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# Involvement of PAPP-A and IGFR1 in Cystic Ovarian Disease in Cattle

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**Contents**
Cystic ovarian disease (COD) is one of the main causes of infertility in dairy cattle. It has been shown that intra-ovarian factors, such as members of the insulin-like growth factor (IGF) system, may contribute to follicular persistence. The bioavailability of IGF to initiate its response by binding to specific receptors (IGFRs) depends on interactions with related compounds, such as pregnancy-associated plasma protein A (PAPP-A). The aim of this study was to determine IGFR1 and PAPP-A expression both in follicles at different stages of development and in cysts, to evaluate the roles in the etiopathogenesis of COD in cattle. The mRNA expression of PAPP-A was higher in granulosa cells of large tertiary follicles than in cysts, whereas the protein PAPP-A present in the follicular fluid from these follicles showed no differences. Although no PAPP-A mRNA expression was detected in smaller tertiary follicles, in their follicular fluid, this protease was detected in lesser concentration than in cysts. The mRNA expression of IGFR1 was lower in granulosa cells from cystic follicles than in those from tertiary ones. However, the protein expression of this receptor presented the highest levels in cystic structures, probably to increase the possibility of IGF response. The data obtained would indicate that animals with COD have an altered regulation of the IGF system in the ovary, which could be involved in the pathogenesis of this disease in cattle.

## Introduction

Cystic ovarian disease (COD) is characterized by large follicular structures that persist in the ovary for 10 days or more in the absence of a corpus luteum, with interruption of the normal oestrous cycle (Silvia et al. 2002; Vanholder et al. 2006). Cystic ovarian disease has a multifactorial aetiology (Vanholder et al. 2006) but an endocrine imbalance in the hypothalamic–hypophyseal–gonadal axis appears to be one of the underlying causes of follicular cyst development (Gümen and Wiltbank 2002). However, there is an increasing interest to understand the factors involved in follicular persistence. Follicular growth and dominance are controlled by different hormonal events that lead to changes in the expression of several intrafollicular regulatory proteins, including the insulin-like growth factor (IGF) system in granulosa and theca cell (Kawashima et al. 2007; Webb and Campbell 2007). The initial response of IGF1 is a result of interactions among molecules of the IGF system, which is constituted by IGF1 and IGF2, six IGF-binding proteins (IGFBP1–6) and IGFBP proteases (Spicer and Echternkamp 1995; Silva et al. 2009; Sanchez et al. 2014). The insulin-like growth factor binding proteins (IGFBPs) bind IGFs with high affinity and consequently regulate their access to IGF receptors. This main mechanism modulates IGF bioavailability during folliculogenesis (Nicholas et al. 2002; Silva et al. 2009). Several reports indicate that changes in the concentrations of IGFBPs in bovine follicles may be regulated by their own gene expression in different ovarian tissues, post-translational degradation by hormone-induced proteases or both (Spicer and Echternkamp 1995; Fortune et al. 2001; Monget et al. 2002). Degradation by proteases has been shown in different species such as humans (Conover et al. 2001), cattle (Mazerbourg et al. 2001; Spicer 2004) and rodents (Hourvitz et al. 2002). Pregnancy-associated plasma protein A (PAPP-A) is a high molecular weight hetero-tetrameric glycoprotein, with two characteristic subunits of 180 kDa (Spicer et al. 2004; Santiago et al. 2005). PAPP-A has been shown to be the main IGFBP protease regulating the levels of free IGF1 in cattle (Mazerbourg et al. 2001; Spicer 2004; Sanchez et al. 2014). Within follicles, PAPP-A is able to degrade IGFBP4 and 5, leaving IGF1 available, and is thus likely an important regulator of follicular growth and selection in cattle (Aad et al. 2006).

The physiological actions of IGFs are triggered by interacting with specific cell surface membrane receptors, IGFR1 and 2 (Giudice 1992; Spicer 2004; Echternkamp et al. 2012). IGFR1 is a membrane glycoprotein mediating most actions of IGF1 and 2 and has an important effect on ovarian follicular development (Giudice 1992; Echternkamp et al. 2004; Yang et al. 2013). IGFR1 preferentially binds IGF1, whereas IGFR2 preferentially binds IGF2 (Spicer and Aad 2007). IGFR1 gene expression increases with follicular diameter in sheep (Perks et al. 1995), and in cattle, the mRNA expression was higher in large tertiary gonadotropin-dependent follicles in cattle (Spicer et al. 1994; Monget et al. 2002; Hastie and Haresign 2008). In both species, that increase in IGFR1 expression is concomitant with an increase in follicle-stimulating hormone (FSH) and luteinizing hormone (LH) receptors of large follicles, suggesting that a positive feedback could take place during early differentiation of follicles (Giudice 1992; Hastie and Haresign 2008).

As the IGF system is a key intra-ovarian regulator of folliculogenesis and ovarian steroidogenesis, a slight modification in this system could be associated with

COD (Ortega et al. 2008; Rey et al. 2010; Probo et al. 2011; Rodríguez et al. 2011, 2013). Therefore, the aim of this study was to determine IGFR1 and PAPP-A expression both in follicles at different stages of development and in cysts.

## Materials and Methods

### Ethical aspects

All the procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Adam, 2010), and the protocol was approved by the ethics and safety committee of the Facultad de Ciencias Veterinarias of the Universidad Nacional del Litoral (Santa Fe, Argentina) under protocol number 61/2010.

### Induction of COD by adrenocorticotropin administration

Ten nulliparous Holstein heifers (18–24 months old, maintained under standard husbandry conditions) with regular oestrous cycles according to prior detection of oestrus, rectal palpation and ultrasonography were used. Their oestrous cycles were synchronized using the Ovsynch protocol as described previously (Ortega et al. 2008; Rodríguez et al. 2013). The beginning of oestrus was confirmed by rectal examination and ultrasonography and designated as Day 0 of the cycle. On Day 15, five heifers received subcutaneous injections of tetracosactrin hexaacetate (1 mg/ml/animal; Synacthen Depot, Novartis, Basel, Switzerland), a synthetic polypeptide with adrenocorticotropin (ACTH) activity, every 12 h for 7 days (Ortega et al. 2008). Five control animals received saline (1 ml) (Dobson et al. 2000; Ortega et al. 2008).

Ovarian ultrasonographic examinations were performed in all animals using a real-time, B-mode scanner equipped with a 5.0 MHz linear-array transrectal transducer (HS-101V; Honda Co., Tokyo, Japan), as previously described (Rodríguez et al. 2013; Matiller et al. 2014). The growth and regression of follicles >5 mm, corpora lutea and follicular cysts were monitored. Daily ovarian ultrasonography was performed throughout a complete oestrous cycle in control heifers (22 ± 1 days) and from Day 14 until ovariectomy in ACTH-treated heifers as described below (Amweg et al. 2013). Cysts detected by ultrasonography in all treated animals were defined as any follicular structure equal to or >20 mm in diameter present for 10 days or more, without ovulation or corpus luteum formation and lack of uterine tonicity (Silvia et al. 2002; Bartolome et al. 2005). The first day of follicular cyst formation was the day that a follicle attained 20 mm or more in diameter, and the ovaries were removed 10 days later by flank laparotomy (47 ± 3 days). Control heifers were ovariectomized, to obtain normal growing follicles (approximately Day 18), when the dominant follicle reached a diameter >10 mm. The samples were processed as previously described (Rodríguez et al. 2013). The follicular fluid (FF) from all the follicles of different size selected to be evaluated was aspirated and stored at −80°C.

### Spontaneous cystic ovaries and control cows

Holstein cows from dairy herds of the milk-producing region of Santa Fe, Argentina, with spontaneous COD were used to obtain whole ovaries by ovariectomy or FF by follicular aspiration. Cows were diagnosed with the disease during the periodic reproductive control by rectal palpation and ultrasonography (B-mode ultrasound equipped with a transrectal 5.0 MHz linear-array transducer, HS-101V; Honda Co.), considering previously described parameters (Bartolome et al. 2005). The ovaries from 10 multiparous cows were removed by transvaginal ovariectomy (Marelli et al. 2014). To prevent the rupture of the follicular cysts during surgery, the FF was aspirated before ovariectomy, using a digital ultrasound system equipped with a microconvex transducer of 5.0 MHz (Chison 8300vet; Chison Medical Imaging Co., Mainland, China) mounted on a transvaginal probe for follicular aspiration (Watanabe Applied Technology Limited, San Pablo, Brazil).

Additionally, 15 cows with spontaneous COD and 10 control cows in proestrus previously synchronized by the Ovsynch protocol were only aspirated to obtain FF rich in follicular cells. The samples obtained were refrigerated and immediately transported to the laboratory for processing.

### Sample processing and follicle classification

The ovaries obtained by ovariectomy of spontaneous COD and control groups were used for RT-PCR and western blot. Follicular fluids from control and cystic follicles were centrifuged at 2000 × ***g*** for 10 min. The pellets of granulosa cells were resuspended in TRIzol LS reagent (Invitrogen, Life Technology, Camarillo, CA, USA) for mRNA evaluation, whereas the remaining FFs were stored at −80°C for hormonal determination to confirm health status (Ortega et al. 2008; Amweg et al. 2013) and PAPP-A evaluation. The remaining follicular walls of follicles of different size, after aspiration of the FF, were washed several times with phosphate-buffered saline (PBS) to remove residual granulosa cells. Then, the theca cells were excised from the surrounding stroma and washed several times with diethylpyrocarbonate (DEPC) water (Amweg et al. 2013; Marelli et al. 2014). The tertiary follicles were classified into three categories according to their diameters: small (<5 mm), medium (5–10 mm) or large (>10 mm) (Parrott and Skinner 1998). Cystic follicles were characterized, as described before, as follicles of >20 mm in diameter without luteinization signs. All samples were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Additional other samples from ovarian tissues were immediately frozen at −80°C until their use in western blotting to determine

the specificity of the antibody used in immunohistochemistry (IHC).

For IHC, the ovaries from ACTH-induced COD and their controls as well as the ovarian tissue remaining after taking the small samples for RT-PCR and western blot from spontaneous COD and controls were fixed in formaldehyde 4% at room temperature for 8–12 h. The fixed tissues were washed in PBS, dehydrated and embedded in paraffin wax. Sections (5 μm thick) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and primarily stained with haematoxylin–eosin for a preliminary observation of the ovarian structures (Salvetti et al. 2009). Follicles were classified into primary, secondary, tertiary follicles (Priedkalns 1998) and follicular cysts (Silvia et al. 2002). Only follicular cysts with a complete granulosa cell layer and without luteinization were analysed.

Follicular health status was confirmed by hormonal concentrations in FF [data not shown; parallel study (Ortega et al. 2008; Amweg et al. 2013)].

### Real-time RT-PCR

Total RNA was isolated from granulosa and theca cells using TRIzol LS reagent (Invitrogen), according to the manufacturer's instructions with slight modifications (Rey et al. 2010; Rodríguez et al. 2011, 2013). Samples of each follicle were processed individually. RNA samples were treated with DNase (Invitrogen) according to the manufacturer's instructions and assessed for quantity with the Qubit RNA HS assay kit with Qubit fluorometer (Invitrogen), aliquoted and stored at −80°C. The reverse transcription conditions were as described previously (Rodríguez et al. 2011, 2013).

To design primers and verify their specificity, the PrimerSelect LASERGENE application program (DNAStar, Madison, WI, USA) was used. All oligonucleotides used were provided by Invitrogen. In turn, the specificity thereof was checked by direct comparison of the sequence against Bos taurus genome using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

To confirm the mRNA purity of bovine granulosa and theca cells (no cross-contamination), primer sequences for cytochrome P450 aromatase (CYP19a1) and cytochrome P450 17a-hydroxylase/17,20-lyase (CYP17a1) were used (Lagaly et al. 2008). Theca cells expressed CYP17a1 and granulosa cells expressed only CYP19a1. Samples with both populations were discarded. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of reverse transcription and reaction efficiency (Fields et al. 2012; Sayasith and Sirois 2014). The primer sequences used are described in Table 1.

StepOne real-time PCR system (Applied Biosystems, Life Technology, Carlsbad, CA, USA) and SYBR Green I (Invitrogen) were used to analyse the mRNA expression of IGFR1 and PAPP-A in both populations by real-time PCR. An optimized protocol was used: initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 15 s; annealing at 58°C for 20 s (IGFR1), 60°C for 25 s (PAPP-A) and 52°C for 30 s (GAPDH); extension at 72°C for 30 s; and fluorescence reading at 74°C. All measurements for each sample were performed in duplicate, and the CVs were calculated as described by Bustin et al. (2009). The efficiency of PCRs and relative quantities were determined from a standard curve, and PCR efficiency was calculated using the StepOne software v2.2 (Life Techologies). The mRNA expression levels were recorded as cycle threshold (Ct) values as described previously (Rodríguez et al. 2013). Negative DNA template controls were included in all the assays and yielded no consistent amplification. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Fold change was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 1. Forward and reverse primer sequences (5′-3′)

| Primer | Sequence 3′-5′ | Reference |
|---|---|---|
| GAPDH | For CAC CCT CAA GAT TGT CAG CA<br>Rev GGT CAT AAG TCC CTC CAC GA | Shibaya et al. (2007) |
| CYP17a1 | For GGAGGCGACCATCAGAGAAGTGC<br>Rev CAGCCGGGACATGAAGAGGAAGAG | Lagaly et al. (2008) |
| CYP19a1 | For TAAAACAAAGCGCCAATCTCTACG<br>Rev GGAACCTGCAGTGGGAAATGA | Lagaly et al. (2008) |
| PAPP-A | For TGGAGAACGCTTCGCTCAACTG<br>Rev ACGCTGGGTCCTGTCTGGCTTT | GenBank: XM_613511.6 |
| IGFR1 | For CACGCCTTGGTCTCCTTGTCCT<br>Rev CGTCACTTCCTCCATGCGGTAAAT | GenBank: NM_001244612.1 |

### Western blotting

The specificity of the primary antibody against IGFR1 used in IHC and the amount of PAPP-A secreted in FF were evaluated by western blot. To test IGFR1 specificity, small samples of follicular walls of tertiary follicles were homogenized in radioimmunoprecipitation assay lysis buffer with a protease inhibitor cocktail (complete mini protease inhibitor cocktail tablets; Roche, Mannheim, Germany). To determine the amount of PAPP-A secreted, complete bovine FF was used. Proteins were separated in SDS-PAGE [10% (w/v) for IGFR1 antibody specificity and 8% (w/v) acrylamide–polyacrylamide for PAPP-A quantification] and then electroblotted on nitrocellulose membranes (Hybond ECL nitrocellulose membrane; GE Healthcare, Buckinghamshire, UK). The membranes were blocked with TBS containing 0.05% (v/v) Tween-20 (TBST; Sigma-Aldrich Corp., St. Louis, MO, USA) and 10% (w/v) non-fat milk and then incubated overnight at 4°C with specific primary antibodies (Table 2). Bound antibodies were detected using anti-rabbit IgG perox-

Table 2. Antibodies, suppliers and dilutions used for immunohistochemistry (IHC) and western blotting (WB)

| Antibodies | Clone/Source | Dilution | |
|---|---|---|---|
| | | IHC | WB |
| Primary antibodies | | | |
| Anti-PAPP-A | SC-50518. Rabbit polyclonal (Santa Cruz Biotechnology, Dallas, TX, USA) | – | 1 : 300 |
| Anti-IGFR1 | SC-712. Rabbit polyclonal (Santa Cruz Biotechnology) | 1 : 100 | 1 : 100 |
| Secondary antibodies | | | |
| Goat Polyclonal | 65-6140 (Zymed, San Francisco, CA, USA) | 6 μg/ml | – |
| HRP anti-rabbit IgG | SC-2004 (Santa Cruz Biotechnology) | – | 1 : 2000 |

idase antibody (Table 2). The immunopositive bands were detected by chemiluminescence, using the ECL-plus system (GE Healthcare) on Hyperfilm ECL film (GE Healthcare).

### Immunohistochemistry for IGFR1

The antibody was assayed in a minimum of five sections of each ovary from each heifer or cow. A streptavidin–biotin immunoperoxidase method was performed as previously described (Salvetti et al. 2010). Briefly, after deparaffinization, the antigen was retrieved by incubating the sections in 0.01 M citrate buffer (pH 6.0) in a domestic microwave oven at 800 W. The endogenous peroxidase activity was inhibited with 3% (v/v) $H_2O_2$ in methanol, and non-specific binding was blocked with 10% (v/v) normal goat serum. Slides were incubated with polyclonal rabbit anti-IGFR1 (Table 2) for 18 h at 4°C and then for 30 min at room temperature with biotinylated secondary antibody (Table 2). IGFR1 was visualized by the ExtrAvidin®-Peroxidase method (Sigma-Aldrich), and 3,3-diaminobenzidine (liquid DAB-Plus substrate kit; Invitrogen) was used as chromogen. Finally, the slides were washed in distilled water and counterstained with activated haematoxylin (Biopur, Rosario, Argentina), dehydrated and mounted. To verify the immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method, replacing primary antibody with rabbit non-immune sera. The specificity of the secondary antibodies was tested by incubation with a primary antibody against human antigens with a proven negative reaction to tissues of cattle: anti-Ki-67 (polyclonal, rabbit anti-human Ki-67; Dako, Carpinteria, CA, USA). To exclude the possibility of non-suppressed endogenous peroxidase activity, some sections were incubated with DAB reagent alone (Ortega et al. 2008). As positive control, human liver and ovary paraffin archived blocks were used.

### Image analysis

Images were digitized using a colour video camera (Motic 2000; Motic China Group, Xiamen, China) mounted on top of a conventional light microscope (Olympus BH-2; Olympus Co., Tokyo, Japan) and then analysed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA) as described and validated previously (Ortega et al. 2009, 2010; Rodríguez et al. 2011). To obtain semi-quantitative data for IHC staining in follicular wall, at least three sections were evaluated for each specimen. The average density (% of immunopositive area) of the IGFR1 antibody was calculated from at least 50 images of each area (granulosa and theca cells) in each section as a percentage of the total area evaluated through colour segmentation analysis, which extracts objects by locating all objects of a specific colour (brown stain). These values were verified and normalized with controls carried across various runs using the same region (verified by image comparison) for calibration. The percentage of immunopositive area was calculated separately for each follicular compartment (granulosa and theca cells). Sections were analysed with the observer blinded to the experimental group.

The main strength of the well-validated imaging approach used in this study is the visualization of *in situ* localization of proteins within cells of interest. Quantification of biological markers using this approach has been successfully applied to quantify immunoreactivity in different tissues (Ranefall et al. 1998; Ortega et al. 2009).

### Statistical analysis

A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used to analyse the data. The distribution of data was tested for normality using the Kolmogorov–Smirnov test. Tests of homogeneity of variance between groups were conducted using Levene's test. The statistical significance of differences between groups of data was assessed by one-way ANOVA, followed by Duncan's multiple range tests as a multiple comparison test. A value of $p < 0.05$ was considered significant. The Student's *t*-test was used to evaluate the levels of changes in PAPP-A mRNA levels between cysts and the control group. The results are expressed as mean ± SD.

## Results

### PAPP-A and IGFR1 gene expression

All genes presented low overall variability (PAPP-A: 0.70%, IGFR1: 1% CV of Ct). GAPDH mRNA expression was similar in the different groups evaluated ($p > 0.05$), which confirmed that the cDNA quantities used were equivalent.

PAPP-A mRNA expression was detected in granulosa cells, but not in theca cells. PAPP-A mRNA expression

was higher in granulosa cells of large tertiary follicles than in those of spontaneous cysts ($p < 0.05$). No mRNA expression was detected in small or medium tertiary follicles (Fig. 1).

IGFR1 mRNA expression in granulosa cells was lower in cysts than in control tertiary follicles of different sizes (Fig. 2). In contrast, no differences were detected in IGFR1 mRNA levels in theca cells of all groups analysed.

### PAPP-A protein expression

The PAPP-A secreted was analysed by western blotting in FF from follicles of different size of control ovaries and spontaneous follicular cysts (Fig. 3A). PAPP-A concentration was higher in FF from spontaneous cysts than in FF from small and medium control follicles (Fig. 3B). No differences were detected between large tertiary follicles and cysts.

### IGFR1 protein expression

IGFR1 protein expression was evaluated by IHC in follicles of different developmental stages, and their expression was detected only in granulosa cells (Fig. 4). Greater levels were shown in spontaneous cysts than in all control follicles analysed (Fig. 5). Furthermore, as the follicles increased in size, an increase in IGFR1 was detected in ovarian follicles from control animals as well as in those from animals with spontaneous COD and ACTH-induced COD. IGFR1 expression was higher in spontaneous cysts than ACTH-induced cysts and control tertiary follicles ($p < 0.05$). It should be observed that although the expression in follicles of animals with induced COD was higher, the pattern of increasing expression in tertiary and cystic follicles was similar.

## Discussion

In agreement with previous reports (Mazerbourg et al. 2001; Spicer et al. 2004; Santiago et al. 2005; Sanchez et al. 2014), in the current study, we detected the presence of PAPP-A mRNA in bovine granulosa cells of tertiary follicles. However, contrarily to that reported by other authors who detected PAPP-A mRNA expression in theca and granulosa cells (Stewart et al. 1996; Hourvitz et al. 2000; Aad et al. 2006), we detected no mRNA expression in theca cells. Santiago et al. (2005) reported that PAPP-A mRNA expression in granulosa cells was greater in dominant follicles than in subordinate follicles, whereas in our study, PAPP-A mRNA was detected in large tertiary follicles and cysts but not in tertiary follicles smaller in sizes. On the other hand, similar to that found by other authors (Mazerbourg et al. 2001; Monget et al. 2003; Rivera and Fortune 2003), we found PAPP-A protein in FF.

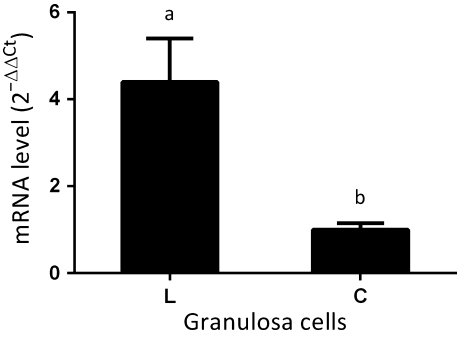


Fig. 1. PAPP-A mRNA expression in granulosa cells of large (L, n = 10) tertiary follicles and spontaneous follicular cysts (C, n = 25). Values represent the mean ± SD. Bars with different superscript letters denote significant differences ($p < 0.05$)

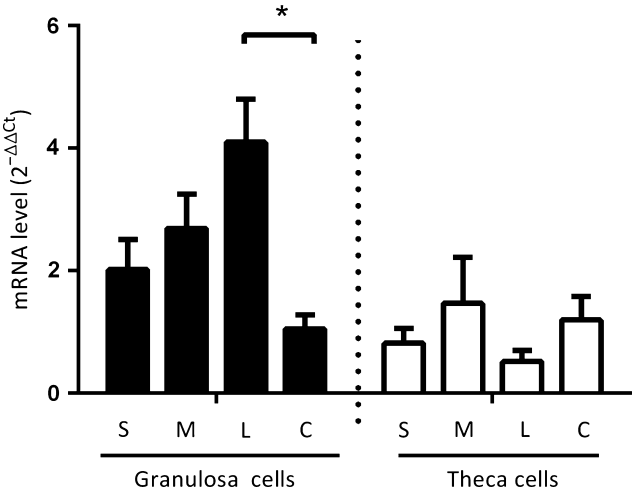


Fig. 2. IGFR1 mRNA expression in granulosa cells (black bars, n = 45) and theca cells (open bars, n = 25) of growing tertiary follicles [small (S), medium (M) and large (L)] and follicular cysts (C). Values represent the mean ± SD. Bars with asterisk denote significant differences ($p < 0.05$)

PAPP-A is responsible for IGFBP degradation (Monget et al. 2003; Spicer 2004), and PAPP-A expression in granulosa cells may contribute to free IGF1 bioavailability for follicle development to the pre-ovulatory phase (Spicer 2004; Sudo et al. 2007). In previous studies, protein and gene expression of IGFBP2, 3, 4 and 5 was detected in granulosa cells and their activities were detected in FF of different follicles (Rodríguez et al. 2011, 2013). The dominant follicles had lower IGF1 binding activities of IGFBP2, 4 and 5 than subordinate follicles (Santiago et al. 2005). In addition, PAPP-A is a key component that degrades IGFBP4 or 5 in FF (Spicer et al. 2004) and shows a tendency to increase as the follicle develops (Santiago et al. 2005). Therefore, the future dominant follicle has higher PAPP-A synthesis and is able to keep lower concentrations of IGFBP4 and 5 (Mazerbourg et al. 2001; Rivera and Fortune 2001; Fortune et al. 2004; Spicer et al. 2004), which results in higher availability of IGF1 for follicular growth and enhances the effects of FSH and oestradiol synthesis (Spicer et al. 1993, 1996, 2004; Fortune et al. 2004). PAPP-A gene expression could be

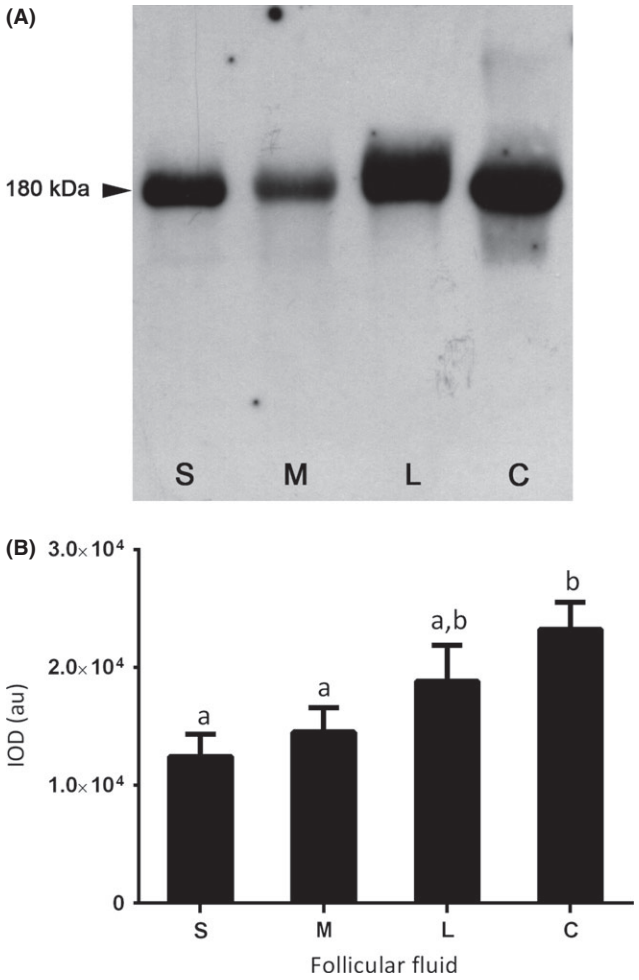


Fig. 3. (A) Representative image of detection of PAPP-A protein secreted to the bovine follicular fluid by western blot of control small (S), medium (M) and large (L) tertiary follicles compared with spontaneous follicular cysts (C). (B) Expression of secreted PAPP-A in follicular fluid of control small (S, n = 5), medium (M, n = 5) and large (L, n = 10) tertiary follicles and spontaneous follicular cysts (C, n = 25). Values represent the mean ± SD. Bars with different superscript letters denote significant differences ($p < 0.05$)

regulated by different ligands or inhibitors in FF (Spicer et al. 2004); then, changes in PAPP-A activity may occur without changes in PAPP-A mRNA levels (Santiago et al. 2005). Also, one control point of the proteolytic activity of PAPP-A depends on the presence of IGFs and is a characteristic of oestrogen-dominant follicles. Another level of control of IGF bioavailability and activity is through eosinophil major basic protein (proMBP), which binds PAPP-A covalently, inhibiting its activity (Kwintkiewicz and Giudice 2009). Therefore, PAPP-A activity could be changed without modifications in PAPP-A protein levels. In our study, although we detected lower expression of PAPP-A mRNA in cysts, PAPP-A was higher in FF of cysts than small and medium tertiary follicles. That difference may be explained by a regulated secretion of this enzyme by the low quantity of IGF1 present in FF of cysts (Ortega et al. 2008), a proteolytic activity independent of protein and gene expression (Santiago et al. 2005; Kwintkiewicz and Giudice 2009), post-translational modifications or a higher stability of protein released to the FF at the moment to detect it. Also, differences between gene expression by some follicles and detection of secreted protease in FF from all follicular structures would indicate that PAPP-A detected in FF might result from local synthesis and/or release from other tissue structures, to act in a paracrine or endocrine way. It was demonstrated expression of PAPP-A in kidney, bone (Conover 2012) and a strong gene and protein expression in corpus luteum of rodents and humans (Mazerbourg et al. 2001; Spicer 2004). Moreover, in bovine and porcine granulosa cells, PAPP-A mRNA expression level was shown to be maximal in differentiated follicles (Mazerbourg et al. 2001). Then, the higher PAPP-A released to FF of cysts compared with small tertiary follicles detected in this study, the subsequent lower concentrations of IGFBP4 and 5 (Mazerbourg et al. 2001; Fortune et al. 2004; Rodríguez et al. 2013; Sanchez et al. 2014) and the lowest IGF1 concentration detected in cysts (Ortega et al. 2008) would indicate the involvement of IGF in growth, maturation and steroidogenesis, thus contributing to follicular persistence.

In this study, in agreement with results obtained by other authors (Hastie and Haresign 2008; Sanchez et al. 2014), IGFR1 mRNA expression was detected in bovine granulosa cells. In addition, it was detected in theca cells of control tertiary and cystic follicles, but with lower expression than that obtained in granulosa cells, consistent with previous studies in sheep (Perks et al. 1995; Hastie and Haresign 2006) and cattle (Perks et al. 1999; Armstrong et al. 2000).

A decrease in IGFR1 gene expression with increasing diameter of the follicle in sheep has been reported (Perks et al. 1995; Hastie and Haresign 2006). In cattle, in agreement with our study, no differences in the levels of IGFR1 mRNA in granulosa cells during antral follicle growth have been shown (Perks et al. 1999; Armstrong et al. 2000).

We detected protein expression of IGFR1 in granulosa cells of follicles in all stages of development with increased levels in tertiary and cystic follicles without expression in theca cells, in accordance with that found by other authors (el-Roeiy et al. 1993; Lucy 2008; Silva et al. 2009). In previous studies, IGFR1 expression in granulosa cells showed constant levels at all developmental stages except in dominant follicles of bovine ovary (Sanchez et al. 2014). Although Stewart et al. (1996) also demonstrated that the numbers of IGFR1 in granulosa cells were not changed by the size or growth of follicles in agreement with our study, Spicer et al. (1994) reported that granulosa cells from tertiary follicles (>8 mm in diameter) have a greater number of IGFR1 than cells from small tertiary follicles (1–5 mm in diameter).

Higher expression of IGFR1 was detected in ACTH-induced cyst related to tertiary control follicles and

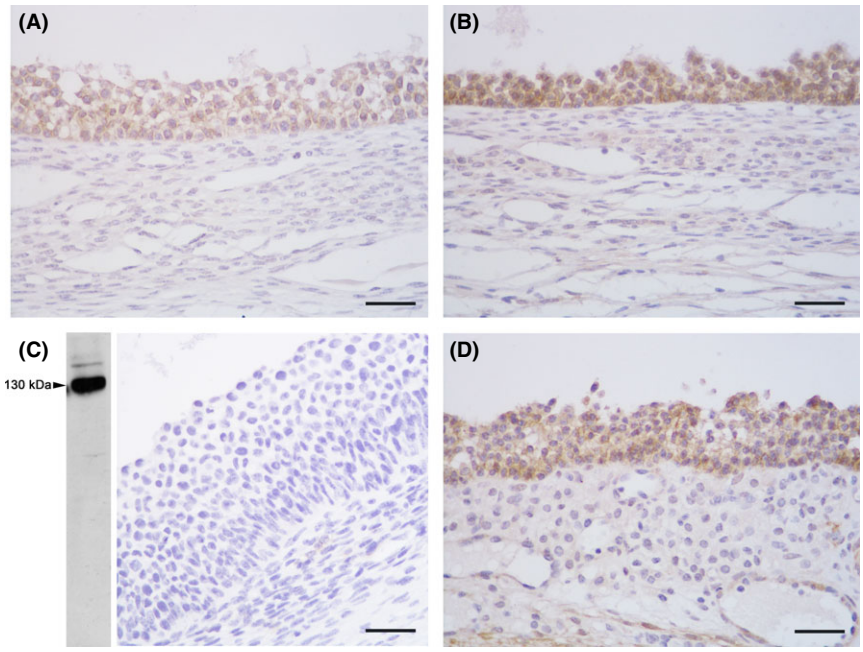


Fig. 4. Representative images of IGFR1 protein localization by immunohistochemistry in follicles. The immunoreactivity was intense in granulosa cells, and no expression was detected in theca cells in the follicular structures analysed. Images represent control tertiary follicles (A) and cystic follicles of induced (B) and spontaneous (D) disease. No staining was observed when the primary antibody was replaced with normal rabbit serum (negative control, C). Verification of antibody specificity by western blot analysis of an ovarian homogenate demonstrating the specificity of the antibody is shown in the left. Bars = 25 μm

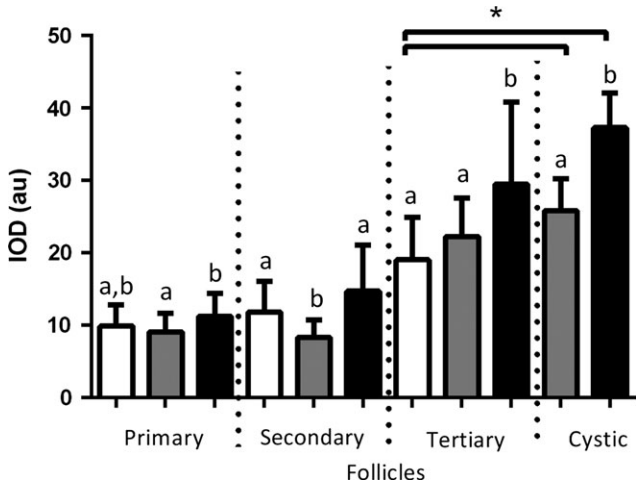


Fig. 5. Immunohistochemical staining of IGFR1, showing the percentage of immunopositive areas in granulosa cells evaluated in developing follicles (primary, secondary and tertiary follicles) of control ovaries (open bars, n = 5), experimentally induced cystic ovaries (grey bars, n = 5) and spontaneous cystic ovaries (black bars, n = 10). Values represent the mean ± SD. Bars with different letters within each follicle category are different ($p < 0.05$). Differences relative to the expression in follicular cysts in relation to control tertiary follicles are also indicated: *$p < 0.05$

spontaneous cyst. Differences between induced and spontaneous cysts could be due to the persistence time of the cystic follicle and different management variables in the animals evaluated. However, it is important to have the experimental model that allows us study changes occurred in the early formation of the cyst.

Differences between gene and protein expression could be due to post-translational modifications, due to altered levels of regulators. IGFR1 acts either via the MAPK or via the PI3K/Akt signalling cascades (LeRoith et al. 1995; Butler et al. 1998; Poretsky et al. 1999). Variations in modulators of the PI3K signalling cascade have been observed (Hein et al. 2015); therefore, changes in regulators would be involved in the variations of IGFR1 levels detected in this work. On the other hand, some studies suggest that oestradiol can stimulate cell proliferation by activating IGFR1 ligand independently (Song et al. 2004; Kamanga-Sollo et al. 2013), whereas others postulate that receptor activity can be ligand dependent (Kavran et al. 2014). Then, the highest levels of IGFR1 in ACTH-induced and spontaneous cysts could be stimulated by constant levels of oestradiol in these structures (Amweg et al. 2013). Thus, cysts would be able to keep IGF1 responses, such as proliferation, modulation of apoptosis (Salvetti et al. 2010) and steroidogenesis (Ortega et al. 2008; Amweg et al. 2013).

## Conclusions

The increased protein expression of IGFR1 and secreted PAPP-A in ACTH-induced and spontaneous follicular cysts shown in this study may indicate the necessity of capturing the low concentrations of IGF1 released present in ovaries from cows with COD (Ortega et al. 2008; Rodríguez et al. 2011, 2013). These data would indicate that animals with COD have an altered regulation of the IGF system in the ovary and thus allow postulating IGFR1 expression and PAPP-A secretion as a modulator of IGF1 in cattle with COD.

### Author contributions

Rodríguez FM has participated in the research design, the acquisition, analysis and interpretation of data and in the drafted paper.

Colombero M, Amweg AN and Huber E have participated in the sample processing, acquisition, analysis or interpretation of data, Gareis NC has participated in the animal treatment, control and ovariectomy, moreover has participate in sample processing, acquisition and analysis of data. Salvetti, NR has participated in the animal treatment, control and ovariectomy, moreover has participated in the research design, interpretation of data and critical revision of the drafted paper. Ortega HH has participated in the research design, interpretation of data and in the critical revision of the drafted paper. Rey F has participated in the research design, interpretation of data and to drafting and revising the paper critically.

## Conflict of interest

None of the authors have any conflict of interest to declare.

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