

*Infertility and ovarian follicle reserve depletion are associated with dysregulation of the FSH and LH receptor density in human antral follicles*

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1 **Infertility and ovarian follicle reserve depletion are associated with**  
2 **dysregulation of the FSH and LH receptor density in human follicles**

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## 20 **Abstract**

21 The low take-home baby rate in older women in Australia (5.8%) undergoing IVF is linked to the  
22 depletion of the ovarian reserve of primordial follicles. Oocyte depletion causes an irreversible change to  
23 ovarian function. We found that the young patient FSH receptor and LH receptor expression profile on the  
24 granulosa cells collected from different size follicles were similar to the expression profile reported in  
25 natural cycles in women and sheep. This was reversed in the older patients with poor ovarian reserve. The  
26 strong correlation of BMPR1B and FSH receptor density in the young was not present in the older women;  
27 whereas, the LH receptor and BMPR1B correlation was weak in the young but was strongly correlated in  
28 the older women. The reduced fertilisation and pregnancy rate was associated with a lower LH receptor  
29 density and a lack of essential down-regulation of the FSH and LH receptor. The mechanism regulating  
30 FSH and LH receptor expression appears to function independently, *in vivo*, from the dose of FSH  
31 gonadotrophin, rather than in response to it. Restoring an optimum receptor density may improve oocyte  
32 quality and the pregnancy rate in older women.

33

34

## 35 **1. Introduction**

36 As women age, the reserve of primordial follicles is depleted, and the quality of oocytes, fertilisation, and  
37 pregnancy rate are reduced. Following their initial recruitment from the ovarian reserve, activated  
38 primordial follicles grow and differentiate into pre-antral and small antral follicles (McGee and Hsueh,  
39 2000). From the onset of puberty, cyclic fluctuations in follicle stimulating hormone (FSH) secretion from  
40 the anterior pituitary reach a threshold point sufficient to rescue a cohort of small antral follicles and  
41 initiate cyclic follicle recruitment (McGee and Hsueh, 2000). The number of antral follicles selected for  
42 dominance and ovulation is largely dependent on the regulatory action and the density of FSH receptors  
43 and LH receptors on the granulosa cell surface (Hillier, 2001, Baird, 1987, Baerwald, Adams and Pierson,  
44 2012).

45  
46 When the FSH level falls, the growth of the smaller follicles is reduced, and only the follicle with  
47 sufficient FSH and LH receptors continue to develop further because of their enhanced capacity to convert  
48 androstenedione to oestrogen for growth (Loumaye, Engrand, Shoham et al., 2003). As the ovarian  
49 primordial follicle reserve declines, the rate of cyclic recruitment of follicles diminishes (Baerwald et al.,  
50 2012, Almog, Shehata, Shalom-Paz et al., 2011). The number of these small antral follicles at the beginning  
51 of each cycle is representative of the ovarian reserve of primordial follicles that remain in the ovary.

52  
53 Older patients, typically, have a slower follicle growth rate and a reduced number of granulosa cells per  
54 follicle (Santoro, Isaac, Neal-Perry et al., 2003). Other ovarian age related changes are associated with  
55 increased mitochondrial deletions in granulosa cells and reduced FSH receptor mRNA expression, which  
56 have been linked with infertility (González-Fernández, Peña, Hernández et al., 2010, Seifer, DeJesus and  
57 Hubbard, 2002, Cai, Lou, Dong et al., 2007). Reduced receptor density may directly contribute to poor  
58 oocyte quality by increasing the number of chromosomal errors (Maman, Yung, Kedem et al.,  
59 2012, Handyside, Montag, Magli et al., 2012).

60

61 In this study, the aim was to comprehensively profile the expression of granulosa FSH receptor and LH  
62 receptor protein in a range of patients of different ages and stages of ovarian primordial follicle depletion,  
63 who were receiving treatment for infertility. An average of ~8000 granulosa cells per follicle was collected  
64 from follicles ranging in size from 4 to 27 mm. Antibody labelling and flow cytometry were used to  
65 evaluate the receptor density. Previous studies of receptor expression have been confined to expression at  
66 the mRNA level, which may not be a reliable indicator of the level of translated 'mature' receptor protein  
67 expressed on the cell surface (Jeppesen, Kristensen, Nielsen et al., 2012, Pidoux, Gerbaud, Tsatsaris et al.,  
68 2007, Ascoli, Fanelli F and DL., 2002). The changes observed in receptor density may explain the adverse  
69 impact that ovarian reserve depletion has on fertility as women age.

70

## 71 **2. Materials and Methods**

### 72 **2.1 Patients**

73 A total of 415 follicles were collected from 56 patients undergoing standard fertility treatment with PIVET  
74 Medical Centre Perth, Western Australia, and are presented in Table 1. Patients were aged between 23 and  
75 45 years, and follicles were collected irrespective of previous aetiology, but limited to exclude unusual  
76 medical conditions, hormonal dysfunction, and polycystic ovarian syndrome.

77

### 78 **2.2 Human IVF: Ovarian stimulation, follicular fluid, and oocyte**

79 Patient treatment consisted of two types of gonadotrophin releasing hormone-LH suppression (Puregon or  
80 Gonal F) in conjunction with commercially prepared recombinant human FSH, from cycle day 2 for ~10  
81 days as previously described (Regan, Knight, Yovich et al., 2016). Ovulation was triggered with 10 000 IU  
82 HCG, and the collection of granulosa cells and oocyte retrieval was 36 hours later by transvaginal oocyte  
83 aspiration (Regan et al., 2016). *In vivo* human studies are more complex and therefore variables such as  
84 BMI have been minimised in this study (Table 1). We initially used the merino sheep model in a natural  
85 cycle (Regan, McFarlane, O'Shea et al., 2015) to establish a comparative data set with the human model  
86 during IVF treatment. The sheep and women both showed prerequisite down-regulation at the two critical  
87 follicle sizes that are equivalent to the size at the time of follicle selection and follicle maturation. Indeed,

88 the administration of the artificial LH surge to induce maturation appears not to alter the receptor  
89 expression in the young patient with a good ovarian reserve based on the similarities between the models  
90 and demonstrated in Fig.5.

91 Furthermore, analysis of the effect of rFSH dose on the receptor expression in a homogeneous group of  
92 patients with all variables controlled (ovarian reserve, age, follicle size, BMI, and AMH) was not  
93 significantly different (Fig. 6,  $p = 0.7$ ).

### 94 **2.3 Ovarian reserve measured by the antral follicle count**

95 Patients received daily FSH according to a long established algorithm based on the patient's profile of age  
96 and ovarian reserve in order to predict the FSH dose required to stimulate multiple preovulatory follicles,  
97 as reported previously (Yovich, Stanger and Hinchliffe, 2012). Ovarian reserve was measured indirectly by  
98 the antral follicle count and was defined as the number of follicles between 2 - 10 mm in size that are  
99 present in total on ~day 2- 5 of a preliminary assessment cycle (Hansen, Hodnett, Knowlton et al., 2011).  
100 The patients were divided into groups levels from A to E; good to poor ovarian reserve, respectively based  
101 on the algorithm, as described previously described (Regan et al., 2016), and a well-established clinical  
102 practice of patient treatment (Yovich et al., 2012): Poly cystic ovarian syndrome (PCOS) patients were  
103 excluded from the study group based on the Rotterdam criteria, initially prepared in 2003, and updated to  
104 reflect the advances in ultrasound technology(Lujan, Jarrett, Brooks et al., 2013): specifically; per ovary >  
105 24 follicles, along with other criteria(Lujan et al., 2013). In the current study the combined ovary follicle  
106 total corresponded to Group A+ = 30-39 small follicles; group A = 20-29 small follicles; group B = 13-19  
107 small follicles; group C = 9-12 small follicles; group D = 5-8 small follicles; group E =  $\leq 4$  small follicles.

### 108 **2.4 Collection of granulosa cells**

109 The diameter of the follicle was calculated using ultrasonography as described previously (Regan et al.,  
110 2016). Flushing of the follicle by the clinician (Quinn's Advantage with Hepes, Sage Media, Pasadena,  
111 California) removed the loosely attached layers of granulosa cells. The cumulus ovarian complex was  
112 removed from the sample by the embryologist. The follicular fluid and flush was then layered onto a ficoll  
113 density gradient (555485; BD Biosciences, Perth, Australia) and centrifuged to isolate the granulosa cells

114 (Regan et al., 2016). Pure follicular fluid were analysed for oestrogen and progesterone using a random  
115 access immunoassay system (Siemens Medical Solutions, Bayswater, Victoria, Australia).

## 116 **2.5 Immunolabelling of granulosa cells**

117 Aliquots of suspended granulosa cells ( $1 \times 10^6$  cells in 100  $\mu$ l) were immunolabelled using a double-indirect  
118 method as previously described (Regan et al., 2016). The antibodies have been used previously in human  
119 studies (Regan et al., 2016, Abir, Ben-Haroush, Melamed et al., 2008, Haÿ, Lemonnier, Fromigué et al.,  
120 2004), including flow cytometry analyses (Regan et al., 2016, Regan et al., 2015, Gao, De Geyter,  
121 Kossowska et al., 2007, Whiteman, Boldt J, Martinez J et al., 1991). Briefly, the cells were incubated with  
122 affinity purified goat polyclonal antibody for the FSH receptor (sc-7798), LH receptor (sc-26341), and  
123 BMPR1B (sc-5679), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then incubated with a  
124 donkey secondary antibody to goat IgG, conjugated to the fluorochrome Alexa 488 (Life Technologies  
125 Australia, Victoria, Australia), as described previously (Regan et al., 2016, Al-Samerria and Almahbobi,  
126 2014 ). The routinely used monoclonal antibody CD45 was added to the LH receptor and BMPR1B to  
127 enable the subtraction of the positive leukocyte common antigen to render a homogeneous population of  
128 granulosa cells (Fig. 1D).

129 Unstained samples or the substitution of a primary antibody with pre-immune goat IgG (Millennium  
130 Science, Surrey Hills, Victoria Australia) at the same concentration as the primary antibody served as a  
131 negative control for auto-fluorescence (Fig. 1A). A blocking peptide for the FSH receptor and BMPR1B  
132 indicated nonspecific binding applied to human granulosa cells (sc-7798P, sc-5679P; Millennium Science,  
133 Surrey Hills, Victoria Australia), (Fig. 1B) as previously published (Cai et al., 2007, Gao et al., 2007). Pre-  
134 absorbed LH (Lutropin, Merck Serono, Frenchs Forest, NSW, Australia) also confirmed binding  
135 specificity (Fig. 1C, D). In the current study, the 'normal' goat IgG and unstained control cells emitted a  
136 similar average mean fluorescent intensity (MFI) and was subtracted from the receptor measurement. The  
137 auto-fluorescence and the nonspecific binding determined by the unstained control for each follicle was  
138 subtracted from each follicle as described previously (Regan et al., 2016). The data were analysed using  
139 FlowJo software (Tree Star Inc., Oregon, USA).



## 140 **2.6 Microscopy**

141 Re-suspended 10 $\mu$ l aliquots of FSH receptor immunolabelled, live granulosa cells were placed on slides  
142 and visualized using an Olympus DP 70 camera fitted to an Olympus BX-51 upright fluorescent  
143 microscope with a 40x UPlan N 0.4 N.A. objective (Olympus Imaging Australia, Macquarie Park,  
144 Australia) as described previously (Regan et al., 2016) (Fig.8B, D). Fluorescent microscopy revealed a  
145 positive staining of the cell membrane-bound FSH receptor as an intermittent, bright, ring-like pattern  
146 around the cells. All control samples showed negative staining. Granulosa cells ranged from 8  $\mu$ m to 25  
147  $\mu$ m, with the average being 15  $\mu$ m. We have validated the FSH receptor and LH receptor antibodies, and  
148 have published these findings (Al-Samerria and Almahbobi, 2014 ). 3D Image Analysis and  
149 immunofluorescent localisation and intensity quantification was performed on 10  $\mu$ m frozen sections in the  
150 sheep. In addition, this antibody has been used successfully by others in humans. Negative and positive  
151 controls were used and natural cell auto-fluorescence has been accounted for and subtracted for each  
152 sample, (Supplementary data Fig. S2) (Cai et al., 2007,Regan et al., 2015).

## 153 **2.7 Flow cytometry**

154 Selective gating of the whole sample to identify a pure granulosa cell population was achieved by graphing  
155 forward scatter to remove doublets (FSC-H versus FSC-A), presented in Fig. 1 and as previously described  
156 (Regan et al., 2016). The resulting population contained a uniform granulosa cell population that revealed  
157 positive staining for the FSH receptor, which is unique to granulosa cells (Hermann and Heckert, 2007).  
158 The data were analysed using FlowJo software (Tree Star Inc., Oregon, USA).

159

## 160 **2.8 Statistics**

161 Mean fluorescent intensity was obtained using ~8000 granulosa cells per individual follicle for the direct  
162 measurement of receptor protein expression. The data were subjected to statistical verification using one-  
163 way ANOVA with an uncorrected Fisher's LSD for follicular size using GraphPad Prism 6. Values in  
164 graphs are means  $\pm$  S.E.M., and differences were considered significant if \* $p$ <0.05, \*\* $p$ <0.01,  
165 \*\*\* $p$ <0.005, and \*\*\*\* $p$ <0.001. The letter, such as 'a', signifies a statistical significant difference to the

166 matching letter (e.g. 'a\*'). The attached asterisk (a\*) indicates the significance level for the size follicle. A  
167 two tailed, student t-test was also used.

## 168 **2.9 Human Ethics**

169 Informed consent was obtained from patients undergoing standard fertility treatment at PIVET Medical  
170 Centre, Perth, Australia, and from three patients undergoing risk reduction removal of the uterus and  
171 ovaries, who were recruited from King Edward Memorial Hospital (KEMH) Perth, Australia. Approval by  
172 the Human Research Ethics Committee of Curtin University of Technology and KEMH Women and  
173 Newborn Health Service ethics committee (WNHS) was obtained for this study (HR RD26-10:2010-  
174 2016), and all methods were performed in accordance with the relevant guidelines and regulations.

175

## 176 **3. Results**

### 177 **3.1 Follicle size and the extent of maturation of granulosa cells in the IVF patient**

178 During an IVF cycle, the granulosa cells from small follicles (8 mm) appear more compact and smaller in  
179 diameter (Fig. 2a). The granulosa cells from large follicles have a heterogeneous group of granulosa cells  
180 in different stages of maturation, which is referred to as luteinisation (Whiteman et al., 1991, Motta, 1969),  
181 (Fig. 2a, b, and c). The more mature granulosa cell has an expanded cytoplasm with prominent lipid  
182 droplets clustered around smooth endoplasmic reticulum, and are closely associated with numerous  
183 mitochondria as well as defined Golgi apparatus in the cytoplasm (Nottola, 1991). The extensive lipid  
184 droplets, observed in granulosa cells as grape-like spaces in the 23 mm follicles, indicate a greater steroid  
185 producing capacity and level of maturation. (Fig. 2c). The largest follicles produced a two fold increase in  
186 oestrogen and progesterone synthesis at the time of collection, Fig. 2d and 1e (Westergaard, Christensen  
187 and McNatty, 1986). High levels of progesterone synthesis by the granulosa cells are indicative of a  
188 greater extent of luteinisation even though all the follicles were exposed to an exogenous LH surge trigger  
189 injection.

190

191 The serum oestrogen peak was similar between the age groups; however, it is not until the ovarian reserve  
192 is used to distinguish the ageing process that more subtle differences are revealed. Peak serum oestrogen  
193 was approximately 6000 pmol/L for all age groups, but declined progressively as the ovarian reserve was  
194 depleted with increasing chronological age (Fig.3a). Age alone was not predictive of peak oestrogen  
195 levels, demonstrating no difference between age groups. There is a reduction in oestrogen output per  
196 follicle as the ovarian reserve is depleted, and is a reflection of the diminished number of follicles. Serum  
197 progesterone concentration was relatively uniform (2-4 nmol/L) but the lowest values were observed in the  
198 severely depleted patient groups of D and/or E, ( $p < 0.05$ , Fig. 3b).

### 199 **3.2 The impact of age on FSH receptor and LH receptor density during follicle growth**

200 In the young (23-30 y) patient group with a good ovarian reserve (ovarian reserve group A+ & A), down-  
201 regulation of the FSH receptor was observed at follicle sizes corresponding to dominant follicle selection  
202 (10 mm,  $p = 0.0201$ ) and during the preovulatory maturation phase (24 mm,  $p = 0.0302$ ; Fig. 4a). The level  
203 of translated 'mature' LH receptor protein was expressed at a constant level on the granulosa cell surface  
204 of those follicles measuring 8 to 19 mm in the young patients, whereas the largest follicles (24 mm)  
205 expressed a significantly lower density of LH receptor ( $p = 0.0237$ , Fig. 4b).

206

207 In the older patients, a high level of granulosa FSH receptor was present in the very small antral follicles  
208 (4-8 mm, 35-40+ y, D & E), which was then reduced in a manner similar to that seen in younger patients  
209 with good ovarian reserve ( $p < 0.001$ , and  $p = 0.0259$ , respectively, Fig. 4a). The initial down-regulation  
210 was followed by a sequential up-regulation of FSH receptor in the largest follicles, in contrast to the  
211 decline observed in the young A+ & A patient group ( $p = 0.0013$ , and  $p = 0.0131$ , respectively, Fig. 4a). In  
212 the older patient group (35-40+ y group D & E) a significant increase in LH receptor was recorded in the  
213 24-26 mm follicles ( $p = 0.0063$ , Fig. 4b). The LH receptor level after dominant follicle selection in the  
214 older patient group was significantly less than the level recorded in the young patient group (16 mm  
215 follicles:  $p = 0.0325$ , Fig. 4b).

### 216 **3.3 The impact of ovarian reserve on receptor expression during follicular growth.**

217 In the 40+ y age group with a good ovarian reserve of B & C, FSH receptor down-regulation at the time of  
218 dominant follicle selection and maturation of the follicles ( $p < 0.01$  and  $p = 0.0182$ , respectively, Fig. 5a),  
219 was similar to the younger patients profile shown in Fig. 4. When the ovarian reserve was depleted within  
220 the same age group, both the FSH receptor and LH receptor expression at the time maturation was reversed  
221 (16 to 24 mm,  $p = 0.0286$  and 10 to 16 mm,  $p = 0.0411$ , respectively). The LH receptor was elevated in the  
222 largest follicles of the poor ovarian reserve 40+ y patient group D & E (19-23 mm,  $p = 0.0324$ , Fig. 5b).

223 The level of recombinant (r) FSH dose in the young was lower than the older patient group ( $p < 0.0001$ ) and  
224 consisted of ~100IU / day, whereas the older patients received an average of 450 IU / day (Fig. 5a). The  
225 effect of rFSH dose on FSH receptor or LH receptor expression was determined by administering either  
226 300 or 600 IU / day for ~10 days during an IVF treatment cycle (Fig. 6b). The patients were matched for  
227 age (40-44y), ovarian reserve (AFC group E,  $< 5$  follicles, both ovaries combined), Anti-Mullerian  
228 hormone (AMH) ( $< 3.2$  p/mol/L), and size and range of follicles (10-22 mm). There was no significant  
229 difference in the effect of dose of rFSH on FSH receptor or LH receptor expression (Fig. 6b).

### 230 **3.4 The relationship between BMPR1B and FSH receptor and LH receptor density as the** 231 **ovarian reserve is depleted**

232 As the patient age increased, and the ovarian reserve declined, the correlation between BMPR1B and LH  
233 receptor density sequentially increased ( $R = 0.872$ ,  $p = 0.0063$ , Fig. 7a). The reversal of this correlation was  
234 observed between FSH receptor and BMPR1B. In the young patient group, the BMPR1B was aligned with  
235 the FSH receptor expression ( $R = 0.75$ ,  $p = 0.0044$ , Fig. 7b), followed by a complete dissociation in the  
236 relationship as ovarian reserve deteriorated ( $p = 0.4$ ). FSH receptor and LH receptor expression were not  
237 strongly correlated (Supplementary data Fig. S1).

238

## 239 **4. Discussion**

240 Depletion of the ovarian reserve of primordial follicles has a considerable impact on young and older  
241 women. High achieving women, in particular, delay having children while they establish careers in  
242 business, elite sport, music, and other pursuits, only to find that the poor quality and reduced quantity of

243 their remaining oocytes prevents them from reproducing (Australian-Bureau-of-Statistics, 2001,Australian-  
244 Bureau-Statistics and 2006). In addition, the one child government policy in China has relaxed, creating  
245 the demand for a second child for older women. This novel, human, *in vivo* analysis reports the change in  
246 granulosa FSH receptor and LH receptor expression that occurs as the ovarian reserve is depleted in the  
247 young compared to the older women during IVF treatment. The present study is robust because of the in  
248 depth analysis of individual follicle sizes rather than pooled sample and the large number of granulosa  
249 cells analysed from each follicle via immunofluorescent flow cytometry.

250

251 The major findings of the study reveal that: 1. the young patient FSH receptor and LH receptor profile of  
252 expression on the granulosa cells collected from different size follicles were similar to the expression  
253 profile reported in natural cycles in women and sheep (Fig. 4) (Jeppesen et al., 2012,Regan et al., 2015).

254 Conversely, in the older patients with poor ovarian reserve, down-regulation of the FSH receptor and LH  
255 receptor was not observed which may indicate reduced or delayed maturation of the granulosa cells in  
256 preparation for ovulation of the oocyte.

257 2. The overall effect of the disruption to receptor expression resulted in a shift in the strong correlation of  
258 BMPR1B and FSH receptor density in the young as the ovarian reserve was depleted. Conversely, the  
259 BMPR1B expression became strongly aligned with the density of LH receptor in the older women. This  
260 change indicates reduced or delayed granulosa cell luteinisation, and confirmed by the reduced  
261 progesterone in the poor ovarian reserve patients (Fig. 3 & 7). 3. In addition, impaired oocyte development  
262 was associated with a lower LH receptor density and a lack of essential down-regulation of the FSH and  
263 LH receptor; however additional contributing factors would also prevail. The poor oocyte quality was  
264 evident by the poor fertilisation rate, reduced pregnancy, and lower live birth rate (Table 1). 4. The  
265 gonadotrophin FSH has been strongly associated with the regulatory control of cyclic folliculogenesis  
266 (Yong, Baird, Yates et al., 1992). However, the mechanism regulating the expression of FSH receptor and  
267 LH receptor functions independently, *in vivo*, from the dose of gonadotrophin rFSH rather than in response  
268 to it in an *in vitro* scenario.

269

270 IVF stimulation of patients using rFSH extends the window of recruitment that promotes multiple  
271 dominant follicles. These follicles have been shown to grow at a similar rate compared to a natural cycle  
272 (Baird, 1987, Fauser and Van Heusden, 1997). We report that in patients matched for ovarian reserve, BMI,  
273 AMH, age, and follicle number and size, the impact of rFSH dose on receptor expression *in vivo* was not  
274 significant (Fig. 6b). Whereas, isolated granulosa cells in culture, responded differently to high  
275 concentrations of rFSH (Zhang and Roy, 2004). The time of dominant follicle selection and steroidogenic  
276 maturation would therefore progress in a similar manner to a natural cycle.

277

278 The high level of FSH receptors in the smallest follicles provides further support of a spatio-temporal  
279 range of differentiation of the follicles at the time of collection in an IVF cycle even though the LH surge  
280 has taken place. Indeed, the small follicles of 4-8 mm were morphologically granular in appearance with a  
281 relatively large nucleus compared to the cytoplasm, which indicates a follicular granulosa cell, and not a  
282 granulosa luteal cell (Fig. 2) (Nottola, Heyn, Camboni et al., 2006). It is therefore reasonable to relate the  
283 observed FSH receptor and LH receptor density profile to the underlying stages of antral follicle  
284 development, as indicated by the size of the follicle. Two critical stages of follicle development are  
285 dominant follicle selection (8 mm) and internalisation of receptors in an 'ovulatory follicle' (largest  
286 follicle).

287

288 The follicles were also shown to have a different follicular fluid steroid output based on size (Fig. 2d and  
289 e). The granulosa cells from a small follicle produced less oestrogen and progesterone compared to a larger  
290 'ovulatory follicle', even though the follicles were exposed to the same gonadotrophin stimulation and the  
291 equivalent LH surge-ovulation-trigger, human chorionic gonadotrophin (HCG) prior to collection.  
292 Therefore, it remains that the mechanism regulating the expression of these receptors is independent, *in*  
293 *vivo*, from the gonadotrophin rFSH dose. In further support of this, post-transcriptional mechanisms have  
294 been identified in the regulation of these receptors (Menon and Menon, 2012).

295

296 In contrast, the clinical administration of HCG or the equivalent natural cycle LH-surge, induces many  
297 maturation changes referred to as luteinisation of the granulosa cell. These changes include degradation or  
298 internalisation of the BMPRI1B (Regan et al., 2016, Regan et al., 2015), LH receptor (Menon and Menon,  
299 2012), and FSH receptor (Regan et al., 2015), cytoskeletal reorganisation of the granulosa cell, cessation  
300 of mitogenic proliferation, cumulus expansion, gap junction closure, resumption of meiosis, and general  
301 maturation of the oocyte (Izadyar, Zeinstra and Bevers, 1998, Fan, Liu, Shimada et al., 2009). Importantly,  
302 the lack of essential down-regulation of the receptors indicate disruption of this process, and may affect the  
303 LH surge-induced changes taking place at this time (Lyga, Volpe, Werthmann et al., 2016, Kash and  
304 Menon, 1998). However, the extent of luteinisation is dependent on the stage of development of the  
305 follicle at the time of the HCG/LH surge, as shown in Fig. 2, and also reported previously (Nottola et al.,  
306 2006). In particular, the level of LH receptor density during follicle development was consistent except for  
307 the largest ‘ovulatory’ follicles (Fig. 4). These large follicles are developmentally receptive to the LH  
308 surge-induced internalisation of the LH receptor (Menon and Menon, 2012) even though they were  
309 exposed to the same dose of HCG/LH surge trigger during IVF treatment (Fig. 2). This difference in  
310 response based on follicle size further supports our finding of differential maturation of the follicles in an  
311 IVF cycle. The ovulatory follicle internalisation of the LH receptor was also evident in natural cycles of  
312 women (Jeppesen et al., 2012) and in sheep studies conducted by our research group using the same  
313 analysis techniques (Regan et al., 2015).

314  
315 The granulosa cells in the current study were collected from individual follicles and analysed immediately  
316 (fresh not frozen) to reduce any potential experiment-induced internalisation of receptors. Whereas,  
317 granulosa cells collected for culture were not responsive to rFSH stimulation (Breckwoldt, Selvaraj,  
318 Aharoni et al., 1996, Gutierrez, Campbell and Webb, 1997). The desensitised cells start to re-express  
319 receptors after 2-5 days of culture (Ophir, Yung, Maman et al., 2014). The lack of responsiveness of the  
320 FSH receptors to rFSH stimulation is consistent with our *in vivo* finding because typically, the largest,  
321 more prominent follicle will be collected for cell culture as it contains the greatest number of cells, and is

322 pooled with any smaller follicles. The pooling of the large and smaller follicles would mask the  
323 differences presented in the current study, and as previously documented (Regan et al., 2016).

324  
325 The acquisition of LH receptors on the granulosa cells coincides with the first down-regulation of FSH  
326 receptors to promote dominant follicle selection (Maman et al., 2012,Jeppesen et al., 2012,Rice, Ojha,  
327 Whitehead et al., 2007,Sen, Prizant, Light et al., 2014). A high expression of the FSH receptor mRNA has  
328 been reported in granulosa cells from small antral follicles of ~6 mm, collected from a wide range of  
329 patients in natural cycles (7-38 years) (Jeppesen et al., 2012). The elevated FSH receptor levels were  
330 followed by a lower level of expression in ~9 and 15 mm follicles. The current study confirms and expands  
331 their data by reporting that at the time of dominant follicle selection (~8 mm follicles), a high level of  
332 granulosa FSH receptor density is observed in both the young and the older women regardless of ovarian  
333 reserve (Fig. 5). This is consistent with a metabolic change from high FSH dependency before dominant  
334 follicle selection and LH receptor expression induced by the high FSH receptor density (Rice et al.,  
335 2007,Sen et al., 2014), in both a natural cycle and a stimulated IVF cycle (Jeppesen et al., 2012).  
336 Therefore, the mechanism regulating early FSH receptor expression appears to be independent of either  
337 pituitary secreted FSH in a natural cycle or dose of rFSH received during IVF treatment.

338  
339 This novel finding is consistent with the *in vivo*, insignificant effect of the dose of rFSH and  
340 receptor expression of either the FSH or LH receptor (Fig. 6b). Furthermore, it may be expected  
341 that if FSH receptors induce LH receptor expression (Rice et al., 2007,Sen et al., 2014), then a  
342 reduced ovarian reserve should not affect the expression of the LH receptors in the older women.  
343 However, it is apparent that the LH receptor density was reduced after dominant follicle selection  
344 when compared to the young patient group (16 mm, Fig. 4b). Therefore, other factors must  
345 influence the expression of LH receptors. Moreover, a reduced LH receptor density in the follicles  
346 in older women may be a contributing factor in the reduced quality of the oocyte and the resulting  
347 poor pregnancy rate (Table 1). The 35-38 year old group of women have a greater percentage of



348 women with the better B grade (45%) of ovarian reserve and a much lower rate of women with  
349 the poor ovarian reserve group of D (32.6%) and E group (5.9%) compared to the older women  
350 from 39-45y, (Fig. 8). Age has a direct impact on the frequency of chromosomal errors and  
351 fertility (Handyside et al., 2012). We demonstrate that the reduced fertility as we age is  
352 associated with a patient's ovarian reserve and the dysregulation of receptor signalling.

353  
354 Expression of the type 1 transforming growth factor beta (TGF $\beta$ ) superfamily receptor, BMPR1B, has  
355 been shown to be down-regulated at the two critical stages of dominant follicle selection and prior to  
356 ovulation (Regan et al., 2016, Regan et al., 2015, Nakamura, Otsuka, Inagaki et al., 2012, Ogura Nose,  
357 Yoshino, Osuga et al., 2012, Miyoshi, Otsuka, Inagaki et al., 2007, Erickson and Shimasaki, 2003). The  
358 BMPR1B is the common receptor for several BMP ligands such as BMP 2, 4, 6, 7, and 15 (Knight and  
359 Glister, 2003), and inhibits progesterone synthesis in favour of oestrogen synthesis during follicular  
360 growth. The BMP-induced inhibition prevents the onset of the LH surge-induced luteinisation (Pierre,  
361 Pisselet, Dupont et al., 2004, Tajima, Dantes, Yao et al., 2003, Val, Lefrançois-Martinez, Veyssi re et  
362 al., 2003, Abdo, Hisheh, Arfuso et al., 2008). In particular, at the time of the LH surge/ HCG trigger, the  
363 granulosa BMPR1B, FSH receptor, and LH receptor density of the largest follicles was shown to be  
364 down-regulated in sheep during a natural cycle (Regan et al., 2015), in women receiving IVF treatment  
365 (Regan et al., 2016), and in the current study (Fig. 4). The lack of down-regulation of the FSH receptor,  
366 LH receptor (Fig. 4), and BMPR1B (Regan et al., 2016) may delay or inhibit the production of  
367 progesterone and expression of progesterone receptors (Shimada, Nishibori, Isobe et al., 2003), (Fig. 3),  
368 which would directly impair germinal vesicle breakdown and meiotic resumption in the oocyte (Shimada  
369 and Terada, 2002). The inhibition of gonadotrophin-induced progesterone receptor formation has been  
370 linked to infertility and porcine oocyte quality (Park-Sarge and Mayo, 1994), and had a negative effect on  
371 the ovarian response to gonadotropin stimulation (Cai et al., 2007). Therefore, it is essential that the BMP  
372 inhibition is attenuated to promote maturation of the oocyte.

373 In the current study, a strong correlation between BMPR1B and FSH receptor expression was observed in  
374 the young patients. The FSH receptor is positively regulated by the BMP ligands 6 and 7 (Chen, Yu, Wang

375 et al., 2008, Shi, Yoshino, Osuga et al., 2010, Shi, Yoshino, Osuga et al., 2009), and as the ovarian reserve  
376 was depleted, the correlation was weakened (Fig. 7a). Significantly, the disruption to the FSH receptor  
377 expression was replaced with a strong correlation between BMPRII and the LH receptor expression in the  
378 older women (Fig. 7b). This shift further supports an association between dysregulated receptor expression  
379 and reduced maturation of the granulosa cells surrounding the oocyte in older women. Future research may  
380 involve restoring the optimum receptor density profile during the maturation phase to improve oocyte  
381 quality.

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### 385 **Authors' roles**

386 SLPR conceived the study, experimental design, conducted all experiments, the analysis and interpretation  
387 of data, wrote the first draft of the manuscript and the final version of the paper. Obtained informed  
388 consent from patients and ethics approval. PK supervised, interpretation of data, contributed to the draft of  
389 the manuscript, interpretation of data, and critically revised the manuscript. JLY supervised, participated in  
390 the study design, participated in obtaining granulosa cells, interpretation of data, and critically revised the  
391 manuscript. JDS supervised, participated in obtaining granulosa cells, participated in the design of the  
392 study, and critically revised the manuscript. YL supervised, participated in the study design, obtained  
393 informed consents from patients and ethics approval, and critically revised the manuscript. FA supervised,  
394 contributed to the draft of the manuscript, interpretation of data, and critically revised the manuscript. AD  
395 supervised, participated in the study design, interpretation of data, contributed to the draft of the  
396 manuscript, and critically revised the manuscript. GA supervised, conceived the study, participated in the  
397 study design, interpretation of data, and critically revised the manuscript.

398

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#### 404 **Conflict of interest**

405 The authors declare that there is no conflict of interest that could be perceived as prejudicing the  
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#### 407 **References**

- 408 [1] McGee, E.A. and Hsueh, A.J.W., 2000. Initial and cyclic recruitment of ovarian follicles,  
409 Endocrine Reviews. 21, 200-214.
- 410 [2] Hillier, S.G., 2001. Gonadotropic control of ovarian follicular growth and development,  
411 Molecular and Cellular Endocrinology. 179, 39-46.
- 412 [3] Baird, D.T., 1987. A model for follicular selection and ovulation: lessons from  
413 superovulation, The Journal of steroid biochemistry. 27, 15-23.
- 414 [4] Baerwald, A.R., Adams, G.P. and Pierson, R.A., 2012. Ovarian antral folliculogenesis  
415 during the human menstrual cycle: a review, Human Reproduction Update. 18, 73-91.
- 416 [5] Loumaye, E., Engrand, P., Shoham, Z., Hillier, S.G. and Baird, D.T., 2003. Clinical  
417 evidence for an LH ceiling?, Human Reproduction. 18, 2719-2720.
- 418 [6] Almog, B., Shehata, F., Shalom-Paz, E., Tan, S.L. and Tulandi, T., 2011. Age-related  
419 normogram for antral follicle count: McGill reference guide, Fertility and Sterility. 95,  
420 663-666.
- 421 [7] Santoro, N., Isaac, B., Neal-Perry, G., Adel, T., Weingart, L., Nussbaum, A., Thakur, S.,  
422 Jinnai, H., Khosla, N. and Barad, D., 2003. Impaired Folliculogenesis and Ovulation in  
423 Older Reproductive Aged Women, The Journal of Clinical Endocrinology & Metabolism.  
424 88, 5502-5509.

- 425 [8] González-Fernández, R., Peña, Ó., Hernández, J., Martín-Vasallo, P., Palumbo, A. and  
426 Ávila, J., 2010. FSH receptor, KL1/2, P450, and PAPP genes in granulosa-lutein cells  
427 from in vitro fertilization patients show a different expression pattern depending on the  
428 infertility diagnosis, *Fertility and Sterility*. 94, 99-104.
- 429 [9] Seifer, D.B., DeJesus, V. and Hubbard, K., 2002. Mitochondrial deletions in luteinized  
430 granulosa cells as a function of age in women undergoing in vitro fertilization, *Fertility  
431 and Sterility*. 78, 1046-1048.
- 432 [10] Cai, J., Lou, H., Dong, M., Lu, X., Zhu, Y., Gao, H. and Huang, H., 2007. Poor ovarian  
433 response to gonadotropin stimulation is associated with low expression of follicle-  
434 stimulating hormone receptor in granulosa cells, *Fertility and Sterility*. 87, 1350-1356.
- 435 [11] Maman, E., Yung, Y., Kedem, A., Yerushalmi, G.M., Konopnicki, S., Cohen, B., Dor, J.  
436 and Hourvitz, A., 2012. High expression of luteinizing hormone receptors messenger  
437 RNA by human cumulus granulosa cells is in correlation with decreased fertilization,  
438 *Fertility and Sterility*. 97, 592-598.
- 439 [12] Handyside, A.H., Montag, M., Magli, M.C., Repping, S., Harper, J., Schmutzler, A.,  
440 Vesela, K., Gianaroli, L. and Geraedts, J., 2012. Multiple meiotic errors caused by  
441 predivision of chromatids in women of advanced maternal age undergoing in vitro  
442 fertilisation, *European Journal of Human Genetics*. 20, 742-747.
- 443 [13] Jeppesen, J.V., Kristensen, S.G., Nielsen, M.E., Humaidan, P., Dal Canto, M., Fadini, R.,  
444 Schmidt, K.T., Ernst, E. and Yding Andersen, C., 2012. Lh-receptor gene expression in  
445 human granulosa and cumulus cells from antral and preovulatory follicles, *The Journal of  
446 Clinical Endocrinology & Metabolism*. 97, E1524-E1531.
- 447 [14] Pidoux, G., Gerbaud, P., Tsatsaris, V., Marpeau, O., Ferreira, F., Meduri, G.,  
448 Guibourdenche, J., Badet, J., Evain-Brion, D. and Frendo, J.-L., 2007. Biochemical

- 449 characterization and modulation of LH/CG—receptor during human trophoblast  
450 differentiation, *Journal of cellular physiology*. 212, 26-35.
- 451 [15] Ascoli, M., Fanelli F and DL., S., 2002. The lutropin/choriogonadotropin receptor, a 2002  
452 perspective, *Endocrine reviews*. Vol.23(2), 141-174
- 453 [16] Regan, S.L.P., Knight, P.G., Yovich, J., Stanger, J., Leung, Y., Arfuso, F., Dharmarajan,  
454 A. and Almahbobi, G., 2016. Dysregulation of granulosa bone morphogenetic protein  
455 receptor 1B density is associated with reduced ovarian reserve and the age-related decline  
456 in human fertility, *Molecular and Cellular Endocrinology*. 425, 84-93.
- 457 [17] Regan, S.L.P., McFarlane, J.R., O'Shea, T., Andronicos, N., Arfuso, F., Dharmarajan, A.  
458 and Almahbobi, G., 2015. Flow cytometric analysis of FSHR, BMRR1B, LHR and  
459 apoptosis in granulosa cells and ovulation rate in merino sheep, *Reproduction*. 150, 151-  
460 163.
- 461 [18] Yovich, J., Stanger, J. and Hinchliffe, P., 2012. Targeted gonadotrophin stimulation using  
462 the PIVET algorithm markedly reduces the risk of OHSS, *Reproductive BioMedicine*  
463 *Online*. 24, 281-292.
- 464 [19] Hansen, K.R., Hodnett, G.M., Knowlton, N. and Craig, L.B., 2011. Correlation of ovarian  
465 reserve tests with histologically determined primordial follicle number, *Fertility and*  
466 *Sterility*. 95, 170-175.
- 467 [20] Lujan, M.E., Jarrett, B.Y., Brooks, E.D., Reines, J.K., Peppin, A.K., Muhn, N., Haider, E.,  
468 Pierson, R.A. and Chizen, D.R., 2013. Updated ultrasound criteria for polycystic ovary  
469 syndrome: reliable thresholds for elevated follicle population and ovarian volume, *Human*  
470 *Reproduction*. 28, 1361-1368.
- 471 [21] Abir, R., Ben-Haroush, A., Melamed, N., Felz, C., Krissi, H. and Fisch, B., 2008.  
472 Expression of bone morphogenetic proteins 4 and 7 and their receptors IA, IB, and II in  
473 human ovaries from fetuses and adults, *Fertility and Sterility*. 89, 1430-1440.

- 474 [22] Haÿ, E., Lemonnier, J., Fromigué, O., Guénou, H. and Marie, P.J., 2004. Bone  
475 morphogenetic protein receptor signaling mediates apoptosis independently of  
476 differentiation in osteoblastic cells, *Journal of Biological Chemistry*. 279, 1650-1658.
- 477 [23] Gao, S., De Geyter, C., Kossowska, K. and Zhang, H., 2007. FSH stimulates the  
478 expression of the ADAMTS-16 protease in mature human ovarian follicles, *Molecular*  
479 *Human Reproduction*. 13, 465-471.
- 480 [24] Whiteman, G., Boldt J, Martinez J and Pantazis C, 1991. Flow cytometric analysis of  
481 induced human graafian follicles.I. Demonstration and sorting of two luteinized cell  
482 populations, *Fertility and Sterility*. 56, 259-263.
- 483 [25] Al-Samerria, S. and Almahbobi, G., 2014 Three-dimensional image analysis to quantify  
484 the temporal-spatial expression of cellular receptors, *Journal of Medical and*  
485 *Bioengineering* 3, 179-182.
- 486 [26] Hermann, B.P. and Heckert, L.L., 2007. Transcriptional regulation of the FSH receptor:  
487 New perspectives, *Molecular and Cellular Endocrinology*. 260–262, 100-108.
- 488 [27] Motta, P.M., 1969. Electron microscope study on the human lutein cell with special  
489 reference to its secretory activity., *Z Zelforsch Mikrosk Anat*. 98, 233-245.
- 490 [28] Nottola, S., Familiari G, Micara G, Aragona C, Motta PM, 1991 The ultrastructure of  
491 human cumulus-corona cells at the time of fertilization and early embryogenesis. A  
492 scanning and transmission electron microscopic study in an in vitro fertilization program,  
493 *Arch Histol Cytol*. . May;54(2):145-61.
- 494 [29] Westergaard, L., Christensen, I.J. and McNatty, K.P., 1986. Steroid levels in ovarian  
495 follicular fluid related to follicle size and health status during the normal menstrual cycle  
496 in women, *Human Reproduction*. 1, 227-232.
- 497 [30] Australian-Bureau-of-Statistics, 2001. Australian social trends 2001 Canberra.

- 498 [31] Australian-Bureau-Statistics and 2006. Births Australia 2005 ABS, Canberra., cat. no.  
499 33010.
- 500 [32] Yong, E.L., Baird, D.T., Yates, R., Reichert, L.E. and Hillier, S.G., 1992. Hormonal  
501 regulation of the growth and steroidogenic function of human granulosa cells, *The Journal*  
502 *of clinical endocrinology and metabolism.* 74, 842-849.
- 503 [33] Fauser, B.C. and Van Heusden, A.M., 1997. Manipulation of human ovarian function:  
504 physiological concepts and clinical consequences, *Endocrine reviews.* 18, 71-106.
- 505 [34] Zhang, Y.-M. and Roy, S.K., 2004. Downregulation of follicle-stimulating hormone (fsh)-  
506 receptor messenger rna levels in the hamster ovary: Effect of the endogenous and  
507 exogenous FSH, *Biology of Reproduction.* 70, 1580-1588.
- 508 [35] Nottola, S.A., Heyn, R., Camboni, A., Correr, S. and Macchiarelli, G., 2006.  
509 Ultrastructural characteristics of human granulosa cells in a coculture system for in vitro  
510 fertilization, *Microscopy Research and Technique.* 69, 508-516.
- 511 [36] Menon, K.M.J. and Menon, B., 2012. Structure, function and regulation of gonadotropin  
512 receptors- a perspective, *Molecular and Cellular Endocrinology.* 356, 88-97.
- 513 [37] Izadyar, F., Zeinstra, E. and Bevers, M.M., 1998. Follicle-stimulating hormone and  
514 growth hormone act differently on nuclear maturation while both enhance developmental  
515 competence of in vitro matured bovine oocytes, *Molecular reproduction and development.*  
516 51, 339-345.
- 517 [38] Fan, H., Liu, Z., Shimada, M., Sterneck, E., Johnson, P.F., Hedrick, S.M. and Richards,  
518 J.S., 2009. MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility,  
519 *Science.*, 324, 938-941.
- 520 [39] Lyga, S., Volpe, S., Werthmann, R.C., Götz, K., Sungkaworn, T., J., L.M. and Calebiro,  
521 D., 2016. Persistent cAMP signaling by internalized LH receptors in ovarian follicles,  
522 *Endocrinology.* 157(4), 1613-1621.

- 523 [40] Kash, J.C. and Menon, K.M.J., 1998. Identification of a hormonally regulated luteinizing  
524 hormone/human chorionic gonadotropin receptor mRNA binding protein: Increased mRNA  
525 binding during receptor down-regulation, *Journal of Biological Chemistry*. 273, 10658-  
526 10664.
- 527 [41] Breckwoldt, M., Selvaraj, N., Aharoni, D., Barash, A., Segal, I., Insler, V. and  
528 Amsterdam, A., 1996. Cell proliferation and apoptosis: Expression of Ad4-BP/cytochrome  
529 P450 side chain cleavage enzyme and induction of cell death in long-term cultures of  
530 human granulosa cells, *Molecular Human Reproduction*. 2, 391-400.
- 531 [42] Gutierrez, C., Campbell, B. and Webb, R., 1997. Development of a long-term bovine  
532 granulosa cell culture system: induction and maintenance of estradiol production, response  
533 to follicle-stimulating hormone, and morphological characteristics, *Biology of*  
534 *Reproduction*. 56, 608 - 616.
- 535 [43] Ophir, L., Yung, Y., Maman, E., Rubinstein, N., Yerushalmi, G.M., Haas, J., Barzilay, E.  
536 and Hourvitz, A., 2014. Establishment and validation of a model for non-luteinized human  
537 mural granulosa cell culture, *Molecular and Cellular Endocrinology*. 384, 165-174.
- 538 [44] Rice, S., Ojha, K., Whitehead, S. and Mason, H., 2007. Stage-specific expression of  
539 androgen receptor, follicle-stimulating hormone receptor, and anti-müllerian hormone  
540 type II receptor in single, isolated, human preantral follicles: Relevance to polycystic  
541 ovaries, *The Journal of Clinical Endocrinology & Metabolism*. 92, 1034-1040.
- 542 [45] Sen, A., Prizant, H., Light, A., Biswas, A., Hayes, E., Lee, H.-J., Barad, D., Gleicher, N.  
543 and Hammes, S.R., 2014. Androgens regulate ovarian follicular development by  
544 increasing follicle stimulating hormone receptor and microRNA-125b expression,  
545 *Proceedings of the National Academy of Sciences*. 111(8), 3008-3013.
- 546 [46] Nakamura, E., Otsuka, F., Inagaki, K., Miyoshi, T., Matsumoto, Y., Ogura, K.,  
547 Tsukamoto, N., Takeda, M. and Makino, H., 2012. Mutual regulation of growth hormone



- 548 and bone morphogenetic protein system in steroidogenesis by rat granulosa cells,  
549 Endocrinology. 153, 469-480.
- 550 [47] Ogura Nose, S., Yoshino, O., Osuga, Y., Shi, J., Hiroi, H., Yano, T. and Taketani, Y.,  
551 2012. Anti-Mullerian hormone (AMH) is induced by bone morphogenetic protein (BMP)  
552 cytokines in human granulosa cells, European journal of obstetrics & gynecology and  
553 reproductive biology. 164, 44-47.
- 554 [48] Miyoshi, T., Otsuka, F., Inagaki, K., Otani, H., Takeda, M., Suzuki, J., Goto, J., Ogura, T.  
555 and Makino, H., 2007. Differential regulation of steroidogenesis by bone morphogenetic  
556 proteins in granulosa cells: Involvement of extracellularly regulated kinase signaling and  
557 oocyte actions in follicle-stimulating hormone-induced estrogen production,  
558 Endocrinology. 148, 337-345.
- 559 [49] Erickson, G. and Shimasaki, S., 2003. The spatiotemporal expression pattern of the bone  
560 morphogenetic protein family in rat ovary cell types during the estrous cycle, Reprod Biol  
561 Endocrinol. 1(9), 1-20.
- 562 [50] Knight, P.G. and Glistler, C., 2003. Local roles of TGF- $\beta$  superfamily members in the  
563 control of ovarian follicle development, Animal Reproduction Science. 78, 165-183.
- 564 [51] Pierre, A., Pisselet, C., Dupont, J., Mandon-Pepin, B., Monniaux, D., Monget, P. and  
565 Fabre, S., 2004. Molecular basis of bone morphogenetic protein-4 inhibitory action on  
566 progesterone secretion by ovine granulosa cells, J Mol Endocrinol. 33, 805 - 814.
- 567 [52] Tajima, K., Dantes, A., Yao, Z., Sorokina, K., Kotsuji, F., Seger, R. and Amsterdam, A.,  
568 2003. Down-regulation of steroidogenic response to gonadotropins in human and rat  
569 preovulatory granulosa cells involves mitogen-activated protein kinase activation and  
570 modulation of DAX-1 and steroidogenic factor-1, The Journal of clinical endocrinology  
571 and metabolism. 88, 2288-2299.

- 572 [53] Val, P., Lefrançois-Martinez, A.-M., Veyssi re, G. and Martinez, A., 2003. SF-1 a  
573 key player in the development and differentiation of steroidogenic tissues, Nuclear  
574 receptor. 1, 8-8.
- 575 [54] Abdo, M., Hisheh, S., Arfuso, F. and Dharmarajan, A., 2008. The expression of tumor  
576 necrosis factor-alpha, its receptors and steroidogenic acute regulatory protein during  
577 corpus luteum regression, Reproductive Biology and Endocrinology : RB&E. 6, 1-11.
- 578 [55] Shimada, M., Nishibori, M., Isobe, N., Kawano, N. and Terada, T., 2003. Luteinizing  
579 hormone receptor formation in cumulus cells surrounding porcine oocytes and its role  
580 during meiotic maturation of porcine oocytes, Biology of Reproduction. 68, 1142-1149.
- 581 [56] Shimada, M. and Terada, T., 2002. FSH and LH induce progesterone production and  
582 progesterone receptor synthesis in cumulus cells: a requirement for meiotic resumption in  
583 porcine oocytes, Molecular Human Reproduction. 8, 612-618.
- 584 [57] Park-Sarge, O.-K. and Mayo, K.E., 1994. Molecular biology of endocrine receptors in the  
585 ovary, in: Findlay, J.K. (Ed.), Molecular Biology of the Female Reproductive System.  
586 Academic Press, San Diego, pp. 153-205.
- 587 [58] Chen, A.Q., Yu, S., Wang, Z., Xu, Z. and Yang, Z., 2008. Stage-specific expression of  
588 bone morphogenetic protein type I and type II receptor genes: Effects of follicle-  
589 stimulating hormone on ovine antral follicles, Animal Reproduction Science. 111, 391-  
590 399.
- 591 [59] Shi, J., Yoshino, O., Osuga, Y., Nishii, O., Yano, T. and Taketani, Y., 2010. Bone  
592 morphogenetic protein 7 (BMP-7) increases the expression of follicle-stimulating  
593 hormone (FSH) receptor in human granulosa cells, Fertility and Sterility. 93, 1273-1279.
- 594 [60] Shi, J., Yoshino, O., Osuga, Y., Koga, K., Hirota, Y., Hirata, T., Yano, T., Nishii, O. and  
595 Taketani, Y., 2009. Bone morphogenetic protein-6 stimulates gene expression of follicle-

596 stimulating hormone receptor, inhibin/activin beta subunits, and anti-Müllerian hormone  
597 in human granulosa cells, *Fertility and Sterility*. 92, 1794-1798.

598

599

**\*Highlights (for review)**

- Reproductive ageing is linked to ovarian cellular function and infertility
- Granulosa FSHR and LHR density from 327 ovarian follicles from IVF patients
- Prerequisite FSHR and LHR down-regulation in older patients was not observed
- Ovarian reserve-impaired fertility was associated with lower granulosa cell LHR
- Ovarian reserve was linked to poor oocyte quality; fertilisation and pregnancy rate

**Table**

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

AGE Year	IVF Patient	Total Follicle	BMI	Ovarian Reserve Group Follicles Collected						Fertility %				
				A+	A	B	C	D	E	Failed Fertilisation	Not Pregnant	Pregnant	Live Birth	
23-30	11	95	24.1±4	31	64						0	36	**64	43
35-45	34	232	24.8±5			88	21	99	24		9	52	**39	18
*39-45	19	131	23.9±5			42	5	66	18		17	72	11	6

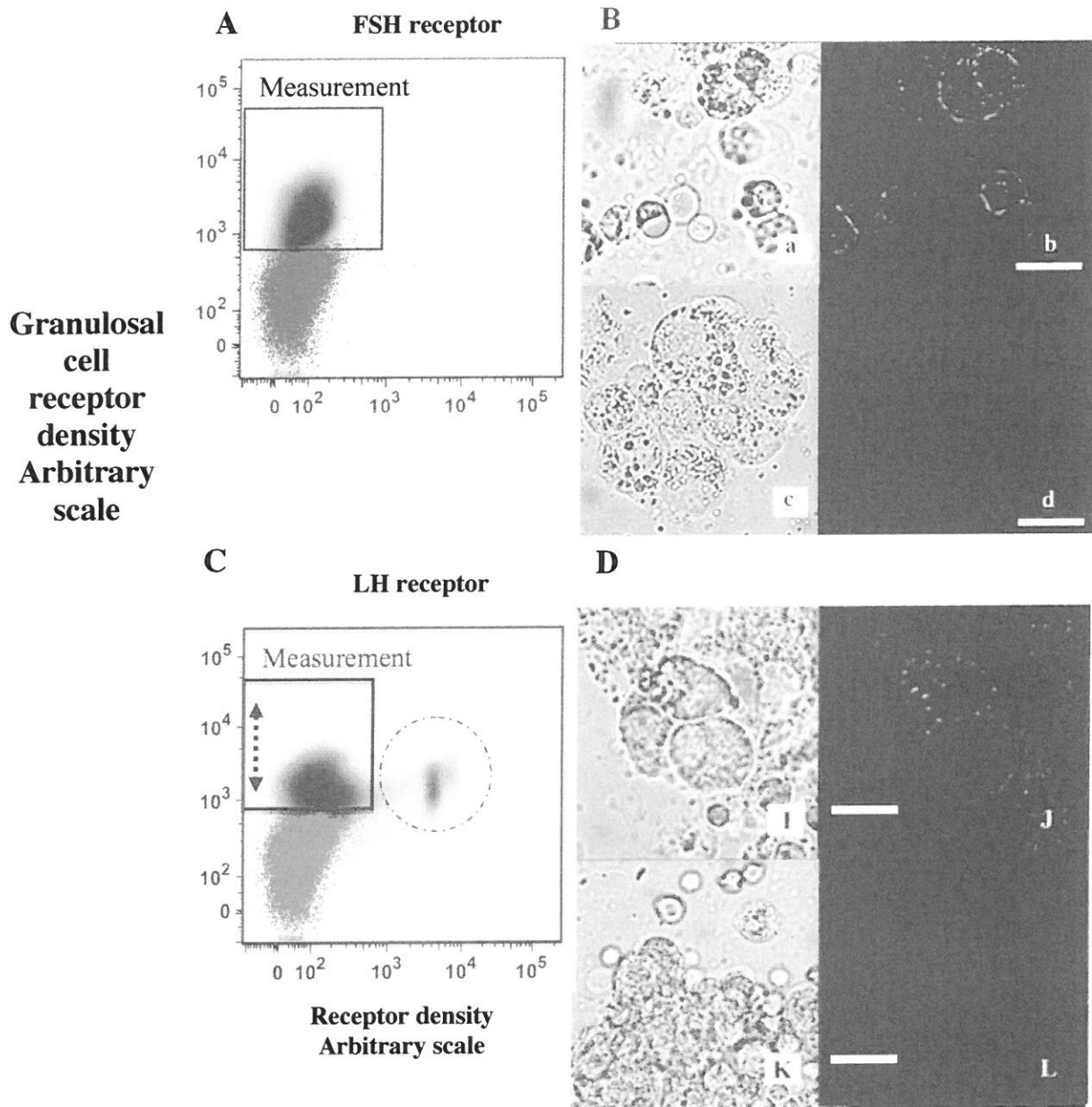
Ovarian reserve measured indirectly by the antral follicle count (AFC). Antral follicle count is the number of

follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12; D =

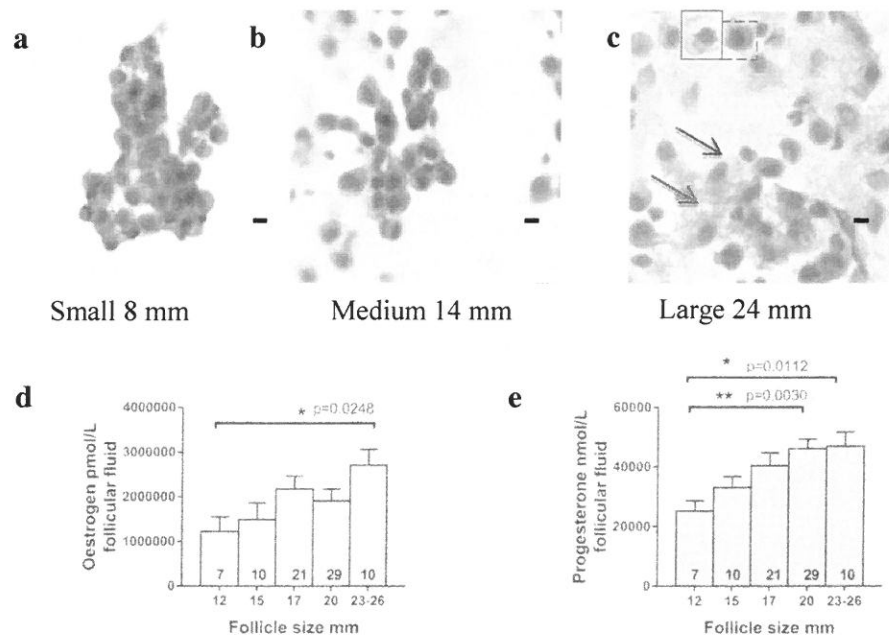
5-8; E = ≤4. Follicle count is based on the combined total from both ovaries. \*Subgroup of older patients.

\*\*1 Ectopic pregnancy. Frozen embryo transfers cycles included.

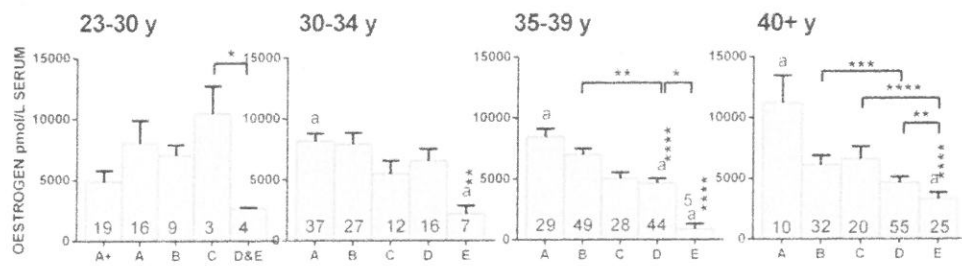
Figure



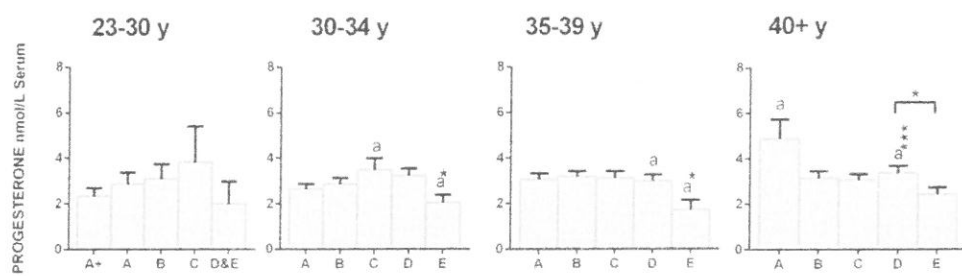
Figure



**a Oestrogen**



**b Progesterone**



**The number of primordial follicles remaining in the ovaries at a given age**

OVARIAN RESERVE

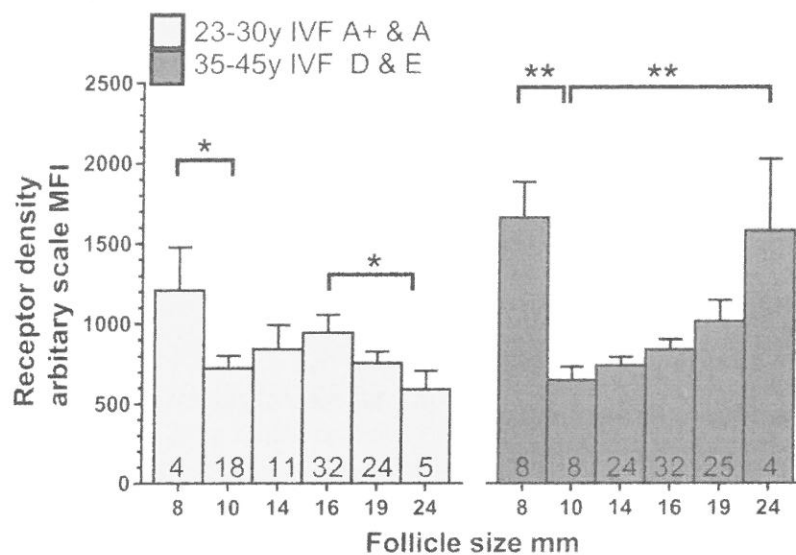
GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR



Figure

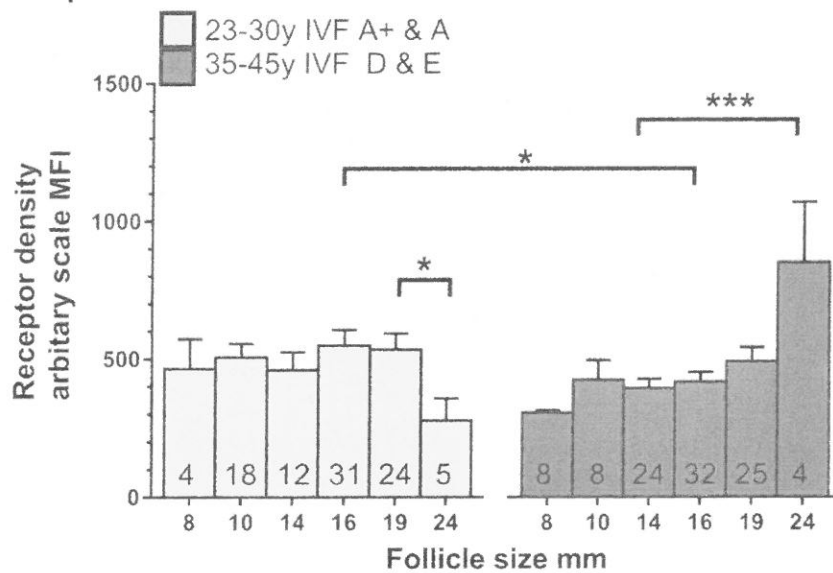
a

FSH receptor



b

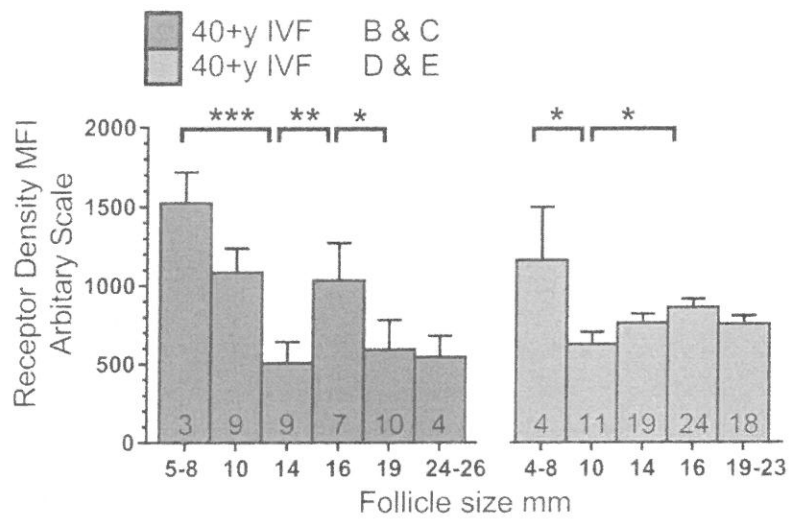
LH receptor



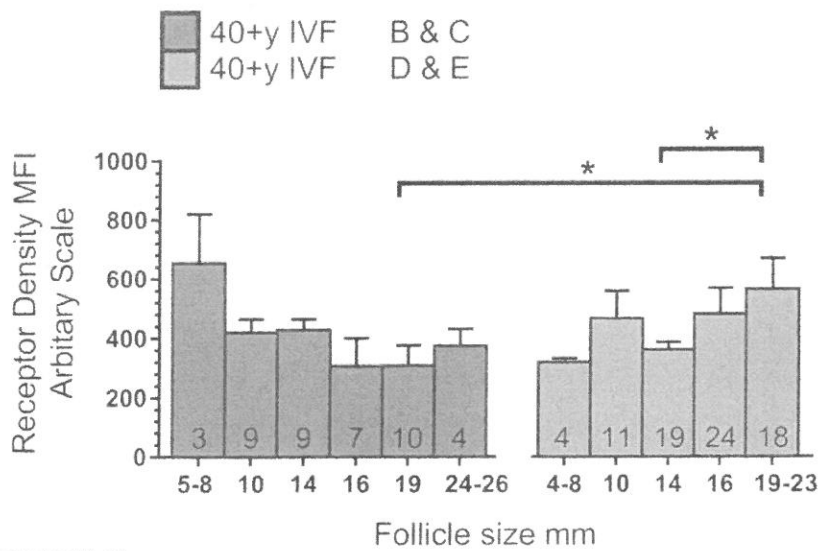
OVARIAN RESERVE

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

**a** FSH Receptor

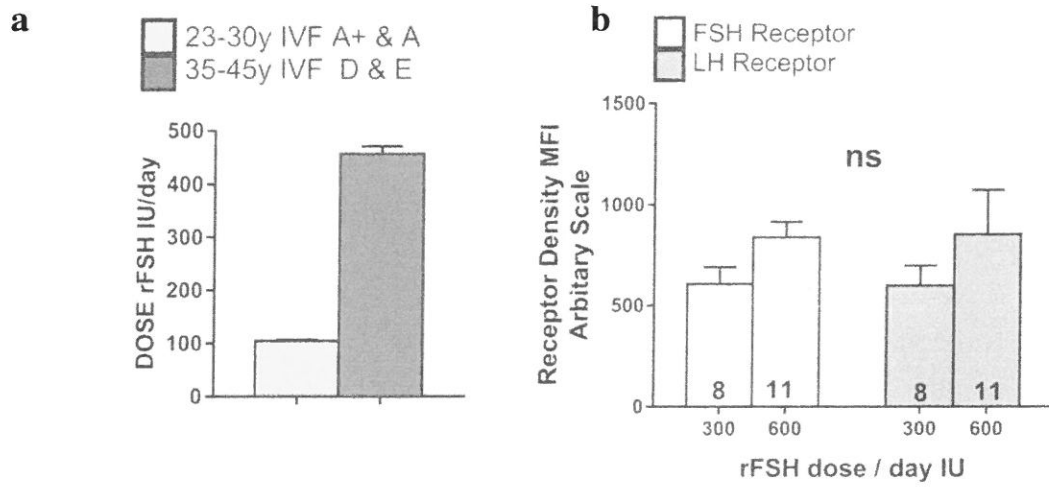


**b** LH Receptor

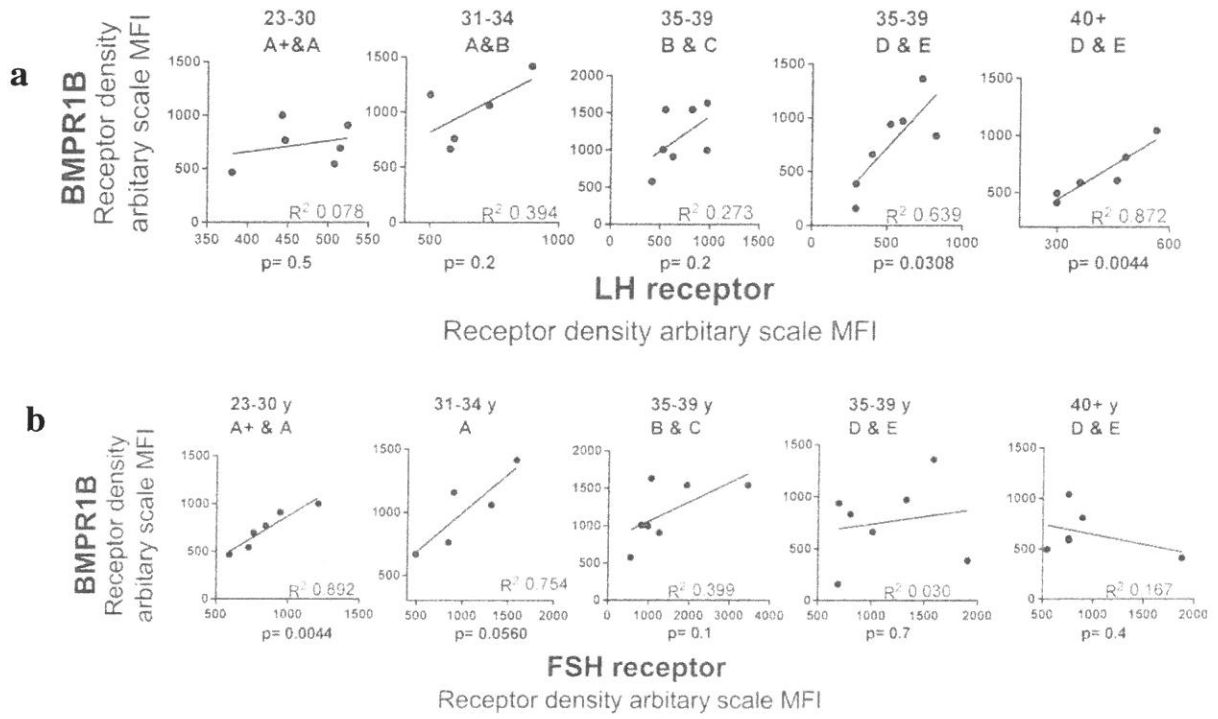


**OVARIAN RESERVE** GOOD **A+** = 30-39 **A** = 20-29 **B** = 13-19 **C** = 9-12 **D** = 5-8 **E** = ≤4 POOR

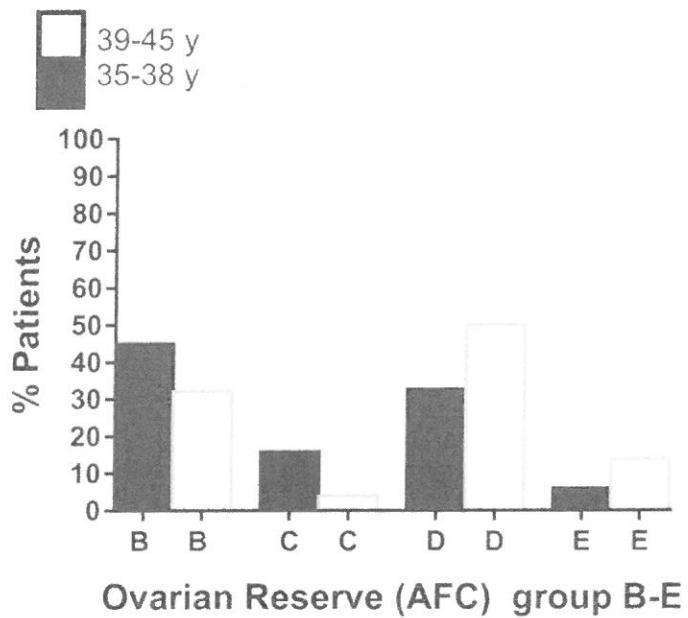
Figure



Figure



Figure



OVARIAN  
RESERVE

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

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

**A.** Live granulosa cells, unstained control for FSH receptor auto-fluorescence (blue) compared to positive fluorescent signal (box). **B.** Live human granulosa cells with positive fluorescence for FSH receptor (a & b), and negative blocking agent for FSH receptor (c & d). Bar 10  $\mu$ m. **C.** Live granulosa cells, unstained control for LH receptor auto-fluorescence (red) compared to positive fluorescent signal (box), Gated and removed CD45 positive cells (circle). **D.** Live human granulosa cells with positive fluorescence for LH receptor (i & j), and negative blocking agent for LH receptor (k & l). Bar 10  $\mu$ m.

**Fig. 2 Comparison of granulosa cells from small, medium, and large human IVF follicles at different stages of maturation.**

a. Small antral follicle of 8 mm, compact morphology with large nucleus compared to cytoplasm. b. Medium size antral follicle of 14 mm, showing some larger granulosa cells, and many still with compact morphology similar to the small antral follicle. c. Granulosa cells from a large antral follicle of 24 mm, showing expanded cytoplasm with many lipid droplet spaces, arrows. Very few granulosa cells with a compact morphology (small dotted box), and the majority with an expanded cytoplasm (larger box). Air dried fresh samples were placed in a fume glass containing formalherhyde for 10 min and then stained with Oil red O, and Harris counterstained. Light microscope image at 40x magnification. Bar = 10  $\mu$ m. d and e. Follicular fluid oestrogen and progesterone concentration from a range of follicle sizes collected 36 hours after the LH ovulation surge induction trigger injection during IVF treatment . The data were subjected to statistical verification using one way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means  $\pm$  SEM. Differences were considered significant if  $p < 0.05$  indicated by an asterisk and \*;  $p < 0.01$  \*\*. The number within the column represents the number of follicles analysed of that size.

**Fig. 3 Peak serum oestrogen (a) and progesterone (b) levels from IVF patients during gonadotrophin stimulated cycles.**

Serum levels were taken at the time of peak oestrogen (just before ovulation and the LH surge) during a stimulated IVF cycle. Ovarian reserve was measured indirectly by the antral follicle count (AFC). Antral

follicle count is the number of follicles between 2-10 mm on day 2-5 of a cycle in both ovaries. The ovarian reserve is then classified into a group level from A to E, good to poor, respectively. The number of patients is indicated, and ranged in age from 23 to 45 years old. The data were subjected to statistical verification using one way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means  $\pm$  SEM. Differences were considered significant if  $p < 0.05$  indicated by an asterisk\*;  $p < 0.01$ \*\*;  $p < 0.005$ \*\*\* and  $p < 0.001$ \*\*\*\*. The letter 'a' was significantly different to the letter a\*\*, with an attached \* indicating the level of significance. The number within the column represents the number of follicles analysed for that group.

**Fig. 4 Granulosa FSH receptor and LH receptor density and ovarian reserve depletion in young compared to older patients.**

**a.** Follicle stimulating hormone (FSH) receptor. **b.** Luteinising hormone (LH) receptor. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). Antral follicle count is the number of follicles from 2-10 mm on day 2-5 of a cycle. Follicle count is based on the combined total from both ovaries. Data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means  $\pm$  S.E.M., and differences were considered significant if \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.005$ . The number within the column represents the number of follicles analysed for that group. The MFI is the average mean fluorescent intensity emitted by the granulosa cells surface receptors. The scale is set the same for all experiments but is arbitrary.

**Fig. 5 Granulosa FSH receptor and LH receptor density and ovarian reserve depletion within the same age group compared to young cohort.**

**a.** Follicle stimulating hormone (FSH) receptor and **b.** luteinising hormone (LH) receptor. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). Antral follicle count is the number of follicles from 2-10 mm on day 2-5 of a cycle. Follicle count is based on the combined total from both ovaries. Data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means  $\pm$  S.E.M., and differences were considered significant if \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.005$ . The number within the column represents the number

of follicles analysed for that group. The MFI is the average mean fluorescent intensity emitted by the granulosa cells surface receptors. The scale is set the same for all experiments but is arbitrary.

**Fig. 6 The comparative effect of rFSH dose on FSH receptor and LH receptor expression**

a. The dose of gonadotrophins rFSH received by young (A+ & A, 23-30y) and older patients groups (D & E, 35-45y) based on ovarian reserve measured by AFC. The number within the column represents the number of follicles analysed for that group. Data were subjected to statistical verification using student t test,  $p = < 0.0001$ . b. The effect of dose of rFSH on granulosa receptor density in patients matched for aged, ovarian reserve, AMH, and size of follicles, 40+ y, with an ovarian reserve of E, an AMH  $< 3.2$ , and follicle size of 10-22 mm. Data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values are means  $\pm$  S.E.M., and differences were considered significant if  $p < 0.05$ . The number within the column represents the number of follicles analysed for that group.

**Fig. 7 Correlation of BMPR1B signalling with FSH receptor and LH receptor as the ovarian reserve is depleted.**

a. FSH receptor and b. LH receptor correlated with BMPR1B signalling. Sequential graphs show increasing age and declining ovarian reserve. Linear regression analysis, R square indicated for each group. Ovarian reserve measured indirectly by the antral follicle count. Antral follicle count is the number of follicles between 2-10 mm on day 2-5 of a cycle. The data points are averages of the receptor expression for that follicle size (ie each dot represents a follicle size from 4 mm 8 mm, 10 mm, 14 mm, 16 mm, 19 mm and 24 mm) and the total number of follicles per age group graph corresponds to: 23-30 y, n= 95 follicles; 31-34 y, n= 43 follicles; 35-39 y, n= 67 follicles; 40+y, n= 77 follicles. Follicle count is based on the combined total from both ovaries. Data were subjected to statistical verification using linear regression Values are means  $\pm$  S.E.M., and differences were considered significant if  $p < 0.05$ .

**Fig. 8 The effect of ovarian reserve measured by AFC on fertility**

The percentage of patients from different age groups based on the remaining primordial reserve of follicles within the ovaries indirectly measured by AFC and grouped from B to E. The younger age range of 35-38 years has a higher percentage of good ovarian reserve (group B) and a smaller percentage of



poor ovarian reserve, and consequently an increased fertility rate (Table 1). The graph shows that the 35-38y group of women have a greater % of women with the best B grade (45%) of ovarian reserve, In addition, this group has a much lower rate of women with the very poor ovarian reserve D (32.6%) and E 5.9%.