

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

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37 **Introduction**

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

47

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

52

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

59

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

67

68 Earlier studies showed GH treatment *in vivo* and *in vitro*, in conjunction with rFSH increased oocyte
69 survival rate and pregnancy rate (19-22). The granulosa cells, including cumulus cells, as well as the
70 oocyte of antral follicles express growth hormone receptor (GHR) and are therefore able to react to
71 pituitary-derived or ovarian sources of GH (23, 24). With regard to the latter, granulosa cells and the
72 oocyte, but not cumulus and theca cells, have been shown to express GH mRNA (23-27). GHRs are
73 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 granulosa 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 granulosa 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 granulosa FSHR, LHR, and BMPRI1B 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

112 **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.

123 **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 μ l) were
130 immunolabelled and incubated separately with an optimised concentration of 4 μ g/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 10 μ l 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 \pm 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 granulosa 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 (Supplementary 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 Granulosa 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 (Supplementary 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 granulosa 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 Granulosa 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 granulosa
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 granulosa 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).

267 **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 proliferative 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 granulosa 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 granulosa 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 granulosa 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 granulosa 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 granulosa 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 granulosa
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 granulosa 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

366 improve follicle development and provide another possible cellular mechanism responsible for the
 367 improved pregnancy and live birth rate. Objectively, we remain uncertain whether the beneficial
 368 action is mediated via improved oocyte quality or other responses such as endometrial receptivity.
 369

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 372 doctors, embryologists, and nursing staff.

373 **Authors' roles**

374 SLPR conceived the study, experimental design, conducted all experiments, the analysis and
 375 interpretation of data, wrote the first draft of the manuscript and the final version of the paper.
 376 Obtained informed consent from patients and ethics approval. PK supervised, interpretation of data,
 377 contributed to the draft of the manuscript, interpretation of data, and critically revised the manuscript.
 378 JLY supervised, participated in the study design, participated in obtaining granulosa cells,
 379 interpretation of data, and critically revised the manuscript. FA supervised, contributed to the draft of
 380 the manuscript, interpretation of data, and critically revised the manuscript. AD supervised,
 381 participated in the study design, interpretation of data, contributed to the draft of the manuscript, and
 382 critically revised the manuscript.
 383

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387 **Conflict of interest**

388 The authors declare that there is no conflict of interest that could be perceived as prejudicing the
 389 impartiality of the research reported.
 390

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

600 A. Subtraction of nonspecific binding (red) and auto-fluorescence (green) at $\sim 10^3$; 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 \pm 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 \pm 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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641 **Table 1 Patient ovarian reserve, based on antral follicle count (AFC)**642 ¹ Typical Ovarian Reserve for age group643 ² Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles
644 between 2-10 mm on day 2-5 of a cycle: group A+ = 30-39 follicles; group A = 20-29

645 follicles; group B = 13-19 follicles; group C = 9-12 follicles, group D = 5-8 follicles; group E = ≤4 follicles.

646 Follicle count is based on the combined total from both ovaries to determine AFC. The number of follicles
647 aspirated from patients from the specified ovarian reserve group.648 ³ CCF-Number of patients with complete failed fertilisation compared to same age group without GH649 ⁴ Percentage per total number of embryos transferred650 ⁵ The average number of oocytes collected at TVOA for the age group651 ^a One patient with an ectopic pregnancy (classed as miscarriage)652 All subsequent frozen embryo cycles (FET) cycles were included in the analysis therefore the data was based
653 on number of embryos transferred.

654 **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)*

655

