



*Dysregulation of granulosa bone morphogenetic protein receptor 1B density is associated with reduced ovarian reserve and the age-related decline in human fertility*

Article

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1 **Dysregulation of granulosa bone morphogenetic protein**  
2 **receptor 1B density is associated with reduced ovarian**  
3 **reserve and the age-related decline in human fertility**

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23

**24 Abstract**

25 Reproductive ageing is linked to the depletion of ovarian primordial follicles, which causes  
26 an irreversible change to ovarian cellular function and the capacity to reproduce. The current  
27 study aimed to profile the expression of bone morphogenetic protein receptor, (BMPRI1B) in  
28 53 IVF patients exhibiting different degrees of primordial follicle depletion. The granulosa  
29 cell receptor density was measured in 403 follicles via flow cytometry. A decline in  
30 BMPRI1B density occurred at the time of dominant follicle selection and during the terminal  
31 stage of folliculogenesis in the 23-30 y good ovarian reserve patients. The 40+ y poor  
32 ovarian reserve patients experienced a reversal of this pattern. The results demonstrate an  
33 association between age-induced depletion of the ovarian reserve and BMPRI1B receptor  
34 density at the two critical time points of dominant follicle selection and pre-ovulatory follicle  
35 maturation. Dysregulation of BMP receptor signalling may inhibit the normal steroidogenic  
36 differentiation required for maturation in older patients.

37

## 38 **1. Introduction**

39 Reproductive ageing is linked to the declining capacity to regenerate cells and tissues,  
40 causing irreversible changes to ovarian cellular dynamics and ultimately reducing the  
41 capacity to reproduce. As the average age of fertility-challenged patients climbs towards 40  
42 years (y), there is an urgency to characterise the cellular changes that occur in the ovary with  
43 time. The response of the ovaries to cyclic recruitment of primordial follicles forms the basis  
44 of the clinical documentation of the antral follicle count (AFC) (Almog, et al. 2011). The  
45 AFC and age are highly correlated to histologically determined ovarian primordial reserve  
46 (Hansen, et al. 2011, van Rooij, et al. 2005). As the primordial follicle reserve declines, the  
47 endocrine, paracrine, and autocrine regulation adapts to a changing environment. It is this  
48 changing landscape that requires further investigation to provide an alternative treatment to  
49 preserve the primordial follicles, and to adjust the cellular regulation to achieve oocyte  
50 competence and improve fertility rates in older patients.

51

52 Earlier research has highlighted the potential role of bone morphogenetic protein (BMP)  
53 signalling in regulating ovulation rate in sheep (Campbell, et al. 2006, Galloway, et al. 2000,  
54 Juengel, et al. 2011), and has led us to further investigate the molecular regulation of  
55 folliculogenesis by the BMPs (Regan, et al. 2015, Ruoss, et al. 2009). During a natural cycle,  
56 small antral follicles with sufficient granulosa follicle-stimulating hormone receptor (FSHR)  
57 expression are recruited in response to the intercycle rise in FSH, and one of these is  
58 subsequently selected to become the dominant follicle. Follicles with reduced FSHR and  
59 luteinising hormone receptor (LHR) become less responsive as the dependence from  
60 pituitary FSH stimulation shifts to LH, and circulating FSH concentrations decline (Lapolt,  
61 et al. 1990, Xu, et al. 1995, Zeleznik, et al. 1974). These subordinate follicles are destined  
62 for atresia. The selected dominant follicle is the one with greatest gonadotrophin

63 responsiveness, and is dependent on the acquisition of FSHR-induced LHR by granulosa  
64 cells; and this follicle continues growing to the ovulatory stage.

65

66 Previous research has shown that, at the time of declining FSH levels, a reduction in BMP6  
67 (Erickson and Shimasaki 2003), BMP15 (Feary, et al. 2007) and the type 1 TGF $\beta$   
68 superfamily receptor BMPR1B (Feary, et al. 2007, Regan, et al. 2015), occurs after  
69 dominant follicle selection. Once selected, follicle growth and cellular proliferation  
70 continues, leading to an increased oestrogen and inhibin production by the granulosa cells.  
71 Together, these hormones suppress pituitary FSH output further, ensuring the demise of  
72 subordinate follicles. When the threshold for oestrogen's positive feedback action on the  
73 hypothalamus-pituitary axis is met, preparation for the ovulation phase begins with a  
74 cessation of cell proliferation, and early luteinisation changes taking place. During cellular  
75 and steroidogenic differentiation, activin (Young, et al. 2012), insulin-like peptide 3 (INSL3)  
76 (Anand-Ivell, et al. 2013), anti-mullerian hormone (AMH) (Andersen, et al. 2010, Ogura  
77 Nose, et al. 2012, Weenen, et al. 2004) and gonadotrophin surge attenuating factor (GnSAF)  
78 activity (Martinez, et al. 2002) declines, from dominant follicle selection to the termination  
79 of folliculogenesis at ovulation.

80

81 The functional role of BMPR1B receptor in follicle development has received considerable  
82 attention in recent years following the discovery that hyper-prolific sheep with the Booroola  
83 (FecB) phenotype have a naturally occurring mutation in the kinase domain of BMPR1B that  
84 perturbs antral follicle development and ovulation rate (Souza et al 2001; Mulsant et al  
85 2001). The BMP ligands, 2, 4, 6, 7, and 15 form a receptor-ligand complex with the type 1  
86 TGF $\beta$  receptor BMPR1B, and recruit the type 2 TGF $\beta$  receptor BMPR2. The complex  
87 initiates phosphorylation of the intracellular substrate molecules, which are the receptor-  
88 regulated Smads. The Smad forms a complex with a common mediator, Smad 4, and

89 translocates to the nucleus where transcription of BMP-responsive genes takes place. Smad  
90 signalling is modulated by repressor and activator molecules in the nucleus, cytoplasm, and  
91 in the extracellular matrix. Alternatively, BMPs activate the non-Smad pathway mitogen-  
92 activated protein kinase (MAPK) such as extracellular signal-regulated kinase (ERK 1/2) or  
93 Ark (Inagaki, et al. 2009, Ryan, et al. 2008)

94

95 In the ovary, granulosa cell signalling induced by the gonadotrophins, FSH and LH, activate  
96 the FSHR or LHR and stimulate cAMP-PKA, which increases the CYP19A1 aromatase to  
97 facilitate oestrogen synthesis. Progesterone synthesis is inhibited by the suppression of  
98 steroidogenic regulatory protein (StAR) (Abdo, et al. 2008, Pierre, et al. 2004, Tajima, et al.  
99 2003, Val, et al. 2003), which is essential for progesterone synthesis in the granulosa cell  
100 (Moore, et al. 2001). Alternatively, or in addition, BMPs inhibit ERK 1/2 signalling, which  
101 provides inhibitory control over the balance of progesterone and oestrogen (Miyoshi, et al.  
102 2007, Nakamura, et al. 2012, Ogura Nose, et al. 2012).

103

104 Given the particular focus of interest on BMPR1B in ovarian function, the current study  
105 aimed to comprehensively profile the expression of granulosa BMPR1B in a range of  
106 patients, of different ages and stages of ovarian primordial follicle depletion, who were  
107 receiving treatment for infertility. Previous reports documenting ovarian BMPR1B  
108 expression have evaluated expression at the mRNA level in pooled follicles from different  
109 size classes (Chen, et al. 2009, Estienne, et al. 2015). However, mRNA expression does not  
110 necessarily reflect expression of translated functional BMPR1B protein on the cell surface.  
111 In contrast, in this study we collected an average of ~ 8000 granulosa cells from each  
112 individual follicle over a comprehensive range of follicle diameters from 4 mm to 27 mm.  
113 Immunofluorescent labelling and flow cytometry were then used to measure the granulosa  
114 cell surface-expressed mature receptor protein density for the BMPR1B receptor.

## 115 2. Materials and Methods

### 116 2.1. Patients

117 A total of 401 follicles were collected from 53 patients undergoing standard fertility  
 118 treatment previously reported in accordance with the PIVET Medical Centre Algorithm, and  
 119 are presented in Table 1 (Yovich, et al. 2012). Follicles were collected irrespective of  
 120 previous aetiology, but limited to exclude unusual medical conditions, hormonal  
 121 dysfunction, and polycystic ovarian syndrome; patients were aged between 23 and 45 y. One  
 122 patient, out of three patients undergoing risk reduction removal of the uterus and ovaries,  
 123 was selected to represent an unstimulated natural healthy cycle prior to the LH surge, and  
 124 was recruited from King Edward Memorial Hospital (KEMH).

125 *Table 1 Patient ovarian reserve, based on antral follicle count (AFC) and the number of*  
 126 *follicles collected per group.*

127 Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number  
 128 of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B =  
 129 13-19; C = 9-12, D = 5-8; E =  $\leq 4$ .

AGE Year	IVF Patient	Total Follicle	Major AFC	Major Group	Number of Follicles Collected Per Group							
					Sub Group	Sub #	Sub Group	Sub #	Sub Group	Sub #		
21-30	8	95	20-40	A+ & A	A+	31	A	64	C	6		
31-34	11	86	13-29	A & B	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	B	50	C	16	D	30	E	6
40-45	18	118	3-8	D & E	D	59	E	19	B	34		
<b>40</b>	<b>1</b>	<b>Natural Cycle Healthy</b>	<b>D</b>	<b>2</b>								

OVARIAN RESERVE    GOOD    A+ = 30-39    A = 20-29    B = 13-19    C = 9-12    D = 5-8    E =  $\leq 4$  POOR

130

131

### 132 2.2. Human IVF: Ovarian stimulation, follicular fluid and oocyte

133 Patient treatment consisted of two types of GnRH-LH suppression in conjunction with rFSH,  
 134 from cycle day 2 for ~ 10 days (Puregon or Gonal F). A GnRH antagonist treatment  
 135 (Cetrotide) (0.25  $\mu$ g/day) was administered from day seven until ovulation induction.



136 Alternatively, a GnRH flare agonist treatment (Lucrin) (0.25 µg/day) was administered in  
137 conjunction with rFSH on day 2, Ovulation was triggered with either 10 000 IU hCG derived  
138 from a urinary preparation (Pregnyl) or a pituitary derived analogue to LH (Ovidrel). Oocyte  
139 retrieval was scheduled for 36 hours post-trigger, by transvaginal oocyte aspiration (Yovich  
140 and Stanger 2010).

### 141 *2.3. Antral follicle count*

142 Patients received rFSH based on the patient's profile of age and AFC, to predict the rFSH  
143 dose required to stimulate multiple pre-ovulatory follicles (Yovich, et al. 2012). The dose of  
144 rFSH was then adjusted to the patient's ovarian response to stimulation. Considerable  
145 overlap in rFSH dose was present between age groups, which allowed for a rFSH dose  
146 comparison between different ovarian reserve patient groups of the same age. Ovarian  
147 reserve was measured indirectly by the antral follicle count (AFC) (Hansen, et al. 2011).  
148 AFC was defined as the number of follicles between 2-10 mm in size that are present on day  
149 2-5 of a cycle. Determination of AFC was ascertained by transvaginal ultrasound and  
150 patients were divided into groups accordingly: Group A+ = 30-39; group A = 20-29; group B  
151 = 13-19; group C = 9-12, group D = 5-8; group E = ≤ 4. The groups were established based  
152 on ovarian response to gonadotrophin hormone stimulation during IVF cycles. (Yovich, et  
153 al. 2012).

### 154 *2.4. Collection of granulosa cells*

155 The diameter of the follicle was calculated using ultrasonography before the clinical  
156 aspiration of individual follicles. The first aspiration was collected without flush medium  
157 into a test tube, and handed to the embryologist to locate the oocyte and attached cumulus  
158 cells if present. Further flushing of the follicle (Quinn's Advantage with Hepes, Sage Media,  
159 Pasadena, California) at ~ 1.24-1.72 MPa removed the loosely attached layers of granulosa  
160 cells. Once the oocyte was located and removed, the clinician proceeded to the next follicle  
161 and repeated the process. The follicular fluid and flush was then layered onto a ficoll density

162 gradient (555485; BD Biosciences, Perth, Australia) and centrifuged to isolate the granulosa  
163 cells

#### 164 *2.5. Natural healthy unstimulated cycle collection*

165 The natural cycle patients scheduled for risk reduction removal of the reproductive organs  
166 was timed to coincide with day 12 of the menstrual cycle (Table 1). Before removal,  
167 ultrasound confirmation of the size of the follicles and the number of follicles present was  
168 made. After removal of the uterus and ovaries, the whole follicle was excised and  
169 transported to the laboratory. The collection of follicular fluid, isolation of the granulosa  
170 cells, and the analysis was performed as described above and below.

#### 171 *2.6. Immunolabelling of granulosa cells*

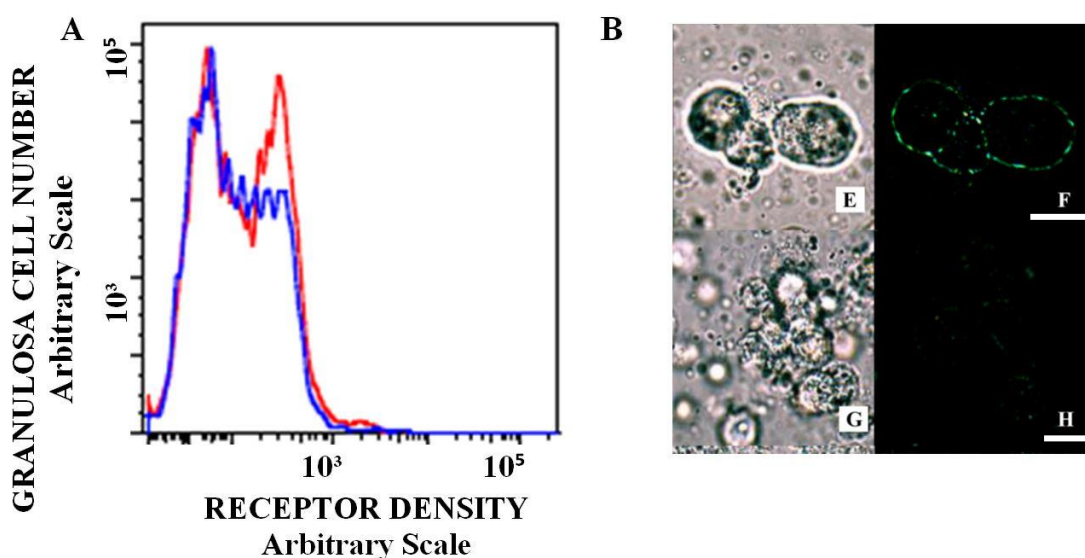
172 Aliquots of suspended granulosa cells ( $1 \times 10^6$  cells in 100  $\mu$ l) were immunolabelled using a  
173 double-indirect method as previously described (Abir, et al. 2008, Cai, et al. 2007, Gao, et al.  
174 2007). The cells were incubated separately with an optimised concentration of 4  $\mu$ g/ml  
175 affinity purified polyclonal antibody to goat BMPR1B (sc-5679), (Santa Cruz  
176 Biotechnology, Santa Cruz, CA, USA), for 25 min at 5°C; washed with PBS and then  
177 incubated with a second antibody, donkey anti-goat conjugated to the fluoro-chrome Alexa  
178 488 (Al-Samerria and Almahbobi 2014 ). The cells were washed again with PBS and  
179 centrifuged at 300 g at 5°C for 5 min. In addition, these antibodies have been used previously  
180 in human studies (Abir, et al. 2008, Haÿ, et al. 2004), including flow cytometry analyses  
181 (Gao, et al. 2007, Regan, et al. 2015, Whiteman, et al. 1991).

182

183 The routinely used monoclonal antibody against CD45 was added to BMPR1B antibody-  
184 containing tubes to enable the subtraction of leukocyte common antigen-positive cells (~  
185 3%) not removed during isolation of the granulosa cells using the ficoll density gradient.  
186 Unstained samples or the substitution of primary antibody with pre-immune goat IgG (Fig.  
187 1A) (Millennium Science, Surrey Hills, Victoria Australia) at the same concentration of the

188 primary antibody served as a negative control for auto-fluorescence; and a blocking peptide  
 189 for BMPR1B also confirmed binding specificity (Fig 1B), (sc-5679P; Millennium Science,  
 190 Surrey Hills, Victoria Australia) and as previously published (Abir, et al. 2008, Al-Samerria  
 191 and Almahbobi 2014 , Haÿ, et al. 2004, Regan, et al. 2015, Weall, et al. 2014)

192 In the current study, the ‘normal’ goat IgG and unstained control cells emitted an average  
 193 MFI that was very similar for each individual follicle but different between follicles and  
 194 patients; therefore, to optimise accuracy, the auto-fluorescence and the nonspecific binding  
 195 determined by the unstained control for each follicle, was subtracted from each individual  
 196 follicle. The data were analysed using FlowJo software (Tree Star Inc., Oregon, USA).



197 **Fig. 1** Validation of immunofluorescent labelling.

198 A. Unstained control (blue) compared to IgG Isotope control (red) for nonspecific binding  
 199 and auto-fluorescence. B. Live human granulosa luteal cells with positive fluorescence for  
 200 BMPR1B (EF), and negative blocking agent for BMPR1B (GH). Bar 10  $\mu$ m.  
 201

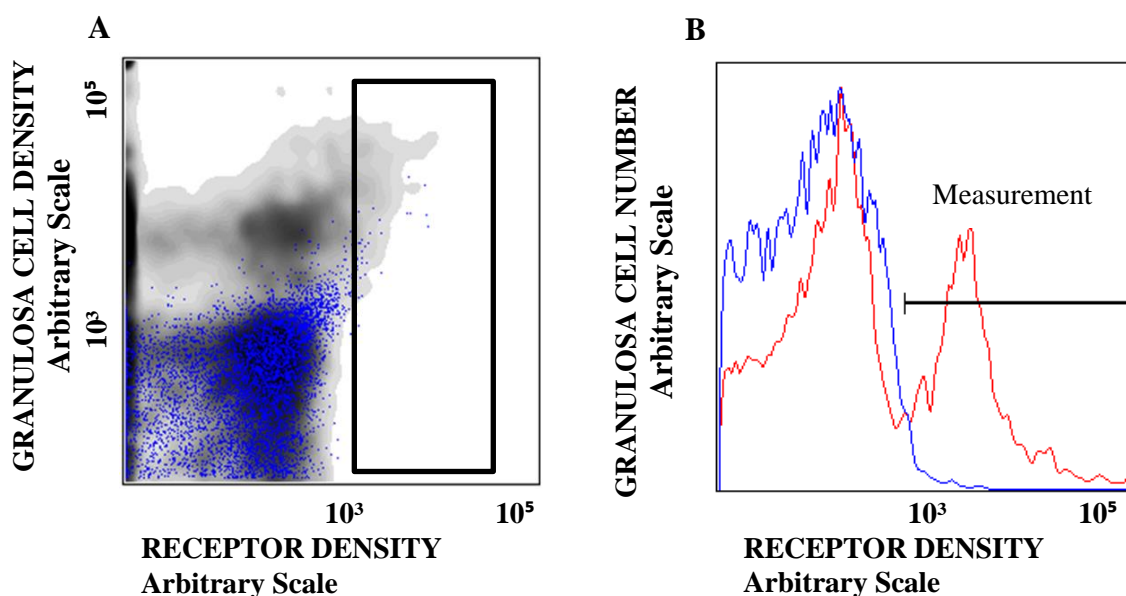
## 202 2.7. Fluorescent microscopy

203 Re-suspended 10 $\mu$ l aliquots of BMPR1B immunolabelled, live granulosa cells were placed  
 204 on slides and visualized using an Olympus DP 70 camera fitted to a Olympus BX-51 upright  
 205 fluorescent microscope with a 40x UPlan N 0.4 N.A. objective; (Olympus Imaging  
 206 Australia, Macquarie Park, Australia), (Fig. 1B). The granulosa cell slides were allowed to air

207 dry to reduce movement during digital capture, which would account for the more clumpy  
 208 appearance compared to the more typical single granulosa cells analysed by flow cytometry.  
 209 Fluorescent microscopy revealed a positive staining of the cell membrane-bound BMPR1B,  
 210 as an intermittent, bright, ring-like pattern around the cells. All control samples showed  
 211 negative staining. Granulosa cells ranged from 8  $\mu\text{m}$  to 25  $\mu\text{m}$ , with the average being 15  
 212  $\mu\text{m}$ .

### 213 2.8. Flow cytometry

214 Selective gating of the whole sample to identify a pure granulosa cell population was  
 215 achieved by graphing forward scatter to remove doublets (FSC-H verses FSC-A) (Regan, et  
 216 al. 2015). Then Alexa Fluor 488 fluorescent intensity was plotted against Allophycocyanin  
 217 (APC) intensity to identify and subtract the cells positive for the leukocyte common antigen  
 218 antibody CD45, which emits in the APC spectrum (Fig. 2A). Auto-fluorescence and  
 219 nonspecific binding were identified by the unstained sample control BMPR1B expression,  
 220 and subtracted from the measurement (Fig. 2B).



221 **Fig. 2** Validation of gating to measure average receptor density in flow cytometry.

222  
 223 A. Unstained control granulosa cells, represented as blue dots (auto-fluorescence) and  
 224 immunostained granulosa cells (grey). A rectangle subtraction gate for the leukocyte  
 225 common antigen CD45 positive cells. B. Subtraction of nonspecific binding and auto-  
 226 fluorescence at  $10^3$ ; mean granulosa cell fluorescent intensity (MFI) measurement.



## 228 2.9. Statistics

229 Mean fluorescent intensity (MFI) was obtained using ~8000 granulosa cells per individual  
230 follicle for the direct measurement of receptor protein expression. The data were subjected to  
231 statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for  
232 follicular size using GraphPad Prism 6. Values in graphs are means  $\pm$  S.E.M., and  
233 differences were considered significant if \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , and  
234 \*\*\*\* $p < 0.001$ . The letter, such as 'a', signifies a statistical difference to the matching letter,  
235 and an attached asterisk (a\*) indicates the significance level for that follicle size category.

## 236 2.10. Human Ethics

237 Informed consent was obtained from 57 patients undergoing standard fertility treatment at  
238 PIVET fertility clinic Perth, Australia, and three patients undergoing risk reduction removal  
239 of the uterus and ovaries which were recruited from King Edward Memorial Hospital  
240 (KEMH) Perth, Australia. Approval by the Human Research Ethics Committee of Curtin  
241 University of Technology and KEMH Women and Newborn Health Service ethics  
242 committee (WNHS) was obtained for this study.

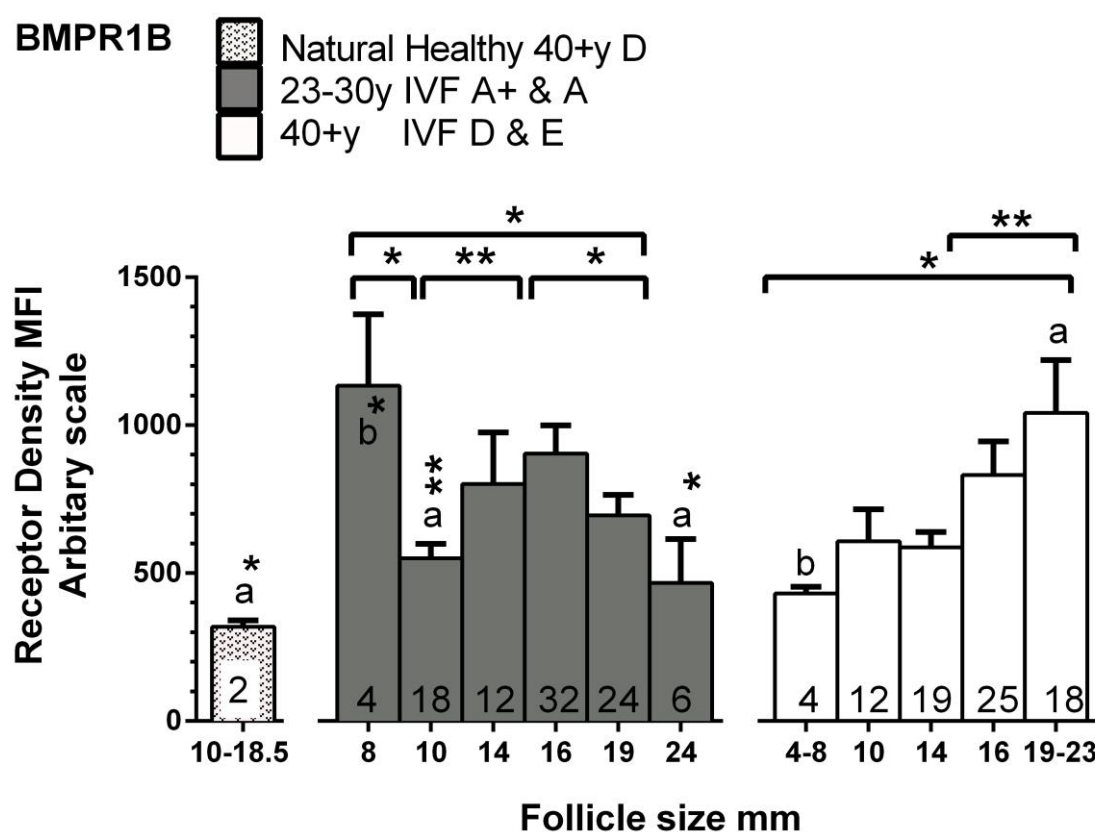
## 243 3. Results

### 244 3.1. Follicle development and ovarian reserve

245 Relative to older (40+y) patients, the level of granulosa BMPR1B expression was lower in  
246 the 23-30 y IVF patients in combined AFC groups A+ & A, and showed a biphasic receptor  
247 density pattern (Fig. 3). The biphasic pattern of receptor density consisted of an initial  
248 decrease in BMPR1B in follicles from 8 mm to 10 mm ( $p < 0.0201$ ), followed by an up-  
249 regulation in the follicles to 16 mm ( $p < 0.0084$ ), which was further followed by a significant  
250 decline in follicles to the terminal-end of folliculogenesis of 24-26 mm ( $p < 0.0301$ , Fig. 3). In  
251 marked contrast, the BMPR1B density increased with follicular size ( $p < 0.0044$ ) in a  
252 monophasic reversed profile in the 40+ y group (Fig. 3). The level of receptor density in the

253 small antral follicles of 8 mm was greater in the young patients than the older patients  
 254 ( $p < 0.0405$ ).

255 In a natural healthy cycle, the granulosa cells were collected from a healthy 40+ y with a  
 256 group D AFC. When combined, the two follicles of 10 mm and 18.5 mm had a significantly  
 257 lower density of BMPR1B compared to the largest follicles of the 40+ y IVF patients with a  
 258 group D & E AFC (Fig. 3). The receptor level in the natural cycle was not significantly  
 259 different to the level recorded in the younger patients and provides a baseline comparison.



OVARIAN RESERVE GOOD **A+** = 30-39 **A** = 20-29 **B** = 13-19 **C** = 9-12 **D** = 5-8 **E** =  $\leq 4$  POOR

260 with the limitation of reduced interpretation.

261  
 262 **Fig. 3** Granulosal BMPR1B density from follicles of different sizes collected from young  
 263 and older IVF patients compared to an unstimulated natural healthy cycle.

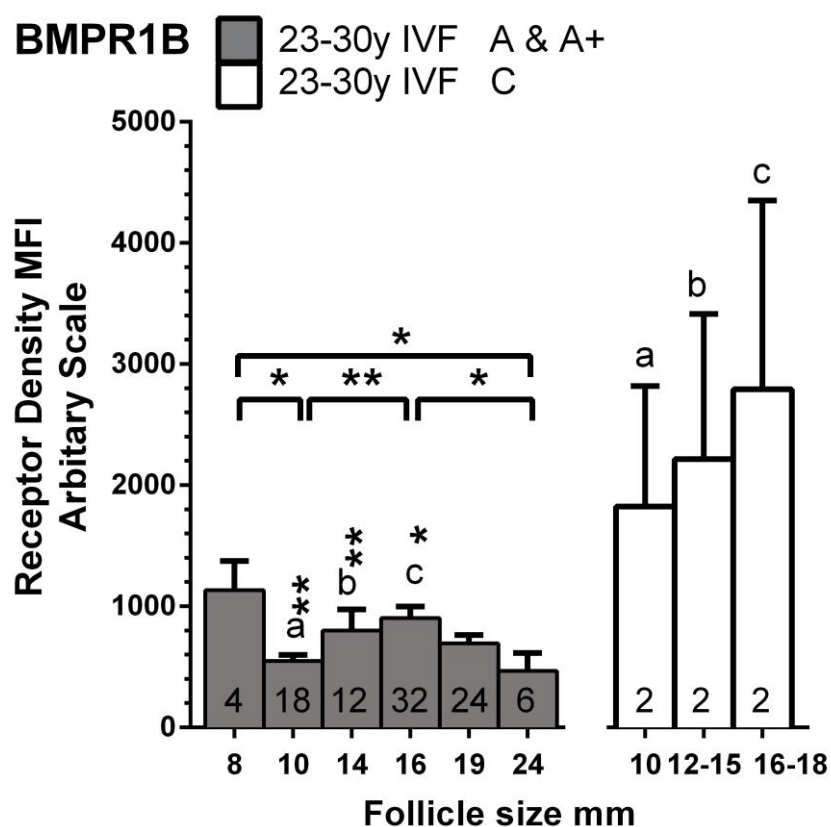
264  
 265 Granulosal BMPR1B protein density and follicle size profile of a natural healthy  
 266 unstimulated patient of 41y with an AFC of D, before the LH surge (patterned bar). Patients,  
 267 23-30 y stimulated, IVF cycle with an AFC of A+ & A, (grey bar). Patients, 40+ y

268 stimulated IVF cycle with an AFC of D & E, (white bar). IVF patients were grouped  
269 according to ovarian reserve measured indirectly by the antral follicle count (AFC). Mean  
270 fluorescent intensity (MFI) was obtained using an average of ~ 8000 granulosa cells per  
271 follicle for the direct measurement of receptor protein expression. The data were subjected to  
272 statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values in  
273 graphs are means  $\pm$  S.E.M., and differences were considered significant if \* $p < 0.05$  and  
274 \*\* $p < 0.01$ . The letter, 'a' signifies a statistical difference to the matching letter with an  
275 attached asterisk(s) (a\*, a\*\*). The number within the column represents the number of  
276 follicles analysed for that group.  
277



278 3.2. *Dysregulation of BMPR1B receptor density young patients with poor ovarian*  
 279 *reserve*

280 In the youngest age group 23-30 y, the majority of the patients had an AFC within groups  
 281 A+ & A (Fig. 4). There was no significant difference between the A+ group and the A group.  
 282 In contrast, the follicles from the C group patients with low AFC had increased BMPR1B  
 283 expression compared to the similar size follicles in the A+ & A group ( $p < 0.05$  to  $p < 0.001$ ,  
 284 Fig. 4), similar to the profile of the 40+y E AFC patients. Young patients with a very poor  
 285 ovarian reserve (group C) for their age do not typically have many follicles available for  
 286 collection. The rFSH dose administered ranged from 87 IU to 150 IU in the A+ & A group,  
 287 and was 190 IU in the C group.



OVARIAN RESERVE      GOOD   A+ = 30-39   A = 20-29   B = 13-19   C = 9-12   D = 5-8   E = ≤4 POOR

288 **Fig. 4** Granulosal BMPR1B density and ovarian reserve depletion in 23-30 year-old patients.  
 289  
 290 Patients were grouped according to ovarian reserve measured indirectly by the antral follicle  
 291 count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. The  
 292 data were subjected to statistical verification using one-way ANOVA with an uncorrected

293 Fisher's LSD. Values are means  $\pm$  S.E.M., and differences were considered significant if  
294 \* $p < 0.05$ . The letter, such as 'a' signifies a statistical difference to the matching letter, and an  
295 attached asterisk (a\*) which indicates the significance level. The number within the column  
296 represents the number of follicles analysed for that group.

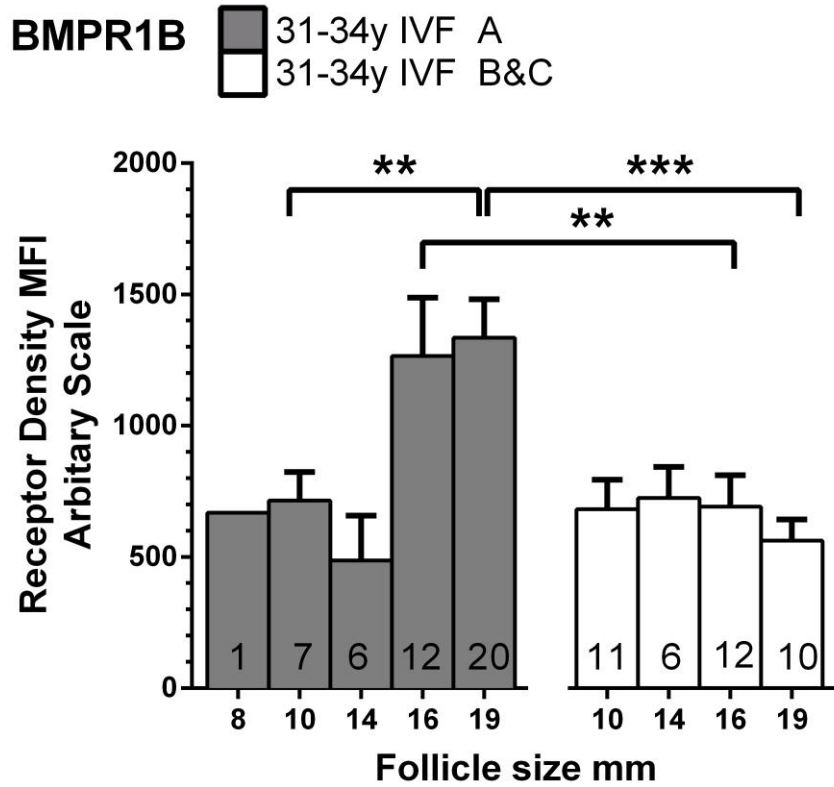
297

298 *3.3. Association between AFC and BMPR1B receptor density in older (31-34y)*  
299 *patients*

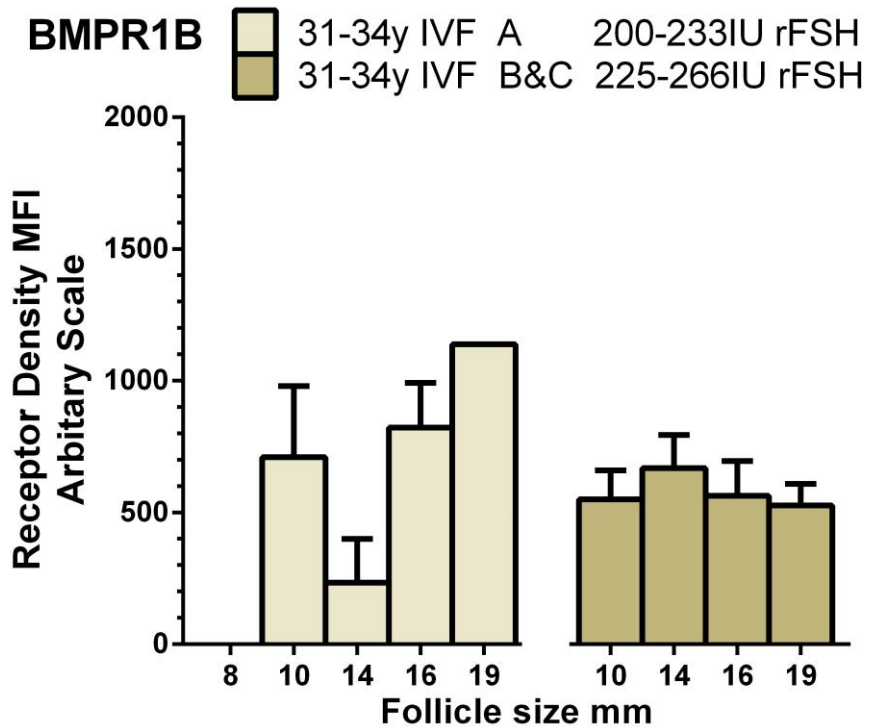
300 In the 31-34 y age group, the decline in ovarian reserve was associated with a loss of  
301 receptor density of BMPR1B in the granulosa cells from a peak in the 19 mm follicles in the  
302 A group to a significantly lower value in the B and C groups ( $p < 0.002$ , Fig. 5A). The rFSH  
303 dose given ranged from 83.5-266 IU, and when the AFC group comparison was restricted to  
304 those patients who received a comparable rFSH dose (200-233 IU), a similar BMPR1B  
305 receptor density profile was observed in the B and C group patients. However, in group A,  
306 the profile was different in the 16-19 mm follicles, which showed reduced receptor density  
307 (Fig. 5B).

308

A



B



OVARIAN RESERVE

GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

309 Fig. 5 Granulosal BMPR1B density and ovarian reserve depletion in 31-34 year-old patients.

310

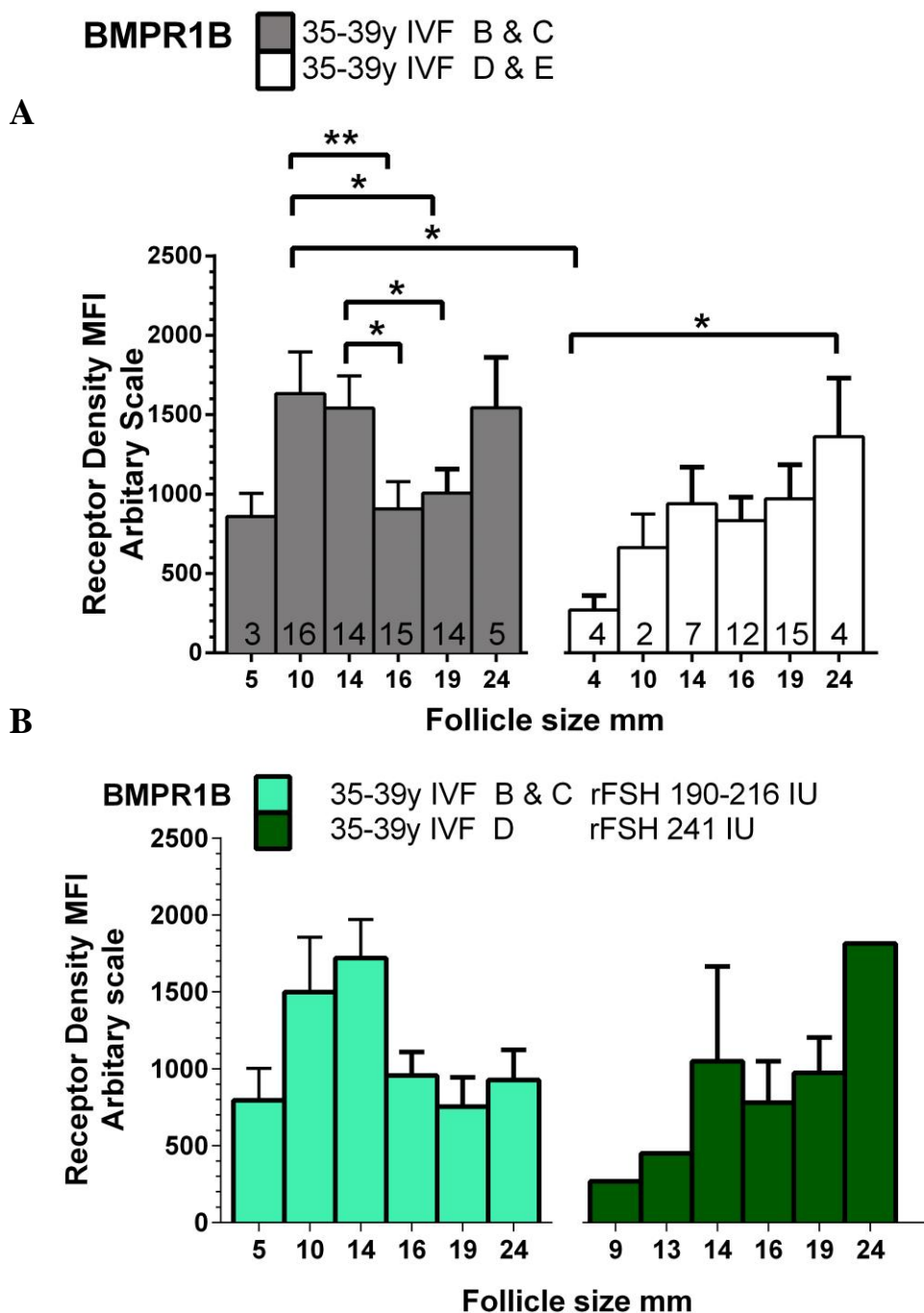
311 A. Patients were grouped according to ovarian reserve measured indirectly by the antral  
312 follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle.

313 B. As above, patients were grouped according to AFC but only those who received an  
314 equivalent rFSH dose were included in the analysis. Data were subjected to statistical  
315 verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means  $\pm$   
316 S.E.M., and differences were considered significant if  $*p < 0.05$ . The letter, such as 'a'  
317 signifies a statistical difference to the matching letter with an attached asterisk (a\*) which  
318 indicates the significance level (ie. 'a' is s.d. to all a\*). The number within the column  
319 represents the number of follicles analysed for that group.

320

### 321 *3.5. BMPR1B receptor density in older patients with declining AFC*

322 The 35-39 y combined B & C group demonstrated a significant reduction in BMPR1B  
323 receptor density in the 10 mm to 16 mm follicles ( $p=0.007$ ), similar to the youngest age  
324 group. With a further decline of the ovarian reserve (group D & E) the receptor density in the  
325 smaller follicles was reduced followed by a steady increase with increasing follicular size  
326 similar to the oldest patients monophasic profile, ( $p=0.037$ ), Fig. 6A). The rFSH dose given  
327 ranged from 83.5-600 IU, and when the AFC group comparison was restricted to those  
328 patients who received a similar FSH dose (190-241 IU), a very similar BMPR1B receptor  
329 density profile was observed (Fig. 6B).



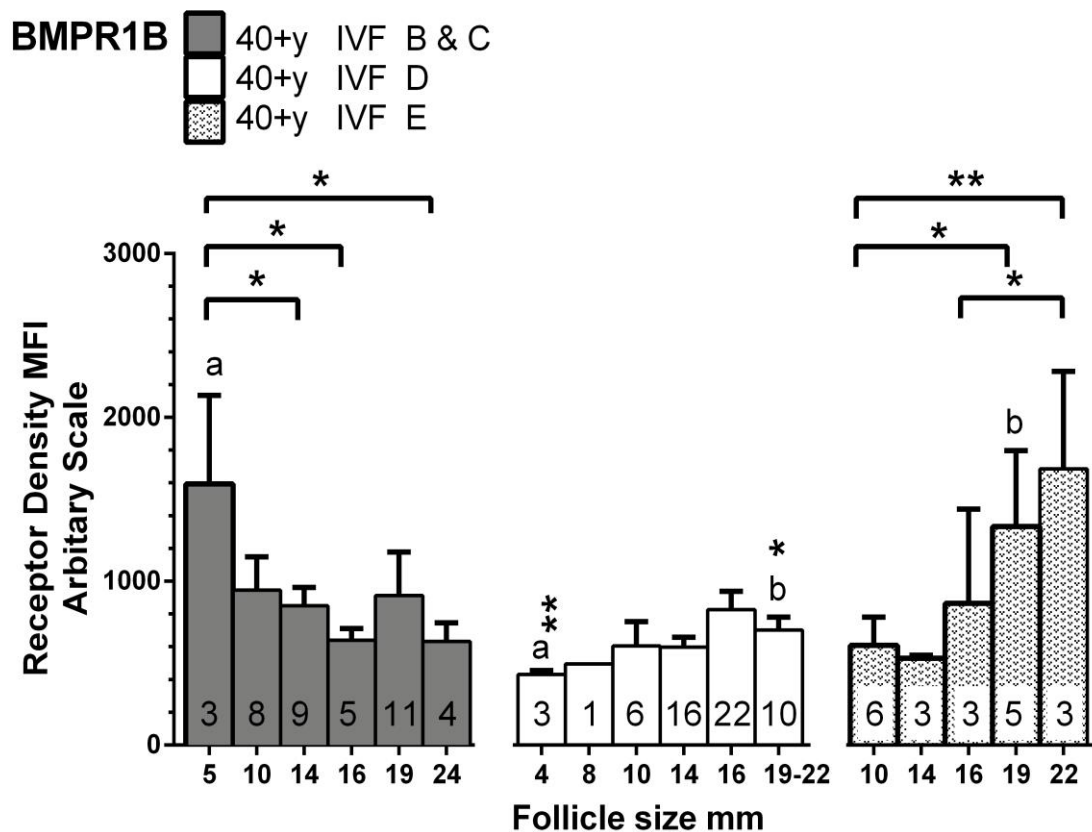
OVARIAN RESERVE GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

330 **Fig. 6** Granulosal BMPR1B density and ovarian reserve depletion in 35-39 year-old patients.  
 331  
 332 A. Patients were grouped according to ovarian reserve measured indirectly by the antral  
 333 follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle.  
 334 B. As above, patients were grouped according to AFC but only those who received a similar  
 335 rFSH dose (190-241 IU) were included in the analysis. Data were subjected to statistical  
 336 verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means ±

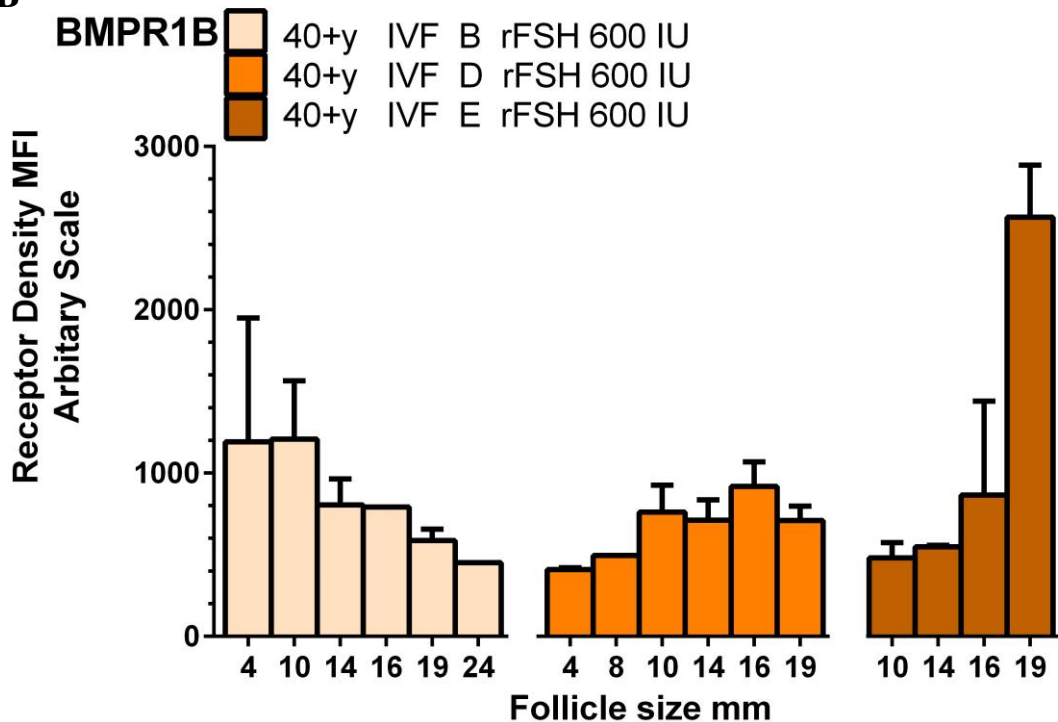
337 S.E.M., and differences were considered significant if  $*p < 0.05$ . The number within the  
338 column represents the number of follicles analysed for that group.

339 The 40+ y patients (40-45 year-old) ranged in AFC from group B to group E (Fig. 7A). The  
340 group B & C patients combined demonstrate a higher BMPR1B receptor density in the small  
341 follicles, followed by significant down-regulation of receptors as follicle size increased  
342 ( $p < 0.0176$ ). With a decline in the ovarian reserve to group D, the receptor density in the  
343 smaller follicles was reduced compared to group B ( $p < 0.0059$ ). With a further decline of  
344 ovarian reserve from D to group E, the receptor density significantly increased (19 mm  
345 follicles,  $p < 0.03$ ); and within the E group increased with follicle size (10 mm to 22 mm,  
346  $p < 0.0058$ , Fig. 7A). This was similar to the ageing effect observed in the youngest group C  
347 patients (Fig 4). The rFSH dose given ranged from 300-600 IU, and when the AFC group  
348 comparison was restricted to those patients who received an identical FSH dose (600 IU), a  
349 very similar BMPR1B receptor density profile was observed (Fig. 7B).

A



B



OVARIAN RESERVE

GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

350 Fig. 7 Granulosa BMP1B density and ovarian reserve depletion in 40+ year-old patients.

351

352 A. Patients were grouped according to ovarian reserve measured indirectly by the antral  
353 follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle.

354 B. As above, patients were grouped according to AFC but only those who received an

355 equivalent rFSH dose (600IU) were included in the analysis. Data were subjected to  
356 statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are  
357 means  $\pm$  S.E.M., and differences were considered significant if \* $p < 0.05$  and \*\* $p < 0.01$ . The  
358 letter, such as 'a' signifies a statistical difference to the matching letter, and an attached  
359 asterisk (a\*) which indicates the significance level (ie. 'a' is s.d. to all a\*). The number  
360 within the column represents the number of follicles analysed for that group.

361

#### 362 **4. Discussion**

363 A continuous process of activation of primordial follicles in the ovary leads to the inevitable  
364 depletion of the ovarian reserve in women (Almog, et al. 2011). The gradual decline in  
365 ovarian reserve can be indirectly measured by the number of small antral follicles at the  
366 beginning of a cycle, which is termed the AFC (Hansen, et al. 2011). The response of the  
367 ovary to exogenous gonadotrophins used to treat infertility declines with age, which is  
368 strongly correlated to the ovarian reserve (Hansen, et al. 2011). Patients with a poor ovarian  
369 reserve are treated with increasing doses of rFSH in an attempt to increase the number of  
370 small antral follicles with sufficient FSHR to develop into pre-ovulatory follicles.

371

372 In sheep carrying the Booroola (FecB) mutation, follicle development was perturbed and  
373 ovulation rate increased as a consequence of a point mutation in the *BMPR1B* gene (Regan,  
374 et al. 2015). Therefore, the potential role of *BMPR1B* within the context of follicle  
375 development, ovarian ageing, and fertility in humans is of considerable interest. In the  
376 present study, the density of expression of mature cell surface protein for *BMPR1B* was  
377 measured by flow cytometric analysis. We found that a reduction in the number of growing  
378 follicles was linked to the sequential disruption of *BMPR1B* density on the surface of  
379 granulosa cells.

380

381 Ovarian reserve depletes sequentially with age in a slow continuous process. The results in  
382 this study show a gradual degradation in the density of receptors, which is perpetuated



383 through the different age groups. The change observed may appear difficult to interpret;  
384 however, within each age group, there is evidence of over-expression followed by a  
385 weakness in expression, a lack of down-regulation, and eventually, reduced receptor density,  
386 ultimately leading to increased levels of BMPR1B in the largest follicles of the oldest,  
387 poorest ovarian patient groups.

388

389 An important finding of this study was that a decline in granulosa BMPR1B receptor  
390 density occurs in follicles of a size that would correspond to the time of cyclic dominant  
391 follicle selection, and again in the largest follicles from the best prognosis IVF patients, aged  
392 23-30 y. In comparison, the older, 40+ y, poor ovarian reserve patients exhibited a reversal  
393 of this pattern (Fig. 3). The emergence of the dominant follicle in a natural cycle and the  
394 multiple 'dominant' follicle cohort of an IVF stimulated cycle occur at the same follicular  
395 size (Baerwald, et al. 2003, Baerwald, et al. 2009). The growth rate of ovulatory follicles  
396 from a major wave was also not significantly different in an IVF cycle (Baerwald, et al.  
397 2009). The rFSH only extends the window of recruitment that promotes multiple dominant  
398 follicles (Baird 1987, Fauser and Van Heusden 1997). Therefore, it is possible to compare  
399 the receptor density to the physiological process indicated by the size of the follicle, such as  
400 dominant follicle selection. It is, therefore, speculated that enhanced BMP signalling arising  
401 from elevated pre-ovulatory BMPR1B levels would inhibit the normal steroidogenic  
402 differentiation required for maturation of the follicle in older patients with reduced AFC. A  
403 decrease in small antral follicle number has been associated with a rise in luteal and start-of-  
404 cycle FSH and LH with a corresponding decrease in inhibin B, AMH, and IGF 1 (Klein, et al.  
405 2000, Pal, et al. 2010). The increase in FSH and LH has been shown to accelerate the early  
406 growth of small follicles, followed by reduced growth rates of the pre-ovulatory follicles in  
407 older patients. Other ovarian age related changes were associated with an increase in  
408 mitochondrial deletions in granulosa cells (Seifer, et al. 2002) and an increase in the number  
409 of chromosomal errors (Handyside, et al. 2012).

410 *4.1. BMPR1B down-regulation and dominant follicle selection*

411 The biphasic down-regulation of the density of the TGF $\beta$  superfamily type I receptor,  
412 BMPR1B, during folliculogenesis was similar to our previous finding in unstimulated young  
413 adult sheep (Regan, et al. 2015). During dominant follicle selection in sheep, and at an  
414 equivalent size in gonadotrophin stimulated humans, granulosa expression of BMPR1B was  
415 reduced, followed by a sequential increase with follicle size (Fig 3). The similarity between  
416 the sheep in natural cycles and the human IVF model suggests that rFSH has minimal impact  
417 on receptor expression levels and on the timing of dominant follicle selection. The addition  
418 of gonadotrophin in the form of rFSH masks the normal physiological pituitary drop in FSH,  
419 allowing a prolonged recruitment phase that enables multiple follicles to grow (Rice, et al.  
420 2007). The process of recruitment and dominant follicle selection should therefore be  
421 comparable to a normal unstimulated IVF cycle.

422

423 In other studies, granulosa cell expression of BMPR1B has been shown to increase with  
424 follicle size (Chen, et al. 2009, Estienne, et al. 2015), which is consistent with our findings.  
425 However, the pre-ovulatory, leading dominant follicle in sheep was pooled with smaller  
426 follicles in these studies, which would effectively mask the down-regulation (Regan, et al.  
427 2015). The down-regulation of granulosa cell BMPR1B expression in the present study was  
428 consistent with findings for sheep dominant follicles compared to the subordinate follicles  
429 reported in another recent study (Gasperin, et al. 2014). The interrelationship between FSH  
430 and BMP regulation has been previously reported (Miyoshi, et al. 2006, Shi, et al. 2009, Shi,  
431 et al. 2010), and the decline in pituitary FSH secretion initiating the dominant follicle  
432 selection process would, therefore, appear to be temporally related to the decline in  
433 BMPR1B expression on the granulosa cell surface. The low levels of receptor expression in  
434 the small antral follicles of older patients with reduced ovarian reserve suggest a possible  
435 cause of poor quality follicles and oocytes typical of older patients. Oocytes surrounded by  
436 cumulus cells with greater levels of *BMP15* mRNA were shown to have an increased

437 pregnancy rate after IVF (Li, et al. 2014), and reduced apoptosis (Hussein, et al. 2005).  
438 Moreover, an association between high levels of BMP15 in the follicular fluid and oocyte  
439 quality has been reported (Li, et al. 2014, Wu, et al. 2007).

#### 440 *4.2. BMPR1B down-regulation and the maturation of pre-ovulatory follicles*

441 The degenerative ageing of granulosa BMPR1B density is highlighted by the observation  
442 that 40+ y patients (groups B & C) with a favourable ovarian reserve for age exhibit a  
443 pattern of declining receptor density with follicle size, whereas the pattern in the 40+ y  
444 patients (groups D & E) with reduced ovarian reserve is reversed (Fig. 7). Similar ovarian  
445 ageing was found in the 35-39 y B & C group with the same steady increasing density in the  
446 reduced ovarian reserve D & E group (Fig. 6). In addition, evidence of ovarian ageing was  
447 seen in the youngest patients with a severely reduced for age ovarian reserve (AFC group C),  
448 where the receptor density was increased substantially (Fig. 4). The over-expression of  
449 BMPR1B was also present in the oldest patients with severe ovarian depletion (AFC E; Fig  
450 7A). In the 31-34 y patient group with an A ovarian reserve, the receptor density increased in  
451 the largest follicles followed by a general decrease in the poorer ovarian reserve patients for  
452 that age group (Fig. 5A). As age increases, and the ovarian reserve declines, fewer follicles  
453 are stimulated; hence, the extra-large follicles are rare. In the younger patients of 31-34 y, it  
454 would be expected that a 24+ mm follicle would be common; however, none were analysed.  
455 The reversal of receptor density provides evidence of a fundamental shift in granulosa  
456 BMPR1B receptor density with ovarian ageing. High levels of BMPR1B in pre-ovulatory  
457 follicles would promote oestrogen synthesis and inhibit progesterone synthesis, which could  
458 potentially suppress maturation of the follicle (Otsuka 2010, Shimasaki, et al. 1999).

#### 459 *4.3. Could the apparent effect of ovarian ageing on BMPR1B receptor density be due* 460 *to different degrees of rFSH stimulation in the treatment cycle?*

461 Patients with declining ovarian reserves are prescribed greater doses of rFSH and this could  
462 potentially confound the interpretation of the present observation that ovarian ageing affects  
463 granulosa BMPR1B receptor density. However, when 'like-with-like' comparisons were

464 made, with only those patients prescribed similar doses of rFSH included in the analyses, the  
465 effect of ovarian ageing on receptor expression persisted. The changes observed in BMPR1B  
466 density are therefore, unlikely to be attributable to the degree of rFSH stimulation that the  
467 patient received during a treatment cycle, at least within patients of a similar chronological  
468 age. In support of this, unpublished findings from one of our laboratories (PGK) have  
469 indicated that treatment of cultured bovine granulosa cells with FSH promotes a marked  
470 increase in CYP19A1 mRNA expression and E2 secretion but has no effect on BMPR1A,  
471 BMPR1B or BMPR2 mRNA expression (C Glistler and PG Knight, unpublished  
472 observations).

#### 473 *4.4. Ovarian gonadotrophin surge attenuating factor (GnSAF) and BMPR1B* 474 *receptor density during folliculogenesis: is there a link?*

475 The BMPs have been described as inhibitors of the LH surge and luteinisation (Otsuka  
476 2010, Shimasaki, et al. 1999). GnSAF is an uncharacterised follicular-derived factor  
477 purported to reduce GnRH-induced pituitary LH secretion (Dimitraki, et al. 2014). An  
478 inverse relationship between GnSAF activity and follicle size has been reported (Fowler, et  
479 al. 2001). Furthermore, the age-related decline in ovarian reserve was associated with  
480 reduced GnSAF activity during follicle development (Martinez, et al. 2002). Could this be  
481 linked to the somewhat similar effect of age on granulosa BMPR1B receptor density shown  
482 in the present study? In the current study the observed changes in granulosa BMPR1B  
483 density in the young and older patients mimic the changes in GnSAF activity described by  
484 Martinez et al. (2002). Martinez et al. (2002) concluded that the GnSAF bioactivity  
485 prevented the premature onset of the LH surge, which bears comparison with the proposed  
486 role of BMPs as an inhibitor of luteinisation as evidenced by others (Otsuka 2010,  
487 Shimasaki, et al. 1999). In this context, it should be mentioned that both BMP ligands and  
488 receptors are expressed at the anterior pituitary gland level and BMP signalling has been  
489 implicated in the regulation of gonadotrophin production (Nicol et al 2008). Thus, it is  
490 tempting to speculate that ovarian GnSAF bioactivity, as yet uncharacterised, might actually

491 be attributed to BMPs synthesized and secreted by ovarian follicles. Clearly, further detailed  
492 experiments would be required to evaluate the tenability of this suggestion.

#### 493 *4.5. Conclusion*

494 Taken together, the results demonstrate the disrupting effect that ageing-induced depletion of  
495 the ovarian reserve has on granulosa BMPR1B receptor density in antral follicles. Age-  
496 induced depletion is associated with a loss of the biphasic down-regulation of granulosa  
497 BMPR1B density during follicle development. The findings extend previous research by the  
498 comprehensive nature of the range of follicle sizes and age groups studied, together with  
499 measurement of the translated mature, BMPR1B protein as opposed to measurement of  
500 receptor expression at the mRNA level (Ascoli, et al. 2002). Further work is needed to  
501 confirm the identity of the locally-produced TGF $\beta$  family ligand(s) (BMP2, BMP4, BMP6,  
502 BMP 7, and BMP15) whose signalling may either promote, or be impacted by this change in  
503 BMPR1B receptor density on the granulosa cell surface, and also to explore the  
504 consequences of altered signalling.

505

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509 **Authors' roles**

510 S.L.P.R. performed the experiments, analysed and interpreted the data, and wrote the  
511 manuscript. All authors contributed to the study design, manuscript revision, and final  
512 approval.

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517 **Conflict of interest**

518 The authors declare that there is no conflict of interest that could be perceived as prejudicing  
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520

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