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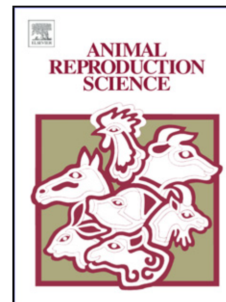

Title: Interaction between IGF1 and IGFBPs in bovine cystic ovarian disease

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PII: S0378-4320(13)00144-9
DOI: http://dx.doi.org/doi:10.1016/j.anireprosci.2013.04.012
Reference: ANIREP 4765

To appear in: *Animal Reproduction Science*

Received date: 15-8-2012
Revised date: 15-4-2013
Accepted date: 25-4-2013

Please cite this article as: Rodríguez, F.M., Salvetti, N.R., Colombero, M., Stangaferro, M.L., Barbeito, C.G., Ortega, H.H., Rey, F., Interaction between IGF1 and IGFBPs in bovine cystic ovarian disease, *Animal Reproduction Science* (2013), http://dx.doi.org/10.1016/j.anireprosci.2013.04.012

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1 **Interaction between IGF1 and IGFBPs in bovine cystic ovarian disease**

2

3 **Short title:** IGF1 and IGFBPs in bovine cystic ovaries

4

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21 **ABSTRACT**

22 Cystic ovarian disease (COD) is one of the main factors responsible for reproductive
23 disorders in cattle. Although the pathogenesis and mechanism of cyst formation are not
24 fully understood, it has been proposed that the IGF system could play an essential role, as
25 it is a key intraovarian regulator. The aim of the present study was to determine whether
26 the altered levels in IGF1 detected in bovines with COD are associated with changes at
27 mRNA level or with differential modulation by IGFBPs. The mRNA levels of the IGF
28 components studied were analyzed by real time PCR and in situ hybridization, and IGFBP
29 expression and activity were assayed by immunohistochemistry and ligand blot
30 respectively. Results showed a decreased *IGF1* mRNA level due to a lower granulosa cell
31 gene expression in cystic follicles ($P < 0.05$). Results also showed variations in IGFBP
32 expression in the intraovarian cellular compartment and concentration in follicular fluid,
33 and suggest that IGFBP3 is a key regulator of intrafollicular IGF1 in animals with COD.

34

35 **Keywords:** cattle, cystic ovarian disease, insulin-like growth factor, ovaries

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37 **INTRODUCTION**

38 Ovarian follicular cysts in cattle are characterized by large anovular structures that persist
39 in the ovary for approximately 10 days in the absence of a corpus luteum, with interruption of
40 the normal estrous cycle (Silvia et al., 2002; Vanholder, 2006). The incidence of this disease has
41 been estimated to occur in up to 30% of dairy cows, and the number of cases has increased in
42 recent years (Garverick, 1997; Silvia et al., 2005; Vanholder et al., 2006), probably due to the
43 selection of high-yielding animals.

44 The pathogenesis and mechanism of cyst formation are not fully understood. One of the
45 main difficulties in investigating ovarian cysts in bovines is that there are different factors
46 contributing to cyst formation (Vanholder et al., 2006). However, a hypothalamic
47 unresponsiveness to an estradiol surge appears to be one of the underlying causes of follicular
48 cyst formation in dairy cows (Gümen and Wiltbank, 2002). It is believed that an altered
49 feedback mechanism of estrogens in the hypothalamic-pituitary axis can result in an aberrant
50 GnRH and LH release and cyst formation, where growth factors play an active role.

51 The insulin-like growth factor (IGF) system is a key intraovarian regulator and the
52 successful development of a healthy oocyte and appropriate granulosa and theca cell
53 steroidogenesis in the estrous cycle depend on multiple factors, including the proper function of
54 this system (Jones and Clemmons, 1995; Spicer, 2004). Disruption of even one component of
55 this system can lead to abnormal follicular development and function, and compromise the
56 reproductive capacity by contributing to the development of follicular cysts (Zulu et al., 2002;
57 Ortega et al., 2008, Rey et al., 2010; Probo et al., 2011).

58 The bioavailability of both IGF1 and IGF2 in the follicle is intimately influenced by the
59 relative proportions of IGF-binding proteins (IGFBPs) (Firth and Baxter, 2002, Rey et al., 2010;

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60 Rodríguez et al., 2011) and by the different affinities between the ligands and individual IGFBPs
61 (Jones and Clemmons, 1995; Rey et al., 2010). Six IGFBPs (IGFBP1 to 6) have been described to
62 bind IGF1 and IGF2, prolong their half-lives, and block their action in most situations (Rechler,
63 1993; Firth and Baxter, 2002). On the other hand, evidence exists for proteolytic enzymes that
64 degrade and inactivate IGFBPs during follicular development in mammals. These protease-
65 induced decreases in IGFBPs likely cause increased levels of free IGFs, which stimulate
66 steroidogenesis and mitogenesis (Spicer, 2004). Therefore, IGFs are regulated at various levels,
67 with a constantly shifting contribution of endocrine, autocrine and paracrine factors.

68 Given the role of the IGF system in the ovarian function, we hypothesized that an
69 imbalance in this system may result in ovarian alterations such as cystic ovarian disease (COD).
70 Therefore, the aim of the present study was to determine whether the altered levels of IGF1
71 previously detected in cattle with COD (Ortega et al., 2008) are associated with changes at
72 mRNA level or with a differential modulation by IGFBPs.

73

74 **MATERIALS AND METHODS**

75 ***Induced cystic follicles and controls***

76 All procedures were carried out according to the Guide for the Care and Use of
77 Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science
78 Societies, 1999) and the protocol was approved by the Ethics and Safety Committee of the
79 Faculty of Veterinary Sciences (Universidad Nacional del Litoral, Argentina). Ten nulliparous
80 Argentinean Holstein heifers (18 to 24 months old; 400 to 450 kg body weight; maintained
81 under standard husbandry conditions) with regular estrous cycles according to prior detection
82 of estrus, rectal palpation and ultrasonography, were used. Their estrous cycles were

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83 synchronized using the Ovsynch protocol as described previously (Ortega et al, 2008; Rodríguez
84 et al., 2011). The day on which estrous behavior was first detected was confirmed by rectal
85 examination and ultrasonography, and designated Day 0 of the cycle (Gümen et al., 2003).
86 Beginning on Day 15 of a synchronized estrous cycle, five heifers received subcutaneous
87 injections of tetracosactrin hexaacetate (1 mg/ml/animal; Synacthen Depot, Novartis, Basel,
88 Switzerland), a synthetic polypeptide with ACTH activity, every 12 h for 7 days (Ortega et al.,
89 2008). Five control animals received saline (1 ml) (Dobson et al., 2000; Ortega et al., 2008).
90 Ultrasonographic ovarian examinations were performed in all animals, as previously described,
91 using a real-time, B-mode scanner equipped with a 7.5 MHz, linear-array, transrectal transducer
92 (Aloka, SSD 500; Wallingford, CT, USA) (Sirois and Fortune, 1988; Rodríguez et al., 2011). Daily
93 ovarian ultrasonography was performed throughout one complete estrous cycle (21 days in the
94 control group) and from Day 14 (Day 0 = day of estrus) until Day 48 (treated group). Cysts
95 detected by ultrasonography were defined as any follicular structure equal to or greater than 20
96 mm in diameter that was present for 10 days without forming a corpus luteum (CL) (Dobson et
97 al., 2000; Silvia et al., 2002). The first day of cyst formation was identified retrospectively, 10
98 days before the daily detection of a follicle equal to or greater than 20 mm in diameter. The
99 ovaries were removed 10 days later, for the identification of follicular cysts, by flank
100 laparatomy. Control heifers were ovariectomized, to obtain normal growing follicles
101 (approximately Day 18), when the dominant follicle reached a diameter greater than 10 mm.
102 During dissection of the ovaries, the follicular diameter was measured using callipers and
103 follicular fluid from each follicle was aspirated and stored separately at -20°C. Tertiary (antral)
104 follicles dissected from normally cycling ovaries were classified as small (< 5 mm), medium-sized
105 (5–10 mm) or large (> 10 mm) (Parrott and Skinner, 1998). Blood samples were obtained

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106 immediately before ovarian excision and centrifuged at 1000 x *g* for 30 min. The serum
107 collected was stored at -20 °C until hormonal analysis (parallel studies Ortega et al., 2008; Rey et
108 al., 2010). Luteinization of cystic follicles was discarded by means of hormonal analysis.
109 Follicular fluid was aspirated from both the preovulatory follicles of control animals and induced
110 cystic follicles and then frozen until analysis. The ovaries were fixed in 4 % neutral buffered
111 formalin.

112

113 ***Spontaneous cystic ovaries***

114 Ovaries with spontaneous cystic follicles were obtained from dairy cows by flank
115 laparotomy. Eight pluriparous (mean 3.1 ± 1.5 lactations, range 2-7), high-yielding (mean 25.12
116 ± 6.23 kg of milk per day at diagnosis) Argentinean Holstein cows affected by COD were
117 previously monitored by ultrasonography, and when a follicle ≥20 mm in diameter persisting for
118 10 days in the absence of a functional CL was detected, animals were ovariectomized. Cystic
119 fluids from animals with COD were aspirated using a probe of the real-time, B-mode scanner
120 equipped with a 7.5 MHz, linear-array, transrectal transducer (Aloka, SSD 500; Wallingford, CT,
121 USA) before ovarian excision. After excision, samples were collected, refrigerated on ice and
122 immediately transported to the laboratory. Follicular cysts were evaluated grossly,
123 microscopically and by hormone analysis. Tissue fractions of ovaries from cystic follicles were
124 immediately frozen at -80 °C until use in gene expression assays. Additional sections of ovarian
125 tissue were fixed in 4 % neutral buffered formalin.

126

127 ***Tissue preparation***

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128 For light microscopy, the fixed tissues were dehydrated and embedded in paraffin wax.
129 Sections (4 µm in thickness) were mounted on slides previously treated with 3-
130 aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and assigned for staining with
131 hematoxylin and eosin for preliminary observations of all ovarian structures (Salvetti et al.,
132 2004) or for use in immunohistochemistry (IHC) or in situ hybridization (ISH). Follicles were
133 classified into primary, secondary, tertiary and cystic follicles (Silvia et al., 2002).

134

135 ***RNA extraction***

136 Total RNA was isolated from the follicular wall of different sized tertiary control follicles
137 and cysts, after treatment with Trizol LS reagent (Invitrogen, Life technology, CA, USA),
138 according to the manufacturer's instructions but with slight modifications (Rey et al., 2010;
139 Rodríguez et al., 2011).

140

141 ***Reverse transcription***

142 To avoid putative genomic DNA contamination, RNA samples were treated with DNAse
143 (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized
144 using a master mix (Moloney Murine Leukemia Virus (MMLV) buffer, dithiothreitol (DTT),
145 RNAout, MMLV reverse transcriptase, deoxyribonucleotide triphosphate (dNTP) and random
146 primers (Invitrogen)). The reverse transcription conditions were as described previously
147 (Rodríguez et al., 2011).

148

149 ***Real Time PCR***

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150 An optimized real time PCR protocol was used to analyze the mRNA expression of *IGF1*,
151 *IGFBP1*, *IGFBP4*, *IGFBP5* and *IGFBP6* using SYBR Green I (Invitrogen) technology in LightCycler
152 (Pfaffl et al., 2001). The *IGFBP2* and *IGFBP3* mRNA had been analyzed in a previous work
153 (Rodríguez et al., 2011).

154 Transcript levels were measured by relative quantitative real time PCR using a StepOne
155 Real Time PCR System (Applied Biosystems, Life technology, CA, USA). An optimized protocol
156 was used: initial denaturation at 95 °C for 10 min, 36 cycles of denaturation at 95 °C for 15 s and
157 annealing 62 °C (*IGF1*), 58 °C (*IGFBP1*), 60 °C (*IGFBP4*, *IGFBP6*), 63 °C (*IGFBP5*), and 52 °C
158 glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) for 20 s, extension at 72 °C for 30 s and
159 fluorescence reading at 74 °C. All measurements for each sample were performed in duplicate.
160 The efficiency of PCRs and relative quantities were determined from a six-point standard curve.
161 Standard curves were constructed from a dilution series of pooled cDNAs (including six dilutions
162 from 1/5 to 1/160), and PCR efficiency was calculated using the StepOne software v2.2. In
163 standard curves, R^2: 0.99, except in the case of *GAPDH*, where R^2: 0.98. About 15 ng cDNA was
164 used for all primers, except for *IGFBP-1*, where 90 ng was used for each real time PCR reaction,
165 and was previously quantified by the Qubit method (Invitrogen). For amplification, 4 µl of cDNA
166 was combined with a PerfeCta SYBR Green SuperMix, ROX (Quanta Biosciences, Inc., MD, USA)
167 in a final volume of 20 µl. The primer sequences used are described in Table 1. The *GAPDH* gene
168 sequence was included as the housekeeping gene.

169 The mRNA expression levels of genes were recorded as cycle threshold (Ct) values that
170 corresponded to the number of cycles at which the fluorescence signal can be detected above a
171 threshold value. The Ct was calculated manually using StepOne v2.2 (Applied Biosystems).
172 Negative DNA template controls were included in all the assays, and yielded no consistent

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173 amplification. Product purity was confirmed by dissociation curves, and random samples were
174 subjected to agarose gel electrophoresis. Fold change was determined using the 2-ΔΔCt method
175 (Livak et al., 2001).

176

177 ***Nucleotide sequencing***

178 The specificities of the PCR products were checked by direct sequencing to ensure
179 amplification of the correct sequences using the Macrogen Sequencing Service (Macrogen,
180 Korea). The resulting sequences were verified using the MegAlign Tool in the LASERGENE
181 software (DNAStar, WI, USA).

182

183 ***In situ hybridization***

184 All reagents and materials were RNase-free. Sections were prepared as described above,
185 but using RNase-free conditions. Single-stranded oligonucleotide probes of 45 bases, end-
186 labeled with biotin (Invitrogen) were used and, together with those detected previously
187 (Rodríguez et al., 2011), we assayed the main IGFBPs described in bovines. Probe sequences are
188 described in Table 2.

189 Probes were diluted to a final concentration of 200 ng/ml in a hybridization solution
190 (Sigma). The sections were dewaxed, hydrated and endogenous peroxidase activity was
191 inhibited with 1 % H_2O_2 in methanol. After washing with Tris-buffered saline (TBS) and sterile
192 water, sections were subjected to microwave pre-treatment in citrate buffer (pH 6.0). After
193 washing with TBS, the corresponding probe was added to each slide and the sections were
194 incubated in a humid chamber overnight at 45 °C (Llewellyn et al., 2007). Following incubation,
195 the slides were washed with TBS and a pre-warmed astringent solution (one wash of saline-

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196 sodium citrate (SSC) 2X-50 % formamide-0.05 % Tween for 5 min, followed by two washes of
197 SSC 2X for 10 min, one wash of SSC 1X for 10 min and one wash of SSC 0.1X for 15 min, all at
198 45 °C; finally one wash with SSC 0.1X for 5 min at room temperature). The slides were then
199 rinsed with distilled water and TBS and incubated with extravidin-peroxidase (1:200, Sigma) for
200 30 min at 25°C. A positive reaction was visualized using 3,3'-diaminobenzidine (DAB, Liquid DAB-
201 Plus Substrate Kit, Zymed, CA, USA) as the chromogen. Finally, the slides were washed in
202 distilled water and then counterstained with Mayer's hematoxylin, dehydrated and mounted.
203 To verify specificity, adjacent negative control sections were subjected to the same procedure,
204 replacing the probe with hybridization solution or incubating with a sense probe. The same
205 hybridization temperature, washing and detection conditions were used for the probes used.
206
207 ***Immunohistochemistry***
208 Protein expression of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 was detected using polyclonal
209 antibodies (conditions in Table 3; Novozymes GroPep Ltd, Australia). The extravidin biotin
210 immunoperoxidase method was carried out as described previously (Ortega et al., 2009; Salvetti
211 et al., 2009). For antigen visualization, DAB was used as the chromogen. Negative control
212 sections in which the primary antibody was replaced by a non-immune rabbit serum were
213 included (Ortega et al., 2009). Some sections were incubated with DAB alone to exclude the
214 possibility that endogenous peroxidase activity had been unsuccessfully blocked.
215
216 ***Western blotting***
217 To test the specificity of the primary antibodies used, bovine tertiary follicles were
218 homogenized in a radio-immunoprecipitation assay lysis buffer with a protease inhibitor cocktail

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219 (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany), as described
220 previously (Rodríguez et al., 2011). To this end, 40 µg of protein, along with pre-stained
221 molecular weight markers (GE Healthcare Buckinghamshire, UK) were separated in SDS-PAGE
222 containing 15 % (w/v) acrylamide-polyacrylamide, according to a procedure previously
223 described (Ortega et al., 2009). After blotting on nitrocellulose membranes (Hybond ECL
224 Nitrocellulose Membrane, GE Healthcare), the membranes were blocked with TBS containing
225 0.05 % v/v Tween20 (TBST, Sigma-Aldrich Corp.) and 5 % w/v non-fat milk, and then incubated
226 overnight at 4ºC with specific primary antibodies. Bound antibody was detected using anti-
227 rabbit IgG peroxidase antibody (Amersham, Buckinghamshire, UK; 1:500) and ECL plus western
228 blotting detection reagents (GE Healthcare).
229

230 ***Western ligand blot***

231 Samples of follicular fluid were separated electrophoretically under non-reducing
232 conditions and transferred onto a nitrocellulose membrane as described previously (Rey et al.,
233 2010). Membranes were blocked with 5 % w/v bovine serum albumin (Sigma-Aldrich) in TBS and
234 then incubated overnight at 4°C with 750 ng/ml of biotinylated IGF1 (Novozymes GroPep).
235 Membranes were washed with TBST, and incubated with a 1:5000 dilution of extravidin-
236 peroxidase (Sigma-Aldrich) in blocking solution. Before washes with TBST, signal was detected
237 by chemiluminescence using the ECL-Plus system (GE Healthcare) on hyperfilm-ECL film (GE
238 Healthcare). Individual binding proteins were identified on the basis of their molecular weights,
239 as reported previously (Jones and Clemmons, 1995; Nicholas et al., 2002). The molecular weight
240 reported for IGFBP3 corresponds to a double band of 40 and 44 kDa. IGFBP2 was detected at 34

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241 kDa, IGFBP4 has been reported to have a glycosylated and a non-glycosylated form, with
242 molecular weights of 29 and 24 kDa, respectively, and IGFBP5 was observed at 31 kDa.

243

244 ***Image analysis***

245 Images were analyzed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver
246 Spring, MA, USA). Images were digitized using a color video camera (Motic 2,000, Motic China
247 Group, China) mounted on top of a conventional light microscope (Olympus BH-2, Olympus Co.,
248 Japan) using an objective magnification of × 40, as described and validated previously (Ortega et
249 al., 2009, 2010; Rodríguez et al., 2011). To obtain quantitative data for IHC and ISH labeling of
250 IGFBPs in the follicular wall, at least three sections were evaluated for each specimen and
251 antibody or oligonucleotide. The average density (% of positive area) of the IGFBPs antibody or
252 oligonucleotide reaction was calculated from at least 20 images of each area (granulosa and
253 theca cells) in each section as a percentage of the total area evaluated through color
254 segmentation analysis, which extracts objects by locating all objects of a specific color (brown
255 stain) (Ortega et al., 2009).

256 For the western ligand blot, the exposed films were scanned at 1200 dpi (scanner HP
257 Officejet J5, 780). The level of each IGFBP was analyzed by densitometry to obtain an integrated
258 optical density (IOD) value, which was compared between follicular fluid samples from healthy
259 and cystic ovaries (Rey et al., 2010).

260

261 ***Statistical analysis***

262 A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois, USA)
263 was used to analyze the data. Tests of homogeneity of variance among groups were conducted

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264 using Levene's test. The statistical significance of differences between groups of data was
265 assessed by one-way ANOVA, followed by Duncan's multiple range tests as a multiple
266 comparison test. A value of $P < 0.05$ was considered significant. The results are expressed as
267 mean ± SD.
268

269 **RESULTS**

270 ***IGF1 and IGFBPs gene expression***

271 The identity of the PCR products was confirmed by sequencing (100% homology with
272 bovine sequences). No differences were detected in GAPDH gene expression between the
273 different groups evaluated ($P > 0.05$).

274 Levels of mRNA of *IGFBP1*, *IGFBP4*, *IGFBP5* and *IGFBP6* in the complete follicular wall of
275 control tertiary follicles of different sizes and cystic follicles were similar ($P > 0.05$) (Figure 1).
276 Moreover, no differences were detected in the levels of *IGF1* of tertiary follicles and cystic
277 structures ($P > 0.05$) (Figure 2A). However, when mRNA expression was analyzed in different
278 follicular cells by ISH, *IGFBP5* was detected mainly in granulosa cells, where the expression
279 determined in cystic structures was lower than that in growing follicles (Figure 3B and 4).
280 Growing follicles from both control and cystic ovaries showed a decreasing expression from
281 primary to tertiary follicles. *IGFBP5* mRNA was detected in theca cells of cystic follicles, but a
282 very low presence or complete absence was detected in theca cells of tertiary follicles.

283 *IGFBP4* was detected in granulosa and theca cells, and no differences were observed in
284 the structures analyzed ($P > 0.05$) (Figure 3). Similarly to that observed in mRNA expression of
285 *IGFBP5*, in granulosa cells, *IGFBP4* mRNA levels decreased as follicles grew, and the lowest levels
286 were detected in cystic structures ($P < 0.05$).

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287 *IGF1* mRNA was detected only in granulosa cells, with similar levels between structures of
288 the same group (control, spontaneous COD and experimentally induced COD). However,
289 reduced levels were detected in growing follicles and cysts from ovaries with induced COD
290 compared with control and spontaneous COD ($P < 0.05$).
291 Structures analyzed in follicles from ovaries with spontaneous COD consistently showed
292 higher positive labeling than follicles from ovaries with experimentally induced COD, and the
293 expression pattern was similar in each structure analyzed (Figure 2B, 3).
294

295 ***Protein expression of IGFPs***

296 Expressions of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 were evaluated in follicles of different
297 developmental stages. Insulin-like growth factor binding protein 4, IGFBP5 and IGFBP6 were
298 detected both in granulosa and theca cells, with greater levels in granulosa cells (Figure 6),
299 whereas IGFBP1 was not observed in either cell type.
300 Both IGFBP4 and IGFBP5 showed the lowest expression levels in granulosa cells of cystic
301 structures, and the highest expression in primary follicles of control ovaries as well as in ovaries
302 with COD ($P < 0.05$) (Figure 7). Theca cells of experimentally induced cystic follicles showed
303 lower expression levels of IGFBP4 than control tertiary follicles ($P < 0.05$) and expression levels
304 similar to those of spontaneous cystic follicles ($P > 0.05$). Expression levels of IGFBP5 were
305 similar in the control and induced structures analyzed ($P > 0.05$), and different from those in
306 spontaneous structures ($P < 0.05$).
307 In contrast, IGFBP6 expression in granulosa cells increased expression from primary to
308 tertiary follicles, with larger levels in cystic follicles of experimentally induced COD ($P < 0.05$)
309 (Figure 7). Once more, the levels were higher in the structures from spontaneous COD and

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increased from primary to secondary follicles, and then remained constant through tertiary and cystic follicles. IGFBP6 was not detected in theca cells.

Follicular fluid content of IGFBPs

The content of IGFBPs in follicular fluid from different sized follicles was identified based on their molecular weight (Nicholas et al., 2002). The western ligand blot allowed for the identification of IGFBP2 (34 kDa), a doublet corresponding to IGFBP3 (40-44kDa), IGFBP4 in its glycosylated (29 kDa) and non-glycosylated forms (24 kDa), and IGFBP5 (31 kDa). Their identities were confirmed by western blot. The patterns for IGFBP2 and IGFBP3 have been shown in a previous work (Rodríguez et al., 2011), whereas those for IGFBP4 and IGFBP5 are shown in Figure 8.

IGFBP2 and IGFBP3 were expressed at higher levels in the follicular fluid of the structures analyzed than the other IGFBPs detected. However, while IGFBP2 levels remained practically constant in tertiary follicles and cysts ($P > 0.05$), IGFBP3 levels were higher in cysts than in small tertiary follicles ($P < 0.05$). In contrast, the glycosylated form of IGFBP4 and IGFBP5 showed higher levels in small tertiary follicles than in cysts and tertiary follicles greater in size ($P < 0.05$). No differences in the non-glycosylated form of IGFBP4 were detected in the follicles analyzed (Figure 8).

DISCUSSION

The study of the etiopathogenesis of COD in dairy cows has several limitations. The main difficulties are the fact that bovine COD is a multifactorial disease and there are few opportunities to follow clinical cases without treatment because farmers need their cows to

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333 receive treatment as soon as possible (Ribadu, 2000; Probo et al., 2011). For this last reason,
334 many studies have been conducted with slaughterhouse material, which lacks the reproductive
335 history and information of cyst persistence. In the present study, we were able to follow the
336 reproductive behavior by ultrasonography and rectal palpation of the dairy cows identified as
337 having COD, and thus to promptly detect persistent follicles and development of cysts.
338 Moreover, since follicular cysts were recovered from experimental animals at known times after
339 cyst emergence, analysis of their structure and function was more accurate (Ortega et al., 2008).

340 In the present study, we determined that *IGFBP4* and *IGFBP5* mRNA levels in granulosa
341 cells decreased from primary to tertiary follicles in the control group, similarly to that observed
342 in sheep (Hastie et al., 2004; Hastie and Haresign, 2006). Interestingly, *IGFBP4* mRNA was
343 detected mainly in granulosa cells, but expressed in lower levels in theca cells, contrarily to that
344 observed by other authors (Armstrong et al., 1998; Roberts and Etcherntkamp, 2003).
345 Moreover, the reduced levels of *IGFBP4* and *IGFBP5* detected in granulosa cells of cystic ovaries
346 were more evident in the experimental model than in spontaneous cysts, probably due to the
347 differences in the environmental conditions and the time of persistence of the cysts, which
348 could influence the gene expression of these IGFBPs. Consistently, IGFBP5 protein expression
349 showed a similar pattern. On the other hand, similar protein levels of IGFBP4 were observed in
350 the structures from ovaries from spontaneous and induced COD. Differences in mRNA levels
351 related to protein expression could be due to the hormonal milieu that participates in the
352 control of the IGFBP system and proteins associated such as IGFBP proteases (Spicer, 2004; Aad
353 et al., 2006; Sudo et al., 2007). Moreover, it is worth mentioning that levels detected by IHC
354 correspond to the glycosylated form of IGFBP4, as demonstrated by western blot.

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355 In support of the control exerted by steroid hormones on IGFBP production, Spicer and
356 Chamberlain (2000) observed that estradiol inhibited IGFBP4 production in bovine granulosa
357 cells in vitro, but did not determine whether this inhibition was due to a change in the
358 proteolysis of IGFBP4. IGF1, FSH, and/or estradiol induce IGFBP4 proteolysis in cultured human
359 granulosa cells (Iwashita et al., 1998). In addition, the reduced levels of IGFBP4 and IGFBP5
360 determined in spontaneous and experimentally induced cystic follicles as compared with those
361 in primary follicles from both control and COD ovaries are probably associated with the lower
362 mRNA levels detected, although control by IGFBP proteases cannot be ruled out. It has been
363 suggested that IGFBP4 production, via changes in intrafollicular mRNA expression, is correlated
364 with LH regulation and IGFBP4 degradation (Mazerbourg et al., 2001; Hastie and Haresing,
365 2010). Moreover, both FSH and LH inhibit the expression of *IGFBP5* mRNA in ovine follicles,
366 determining a decrease in the levels of *IGFBP5* mRNA (Besnard et al., 1996a; Hastie and
367 Haresign, 2006a) and protein (Monget et al., 1993; Spicer and Chamberlain, 2002) in follicles as
368 they increase in diameter (Hastie and Haresing, 2010).

369 Since there is limited information about the patterns of *IGFBP1* and *IGFBP6* mRNA and
370 protein expression in the ovary of ruminants, little is known about the elements that regulate
371 these binding proteins. In the present study, we found no changes in *IGFBP1* and *IGFBP6* mRNA
372 levels in the follicular wall of the tertiary and cystic structures analyzed, and detected no protein
373 expression in any of the follicles analyzed. Because lower levels of *IGFBP1* and *IGFBP6* mRNA
374 detected, not expression of those binding proteins mRNA were analyzed by situ hybridization. It
375 has been reported that IGFBP1 concentrations in serum and follicular fluid are decreased in
376 women with polycystic ovary syndrome (Thierry van Dessel et al. 1999). In bovines, IGFBP1 is
377 detected in small quantities in the follicular fluid, probably because it is either poorly expressed

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378 or not synthesized within the follicle compartment, entering the basal membrane as a result of
379 changes (Nicholas et al. 2002). Probably, these changes could explain why we detected mRNA
380 but not protein. This lack of detection of protein may also be due to the methodology used
381 (IHC). This may also be true because we failed to detect both IGFBP1 and IGFBP6 in follicular
382 fluid by ligand blot (Nicholas et al., 2002), but found IGFBP6 in granulosa cells.

383 Although IGFBPs were synthesized intrafollicularly (Armstrong et al., 1998; Voge et al.,
384 2004; Rodríguez et al., 2011), we analyzed the levels of IGFBPs in follicular fluid, where IGF
385 availability is effectively regulated. Changes in IGFBP concentrations in bovine follicular fluid
386 have been documented during follicle growth and development in cattle (de la Sota et al., 1996;
387 Stewart et al., 1996), sheep (Perks and Wathes, 1996), and pigs (Yuan et al., 1996). Typically,
388 dominance is associated with decreased follicular fluid concentrations of the binding proteins
389 with lower molecular weight, i.e. IGFBP4 and IGFBP5 (Austin et al., 2001), in agreement with our
390 present results, which showed lower protein concentrations in tertiary follicles increasing in
391 size. The changes in the IGFBP content of follicular fluid could be due to the regulation of IGFBP
392 production, changes in the activity of specific IGFBP proteases, or changes in the selective
393 uptake of IGFBPs from the circulation during follicle growth (Armstrong et al., 1998). In fact, we
394 detected lower gene expression of *IGFBP4* and *IGFBP5,* which was reflected in lower protein
395 synthesis, while follicles were growing from primary to tertiary and cystic follicles. However, no
396 changes were detected in mRNA or protein expression levels of control tertiary follicles when
397 compared with spontaneous cystic follicles. In follicular fluid, a decreasing concentration of
398 IGFBP5 was detected through tertiary follicles growing in size until cystic structures. Similar
399 results were obtained with the 29-kDa glycosylated form of IGFBP4, although no changes were

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400 detected with the 24-kDa non-glycosylated form. The lower levels detected could result from an
401 increasing activity or concentration of IGFBP protease.

402 Previously, we detected similar protein expression of IGFBP2 and IGFBP3 in tertiary
403 control and cystic follicles (Rodríguez et al., 2011). However, the concentration of IGFBP3 in
404 follicular fluid increased as the control tertiary follicles increased in size, with the highest
405 concentration found in cystic structures. Therefore, the differences detected in IGFBP3 content
406 in follicular fluid support the hypothesis of an extrafollicular origin (Echternkamp et al., 1994;
407 Santiago et al., 2005; Rey et al., 2010), probably responsible for the bioavailability of IGF1.

408 In previous studies, we detected similar serum IGF1 concentrations in controls and
409 animals with spontaneous or induced cystic ovarian disease (Ortega et al., 2008). Part of the
410 lack of a direct effect on ovarian activity depends on how much of the circulating IGF1
411 concentration is truly reflected in the ovaries. IGF1 concentration in follicular fluid has been
412 found to be lower (Echternkamp et al., 1990; Spicer et al., 1991), equal to (Spicer et al., 1992),
413 or greater (Spicer et al., 1992; Ortega et al., 2008) than peripheral IGF1 concentration, and
414 although they are correlated, in some circumstances, such as short-term fasting, liver-derived
415 IGF1 can be decreased significantly without affecting intrafollicular IGF1 concentration (Spicer et
416 al., 1992; Velazquez et al., 2008). Furthermore, it has been suggested that IGF1 is involved not
417 only in the pathogenesis but also in the maintenance of COD in cattle (Zulu et al., 2002; Probo et
418 al., 2011). In this pathological condition, while IGF1 concentrations are known to be lower
419 during ovarian cyst formation (Beam and Butler 1997, 1998; Zulu et al. 2002), intrafollicular IGF1
420 can be even lower than serum concentrations (Ortega et al., 2008). Considering the alterations
421 in the expression of IGFBP2 and IGFBP3 reported previously (Rodríguez et al., 2011) and the
422 results of the present study, we suggest that the decreased IGF1 levels could result from

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423 alterations in the diffusion rate between the blood and the follicular fluid. These alterations
424 could be due to differences in the interaction with IGFBPs related to gene and protein
425 expression and/or to induced changes attributable to a decreased *IGF1* mRNA in ovaries with
426 induced COD, causing an imbalance in the IGF system that could modify the circulating levels of
427 the free fraction of IGF (Rechler and Clemmons, 1998; Hastie and Haresing, 2006; Thomas et al.,
428 2007).
429

430 **CONCLUSION**

431 The results of the present study support that the IGF system has a clear influence in cows
432 with COD. We suggest that IGF1 is influenced by the action of IGFBPs, mainly by IGFBP3 and by
433 the decreased level of *IGF1* mRNA during a COD condition.
434

435 **Acknowledgements**

436 We are grateful to the staff of the Large Animal Health Hospital (FCV-UNL) for animal care and
437 help with the generation of experimental animals. Assistance of the staff members of the
438 Laboratory of Cellular Biology (FCV-UNL) and the provision of drugs by Novartis Laboratories
439 (Buenos Aires, Argentina) is gratefully acknowledged. This work was supported by grants (PICT
440 2007-01202) from the Argentine National Agency for the Promotion of Science and Technology
441 (ANPCyT). HHO, CGB, NRS and FR are Research Career Members, and FMR in Fellow of the
442 National Scientific Research Council (CONICET, Argentina).
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444

Accepted Manuscript

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444

445 **LITERATURE CITED**

446 Aad, P. Y., Voge, J. L., Santiago, C. A., Malayer J. R., Spicer, L. J., 2006. Real-time RT-PCR
447 quantification of pregnancy-associated plasma protein-A mRNA abundance in bovine
448 granulosa and theca cells: effects of hormones in vitro. Domest. Anim. Endocrinol. 31:357-
449 372.

450 Armstrong, D. G., Baxter, G., Gutierrez, C. G., Hogg, C. O., Glazyrin, A. L., Campbell, B. K.,
451 Bramley, T. A., Webb, R., 1998. Insulin-like growth factor binding protein -2 and -4
452 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins
453 and developmental status. Endocrinology 139:2146-2154.

454 Armstrong, D. G., Gutierrez, C. G., Baxter, G., Glazyrin, A. L., Mann, G. E., Woad, K. J., Hogg, C.
455 O., Webb, R., 2000. Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in
456 bovine ovarian follicles. J. Endocrinol. 165:101-113.

457 Austin, E. J., Mihm, M., Evans, A. C. O., Knight, P. G., Ireland, J. L. H., Ireland, J. J., Roche, J. F.,
458 2001. Alterations in intrafollicular regulatory factors and apoptosis during selection of
459 follicles in the first follicular wave of the bovine estrous cycle. Biol. Reprod. 64:839–848.

460 Beam, S., Butler, W., 1998. Energy balance, metabolic hormones, and early post partum
461 follicular development in dairy cows fed prilled lipid. J. Dairy. Sci. 81:121-123.

462 Besnard, N., Pisselet, C., Monniaux, D., Locatelli, A., Benne, F., Gasser, F., Hatey, F., Monget, P.,
463 1996. Expression of messenger ribonucleic acids of insulin-like growth factor binding
464 protein-2, -4, and -5 in the ovine ovary: localization and changes during growth and atresia
465 of antral follicles. Biol. Reprod. 55:1356–1367

Accepted Manuscript

ACCEPTED MANUSCRIPT

466 de la Sota, R. L., Simmen, F. A., Diaz, T., Thatcher, W. W., 1996. Insulin-like growth factor system
467 in bovine first-wave dominant and subordinate follicles. Biol. Reprod. 55:803-812.
468 Dobson, H., Ribadu, A. Y., Noble, K. M., Tebble, J. E., Ward, W. R., 2000. Ultrasonography and
469 hormone profiles of adrenocorticotrophic hormone (ACTH)-induced persistent ovarian
470 follicles (cysts) in cattle. J. Reprod. Fertil. 120:405-410.
471 Echternkamp, S. E., Spicer, L. J., Gregory, K. E., Canning, S. F., Hammond, J. M., 1990.
472 Concentrations of insulin-like growth factor-I in blood and ovarian follicular fluid of cattle
473 selected for twins. Biol. Reprod. 43:8–14.
474 Echternkamp, S. E., Spicer, L. J., Klindt, J., Vernon, R. K., Yen, J. T., Buonomo, F. C., 1994.
475 Administration of porcine somatotropin by a sustained-release implant: effect on follicular
476 growth, concentrations of steroids and insulin-like growth factor I, and insulin-like growth
477 factor binding protein activity in follicular fluid of control, lean, and obese gilts. J. Anim. Sci.
478 72:2431-2440.
479 Firth, S. M., Baxter, R. C., 2002. Cellular actions of the insulin-like growth factor binding proteins.
480 Endocr. Rev. 23:824-854.
481 Gümen, A., Wiltbank, M. C., 2002. An alteration in the hypothalamic action of estradiol due to
482 lack of progesterone exposure can cause follicular cysts in cattle. Biol. Reprod. 66:1689–
483 1695.
484 Gümen, A., Guenther, J. N., Wiltbank, M. C., 2003. Follicular size and response to Ovsynch
485 versus detection of estrus in anovular and ovular lactating dairy cows. J. Dairy Sci. 86:3184-
486 3194.
487 Hastie, P. M., Onagbesan, O. M., Haresign, W., 2004. Co-expression of messenger ribonucleic
488 acids encoding IGF-I, IGF-II, type I and II IGF receptors and IGF-binding proteins (IGFBP-1 to -

ACCEPTED MANUSCRIPT

489 6) during follicular development in the ovary of seasonally anoestrous ewes. Anim. Reprod.
490 Sci. 84:93-105.
491 Hastie, P. M., Haresign, W., 2006. Expression of mRNAs encoding insulin-like growth factor (IGF)
492 ligands, IGF receptors and IGF binding proteins during follicular growth and atresia in the
493 ovine ovary throughout the oestrous cycle. Anim. Reprod. Sci. 92:284-299.
494 Hastie, P. M., Haresign, W., 2010. Modulating peripheral gonadotrophin levels affects follicular
495 expression of mRNAs encoding insulin-like growth factor binding proteins in sheep. Anim.
496 Reprod. Sci. 119:198-204.
497 Iwashita, M., Sakai, K., Kudo, Y., Takeda, Y., 1998. Phosphoisoforms of insulin-like growth factor
498 binding protein-1 in appropriate-for-gestational-age and small-for-gestational-age fetuses.
499 Growth Horm. IGF Res. 8:487-493.
500 Jones, J. I., Clemmons, D. R., 1995. Insulin-like growth factors and their binding proteins:
501 biological actions. Endocr. Rev. 16:3-34.
502 Livak, K. J., Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time
503 quantitative PCR and the 2(-Delta Delta C (T)) Method. Methods 25:402-408.
504 Llewellyn, S., Fitzpatrick, R., Kenny, D. A., Murphy, J. J., Scaramuzzi, R. J., Wathes, D. C., 2007.
505 Effect of negative energy balance on the insulin-like growth factor system in pre-
506 recruitment ovarian follicles of post partum dairy cows. Reprod. 133:627-639.
507 Llewellyn, S., Fitzpatrick, R., Kenny, D. A., Patton, J., Wathes, D. C., 2008. Endometrial expression
508 of the insulin-like growth factor system during uterine involution in the postpartum dairy
509 cow. Domest. Anim. Endocrinol. 34:391–402.
510 Mazerbourg, S., Overgaard, M. T., Oxvig, C., Christiansen, M., Conover, C. A., Laurendeau, I.,
511 Vidaud, M., Tosser-Klopp, G., Zapf, J., Monget, P., 2001. Pregnancy-associated plasma

Accepted Manuscript

ACCEPTED MANUSCRIPT

512 protein-A (PAPP-A) in ovine, bovine, porcine, and equine ovarian follicles: involvement in
513 IGF binding protein-4 proteolytic degradation and mRNA expression during follicular
514 development. Endocrinology 142:5243-5253.
515 Monget, P., Monniaux, D., Pisselet, C., Durand, P., 1993. Changes in insulin-like growth factor-I
516 (IGF-I), IGF-II, and their binding proteins during growth and atresia of ovine ovarian follicles.
517 Endocrinology 132:1438–1446.
518 Moser, D. R., Lowe Jr, W. L., Dake, B. L., Booth, B. A., Boes, M., Clemmons, D. R., Bar, R. S., 1992.
519 Endothelial cells express insulin-like growth factor-binding proteins 2 to 6. Mol. Endocrinol.
520 6:1805-1814.
521 Nicholas, B., Scougall, R. K., Armstrong, D. G., Webb, R., 2002. Changes in insulin-like growth
522 factor binding protein (IGFBP) isoforms during bovine follicular development. Reproduction
523 124:439-446.
524 Ortega, H. H., Palomar, M. M., Acosta, J. C., Salvetti, N. R., Dallard, B. E., Lorente, J. A., Barbeito,
525 C. G., Gimeno, E. J., 2008. Insulin-like growth factor I in ovarian follicles and follicular fluid of
526 cows with spontaneous and induced cystic ovarian disease. Res. Vet. Sci. 84:419-427.
527 Ortega, H. H., Salvetti, N. R., Padmanabhan, V., 2009. Developmental programming: prenatal
528 androgen excess disrupts ovarian steroid receptor balance. Reproduction 137:865-877.
529 Ortega, H. H., Rey, F., Velazquez, M. M., Padmanabhan, V., 2010. Developmental programming:
530 effect of prenatal steroid excess on intraovarian components of insulin signaling pathway
531 and related proteins in sheep. Biol. Reprod. 82:1065-1075.
532 Parrott, J. A., Skinner, M. K., 1998. Developmental and hormonal regulation of hepatocyte
533 growth factor expression and action in the bovine ovarian follicle. Biol. Reprod. 59:553-560.

Accepted Manuscript

ACCEPTED MANUSCRIPT

534 Perks, C. M., Wathes, D. C., 1996. Expression of mRNAs for insulin-like growth factor binding
535 proteins-2, -3 and -4 in the ovine ovary throughout the oestrous cycle. J. Endocrinol.
536 151:241-249.
537 Pfaffl, M. W., 2001. A new mathematical model for relative quantification in real-time RT-PCR.
538 Nucleic Acids Res. 29:45.
539 Pfaffl, M. W., Georgieva, T. M., Georgiev, I. P., Ontsouka, E., Hageleit, M., Blum, J. W., 2002.
540 Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2,
541 IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1, 2 and 3
542 in the bovine species. Domest. Anim. Endocrinol. 22:91-102.
543 Plath-Gabler, A., Gabler, C., Sinowatz, F., Berisha, B., Schams, D., 2001. The expression of the IGF
544 family and GH receptor in the bovine mammary gland. J. Endocrinol. 168:39-48.
545 Probo, M., Comin, A., Cairoli, F., Faustini, M., Kindahl, H., De Amicis, I., Veronesi, M. C., 2011.
546 Selected metabolic and hormonal profiles during maintenance of spontaneous ovarian cysts
547 in dairy cows. Reprod. Domest. Anim. 46:448-454.
548 Rechler, M. M., 1993. Insulin-like growth factor binding proteins. Vitam. Horm. 47:1-114.
549 Rechler, M. M., D. R. Clemmons. 1998. Regulatory Actions of Insulin-like Growth Factor-
550 binding Proteins. Trends. Endocrinol. Metab. 9:176-183.
551 Rey, F., Rodríguez, F. M., Salvetti, N. R., Palomar, M. M., Barbeito, C. G., Alfaro, N. S., Ortega, H.
552 H., 2010. Insulin-like Growth Factor-II and Insulin-like Growth Factor Binding Proteins in
553 Bovine Cystic Ovarian Disease. J. Comp. Pathol. 142:193-204.
554 Ribadu, A. Y., Nakada, K., Moriyoshi, M., Zhang, W. C., Tanaka, Y., Nakao, T. 2000. The role of LH
555 pulse frequency in ACTH-induced ovarian follicular cysts in heifers. Anim. Reprod. Sci.
556 64:21-31.

Accepted Manuscript

ACCEPTED MANUSCRIPT

557 Roberts, A. J., Echternkamp, S. E., 2003. Insulin-like growth factor binding proteins in granulosa
558 and thecal cells from bovine ovarian follicles at different stages of development. J. Anim.
559 Sci. 81:2826-2839.
560 Rodríguez, F. M., Salvetti, N. R., Panzani, C. G., Barbeito, C. G., Ortega, H. H., Rey, F., 2011.
561 Influence of insulin-like growth factor-binding proteins-2 and -3 in the pathogenesis of
562 cystic ovarian disease in cattle. Anim. Reprod. Sci. 128:1-10.
563 Salvetti, N. R., Gimeno, E. J., Lorente, J. A., Ortega, H. H., 2004. Expression of cytoskeletal
564 proteins in the follicular wall of induced ovarian cysts. Cells Tissues Organs 178:117-125.
565 Salvetti, N. R., Baravalle, C., Mira, G. A., Gimeno, E. J., Dallard, B. E., Rey, F., Ortega, H. H., 2009.
566 Heat shock protein 70 and sex steroid receptors in the follicular structures of induced
567 ovarian cysts. Reprod. Domest. Anim. 44:805-814.
568 Santiago, C. A., Voge, J. L., Aad, P. Y., Allen, D. T., Stein, D. R., Malayer, J. R., Spicer, L. J., 2005.
569 Pregnancy-associated plasma protein-A and insulin-like growth factor binding protein
570 mRNAs in granulosa cells of dominant and subordinate follicles of preovulatory cattle.
571 Domest. Anim. Endocrinol. 28:46-63.
572 Shibaya, M., Matsuda, A., Hojo, T., Acosta, T. J., Okuda, K., 2007. Expressions of estrogen
573 receptors in the bovine corpus luteum: cyclic changes and effects of prostaglandin F2alpha
574 and cytokines. J. Reprod. Dev. 53:1059-1068.
575 Silvia, W. J., Alter, T. B., Nugent, A. M., Laranja da Fonseca, L. F., 2002. Ovarian follicular cysts in
576 dairy cows: an abnormality in folliculogenesis. Domest. Anim. Endocrinol. 23:167-177.
577 Silvia, W. J., McGinnis, A. S., Hatler, T. B., 2005. A comparison of adrenal gland function in
578 lactating dairy cows with or without ovarian follicular cysts. Reprod. Biol. 5:19-29.

ACCEPTED MANUSCRIPT

579 Sirois, J., Fortune, J. E., 1988. Ovarian follicular dynamics during the estrous cycle in heifers
580 monitored by real-time ultrasonography. Biol. Reprod. 39:308-317.
581 Spicer, L. J., Enright, W. J., Murphy, M. G., Roche, J. F., 1991. Effect of dietary intake on
582 concentrations of insulin-like growth factor-I in plasma and follicular fluid, and ovarian
583 function in heifers. Domest. Anim. Endocrinol. 8:431-437.
584 Spicer, L. J., Crowe, M. A., Prendiville, D. J., Goulding, D., Enright, W. J., 1992. Systemic but not
585 intraovarian concentrations of insulin-like growth factor-I are affected by short-term
586 fasting. Biol. Reprod. 46:920-925.
587 Spicer, L. J., Chamberlain, C. S., 2000. Production of insulin-like growth factor-I by granulosa cells
588 but not thecal cells is hormonally responsive in cattle. J. Anim. Sci. 78:2919-2926.
589 Spicer, L. J., Chamberlain, C. S., Maciel, S. M., 2002. Influence of gonadotropins on insulin- and
590 insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells.
591 Domest. Anim .Endocrinol. 22:237-254.
592 Spicer L. J., 2004. Proteolytic degradation of insulin-like growth factor binding proteins by
593 ovarian follicles: a control mechanism for selection of dominant follicles. Biol. Reprod.
594 70:1223-1230.
595 Stewart, R. E., Spicer, L. J., Hamilton, T. D., Keefer, B. E., Dawson, L. J., Morgan, G. L.,
596 Echternkamp, S. E., 1996. Levels of insulin-like growth factor (IGF) binding proteins,
597 luteinizing hormone and IGF-I receptors, and steroids in dominant follicles during the first
598 follicular wave in cattle exhibiting regular estrous cycles. Endocrinology 137:2842–2850.
599 Sudo, N., Shimizu, T., Kawashima, C., Kaneko, E., Tetsuka, M., Miyamoto, A., 2007. Insulin-like
600 growth factor-I (IGF-I) system during follicle development in the bovine ovary: relationship

Accepted Manuscript

ACCEPTED MANUSCRIPT

601 among IGF-I, type 1 IGF receptor (IGFR-1) and pregnancy-associated plasma protein-A
602 (PAPP-A). Mol. Cell. Endocrinol. 264:197-203.
603 Thierry van Dessel, H. J., Lee, P. D., Faessen, G., Fauser, B. C., Giudice, L. C., 1999. Elevated
604 serum levels of free insulin-like growth factor I in polycystic ovary syndrome. J. Clin.
605 Endocrinol. Metab. 84:3030-3035.
606 Thomas, F. H., Campbell, B. K., Armstrong, D. G., Telfer, E. E., 2007. Effects of IGF-I bioavailability
607 on bovine preantral follicular development in vitro. Reproduction. 133:1121-8.
608 Vanholder, T., Opsomer, G., de Kruif, A., 2006. Aetiology and pathogenesis of cystic ovarian
609 follicles in dairy cattle: a review. Reprod. Nutr. Dev. 46:105-119.
610 Velazquez, M. A., Spicer, L. J., Wathes, D. C., 2008. The role of endocrine insulin-like growth
611 factor-I (IGF-I) in female bovine reproduction. Domest.Anim. Endocrinol. 35:325-42.
612 Voge, J. L., Aad, P. Y., Santiago, C. A., Goad, D. W., Malayer, J. R., Allen, D., Spicer, L. J., 2004.
613 Effect of insulin-like growth factors (IGF), FSH, and leptin on IGF-binding-protein mRNA
614 expression in bovine granulosa and theca cells: quantitative detection by real-time PCR.
615 Peptides 25:2195-2203.
616 Yuan, W., Lucy, M. C., Smith, M. F., 1996. Messenger ribonucleic acid for insulin-like growth
617 factors-I and -II, insulin-like growth factor-binding protein-2, gonadotropin receptors, and
618 steroidogenic enzymes in porcine follicles. Biol. Reprod. 55:1045-1054.
619 Zulu, V.C., Sawamukai, Y., Nakada, K., Kida, K., Moriyoshi, M., 2002. Relationship among insulin-
620 like growth factor-I, blood metabolites and postpartum ovarian function in dairy cows. J.
621 Vet. Med. Sci. 64:879-885.

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FIGURE LEGENDS

Figure 1: Expression of *IGFBP1, IGFBP4, IGFBP5* and *IGFBP6* mRNA in small (S), medium (M) and large (L) tertiary follicles and cysts (C). No differences were detected in the quantification of the PCR products of *IGFBP1* (**A**), *IGFBP4* (**B**), *IGFBP5* (**C**) and *IGFBP6* (**D**). Values represent the mean ± SD.

Figure 2: Expression of *IGF1* mRNA levels in follicular structures. (**A**) Quantification of *IGF1* mRNA in ovarian follicular wall of small (S), medium (M) and large (L) tertiary follicles and cysts (C) by real time PCR. No differences were detected in the PCR products of *IGF1* mRNA. (**B**) Analysis of cell population by in situ hybridization showed decreased expression of *IGF1* in experimentally induced cysts (black bars) related to control follicles (open bars) without differences in spontaneous cystic ovaries (hatched bars). Values represent the mean ± SD. Bars with different superscript letters denote significant differences ($P < 0.05$).

Figure 3: Expression of *IGFBP4* and *IGFBP5* mRNA levels by in situ hybridization. Quantification of *IGFBP4* mRNA in granulosa and theca interna cells (**A**) and quantification of *IGFBP5* mRNA in granulosa cells (**B**) in developing follicles of control ovaries (open bars), experimentally induced cystic ovaries (black bars) and spontaneous cystic ovaries (hatched bars). Values represent the mean ± SD. Bars with different superscript letters denote significant differences ($P < 0.05$). C: control, I: induced cystic ovaries, S: spontaneous cystic ovaries.

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Figure 4: Representative images of *IGFBP4* (**A-C**) and *IGFBP5* (**D-F**) mRNA localization by in situ hybridization in bovine follicles. The immunoreactivity was intense in granulosa cells of tertiary control follicles (A, D) and weaker in cystic follicles (B, E). No staining was observed when the sense probe was pre-incubated with the antisense probe as negative control (C, F). In the right column, the immunopositive areas of A and D were segmented by digital image analysis, showing in black the differential cytoplasmic immunostaining pattern. Bars = 25 µm

Figure 5: Representative images of *IGF1* mRNA localization by in situ hybridization in bovine follicles. The immunoreactivity was intense in granulosa cells of secondary (**A**) and tertiary (**B**) control follicles and weaker in cystic follicles (**C**). No staining was observed when the sense probe was pre-incubated with the antisense probe as negative control (**D**). Bars = 25 µm.

Figure 6: Representative images of IGFBP4 (**A-C**), IGFBP5 (**D-F**) and IGFBP6 (**G-I**) protein localization by immunohistochemistry in bovine follicles. The immunoreactivity was intense in granulosa cells and reduced in theca cells in the follicular structures analyzed. Images represent tertiary control follicles (A, D, G) and cystic follicles (B, E, H). No staining was observed when the primary antibody was replaced with normal rabbit serum (negative control C, F, I). Verification of antibody specificity by western blot analysis of an ovarian homogenate demonstrating the specificity of the antibody is shown on the left. In the right column, the immunopositive areas of A, D and G were segmented by digital

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image analysis, showing in black the differential cytoplasmic immunostaining pattern. Bars = 25 µm

Figure 7: Immunohistochemical staining of IGFBP4 (**A**), IGFBP5 (**B**) and IGFBP6 (**C**), showing the percentage of immunopositive areas in granulosa and theca interna cells evaluated in primary, secondary, and tertiary follicles of control ovaries (open bars), experimentally induced cystic ovaries (black bars) and spontaneous cystic ovaries (hatched bars). Values represent the mean ± SD. Bars with different superscript letters denote significant differences ($P < 0.05$) in separate granulosa and theca cell compartments. The theca interna was not labeled for IGFBP6.

Figure 8: Detection of IGFBPs in follicular fluid by western ligand blot of healthy small (S), medium (M) and large (L) tertiary follicles compared with cystic follicles (C). **A**: Quantification of IGFBP subtypes as determined by molecular weight: IGFBP4 non-glycosylated form of 24 kDa (open bars), IGFBP4 glycosylated form of 29 kDa (squared bars), IGFBP5 (horizontally hatched bars) of 31 kDa, IGFBP2 (black bars) of 34 kDa and IGFBP3 (diagonally hatched bars). Values represent the mean ± SD. Bars with different superscript letters denote significant differences ($P < 0.05$) between different follicle types. **B**: Representative western ligand blot showing the IGFBPs that bind to IGF1.

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Table 1: Forward and reverse primer sequences (5'→3')

Primer	Sequence (5'→3')	Length, bp	Reference
IGF1	For[1] TCG CAT CTC TTC TAT CTG GCC CTG T Rev[2] GCA GTA CAT CTC CAG CCT CCT CAG A	240	Pfaffl et al. (2002)
IGFBP1	For TCA AGA AGT GGA AGG AGC CCT Rev AAT CCA TTC TTG TTG CAG TTT	123	Pfaffl et al. (2002)
IGFBP4	For GCC CTG TGG GGT GTA CAC Rev TGC AGC TCA CTC TGG CAG	342	Plath- Gabler et al. (2001)
IGFBP5	For TGC GAG CTG GTC AAG GAG Rev TCC TCT GCC ATC TCG GAG	257	Plath- Gabler et al. (2001)
IGFBP6	For AGA AAG AGG ATT TGC CTT TGC Rev TCC GGT AGA AGC CCC TAT G	324	Plath- Gabler et al. (2001)
GAPDH	For CAC CCT CAA GAT TGT CAG CA Rev GGT CAT AAG TCC CTC CAC GA	103	Shibaya et al. (2007)

[1] Forward (For)

[2] Reverse (Rev)

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Table 2: Probe sequences (5'→3')

Probe	Sequence (5'→3')	Reference
IGF1	TCACATCCTCCTCGCATCTCTTCTATCTGGCCCTGTGCTTGCTCG	(Llewellyn et al., 2008)
IGFBP4	AAGACGGGAGTGAAGCTTCCGGGGGGCCTGGAGCCGAAGGGGGAG	901–945 of bovine IGFBP4 mRNA (Moser et al. 1992)
IGFBP5	CTACTCGCCCAAGATCTTCCGGCCCAAGCACACCCGCATCTCCGA	291–335 of bovine IGFBP5 gene (Moser et al. 1992)

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Table 3: Antibodies, suppliers and dilutions used

Antibodies	Type	Supplier	Dilution
Primary antibodies			
IGFBP1	Polyclonal	Novozymes GroPep Ltd, Australia	--
IGFBP4	Polyclonal	Novozymes GroPep Ltd, Australia	1:100
IGFBP5	Polyclonal	Novozymes GroPep Ltd, Australia	1:75
IGFBP6	Polyclonal	Novozymes GroPep Ltd, Australia	1:200
Secondary antibody			
Anti-rabbit IgG	Polyclonal	Zymed, CA, USA	1:200

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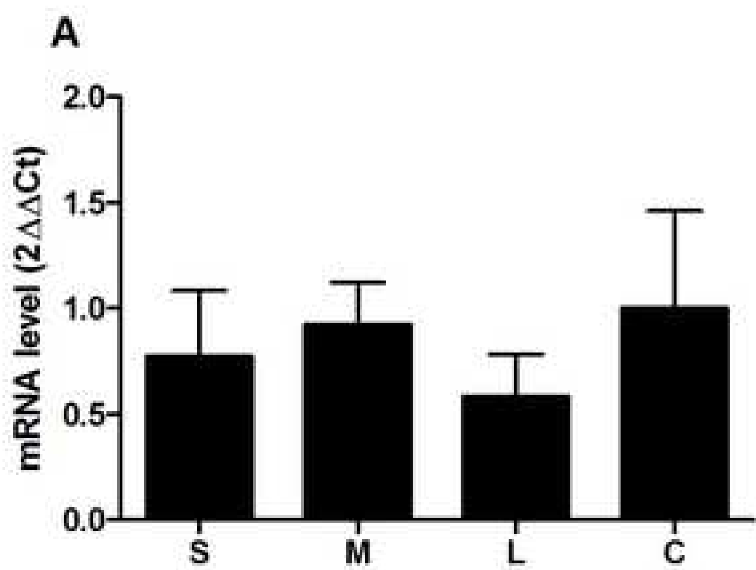
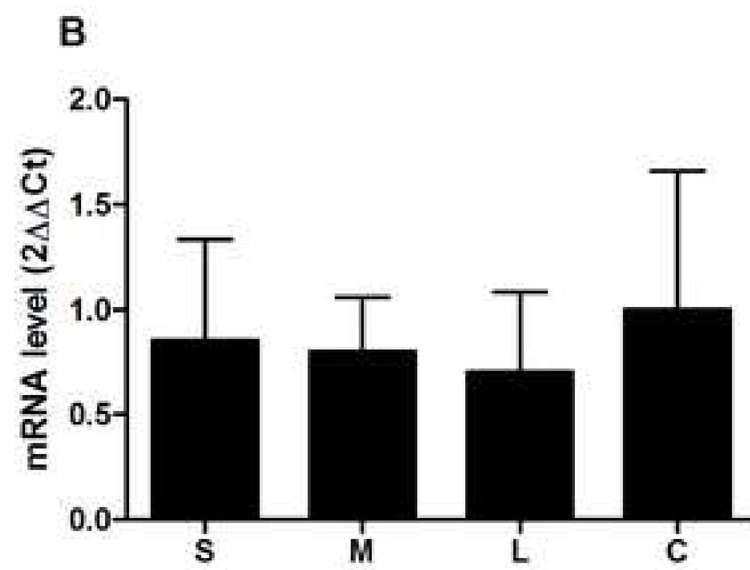
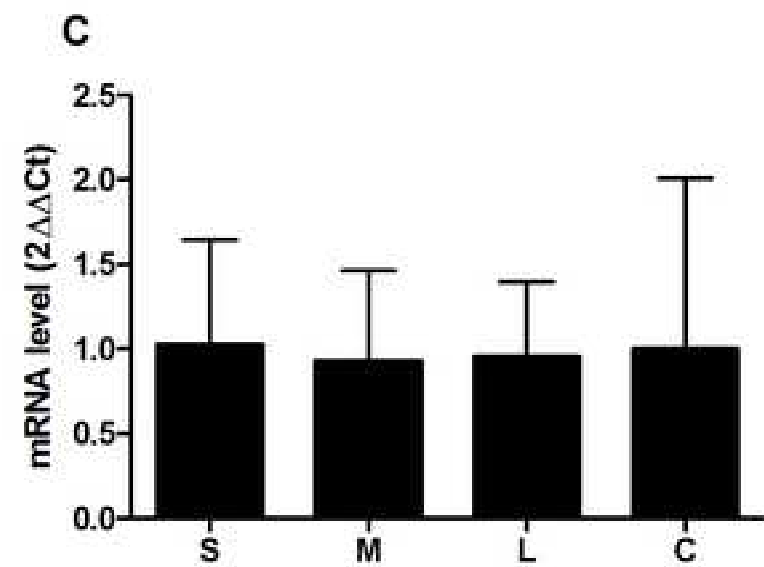
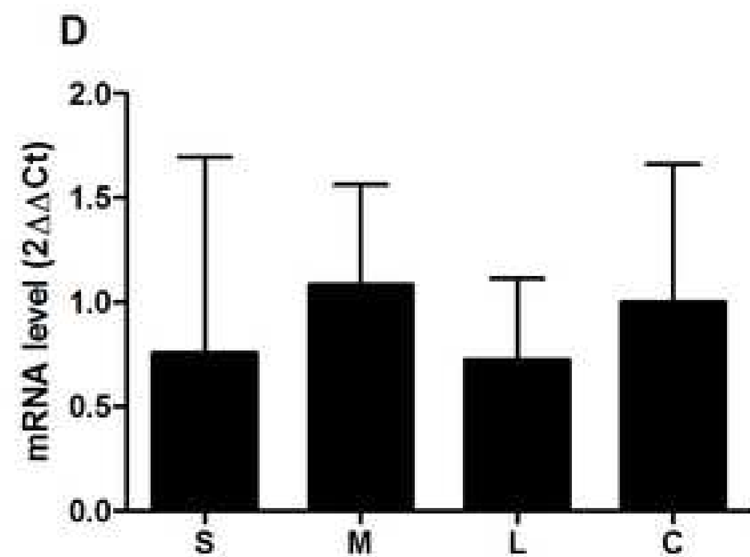
Figure 1









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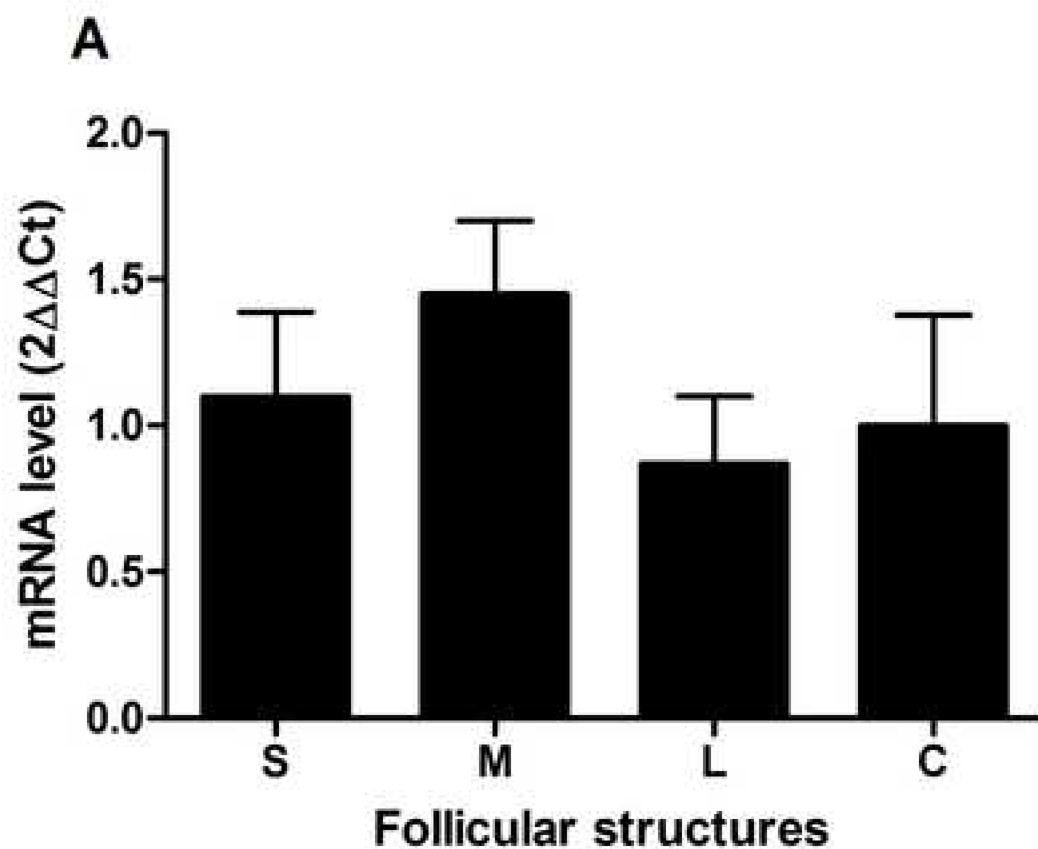

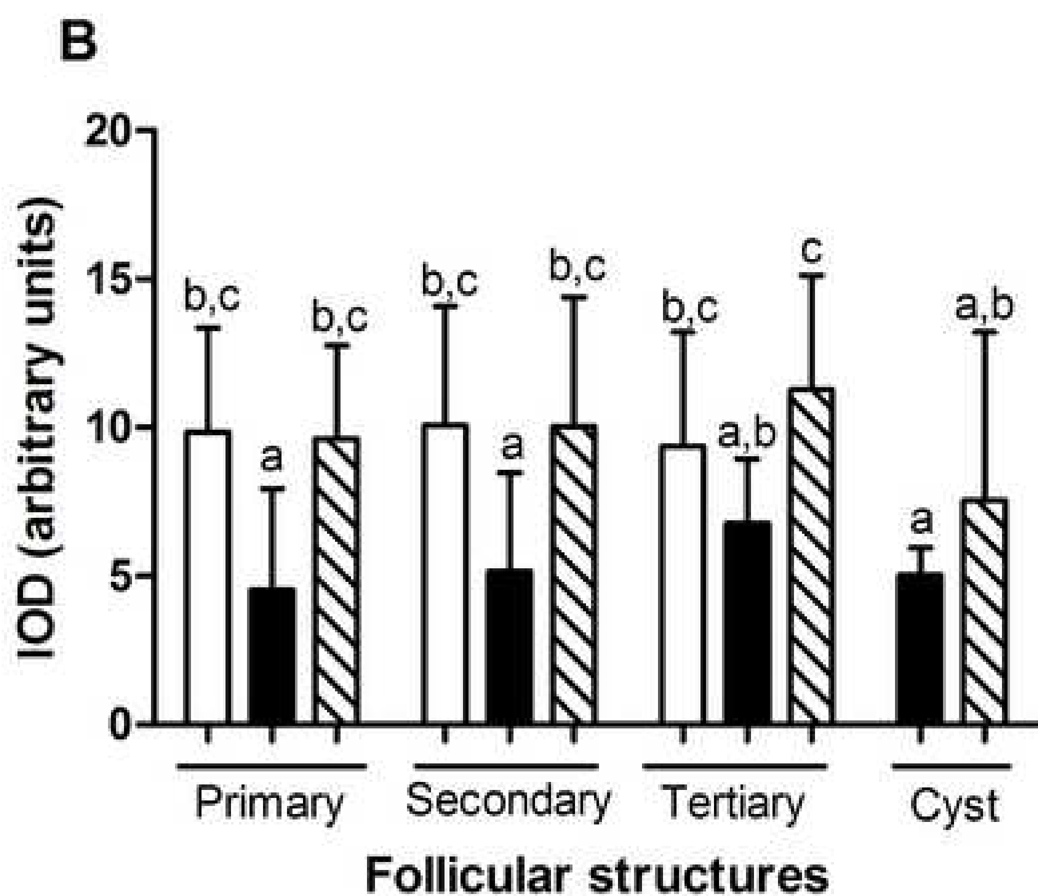

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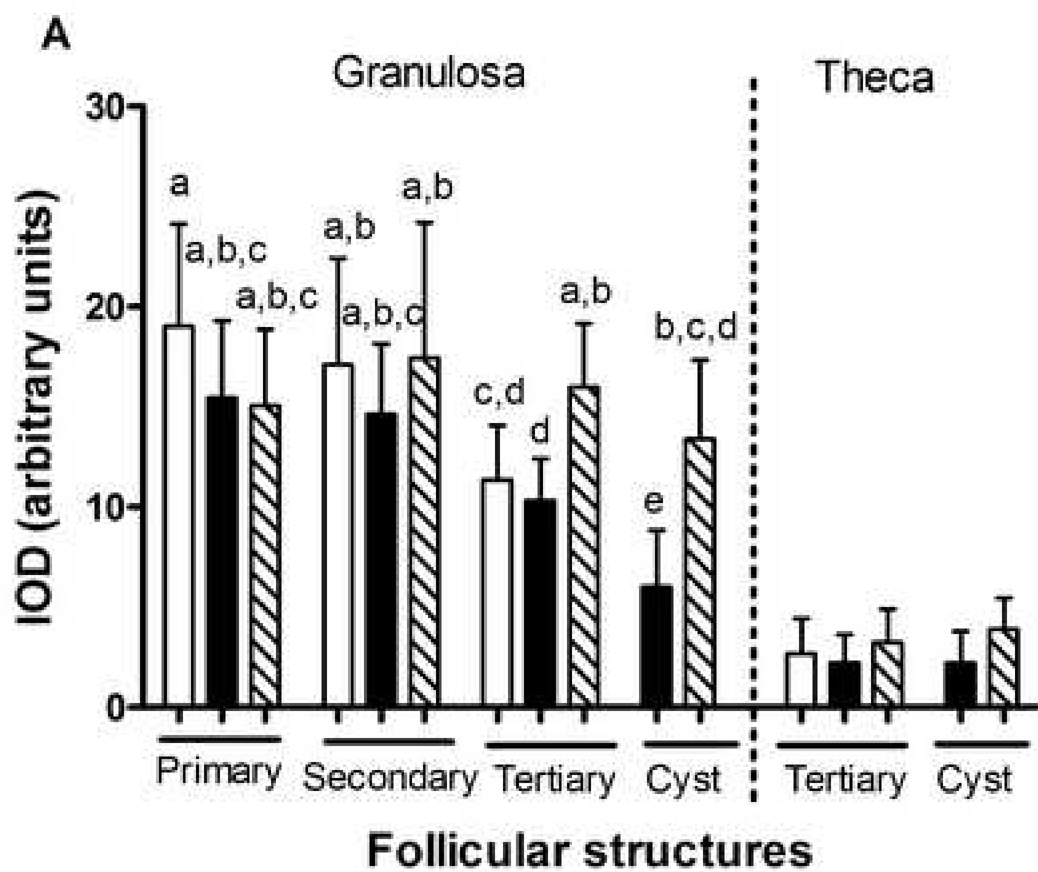


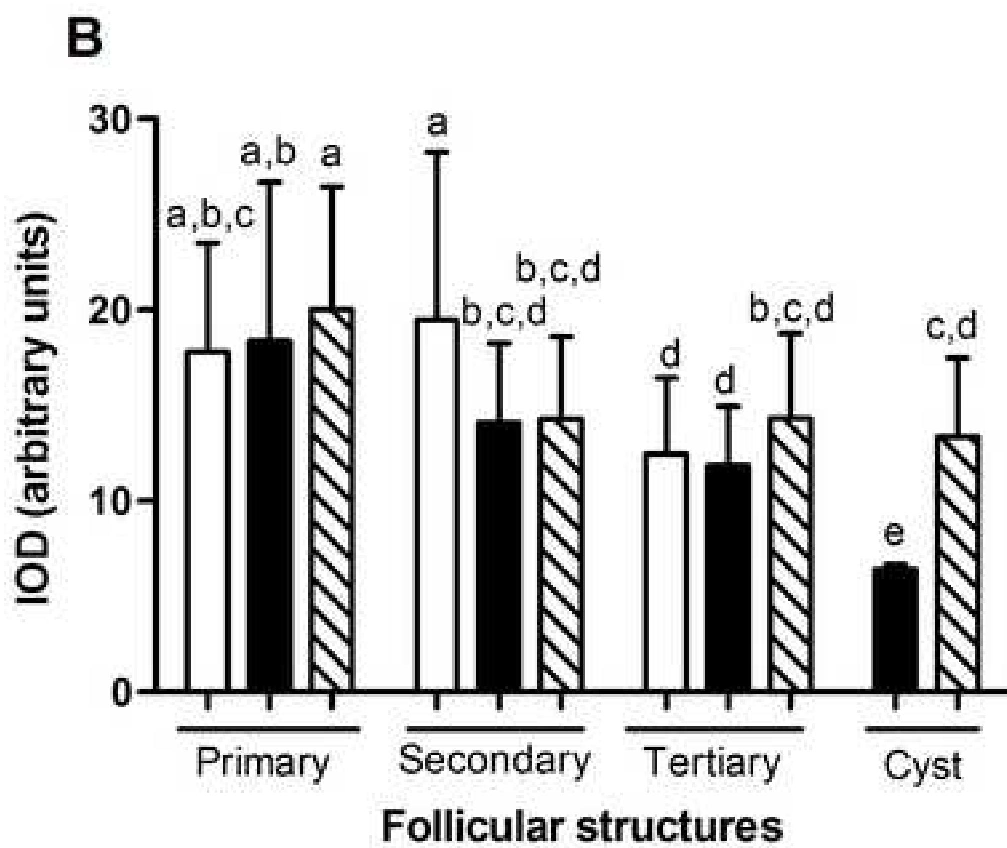


Figure 4

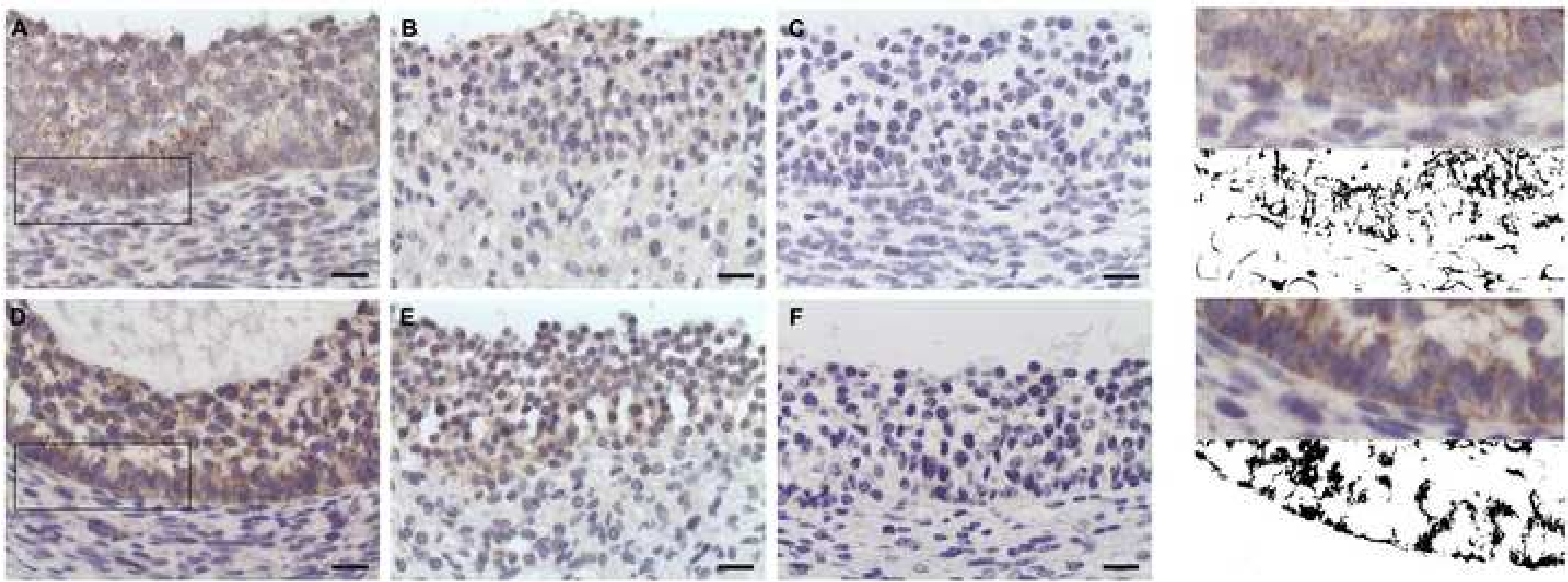
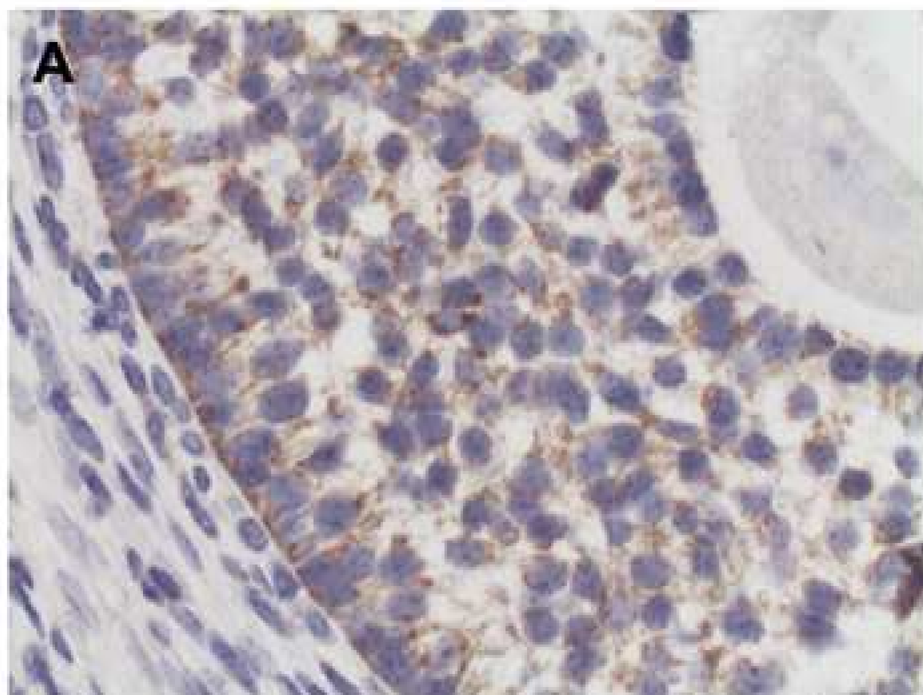
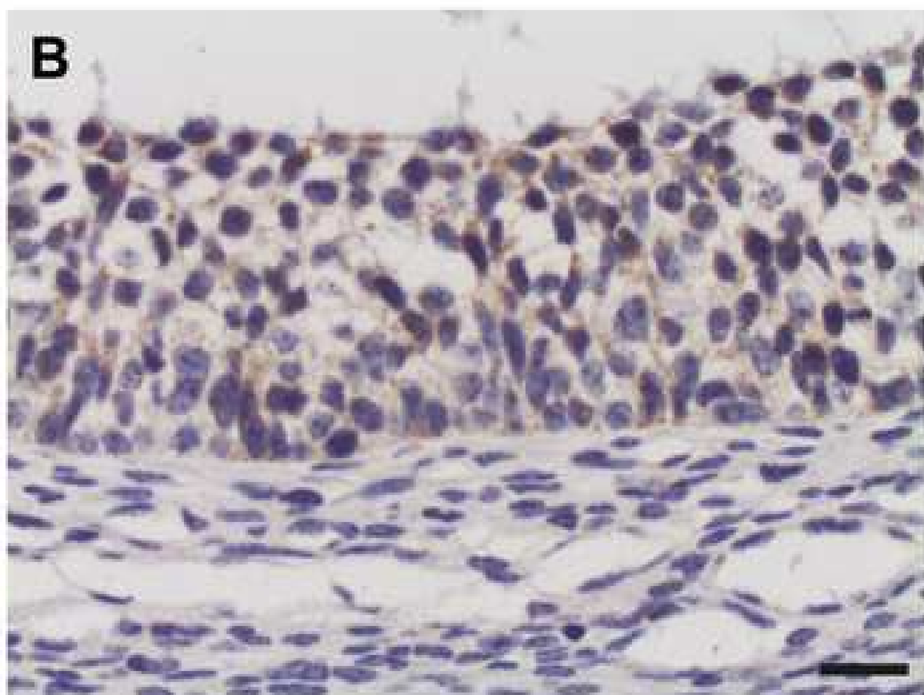
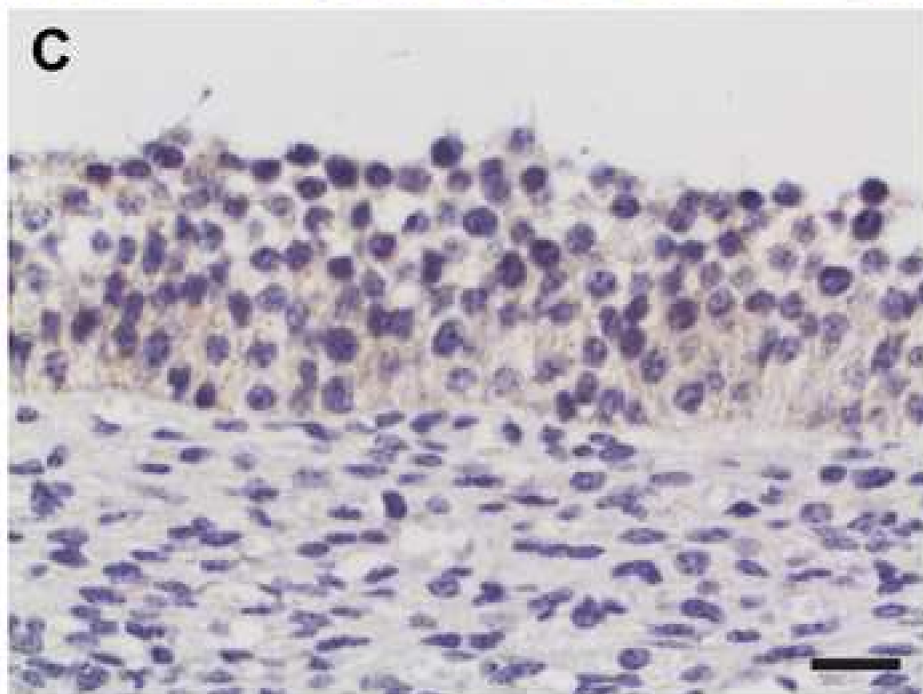


Figure 5







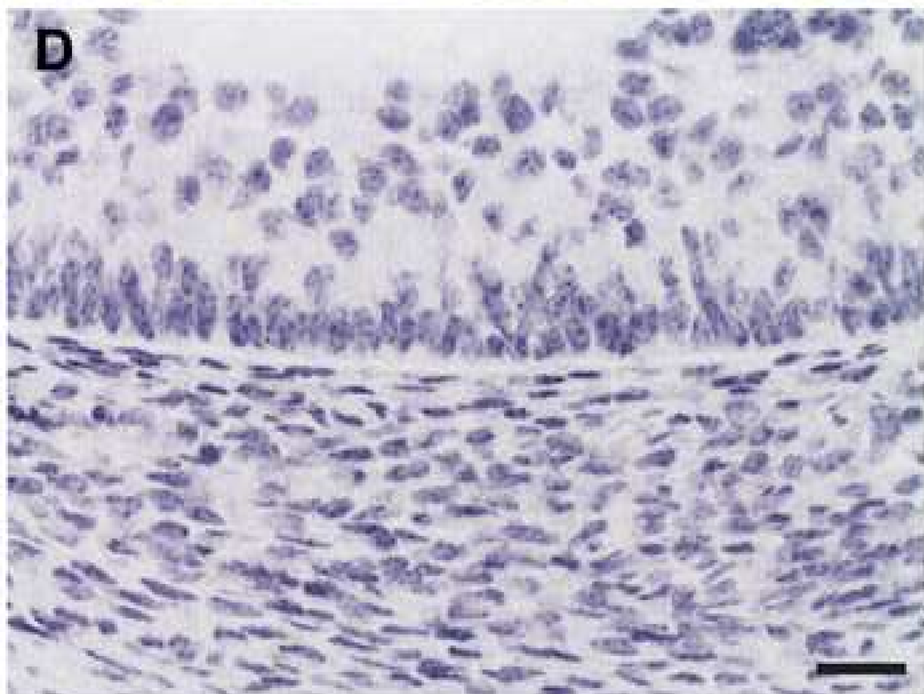
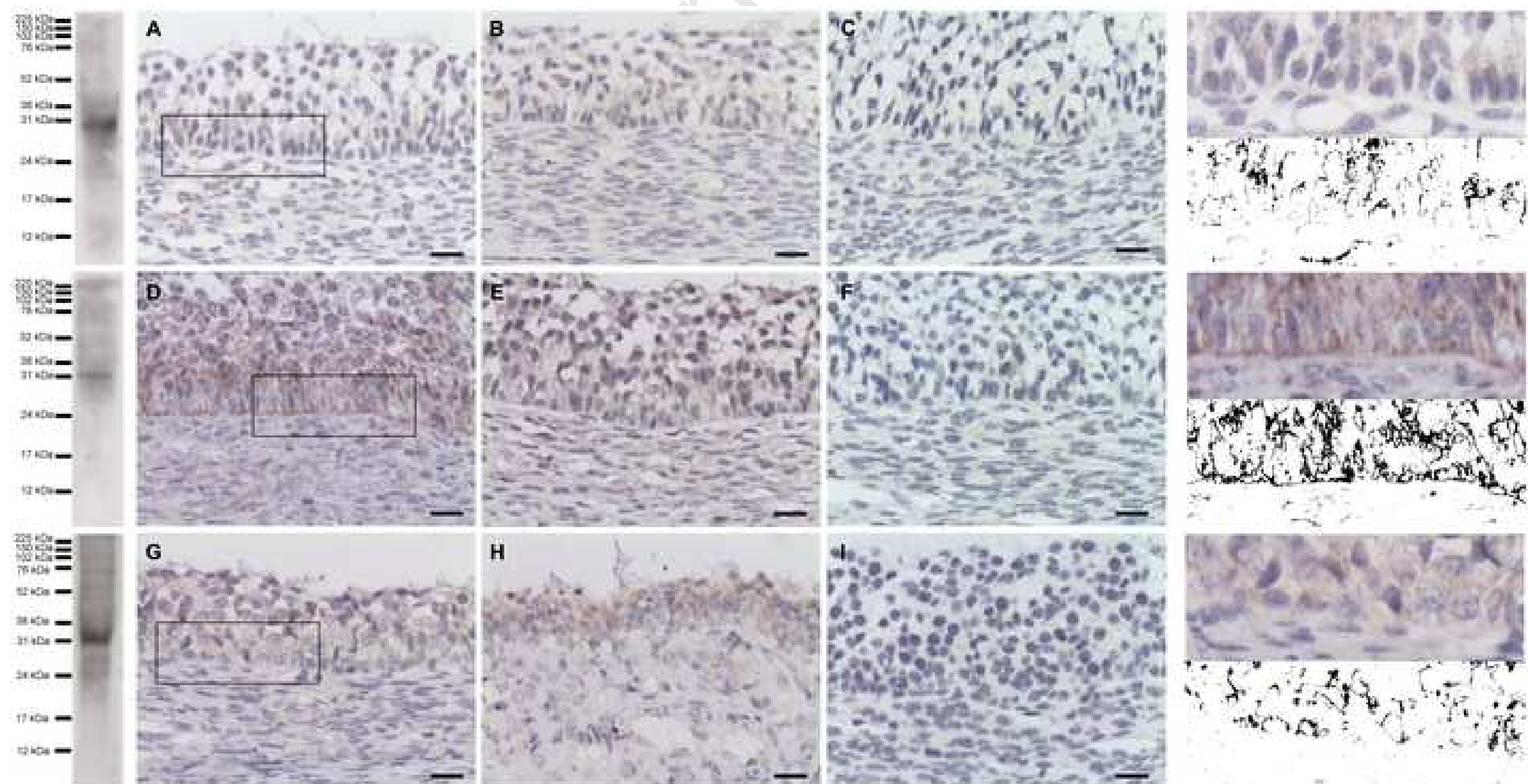


Figure 6



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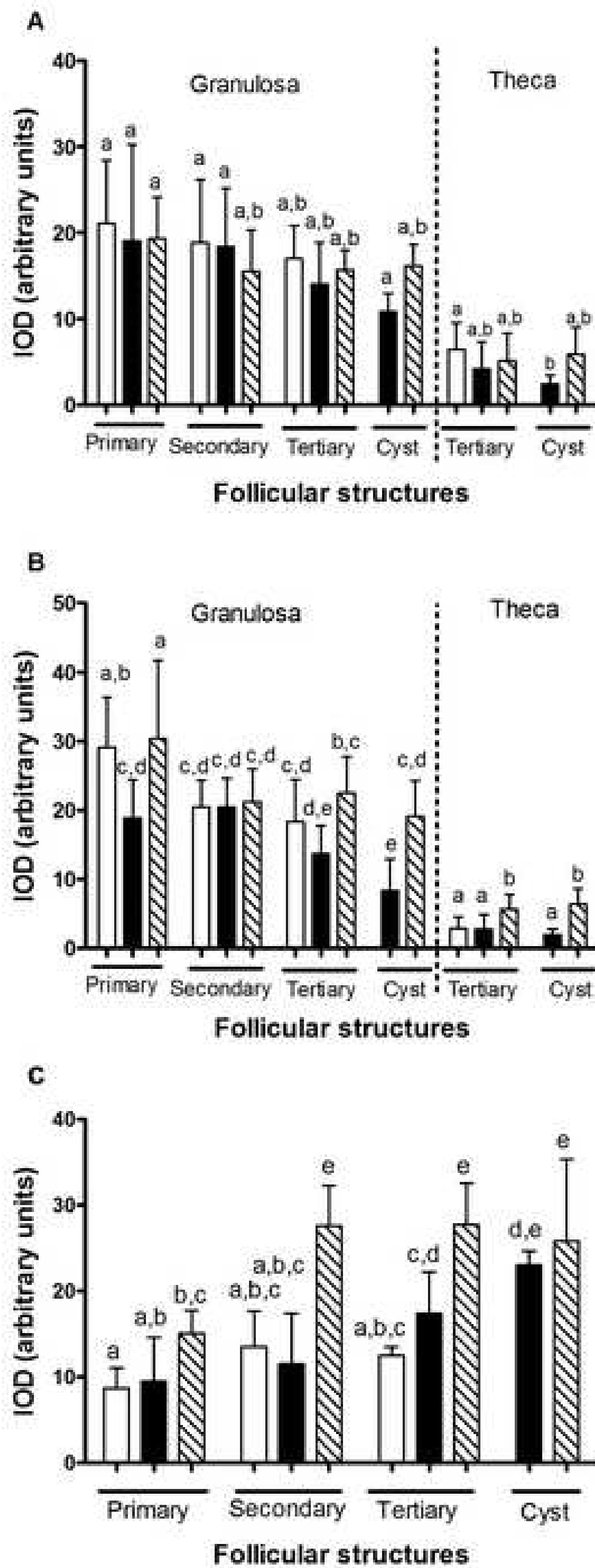

Figure 8

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A

IOD (arbitrary units)

80
60
40
20
0

a b b b a b b b a b b b

S M L C S M L C S M L C S M L C S M L C

24 kDa 29 KDa 31 KDa 34 KDa 40-44 KDa

B