
1160	Q3T077	Tubulin polymerization-promoting protein family member 2	ASEAERTFQRFAVFGESSSSGTEMNNKNFSKLCKDCGIMDGKTVTSTDVDIVFSKVKAKNARTITFQQFQEAMKELGQKRFKGKSPDEALENIYKLMEGKDPATTGVTKATTVGGVSRLTDTSKYTGTHKERFDESGKGKGIAGREDVTDNSGYVS	N-Term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	LF	-6,64	602,6932	5,25E-30	9,5
9449	Q3T077	Tubulin polymerization-promoting protein family member 2	PATTGVTKATTVGGVSRLTDTSKYTGTHKERFDESGKGKGIAGREDVTDNSGYVSGYKGAGTYDKKGSN		Truncated	HCD/EthCD	LF	-6,64	493,8133	2,32E-09	45,6

ST2_enriched_pathway: KEGG, Reactome and WikiPathways pathways specifically enriched (FDR<0.01 and n° genes>5) by processing protein interactors (STRING score >0.5) of uniquely expressed proteoform families in high (HF) and low freezability (LF) seminal plasma (SP).

<table>
<tr><th></th><th></th><th></th><th></th><th colspan="2">n° genes</th><th colspan="2">FDR</th><th colspan="2">p-value</th><th colspan="2"># genes</th></tr>
<tr><th>Category</th><th>Term name</th><th>Description</th><th># background genes</th><th>SP HF</th><th>SP LF</th><th>SP HF</th><th>SP LF</th><th>SP HF</th><th>SP LF</th><th>SP HF</th><th>SP LF</th></tr>
<tr><td>KEGG Pathways</td><td>bta03020</td><td>RNA polymerase</td><td>29</td><td>27</td><td></td><td>1,09E-40</td><td></td><td>3,27E-43</td><td></td><td>POLR2H|POLR2C|POLR2F|POLR3H|POLR2J|POLR1A|POLR3F|POLR2G|POLR3D|POLR2D|POLR1E|POLR3A|POLR1B|POLR2I|POLR1C|POLR2B|POLR3K|POLR2E|POLR3C|POLR2K|POLR3B|POLR2L|ZNRD1|POLR3E|POLR1D|TWISTNB|POLR2A</td><td></td></tr>
<tr><td>KEGG Pathways</td><td>bta03022</td><td>Basal transcription factors</td><td>40</td><td>21</td><td></td><td>1,17E-27</td><td></td><td>7,03E-30</td><td></td><td>ERCC2|GTF2A1L|GTF2H1|GTF2H3|TBP|GTF2H5|GTF2A2|CDK7|GTF2E2|GTF2A1|TBPL2|ERCC3|ENSBTAP00000027988|CCNH|GTF2H2|MNAT1|GTF2B|LOC511494|GTF2F2|TBPL1|GTF2H4</td><td></td></tr>
<tr><td>KEGG Pathways</td><td>bta03420</td><td>Nucleotide excision repair</td><td>43</td><td>15</td><td></td><td>1,99E-17</td><td></td><td>1,80E-19</td><td></td><td>ERCC2|GTF2H1|GTF2H3|GTF2H5|RFC3|CDK7|CUL4A|ERCC3|CCNH|GTF2H2|RBX1|MNAT1|ERCC6|LOC780968|GTF2H4</td><td></td></tr>
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KEGG Pathways	bta04623	Cytosolic DNA-sensing pathway	60	15		1,03E-15		1,24E-17		POLR2H|POLR2F|POLR3H|POLR3F|POLR3D|POLR3A|POLR1C|POLR3K|POLR2E|POLR3C|POLR2K|POLR3B|POLR2L|POLR3E|POLR1D	
KEGG Pathways	bta04710	Circadian rhythm	28	5		2,00E-04		4,73E-06		BTRC|CUL1|SKP1|RBX1|LOC780968	
KEGG Pathways	bta04066	HIF-1 signaling pathway	102	6		0,0053		1,80E-04		TCEB1|TCEB2|VHL|RBX1|LOC780968|CUL2	
KEGG	bta04713	Circadian	90		7		0,0048		3,10E-04		RYR1|ENSBTAP00
KEGG Pathways	bta04217	Necroptosis	144		9		0,0034		2,10E-04		TNFAIP3|CYLD|ENSBTAP00000016813|FAF1|SQSTM1|CAMK2A|USP21|CAMK2D|RNF31
KEGG Pathways	bta03460	Fanconi anemia pathway	48		6		0,0013		7,68E-05		WDR48|POLH|FANCI|POLI|FANCD2|REV1
KEGG Pathways	bta04020	Calcium signaling pathway	187		11		0,0013		6,92E-05		PDE1B|NOS2|RYR1|ENSBTAP00000016813|PPIF|CAMK2A|RYR3|CAMK2D|CALM|PDE1A|RYR2
KEGG Pathways	bta03013	RNA transport	152		10		0,0012		6,15E-05		POP4|RPP30|RPP40|POP5|RPP38|POP1|RPP25L|RPP14|RPP25|POP7
KEGG Pathways	bta04722	Neurotrophin signaling pathway	107		10		7,41E-05		3,57E-06		TP53|NTRK1|NGF|SORT1|ENSBTAP00000016813|TRAF6|CAMK2A|NGFR|CAMK2D|CALM
KEGG Pathways	bta04141	Protein processing in endoplasmic reticulum	154		13		8,04E-06		3,63E-07		UBQLN4|RAD23B|ATXN3|NSFL1C|CUL1|PLAA|UBQLN1|VCP|RAD23A|YOD1|NPLOC4|UBE2D1|UBE2G2

KEGG Pathways	bta03008	Ribosome biogenesis in eukaryotes	70		10		2,40E-06		1,01E-07		POP4|RPP30|RPP40|POP5|NHP2L1|RPP38|POP1|RPP25L|RPP25|POP7
KEGG Pathways	bta04144	Endocytosis	221		17		5,20E-07		2,03E-08		HGS|STAM2|STAM|SH3KBP1|VPS36|CBL|STAMBP|RABEP1|EPS15L1|CBLB|TSG101|NEDD4|NEDD4L|TRAF6|USP8|MDM2|EPS15
KEGG Pathways	bta04714	Thermogenesis	221		18		9,28E-08		3,35E-09		ATP5D|COX17|ATP5A1|ATP5G3|ATP5G2|ATP5F1|ATP5L|ATP5B|ATP5C1|ATP5I|ATP5O|ATP5G1|ATP5H|ATP5J|ENSBTAP00000035213|ATP5J2|ATP8|ATP6
KEGG Pathways	bta04137	Mitophagy - animal	61		12		6,09E-09		2,02E-10		TP53|UBA52|NBR1|SQSTM1|OPTN|UBB|HIF1A|PINK1|USP30|RPS27A|TAX1BP1|USP8
KEGG Pathways	bta00190	Oxidative phosphorylation	130		18		3,83E-11		1,15E-12		ATP5D|COX17|ATP5A1|ATP5G3|ATP5G2|ATP5F1|ATP5L|ATP5B|ATP5C1|ATP5I|ATP5O|ATP5G1|ATP5H|ATP5J|ENSBTAP00000035213|ATP5J2|ATP8|ATP6
KEGG Pathways	bta04120	Ubiquitin mediated proteolysis	133	18	28	6,48E-15	8,92E-22	9,75E-17	2,15E-23	TCEB1|BTRC|UBA52|CUL1|UBE2M|UBE2L3|UBA3|TCEB2|CUL4A|CUL3|VHL|RPS27A|SKP1|UBE2F|RBX1|LOC780968|CUL2|CUL5	UBE2R2|CDC34|HUWE1|CBL|UBA6|UBA52|UBE3C|CUL1|UBE2S|CDC20|UBE2L3|CBLB|NEDD4|NEDD4L|TRAF6|UBA1|UBB|UBE2K|UBR5|RPS27A|UBE2F|TRIP12|UBE2B|MDM2|UBE2D1|UBE2N|UBE2G2|UBE2A

KEGG Pathways	bta03050	Proteasome	45	34	3,94E-41	2,38E-43	PSMA2|PSMB2|PSMD12|PSMA3|PSMB8|ADRM1|PSMB7|PSMB3|PSMD1|PSMA7|PSMC1|PSMD2|PSMD4|PSMA1|PSMB1|PSMB9|PSMC4|PSMA6|PSMB5|PSMB6|PSMA4|PSMC6|PSMA8|PSMB10|PSMD7|PSMD11|PSMA5|PSMC5|PSMB4|PSMD3|PSMC3|PSMD8|PSMD14|PSMC2
Reactome Pathways	BTA-74160	Gene expression (Transcription)	409	33	1,95E-20	1,73E-23	BDP1|POLR2H|POLR2C|TCEB1|ENSBTAP00000003296|SUPT4H1|RRN3|POLR2J|POLR1A|UBA52|CUL1|POLR2G|POLR2D|GTF2A2|CDK7|STK11|POLR1E|GTF2A1|POLR1B|WHSC2|POLR1C|TCEB2|ENSBTAP00000027988|NELFB|POLR2K|SKP1|BRF2|POLR2L|ZNRD1|TWISTNB|GTF2F2|LOC780968|POLR2A
Reactome Pathways	BTA-674695	RNA Polymerase II Pre-transcription Events	22	15	3,66E-20	6,49E-23	POLR2H|POLR2C|TCEB1|SUPT4H1|POLR2J|POLR2G|POLR2D|WHSC2|TCEB2|ENSBTAP00000027988|NELFB|POLR2K|POLR2L|GTF2F2|POLR2A

Reactome Pathways	BTA-113418	Formation of the Early Elongation Complex	16	13	3,82E-18	1,02E-20	POLR2H|POLR2C|SUPT4H1|POLR2J|POLR2G|POLR2D|WHSC2|ENSBTAP00000027988|NELFB|POLR2K|POLR2L|GTF2F2|POLR2A
Reactome Pathways	BTA-6807505	RNA polymerase II transcribes snRNA genes	27	14	7,72E-18	3,42E-20	POLR2H|POLR2C|ENSBTAP00000003296|POLR2J|POLR2G|POLR2D|GTF2A2|CDK7|GTF2A1|ENSBTAP00000027988|POLR2K|POLR2L|GTF2F2|POLR2A
Reactome Pathways	BTA-73762	RNA Polymerase I Transcription Initiation	15	11	4,44E-15	2,36E-17	POLR2H|ENSBTAP00000003296|RRN3|POLR1A|POLR1E|POLR1B|POLR1C|POLR2K|POLR2L|ZNRD1|TWISTNB
Reactome Pathways	BTA-8951664	Neddylation	75	15	3,63E-14	2,25E-16	TCEB1|COPS5|COPS8|COPS4|UBA52|CUL1|NAE1|COPS2|GPS1|TCEB2|VHL|SKP1|UBE2F|LOC780968|CUL5
Reactome Pathways	BTA-73857	RNA Polymerase II Transcription	381	24	8,25E-13	5,85E-15	POLR2H|POLR2C|TCEB1|ENSBTAP00000003296|SUPT4H1|POLR2J|UBA52|CUL1|POLR2G|POLR2D|GTF2A2|CDK7|STK11|GTF2A1|WHSC2|TCEB2|ENSBTAP00000027988|NELFB|POLR2K|SKP1|POLR2L|GTF2F2|LOC780968|POLR2A

Reactome Pathways	BTA-5250924	B-WICH complex positively regulates rRNA expression	45	10		1,06E-09		1,04E-11		POLR2H|ENSBTAP00000003296|POLR1A|POLR1E|POLR1B|POLR1C|POLR2K|POLR2L|ZNRD1|TWISTNB	
Reactome Pathways	BTA-5696394	DNA Damage Recognition in GG-NER	13	7		7,30E-09		9,06E-11		COPS5|COPS8|COPS4|UBA52|COPS2|GPS1|LOC780968	
Reactome Pathways	BTA-1234176	Oxygen-dependent proline hydroxylation of Hypoxia-inducible Factor Alpha	11	5		6,41E-06		9,66E-08		TCEB1|UBA52|TCEB2|VHL|LOC780968	
Reactome Pathways	BTA-5358351	Signaling by Hedgehog	20	5		5,46E-05		1,11E-06		UBA52|CUL1|CDC73|SKP1|LOC780968	
Reactome Pathways	BTA-8856825	Cargo recognition for clathrin-mediated endocytosis	25	5		1,20E-04		2,89E-06		COPS5|COPS8|COPS4|COPS2|GPS1	
Reactome Pathways	BTA-73887	Death Receptor Signalling	24		5		0,0013		3,57E-05		UBA52|OTUD7B|TRAF6|UBB|RNF31
Reactome Pathways	BTA-168249	Innate Immune System	340		17		2,60E-04		6,01E-06		PSMA2|PSMD12|PSMD1|CLU|UBA52|CUL1|B2M|TAB2|TRAF6|UBB|PSMA5|PSMD3|PSMC3|PSMD14|DDX58|TRIM21|PSMC2
Reactome Pathways	BTA-1280218	Adaptive Immune System	222		14		1,70E-04		3,38E-06		UBE2R2|TRIM39|UBA52|FKBP1A|CUL1|B2M|CBLB|TRAF6|UBB|UBE2K|UBE2F|UBE2B|UBE2D1|TRIM21

Reactome Pathways	BTA-69275	G2/M Transition	13	5	1,60E-04	2,90E-06	TP53|UBA52|CUL1|OPTN|UBB
Reactome Pathways	BTA-110320	Translesion Synthesis by POLH	10	5	5,93E-05	1,05E-06	POLH|PCNA|UBA52|UBB|NPLOC4
Reactome Pathways	BTA-168256	Immune System	618	26	2,90E-05	4,88E-07	UBE2R2|PSMA2|PSMD12|PSMD1|CLU|ARIH1|TRIM39|UBA52|FKBP1A|CUL1|B2M|CBLB|TAB2|TRAF6|UBB|UBE2K|PSMA5|PSMD3|PSMC3|UBE2F|UBE2B|PSMD14|UBE2D1|DDX58|TRIM21|PSMC2
Reactome Pathways	BTA-983169	Class I MHC mediated antigen processing & presentation	123	12	1,73E-05	2,45E-07	UBE2R2|TRIM39|UBA52|CUL1|B2M|CBLB|UBB|UBE2K|UBE2F|UBE2B|UBE2D1|TRIM21
Reactome Pathways	BTA-110313	Translesion synthesis by Y family DNA polymerases bypasses lesions on DNA template	14	6	1,35E-05	1,68E-07	POLH|PCNA|UBA52|UBB|NPLOC4|REV1
Reactome Pathways	BTA-8866652	Synthesis of active ubiquitin: roles of E1 and E2 enzymes	7	6	8,25E-07	8,04E-09	USP7|UBA6|UBA52|UBB|USP5|UBE2D1
Reactome Pathways	BTA-5689877	Josephin domain DUBs	6	6	4,94E-07	4,38E-09	RAD23B|ATXN3|JOSD2|UBA52|JOSD1|UBB

<table>
<tr><td>Reactome Pathways</td><td>BTA-5689880</td><td>Ub-specific processing proteases</td><td>51</td><td></td><td>12</td><td></td><td>4,64E-09</td><td></td><td>3,29E-11</td><td></td><td>TP53|USP7|TRRAP|UBA52|USP22|CDC20|UBB|IDE|USP5|USP30|USP13|DDX58</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-5689896</td><td>Ovarian tumor domain proteases</td><td>14</td><td></td><td>10</td><td></td><td>5,21E-11</td><td></td><td>2,31E-13</td><td></td><td>TP53|OTUB1|UBA52|TNIP1|OTUB2|TNIP2|OTUD7B|TRAF6|UBB|UBE2D1</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-8949613</td><td>Cristae formation</td><td>10</td><td></td><td>10</td><td></td><td>1,05E-11</td><td></td><td>2,28E-14</td><td></td><td>ATP5D|ATP5G3|ATP5C1|ATP5I|ATP5O|ATP5G1|ATP5H|ATP5E|ATP8|ATP6</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-163210</td><td>Formation of ATP by chemiosmotic coupling</td><td>10</td><td></td><td>10</td><td></td><td>1,05E-11</td><td></td><td>2,28E-14</td><td></td><td>ATP5D|ATP5G3|ATP5C1|ATP5I|ATP5O|ATP5G1|ATP5H|ATP5E|ATP8|ATP6</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-597592</td><td>Post-translational protein modification</td><td>400</td><td>16</td><td>31</td><td>7,45E-06</td><td>1,05E-11</td><td>1,19E-07</td><td>1,86E-14</td><td>MGAT1|TCEB1|COPS5|COPS8|COPS4|UBA52|CUL1|NAE1|COPS2|GPS1|TCEB2|VHL|SKP1|UBE2F|LOC780968|CUL5</td><td>TP53|RAD23B|OTUB1|USP7|ATXN3|PCNA|IKBKG|JOSD2|UBA6|TRRAP|UBA52|CUL1|USP22|CDC20|JOSD1|TNIP1|OTUB2|TNIP2|RBBP5|OTUD7B|TRAF6|UBB|IDE|USP5|ENSBTAP00000028591|USP30|UBE2F|PSMD14|UBE2D1|USP13|DDX58</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-5688426</td><td>Deubiquitination</td><td>71</td><td></td><td>25</td><td></td><td>1,29E-22</td><td></td><td>1,15E-25</td><td></td><td>TP53|RAD23B|OTUB1|USP7|ATXN3|JOSD2|TRRAP|UBA52|USP22|CDC20|JOSD1|TNIP1|OTUB2|TNIP2|OTUD7B|TRAF6|UBB|IDE|USP5|ENSBTAP00000028591|USP30|PSMD14|UBE2D1|USP13|DDX58</td></tr>
</table>

WikiPathways	WP1066	Eukaryotic transcription initiation	37	30	2,73E-44	1,01E-46	POLR2H|POLR2C|ERCC2|POLR2F|GTF2H1|POLR3H|POLR1A|GTF2H3|POLR2G|POLR3D|GTF2A2|CDK7|POLR1E|GTF2E2|POLR1B|POLR2I|POLR2B|POLR3K|POLR2E|ERCC3|POLR2K|CCNH|POLR3B|GTF2H2|MNAT1|GTF2B|POLR1D|GTF2F2|GTF2H4|POLR2A
WikiPathways	WP3212	Membrane trafficking	61	7	0,0014	3,19E-05	HGS|STAM|VPS36|UBA52|TSG101|UBB|RPS27A
WikiPathways	WP1045	TGF-beta signaling pathway	106	8	0,0041	1,40E-04	HGS|TP53|STAMBPL1|CUL1|UCHL5|NEDD4L|TRAF6|APP
WikiPathways	WP3141	BDNF signaling pathway	114	9	0,0015	3,84E-05	NTRK1|IKBKG|NGF|SORT1|SQSTM1|TRAF6|CAMK2A|NGFR|KCNN2
WikiPathways	WP3132	Regulation of toll-like receptor signaling pathway	114	9	0,0015	3,84E-05	TNFAIP3|OTUD5|USP7|IKBKG|CYLD|TAB2|SQSTM1|TRAF6|RNF31
WikiPathways	WP994	Oxidative phosphorylation	54	12	3,94E-09	5,86E-11	ATP5D|ATP5A1|ATP5G3|ATP5G2|ATP5F1|ATP5B|ATP5O|ATP5G1|ATP5H|ATP5J|ATP5J2|ATP6
WikiPathways	WP978	EGF/EGFR signaling pathway	132	13	3,74E-06	6,95E-08	HGS|STAM|SH3KBP1|PCNA|CBL|STAMBP|EPS15L1|CBLB|NEDD4|PEBP1|CAMK2A|USP8|EPS15

<table>
<tr><td>WikiPathways</td><td>WP1002</td><td>Electron transport chain</td><td>90</td><td>17</td><td>5,02E-12</td><td>5,60E-14</td><td>ATP5D|COX17|ATP5A1|ATP5G3|ATP5G2|ATPIF1|ATP5F1|ATP5L|ATP5B|ATP5C1|ATP5O|ATP5G1|ATP5J|ENSBTAP00000035213|ATP5J2|ATP8|ATP6</td></tr>
<tr><td>WikiPathways</td><td>WP3199</td><td>Parkin-ubiquitin proteasomal system pathway</td><td>60</td><td>19</td><td>6,27E-17</td><td>4,66E-19</td><td>PSMD12|PSMD1|PSMC1|PSMD2|PSMD4|CUL1|PSMC4|UBE2L3|PSMC6|SNCA|UBA1|PSMD7|PSMD11|PSMC5|PSMD3|PSMC3|PSMD8|PSMD14|PSMC2</td></tr>
<tr><td>WikiPathways</td><td>WP1079</td><td>Proteasome degradation</td><td>53</td><td>38</td><td>2,45E-45</td><td>9,13E-48</td><td>PSMA2|PSMB2|PSMD12|PSMA3|PSMB8|PSMB7|PSMB3|PSMD1|PSMA7|PSMC1|PSMD2|PSMD4|PSMB1|PSMB9|PSMC4|PSMA6|PSMB5|PSMB6|NEDD4|PSMA4|PSMC6|UBA1|UBB|PSMB10|PSMD7|PSMD11|PSMA5|PSMC5|PSMB4|PSMD3|PSMC3|UCHL3|UBE2B|PSMD8|UCHL1|UBE2D1|UBE2A|PSMC2</td></tr>
</table>

ST3_Sperm proteoforms: Sperm proteoforms of high (HF) and low freezability (LF) bulls.

PRF ID	Protein Accession	Description	Sequence	Modification	Type	Activation type	Group	Log2 FC	C-Score	Q-value	% Residue Cleavages
502	P61603	10 kDa heat shock protein, mitochondrial	AGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVVAVGSGSKGKGGEIQPVSVKVGDKVLLPEYGGTKVVLDDKDYFLFRDGDILGKYVD	K7(N6-acetyl-L-lysine)	Non-truncated	HCD/EthCD	Both	0,83	41,5695999	8,75E-66	33,00
503	P61603	10 kDa heat shock protein, mitochondrial	AGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVVAVGSGSKGKGGEIQPVSVKVGDKVLLPEYGGTKVVLDDKDYFLFRDGDILGKYVD	N-term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	Both	0,83	43,0442957	7,78E-66	33,00
509	P61603	10 kDa heat shock protein, mitochondrial	AGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGKVLQATVVAVGSGSKGKGGEIQPVSVKVGDKVLLPEYGGTKVVLDDKDYFLFRDGDILGKYVD	N-term(alpha-amino acetylated residue); K7(N6-acetyl-L-lysine); K85(N6-acetyl-L-lysine)	Non-truncated	HCD	LF	NA	21,6758001	3,15E-07	7,00
2468	P82922	28S ribosomal protein S29, mitochondrial	GKTLSLCHIIHFCAKQDWLILHIPDAHLWVKNCRDLLQSTYNKQRFDQPLEASIWLKNFKTANERFL	K43(N6-acetyl-L-lysine)	Truncated	EthCD	Both	-0,65	23,5483742	0,017724	15,15
8411	P82908	28S ribosomal protein S36, mitochondrial	SAGLPSHTSSISQHSKGSKSPDWLMHQGPPDTAEMIKTLPQKYR		Truncated	HCD	Both	-0,57	274,043692	0,000588	20,93
9657	P82908	28S ribosomal protein S36, mitochondrial	VVQVVKPHTPLIRFPDRRDNPKPNVSEVLR		Truncated	HCD/EthCD	Both	-0,11	353,11285	1,21E-09	62,07
8689	A5PJD0	2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase	SKRPFSGLGDLEKHFFAFIDGLPLSGQEGVLRCHPELAGRQLPWGRLTAESQREQSAAGLQNLGAAERLRFTE		Truncated	EthCD	Both	-0,65	18,0114302	0,033846	12,50
3004	Q3T0R7	3-ketoacyl-CoA thiolase, mitochondrial	KGKQTMQVDEHPRPQTTMEQLNKLPPVFKKEGTVTAGNASGVSDGAGAVIIASEDAVKKHNFTPLAR		Truncated	HCD	HF	6,64	267,213503	0,000586	15,15
3006	P31081	60 kDa heat shock protein, mitochondrial	KGVITVKDGKTLNDELEIIEGMKFDR		Truncated	HCD	HF	NA	194,695833	3,92E-05	40,00

1407	P13536	Acetylcholine receptor subunit gamma	DGCVYWLPPAIFRSSCPVSVTFFPFDWQNCSLIFQSQTYSTNEINLQLSQEDGQTIEWIFIDPEAFTENGEWAI	C16(half cystine); C30(half cystine)	Truncated	EthCD	Both	1,45	31,0079562	0,022012	13,70
1141	Q32KZ2	Actin-like protein 7A	AVWVRRDHSEPEPTTSPEVKKPKLELTK		Truncated	HCD/EthCD	Both	NA	366,833855	1,02E-10	55,56
1438	Q32KZ2	Actin-like protein 7A	DHSEPEPTTSPEVKKPKLELTK		Truncated	HCD	LF	NA	199,007219	0,000104	52,38
9245	Q2TA43	Actin-related protein T2	TPLTGANQKKYFVGEEALHRHEVLQLHYPIER		Truncated	HCD	Both	-1,77	213,182128	0,00033	32,26
8789	P07107	Acyl-CoA-binding protein	SQAEFDKAAEEVKHLKTKPADEEMLFIYSHYKQATVGDINTERPGMLDFKGKAKWDAWNELKGTSKEDAMKAYIDKVEELKKKYGI	N-term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	Both	1,34	53,5673323	2,45E-85	29,41
8790	P07107	Acyl-CoA-binding protein	SQAEFDKAAEEVKHLKTKPADEEMLFIYSHYKQATVGDINTERPGMLDFKGKAKWDAWNELKGTSKEDAMKAYIDKVEELKKKYGI	K7(N6-acetyl-L-lysine)	Non-truncated	HCD	Both	1,34	4,01029996	2,25E-15	8,24
7529	A6QLP2	Adenosylhomocysteinase 3	PSEAEPQLGLSTAAVSAMAPPAGGGDPEAPAPAAERPPAPGPGSGPA		Truncated	HCD	LF	NA	31,5682202	0,023911	13,04
7200	Q8SQH5	ADP/ATP translocase 2	NVSVQGIIIYRAAYFGIYDTAKGMLPDPKNTHIFISWMIAQSVTAVAGLTSYPFDT		Truncated	HCD	Both	-0,01	60,1707025	0,02939	12,73
3335	Q9XSJ4	Alpha-enolase	LAKYNQILRIEEELGSKAKFAGRSFRNPLAK		Truncated	HCD/EthCD	Both	0,51	563,108689	3,34E-17	86,67
2485	Q24K15	Angiopoietin-4	GLERTLRAASSNSSLLQRQQHQLLESVQ		Truncated	HCD	Both	-0,73	25,6585214	0,032357	22,22
3333	Q8WMX8	Ankyrin repeat, SAM and basic leucine zipper domain-containing protein 1	LAKNGITSRDQQKIMAALKELEVEEIKFGELPEVAKL		Truncated	HCD	Both	-0,6	49,3279638	0,018949	19,44
2495	Q3ZC08	Annexin A9	GIIDYNLAAQDVQALKQAEGPSTERTWVLVFTQRNPEHLVRVLNQYQWYTGHGLEKTVRARFHGAACVALL		Truncated	EthCD	Both	-0,65	6,09511965	0,017733	15,71
3955	P00829	ATP synthase subunit beta, mitochondrial	ITTTKKGSITSVQAIYVPADDLTDPAPATTFAHLDATTVLSR		Truncated	HCD	LF	NA	278,560678	0,00059	24,39

9543	Q00361	ATP synthase subunit e, mitochondrial	VPPVQVSPLIKLGRYSALFLGMAYGAKRYNYLKPRAEEERRLAAEEKKKRDEQKRIERELAEAQEDTILK	Non-truncated	HCD/EthCD	Both	0,83	1199,00024	5,70E-39	24,64
7037	P02721	ATP synthase-coupling factor 6, mitochondrial	NKELDPVQKLFVDKIREYRTKRQTSGGPVDAGPEYQQDLDRELFKLKQMYGKADMNTFPNFTFEDPKFEVVEKPQS	Non-truncated	HCD/EthCD	Both	-0,34	801,668229	1,31E-125	61,33
7593	P02721	ATP synthase-coupling factor 6, mitochondrial	PVQKLFVDKIREYRTKRQTSGGPVDAGPEYQQDLDRELFKLKQMYGKADMNTFPNFTFEDPKFEVVEKPQS	Truncated	HCD/EthCD	Both	1,59	522,401524	6,24E-10	24,29
2917	P02721	ATP synthase-coupling factor 6, mitochondrial	KELDPVQKLFVDKIREYRTKRQTSGGPVDAGPEYQQDLDRELFKLKQMYGKADMNTFPNFTFEDPKFEVVEKPQS	Truncated	EthCD	LF	-6,64	323,707405	1,05E-14	48,65
581	P01096	ATPase inhibitor, mitochondrial	AKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD	Truncated	HCD/EthCD	Both	0,49	760,873192	2,55E-20	50,00
920	P01096	ATPase inhibitor, mitochondrial	ARAKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD	Truncated	HCD/EthCD	Both	0,91	630,87419	3,94E-36	77,08
1301	P01096	ATPase inhibitor, mitochondrial	DAGGAFGKREQAEEERYFRARAKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD	Truncated	HCD/EthCD	Both	-0,69	986,742659	2,14E-50	70,15
2344	P01096	ATPase inhibitor, mitochondrial	GDNVRSSAGAVRDAGGAFGKREQAEEERYFRARAKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD	Truncated	HCD/EthCD	Both	-1,41	494,941533	2,07E-18	34,18
8601	P01096	ATPase inhibitor, mitochondrial	SGDNVRSSAGAVRDAGGAFGKREQAEEERYFRARAKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD	Truncated	HCD/EthCD	Both	-1,52	412,892749	1,55E-16	37,50
8864	P01096	ATPase inhibitor, mitochondrial	SSAGAVRDAGGAFGKREQAEEERYFRARAKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD	Truncated	HCD/EthCD	Both	-0,45	391,066117	4,69E-20	41,89
9612	P01096	ATPase inhibitor, mitochondrial	VRSSAGAVRDAGGAFGKREQAEEERYFRARAKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD	Truncated	HCD	Both	-2,76	82,1840921	0,005829	11,84
9775	P01096	ATPase inhibitor, mitochondrial	YFRARAKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD	Truncated	HCD/EthCD	Both	0,59	671,193489	4,35E-34	68,63

6231	Q58CU2	Band 4.1-like protein 5	MRKQAEKDRLREAQRAA THIPAAGDARAVITCR		Truncated	EthCD	Both	-0,61	24,9555561	0,022002	21,88
7807	P46168	Beta-defensin 10	QGVRSYLSCWGNRGICLL NRCPGRMRQIGTCLAPRV KCCR	N-Term(alpha-amino acetylated residue); Q1(2-pyrrolidone-5-carboxylic acid (Gln)); C9(half cystine); C21(half cystine); C38(half cystine); C39(half cystine)	Non-truncated	EthCD	LF	-6,64	9,89140893	0,00185	15,38
7826	P21809	Biglycan	QKLYISKNHLVEIPPNLPS SLVELRIHDNRIRKVPKG VFSGLRNM		Truncated	EthCD	Both	0,15	10,896323	0,01153	18,18
2915	Q3SX22	BSD domain-containing protein 1	KEKLTTEGSSGATEKMKK GLSDFLGVISDTFAPSPDK TIDCDVITLM		Truncated	HCD	Both	-0,18	77,1588404	0,004908	17,39
9230	Q3MHJ9	Calcium/calmodulin-dependent protein kinase type II subunit beta	TMGLVEQAKSLLNKKAD GVKPQTNSTKNSAAATSP KGTLPPAALEPQTTVIHNP V		Truncated	EthCD	Both	2,41	32,6570971	0,02015	11,11
2123	O18737	Calcium-binding and coiled-coil domain-containing protein 2	FCYVDQDGVVRGASIPFQ FRPENEEDILVVTTQSEVE EIEQHNKELCKENRELKD SCVSLQKQNSDMQATLQ KKQEELETLKSINKKLE		Truncated	EthCD	Both	1,75	47,6108812	0,024461	9,09
307	P62157	Calmodulin	ADQLTEEQIAEFKEAFSLF DKDGDGTITTKELGTVM RSLGQNPTEAELQDMINE VDADGNGTIDFPEFLTMM ARKMKDTDSEEEIREAFR VFDKDGNGYISAAELRHV MTNLGEKLTDEEVDEMI READIDGDGQVNYEEFV QMMTAK	N-term(alpha-amino acetylated residue); K21(N6-acetyl-L-lysine)	Non-truncated	HCD/EthCD	Both	-0,45	494,949605	6,19E-09	16,33
308	P62157	Calmodulin	ADQLTEEQIAEFKEAFSLF DKDGDGTITTKELGTVM RSLGQNPTEAELQDMINE VDADGNGTIDFPEFLTMM ARKMKDTDSEEEIREAFR VFDKDGNGYISAAELRHV MTNLGEKLTDEEVDEMI READIDGDGQVNYEEFV QMMTAK	N-term(alpha-amino acetylated residue); K115(N6-acetyl-L-lysine)	Non-truncated	HCD/EthCD	Both	-0,45	282,04729	3,76E-120	36,73

317	P62157	Calmodulin	ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAK	N-Term(alpha-amino acetylated residue); K115(N6-acetyl-L-lysine); Y138(O4'-phospho-L-tyrosine)	Non-truncated	HCD/EthCD	Both	-0,91	68,2384217	1,04E-22	8,16
316	P62157	Calmodulin	ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAK	N-Term(alpha-amino acetylated residue); K21(N6-acetyl-L-lysine); Y138(O4'-phospho-L-tyrosine)	Non-truncated	HCD	LF	NA	227,479909	0,00059	6,80
297	P62157	Calmodulin	ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIR	N-Term(alpha-amino acetylated residue)	Truncated	HCD	Both	NA	151,022196	1,55E-25	44,71
298	P62157	Calmodulin	ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFR	N-Term(alpha-amino acetylated residue)	Truncated	HCD	Both	-3,14	90,1469375	2,23E-20	43,82
299	P62157	Calmodulin	ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFD	N-Term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	Both	-3,19	212,448816	7,73E-17	32,61
300	P62157	Calmodulin	ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELR	N-Term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	Both	-2,75	203,551063	1,24E-22	35,24
1003	P06833	Caltrin	ASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGN		Truncated	HCD	Both	0,64	200,143245	4,38E-06	30,77
1004	P06833	Caltrin	ASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNR		Truncated	HCD/EthCD	Both	3,76	325,985205	4,79E-14	52,50
1005	P06833	Caltrin	ASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV		Truncated	HCD/EthCD	Both	1,02	252,95324	5,70E-08	38,10
1338	P06833	Caltrin	DEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSV		Truncated	EthCD	Both	0,3	77,6017165	0,023816	15,56

2258	P06833	Caltrin	FSLSRYAKLANRLANPKL LETFLSKWIGDRGNR	Truncated	HCD/EthCD	Both	0,73	806,16155	1,58E-33	87,50
2259	P06833	Caltrin	FSLSRYAKLANRLANPKL LETFLSKWIGDRGNRSV	Truncated	HCD/EthCD	Both	0,33	710,209058	1,89E-24	76,47
7523	P06833	Caltrin	PSDEKASPDKHHRFSLSR YAKLANRLANPKLLETFL SKWIGDRGN	Truncated	EthCD	Both	1,83	206,333118	0,000591	29,55
7524	P06833	Caltrin	PSDEKASPDKHHRFSLSR YAKLANRLANPKLLETFL SKWIGDRGNR	Truncated	HCD/EthCD	Both	0,3	212,398893	4,70E-07	42,22
7525	P06833	Caltrin	PSDEKASPDKHHRFSLSR YAKLANRLANPKLLETFL SKWIGDRGNRSV	Truncated	HCD/EthCD	Both	0,83	347,466701	2,97E-18	59,57
8464	P06833	Caltrin	SDEKASPDKHHRF	Truncated	HCD/EthCD	Both	NA	643,52551	3,91E-14	91,67
8469	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANRLANPKLLETFLS KWIGD	Truncated	EthCD	Both	1,72	117,291535	0,024606	25,00
8470	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANRLANPKLLETFLS KWIGDRGN	Truncated	HCD/EthCD	Both	0,07	676,922027	8,33E-25	53,49
8471	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANRLANPKLLETFLS KWIGDRGNR	Truncated	HCD/EthCD	Both	0,17	918,60115	2,61E-47	84,09
8472	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANRLANPKLLETFLS KWIGDRGNRSV	Truncated	HCD/EthCD	Both	1,61	783,915976	5,98E-36	73,91
8473	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANRLANPKLLETFLS KWIGDRGNRSVK	Truncated	HCD/EthCD	Both	-0,8	691,56166	2,46E-62	68,09
9747	P06833	Caltrin	YAKLANRLANPKLLETFL SKWIGDRGNR	Truncated	HCD/EthCD	Both	-1,61	942,649116	1,07E-34	96,30
8465	P06833	Caltrin	SDEKASPDKHHRFS	Truncated	HCD	HF	NA	130,349429	0,013512	38,46
8466	P06833	Caltrin	SDEKASPDKHHRFSL	Truncated	HCD	LF	NA	122,011829	0,000588	50,00
8467	P06833	Caltrin	SDEKASPDKHHRFSLSR	Truncated	HCD/EthCD	LF	NA	465,494129	4,33E-11	87,50
8468	P06833	Caltrin	SDEKASPDKHHRFSLSRY AKLANR	Truncated	HCD	LF	NA	198,255757	1,16E-06	43,48
9748	P06833	Caltrin	YAKLANRLANPKLLETFL SKWIGDRGNRSV	Truncated	HCD/EthCD	LF	NA	465,979845	7,44E-15	62,07
9263	Q2YDG3	Cell division cycle protein 123 homolog	TRDFTQPFIHCTDDSPDPC MEYELVLRKWCELIPGAE FRCFVKENKLIGISQRDYT QYYDHISKQKEEICRCIQD FFKKHIQYK	Truncated	HCD/EthCD	Both	-2,06	30,2570366	0,03698	7,23
1444	Q2NKU0	Centromere protein X	DKKTKVSGDALQLVAEL LKIFVVEAAIRSVRQAQA EGLAHVDVEQLEKVLP	Truncated	HCD	HF	6,64	28,4125198	0,014667	10,00

548	Q3ZBF8	Ceramide synthase 2	AGTAVIVDKPWFYDLRKVWEGYPIQSIIPSQY		Truncated	HCD	Both	-1,33	47,5852033	0,02487	16,13
9439	Q8HXM1	Cleavage stimulation factor subunit 2	VHSAGPGSGSSVSMNQQNPQTPQAQTLSGMHVNGAPPLMQASLQAGVAAPGQIPATVTGPGPGSLAPAGGMQAQVGMPGSGPVSMERGQVPMQDPRAAMQRGPLP		Truncated	EthCD	Both	-2,06	221,444715	0,010721	5,77
7196	P17697	Clusterin	NVMPFPLLEPFNFHDVFQPFYDMIHQAQQAMDAHLQRTPYHFPTMEFTENNDRTVCKEIRHNSTGCLRMKDQCEKCQEILEVDCSASNPTQTLLRQQLNASLQLAEKFSRLYDQLLQSYQQKMLNTSALLKQLNEQFTWVSQLANLTQSDDQHYLQVFTVNSHNSDPSIPSGLTKVIVKLFNSFPITVTVPQEVSSPNFMENVAEKALQQYRRKSQEE		Truncated	HCD	Both	NA	551,994931	1,83E-15	7,37
570	Q2TBP7	c-Myc-binding protein	AHYKAADSKREQFRRYLEKSGVLDTLTKVLVALYEEPEKPNSALDFLKHHLGAATPENPEIELLRLELAEMKEKYEAIVEENKKLKTKLAQYEPPQEEKRAE	N-Term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	Both	-1,68	554,367296	7,21E-53	20,79
65	Q2TBP7	c-Myc-binding protein	AADSKREQFRRYLEKSGVLDTLTKVLVALYEEPEKPNSALDFLKHHLGAATPENPEIELLRLELAEMKEKYEAIVEENKKLKTKLAQYEPPQEEKRAE		Truncated	HCD	Both	-0,2	699,719036	2,67E-11	17,53
2420	Q2YDN4	Coiled-coil domain-containing protein 105	GGSTMEKPPPGEGVTLWKSKMKPPAWHARLPLPMHR		Truncated	EthCD	HF	NA	88,3230347	0,027012	28,57
2368	P23206	Collagen alpha-1(X) chain	GEMGHCTPCRPGERGLPGPQGPTGPPGPPGVGKRGENGLPGQPGLKGDQGVPGERGAAGPSGPQGPPGEQGPEGIGKPGAPGIPGQPGIPGMKGQPGAPGTAG	C6(half cystine); C9(half cystine)	Truncated	EthCD	Both	0,15	51,8823217	0,021993	9,80
2370	P23206	Collagen alpha-1(X) chain	GEMGHCTPCRPGERGLPGPQGPTGPPGPPGVGKRGENGLPGQPGLKGDQGVPGERGAAGPSGPQGPPGEQGPEGIGKPGAPGIPGQPGIPGMKGQPGAPGTAG	C6(half cystine)	Truncated	EthCD	Both	0,15	13,5236869	0,016129	10,78

1502	P55206	C-type natriuretic peptide	DLRVDTKSRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C37(half cystine); C53(half cystine)	Non-truncated	HCD	Both	2,68	205,36885	3,01E-24	23,08
201	P55206	C-type natriuretic peptide	AAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C28(half cystine); C44(half cystine)	Truncated	HCD	Both	-0,85	98,5756265	0,004905	13,95
444	P55206	C-type natriuretic peptide	AGGGQKKGDKTPGGGGANLKDDR		Truncated	HCD/EthCD	Both	NA	760,192839	9,07E-21	90,91
843	P55206	C-type natriuretic peptide	APPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDR		Truncated	EthCD	Both	-4,03	443,7521	2,86E-19	65,85
2308	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDR		Truncated	HCD/EthCD	Both	-1,25	855,700616	4,03E-48	88,10
2309	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRS		Truncated	EthCD	Both	-2,72	217,637019	2,20E-09	53,49
2310	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSR		Truncated	HCD/EthCD	Both	0,15	254,028156	6,90E-07	43,18
2311	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	HCD/EthCD	Both	-1,43	271,109571	5,62E-09	39,13
2312	P55206	C-type natriuretic peptide	GAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLLR		Truncated	HCD	Both	-4,96	165,383814	2,84E-05	23,40
2404	P55206	C-type natriuretic peptide	GGGQKKGDKTPGGGGANLKDDR		Truncated	EthCD	Both	NA	411,428579	4,83E-13	76,19
2414	P55206	C-type natriuretic peptide	GGQKKGDKTPGGGGANLKDDR		Truncated	HCD/EthCD	Both	NA	210,919574	4,31E-05	55,00
3632	P55206	C-type natriuretic peptide	LLHEHPNARKYKGGNKKGLS		Truncated	HCD/EthCD	Both	NA	436,016148	5,06E-11	63,16
3633	P55206	C-type natriuretic peptide	LLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C23(half cystine); C39(half cystine)	Truncated	HCD	Both	-0,51	139,914633	0,000158	26,32
7472	P55206	C-type natriuretic peptide	PQAAGGGQKKGDKTPGGGGANLKDDR		Truncated	HCD/EthCD	Both	NA	500,882243	2,44E-11	80,00
8816	P55206	C-type natriuretic peptide	SRAAWTRLLHEHPNARKYKGGNKKGLSKGCFGLKLDRIGSMSGLGC	C30(half cystine); C46(half cystine)	Truncated	HCD	Both	1,1	100,189678	0,01585	13,33
9249	P55206	C-type natriuretic peptide	TPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDR		Truncated	HCD	Both	-0,8	118,655176	0,000589	23,53
9250	P55206	C-type natriuretic peptide	TPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSR		Truncated	EthCD	Both	0,49	219,990211	0,000381	33,33
1993	P55206	C-type natriuretic peptide	EPQAAGGGQKKGDKTPGGGGANLKDDR		Truncated	HCD	HF	NA	61,719405	0,030877	23,08

3631	P55206	C-type natriuretic peptide	LLHEHPNARKYKGGNKKGL		Truncated	EthCD	LF	NA	109,519529	0,027022	38,89
7295	P55206	C-type natriuretic peptide	PGAPPKVPRTPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDR		Truncated	HCD	LF	NA	246,996521	1,23E-06	27,91
9251	P55206	C-type natriuretic peptide	TPSGEEVAEPQAAGGGQKKGDKTPGGGGANLKDDRSRLL		Truncated	HCD	LF	NA	126,517468	0,010301	18,42
7449	Q28181-1	Cyclic nucleotide-gated cation channel beta-1	PPASQERPEGDKDAARPEEHPVRIHVTLGPDPSEQILLVEVPEKQEEKEKKEEETEEKEEGEEARKEKEE		Truncated	HCD	Both	-0,03	12,7850254	0,036797	11,59
2227	Q28092	Cylicin-2	FQKINFGAYDNYVPVSELSKKSWNQQHFALVFPKPPRPGKRR		Truncated	HCD	Both	0,68	283,845233	4,96E-05	36,59
2229	Q28092	Cylicin-2	FQKINFGAYDNYVPVSELSKKSWNQQHFALVFPKPPRPGKRRRSKPSLLQENTSPKYDAEKLRGDRKQPLWMHR		Truncated	HCD/EthCD	Both	1,98	514,132098	3,87E-17	43,84
8685	Q28092	Cylicin-2	SKPSLLQENTSPKYDAEKLRGDRKQPLWMHR		Truncated	HCD/EthCD	Both	1,76	229,216713	3,71E-07	43,33
8723	Q28092	Cylicin-2	SLMRISERPSVYLAARSRHPQKETPPSQEDAKQAAKPSSPKVKKSKEDKDKSDSEAESIVSKEKPR		Truncated	HCD/EthCD	Both	0,66	338,689422	4,02E-07	20,00
8828	Q28092	Cylicin-2	SRHPQKETPPSQEDAKQAAKPSSPKVKKSKEDKDKSDSEAESIVSKEKPR		Truncated	HCD/EthCD	Both	1,07	512,397724	1,61E-19	44,90
9050	Q28092	Cylicin-2	SVPRFQKINFGAYDNYVPVSELSKKSWNQQHFALVFPKPPRPGKRRRSKPSLLQENTSPKYDAEKLRGDRKQPLWMHR	N-Term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	Both	1,69	622,50605	1,02E-25	48,05
9073	Q28092	Cylicin-2	SWNQQHFALVFPKPPRPGKRRRSKPSLLQENTSPKYDAEKLRGDRKQPLWMHR		Truncated	HCD	Both	-0,45	152,290128	0,010295	13,46
2226	Q28092	Cylicin-2	FQKINFGAYDNYVPVSELSKKSWNQQHFALVFPKPPRPGKR		Truncated	HCD	HF	6,64	303,572053	7,57E-08	35,00
2228	Q28092	Cylicin-2	FQKINFGAYDNYVPVSELSKKSWNQQHFALVFPKPPRPGKRRR		Truncated	HCD	HF	6,64	457,299812	2,55E-12	47,62
9049	Q28092	Cylicin-2	SVPRFQKINFGAYDNYVPVSELSKKSWNQQHFALVFPKPPRPGKRRR	N-Term(alpha-amino acetylated residue)	Truncated	HCD	HF	6,64	424,345646	5,63E-12	41,30
8302	Q28092	Cylicin-2	RSKPSLLQENTSPKYDAEKLRGDRKQPLWMHR		Truncated	EthCD	LF	NA	121,909937	0,010288	32,26
8413	Q28092	Cylicin-2	SAKKDAKKDAKKDAKKDAKKDAKKDAKKGK		Truncated	EthCD	LF	-6,64	892,207751	3,80E-27	100,00

8706	P23004	Cytochrome b-c1 complex subunit 2, mitochondrial	SLKVAPKVKATEAPAGVPPHPQDLEFTRLPNGLVIASLENYAPASRIGLFIKAGSRYENSNNLGTSHLLRLASSLTTKGASSFKITR		Truncated	HCD/EthCD	Both	0,88	379,483732	9,13E-17	30,23
3525	P23004	Cytochrome b-c1 complex subunit 2, mitochondrial	IGLFIKAGSRYENSNNLGTSHLLRLASSLTTKGASSFKITR		Truncated	HCD	LF	NA	105,524721	0,018381	20,00
8705	P23004	Cytochrome b-c1 complex subunit 2, mitochondrial	SLKVAPKVKATEAPAGVPPHPQDLEFTRLPNGLVIASLENYAPASR		Truncated	HCD	LF	NA	291,003513	4,44E-07	35,56
527	P00129	Cytochrome b-c1 complex subunit 7	AGRPAVSASSRWLEGIRKWYYNAAGFNKLGLMRDDTIHENDDVKEAIRRLPENLYNDRVFRIKRALDLSMRQQILPKEQWTKYEEDKSYLEPYLKEVIRERKEREEWAKK	N-Term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	Both	0,88	369,164649	1,44E-17	10,09
9349	P00130	Cytochrome b-c1 complex subunit 9	VAPTLTARLYSLLFRRTSTFALTIVVGALFFERAFDQGADAIYEHINEGKLWKHIKHKYENKE		Non-truncated	HCD/EthCD	HF	6,64	756,443541	2,90E-61	33,87
962	P00428	Cytochrome c oxidase subunit 5B, mitochondrial	ASGGGVPTDEEQATGLEREVMLAARKGQD		Truncated	HCD/EthCD	Both	NA	701,156817	2,16E-20	85,71
963	P00428	Cytochrome c oxidase subunit 5B, mitochondrial	ASGGGVPTDEEQATGLEREVMLAARKGQDPYNILAPKATSGTKED		Truncated	HCD/EthCD	Both	-1,37	735,572717	1,48E-20	65,91
964	P00428	Cytochrome c oxidase subunit 5B, mitochondrial	ASGGGVPTDEEQATGLEREVMLAARKGQDPYNILAPKATSGTKEDPNLVPSITNKRIVGCICEEDNSTVIWFWLHKGEAQRCPSCGTHYKLVPHQLAH		Truncated	HCD/EthCD	Both	3,44	179,116565	8,78E-12	17,53
967	P00428	Cytochrome c oxidase subunit 5B, mitochondrial	ASGGGVPTDEEQATGLEREVMLAARKGQDPYNILAPKATSGTKEDPNLVPSITNKRIVGCICEEDNSTVIWFWLHKGEAQRCPSCGTHYKLVPHQLAH	K55(N6-acetyl-L-lysine)	Truncated	HCD/EthCD	Both	NA	38,5999954	2,52E-09	18,56
8605	P00428	Cytochrome c oxidase subunit 5B, mitochondrial	SGGGVPTDEEQATGLEREVMLAARKGQD		Truncated	HCD/EthCD	Both	NA	349,970459	1,06E-07	59,26
8606	P00428	Cytochrome c oxidase subunit 5B, mitochondrial	SGGGVPTDEEQATGLEREVMLAARKGQDPYNILAPKATSGTKED		Truncated	EthCD	LF	NA	286,375866	0,000591	27,91

8877	P13182	Cytochrome c oxidase subunit 6A1, mitochondrial	SSGAHGEEGSARMWKALTYFVALPGVGVSMLNVFLKSHHGEEERPEFVAYPHLRIRSKPFPWGDGNHTLFHNPHVNPLPTGYEDE		Non-truncated	HCD/EthCD	Both	1,41	639,324388	1,27E-48	38,10
2554	P13182	Cytochrome c oxidase subunit 6A1, mitochondrial	GNHTLFHNPHVNPLPTGYEDE		Truncated	HCD	LF	NA	119,877236	0,035263	30,00
345	P00429	Cytochrome c oxidase subunit 6B1	AEDIQAKIKNYQTAPFDSRFPNQNQTRNCWQNYLDFHRCEKAMTAKGGDVSVCEWYRRVYKSLCPISWVSTWDDRRAEGTFPGKI	N-term(alpha-amino acetylated residue); C29(half cystine); C39(half cystine); C53(half cystine); C64(half cystine)	Non-truncated	HCD/EthCD	Both	-0,12	70,8733084	1,26E-23	14,29
2133	P13184	Cytochrome c oxidase subunit 7A2, mitochondrial	FENKVPEKQKLFQEDNGIPVHLKGGIADALLYRATLILTVGGTAYAMYELAVASFPKKQD		Truncated	HCD/EthCD	Both	-2,37	9999	3,73E-116	64,41
9252	P00727-1	Cytosol aminopeptidase	TPTKFAEIVEENLKSASIKTDVFIRPKSWIEEQEMGSFLSVAKGSEEPPVFLEIHYKGSPNASEPPLVFVGKG		Truncated	EthCD	Both	-0,65	19,7254495	0,029912	13,89
3102	Q58DC2	DDB1- and CUL4-associated factor 4	KRYFRLLPGHNNCNPLTKESIRQKEMERKRLRLLEEEEQQGKKIA		Truncated	EthCD	Both	0,83	8,01463515	0,023537	15,91
625	Q9MZG3	Diazepam-binding inhibitor-like 5	AKWEAWNENKGMSKMDAMRIYIAKVEELKKNEAG		Truncated	HCD/EthCD	Both	1,37	491,271507	2,01E-12	48,48
1512	Q58D13	DNA polymerase alpha subunit B	DIVSIQELIEVEEEEETLLNSYTTPSKGSQKRTITTPETPLTKRSVSARSPHQLLSP	T39(O-phospho-L-threonine)	Truncated	EthCD	Both	0,89	17,2583099	0,030887	14,29
571	Q2KID4	Dynein axonemal light chain 1	AKATTIKEALARWEEKTSQKPSEAR	N-term(alpha-amino acetylated residue)	Truncated	HCD	HF	NA	166,92348	0,033302	25,00
1186	P61285	Dynein light chain 1, cytoplasmic	CDRKAVIKNADMSEEMQQDSVECATQALEKYNIEKDIAAHIKKEFDKKYNPTWHCIVGRNFGSYVTHETKHFIYFYLGQVAILLFKSG	N-term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	Both	0,74	95,8354096	1,99E-49	18,39
1187	P61285	Dynein light chain 1, cytoplasmic	CDRKAVIKNADMSEEMQQDSVECATQALEKYNIEKDIAAHIKKEFDKKYNPTWHCIVGRNFGSYVTHETKHFIYFYLGQVAILLFKSG	K35(N6-acetyl-L-lysine)	Non-truncated	HCD/EthCD	Both	0,74	4,01029996	2,10E-49	18,39

1189	P61285	Dynein light chain 1, cytoplasmic	CDRKAVIKNADMSEEMQQDSVECATQALEKYNIEKDIAAHIKKEFDKKYNPTWHCIVGRNFGSYVTHETKHFIYFYLGQVAILLFKSG	N-term(alpha-amino acetylated residue); S87(O-phospho-L-serine)	Non-truncated	EthCD	LF	NA	29,0388407	3,77E-08	10,34
6438	Q3MHR3	Dynein light chain 2, cytoplasmic	MSDRKAVIKNADMSEDMQQDAVDCATQAMEKYNIEKDIAAYIKKEFDKKYNPTWHCIVGRNFGSYVTHETKHFIYFYLGQVAILLFKSG	N-term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	Both	1,31	612,503769	3,00E-30	22,73
8508	Q3MHR3	Dynein light chain 2, cytoplasmic	SDRKAVIKNADMSEDMQQDAVDCATQAMEKYNIEKDIAAYIKKEFDKKYNPTWHCIVGRNFGSYVTHETKHFIYFYLGQVAILLFKSG	N-term(alpha-amino acetylated residue)	Non-truncated	HCD/EthCD	Both	1,53	2209,09809	3,36E-203	51,72
8506	Q3MHR3	Dynein light chain 2, cytoplasmic	SDRKAVIKNADMSEDMQQDAVDCATQAMEKYNIEKDIAAYIKKEFDKKYNPTWHC	N-term(alpha-amino acetylated residue)	Truncated	HCD	Both	-1,29	653,740813	1,03E-07	24,07
1913	Q2T9V2	Dynein regulatory complex protein 9	EIGGFKMPKDKDDSKDVKGKGKEKDKRRGKK		Truncated	EthCD	LF	NA	235,311017	2,18E-05	36,67
3701	Q2TBV3	Electron transfer flavoprotein subunit beta	LNEPRYATLPNIMKAKKKIEVIKAGDLGVDLTSKLSVISVEDPPQRT		Truncated	EthCD	Both	0,67	50,6228876	0,024616	14,89
3759	Q2KIG0	Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial	LPMNNHGNYIVRLGHLVSWMGEQAEALGVEVYPGYAAAE		Truncated	EthCD	Both	-0,05	19,0160575	0,024321	21,05
9693	P49410	Elongation factor Tu, mitochondrial	WDMACRIILPPGKELAMPGEDLKLTLILRQPMILEKGQRFTLRDGNRTIG	K36(N6-acetyl-L-lysine)	Truncated	HCD	HF	NA	62,3749026	0,02394	14,29
3327	A1A4N1	Equilibrative nucleoside transporter 3	LAEATGVVMTFYMGLGLVLGSACSALLVHLI		Truncated	EthCD	Both	-0,73	8,28042539	0,037142	13,33
7598	A0JN51	ETS-related transcription factor Elf-1	PVVVSPGNQHLHTVTLQTVPITTVIASADPSSAAGSQKFILQAIPSSQPMTV		Truncated	EthCD	Both	0,3	43,6431856	0,005413	15,69
955	Q2YDH8	Fibronectin type III domain-containing protein 8	ASETFYKVGDGEEAMLKKETLNVLNALDQMSKPFPNPKSMNRTVTTKSLPLSSR	N-term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	Both	NA	666,995272	1,32E-20	56,60
9416	Q2YDH8	Fibronectin type III domain-containing protein 8	VGDGEEAMLKKETLNVLNALDQMSKPFPNPKSMNR		Truncated	HCD	HF	6,64	319,2789	2,37E-09	47,06

953	Q2YDH8	Fibronectin type III domain-containing protein 8	ASETFYKVGDGEEAMLKKETLNVLNALDQMSKPFPNPKSMNR	N-term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	LF	-6,64	961,568178	3,85E-40	78,05
954	Q2YDH8	Fibronectin type III domain-containing protein 8	ASETFYKVGDGEEAMLKKETLNVLNALDQMSKPFPNPKSMNRTVTTK	N-term(alpha-amino acetylated residue)	Truncated	HCD	LF	NA	483,17685	1,38E-13	41,30
1431	Q2KJE5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	DGRGAHQNIIPASTGAAKAVGKVIPDLKGKLTGMAFR		Truncated	HCD/EthCD	Both	0,63	814,650322	7,78E-29	72,22
2152	Q2KJE5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	FGILEGLMTTVHSYTATQKTVDGPSKKAWR		Truncated	HCD/EthCD	Both	0,64	893,617436	6,09E-36	79,31
2153	Q2KJE5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	FGILEGLMTTVHSYTATQKTVDGPSKKAWRDGR		Truncated	HCD/EthCD	Both	1,35	862,608333	9,57E-28	75,00
2303	Q2KJE5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	GAHQNIIPASTGAAKAVGKVIPDLKGKLTGMAFR		Truncated	HCD/EthCD	Both	-1,16	658,240957	2,48E-23	84,85
9399	Q2KJE5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	VEAEPEPPAQPQPQPEPIKEEVPPPPPPPPAPKKVR		Truncated	HCD/EthCD	HF	6,64	611,181543	6,59E-18	62,86
9400	Q2KJE5	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	VEAEPEPPAQPQPQPEPIKEEVPPPPPPPPAPKKVRELIVGINGFGR		Truncated	HCD	HF	NA	591,568234	8,46E-12	36,96
7026	O18836	Growth/differentiation factor 8	NGHDLAVTFPEPGEDGLTPFLEVKVTDTPKRSRRDFGLDCDEHSTESRCCRYPLTVDFEAFGWDWIIAPKRYKANYCSGEYEFVFLQKY		Truncated	HCD	Both	1,33	189,164615	0,011516	6,82
7426	Q08DJ8	Heat shock factor protein 1	PLSSSPLVRVKEEPPSPPQSPRAEGASPGRPSSMVETPLSPTTLIDSILRESEPTPVASTTPLVDTGGRPP		Truncated	EthCD	Both	1,84	119,609588	0,015858	8,57
3469	A4IF94	Hippocampus abundant transcript-like protein 1	LFGACIVFMSFLVAVFIPEYSKGGIQKHSNSISGSLANTPERGS		Truncated	HCD	Both	0,37	83,4341516	0,006317	18,60

8636	P0C0S9	Histone H2A type 1	SGRGKQGGKARAKAKTRSSR	N-term(alpha-amino acetylated residue)	Truncated	EthCD	LF	NA	181,665522	0,000586	68,42
9177	Q8MJD5	Homeodomain-only protein	TETASGPTEDQVEILEYNFNKVNKHPDPTTLCLIAAEAGLSE		Truncated	EthCD	LF	-6,64	29,9657326	0,024312	19,51
7232	Q5E9X7	Inactive serine protease 35	PAPSLSDLEDSLSYETVFENGSRTLTRVKVQGWVPEPTQNLTSQGA		Truncated	EthCD	Both	-0,18	56,4975688	0,016137	20,00
9055	Q0P5I2	Interleukin-1 receptor-associated kinase-like 2	SVRNTEDKQETGQPVRPPTFPGSGPAAVRVNLPAPPEDASS		Truncated	EthCD	HF	NA	1,49318123	0,023367	15,00
7708	P26895	Interleukin-7	QGTLTLLNCTSKGKGRKPPSLSEAQPTKNLEENKSSK		Truncated	HCD	HF	NA	100,746798	0,011523	16,67
5011	Q3SYS7	IQ domain-containing protein F1	METDQPKTVDENTENKPQENKGLVENSLPSEPSPTLEKAEAQQENKAATK	N-term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	Both	NA	230,921934	3,63E-10	38,78
5012	Q3SYS7	IQ domain-containing protein F1	METDQPKTVDENTENKPQENKGLVENSLPSEPSPTLEKAEAQQENKAATKGADDADKR	N-term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	Both	-0,61	679,476287	5,91E-36	77,19
2539	Q3SYS7	IQ domain-containing protein F1	GLVENSLPSEPSPTLEKAEAQQENKAATKGADDADKR		Truncated	HCD/EthCD	HF	6,64	216,244597	3,88E-06	41,67
8657	Q3SYS7	IQ domain-containing protein F1	SGVVTKPQKKPPVSDPEAVKIQAWWR		Truncated	HCD	HF	NA	243,338628	1,91E-05	44,00
8658	Q3SYS7	IQ domain-containing protein F1	SGVVTKPQKKPPVSDPEAVKIQAWWRGTLVRRTLLHAALRVWIIQAWWRVKLARLQDSRRR		Truncated	HCD	HF	NA	361,11807	0,000502	13,33
1498	Q5E9A7	Kelch domain-containing protein 2	DLREDELPGPAYEGYESAELACPAERSGHVAVSDGRHMFVWGGYKSNQVRGLYDFYLPREELWIYNMETGRWKKINTEGDVPPSMSGSCAVCVDRVLYLFGGHHSRGNTNKFYMLDSRSTDRVLHWERIDCQGVPPSSKDKLGVWVYKNKLIFFGGYGYLPEDKVLGTFEFDETSFWNSSHPRGWNDHVHILDTETFI		Truncated	HCD	LF	NA	120,791043	0,018715	3,05
7189	Q2HJJ0	Kinesin light chain 4	NTTLRNLGALYRRQGKLEAAETLEECALRSRKQGTDPISQTKVAELLGEGDSGRTSQE		Truncated	EthCD	Both	0,54	20,6671334	0,032368	12,28

7053	Q05443	Lumican	NKISNIPDEYFKRFSALQY LRLSHNELADSGVPGNSF NVSSLLELDLSYNKLKSIP TVNENL		Truncated	EthCD	Both	0,3	28,460479	0,036351	11,48
8043	Q05204	Lysosome-associated membrane glycoprotein 1	RDAAIQAYLSSSNFSREET RCEQDLPTPTTPPQPAPTP APASPAVFRYNVSGSNGT CLLASMGLQLNVTYRRV DNKTVTREFNVNPNKTTF GGNCSATLATLELHSENL LLLALQFVMNESSSRVFL QGVQLNLTLPDAKEGSFT ATNSSLRALQATAGNSYK CNAEQRLRVTSSFSLNMF RVWLQAFRVDGDKFGPV EECQLDENSMLIPIAVGG ALAGLVLIVLLAYLIGRK RSHAGYQT	C(201half cystine)	Truncated	HCD	LF	NA	268,774466	0,002257	2,49
620	Q32LG3	Malate dehydrogenase, mitochondrial	AKVAVLGASGGIGQPLSL LLKNSPLVSRLTLYDIAHT PGVAADLSHIETR		Truncated	EthCD	Both	1,07	780,537425	3,74E-29	65,31
1302	Q3SZB4	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	DAKIYQIYEGTAQIQRLII AREHIGRYKK		Truncated	EthCD	Both	NA	495,594812	5,50E-14	60,71
1167	Q5E9T8	Mevalonate kinase	AYSVCLAAALLTACEEIP NPLKDGEAAGRWTEENL ELINKWAFQGERVIHGNP SGVDNAVSTWGGALR		Truncated	EthCD	Both	0,23	5,98086759	0,030571	13,43
8882	Q148H0	MICOS complex subunit MIC26	SSKKDSPHKDTVKVNELS LYSVPEHQSKYVEEPRTQ LEESISHLR		Truncated	HCD	Both	-0,71	176,052646	0,005837	18,18
741	P10522	Myelin protein P0	AMEKGKLHKTAKDASKR GRQTPVLYAMLDHSRST KAASEKKTKGLGES		Truncated	HCD	Both	-0,82	31,0660853	0,022021	12,77
9041	Q32KY3	Myeloid leukemia factor 1	SVAHENSGSRELKRREKH HQSPAIEHGR		Truncated	EthCD	LF	NA	92,1625585	0,013505	25,93
1727	Q05752	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	EAMPPSIVMSSQKVLVAG KPAESSAVAASEKKAVSP APPIKRWELSQDEPYL		Truncated	EthCD	Both	NA	207,648055	0,00059	19,61
8399	P25712	NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial	SAESGKNEKGLPPNPKKQ SPPKKPVSAAPTEPFDNTT YKNLQHHDYSTYTFLDL NLDLSKFRMPQPSSGRES PRH		Non-truncated	HCD/EthCD	Both	-2,35	9999	1,92E-38	17,57

3277	P23934	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	KVRFVGRQKEVNENFAIDLIAEQPVSQVGSR	Truncated	HCD	LF	NA	157,059756	0,013732	26,67
7281	Q2TBI1	Non-structural maintenance of chromosomes element 4 homolog A	PERPGLEDTEPSDSGDEMIDPAS	Truncated	EthCD	LF	NA	177,604961	0,011869	27,27
9192	Q2TBH0	Outer dense fiber protein 3	TGKDLGPAYSILGRYHTKTTLTPGPGDYFPEKSTKHVFDSAPSHSISAR	Truncated	HCD/EthCD	Both	-1	369,726709	1,60E-10	37,50
9214	Q2TBH0	Outer dense fiber protein 3	TKTFRVDSTPGPAAYMLPMVMGPHTIGKVSQPSFSIKGRSKLGSFSDDLHKTPGPAAYR	Truncated	HCD	Both	-2,91	285,91796	0,000589	13,79
9872	Q2TBH0	Outer dense fiber protein 3	YSVNPKILRTGKDLGPAYSILGRYHTKTTLTPGPGDYFPEKSTKHVFDSAPSHSISAR	Truncated	HCD/EthCD	Both	-0,24	476,254954	6,32E-10	29,82
9213	Q2TBH0	Outer dense fiber protein 3	TKTFRVDSTPGPAAYMLPMVMGPHTIGKVSQPSFSIKGR	Truncated	HCD/EthCD	LF	-6,64	554,951023	3,15E-09	34,21
9791	Q2TBH0	Outer dense fiber protein 3	YHTKTTLTPGPGDYFPEKSTKHVFDSAPSHSISAR	Truncated	HCD	LF	-6,64	169,069505	0,000589	26,47
2520	A6QQJ3	Peripherin	GLRSSVSSTSYRRTFGPPPSLSPGAFSYSSSSRFSSSRLL	Truncated	EthCD	Both	-0,05	16,810401	0,000587	23,08
9730	Q32L96-1	Phytanoyl-CoA hydroxylase interacting protein-like	WSEIIEFCTADYSKVHLTQLLEKAEVIAGRMLKFSVFYRNQHKEYFDYIREHHGNAMQPSVKDNSGSHGSPISGKLEGIFFSCSTEFNTG	Truncated	EthCD	Both	1,73	77,3676519	0,02513	8,99
3548	Q9N2I2	Plasma serine protease inhibitor	LGSQRIVFNRPFLVLIVKNSKHILFLGKVTRP	Truncated	EthCD	LF	NA	409,900992	0,030867	22,58
8431	Q9N2I2	Plasma serine protease inhibitor	SARLGSQRIVFNRPFLVLIVKNSKHILFLGKVTRP	Truncated	HCD	LF	-6,64	758,058158	1,30E-08	35,29
6145	P0CG53	Polyubiquitin-B	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG	Truncated	HCD/EthCD	Both	-1,55	816,081707	6,34E-135	46,67
2237	Q2KHV4	Presenilins-associated rhomboid-like protein, mitochondrial	FRKAPRKVEPRRSDTSSEAYKR	Truncated	EthCD	HF	NA	249,659622	0,000101	57,14

2494	Q0P5H7	Probable arginine--tRNA ligase, mitochondrial	GLKSELFSGLPKKRIVVEFSSPNVAKKFHVGHLRSTIIGNFIANLKE		Truncated	EthCD	Both	0,15	1,0329803	0,036374	13,04
7120	P01211	Proenkephalin-A	NPLACTLECEGKLPSLKTWETCKELLQLTKLELPPDATSALSKQEESHLLAKKYGGFMKRYGGFMKKMDELYPLEVEEEANGG		Truncated	EthCD	Both	-1,52	34,5038778	0,036969	10,98
8268	Q3SX30	Proteasome inhibitor PI31 subunit	RRGGMIVDPLRSGFPRALIDPSSGLPNRLPPGAVPPGARFDPFGPIGTSPSG	R28(omega-N-methyl-L-arginine)	Truncated	EthCD	Both	0,3	12,6132182	0,007882	15,69
7025	Q2TBR5	Protein FAM166B	NGGQGSQELPKEAKGEKDVEKDQEPKPEVEKEPELGQEAEQASPYSMDDRDPRKFFMSGFTGYVPRARFLFGSSFPVLSNQALQEFGEMKSPG		Truncated	EthCD	Both	1,75	23,9007696	0,024331	7,61
2180	A8QW39	Protein FAM183A	FLNLIHHAAQGPRKKYPETQTEGQEIGWDSEPLVNPQRDDR		Truncated	HCD/EthCD	Both	-0,41	312,116114	1,63E-06	32,50
3773	Q32LN6	Protein FAM205C	IPVNQHVKKKSNSEGILKKQEAVEADLGNKLK		Truncated	HCD/EthCD	Both	0,72	214,353586	1,01E-05	41,94
7592	Q32LN6	Protein FAM205C	PVNQHVKKKSNSEGILKKQEAVEADLGNKLKHFTQWINPDMKGQGRKECILRCKDE		Truncated	HCD	Both	0,89	41,1279998	0,034386	12,73
1053	Q3SZT6	Protein Flattop	ATNYSANQYEKPFSPKYLQNWSLAKPTKERISSHEGYTQIIANDR	N-term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	Both	-0,56	672,773812	3,40E-21	54,55
9094	Q3SZT6	Protein Flattop	TAAGAASLTRWIQKNPDLLKASNGLRPEIFGKPHDPDSQKKLR		Truncated	HCD/EthCD	Both	NA	362,005311	7,74E-06	26,19
9095	Q3SZT6	Protein Flattop	TAAGAASLTRWIQKNPDLLKASNGLRPEIFGKPHDPDSQKKLRK		Truncated	HCD/EthCD	Both	-2,39	606,060653	1,67E-27	69,77
9716	Q3SZT6	Protein Flattop	WIQKNPDLLKASNGLRPEIFGKPHDPDSQKKLR	N-term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	Both	NA	179,874308	6,95E-05	37,50
9717	Q3SZT6	Protein Flattop	WIQKNPDLLKASNGLRPEIFGKPHDPDSQKKLRK		Truncated	HCD/EthCD	Both	NA	323,089297	8,07E-11	54,55
3259	Q2T9T0	Protein phosphatase 1 regulatory subunit 32	KVHFDTQDYGPQAITGLEPKDAPLLHQQQNKGSLEWENAGHGPR		Truncated	HCD	HF	NA	153,325114	0,002259	13,95
1630	P50397	Rab GDP dissociation inhibitor beta	DRVEKVGQVIRVICILSHPIKNTNDANSCQIIIPQNQVNRKSDIYVCMISSAHNVAAQ		Truncated	EthCD	Both	0,89	132,284828	0,022031	10,53

8436	A4FUG8	Rab GTPase-binding effector protein 2	SASISSFSLGGGASGRASLPRSRQGLSPEQEETASLVSTGTLVPEGIYLP		Truncated	HCD	HF	6,64	1,31997317	0,026011	16,33
2805	A8E4N3	Radial spoke head protein 3 homolog	HASVRPENGLGGPGGTREPLVGFESQDQGASQAQRPLPDRDSLQR		Truncated	HCD	Both	-0,62	91,9109014	0,011876	15,91
9635	A5D7J5	Rho-related GTP-binding protein RhoU	VSVDGRPVKLQLCDTAGQDEFDKLRPLCYTNADIFLLC		Truncated	EthCD	Both	-0,05	1,78623074	0,036339	16,22
9329	Q17QD3	RNA binding protein fox-1 homolog 1	TVSGTATTDDSAPTDGQPQTQPSEN		Truncated	EthCD	LF	NA	130,848871	0,001746	16,67
1345	P04557	Seminal plasma protein A3	DEQLSEDNVILPKEKKD		Truncated	HCD/EthCD	Both	-1,3	671,411564	5,24E-14	81,25
1346	P04557	Seminal plasma protein A3	DEQLSEDNVILPKEKKDPASGAETKD		Truncated	HCD/EthCD	Both	NA	212,138705	0,000589	44,00
1362	P04557	Seminal plasma protein A3	DEQLSEDNVILPKEKKDPASGAETKDNKCVFPFIYGNKKYFDCTLHGSLFLWCSLDADYTGRWKYCTKNDYAKCVFPFIYEGKSYDTCIIIGSTFMNYWCSLSSNYDEDGVWKYC	C115(half cystine)	Truncated	EthCD	HF	NA	4,01029996	0,000587	10,53
2006	P04557	Seminal plasma protein A3	EQLSEDNVILPKEKKD		Truncated	HCD	LF	NA	165,901246	0,000587	60,00
1579	P02784	Seminal plasma protein PDC-109	DQDEGVSTEPTQDGPAELPEDEECVFPFVYRNRKHFDCTVHGSLFPWCSLDADYVGRWKYCAQRDYAKCVFPFIYGGKKYETCTKIGSMWMSWCSLSPNYDKDRAWKYC	C24(half cystine); C38(half cystine); C48(half cystine); C61(half cystine); C69(half cystine); C83(half cystine); C94(half cystine); C109(half cystine)	Non-truncated	HCD/EthCD	Both	0,24	218,55354	3,33E-38	15,74
1577	P02784	Seminal plasma protein PDC-109	DQDEGVSTEPTQD		Truncated	EthCD	LF	NA	465,41858	8,98E-06	75,00
956	Q2KJB1	Septin-10	ASEVARHLLFQSHITTKTAHTSSQVSDHEQKQKDSPR	N-term(alpha-amino acetylated residue)	Truncated	HCD/EthCD	Both	-2,68	280,114168	6,28E-05	38,89
3476	P01001	Serine protease inhibitor Kazal-type 6	LFQVDCAEFKDPKVYCTRESNPHCGSDGQTYGNKCAFCKAVMKSGGKINLKHRGKC	C6(half cystine); C16(half cystine); C24(half cystine); C35(half cystine); C38(half cystine); C56(half cystine)	Non-truncated	HCD/EthCD	Both	1,67	84,1057314	5,63E-09	10,91

7674	P01001	Serine protease inhibitor Kazal-type 6	QGAQVDCAEFKDPKVYCTRESNPHCGSDGQTYGNKCAFCKAVMKSGGKINLKHRGKC	Q1(2-pyrrolidone-5-carboxylic acid (Gln)); C7(half cystine); C17(half cystine); C25(half cystine); C36(half cystine); C39(half cystine); C57(half cystine)	Non-truncated	HCD/EthCD	Both	0,41	114,837027	0,002924	5,36
9701	Q9GMB8	Serine--tRNA ligase, cytoplasmic	WFPGSGAFRELVSCSNCTDYQARRLRIRYGQTKKMM		Truncated	HCD	LF	NA	1,31225609	0,037473	11,43
7186	A2I7N0	Serpin A3-4	NTDFAFSLYKQLALKDPNKNVMFSPLSVSMALAFLSLGARGPTLTEILEGLKFNPTEIQETQIHQGFQHLLQ		Truncated	EthCD	Both	-0,65	14,109519	0,031424	16,90
8358	A0RZB4	Shadow of prion protein	RVRVRPAPRYAGSSMRVAAGAAAGAAAGAAAGLAAGSSW		Truncated	HCD	LF	NA	48,2785659	0,023902	7,89
7822	A6QQL9	Signal recognition particle 14 kDa protein	QKCRLSGSVFITLKKYDGRTKPIPRKGSVEGFEPSDNKCLLRATDGK		Truncated	EthCD	Both	0,17	2,22318442	0,037314	15,22
8027	Q5J316	Solute carrier family 2, facilitated glucose transporter member 12	RAMALTSSMNWGINLLISLTFLTVTDLIGLPWVCFIYTVMSLASLVFV		Truncated	EthCD	Both	0,3	27,3615807	0,030592	17,02
8772	P58353	Solute carrier family 2, facilitated glucose transporter member 5	SNVVPMYLGELAPKNWRGALGVVPQLFITIGILVAQIFGLRSLLANE		Truncated	HCD	LF	NA	64,224195	0,018958	13,04
1263	Q58D31	Sorbitol dehydrogenase	CRRAGVTLGNKVLVCGAGPIGLVSLLAAKAMGAAQVVVTDLSASRLSKAKEVG		Truncated	EthCD	Both	0,15	8,19583134	0,02393	9,62
8360	A6QQ77	Sperm acrosome membrane-associated protein 3	RVYSRCELARVLQDFGLEGYRGYSLADWICLAYFASGFNTGAVDHEADGSTNSGIFQINSRKWCKNLNPNVPNLCQMYCSDLLNPNLKDTVICAMKITQEPQGLGSWEAWRHHCQGKDLSDWVDGCEL	C6(half cystine); C30(half cystine); C64(half cystine); C75(half cystine); C79(half cystine); C93(half cystine); C114(half cystine); C123(half cystine)	Non-truncated	HCD/EthCD	Both	0	130,412216	4,46E-05	3,94
3325	Q32KL7	Sperm equatorial segment protein 1	IADINSQLHHVPLPESYKPEYRADIRASKEHLKR		Truncated	HCD	Both	-2,42	85,2658625	0,012707	21,21

9442	Q32KL7	Sperm equatorial segment protein 1	VKELKYTHDVGPGDNDV LINPVSEETTTFPTR		Truncated	HCD/EthCD	Both	NA	248,898901	0,000473	32,26
9548	Q32KL7	Sperm equatorial segment protein 1	VPTKEPGRQKKSKSPNNA NFIGPR		Truncated	HCD	Both	NA	272,66739	1,87E-10	60,87
9550	Q32KL7	Sperm equatorial segment protein 1	VPTKEPGRQKKSKSPNNA NFIGPRVSRVKELKYTHD VGPGDNDVLINPVSEETT TFPTR		Truncated	HCD/EthCD	Both	-1,14	230,21287	1,41E-06	25,86
9631	Q32KL7	Sperm equatorial segment protein 1	VSRVKELKYTHDVGPGD NDVLINPVSEETTTFPTR		Truncated	HCD/EthCD	Both	-0,65	530,528056	2,55E-19	67,65
8863	Q32KL7	Sperm equatorial segment protein 1	SRVKELKYTHDVGPGDN DVLINPVSEETTTFPTR		Truncated	HCD	HF	NA	213,281347	2,81E-05	36,36
9549	Q32KL7	Sperm equatorial segment protein 1	VPTKEPGRQKKSKSPNNA NFIGPRVS		Truncated	EthCD	HF	NA	126,211885	0,002255	36,00
4886	P29392	Spermadhesin-1	MDWLPRNTNCGGILKEES GVIATYYGPKTNCVWTIQ MPPEYHVRVSIQYLQLNC NKESLEIIDGLPGSPVLGK ICEGSLMDYRSSGSIMTV KYIREPEHPASFYEVLYFQ DPQA	C10(half cystine); C31(half cystine); C54(half cystine); C75(half cystine)	Non-truncated	HCD/EthCD	Both	-1,38	264,948241	6,11E-47	19,47
7460	Q32KP0	Spermatid-specific manchette-related protein 1	PPLWAWEANKFVTPGLT HTAQR		Truncated	HCD	HF	NA	252,44925	5,00E-07	52,38
872	Q32KP0	Spermatid-specific manchette-related protein 1	APTSIKEVYKDPPLWAWE ANKFVTPGLTHTAQR		Truncated	HCD	LF	NA	427,392386	7,42E-14	59,38
3074	Q17QX9	Spliceosome-associated protein CWC27 homolog	KNFSLLSFGEEAEEEEEEV NRVSQSMKGKSKSS		Truncated	HCD	Both	0,42	33,9053686	0,026396	21,88
5104	Q58DR8	Succinate--CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	MGHAGAIIAGGKGGAKE KIAALQSAGVVVSMSPAQ LGTTMYKEFEKRKML		Truncated	HCD/EthCD	Both	1,77	757,790825	5,43E-30	57,14
3964	A0JNN2	Surfactant-associated protein 2	LVGSSQGAGPGMTLQLK LKNSLLANSSYNSSFLDFL QKFCLLLHLPLGTNVTL		Truncated	EthCD	HF	NA	44,9726461	0,032296	13,46
8694	Q2YDD6	Synaptogyrin-4	SLDEGGVVLTSLSPPSAAS PVNTPTTGPHGPSYASSSL SPYLSTPKAPR		Truncated	HCD	LF	NA	384,454432	7,56E-13	43,75
1334	P61763	Syntaxin-binding protein 1	DEDDDLWIALRHKHIAEV SQEVTRSLKDFSSSKRMN		Truncated	EthCD	Both	-0,05	10,2985469	0,025767	20,00
3292	A8SMG2	Testis-expressed protein 43	KVVLKKGPPEIKIADMPL HSPLSRYQSTVISHGFRRR LI		Truncated	HCD	LF	-6,64	463,53708	4,27E-11	44,74

1400	Q3T0I4	THO complex subunit 4	DFGVSDADIQELFAEFGT LKKAAVHYDRSGRSLGT ADVHFERKADALKAMKQ YNGVPLDGRPMNIQ		Truncated	EthCD	Both	1,87	24,4089189	0,022297	15,38
7819	Q29S07	Transcription factor IIIB 50 kDa subunit	QHRHMLVRKAFRDGTAE MDAGEKELQGQGQGQGL GDEDVGSSSLELPAG		Truncated	EthCD	LF	NA	40,3048534	0,030582	12,50
3405	Q3ZBY2	Transgelin-3	LCKLINSLYPPGQEPIPKIS ESKMAFKQMEQISQFLKA AEIYGVRTTDIFQTVDLW		Truncated	EthCD	Both	0,89	33,9240011	0,036991	16,36
1010	O46629	Trifunctional enzyme subunit beta, mitochondrial	ASQTKSKKTLAKPNIRNIV VVDGVRTPFLLSGTSYKD LMPHDLAR		Truncated	HCD/EthCD	Both	0,01	424,587908	6,32E-11	40,91
4166	P81947	Tubulin alpha-1B chain	MACCLLYRGDVVPKDVN AAIATIKTKR		Truncated	HCD	LF	NA	178,878209	0,00226	42,31
6199	P81947	Tubulin alpha-1B chain	MRECISIHVGQAGVQIGN ACWELYCLEHGIQPDGQ MPSDKTIGGGDDSFNTFF SETGAGKHVPR	N-term(alpha-amino acetylated residue)	Truncated	EthCD	LF	NA	283,240547	0,013739	14,29
6200	Q3MHM5	Tubulin beta-4B chain	MREIVHLQAGQCGNQIG AKFWEVISDEHGIDPTGT YHGDSDLQLER		Truncated	HCD	Both	-1,24	514,960631	5,69E-18	60,00
6201	Q3MHM5	Tubulin beta-4B chain	MREIVHLQAGQCGNQIG AKFWEVISDEHGIDPTGT YHGDSDLQLERINVYYNE ATGGKYVPR		Truncated	HCD/EthCD	Both	-0,06	456,412107	6,21E-17	49,18
7556	Q3MHM5	Tubulin beta-4B chain	PTGTYHGDSDLQLERINV YYNEATGGKYVPR		Truncated	HCD	Both	NA	637,008066	2,66E-29	90,00
2277	Q3MHM5	Tubulin beta-4B chain	FWEVISDEHGIDPTGTYH GDSDLQLERINVYYNEAT GGKYVPR		Truncated	HCD	LF	NA	88,4590694	0,023546	23,81
7555	Q3MHM5	Tubulin beta-4B chain	PTGTYHGDSDLQLER		Truncated	HCD	LF	NA	138,256487	0,003328	57,14
9381	Q2KJD0	Tubulin beta-5 chain	VDLEPGTMDSVRSGPFGQ IFRPDNFVFGQSGAGNNW AKGHYTEGAELVDSVLD VVRKEAESCDCLQGFQLT HSLGGGTGSGMGTLLISK IREEYPDR		Truncated	HCD	Both	1,96	221,520078	0,023893	6,25
3031	Q2KJD0	Tubulin beta-5 chain	KLAVNMVPFPRLHFFMPG FAPLTSRGSQQYR		Truncated	HCD/EthCD	LF	NA	321,860696	1,59E-07	40,00
3939	Q3T077	Tubulin polymerization-promoting protein family member 2	LTDTSKYTGTHKERFDES GKGKGIAGREDVTDNSG YVSGYK		Truncated	HCD/EthCD	Both	1,31	291,847715	1,34E-06	30,00

3940	Q3T077	Tubulin polymerization-promoting protein family member 2	LTDTSKYTGTHKERFDESGKGKGIAGREDVTDNSGYVSGYKGAGTYDKKGSN		Truncated	HCD/EthCD	Both	-0,02	688,841081	1,22E-22	47,06
7241	Q3T077	Tubulin polymerization-promoting protein family member 2	PATTGVTKATTVGGVSR		Truncated	HCD	LF	NA	214,230377	0,002801	62,50
7171	Q3T131	Ubiquinone biosynthesis O-methyltransferase, mitochondrial	NRIMWFKSYSITFACLNWMKSYRLP		Truncated	HCD	Both	-0,51	60,6626071	0,013724	20,83
5345	Q2KIJ6	UBX domain-containing protein 6	MKKFFQEIKADIKFKSAGPGQKLTESVGEKAPKEKPSQPPVRQPR	N-term(alpha-amino acetylated residue)	Truncated	HCD	Both	1,01	255,15378	0,001744	15,91
424	Q2T9X5	Uncharacterized protein C7orf61 homolog	AFKVSETFKLVEPPKEAKVSKMDVSPK		Truncated	EthCD	LF	NA	284,048445	9,73E-05	61,54
212	P00744	Vitamin K-dependent protein Z	ADADFADSVLLPQPGVLGGWTLRGREMVPLRLRVTHVEPAECGRALNAT		Truncated	HCD	HF	6,64	16,2608951	0,023921	8,33
9803	Q9MZL7-1	Voltage-dependent L-type calcium channel subunit beta-1	YLEAYWKATHPPSSTPPNPLLNRTMATAALAASPAPVSNLQGPYLASGDQSLERATGEHASVHEYPGELGQPPGLYPSSHPPGRAG		Truncated	HCD	Both	-0,34	25,8598564	0,020884	5,88
886	Q17QX2	Zinc finger CCCH-type with G patch domain-containing protein	AQEDAEPLALQNAIAETAEVPVAPGAELETVPSRETGPGPTE		Truncated	EthCD	LF	NA	43,054023	0,02447	19,51
8996	A6QQM4-1	Zinc finger protein 474	STLQQSFHHSKEPTFLINQAVLFGESHSSFLPEIERD		Truncated	EthCD	Both	-0,05	22,4295639	0,027032	19,44

ST4_enriched_pathway: KEGG, Reactome and WikiPathways pathways specifically enriched (FDR<0.01 and n° genes>5) by processing protein interactors (STRING score >0.5) of uniquely expressed proteoform families in high (HF) and low fertility (LF) sperm.

<table>
<tr><th></th><th></th><th></th><th></th><th colspan="2">n° genes</th><th colspan="2">FDR</th><th colspan="2">p-value</th><th colspan="2"># genes</th></tr>
<tr><th>Category</th><th>Term name</th><th>Description</th><th># background genes</th><th>SP HF</th><th>SP LF</th><th>SP HF</th><th>SP LF</th><th>SP HF</th><th>SP LF</th><th>SP HF</th><th>SP LF</th></tr>
<tr><td>KEGG Pathways</td><td>bta04630</td><td>JAK-STAT signaling pathway</td><td>176</td><td>46</td><td></td><td>1,11E-32</td><td></td><td>3,69E-34</td><td></td><td>IL5RA|IL22RA1|IFNLR1|GHR|IL4R|JAK1|MPL|IL10RA|LEPR|IL15RA|PDGFRA|IL9R|IL2RG|EPOR|IL20RB|CSF2RB|IL12RB2|LIFR|PRLR|EGFR|JAK2|IFNGR1|IFNGR2|IL23R|IFNAR2|IL20RA|IL7|IL27RA|IL22RA2|IL6ST|IL6R|IL13RA1|IL10RB|IL21R|IL7R|LOC616008|CRLF2|IL2RA|JAK3|IFNAR1|IL11RA|CNTFR|PDGFRB|OSMR|CSF2RA|IL12RB1</td><td></td></tr>
<tr><td>KEGG Pathways</td><td>bta04060</td><td>Cytokine-cytokine receptor interaction</td><td>283</td><td>45</td><td></td><td>5,47E-24</td><td></td><td>1,98E-25</td><td></td><td>IL5RA|IL22RA1|IFNLR1|IL1B|GHR|IL4R|MPL|IL10RA|IL1R1|LEPR|IL15RA|IL9R|IL2RG|EPOR|IL20RB|CSF2RB|IL12RB2|IL1A|LIFR|PRLR|IFNGR1|IFNGR2|IL23R|IFNAR2|IL20RA|IL7|IL27RA|IL6ST|IL6R|IL13RA1|IL10RB|IL21R|IL1RN|IL7R|LOC616008|CRLF2|IL2RA|NGFR|IFNAR1|IL11RA|IL1RAP|CNTFR|OSMR|CSF2RA|IL12RB1</td><td></td></tr>
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<table>
<tr><td>KEGG Pathways</td><td>bta03460</td><td>Fanconi anemia pathway</td><td>48</td><td>25</td><td>1,41E-23</td><td>5,51E-25</td><td>FANCA|FANCM|CENPX|SLX4|UBE2T|FANCL|RPA2|FANCF|SLX1A|FANCI|RPA1|FANCD2|APITD1|FANCB|EME2|FANCC|ERCC1|C17orf70|BLM|FANCE|ERCC4|FANCG|EME1|FAAP24|HES1</td></tr>
<tr><td>KEGG Pathways</td><td>bta03010</td><td>Ribosome</td><td>134</td><td>33</td><td>1,13E-22</td><td>4,77E-24</td><td>MRPL9|MRPS16|MRPL32|MRPL19|RPS18|RPS3|RPL35|RPL23|MRPL21|MRPL20|MRPS2|MRPS9|RPS9|UBA52|MRPS5|RPS2|RPSA|MRPS6|MRPL2|RPS11|MRPS10|RPS5|MRPS7|RPS20|RPS15|RPL11|MRPL27|MRPL17|RPL8|MRPL22|LOC506989|MRPL36|MRPL33</td></tr>
<tr><td>KEGG Pathways</td><td>bta00280</td><td>Valine, leucine and isoleucine degradation</td><td>47</td><td>21</td><td>8,74E-19</td><td>4,21E-20</td><td>HADH|ACAT2|ACAA2|HMGCS2|DBT|ACADS|HADHB|PCCA|HMGCS1|ACAT1|HMGCLL1|HADHA|PCCB|HSD17B10|ACADSB|ACAA1|ALDH6A1|HMGCL|ACADM|OXCT1|EHHADH</td></tr>
<tr><td>KEGG Pathways</td><td>bta00640</td><td>Propanoate metabolism</td><td>30</td><td>18</td><td>7,07E-18</td><td>3,62E-19</td><td>ACAT2|ECHDC1|ACSS1|SUCLG1|DBT|SUCLA2|ACADS|SUCLG2|PCCA|ACAT1|ACSS2|HADHA|PCCB|ALDH6A1|ACSS3|ACOX1|ACOX3|EHHADH</td></tr>
<tr><td>KEGG Pathways</td><td>bta00071</td><td>Fatty acid degradation</td><td>41</td><td>17</td><td>8,11E-15</td><td>4,40E-16</td><td>HADH|ACAT2|ACAA2|ACADVL|ACADS|ECI1|HADHB|ACADL|ACAT1|HADHA|ECI2|ACADSB|ACAA1|ACOX1|ACADM|ACOX3|EHHADH</td></tr>
</table>

KEGG Pathways	bta01212	Fatty acid metabolism	57	17	6,75E-13	3,86E-14	HADH|ACAT2|ACAA2|ACADVL|HSD17B4|ACADS|HADHB|ACADL|ACAT1|HADHA|MECR|ACADSB|ACAA1|ACOX1|ACADM|ACOX3|EHHADH
KEGG Pathways	bta04659	Th17 cell differentiation	107	21	8,02E-13	4,83E-14	IL1B|IL4R|JAK1|IL1R1|IKBKG|HSP90AA1|CHUK|IKBKB|IL2RG|JAK2|IFNGR1|IFNGR2|IL23R|IL27RA|IL6ST|IL6R|IL21R|IL2RA|JAK3|IL1RAP|IL12RB1
KEGG Pathways	bta01200	Carbon metabolism	108	21	8,96E-13	5,67E-14	ACAT2|ACSS1|CS|SUCLG1|SUCLA2|ACADS|SDHB|SUCLG2|PCCA|DLAT|ACAT1|ACSS2|HADHA|PCCB|SDHC|SDHD|ALDH6A1|ACOX1|ACOX3|EHHADH|SDHA
KEGG Pathways	bta03040	Spliceosome	123	22	9,59E-13	6,36E-14	SMNDC1|PUF60|SF3B2|SF3A1|SF3B14|CHERP|SNRPG|SF3A3|LOC786829|DHX15|SF3B1|U2SURP|SF3B3|U2AF1|SNRPD2|SRSF9|SNRPF|SNRPD3|U2AF1L4|SF3A2|SRSF1|SNRPE
KEGG Pathways	bta00650	Butanoate metabolism	26	11	8,88E-10	6,15E-11	HADH|ACAT2|HMGCS2|ACADS|HMGCS1|ACAT1|HMGCLL1|HADHA|HMGCL|OXCT1|EHHADH
KEGG Pathways	bta04620	Toll-like receptor signaling pathway	98	17	9,92E-10	7,17E-11	MYD88|IL1B|TLR4|IKBKG|CHUK|IKBKB|TLR2|TOLLIP|LY96|CD14|IFNAR2|IRAK1|TRAF6|IRAK4|TIRAP|IFNAR1|TLR6
KEGG Pathways	bta04217	Necroptosis	144	18	2,76E-08	2,33E-09	IL1B|JAK1|TLR4|HSP90AA1|LOC617905|IL1A|JAK2|IFNGR1|IFNGR2|IFNAR2|H2AFV|HMGB1|JAK3|IFNAR1|H2AFJ|HIST2H2AB|HIST1H2AC|LOC528006

<table>
<tr><td>KEGG Pathways</td><td>bta00020</td><td>Citrate cycle (TCA cycle)</td><td>29</td><td>10</td><td>2,76E-08</td><td>2,33E-09</td><td>CS|SUCLG1|SUCLA2|SDHB|SUCLG2|DLAT|SDHC|SDHD|ACLY|SDHA</td></tr>
<tr><td>KEGG Pathways</td><td>bta00072</td><td>Synthesis and degradation of ketone bodies</td><td>9</td><td>7</td><td>9,56E-08</td><td>8,93E-09</td><td>ACAT2|HMGCS2|HMGCS1|ACAT1|HMGCLL1|HMGCL|OXCT1</td></tr>
<tr><td>KEGG Pathways</td><td>bta04640</td><td>Hematopoietic cell lineage</td><td>92</td><td>14</td><td>1,60E-07</td><td>1,55E-08</td><td>IL5RA|IL1B|IL4R|IL1R1|IL9R|EPOR|IL1A|CD14|IL7|IL6R|IL7R|IL2RA|IL11RA|CSF2RA</td></tr>
<tr><td>KEGG Pathways</td><td>bta04151</td><td>PI3K-Akt signaling pathway</td><td>331</td><td>25</td><td>2,81E-07</td><td>2,79E-08</td><td>GHR|IL4R|JAK1|TLR4|IKBKG|HSP90AA1|PDGFRA|CHUK|IKBKB|IL2RG|TLR2|EPOR|PRLR|EGFR|JAK2|IFNAR2|IL7|IL6R|IL7R|IL2RA|JAK3|NGFR|IFNAR1|PDGFRB|OSMR</td></tr>
<tr><td>KEGG Pathways</td><td>bta04064</td><td>NF-kappa B signaling pathway</td><td>101</td><td>14</td><td>4,23E-07</td><td>4,46E-08</td><td>MYD88|IL1B|IL1R1|TLR4|IKBKG|CHUK|IKBKB|LY96|CD14|IRAK1|TRAF6|IRAK4|TIRAP|DDX58</td></tr>
<tr><td>KEGG Pathways</td><td>bta04658</td><td>Th1 and Th2 cell differentiation</td><td>92</td><td>13</td><td>9,18E-07</td><td>1,11E-07</td><td>IL4R|JAK1|IKBKG|CHUK|IKBKB|IL2RG|IL12RB2|JAK2|IFNGR1|IFNGR2|IL2RA|JAK3|IL12RB1</td></tr>
<tr><td>KEGG Pathways</td><td>bta05161</td><td>Hepatitis B</td><td>158</td><td>16</td><td>2,11E-06</td><td>2,61E-07</td><td>MYD88|JAK1|TLR4|IKBKG|CHUK|IKBKB|TLR2|IFIH1|JAK2|IRAK1|TRAF6|JAK3|IRAK4|TIRAP|IFNAR1|DDX58</td></tr>
<tr><td>KEGG Pathways</td><td>bta00630</td><td>Glyoxylate and dicarboxylate metabolism</td><td>28</td><td>7</td><td>2,89E-05</td><td>3,83E-06</td><td>ACAT2|ACSS1|CS|PCCA|ACAT1|ACSS2|PCCB</td></tr>
<tr><td>KEGG Pathways</td><td>bta04380</td><td>Osteoclast differentiation</td><td>113</td><td>12</td><td>3,80E-05</td><td>5,26E-06</td><td>IL1B|JAK1|IL1R1|IKBKG|CHUK|IKBKB|IL1A|IFNGR1|IFNGR2|IFNAR2|TRAF6|IFNAR1</td></tr>
<tr><td>KEGG Pathways</td><td>bta03320</td><td>PPAR signaling pathway</td><td>77</td><td>10</td><td>4,57E-05</td><td>6,47E-06</td><td>HMGCS2|ACOX2|ACADL|HMGCS1|UBB|ACAA1|ACOX1|ACADM|ACOX3|EHHADH</td></tr>
<tr><td>KEGG Pathways</td><td>bta04146</td><td>Peroxisome</td><td>79</td><td>10</td><td>5,50E-05</td><td>7,95E-06</td><td>ACOX2|HSD17B4|HMGCLL1|ECI2|ACAA1|HMGCL|ACOX1|SOD1|ACOX3|EHHADH</td></tr>
</table>

KEGG Pathways	bta04061	Viral protein interaction with cytokine and	83	9		4,50E-04		6,85E-05		IL22RA1|IL10RA|IL2RG|IL20RB|IL20RA|IL6ST|IL6R|IL10RB|IL2RA	
KEGG Pathways	bta00410	beta-Alanine metabolism	32	6		4,90E-04		7,87E-05		ACADS|HADHA|ALDH6A1|ACOX1|ACOX3|EHHADH	
KEGG Pathways	bta04010	MAPK signaling pathway	266	16		7,30E-04		1,20E-04		MYD88|IL1B|IL1R1|IKBKG|PDGFRA|CHUK|IKBKB|IL1A|EGFR|CD14|IRAK1|TRAF6|NGFR|IRAK4|IL1RAP|PDGFRB	
KEGG Pathways	bta04621	NOD-like receptor signaling pathway	163	12		9,00E-04		1,50E-04		MYD88|IL1B|JAK1|TLR4|IKBKG|HSP90AA1|CHUK|IKBKB|IFNAR2|TRAF6|IRAK4|IFNAR1	
KEGG Pathways	bta00062	Fatty acid elongation	28	5		0,0023		3,90E-04		HADH|ACAA2|HADHB|HADHA|MECR	
KEGG Pathways	bta00620	Pyruvate metabolism	36	5		0,0059		0,0011		ACAT2|ACSS1|DLAT|ACAT1|ACSS2	
KEGG Pathways	bta04657	IL-17 signaling pathway	83	7		0,009		0,0017		IL1B|IKBKG|HSP90AA1|CHUK|IKBKB|TRAF6|SRSF1	
KEGG Pathways	bta00260	Glycine, serine and threonine	42		5		0,0074		5,80E-04		CBS|BHMT|SDSL|SDS|CTH
KEGG Pathways	bta01230	Biosynthesis of amino acids	70		8		2,50E-04		1,72E-05		CBS|IDH3G|IDH3A|SDSL|MTR|IDH3B|SDS|CTH
KEGG Pathways	bta04142	Lysosome	122		11		6,12E-05		3,87E-06		CD68|LAMP3|NAPSA|ATP6V0A2|CTSD|ATP6AP1|ATP6V0D1|ATP6V0A1|ATP6V0D2|ATP6V0C|LAMP1
KEGG Pathways	bta04962	Vasopressin-regulated water reabsorption	43		8		1,09E-05		6,56E-07		DYNC1LI2|DYNC1I2|PRKACA|DYNC1LI1|DYNC1I1|DYNC2H1|DYNC1H1|PRKACB
KEGG Pathways	bta04721	Synaptic vesicle cycle	70		10		4,36E-06		2,36E-07		ATP6V1G1|ATP6V0A2|ATP6V1B1|ATP6V1C1|ATP6V0D1|ATP6V1D|ATP6V0A1|ATP6V0D2|ATP6V0C|ATP6V1A
KEGG Pathways	bta04623	Cytosolic DNA-sensing pathway	60		11		1,14E-07		5,82E-09		POLR2H|POLR2F|POLR3H|POLR3F|POLR3D|POLR3A|POLR1C|POLR2E|POLR3C|POLR3B|POLR3E
KEGG Pathways	bta04540	Gap junction	81		13		2,09E-08		1,01E-09		TUBB3|GJA1|TUBB2B|TUBB4B|PRKACA|TUBB|TUBB4A|TUBA1B|TUBB1|TUBB2A|TUBB6|LOC533307|PRKACB

<table>
<tr><td>KEGG Pathways</td><td>bta04966</td><td>Collecting duct acid secretion</td><td>24</td><td>10</td><td>7,49E-10</td><td>3,38E-11</td><td>ATP6V1G1|ATP6V0A2|ATP6V1B1|ATP6V1C1|ATP6V0D1|ATP6V1D|ATP6V0A1|ATP6V0D2|ATP6V0C|ATP6V1A</td></tr>
<tr><td>KEGG Pathways</td><td>bta03020</td><td>RNA polymerase</td><td>29</td><td>11</td><td>1,86E-10</td><td>7,82E-12</td><td>POLR2H|POLR2F|POLR3H|POLR3F|POLR3D|POLR3A|POLR1C|POLR2E|POLR3C|POLR3B|POLR3E</td></tr>
<tr><td>KEGG Pathways</td><td>bta00270</td><td>Cysteine and methionine metabolism</td><td>47</td><td>15</td><td>2,36E-13</td><td>9,24E-15</td><td>CBS|CCBL2|AHCYL2|BHMT|DNMT1|DNMT3B|SDSL|MDH2|MTR|AHCY|SDS|BHMT2|CCBL1|AHCYL1|CTH</td></tr>
<tr><td>KEGG Pathways</td><td>bta04145</td><td>Phagosome</td><td>149</td><td>32</td><td>4,24E-24</td><td>1,41E-25</td><td>ATP6V1G1|TUBB3|DYNC1LI2|DYNC1I2|TUBB2B|TUBB4B|TUBB|ATP6V0A2|RAB7A|ATP6V1B1|TUBB4A|ATP6AP1|ENSBTAP00000016194|TUBA1B|RAB7B|ATP6V1C1|ATP6V0D1|DYNC1LI1|DYNC1I1|ATP6V1D|TUBB1|ATP6V0A1|DYNC2H1|ATP6V0D2|TUBB2A|ATP6V0C|TUBB6|DYNC1H1|LAMP1|LOC533307|RILP|ATP6V1A</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-1428517</td><td>The citric acid (TCA) cycle and respiratory electron transport</td><td>78</td><td>38</td><td>5,17E-34</td><td>1,37E-36</td><td>NDUFB8|NDUFB5|NDUFA8|CS|COX18|NDUFA9|NDUFB6|NDUFA1|NDUFA3|NDUFA13|NDUFA5|NDUFB10|NDUFS5|NDUFA4|NDUFB7|NDUFB3|COX6A1|NDUFA2|COX5A|NDUFC2|NDUFA11|NDUFS7|NDUFB9|NDUFB2|NDUFS1|COX20|NDUFS6|ENSBTAP00000048274|COX4I1|COX7CP1|ENSBTAP00000052707|NDUFV2|ND4|COX2|ND6|ND3|NDUFB11|NDUFA7</td></tr>
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Reactome Pathways	BTA-606279	Deposition of new CENPA-containing nucleosomes at the centromere	39	32	7,16E-34	2,54E-36	CENPC1|MIS18BP1|CENPX|RBBP4|CENPL|LOC617905|OIP5|APITD1|MIS18A|H2AFV|CENPQ|RUVBL1|CENPO|HJURP|H2AFJ|HIST2H2AB|HIST1H2BL|CENPW|ENSBTAP00000046113|ENSBTAP00000046382|LOC507211|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|CENPK|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-73894	DNA Repair	104	37	1,74E-29	1,18E-31	FANCA|DCLRE1B|CENPX|SLX4|FANCL|RPA2|LOC617905|FANCF|UBA52|SLX1A|FANCD2|APITD1|FANCB|EME2|H2AFV|UBB|ERCC1|DCLRE1A|H2AFJ|HIST2H2AB|HIST1H2BL|ERCC4|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|FANCG|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|MUS81|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|EME1|FAAP24|LOC528006

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<tr><td>Reactome Pathways</td><td>BTA-1640170</td><td>Cell Cycle</td><td>136</td><td>36</td><td>5,70E-25</td><td>4,55E-27</td><td>CENPC1|MIS18BP1|CENPX|RBBP4|RPA2|CENPL|LOC617905|UBA52|OIP5|JAK2|APITD1|MIS18A|H2AFV|UBB|CENPQ|RUVBL1|CENPO|HJURP|H2AFJ|HIST2H2AB|HIST1H2BL|CENPW|ENSBTAP00000046113|ENSBTAP00000046382|LOC507211|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|CENPK|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-6783310</td><td>Fanconi Anemia Pathway</td><td>20</td><td>20</td><td>3,07E-22</td><td>2,72E-24</td><td>FANCA|DCLRE1B|CENPX|SLX4|FANCL|FANCF|UBA52|SLX1A|FANCD2|APITD1|FANCB|EME2|UBB|ERCC1|DCLRE1A|ERCC4|FANCG|MUS81|EME1|FAAP24</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-449147</td><td>Signaling by Interleukins</td><td>81</td><td>25</td><td>1,74E-18</td><td>1,70E-20</td><td>IFNLR1|JAK1|IL1R1|IL15RA|IL9R|IKBKB|UBA52|IL20RB|CSF2RB|LIFR|JAK2|IL23R|IL20RA|TRAF6|IL27RA|UBB|IL6ST|IL6R|IL13RA1|IL7R|CRLF2|JAK3|IRAK4|CSF2RA|IL12RB1</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-1280215</td><td>Cytokine Signaling in Immune system</td><td>142</td><td>28</td><td>2,22E-16</td><td>2,36E-18</td><td>IFNLR1|JAK1|IL1R1|IL15RA|IL9R|IKBKB|UBA52|IL20RB|CSF2RB|LIFR|JAK2|IFNGR2|IL23R|IL20RA|TRAF6|IL27RA|UBB|IL6ST|IL6R|IL13RA1|IL7R|CRLF2|JAK3|IRAK4|IFNAR1|DDX58|CSF2RA|IL12RB1</td></tr>
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Reactome Pathways	BTA-212300	PRC2 methylates histones and DNA	30	17	6,42E-16	7,40E-18	RBBP4|LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-427413	NoRC negatively regulates rRNA expression	25	16	1,33E-15	1,65E-17	LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-73728	RNA Polymerase I Promoter Opening	26	16	1,97E-15	2,62E-17	LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-427359	SIRT1 negatively regulates rRNA expression	26	16	1,97E-15	2,62E-17	LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006

Reactome Pathways	BTA-110330	Recognition and association of DNA glycosylase with site containing an affected purine	28	16	3,97E-15	6,33E-17	LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-9670095	Inhibition of DNA recombination at telomere	29	16	5,73E-15	9,65E-17	LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-110331	Cleavage of the damaged purine	30	16	8,20E-15	1,45E-16	LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-2299718	Condensation of Prophase Chromosomes	30	16	8,20E-15	1,45E-16	LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006

Reactome Pathways	BTA-201681	TCF dependent signaling in response to WNT	47	18	8,99E-15	1,83E-16	LOC617905|UBA52|H2AFV|UBB|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-201722	Formation of the beta-catenin:TCF transactivating complex	31	16	1,02E-14	2,17E-16	LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-2559580	Oxidative Stress Induced Senescence	41	17	1,84E-14	4,40E-16	RBBP4|LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006
Reactome Pathways	BTA-212165	Epigenetic regulation of gene expression	54	18	5,25E-14	1,35E-15	RBBP4|LOC617905|SF3B1|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006

<table>
<tr><td>Reactome Pathways</td><td>BTA-8936459</td><td>RUNX1 regulates genes involved in megakaryocyte differentiation and platelet function</td><td>36</td><td>16</td><td>5,25E-14</td><td>1,37E-15</td><td>LOC617905|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-5250924</td><td>B-WICH complex positively regulates rRNA expression</td><td>45</td><td>17</td><td>5,59E-14</td><td>1,54E-15</td><td>LOC617905|SF3B1|H2AFV|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-69278</td><td>Cell Cycle, Mitotic</td><td>101</td><td>19</td><td>4,20E-11</td><td>1,53E-12</td><td>LOC617905|UBA52|JAK2|H2AFV|UBB|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-2262752</td><td>Cellular responses to stress</td><td>119</td><td>20</td><td>6,64E-11</td><td>2,47E-12</td><td>RBBP4|LOC617905|UBA52|HSPA9|H2AFV|UBB|H2AFJ|HIST2H2AB|HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006</td></tr>
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<tr><td>Reactome Pathways</td><td>BTA-5693532</td><td>DNA Double-Strand Break Repair</td><td>43</td><td>14</td><td>6,92E-11</td><td>2,64E-12</td><td>SLX4|SLX1A|EME2|HIST1H2BL|ENSBTAP00000046113|LOC526915|ENSBTAP00000049501|HIST1H2BB|HIST3H2BB|MUS81|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|EME1</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-73857</td><td>RNA Polymerase II Transcription</td><td>381</td><td>32</td><td>5,90E-10</td><td>2,41E-11</td><td>COX18|SNRPG|LOC617905|UBA52|ENSBTAP00000011044|NDUFA4|COX6A1|SNRPF|H2AFV|ENSBTAP00000022848|UBB|COX5A|H2AFJ|HIST2H2AB|HIST1H2BL|COX20|ENSBTAP00000046113|LOC507211|HIST1H2AC|LOC526915|ENSBTAP00000048274|ENSBTAP00000049501|COX4I1|COX7CP1|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|COX2|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-9018519</td><td>Estrogen-dependent gene expression</td><td>25</td><td>10</td><td>1,73E-08</td><td>7,20E-10</td><td>HIST1H2BL|ENSBTAP00000046113|HIST1H2AC|LOC526915|ENSBTAP00000049501|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313</td></tr>
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<tr><td>Reactome Pathways</td><td>BTA-212436</td><td>Generic Transcription Pathway</td><td>345</td><td>28</td><td>2,14E-08</td><td>9,10E-10</td><td>COX18|LOC617905|UBA52|NDUFA4|COX6A1|H2AFV|UBB|COX5A|H2AFJ|HIST2H2AB|HIST1H2BL|COX20|ENSBTAP00000046113|LOC507211|HIST1H2AC|LOC526915|ENSBTAP00000048274|ENSBTAP00000049501|COX4I1|COX7CP1|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|COX2|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313|LOC528006</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-5689880</td><td>Ub-specific processing proteases</td><td>51</td><td>12</td><td>5,34E-08</td><td>2,32E-09</td><td>LOC617905|UBA52|IFIH1|UBB|HIST2H2AB|HIST1H2BL|HIST1H2AC|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|DDX58</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-5688426</td><td>Deubiquitination</td><td>71</td><td>13</td><td>1,61E-07</td><td>7,13E-09</td><td>LOC617905|UBA52|IFIH1|TRAF6|UBB|HIST2H2AB|HIST1H2BL|HIST1H2AC|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|DDX58</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-5693565</td><td>Recruitment and ATM-mediated phosphorylation of repair and signaling proteins at DNA double strand breaks</td><td>27</td><td>9</td><td>4,23E-07</td><td>1,91E-08</td><td>HIST1H2BL|ENSBTAP00000046113|LOC526915|ENSBTAP00000049501|HIST1H2BB|HIST3H2BB|LOC505903|ENSBTAP00000055166|ENSBTAP00000055313</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-3214815</td><td>HDACs deacetylate histones</td><td>31</td><td>9</td><td>1,08E-06</td><td>5,19E-08</td><td>RBBP4|LOC617905|HIST2H2AB|HIST1H2BL|HIST1H2AC|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-451927</td><td>Interleukin-2 family signaling</td><td>15</td><td>7</td><td>2,47E-06</td><td>1,21E-07</td><td>JAK1|IL15RA|IL9R|CSF2RB|JAK2|JAK3|CSF2RA</td></tr>
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Reactome Pathways	BTA-3214847	HATs acetylate histones	26	8	4,04E-06	2,00E-07	LOC617905|HIST2H2AB|HIST1H2BL|HIST1H2AC|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB
Reactome Pathways	BTA-447115	Interleukin-12 family signaling	10	6	6,46E-06	3,26E-07	JAK1|JAK2|IL23R|IL27RA|IL6ST|IL12RB1
Reactome Pathways	BTA-8854691	Interleukin-20 family signaling	10	6	6,46E-06	3,26E-07	IFNLR1|JAK1|IL20RB|JAK2|IL20RA|JAK3
Reactome Pathways	BTA-512988	Interleukin-3, Interleukin-5 and	19	7	8,18E-06	4,35E-07	JAK1|UBA52|CSF2RB|JAK2|UBB|JAK3|CSF2RA
Reactome Pathways	BTA-5693568	Resolution of D-loop Structures through Holliday Junction Intermediates	5	5	1,09E-05	5,90E-07	SLX4|SLX1A|EME2|MUS81|EME1
Reactome Pathways	BTA-5689901	Metalloprotease DUBs	15	6	3,48E-05	2,04E-06	LOC617905|UBA52|UBB|HIST2H2AB|HIST1H2AC|ENSBTAP00000052035
Reactome Pathways	BTA-8953854	Metabolism of RNA	109	12	6,21E-05	3,75E-06	RPS3|RPL35|RPL23|SNRPG|UBA52|LOC786829|ENSBTAP00000011044|RPS11|SNRPF|ENSBTAP00000022848|RPS20|RPL8
Reactome Pathways	BTA-912526	Interleukin receptor SHC signaling	10	5	1,10E-04	6,49E-06	JAK1|CSF2RB|JAK2|JAK3|CSF2RA
Reactome Pathways	BTA-6783589	Interleukin-6 family signaling	10	5	1,10E-04	6,49E-06	JAK1|LIFR|JAK2|IL6ST|IL6R
Reactome Pathways	BTA-191859	snRNP Assembly	15	5	4,50E-04	3,10E-05	SNRPG|LOC786829|ENSBTAP00000011044|SNRPF|ENSBTAP00000022848
Reactome Pathways	BTA-392499	Metabolism of proteins	535	24	0,0028	2,20E-04	RPS3|RPL35|RPL23|IKBKG|LOC617905|UBA52|IFIH1|CSF2RB|RPS11|TRAF6|UBB|RPS20|BLM|RPL8|HIST2H2AB|HIST1H2BL|LOC507211|HIST1H2AC|ENSBTAP00000049501|ENSBTAP00000052035|HIST1H2BB|HIST3H2BB|DDX58|CSF2RA

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<tr><td>Reactome Pathways</td><td>BTA-913531</td><td>Interferon Signaling</td><td>30</td><td>5</td><td></td><td>0,0061</td><td></td><td>5,10E-04</td><td></td><td>JAK1|JAK2|IFNGR2|IFNAR1|DDX58</td><td></td></tr>
<tr><td>Reactome Pathways</td><td>BTA-8978868</td><td>Fatty acid metabolism</td><td>70</td><td>7</td><td></td><td>0,0076</td><td></td><td>6,90E-04</td><td></td><td>HADH|ACOX2|ECI1|ACADL|ACAA1|ACADM|ACOX3</td><td></td></tr>
<tr><td>Reactome Pathways</td><td>BTA-72706</td><td>GTP hydrolysis and joining of the 60S ribosomal subunit</td><td>73</td><td>7</td><td></td><td>0,0093</td><td></td><td>8,60E-04</td><td></td><td>RPS3|RPL35|RPL23|UBA52|RPS11|RPS20|RPL8</td><td></td></tr>
<tr><td>Reactome Pathways</td><td>BTA-72689</td><td>Formation of a pool of free 40S subunits</td><td>74</td><td>7</td><td></td><td>0,0099</td><td></td><td>9,30E-04</td><td></td><td>RPS3|RPL35|RPL23|UBA52|RPS11|RPS20|RPL8</td><td></td></tr>
<tr><td>Reactome Pathways</td><td>BTA-6807505</td><td>RNA polymerase II transcribes snRNA genes</td><td>27</td><td></td><td>6</td><td></td><td>4,90E-04</td><td></td><td>7,02E-06</td><td></td><td>SNAPC4|POLR2H|ENSBTAP00000003296|SNAPC5|SNAPC1|SNAPC2</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-6798695</td><td>Neutrophil degranulation</td><td>227</td><td></td><td>18</td><td></td><td>1,66E-06</td><td></td><td>2,06E-08</td><td></td><td>CD68|LILRB3|ENSBTAP00000007682|TUBB4B|TUBB|SIRPB1|ENSBTAP00000016194|DYNC1LI1|BOLA-NC1|NDUFC2|SIGLEC14|LOC617909|MGC126945|LAMP1|ENSBTAP00000048263|ENSBTAP00000051064|LILRA6|ENSBTAP00000054360</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-168249</td><td>Innate Immune System</td><td>340</td><td></td><td>22</td><td></td><td>1,52E-06</td><td></td><td>1,75E-08</td><td></td><td>CD68|ATP6V1G1|LILRB3|ENSBTAP00000007682|TUBB4B|TUBB|ATP6V0A2|SIRPB1|ENSBTAP00000016194|ATP6V1C1|DYNC1LI1|ECSIT|BOLA-NC1|NDUFC2|SIGLEC14|LOC617909|MGC126945|LAMP1|ENSBTAP00000048263|ENSBTAP00000051064|LILRA6|ENSBTAP00000054360</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-76071</td><td>RNA Polymerase III Transcription Initiation From Type</td><td>7</td><td></td><td>7</td><td></td><td>4,44E-08</td><td></td><td>3,93E-10</td><td></td><td>SNAPC4|BDP1|ENSBTAP00000003296|SNAPC5|SNAPC1|SNAPC2|BRF2</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-8949613</td><td>Cristae formation</td><td>10</td><td></td><td>10</td><td></td><td>1,07E-11</td><td></td><td>5,70E-14</td><td></td><td>ATP5D|ATP5G3|ATP5C1|ATP5I|ATP5O|ATP5G1|ATP5H|ATP5E|ATP8|ATP6</td></tr>
<tr><td>Reactome Pathways</td><td>BTA-163210</td><td>Formation of ATP by chemiosmotic coupling</td><td>10</td><td></td><td>10</td><td></td><td>1,07E-11</td><td></td><td>5,70E-14</td><td></td><td>ATP5D|ATP5G3|ATP5C1|ATP5I|ATP5O|ATP5G1|ATP5H|ATP5E|ATP8|ATP6</td></tr>
</table>

<table>
<tr><td>Reactome Pathways</td><td>BTA-163200</td><td>Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.</td><td>67</td><td>50</td><td>2,65E-58</td><td>2,35E-61</td><td>NDUFB8|ATP5D|NDUFB5|ATP5G3|NDUFAF4|NDUFA8|NDUFA9|NDUFB6|NDUFA1|NDUFAF6|NDUFA3|NDUFA13|NDUFA5|NDUFB10|NDUFS5|NDUFAF1|NDUFA4|NDUFB7|NDUFB3|COX6A1|ATP5C1|NDUFA2|ECSIT|COX5A|ATP5I|NDUFC2|ATP5O|ATP5G1|NDUFAF3|NDUFA11|NDUFS7|NDUFB9|TMEM126B|ATP5H|NDUFB2|NDUFS1|NDUFS6|ENSBTAP00000048274|COX4I1|ATP5E|ENSBTAP00000052707|NDUFV2|ATP8|ND4|COX2|ATP6|ND6|ND3|NDUFB11|NDUFA7</td></tr>
<tr><td>WikiPathways</td><td>WP1067</td><td>Toll-like receptor signaling pathway</td><td>81</td><td>17</td><td>4,54E-10</td><td>5,06E-12</td><td>MYD88|IL1B|TLR4|IKBKG|CHUK|IKBKB|TLR2|TOLLIP|LY96|CD14|IFNAR2|IRAK1|TRAF6|IRAK4|TIRAP|IFNAR1|TLR6</td></tr>
<tr><td>WikiPathways</td><td>WP3271</td><td>IL-1 signaling pathway</td><td>46</td><td>14</td><td>4,54E-10</td><td>5,65E-12</td><td>MYD88|IL1B|IL1R1|IRAK2|IKBKG|CHUK|IKBKB|TOLLIP|IL1A|IRAK1|TRAF6|IRAK4|IRAK3|IL1RAP</td></tr>
<tr><td>WikiPathways</td><td>WP3132</td><td>Regulation of toll-like receptor signaling pathway</td><td>114</td><td>19</td><td>5,39E-10</td><td>1,00E-11</td><td>MYD88|IL1B|IRAK2|TLR4|IKBKG|CHUK|IKBKB|TLR2|TOLLIP|LY96|CD14|IFNAR2|IRAK1|TRAF6|IRAK4|TIRAP|IFNAR1|IRAK3|TLR6</td></tr>
<tr><td>WikiPathways</td><td>WP1071</td><td>Cytoplasmic ribosomal proteins</td><td>61</td><td>14</td><td>6,30E-09</td><td>1,41E-10</td><td>MRPL19|RPS18|RPS3|RPL35|RPL23|RPS9|UBA52|RPS2|RPSA|RPS11|RPS5|RPS20|RPS15|RPL11</td></tr>
<tr><td>WikiPathways</td><td>WP3178</td><td>Interleukin-1 (IL-1) structural pathway</td><td>41</td><td>11</td><td>1,29E-07</td><td>3,36E-09</td><td>MYD88|IL1R1|IRAK2|CHUK|IKBKB|TOLLIP|IL1A|IRAK1|TRAF6|IRAK4|IL1RAP</td></tr>
<tr><td>WikiPathways</td><td>WP1061</td><td>Fatty acid beta-oxidation</td><td>32</td><td>10</td><td>1,72E-07</td><td>5,12E-09</td><td>HADH|ACADVL|ACADS|ECI1|HADHB|ACADL|ACAT1|ACSS2|HADHA|ACADM</td></tr>
</table>

WikiPathways	WP989	Mitochondrial long chain fatty acid beta-oxidation	17	8		4,15E-07		1,39E-08		HADH|ACADVL|ACADS|ECI1|ACADL|HADHA|ACADM|EHHADH	
WikiPathways	WP1023	mRNA Processing	107	13		1,31E-05		5,36E-07		SF3B2|SF3A1|SF3A3|DHX15|SF3B1|SF3B3|U2AF1|SNRPD2|SRSF9|SNRPF|SNRPD3|SRSF1|SNRPE	
WikiPathways	WP1020	Fatty acid biosynthesis	20	6		1,80E-04		7,98E-06		HADH|ACAA2|ECHDC1|ACSS2|ACLY|MECR	
WikiPathways	WP1012	IL-7 signaling pathway	20	6		1,80E-04		7,98E-06		JAK1|IL2RG|IL7|IL7R|IL2RA|JAK3	
WikiPathways	WP3124	AGE/RAGE pathway	54	8		4,20E-04		2,34E-05		MYD88|CHUK|IKBKB|EGFR|JAK2|IRAK4|TIRAP|SOD1	
WikiPathways	WP3274	Leptin signaling pathway	62	8		9,60E-04		5,71E-05		IL1B|JAK1|LEPR|IKBKG|CHUK|IKBKB|JAK2|IL1RN	
WikiPathways	WP1055	IL-4 signaling pathway	45	7		9,60E-04		5,76E-05		IL4R|JAK1|CHUK|IKBKB|IL2RG|JAK2|JAK3	
WikiPathways	WP984	EBV LMP1 signaling	19	5		0,0012		7,97E-05		HSP90AA1|CHUK|IKBKB|IRAK1|TRAF6	
WikiPathways	WP3159	Interleukin-11 signaling pathway	35	6		0,0017		1,20E-04		JAK1|CHUK|IKBKB|JAK2|IL6ST|IL11RA	
WikiPathways	WP3176	IL-17 signaling pathway	21	5		0,0017		1,20E-04		JAK1|IKBKG|IKBKB|JAK2|TRAF6	
WikiPathways	WP3160	IL1 and megakaryotyces in	23	5		0,0022		1,70E-04		MYD88|IL1B|IL1R1|TLR2|IRAK1	
WikiPathways	WP3277	Oncostatin M signaling pathway	51	6		0,0079		7,60E-04		JAK1|LIFR|JAK2|IL6ST|JAK3|OSMR	
WikiPathways	WP1066	Eukaryotic transcription	37		6		9,20E-04		3,42E-05		POLR2H|POLR2F|POLR3H|POLR3D|POLR2E|POLR3B
WikiPathways	WP1030	Selenium metabolism and	34		6		6,70E-04		2,23E-05		VIMP|SARS2|SELENBP1|SARS1|PSTK|CTH
WikiPathways	WP1026	One-carbon metabolism	26		6		2,00E-04		5,81E-06		AHCYL2|BHMT|DNMT1|DNMT3B|MTR|AHCY
WikiPathways	WP3145	Trans-sulfuration pathway	10		5		6,51E-05		1,65E-06		CBS|DNMT1|MTR|AHCY|CTH
WikiPathways	WP3217	Iron uptake and transport	87		10		6,51E-05		1,45E-06		ATP6V1G1|ATP6V0A2|ATP6V1B1|ATP6V1C1|ATP6V0D1|ATP6V1D|ATP6V0A1|ATP6V0D2|ATP6V0C|ATP6V1A

<table>
<tr><td>WikiPathways</td><td>WP3199</td><td>Parkin-ubiquitin proteasomal system pathway</td><td>60</td><td>10</td><td>3,48E-06</td><td>6,46E-08</td><td>TUBB3|TUBB2B|TUBB4B|TUBB|TUBB4A|TUBA1B|SNCA|TUBB1|TUBB2A|TUBB6</td></tr>
<tr><td>WikiPathways</td><td>WP3157</td><td>Pathogenic Escherichia coli infection</td><td>49</td><td>10</td><td>7,92E-07</td><td>1,18E-08</td><td>YWHAZ|TUBB3|TUBB2B|TUBB4B|TUBB|TUBB4A|TUBA1B|TUBB1|TUBB2A|TUBB6</td></tr>
<tr><td>WikiPathways</td><td>WP3135</td><td>Trans-sulfuration and one-carbon metabolism</td><td>28</td><td>9</td><td>1,95E-07</td><td>2,18E-09</td><td>CBS|AHCYL2|BHMT|DNMT1|DNMT3B|MTR|AHCY|AHCYL1|CTH</td></tr>
</table>

5. GENERAL CONCLUSION

The results of this thesis indicate that, even after a comprehensive and systematic review of the literature, it is still not feasible to state one (or more) biomarker(s) of bull fertility in semen. Although some proteins have enhancer activity in some sperm parameters, functional and validation studies are still needed to determine the accuracy of biomarkers. This thesis also presents, for the first time in any species, an atlas of intact proteins (<30kDa) of seminal plasma and sperm from bulls with contrasting freezeability status, based on post-thaw sperm viability membrane. The results presented here support the idea that the way fertility seminal biomarkers of bull have been investigated in the last three decades needs to be better standardized to reduce the risk of bias. Furthermore, the characterization of proteins in their intact form is crucial since their structures are dynamic and governs their function. The strategies of investigations applied thesis can be applied to studies in other species, as proteoform signatures emerge as vital for clinical investigation, drug screening, as therapeutic targets and fertility markers.

6. FINAL CONSIDERATIONS

- Despite the vast literature, carefully and systematically evaluated, regarding the role of seminal plasma proteins on bull fertility, there is great methodological variability among studies carried out over decades, which makes it difficult to compile and compare results. A standardization of methodologies to study the effect of seminal plasma on male fertility may minimize the sources of biases.
- The results of associations detected between seminal proteins and fertility parameters should be validated in additional studies with larger groups of animals with the same phenotypes. Moreover, fertility-related proteins must be tested using *in vitro* assays for complete understanding the biological role.
- Our qualitative analysis showed that the exposure to a single protein, such as osteopontin (OPN), binder of sperm proteins (BSPs), and heparin binding proteins (HBPs), can increment sperm motility, capacitation, and fertilizing ability by modulating intracellular calcium concentrations, removing lipids from sperm membranes, and regulating the acrosome reaction.
- The quantitative analysis revealed that SP proteins improved the motility and quality of *Bos taurus* sperm collected by artificial vagina, mainly in the presence of HBPs. BSPs and beta-defensin 126 highly favored sperm protection when cells were collected from the epididymis by retrograde flux and analyzed under room temperature conditions.
- Using top-down proteomics approach we provided novel information about the diversity of truncated units of seminal and sperm proteoforms. Seminal caltrin and C-type natriuretic peptide, that play opposite roles intracellular calcium regulation, were the proteins with the greater number of proteoforms.
- A truncated proteoform of beta-defensin 10 with pyrrolidone carboxylic acid in the N-terminal was exclusively identified in the SP of HF bulls and may be further investigated to be pointed out as a potential biomarker.
- Eight truncations of seminal OPN, including one with a serine phosphorylation site, were uniquely expressed in the LF bulls, and acetylation in histone H2A type 1 and in c-Myc protein binding expressed in the sperm of LF sires, which may indicate a deficiency in DNA packing and/or nuclear activity.

- In general, there is greater abundance of post-translational modifications in the proteoforms of seminal plasma and sperm from LF bulls, probably indicating an increased cell activity and metabolism rate, which is prejudicial to post-thaw sperm membrane viability.
- This pilot study is the first characterization of seminal plasma and sperm proteoform atlas of any species and can direct future trials for the determination of fertility markers in the semen of bulls.
- Specific seminal proteins could be used as biomarkers of fertility, being an additional parameter employed to select superior sires. Alternatively, selected seminal proteins could be used as fertility enhancers, being added into extenders to improve sperm cryotolerance and fertilizing capacity.
- The knowledge of seminal molecular configuration will contribute to the efficiency of assisted reproductive technologies in the bovine species and may support reproductive biology science in other species.