

## Research Article

# Quantitative proteomic profiling of bovine follicular fluid during follicle development<sup>†</sup>

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## Abstract

Bovine follicular fluid (FF) constitutes the microenvironment of follicles and includes various biologically active proteins. We performed a study involving 18 healthy nonlactating Holstein cows to determine the protein expression profile of FF at key stages of follicular development. Follicles were individually aspirated in vivo at predeviation (F1 ~ 7.0 mm), deviation (F1 ~ 8.5 mm), postdeviation (F1 ~ 12.0 mm), and preovulatory stages of follicle development, which were confirmed by measurement of follicular estradiol and progesterone concentrations. The FFs from nine cows were selected for proteomic analysis. After albumin depletion, triplicates of pooled FF were reduced, alkylated, and digested with trypsin. The resulting peptides were labeled with TMTsixplex and quantified using liquid chromatography-mass spectrometry/mass spectrometry. A total of 143 proteins were identified and assigned to a variety of biological processes, including response to stimulus and metabolic processes. Twenty-two differentially ( $P < 0.05$ ) expressed proteins were found between stages indicating intrafollicular changes over development, with expected deviation time critical to modulate the protein expression. For instance, high concentrations of follistatin, inhibin, serglycin, spondin-1, fibrinogen, and anti-testosterone antibody were found during early stages of follicular development. In contrast, apolipoprotein H, alpha-2-macroglobulin, plasminogen, antithrombin-III, and immunoglobulins were increased after deviation. Among the differentially abundant proteins, 19 were found to be associated with steroidogenesis. Pathway analysis identified proteins that were mainly associated with the acute phase response signaling, coagulation system, complement system, liver/retinoid X receptor activation, and biosynthesis of nitric oxide and reactive oxygen. The differentially expressed proteins provide insights into

the size-dependent protein changes in the ovarian follicle microenvironment that could influence follicular function.

### Summary Sentence

The protein dynamic changes in ovarian follicle microenvironment during the follicle development are critical for follicular maturation and influence follicular function in cows.

**Key words:** folliculogenesis, proteomic, steroid hormone, mass spectrometry, Holstein cow.

### Introduction

Follicular fluid (FF) is produced during follicular growth and provides the microenvironment for oocyte development and maturation. The main origins of FF are circulating blood, which diffuses through the follicular wall into an antrum of a follicle, and secretions by granulosa, theca cells, and oocytes [1]. The composition of FF is very complex, and among other biological active molecules it contains regulatory proteins that may affect follicular growth, regulate permeability of follicular wall, follicular maturation, and ovulation.

Protein composition of FF could be used as an indicator of secretory activities and metabolism of follicular cells, which regulate follicle quality [2]. As proteins are vital elements in cell-function control, the alterations in protein abundance depict biological processes over the estrous cycle. A number of studies have reported proteome profiles in human FF obtained from healthy fertile women [3–5], women who have had or not successful in vitro fertilization [6–8], women undergoing controlled ovarian hyperstimulation [9], and women in pathological states such as polycystic ovary syndrome [10, 11], endometriosis [12], or repetitive pregnancy loss [13]. However, in cows it is still poorly characterized with only two previous studies [14, 15], addressing issues based on reproductive disorders. Additionally, the majority of such researches were mainly limited to the identification and description of the fluid components, without providing an integrated overview of protein changes. Therefore, knowledge of the temporal characteristics of proteome profile of bovine FF could contribute to our better understanding of the highly coordinated process involved during folliculogenesis biology.

Due to the complex composition of FF, sensitive and specific techniques to identify the proteins in FF are essential. Quantitative proteomics can elucidate the regulation of folliculogenesis within a time-dependent context by measuring the change in abundance of different proteins. Protein abundance could more accurately demonstrate cellular activities than messenger RNA quantification, because messenger RNA and protein levels do not necessarily correlate [16]. Recent advances in relative quantification methods, using isobaric labeling approaches such as tandem mass tags (TMT), have improved measurement precision, accuracy, and reproducibility [17, 18], surpassing label-free quantification methods such as spectral counting [19].

In order to clarify the follicular development process, the aims of this study were to investigate the proteome profile and the functional pathways with biological relevance of bovine FF during different stages of follicle development. We further used quantitative proteomic analyses to characterize the association of the abundance of the identified proteins with local steroids concentrations.

### Materials and methods

#### Animals and experimental design

This study was approved by the Ethical Committee for the Use of Animals in Research of the FMVZ–UNESP (Permit number: 86/2013–

CEUA). The experiment was conducted at the Lageado Experimental Farm, School of Veterinary Medicine and Animal Science, São Paulo State University (UNESP), Botucatu, SP, Brazil. Eighteen nonlactating, clinical healthy, and cycling Holstein cows (2–7 years old) were used in this experiment. The study was conducted during the winter to avoid effects of heat stress. All cows were initially subjected to ovulation synchronization using the Ovsynch protocol [20] added with an intravaginal progesterone device (Sincrogest, Ourofino, Cravinhos, Brazil). Of the 18 cows, 13 successfully responded to the estrus synchronization and were used in the experiment. From the day of ovulation, ovarian ultrasonography (MyLab30 Vet Gold with a 7.5 MHz linear array transducer, Esaote, Genova, Italy) was performed twice daily (every 12 h) by a single operator to monitor the emergence and development of a new follicular wave. Transrectal ultrasound was used to confirm ovulation and to determine the size and location of the follicles and corpus luteum. Ultrasound measurements were used to calculate the average follicular diameter in accordance with Sartori, Rosa and Wiltbank [21]. The ovarian follicular dynamics were monitored, and the FF from the largest follicle (F1) was individually aspirated at predeviation (F1 ~ 7.0 mm [22]). After the target follicle was aspirated, all remaining visible follicles were ablated to allow the emergence of a new follicular wave. Then, at the expected beginning of deviation (F1 ~ 8.5 mm [23]), both the F1 and the second largest follicle (F2) were aspirated. Again, all remaining visible follicles were ablated to allow the emergence of a new follicular wave, and the F1 was aspirated after acquisition of ovulatory capacity (F1 ~ 12.0 mm [24]). All cows were subjected to a new synchronization protocol, as previously reported [25]. Ovaries were examined at the time of GnRH injection by transrectal ultrasonography to ensure a minimal diameter of 12 mm. Only cows that presented a single preovulatory follicle with a diameter >12 mm were used. At preovulatory stage, 13 cows successfully responded to the estrus synchronization, and the F1 was aspirated 24 h after the intramuscular GnRH injection. Collectively, four time points characterized the experimental groups: predeviation (PreDev), deviation (DevF1 and DevF2), postdeviation (PostDev), and preovulatory (PreOv).

#### In vivo transvaginal follicular aspiration

Transvaginal follicular aspiration was performed using an ultrasound equipment (Mindray DP-3300 Vet, Mindray Bio-Medical Electronics Co. Ltd, Sheuzheu, China) equipped with a micro-convex 5 MHz transducer coupled to a needle guide system (WTA, Watanabe Applied Technology, Cravinhos, Brazil) and connected to an aspiration line (WTA) and a 20G disposable needle (WTA). Instead of a pump, the differential pressure applied to the system to recover the FF was created using a 10 mL syringe (Descarpack, São Paulo, Brazil). Caudal epidural anesthesia was induced with 5 mL of 2% lidocaine (Lidovet, Bravet, Rio de Janeiro, Brazil), the perineal area was scrubbed and the aspiration guide with the transducer was inserted via the transvaginal route. The ovary containing the target follicle was positioned via transrectal against the vaginal wall over

the transducer face, so that the targeted follicle was transected by the built-in line on the ultrasound image representing the projected needle path. The needle trajectory course to the follicle was positioned so that it transected the ovarian stroma but not other detectable follicles or luteal tissue [26]. The needle was then advanced until the image of the needle tip was centered within the targeted follicle and aspiration of FF was done. Immediately after follicle collapse, the needle was withdrawn from the ovary and the system was checked for the presence of FF and blood contamination. Each collected fluid sample was derived from a single follicle. Only the FF samples without macroscopic blood contamination were used in this study.

After collection, the FF samples were centrifuged at 2000 g at 4°C for 10 min to eliminate cells and debris, a protease inhibitor cocktail added (0.8 mmol EDTA, 1.0 µg/mL aprotinin, 1.0 µg/mL leupeptin, and 35.0 µg/mL phenylmethylsulfonyl fluoride) [27] and stored at -80°C until processing for hormone dosage assays and proteomic analysis.

### Hormone analysis and sample preparation

In order to confirm the follicle viability in accordance with the stage of follicle development, the concentrations of estradiol (E2) and progesterone (P4) in the FF were determined by a manual kit-based method for enzyme immunoassay. Procedures were similar to those previously described for the determination of serum E2 and P4 [28]. Briefly, 96-well plates were coated with goat antimouse immunoglobulin (Ig) G for P4 assay and goat antisheep IgG for E2 assay. Plates were then washed, and a mouse antibody against ovalbumin was added for P4 assay (Biostride Inc., Palo Alto, CA, USA) and a sheep antibody against E2 for E2 assay (Animal Reproduction and Biotechnology Laboratory, Colorado State University, USA). Horseradish peroxidase was conjugated to P4 and E2 and used in competitive assays with the steroids from a 1:2 to 1:1000 dilution of FF in assay buffer. Subsequently, 3,3',5,5'-tetramethylbenzidine was used to quantify horseradish peroxidase activity. Color intensity of the enzyme substrate was inversely proportional to the steroids concentrations. Assay limit of detection was 0.02 ng/mL for both hormones, and the intra-assay coefficients of variation for quality control samples were <10% for all assays.

Nine samples of each experimental group were used for proteomic analysis. The criteria for selecting the samples were as follows: macroscopically blood-free fluids; intrafollicular estradiol:progesterone ratio [29]  $\geq 1$  for PreDev, DevF1, and PostDev groups, and <1 for PreOv; and the inclusion of the same cows in all experimental groups. For each of the five stages, three pools of FF samples from three cows were prepared in order to provide replicates for statistical analysis. Aliquots of all selected samples (9 samples  $\times$  5 stages = 45 samples) were mixed and used as control group for relative quantification. The pooled FF samples were processed using a trichloroacetic acid/acetone precipitation method [30] to deplete albumin and thus to enhance the detection of low-abundance proteins from FF.

### Protein in-solution digestion and peptide labeling

The protein concentration of the FF samples was quantified using a bicinchoninic acid protein assay kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific Inc. #23227, Waltham, MA, USA) according to the manufacturer's instructions. A quantity of 100 µg protein was reduced with 5 µL of 200 mM 1,4-dithiothreitol, and the filter-aided sample preparation method (FASP Protein Digestion Kit, Expedon, San Diego, CA, USA) was then used [31] with a minor

modification by substituting sodium chloride with 10% v/v acetonitrile for elution of the proteins from the spin columns to avoid a desalting step. Alkylation with 10 µL of 0.05 M iodoacetamide solution was performed in the dark at room temperature (23°C) for 20 min. Protein digestion was carried out using trypsin (Promega, Madison, WI, USA) to a final enzyme:protein ratio of 1:100 at 37°C for 16 h.

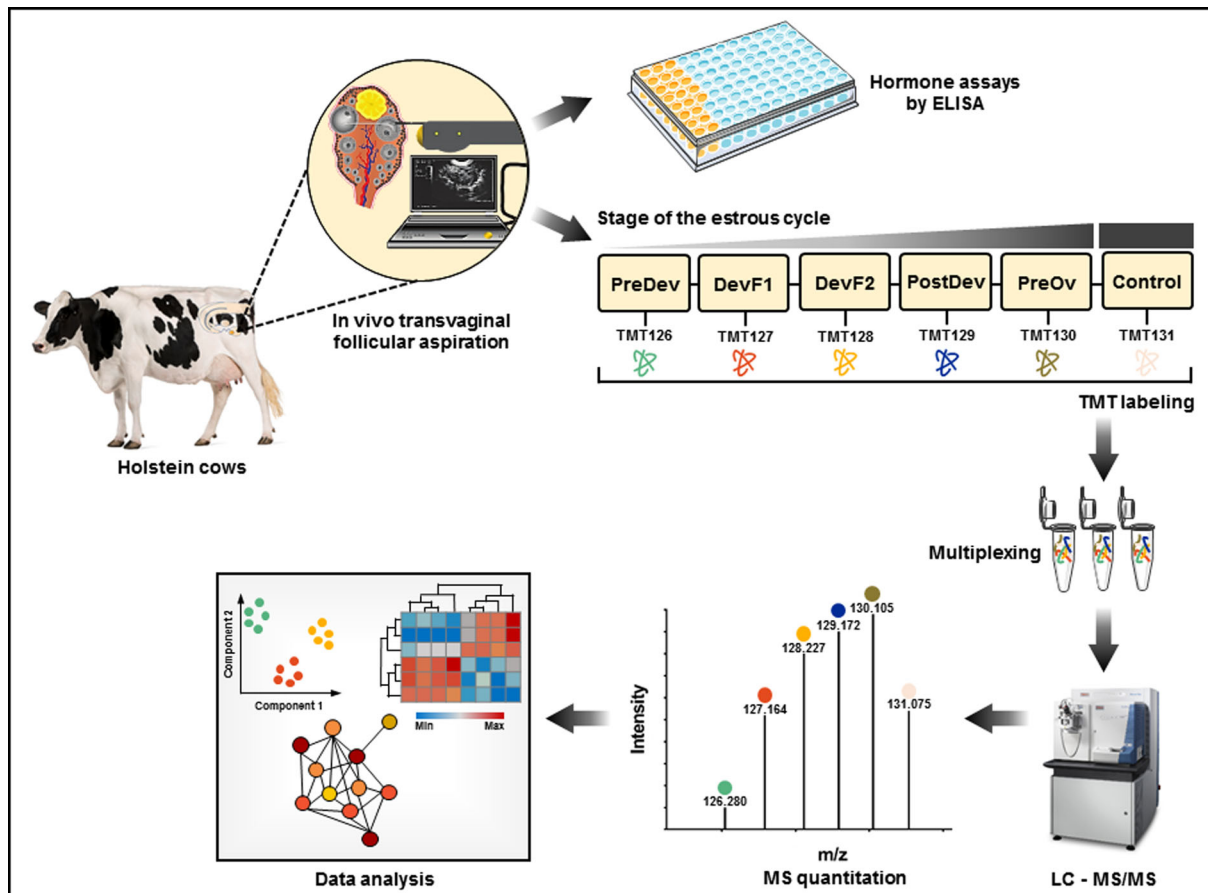
After protein digestion, the samples were labeled with TMTsix-plex Isobaric Label Reagent Set (Thermo #90062) according to the manufacturer's instructions. Briefly, TMT reagents were dissolved in 41 µL of acetonitrile, added to samples, and the reactions were incubated for 1 h at room temperature. To quench the reaction, 8 µL of 5% (v/v) hydroxylamine was added to the samples and incubated for 15 min at room temperature. The samples were then combined in equal volumes, vacuum-dried, and stored at -20°C until analysis. Three experimental replicates per group were labeled with TMT tags to maximize the accuracy of quantitative proteomics. Figure 1 shows the workflow for the isobaric labeling-based relative quantification of bovine FF.

### Mass spectrometry analysis

Samples were analyzed in an LTQ Orbitrap Elite Mass Spectrometer (Thermo Scientific Inc., San Jose, CA, USA) coupled to an UltiMate 3000 RSLC nano chromatography system (Dionex, Camberley, UK). TMT-labeled peptide mixtures were reconstituted in buffer A (2% v/v acetonitrile in 0.1% v/v formic acid). Three micrograms were loaded on the trapping column C18 PepMap100 (5 µm, 100 Å, 300 µm  $\times$  5 mm) and then separated on an Acclaim PepMap RSLC C18 Nanocolumn (15 cm  $\times$  75 µm) with a linear gradient of 5–35% solvent B (80% acetonitrile/0.1% formic acid) over 135 min at a flow rate of 300 nL/min. The eluate from the column was introduced to the mass spectrometer. The ionization voltage was set to 1.7 kV and the ion transfer tube temperature to 220°C. The mass spectrometer was set up in positive ion mode using collision-induced dissociation (CID)/high-energy collision-induced dissociation (HCD) fragmentation methods for the second stage of mass spectrometry (MS2). Full scan fourier transform mass analyzers (FTMS) spectra were acquired in range from 380 to 1800 m/z, with resolution of 60 000. The maximum injection time for FTMS full scan was set as 200 ms reaching an automatic gain control (AGC) target value of  $1 \times 10^6$ . For the identification of TMT-labeled peptides, the three most abundant peaks from the MS spectra were selected for each fragmentation mode. The HCD MS/MS scan was fixed to start from 100 m/z, with resolution of 15 000, using MS2 AGC target of  $5 \times 10^4$ . The collision energy was set as 40% normalized collision energy (NCE). Isolation window of  $\pm 1.5$  Da was applied to isolate precursor ions with dynamic exclusion of 20 s. The Ion trap mass spectrometry CID MS/MS scan spectra were acquired with 35% NCE and an AGC target of  $1 \times 10^4$ .

### Database searching and bioinformatics analysis

Raw data files were loaded into Proteome Discoverer software (version 2.1, Thermo Fisher Scientific, Bremen, Germany) and searched against the *Bos taurus* NCBI protein database using both the Sequest and Mascot algorithms. The parameters used for database searches included trypsin as protease with one missed trypsin cleavage allowed, carbamidomethylation (C) as fixed modification, oxidation (M), and TMT-6plex (Y) as variable modification. The mass tolerance of precursor ions was set at 10 ppm and 0.6 Da for fragment ion matches. Only peptides that were filtered with a confidence level of 95% and at least one unique peptide was accepted. The false



**Figure 1.** Schematic representation of isobaric labeling-based relative quantification of bovine FF during different stages of the follicle development. See methods for details.

discovery rate (FDR) was calculated using peptide validator based on decoy database searching. Labeling efficiency, calculated as the percentage of the total number of unique peptides that were modified by TMT labeling, was greater than 99%. The peptide ratios were calculated from median peptide spectrum matches. These peptide ratios were normalized to the total intensity and then assembled (median) to protein groups. Gene ontology (GO) analysis of the identified proteins was performed by GO-term annotation step directly in Proteome Discoverer software.

### Statistical analysis

The normality and homoscedasticity of the data were evaluated using the Kolmogorov-Smirnov and Levene's tests, respectively. Hormone concentration data were log-transformed to attain normality. Comparisons of diameters, steroids and protein concentrations, and E2:P4 ratio between stages of follicle development were performed by PROC GLM procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) and post hoc comparisons with Student-Newman-Keuls test. Results are reported as least square means, and a  $P$ -value  $< 0.05$  was considered as significant. For proteomic data, fold changes were calculated based on the ratio of the case versus control samples. To identify differentially expressed proteins, PROC GLM was performed in normalized relative abundances, with the stage of follicle development as factor. Proteins with FDR-adjusted

$P$ -value  $< 0.05$  were considered differentially expressed and were included in the protein lists. The potential associations between mean FF concentrations of E2 and P4 and normalized relative abundance of differentially expressed proteins among the stages were analyzed by Pearson's correlation test and linear regression. For data exploratory analysis, hierarchical clustering using the Euclidian algorithm for dissimilarity with Ward's linkage and principal component analysis (PCA) were performed using GeneSpring software (version 13.0, Agilent Technologies Inc., Santa Clara, CA, USA).

### Pathway and network analysis

Enrichment of canonical pathways and networks of identified proteins were generated using Ingenuity Pathway Analysis software (IPA, QIAGEN Redwood City, CA, USA). The datasets contained respective gene symbols and the normalized relative abundances of proteins. Networks of these genes were generated based on information contained in the Ingenuity Knowledge Base, considering direct and indirect relationships (as general settings), experimentally observed, highly predicted interaction networks, with no restriction for tissues/cell lines or mutations. The Fisher's exact test was used by the IPA to calculate  $P$ -values and identify statistically significant pathways and networks. The direction of regulation, i.e., up- or downregulation, was inferred from the activation  $z$ -score in the IPA.



**Table 1.** Morphological, hormonal, and biochemical characteristics of the in vivo aspirated bovine follicles on different stages of follicle development.

Parameter <sup>1</sup>	Stage of follicle development				
	PreDev	DevF1	DevF2	PostDev	PreOv
Follicle diameter (mm)	7.20 ± 0.23 <sup>d</sup>	8.64 ± 0.09 <sup>c</sup>	7.44 ± 0.15 <sup>d</sup>	12.13 ± 0.14 <sup>b</sup>	14.08 ± 0.71 <sup>a</sup>
Time of aspiration (day) <sup>2</sup>	3.50 ± 0.71 <sup>*</sup>	4.87 ± 1.19 <sup>#</sup>	4.87 ± 1.19 <sup>#</sup>	6.22 ± 0.97 <sup>#</sup>	24 h after GnRH
Estradiol (ng/mL)	21.27 ± 5.60 <sup>b</sup>	26.42 ± 7.72 <sup>b</sup>	12.24 ± 3.03 <sup>b</sup>	105.15 ± 20.89 <sup>a</sup>	17.60 ± 3.08 <sup>b</sup>
Progesterone (ng/mL)	5.96 ± 1.42 <sup>c</sup>	13.40 ± 2.40 <sup>b</sup>	16.17 ± 3.27 <sup>b</sup>	38.35 ± 6.66 <sup>b</sup>	158.13 ± 51.94 <sup>a</sup>
E2:P4 ratio <sup>3</sup>	5.16 ± 2.02 <sup>a</sup>	1.64 ± 0.41 <sup>b,c</sup>	0.93 ± 0.22 <sup>c</sup>	2.76 ± 0.46 <sup>a,b</sup>	0.14 ± 0.04 <sup>d</sup>
Total protein (mg/mL)	19.41 ± 2.42 <sup>b</sup>	64.51 ± 24.52 <sup>a,b</sup>	47.26 ± 11.18 <sup>a,b</sup>	80.83 ± 9.32 <sup>a</sup>	71.43 ± 11.98 <sup>a</sup>

<sup>a-d</sup>Values with different superscripts in the same row differ significantly ( $P < 0.05$ ) by the Student-Newman-Keuls test.

<sup>1</sup>Refers to nine samples in each stage of follicle development that were used for proteomic analysis.

<sup>2</sup>Number of days after GnRH injection\* or follicular aspiration<sup>#</sup> was considered as Day 0.

<sup>3</sup>Estradiol:progesterone concentration ratio.

## Results

### Morphological, hormonal, and biochemical characteristics of the bovine follicles

Mean diameters of follicles ranged from 7.2 mm in the PreDev to 14.1 mm in the PreOv and were affected ( $P < 0.001$ ) by the stage of follicular development. Follicular fluid concentration of E2 was higher ( $P < 0.001$ ) in the PostDev compared with the other groups. Progesterone concentration in FF was dramatically lower ( $P < 0.001$ ) in the PreDev follicles and the increase in intrafollicular P4 concentration after the Luteinizing hormone (LH) surge (PreOv) was highly significant ( $P < 0.001$ ). The estradiol:progesterone ratio in the FF, widely used as follicular viability criteria, indicated that the PreDev, DevF1, and PostDev groups were estrogenically active. Total protein concentration was higher ( $P < 0.05$ ) after deviation (PostDev and PreOv) and was mainly associated with E2 levels ( $r = 0.67$ ;  $P < 0.001$ ) (Table 1).

### Proteins identified in bovine follicular fluid

We acquired a total of 50 282 MS/MS spectra and matched to 774 unique peptides, corresponding to 524 proteins. In total, 143 protein groups were identified with high confidence and were used for further analysis [32]. A complete list of proteins identified in FF is provided in Supplement File S1. In the distribution of protein molecular weight (MW) and isoelectric point (pI), most MWs ranged from 10 to 80 kDa (Figure 2A) and pI values ranged from 5 to 8 (Figure 2B). Sequence coverage of 79% (113/143) of the proteins was above 5%; the average sequence coverage was 16% (Figure 2C). In addition, 56% (80/143) of the proteins had more than one unique peptide (Figure 2D).

To explore the dataset, we used a heat map to provide a first overall view of the expression of 143 proteins that were quantified from the cow reference proteome. Our results suggest that physiological processes involved during folliculogenesis are coordinated through relatively modest proteomics changes. Hierarchical clustering analysis using Euclidean and Ward's linkage showed two major clusters in the rows dendrogram, corresponding to different time points of follicle development. There was a distinct change in FF proteome pre- and postdeviation (Figure 3A). PCA was used to discriminate the FF proteome dataset according to the stage of follicle development. The results showed a distinguished pattern for PreDev, PostDev, and PreOv stages, while DevF1 and DevF2 were not clearly discriminated. Moreover, PCA revealed clustering samples by time point (Figure 3B). For further evaluation, we plotted the

stages of follicle development according to their correlation with the principal components. A positive correlation between the PostDev and PreOv was found, with no association with samples collected at the first stages of follicle development (PreDev, DevF1, and DevF2) (Figure 3C).

### Gene ontology analysis

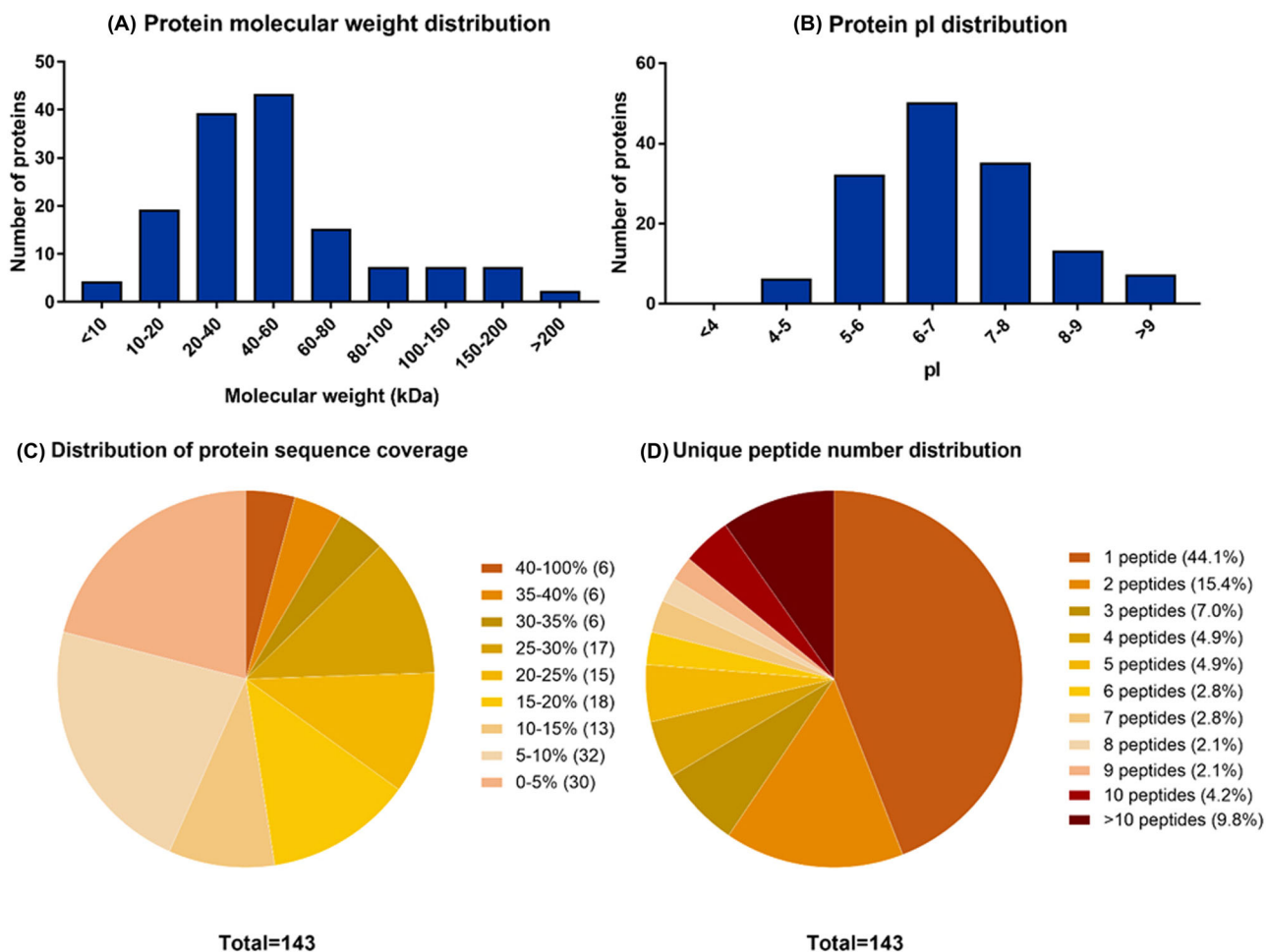
The FF proteome was classified according to GO annotations to cellular localization, biological processes, and molecular function. Classification based on cellular localization indicated that 60% of proteins were extracellular (Figure 4A). The majority of the FF proteins detected were involved in the regulation of biological process (29%), response to stimulus (20%), metabolic process (14%), defense response (11%), and transport (7%) (Figure 4B). The top molecular function categories were protein binding (38%), enzyme regulator activity (25%), metal ion binding (14%), catalytic (9%), and transporter activity (4%) (Figure 4C).

### Differentially abundant proteins over follicle development

Numerous proteins identified in FF are known to originate from bovine plasma, such as albumin, immunoglobulins, coagulation, and complement components. It was not possible to correlate a specific protein with a particular stage of follicular development. However, we found 22 differentially ( $P < 0.05$ ) expressed proteins between the stages of follicle development (Table 2). Of these proteins, follistatin, inhibin, serglycin, spondin-1, fibrinogen, and antitestosterone antibody were found to be relatively more abundant during early stages of follicular development. On the other hand, apolipoprotein H, alpha-2-macroglobulin, plasminogen, antithrombin-III, and immunoglobulins were increased after follicle deviation (Figure 5). The fold-change estimates of the differentially expressed proteins are provided in Supplementary Figure S1.

### Correlations between differentially abundant proteins and steroids content in the bovine follicular fluid

Intrafollicular E2 concentration was positively correlated with plasminogen. On the other hand, a negative correlation was observed between E2 and modified bovine fibrinogen and spondin-1 (Figure 6A). There were associations between FF concentration of P4 and 16 other proteins. Of these, the six strongest correlations are presented in Figure 6B, with additional correlations presented in Supplementary Figure S2. Serglycin, follistatin, interalpha trypsin in-



**Figure 2.** Distribution of the protein molecular weight (A), protein isoelectric point (pI) values (B), protein sequence coverage (C), and number of unique peptide (D) of the identified proteins in the FF.

hibitor, and spondin-1 were negatively correlated with P4, whereas alpha-2-macroglobulin and immunoglobulin M heavy chain were positively correlated with P4.

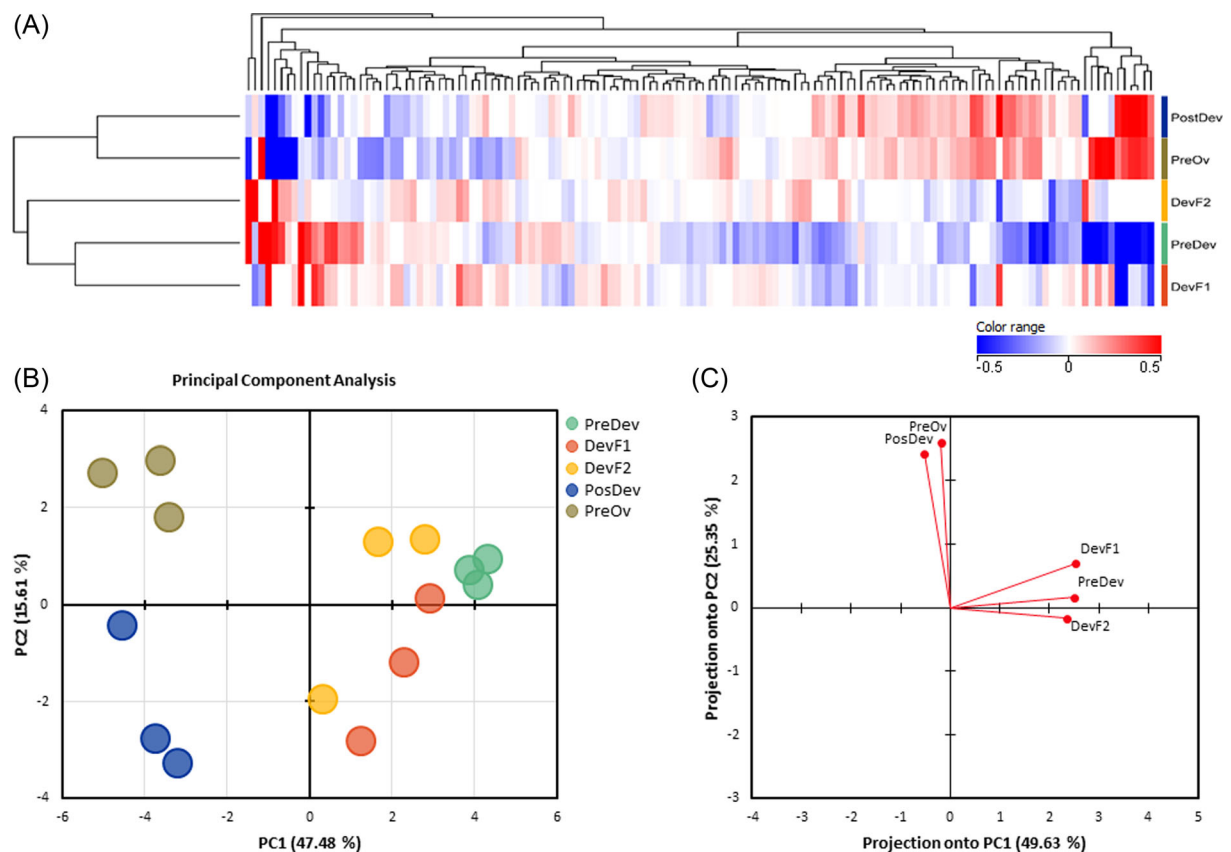
### Pathway analysis of differentially abundant proteins in the bovine follicular fluid

In order to determine the major pathways, biological relevance, and networks related to the relative abundance of the proteins identified in bovine FF over follicle development, functional enrichment analysis using IPA software was performed. The top canonical pathways detected were primarily associated with the acute phase response, coagulation system, complement system, liver X receptor (LXR) and retinoid X receptor (RXR) activation, and the production of nitric oxide (NO) and reactive oxygen species (ROS). Activated (orange) and inhibited (blue) pathways over different stages of follicle development obtained from IPA are depicted on Figure 7. Notably, the most upregulated pathway over follicle development was the acute phase response signaling, with the highest z-score at DevF1. The coagulation system pathway was downregulated at DevF1 and PreOv. The complement system pathway was inhibited during deviation (DevF1 and DevF2), and the production of NO and ROS pathway changed from downregulation at PreDev to upregulation at PostDev and PreOv. The network implicated in the acute phase response

signaling integrated 21 proteins, including several key regulatory molecules as complement components, inter-alpha-trypsin inhibitor (ITI) heavy chain, and fibrinogen (Figure 8). A second network was involved in coagulation system and is shown in Figure 9. Supplementary Table S1 summarizes the networks, genes, and proteins that correspond to the nodes of the statistically significant pathway networks.

### Discussion

Follicular fluid contains proteins involved in follicle activity, cell proliferation and differentiation, and oocyte maturation. The expression pattern and functional characterization of the FF proteome during the follicle development may contribute to further understanding of the physiological mechanisms underlying folliculogenesis. A field study was designed to determine the proteome profile of bovine FF according to the stage of follicle development and to evaluate the association of the identified proteins with FF E2 and P4 concentrations. We used a gel-free isobaric labeling proteomic technology to characterize for the first time a comprehensive overview of multiple protein shifts during bovine follicle development. Numerous differentially ( $P < 0.05$ ) regulated proteins involved in several essential biological processes played an



**Figure 3.** (A) Hierarchical clustering heat map showing different expression pattern of 143 proteins identified by LC-MS/MS in bovine FF over follicle development. The heat map indicates high (red), low (blue), and intermediate (white) concentrations. The rows represent different stages of follicle development. The columns represent individual proteins. (B) PCA plots of the bovine FF proteome reveal grouping according to stage of follicle development. The data points refer to FF samples obtained at different stages of follicle development. Time points are identified by colors shown in the legend. (C) PCA biplot with proteins plotted in two dimensions using their projections onto the first two principal components, and stages of follicle development plotted using their weights for the components (red points). (Please see the online version for the color figure).

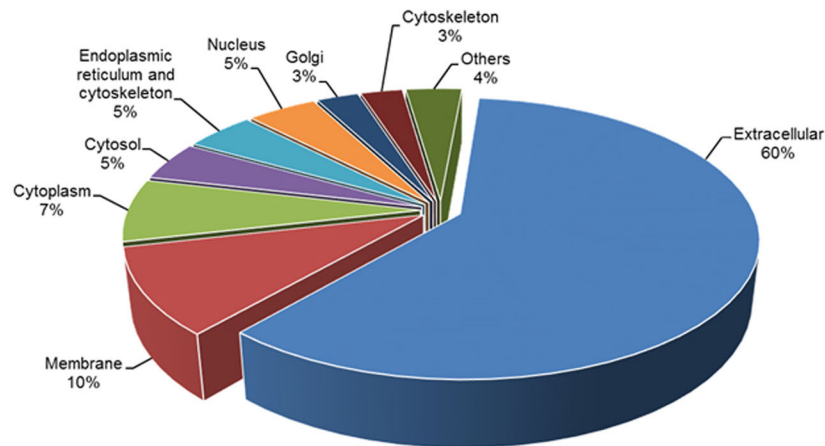
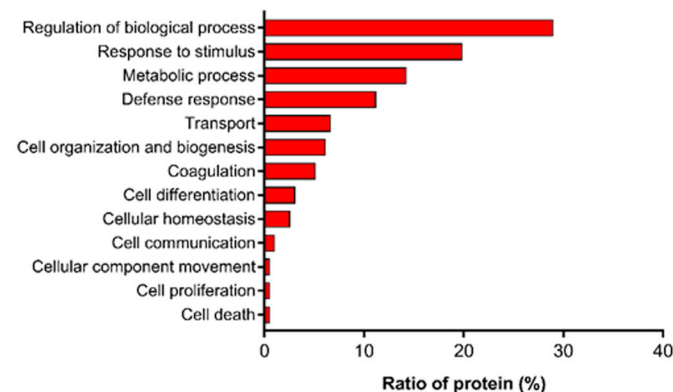
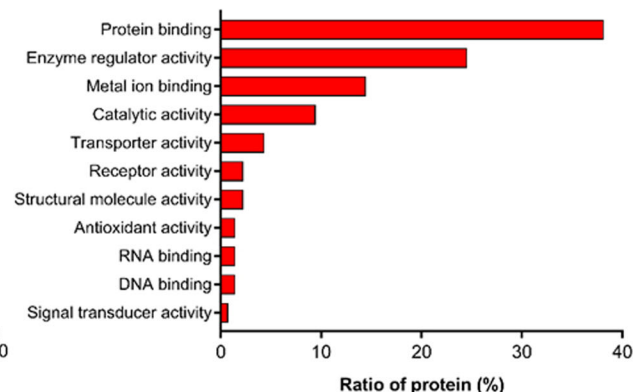
important role in promoting the maturation of bovine follicles, with expected deviation time critical to determine the protein expression of the FF.

In our experimental model, we precisely monitored the morphological dynamics of ovarian follicles using transrectal real-time ultrasonography, and the stage of follicle development was confirmed endocrinologically by measuring the E2 and P4 concentrations in FF samples. In addition, the primary essential prerequisite for inclusion of FF samples in this study was the absence of blood contamination. All the fluids were macroscopically analyzed and thus retained blood-free. Consequently, there is an improbable correlation between the FF proteins and blood contamination of the collected FFs. For proteomic analysis, our first focus was to eliminate the potential interference of high-abundance proteins, which have a masking effect on less-abundant proteins and hinder their identification. To overcome this limitation and for better coverage of low-abundance proteins, we depleted albumin, the most abundant protein in FF [33].

Ovarian steroid hormones are major regulators of the physiology of the reproductive events. The determination of E2 and P4 concentrations in bovine FF evidenced important variations related to the stage of the follicle development. These findings are largely in accordance with E2 and P4 concentrations in FF previously reported in cows [34]. Follicles with an E2:P4 ratio greater than 1 are estrogen active and healthy and follicles with an E2:P4 ratio

less than 1 are estrogen inactive and atretic [29]. However, near the time of ovulation, an LH peak induces a switch from E2 dominance to P4 dominance in the FF of preovulatory follicles [35]. Additionally, differing from other studies [36, 37], we observed an increase in intrafollicular total protein contents across follicle development. This finding could indicate a tight protein coordination for secretory activity and metabolism of follicular cells, such as modulation of enzymatic activities in follicle development and in acquiring oocyte competence. Secretions from granulosa and theca cells and from capillary diffusion compose bovine FF [38]. During folliculogenesis, the ovarian blood-follicle barrier plays a central function in the regulation of protein transfer [39] and becomes more permeable to plasma molecular components particularly at the terminal phase of follicle development [40].

Gene ontology enrichment analysis revealed that the majority of FF proteins identified in this study were classified as extracellular (60%) and belonged to a variety of biological processes and molecular functions that are known to be essential in the physiological functioning of the ovary. Intracellular proteins in the FF are related to cellular apoptosis, which usually occurs during follicular development [41], leading to the release of their cellular component into the FF. Evidence suggests that FF also contains proteins from epithelial shedding, because the ovarian surface epithelial cells surround follicles as oocytes grow and follicles expand [42], and from

**(A) Cellular localization****(B) Biological processes****(C) Molecular function**

**Figure 4.** Gene ontology analysis of the proteins identified in bovine FF. Proteins were classified according to (A) cellular localization, (B) biological processes, and (C) molecular function. Results are displayed as percent of genes classified to a category over the total.

extracellular vesicles [43]. In addition, the procedure of FF collection probably could lead to some cellular damage.

In the current study, we used a powerful proteomic approach to quantify changes in protein expression at key stages of follicle development (emergence, deviation, dominance, and preovulatory). The stage of follicle development affected the protein expression of the FF. The 22 proteins that were found to be differentially ( $P < 0.05$ ) expressed extend our understanding of the molecular changes accountable for remodeling of the follicle as well as oocyte development and maturation. Interestingly, the differential change in protein profile was associated with the presumed deviation phenomenon. Follicle selection is the process wherein one follicle develops from a wave of growing follicles and becomes the only follicle with ovulatory capacity [44]. The attainment of dominance by a single follicle is characterized by its largest size, highest intrafollicular E2 concentrations, and continued growth despite low systemic Follicle-stimulating hormone (FSH) concentrations [45]. In cattle, the literature strongly supports a role for systemic regulation involving both FSH and LH in the development of follicle dominance [44, 46], and the present results, at least partially, implicate intrafollicular protein expression acting synergistically with gonadotropins. Specifically, we found increased protein expression for antitestosterone antibody, follistatin, inhibin beta A chain, fibrinogen gamma-B chain, and apolipoprotein H in

the largest follicle at expected deviation time, but decline of them after acquisition of dominance.

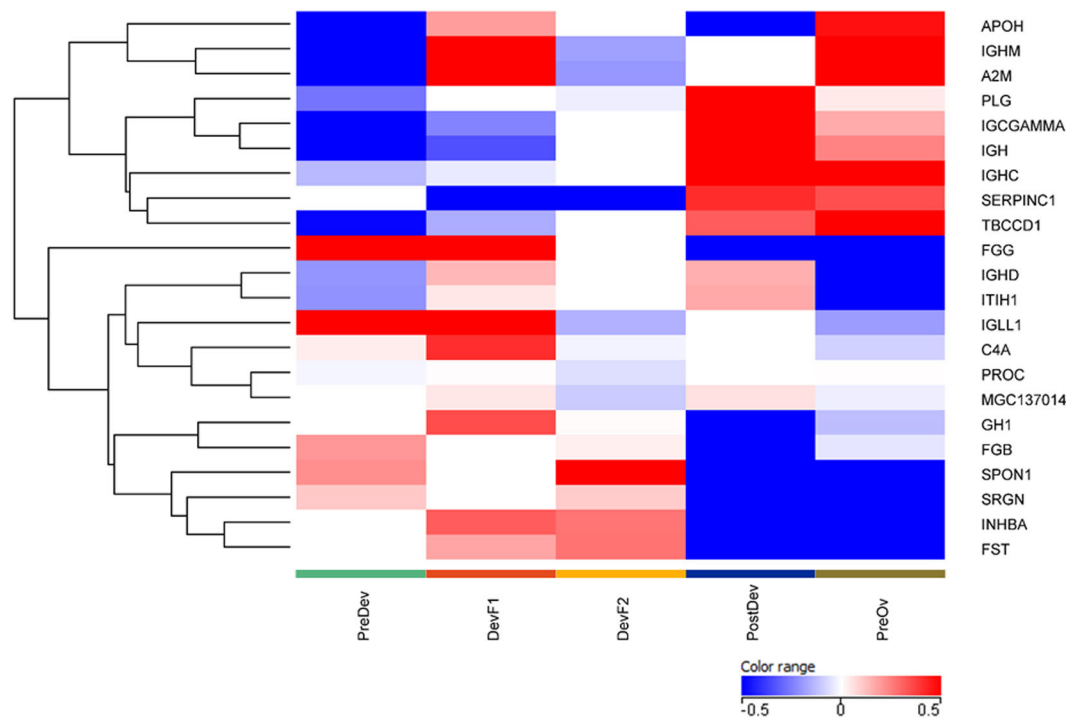
To the best of our knowledge, no study has been published about the association between these proteins and follicular development stage in cows. Follicular fluid of cows contains proteins that are required for growth and maturation of ovarian follicles and the oocyte. Alterations in expressed proteins in the FF, for instance, have been correlated with reproductive failure in dairy cows [15]. Consequently, these results represent an attractive target to line out excellent folliculogenesis models in future that, ultimately, could assist to better explore the reproductive potential of the cow and to obtain better results in the use of reproductive technologies. Moreover, these proteins might serve as potential biomarkers of follicle maturation and oocyte competence, which is in line with the efforts in human research for noninvasive assessment of oocyte quality and ovarian stimulation outcomes.

Most of the proteins differentially expressed between the stages of follicle development, including fibrinogen, A-2 macroglobulin, complement C4, serine protease inhibitor, and immunoglobulins, were associated with the inflammatory response and may play an essential role in bovine female reproduction. These proteins were mainly related to the advanced stages of follicle maturation, prior to ovulation and subsequent luteinization. Previous studies also



**Table 2.** Differentially expressed proteins in bovine FF over follicle development.

Protein name	Accession	Gene symbol	P-value
Apolipoprotein H	P17690	APOH	0.0002
Immunoglobulin M heavy chain	24496448	IGHM	0.0009
Vitamin K-dependent protein C	131065	PROC	0.0011
Anti-testosterone antibody	432627	GH1	0.0014
Immunoglobulin IgG2a(A1)	252887	IGCGAMMA	0.0018
Spondin-1	Q9GLX9	SPON1	0.0023
Serglycin	70778776	SRGN	0.0034
Alpha-2-macroglobulin	Q7SIH1	A2M	0.0035
Plasminogen	27806815	PLG	0.0038
Immunoglobulin alpha heavy chain	509264946	IGH	0.0041
Complement C4	982963988	C4A	0.0076
Follistatin	P50291	FST	0.0083
Immunoglobulin lambda-like polypeptide 1	741957421	IGLL1	0.0085
Inhibin beta A chain	27805949	INHBA	0.0154
ITI heavy chain H1	Q0VCM5	ITIH1	0.0166
Antithrombin-III	555957525	SERPINC1	0.0173
Immunoglobulin gamma3 heavy chain	1040566762	IGHC	0.0175
TBCC domain-containing protein 1	A4IF93	TBCCD1	0.0181
Bovine antibody Blv1h12 With Ultralong Cdr H3	513137422	IGHD	0.0182
Protein HP-20 homolog	114051225	MGC137014	0.0211
Fibrinogen gamma-B chain	27806893	FGG	0.0336
Modified bovine fibrinogen	6980814	FGB	0.0376



**Figure 5.** Hierarchical clustering heat map showing the effect of the stage of follicle development on protein expression profiling of 22 differentially expressed proteins in bovine FF. The heat map indicates high (red), low (blue), and intermediate (white) concentrations. Individual proteins are represented by a single row, and each stage of follicle development is represented by a single column. (Please see the online version for the color figure).

identified similar proteins in human FF. For example, fibrinogen, which is an inflammatory mediator, is involved in maintaining early pregnancy [13] and was increased after ovarian hyperstimulation [9]. Therefore, the immune system plays a crucial role in follicle development and supports the hypothesis that folliculogenesis and ovulation could be regarded as a hormone-induced inflammatory process [47].

Moreover, during follicular development, proteins associated with protease inhibition (i.e., SERPINC1 and A2M) increased gradually, suggesting that protein degradation was restrained to support the growth and maturation of oocytes.

Our results provide compelling evidence that the proteins belonging to the coagulation cascade system of FF are regulated according

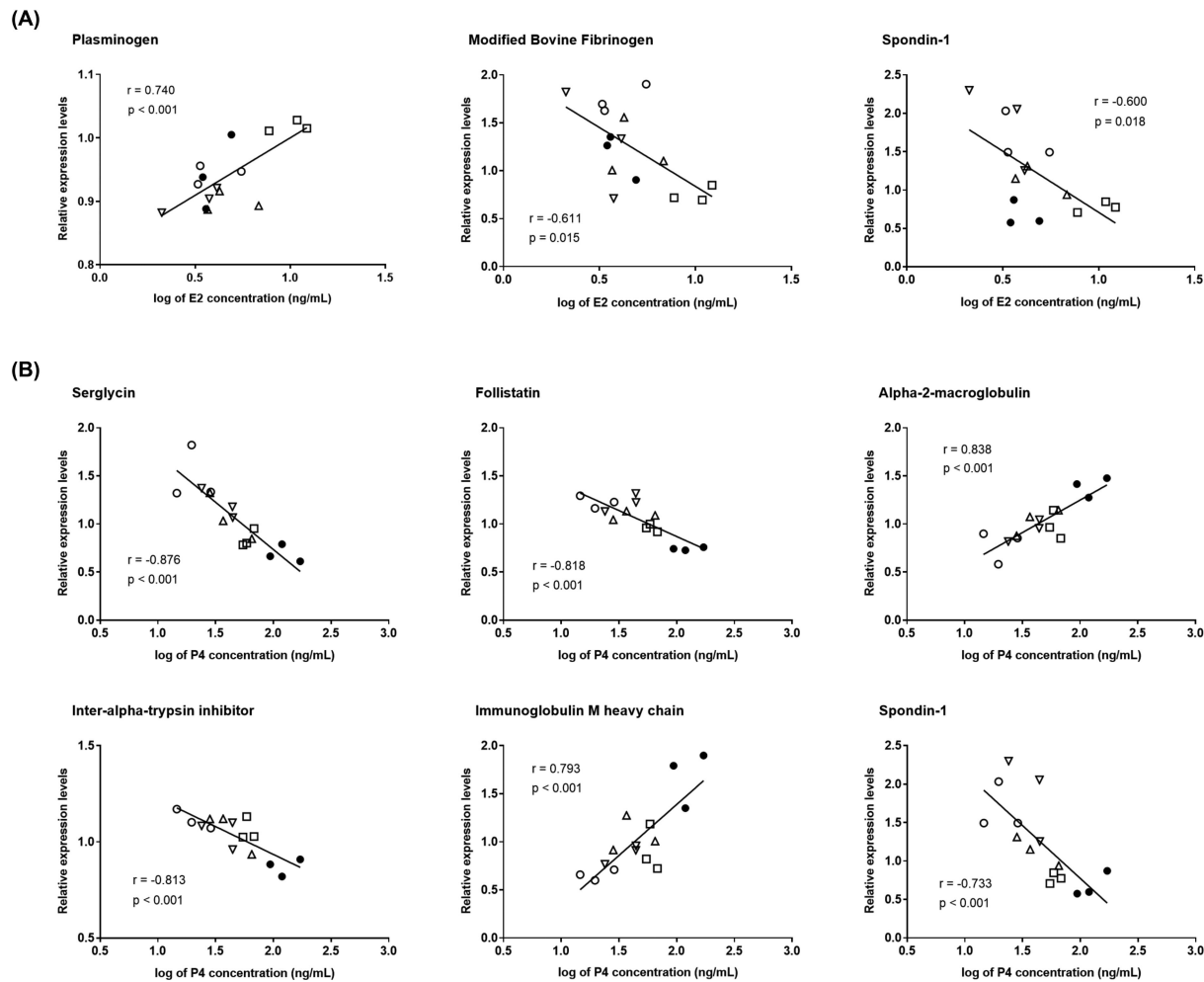


Figure 6. Correlation between abundance of proteins and estradiol (A) and progesterone (B) concentrations in bovine FF over follicle development.

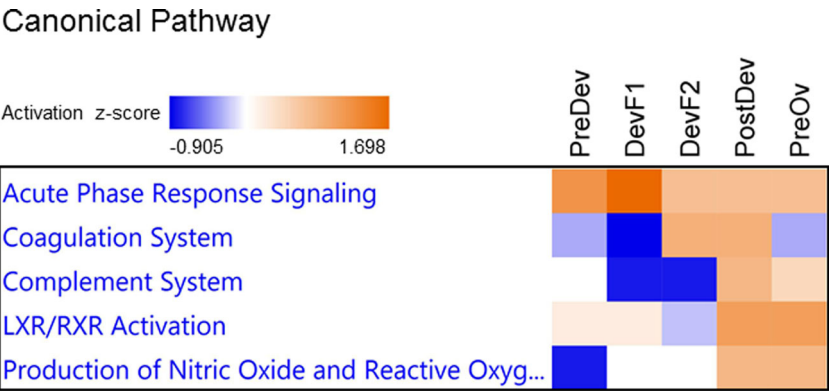
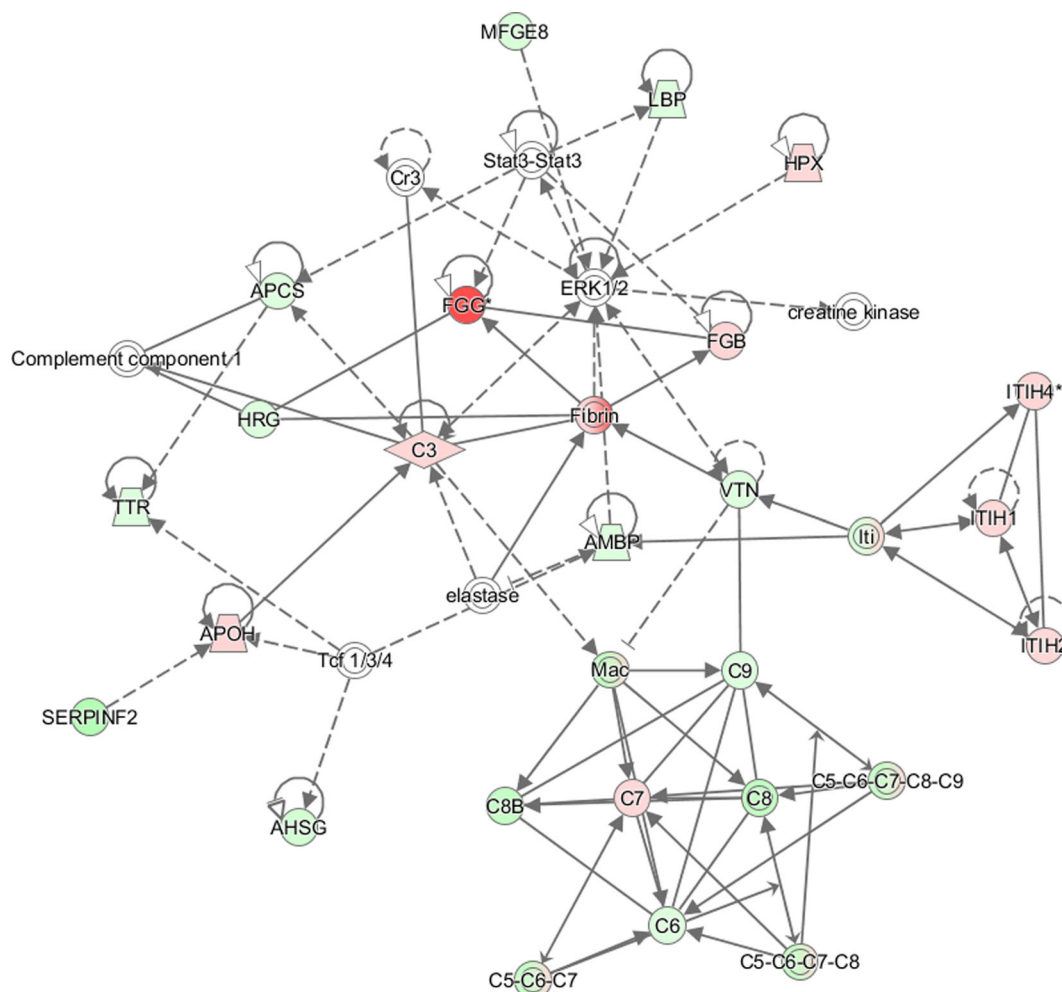


Figure 7. List of the most significant canonical pathways and their respective scores obtained from Ingenuity Pathway Analysis software. Color intensity indicates the magnitude of regulation inferred from z-score (orange: upregulation, blue: downregulation, white: z-score = 0, indicating upregulation of some proteins and downregulation of other). LXR = liver X receptor; RXR = retinoid X receptor. (Please see the online version for the color figure).

to the stage of follicle development. Agostini et al. [48] showed an anticoagulant state during follicle growth (the follicular phase) and rupture (the ovulatory phase). These authors stated that prevention of clotting after ovulation ensures that FF can serve as a conduit for delivery of the oocyte to the oviduct. In addition, thrombin

was demonstrated to be an intraovarian signal for optimal follicular luteinization in mice [49], and decreased levels of antithrombin in the FF were associated with improved pregnancy outcome in humans [6], confirming their crucial role in female reproduction. Thus, our findings signaling that the coagulation factors are potentially



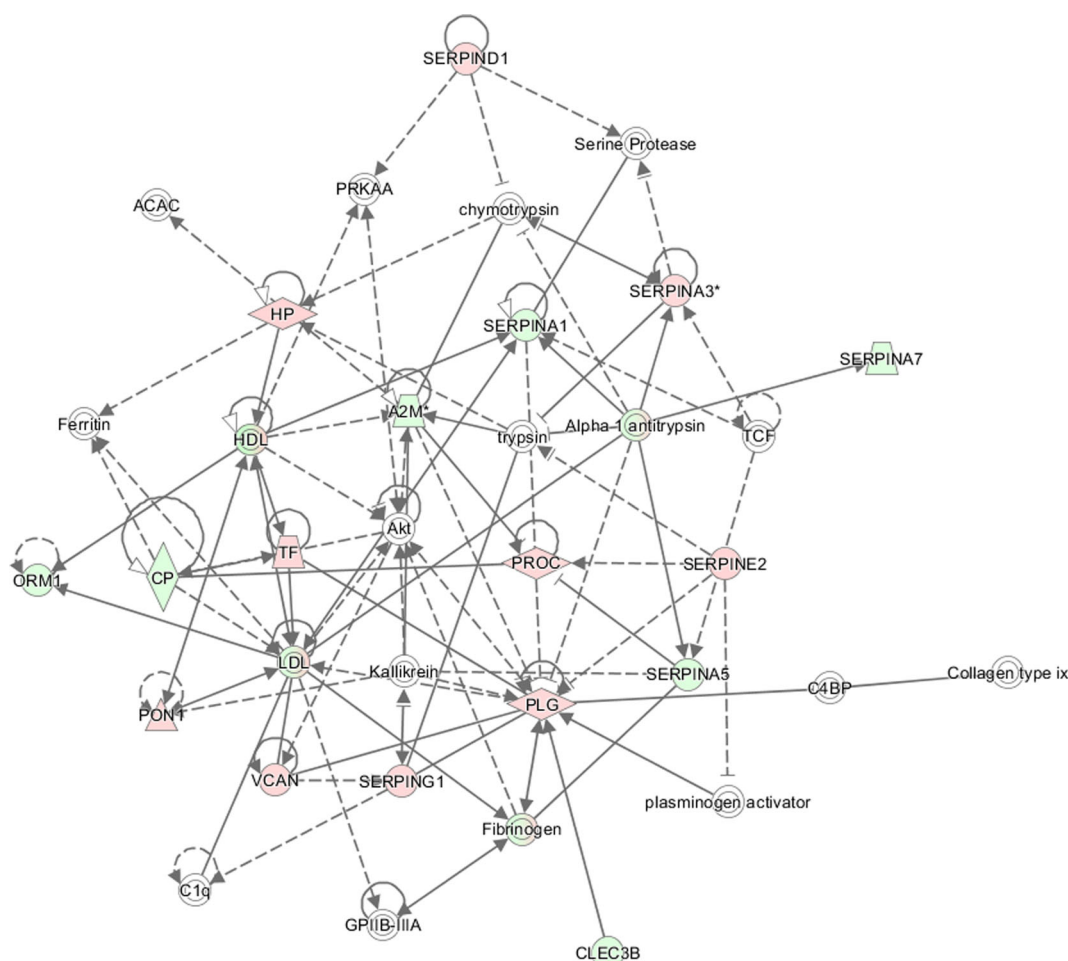
**Figure 8.** Network of proteins involved in the acute phase response. The network was generated in Ingenuity Pathway Analysis software. Entire lines represent direct and evidence-based interactions and dash lines represent presumed interactions between proteins. Colors indicate the nature of the expression: red are upregulated and green are downregulated. Uncolored nodes mean unspecified effect in our experiment and were integrated into the computationally generated network based on the evidence stored in the IPA knowledge memory indicating a relevance to this network. Molecules are named according to IPA software. AHSG = alpha 2-HS glycoprotein; AMBP = alpha-1-microglobulin/bikunin precursor; APCS = serum amyloid P-component precursor; APOH = apolipoprotein H; C3, C5, C6, C7, C8, C8B, C9 = complement factors; Cr3 = complement receptor 3; FGB = fibrinogen beta chain; FGG = fibrinogen gamma chain; HPX = hemopexin; HRG = histidine-rich glycoprotein; Iti = ITI; ITIH1, ITIH2, ITIH4 = inter-alpha-trypsin inhibitor heavy chain; LBP = lipopolysaccharide-binding protein; Mac = membrane attack complex; MFGE8 = lactadherin; SERPINF2 = alpha-2-antiplasmin precursor; Stat3-Stat3 = signal transducer and activator of transcription 3; Tcf 1/3/4 = transcription factor; TTR = transthyretin; VTN = vitronectin. (Please see the online version for the color figure).

correlated to the inflammatory-related proteins present in FF and have relevant roles in the follicular microenvironment.

Abundance of several proteins was found to be correlated with steroid concentration in FF, which suggests that these follicular proteins are associated with endocrine phenomena in the ovary and modulate diverse ovarian physiological processes, including granulosa and theca cells differentiation, oocyte maturation, cumulus cell expansion, luteinization, and early embryo development. However, as pointed out by Kushnir et al. [7], it is yet unclear if the associations between abundance of proteins and local concentrations of steroids are “cause and effect” relationships or if they represent covariance of unrelated events. Proteomic molecular approaches have emerged as a complement promising tool to investigate the complex female reproductive physiology. Future studies that precisely characterize the follicular diameter and stage are necessary to further evaluate these associations and should contribute to

increase the understanding of their role in ovarian folliculogenesis biology.

The functional enrichment analysis showed a particular balance of functions related to the acute phase response signaling and the coagulation system. Several proteins identified in this study were involved in both generated networks, which indicates that inflammation and coagulation cascade are intimately linked processes throughout follicular development. The effectors and inhibitors act in a controlled and balanced time-dependent manner. Among these compounds, complement components, ITI heavy chain, and fibrinogen are essentially involved in the acute phase response pathway and were the principal hubs of the generated network (Figure 8). Our findings therefore support a role for the complement system in the associated inflammatory response and suggest that this role is of relevance in folliculogenesis. The family of ITIs is a long-known family of serine protease inhibitors, composed of a light chain



**Figure 9.** Network of proteins involved in the coagulation system. The network was generated in Ingenuity Pathway Analysis software. Entire lines represent direct and evidence-based interactions and dash lines represent presumed interactions between proteins. Colors indicate the nature of the expression: red are upregulated and green are downregulated. Uncolored nodes mean unspecified effect in our experiment and were integrated into the computationally generated network based on the evidence stored in the IPA knowledge memory indicating a relevance to this network. Molecules are named according to IPA software. A2M = alpha-2-macroglobulin; ACAC = acetyl-CoA carboxylase 1; AKT = RAC-alpha serine/threonine-protein kinase; C1q = complement subcomponent C1q; C4BP = C4b-binding protein alpha chain; CLEC3B = tetranectin; CP = ceruloplasmin; GPIIB-IIIA = glycoprotein IIb/IIIa; HDL = high-density lipoprotein; HP = haptoglobin; LDL = low-density lipoprotein; ORM1 = alpha-1-acid glycoprotein; PLG = plasminogen; PON1 = paraoxonase 1; PRKAA = AMP-activated protein kinase catalytic; PROC = vitamin K-dependent protein C; SERPINA1, 3, 5, 7 = alpha-1-antiproteinase members; SERPIND1 = serpin family D member 1; SERPINE2 = serine protease inhibitor clade E member 2; SERPING1 = serpin family G member 1; TCF = transcription factor; TF = tissue factor; VCAN = versican core protein. (Please see the online version for the color figure).

with antiproteolytic activity (bikunin) and different homologous heavy chains, which contribute to the stability of the extracellular matrix. There have been many studies on biological effects of the ITI molecules, proposing an involvement in various acute-phase processes, such as inflammation or cancer [50]. In the acute phase response pathway, our results suggest that fibrinogen is involved in controlling, modulating, and balancing inflammation. Fibrinogen is primarily synthesized in the liver, although locally produced fibrinogen by granulosa cells from ovarian follicles was also identified [51]. In fact, fibrinogen contributes to inflammation not only inducing leukocyte migration, but also stimulating the expression of proinflammatory cytokines [52] which are, in the last instance, required for normal follicular development [53].

Our comparative analysis showed that the coagulation system pathway is upregulated at the dominance stage and in the second largest follicle from the deviation stage. The coagulation pathway was tightly regulated by several molecules including antithrombin-

III, alpha-2-macroglobulin, vitamin K-dependent protein C, and plasminogen, which have been previously reported as important mediators of extracellular matrix remodeling, modulating follicular development, ovulation, and corpus luteum formation [4, 54–56]. In fact, coagulation proteins are profoundly integrated in the follicle microenvironment [57], performing, controlling, and triggering several biochemical and molecular processes essential in the follicular maturation. However, the orchestrated coagulation/fibrinolysis molecular mechanisms in the FF are part of the complex shifts in the follicle development that remain poorly understood.

The pathways related to the biosynthesis of NO and ROS were also identified in this study and could play an essential role in folliculogenesis, especially during intense metabolism in advanced stages of follicular maturation (dominance and preovulatory). Nitric oxide is a chemical messenger produced by the activity of NO synthase (NOS), which is expressed in the ovary in endothelial (eNOS) and inducible isoforms. A body of evidence suggests an involvement of the



NOS/NO system in the bovine follicular development [58–60] and ovulation [61]. Indeed, higher degrees of eNOS protein expression in granulosa and theca cells has been reported in E2 active follicles [62], which is consistent with our findings. The ROS pathway has been described to be active in the female reproductive system [63]. However, ROS abundance may have deleterious effects on cellular function by inducing oxidative damage of intracellular components and inducing apoptosis [64]. Thus, a delicate equilibrium between ROS and antioxidant in the follicle microenvironment is required to counter the potentially harmful effects of ROS and to maintain the cellular functions. Follicular fluid milieu of dominant follicles is characterized by elevated concentrations of  $H_2O_2$ , a component of ROS system [65]. Previous studies show that ovulation rates and ovulatory events are perturbed upon ROS reduction in the mouse and rabbit [66, 67]. Therefore, an increase of ROS pathway observed in our study seems to be an important preparatory step for ovulation that commences upon dominant follicle selection. Collectively, these results clearly indicated the involvement of NO and ROS pathways at advanced stages of follicle maturation and ovulation and highlight that appropriate concentrations of these products are necessary to maintain normal healthy functional cells.

In this study, we confirmed that temporal regulation of protein expression modulates follicular development and highlights potential biomarkers for follicular health, dominance, and maturity. The understanding of ovarian folliculogenesis may have profound implications in the improvement of reproductive technologies. Although there is much information on attempting to generate specific biomarkers for follicle development, oocyte quality, and clinical outcome in humans, ethical concerns and scarcely available human FF [68] make it difficult to perform detailed assessments of the temporal changes of the proteome profile during folliculogenesis. Additionally, logistical limitations also restrict potential experiments with frequent sampling or observations when working with humans [69]. Based on similarities in ovarian follicular dynamics and endocrine control in cattle and women, the bovine model has been proposed for studying reproductive events in women [70]. Therefore, our results provide an additional layer of understanding on size-dependent protein dynamics in the ovarian follicle microenvironment, associated with changes in local steroid concentrations.

In summary, we demonstrated numerous differentially expressed proteins in bovine FF according to the stage of follicle development. The follicle selection process and the development of dominance depend on a coordinated activity of gonadotropins, steroid hormones, as well as intraovarian factors. The obtained data provide new insights into the size-dependent protein changes in ovarian follicle microenvironment, associated with changes in local steroid concentrations, which are critical for follicular maturation and may influence follicular function. Comparative pathways analysis highlighted the occurrence in FF of functional networks in a differential temporal balance and control, including activation/inhibition of the acute phase response, coagulation system, complement system, LXR/RXR activation, and NO and ROS production. Together, these results provide a basis for better understanding of folliculogenesis process and shed light on the molecular biology that potentially influences besides follicular development, oocyte competence, and fertilization outcome.

## Supplementary data

Supplementary data are available at [BIOLRE](https://academic.oup.com/biolreprod/article-abstract/97/6/835/4631041) online.

Supplement File S1.

**Supplementary Figure S1.** Quantitative evaluation of the differentially expressed proteins in bovine follicular fluid over follicle development. Proteins are expressed as log2-ratio in samples from different stages of the follicle development relative to control.

**Supplementary Figure S2.** Correlation of abundance of proteins with progesterone concentration in bovine follicular fluid over follicle development.

**Supplementary Table S1.** Signaling pathway networks associated with proteins identified in bovine follicular fluid.

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