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Growth hormone during in vitro fertilization in older women modulates the density of receptors in granulosa cells, with improved pregnancy outcomes.

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Introduction

Ovarian depletion of primordial follicles is a continual natural process from gestation to adulthood, which culminates in the loss of ovarian function and which eventuates in the state of menopause (1, 2). When activated, the primordial follicles grow and develop into small antral follicles, the majority of which succumb to apoptosis (3, 4). At puberty, cyclic increases in circulating follicle stimulating hormone (FSH) recruit a cohort of small antral follicles at the start of each menstrual cycle (3, 5). The follicles grow under the influence of FSH, and express follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR). The activation of FSH and the FSHRs stimulates oestrogen synthesis, which subsequently stimulates proliferation of the granulosa cells and development of the oocyte. (6-9).

The ovulation rate is determined by the stage-specific decrease in pituitary secreted FSH, and results in follicles with insufficient LHRs that succumb to apoptosis (10-12). The follicle continues to grow until pre-ovulatory maturation when proliferation ceases and granulosa cell differentiation occurs in preparation for ovulation of the oocyte.

As the ovarian reserve of primordial follicles is depleted over the reproductive lifespan, regulation of folliculogenesis is altered, which results in decreased fertility (13). Ovarian depletion can be indirectly measured by the number of small antral follicles present at the beginning of a cycle, and is highly correlated to chronological age (14). During IVF treatment, high doses of recombinant human (r) FSH are administered to recruit more of the small antral follicles, and to maintain their growth during pituitary FSH down-regulation (15).

Infertility patients with a poor ovarian reserve have fewer small antral follicles available for recruitment, and higher doses of rFSH are used but with diminishing effectiveness in recruiting more follicles during IVF cycles. In an attempt to improve the pregnancy rate, patients have been offered co-treatment with growth hormone (GH) (15, 16). The patients with a poor response to rFSH treatment represent a large group of patients with critically diminishing ovarian reserve (17, 18). The challenge remains to identify the changes taking place as the ovarian reserve declines, and to find alternative stimulation to provide high quality oocytes for fertilisation.

Earlier studies showed GH treatment *in vivo* and *in vitro,* in conjunction with rFSH increased oocyte survival rate and pregnancy rate (19-22). The granulosa cells, including cumulus cells, as well as the oocyte of antral follicles express growth hormone receptor (GHR) and are therefore able to react to pituitary-derived or ovarian sources of GH (23, 24). With regard to the latter, granulosa cells and the oocyte, but not cumulus and theca cells, have been shown to express GH mRNA (23-27). GHRs are activated by GH, which changes the conformation of the receptor, promoting formation of a complex

74 with janus kinase (JAK)2 (28). The GHR-JAK2 complex can elicit numerous cellular responses in

75 the body, such as cell differentiation and oocyte maturation in the ovary (29).

76

77 The cellular mechanism underpinning the GH-induced improvement in oocyte quality and reduced

78 miscarriage rate has not been reported in human studies. However, many attempts have been made to

79 delineate the indirect changes taking place to serum and follicular fluid hormone levels. Previously,

80 we have presented comprehensive results on the granulosal cell surface receptor density profiles of

81 patients during ovarian ageing (30, 31). Ovarian granulosa cell receptor expression was found to

82 fluctuate at the two critical times of dominant follicle selection and again at the terminal end of

83 folliculogenesis in preparation for ovulation. Lower levels of receptor density and a reversal of this

84 regulatory pattern was associated with reduced fertility and ovarian reserve in older patients. In the

85 present study, we report the granulosal GHR density in different sized follicles from IVF patients

86 undergoing conventional ovarian stimulation, with rFSH alone and with rFSH combined with GH co-

87 treatment in young compared to older women with a reduced ovarian reserve. In addition, we report

88 the granulosal FSHR, LHR, and BMPR1B receptor density in older, poor ovarian reserve patients

89 treated with GH.

90 ## Materials and Methods

91 ### Patients

92 Patients (women) were selected randomly in a prospective regimen, and aged between 23 and 45

93 years, with a range of infertility factors, but limited to exclude unusual medical conditions, endocrine

94 dysfunction, polycystic ovarian syndrome and endometriosis. Infertility issues were comprised of

95 male factor, low ovarian reserve, donor sperm or unexplained fertility; and fertilisation was via

96 intracytoplasmic sperm injection (ICSI). A total of 483 follicles were collected from 64 patients

97 undergoing standard fertility treatment at PIVET Medical Centre Perth, Western Australia, (Table 1).

98

99 ### Human IVF: Ovarian stimulation, follicular fluid, and oocyte

100 Patient treatment consisted of two types of gonadotrophin releasing hormone-LH suppression

101 (Orgalutran; MSD and Cetrotide; Merck Serono) in conjunction with commercially prepared

102 recombinant human (r) FSH (Puregon; MSD and Gonal-f; Merck Serono), from cycle day 2 for ~10

103 days, as described by Regan et al. (2015). Ovulation was triggered with 10 000 IU human chorionic

104 gonadotrophin (hCG: Pregnyl; MSD), and oocyte retrieval was 36 hours later by transvaginal oocyte

105 aspiration (30). Patients classified as poor prognosis due to poor ovarian response or with three or

106 more failed attempts to conceive through IVF treatment with gonadotrophin alone, were co-treated

107 with a total of 60 IU GH (Saizen, Serono, Australia) over a period of 20-24 days in the lead-up to

108 IVF. GH was administered to 11 patients starting on day 21 of the previous cycle, and on day 2, 6, 8,

109   10, and 12 (10 IU per injection, a total of 60 IU) of the current cycle to women aged ≥39 years who

110   had at least one failed IVF cycle (15).

111

Antral follicle count

113   Patients received daily rFSH according to a long established algorithm based on the patient's profile

114   of age and ovarian reserve in order to determine the rFSH dose required to stimulate 8-12

115   preovulatory follicles, (32). Ovarian reserve was measured indirectly by the antral follicle count, and

116   was defined as the number of follicles between 2-10 mm in diameter, combining the number

117   collected from both ovaries; that were present on ~day 5 of a preliminary assessment cycle, without

118   rFSH (14). The patients were divided by age and ovarian reserve into groups based on the algorithm,

119   as described previously by Regan et al. (2016) and a well-established clinical practice of patient

120   treatment (32, 33): Group A+ = 30-39 small follicles; group A = 20-29 small follicles; group B = 13-

121   19 small follicles; group C = 9-12 small follicles, group D = 5-8 small follicles; group E = ≤ 4 small

122   follicles.

Immunolabelling of granulosa cells

124   The ovarian follicles studied ranged in diameter from 4 to 27 mm, and an average of ~8000

125   granulosa cells per individual follicle were analysed. Cell surface-expressed mature GHR protein

126   density was measured by immunofluorescent labelling and flow cytometry. The diameter of the

127   follicle was calculated using ultrasonography as described previously (30, 31, 34). Flushing of the

128   follicle (Quinn's Advantage with Hepes, Sage Media, Pasadena, California) removed the loosely

129   attached layers of granulosa cells. Aliquots of suspended granulosa cells (1×10^6 cells in 100 μl) were

130   immunolabelled and incubated separately with an optimised concentration of 4 μg/ml affinity

131   purified polyclonal antibody to bone morphogenetic hormone receptor (BMPR1B), FSHR, LHR or

132   GHR for 25 min at 5 ˚C.

133   3D image analysis using immunofluorescence detection has established the specificity of the

134   antibodies in sheep, polyclonal goat anti-BMPR1B (sc-5679), goat anti-FSHR (sc-7798), and goat

135   anti-LHR (sc-26341) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), (35); and GHR (AF1210;

136   Life Technologies, Victoria, Australia) (36). In addition, use of these antibodies has been previously

137   reported in human studies (37-44) and for use in flow cytometry (38). The cells were washed with

138   PBS and centrifuged at 300 g at 5˚C for 5 min. To render a homogeneous population of granulosa

139   cells the monoclonal antibody CD45 was added to BMPR1B, GHR, and LHR tubes to enable the

140   subtraction of the positive leukocyte common antigen (~ 3%) not removed during isolation of the

141   granulosa cells with the ficoll gradient (555485; BD Biosciences, Perth, Australia), (Fig. 1A and 1B).

142

143   Unstained samples or the substitution of a primary antibody with pre-immune goat IgG (Millennium

144   Science, Surrey Hills, Victoria Australia) at the same concentration as the primary antibody served as

145   a negative control for auto-fluorescence (Fig. 1A). A blocking peptide for FSH receptor and bone

146   morphogenetic protein receptor 1B indicated nonspecific binding applied to human granulosa cells

147   (sc-7798P, sc-5679P; Millennium Science, Surrey Hills, Victoria Australia), (Fig. 1B), and as

148   previously published (37, 38). Pre-absorbed LH (Lutropin, Merck Serono, Frenchs Forest, NSW,

149   Australia), and GH (Saizen, Merck Serono, Australia) also confirmed binding specificity. In the

150   current study, the 'normal' goat IgG and unstained control cells emitted an average mean fluorescent

151   intensity (MFI) that was classified as non-specific auto-fluorescence. The auto-fluorescence and the

152   nonspecific binding determined by the unstained control for each follicle was subtracted from each

153   follicle (Fig. 1B), and as described previously (30, 31).

154   Re-suspended 10μl aliquots of GHR immunolabelled, live granulosa cells were placed on slides and

155   visualized using an Olympus DP 70 camera fitted to an Olympus BX-51 upright fluorescent

156   microscope with a 40x UPlan N 0.4 N.A. objective (Olympus Imaging Australia, Macquarie Park,

157   Australia), (Fig.1C). Fluorescent microscopy revealed a positive staining of the cell membrane-

158   bound GHR as an intermittent, bright, ring-like pattern around the cells (Fig .1C). Pre-absorbed GH

159   was used as a negative control. A pure granulosa cell population was identified by graphing forward

160   scatter to remove doublets (FSC-H verses FSC-A), as previously described (30, 31, 34). The uniform

161   granulosa cell population revealed positive staining for FSHR, which is unique to granulosa cells

162   (45). The data were analysed using FlowJo software (Tree Star Inc., Oregon, USA).

163

164   Serum and Follicular fluid assessment

165   The peak oestrogen concentration in serum was used to predict the follicular health of the follicle as

166   opposed to the serum levels collected at the time of follicle aspiration. Serum was analysed using

167   biochemical analysis on the days leading up to collection and on the day of collection. IVF patients

168   undergoing treatment were examined in a natural cycle and during exogenous rFSH stimulated

169   cycles. Follicular fluid collected from follicles 17 to 23 mm were analysed for testosterone, FSH, and

170   LH using a random access immunoassay system (Siemens Medical Solutions, Bayswater, Victoria,

171   Australia). Follicular fluid, testosterone, FSH, and LH were analysed undiluted, whereas oestrogen

172   and progesterone were diluted manually 1:1000 with a multi-diluent and, when required, a further

173   manual dilution of progesterone 10 x and oestrogen; 5 x. Percentage coefficient of variance (CV) for

174   a concentration range 137.4 pmol/L to 3257 pmol/L was oestrogen = 5.2; LH = 3.9; FSH = 2.9;

175   testosterone = 5.9; progesterone = 9.4.

176

177   Statistics

178   Mean fluorescent intensity was obtained using ~8000 granulosa cells per individual follicle for the

179   direct measurement of receptor protein expression. The data were subjected to statistical verification

180   using one-way ANOVA with an uncorrected Fisher's LSD for follicular size using GraphPad Prism

181   6. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05,

182   **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a', signifies a statistical significant

183   difference to the matching letter (e.g. 'a*'). The attached asterisk (a*) indicates the significance level

184   for the size follicle. A two tailed, student t-test and chi squared was also used.

185   **Human Ethics**

186   Patients undergoing standard fertility treatment at PIVET Medical Centre, Perth, Australia provided

187   informed consent according to Curtin University Human Research Committee (HR RD26-10:2010

188   and 2016); and all methods were performed in accordance with the relevant guidelines and

189   regulations under State Legislation and National Accreditation processes.

190

191   # Results

192   **GHR density without growth hormone co-treatment and ovarian reserve depletion**

193   In the youngest patients with good ovarian reserve, a constant level of granulosal GHR was

194   expressed during follicular growth in both the A+ and A groups, both of which are typical for a

195   patient in this age group (Fig. 2A).

196

197   GHR density was significantly decreased as the ovarian reserve was depleted in all of the three older

198   age groups. In the 31-34 y patient group, GHR density on the granulosa cells from follicles of the

199   same size was significantly reduced in the patients with a reduced ovarian reserve for the age group

200   (p=0.039, 14 mm follicles; 0.0037, 16 mm follicles, Fig. 2B). This trend was also found in the 35-38

201   y patient group; (p=0.029, 4 mm follicles;, Fig. 2C) and in the 39+ y patient group (p=0.0001, 4 mm

202   follicles; p=0.0012 14 mm follicles, Fig. 2D). In the older patients (39+ y), with a comparatively

203   better ovarian reserve of B or C, the level of GHR was significantly reduced in the larger follicles to

204   the level observed in the poorer D and E ovarian reserve group (p<0.001; Fig. 2D).

205

206   **GHR receptor density profile independent of patient age**

207   The patient data were analysed based on ovarian follicle reserve, independent of chronological age

208   (Suplemetary Fig. 1). In patients with good ovarian reserve, an initial high level of GHR in the

209   smaller follicles was followed by a decline as the follicles increased in size (14 to 23 mm follicles,

210   p=0.0005). This pattern was reversed in the poorer ovarian reserve patient groups of D & E (p<0.05).

211   Granulosal GHR receptor density was greater in the 10 mm (p<0.01) and 14 mm (p<0.005) follicles

212   in the good ovarian reserve patient group compared to patients with the poorest ovarian reserve

213   (Suplemetary Fig. 1).

214

215   **Growth hormone co-treatment restores preovulatory down-regulation of FSHR**

216   **BMPR1B and LHR**

217 The level of GHR was significantly increased in IVF patients receiving GH co-treatment in follicles
218 from 10 to 23 mm compared to the same age patients of 39+ y with an ovarian reserve of D & E
219 (p<0.01 to p<0.001, Fig.3A). The level of GHR expression in different sized follicles was not
220 significantly different in patients treated with GH (Fig 4A).

221

222 The level of FSHR was significantly increased in IVF patients receiving GH in 16 mm follicles
223 compared to the same age patients of 39+ y with an ovarian reserve of D & E without GH (p<0.001,
224 Fig.4B).The level of FSHR in GH treated patients was also increased in the larger follicles from 4
225 mm to 16 mm (Fig. 3B, p<0.005). This was followed by a significant down-regulation of the largest
226 preovulatory follicles (p<0.01, 19 mm).

227

228 The level of LHR was significantly increased in IVF patients receiving GH in 16 mm follicles
229 compared to the same age patients of 39+ y with an ovarian reserve of D & E without GH (p<0.005,
230 Fig.4C). The LHR density of the granulosa cells collected from patients who received GH co-
231 treatment during an IVF cycle was also significantly elevated in the 10 to 16 mm follicles (p<0.01,
232 Fig. 3C). In contrast to the untreated group, GH co-treated patients showed down-regulation of
233 granulosal LHR density in follicles between 16 and 19 mm in diameter (p<0.005, Fig. 3C).

234

235 The level of BMPR1B was significantly increased in IVF patients receiving GH in 10 mm, 14 mm
236 and 16 mm follicles compared to the same age patients of 39+ y with an ovarian reserve of D & E
237 without GH (p<0.001, p<0.005, p<0.05, respectively; Fig.4D).
238 Granulosal BMPR1B density was significantly higher in 10 mm, follicles from the GH co-treated
239 patients compared to the larger pre-ovulatory follicles of either 16 mm or 19 mm (p< 0.05, Fig. 3D).
240 In contrast, to the untreated group, GH co-treated patients showed down-regulation of granulosal
241 BMPR1B density in the largest follicles of 16 to 19 mm (p<0.05, p<0.05, respectively; Fig. 3D)

242

243 When the follicles sizes are combined, the average granulosal density for GHR, FSHR, LHR and
244 BMPR1B was significantly higher in the GH treated group with the same ovarian reserve and age
245 (Fig. 3 A-D Inset, p<0.005).

246
247 **Growth hormone co-treatment and pregnancy rate in IVF patients**
248 The number of pregnancies was calculated based on the number of embryos that were transferred to
249 the patients, which included subsequent FET cycles of cryo preserved embryos.

250 The number of FET cycles was not significantly different between groups of patient. There was a
251 significant difference in the pregnancy rate in GH treated patients compared to the same age and
252 ovarian reserve patients without GH co-treatment (p=0.003; Fig. 5.). The number of live births per

253   embryo transfer was also significantly greater in the GH co-treated older age group compared to the

254   equivalent age and ovarian reserve (p=0.0406, Fig. 6). The level of oestrogen and progesterone in

255   serum and follicular fluid was not significantly different when comparing GH treatment in the

256   equivalent older patient group of 39+ y.

257

258   **Serum & Follicular Fluid & GH co-treatment**

259   The results from the current study indicate that the GH co-treatment did not alter the oestrogen level

260   of the 39+ year group cohort with an ovarian reserve of D or E during an IVF cycle (Supplementary

261   Fig 2A). Furthermore, neither the ratio of oestrogen was not altered, nor the levels of oestrogen

262   secreted, based on either the total number of follicles or the number of follicles greater than 14 mm

263   present in the ovary at the time of collection, which were not significantly different. In addition, the

264   follicular fluid concentration of oestrogen, progesterone, FSH, or testosterone was not significantly

265   different to the age matched patients with a similar ovarian reserve that were co-treated with GH

266   (Supplementary Fig 2B).

Discussion

268   GHRs are predominantly found on the granulosa cell membrane surface and in the endoplasmic

269   reticulum, and to a lesser degree, but commonly, in the nuclear membrane of highly prolific cells (46,

270   47). The GHR is regulated by GH binding proteins, ~~which are secreted from the GHR~~, and by other

271   growth factors indirectly such as FSH, BMPs and somatostatin (48, 49). In the current study, GH

272   treatment induced a direct change to the receptor expression of GHR itself and indirectly to the other

273   receptors FSHR, LHR and BMPR1B.

274

275   In support of the clinical data on ageing, human granulosa receptor density and dysregulation of

276   FSHR, LHR and BMPR1B has been associated with ovarian depletion and reduced fertility (30, 31).

277   We now provide additional data in support of a reversal of the dysregulated receptor expression

278   observed in older patients that occurs when they are treated with GH. In addition, depletion of the

279   ovarian reserve was accompanied by a reduction in GHR density, whereas GH co-treatment during

280   IVF increased the receptor density in older women who had a reduced ovarian reserve. These

281   findings provide a possible cellular regulatory mechanism involved in the poor pregnancy and live

282   birth rate in the older 39-45 y patients and reported by others (15, 16, 50-55) and reviewed by (56-

283   58).

284

285   Evidence from our previously published work and the current study suggest that ovarian reserve and

286   age are associated with reduced and dysregulated levels of receptor expression on granulosa cells.

287   Therefore, the influence of age and ovarian reserve of subjects or animals needs to be considered as a

288   confounding variable in previous studies. In heifers, GH may not have resulted in any change to

289   FSH and LH receptor binding because the cows were young, with an uncompromised ovarian reserve

290   and a sufficient receptor density (59). The effect of GH co-treatment on receptor density in patients

291   with a good ovarian reserve for age remains at this time unknown.

292

293   While GH increased the receptor expression on granulosa cells from the larger follicles, it had no

294   effect on the FSHR and LHR density at the critical time of dominant follicle selection (smallest

295   follicles of 4 mm). Previously, a poor ovarian response to rFSH stimulation has been associated with

296   reduced granulosal FSHR expression (37). However, GH co-treatment was found not to alter the

297   FSHR density in small bovine follicles, which is consistent with our findings for small human

298   follicles (60). The lack of effect on FSHR and LHR expression of small pre-ovulatory follicles may

299   explain why the number of oocytes collected was not increased in the current human model and

300   others (56).

301

302   Conversely, animal studies have reported an increase in oocyte number (61-65) . For example, even

303   though more small bovine antral follicles were produced after 45 days of GH treatment in a natural

304   cycle, the granulosal FSHRs and LHRs from pre-ovulatory bovine follicles were not affected (59,

305   60). This is surprising; however, the receptor binding studies were determined only for the three

306   largest follicles from each cow. Therefore, the expected pre-ovulatory down-regulation of these large

307   follicles would have reduced FSHR expression which would confound these results. Added to this

308   the receptor binding was not measured in any of the smaller follicles. In other studies, GH treatment

309   increased the receptors in the rat (66) however; in the pig the receptor expression was reduced (67).

310   In our human model, small antral follicles had high levels of FSHR followed by down-regulation

311   which coincides with dominant follicle selection. The high level of FSHRs induce LHR expression in

312   a natural cycle to ensure recruitment to the dominant cohort of follicles (68). In a natural cycle,

313   pituitary secreted FSH is reduced at this critical time, whereas in an IVF cycle; rFSH is abundant;

314   therefore the densities of the gonadotrophin receptors (FSHR and LHR) are pivotal in regulating

315   follicle growth and dominance.

316

317   Patients with a reduced ovarian reserve have a poorer response to rFSH treatment in IVF, and

318   produce oocytes of poorer quality (37). The poor responder group of patients also have an associated

319   high risk of foetal aneuploidy that has been correlated to ovarian reserve (69). Recently published

320   data have shown that GH co-treatment increases the pregnancy rate by a suggested improvement in

321   oocyte quality, rather than the quantity of follicles recruited (15, 16).

322

323   If the oocyte number is not significantly different in the GH treated older women, then the focus

324   shifts to the effect of GH on the quality of the oocyte. Regulation of proliferation, steroid production,

325   luteinisation, ovulation, and recommencement of meiosis fundamentally resides with the functional

326   expression of receptors in the follicle cells and oocyte.

327

328 A decline in granulosal BMPR1B and FSHR density occurred at the time of cyclic dominant follicle
329 selection, and again during the terminal stage of folliculogenesis, in young (23-30 years) IVF patients
330 with good ovarian reserve (30, 31). Older patients (39+ years) with poor ovarian reserve experienced
331 a reversal of this pattern (30, 31). In addition, the LHR density failed to be down-regulated during
332 pre-ovulatory maturation in the 39+ year group, and was reduced with ovarian reserve (31).
333
334 In the present study, we report increased granulosa cell GHR density in different sized follicles from
335 IVF patients undergoing conventional ovarian stimulation in young compared to older women with a
336 reduced ovarian reserve. In addition, we report increased granulosal GHR, FSHR, LHR, and
337 BMPR1B receptor density in older, poor ovarian reserve patients treated with GH. Importantly, the
338 women treated with GH demonstrated receptor expression down-regulation in the largest follicles.
339 The down-regulation would be essential for maturation of the ovulatory follicles, luteinisation and a
340 shift to the luteal phase.
341
342 In addition, the increased granulosal LHR density observed in the GH co-treated patients would have
343 the potential to increase the sensitivity during the hCG/LH surge to trigger final maturation and
344 ovulation of the oocyte (70, 71). The improved sensitivity may give rise to improved oocyte quality
345 and live birth rate. In support of the link between receptor density and maturation, a previous
346 electron microscopy study revealed that oocytes that did not fertilise had reduced levels of granulosal
347 luteinisation and were less responsive to hCG, which binds to the LHR (72).
348

349 **Conclusion**

350 The complexity and limitations of a largely observational, *in vivo* study in humans makes it difficult
351 to define the cellular mechanism through which numerous growth factors and pathways contribute to
352 the regulation of follicular growth and differentiation. However, the present study has generated
353 evidence suggesting several cellular mechanisms that could contribute to the improved oocyte quality
354 observed in GH co-treated IVF patients with a poor ovarian reserve.
355
356 GH co-treatment increased granulosal GHR density that would increase GHR-JAK-STAT activity,
357 and result in an increase in the intermediate products of transcription. This, in turn, could be
358 mechanistically linked to the corresponding increase in gonadotrophin receptors and BMPR1B
359 density observed in GH co-treated patients. GH co-treatment did not alter the gonadotrophin receptor
360 density of the small follicles, and would therefore account for the lack of improvement in the number
361 of follicles recruited during dominant follicle selection.
362
363 In contrast, GH co-treatment also restored the pre-ovulatory down-regulation of FSHR, BMPR1B
364 and LHR density, which may improve the maturation process of luteinisation in GH co-treated
365 patients with reduced ovarian reserve. Combined with the latter, an increase in LHR density may

improve follicle development and provide another possible cellular mechanism responsible for the improved pregnancy and live birth rate. Objectively, we remain uncertain whether the beneficial action is mediated via improved oocyte quality or other responses such as endometrial receptivity.

Authors' roles

SLPR conceived the study, experimental design, conducted all experiments, the analysis and interpretation of data, wrote the first draft of the manuscript and the final version of the paper. Obtained informed consent from patients and ethics approval. PK supervised, interpretation of data, contributed to the draft of the manuscript, interpretation of data, and critically revised the manuscript. JLY supervised, participated in the study design, participated in obtaining granulosa cells, interpretation of data, and critically revised the manuscript. FA supervised, contributed to the draft of the manuscript, interpretation of data, and critically revised the manuscript. AD supervised, participated in the study design, interpretation of data, contributed to the draft of the manuscript, and critically revised the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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

600 A. Subtraction of nonspecific binding (red) and auto-fluorescence (green) at ~10^3; granulosa cell. B. Live

601 granulosa cells, unstained control for GHR auto-fluorescence (blue) compared to positive fluorescent signal

602 measurement (box). Gated and removed CD45 positive cells (circle) also confirmed binding specificity

603 (Saizen, Merck Serono, Australia). C. Live human granulosa cells with positive fluorescence for GH receptor

604 (a & b), and pre-absorbed GH for negative control and binding specificity of GHR (c & d). Bar 10 μm.

605

606 **Fig. 2 Granulosal GHR density and ovarian reserve depletion.**

607 GHR expression density on granulosa cells collected from patients during IVF treatment with a range of

608 ovarian reserves of follicles, A. 23-30 y patient group, B. 31-34 y patient group, C. 35-38 y patient group, D.

609 39+ y patient group. Ovarian reserve was measured indirectly by the antral follicle count (AFC). AFC is the

610 number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained

611 using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein

612 expression. The number within the column represents the number of follicles analysed for that group. The data

613 were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for

614 follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$,

615 **$p<0.01$ and ***$p<0.005$.

616

617 **Fig. 3 Follicle size and the granulosa cell density of GHR, FSHR, BMPR1B and LHR in**

618 **poor response** 39+ **y patients co-treated with GH**

619 Follicles of different sizes were individually collected and analysed. Granulosa receptor density during an IVF

620 cycle with or without GH co-treatment was measured by flow cytometry. A. GHR, B. FSHR, C. LHR and D.

621 BMPR1B. The number within the column represents the number of follicles analysed for that follicle size. Inset

622 A Combined follicles of different sizes-GHR. Inset B FSHR, Inset C LHR, and Inset D. The number within the

623 column represents the number of follicles analysed. Ovarian reserve measured indirectly by the antral follicle

624 count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent

625 intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement

626 of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with

627 an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were

628 considered significant if *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$.

629

630 **Fig. 4 GH associated pregnancy and live birth outcome**

631 A. The effect of GH treatment on pregnancy rate during IVF treatment. B. The effect of GH treatment on

632 pregnancy rate during IVF treatment. The data were subjected to statistical verification using chi square. The

633 chi-square statistic *p*-value is p=0.0033. The data were based on the number of embryos transferred per patient

634 age group or treatment, including subsequent frozen embryo cycles (FET). One patient with an ectopic

635 pregnancy (classed as miscarriage) was present in the 23-30 y and the 35-38 y groups. Patients were selected

636 randomly in a prospective regimen.

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Table 1 Patient ovarian reserve, based on antral follicle count (AFC)

[1] Typical Ovarian Reserve for age group
[2] Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: group A+ = 30-39 follicles; group A = 20-29 follicles; group B = 13-19 follicles; group C = 9-12 follicles, group D = 5-8 follicles; group E = ≤4 follicles. Follicle count is based on the combined total from both ovaries to determine AFC. The number of follicles aspirated from patients from the specified ovarian reserve group.
[3] CCF-Number of patients with complete failed fertilisation compared to same age group without GH
[4] Percentage per total number of embryos transferred
[5] The average number of oocytes collected at TVOA for the age group
[a] One patient with an ectopic pregnancy (classed as miscarriage)
All subsequent frozen embryo cycles (FET) cycles were included in the analysis therefore the data was based on number of embryos transferred.
**p=0.003, *p=0.041 Chi square test (d) =+GH 39+ y compared to (c) = 39+ y patient groups.

Age years	IVF patient	Total follicle	Ovarian reserve[1]	Ovarian Reserve Group[2] Number of follicles collected per group						Oocyte quality			Fertility N (%)[4]			
				A+	A	B	C	D	E	#[5]	CCF[3]	ET	Not Pregnant	Pregnant	Miscarriage	Live Birth
21-30[a]	10	68	20-40	26	42	-	-	-	-	10	0	12	4(33)	8(67)	3(37)	5(42)
31-34	12	96	13-29	-	48	23	16	9	-	8	0	15	9(60)	7(47)	1(14)	6(40)
35-38[a]	12	108	9-19	-	6	46	17	34	-	9	0	16	5(31)	11(68)	5(46)	4(25)
39-45	19	131	3-8	-	-	42	5	64	19	7	3	25	22(88)	3(12[c])	2(68)	1(4[c])
+GH39-45	11	48	3-8	-	-	-	-	25	23	4.5	3	10	4(40)	6(60[d])**	4(68)	2(20[d])*

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