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

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

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Abstract

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

1. Introduction

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

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

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

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

2. Materials and Methods

2.1 Patients

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

2.2 Human IVF: Ovarian stimulation, follicular fluid, and oocyte

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

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

2.5 Immunolabelling of granulosa cells

Aliquots of suspended granulosa cells ($1x10^6$ cells in 100 µl) were immunolabelled using a double-indirect method as previously described (Regan et al., 2016). The antibodies have been used previously in human studies (Regan et al., 2016,Abir, Ben-Haroush, Melamed et al., 2008,Haÿ, Lemonnier, Fromigué et al., 2004), including flow cytometry analyses (Regan et al., 2016,Regan et al., 2015,Gao, De Geyter, Kossowska et al., 2007,Whiteman, Boldt J, Martinez J et al., 1991). Briefly, the cells were incubated with affinity purified goat polyclonal antibody for the FSH receptor (sc-7798), LH receptor (sc-26341), and BMPR1B (sc-5679), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then incubated with a donkey secondary antibody to goat IgG, conjugated to the flurochrome Alexa 488 (Life Technologies Australia, Victoria, Australia), as described previously (Regan et al., 2016,Al-Samerria and Almahbobi, 2014). The routinely used monoclonal antibody CD45 was added to the LH receptor and BMPR1B to enable the subtraction of the positive leukocyte common antigen to render a homogeneous population of granulosa cells (Fig. 1D).

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

2.6 Microscopy

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

2.7 Flow cytometry

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

2.8 Statistics

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

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

2.9 Human Ethics

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

3. Results

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

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

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 granulosal 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.**

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

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

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

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

4. Discussion

Depletion of the ovarian reserve of primordial follicles has a considerable impact on young and older women. High achieving women, in particular, delay having children while they establish careers in 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 granulosal 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

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

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

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

In contrast, the clinical administration of HCG or the equivalent natural cycle LH-surge, induces many maturation changes referred to as luteinisation of the granulosa cell. These changes include degradation or internalisation of the BMPR1B (Regan et al., 2016,Regan et al., 2015), LH receptor (Menon and Menon, 2012), and FSH receptor (Regan et al., 2015), cytoskeletal reorganisation of the granulosa cell, cessation of mitogenic proliferation, cumulus expansion, gap junction closure, resumption of meiosis, and general maturation of the oocyte (Izadyar, Zeinstra and Bevers, 1998,Fan, Liu, Shimada et al., 2009). Importantly, the lack of essential down-regulation of the receptors indicate disruption of this process, and may affect the LH surge-induced changes taking place at this time (Lyga, Volpe, Werthmann et al., 2016,Kash and Menon, 1998). However, the extent of luteinisation is dependent on the stage of development of the follicle at the time of the HCG/LH surge, as shown in Fig. 2, and also reported previously (Nottola et al., 2006). In particular, the level of LH receptor density during follicle development was consistent except for the largest 'ovulatory' follicles (Fig. 4). These large follicles are developmentally receptive to the LH surge-induced internalisation of the LH receptor (Menon and Menon, 2012) even though they were exposed to the same dose of HCG/LH surge trigger during IVF treatment (Fig. 2). This difference in response based on follicle size further supports our finding of differential maturation of the follicles in an IVF cycle. The ovulatory follicle internalisation of the LH receptor was also evident in natural cycles of women (Jeppesen et al., 2012) and in sheep studies conducted by our research group using the same analysis techniques (Regan et al., 2015).

The granulosa cells in the current study were collected from individual follicles and analysed immediately (fresh not frozen) to reduce any potential experiment-induced internalisation of receptors. Whereas, granulosa cells collected for culture were not responsive to rFSH stimulation (Breckwoldt, Selvaraj, Aharoni et al., 1996,Gutierrez, Campbell and Webb, 1997). The desensitised cells start to re-express receptors after 2-5 days of culture (Ophir, Yung, Maman et al., 2014). The lack of responsiveness of the FSH receptors to rFSH stimulation is consistent with our *in vivo* finding because typically, the largest, 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 granulosal 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 (TGFβ) 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, LefranÃ*f* ois-Martinez, VeyssiÃ*f* re et
362 al., 2003,Abdo, Hisheh, Arfuso et al., 2008). In particular, at the time of the LH surge/ HCG trigger, the
363 granulosal 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

et al., 2008,Shi, Yoshino, Osuga et al., 2010,Shi, Yoshino, Osuga et al., 2009), and as the ovarian reserve was depleted, the correlation was weakened (Fig. 7a). Significantly, the disruption to the FSH receptor expression was replaced with a strong correlation between BMPR1B and the LH receptor expression in the older women (Fig. 7b). This shift further supports an association between dysregulated receptor expression and reduced maturation of the granulosa cells surrounding the oocyte in older women. Future research may involve restoring the optimum receptor density profile during the maturation phase to improve oocyte quality.

Acknowledgements

The authors thank all the participants who generously donated their samples to this study, the clinical doctors, embryologists, and nursing staff.

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. JDS supervised, participated in obtaining granulosa cells, participated in the design of the study, and critically revised the manuscript. YL supervised, participated in the study design, obtained informed consents from patients and ethics approval, 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. GA supervised, conceived the study, participated in the study design, interpretation of data, and critically revised the manuscript.

Funding

SLPR was a recipient of an Australian Postgraduate Award and a Curtin University Postgraduate Scholarship. This work was supported by additional private external funding, which was gratefully accepted from Denby Macgregor. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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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598

599

***Highlights (for review)**

- Reproductive ageing is linked to ovarian cellular function and infertility
- Granulosal 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

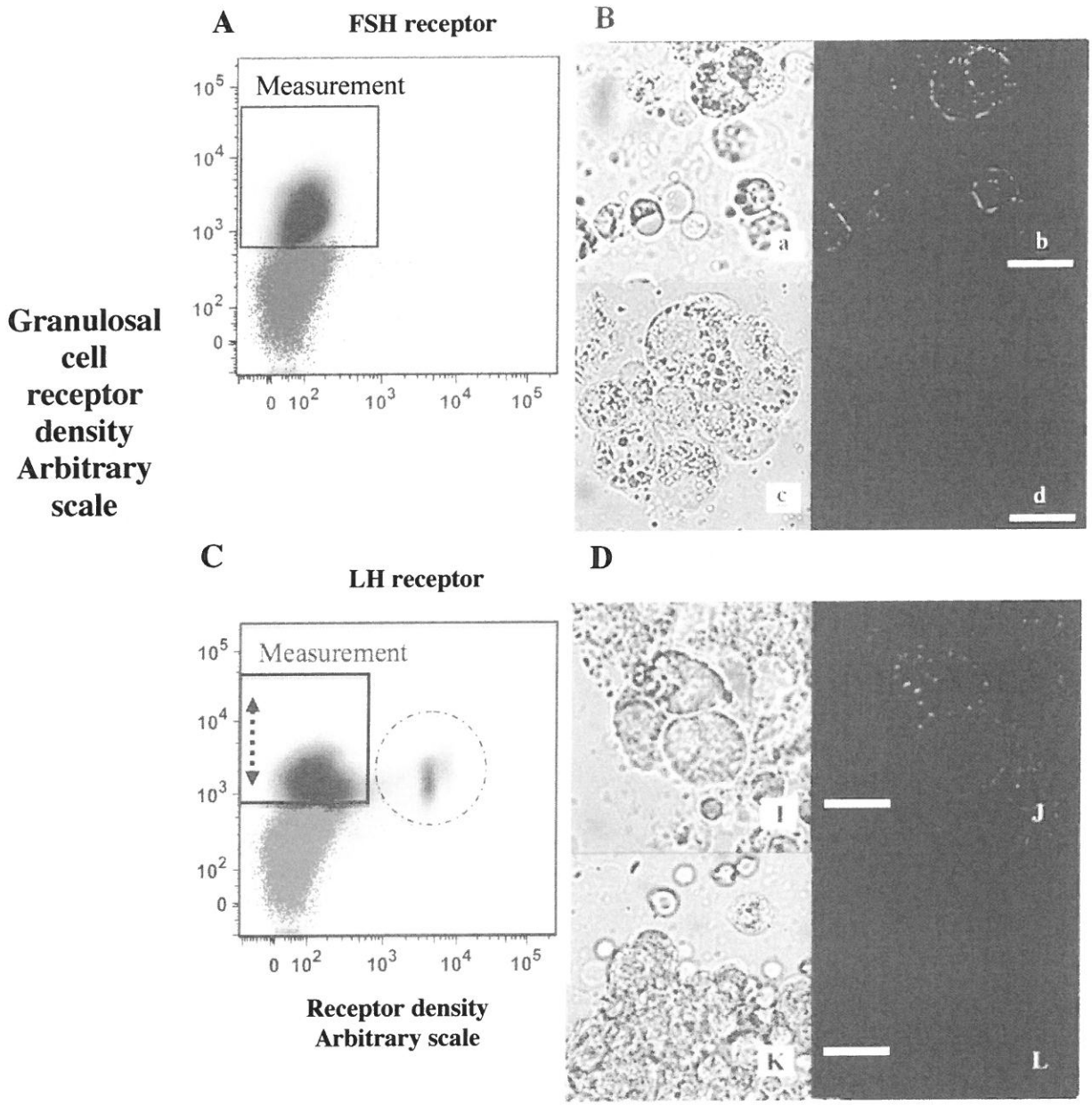
Table 1 Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

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

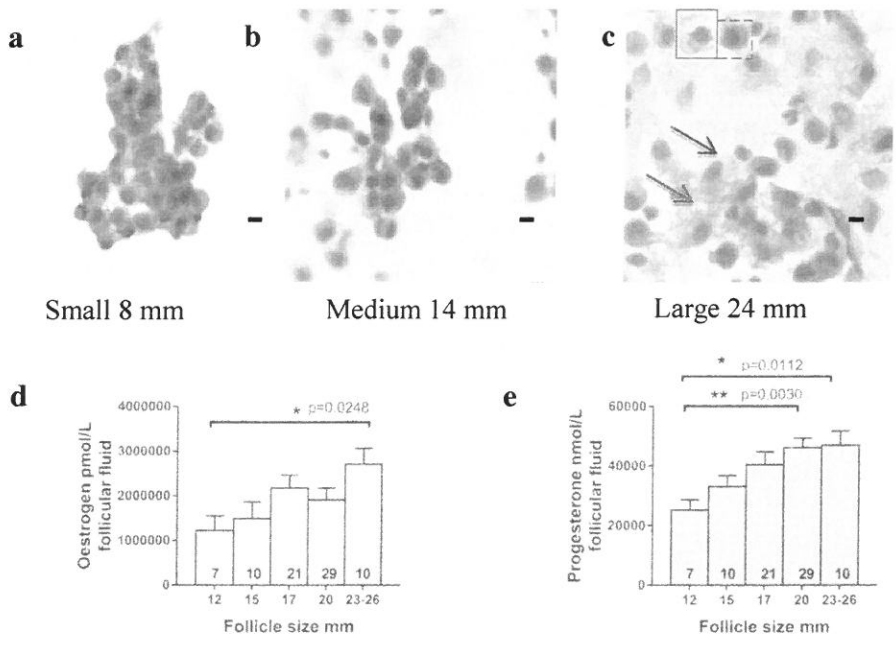
Ovarian reserve measured indirectly by the antral follicle count (AFC). Antral follicle count is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12; D = 5-8; E = ≤4. Follicle count is based on the combined total from both ovaries. *Subgroup of older patients.

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

Figure

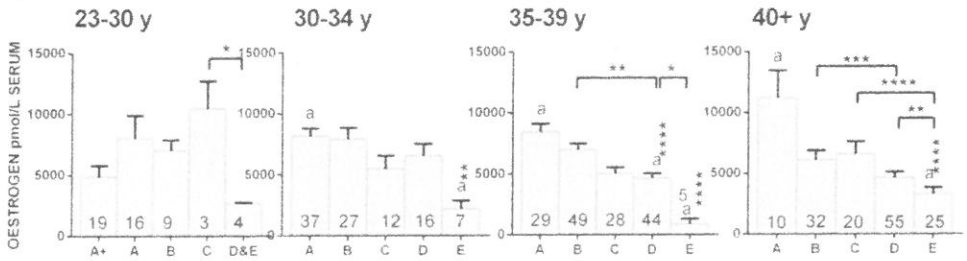

A
FSH receptor
Measurement
10^5
10^4
10^3
10^2
0
0 10^2 10^3 10^4 10^5
B
a
b
c
d
Granulosal cell receptor density Arbitrary scale
C
LH receptor
Measurement
10^5
10^4
10^3
10^2
0
0 10^2 10^3 10^4 10^5
Receptor density Arbitrary scale
D
I
J
K
L

Figure

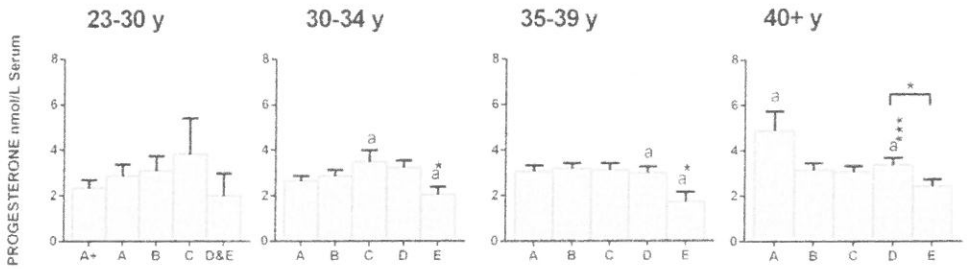

a
b
c
Small 8 mm
Medium 14 mm
Large 24 mm
d
Oestrogen pmol/L follicular fluid
4000000
3000000
2000000
1000000
0
* p=0.0248
7
10
21
29
10
12
15
17
20
23-26
Follicle size mm
e
Progesterone nmol/L follicular fluid
60000
40000
20000
0
* p=0.0112
** p=0.0030
7
10
21
29
10
12
15
17
20
23-26
Follicle size mm

Figure

a **Oestrogen**



b **Progesterone**

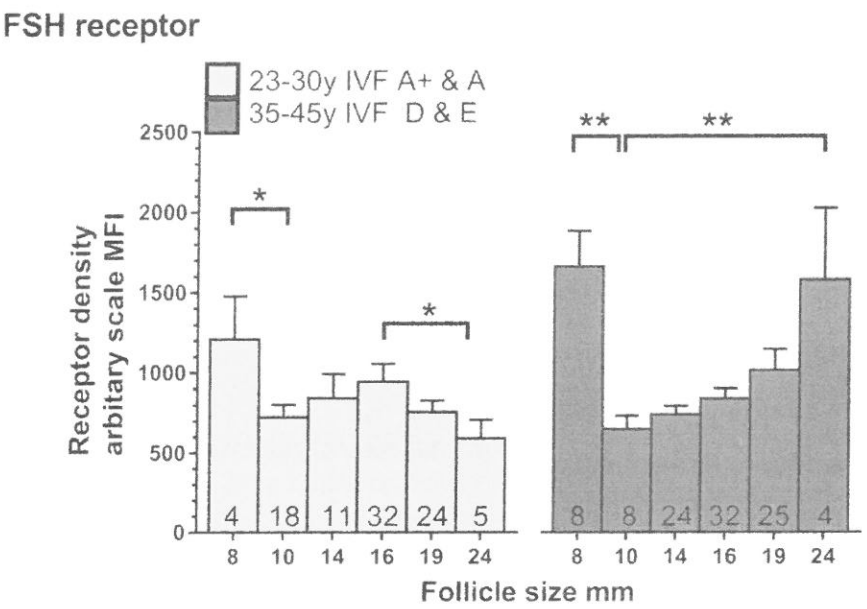


The number of primordial follicles remaining in the ovaries at a given age

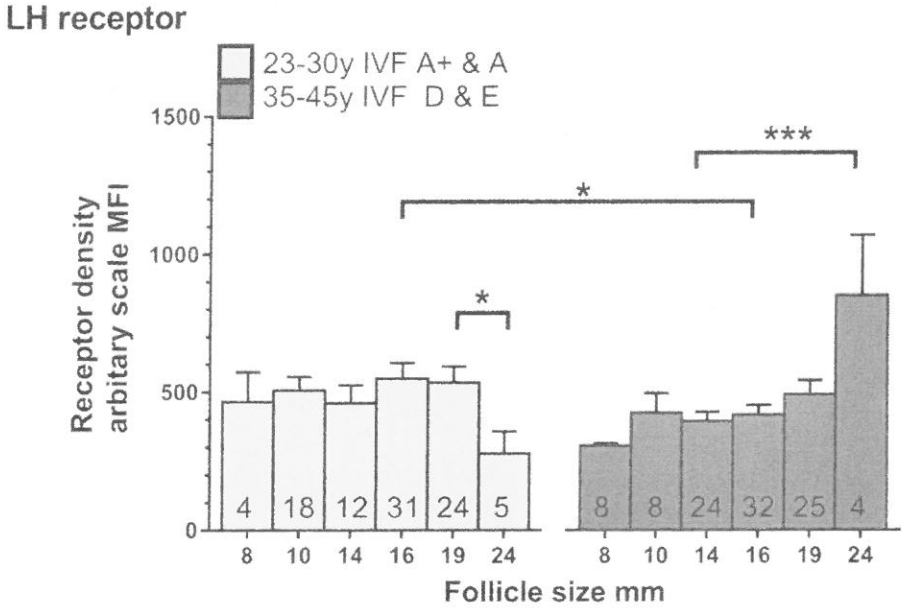
OVARIAN RESERVE GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

Figure

a



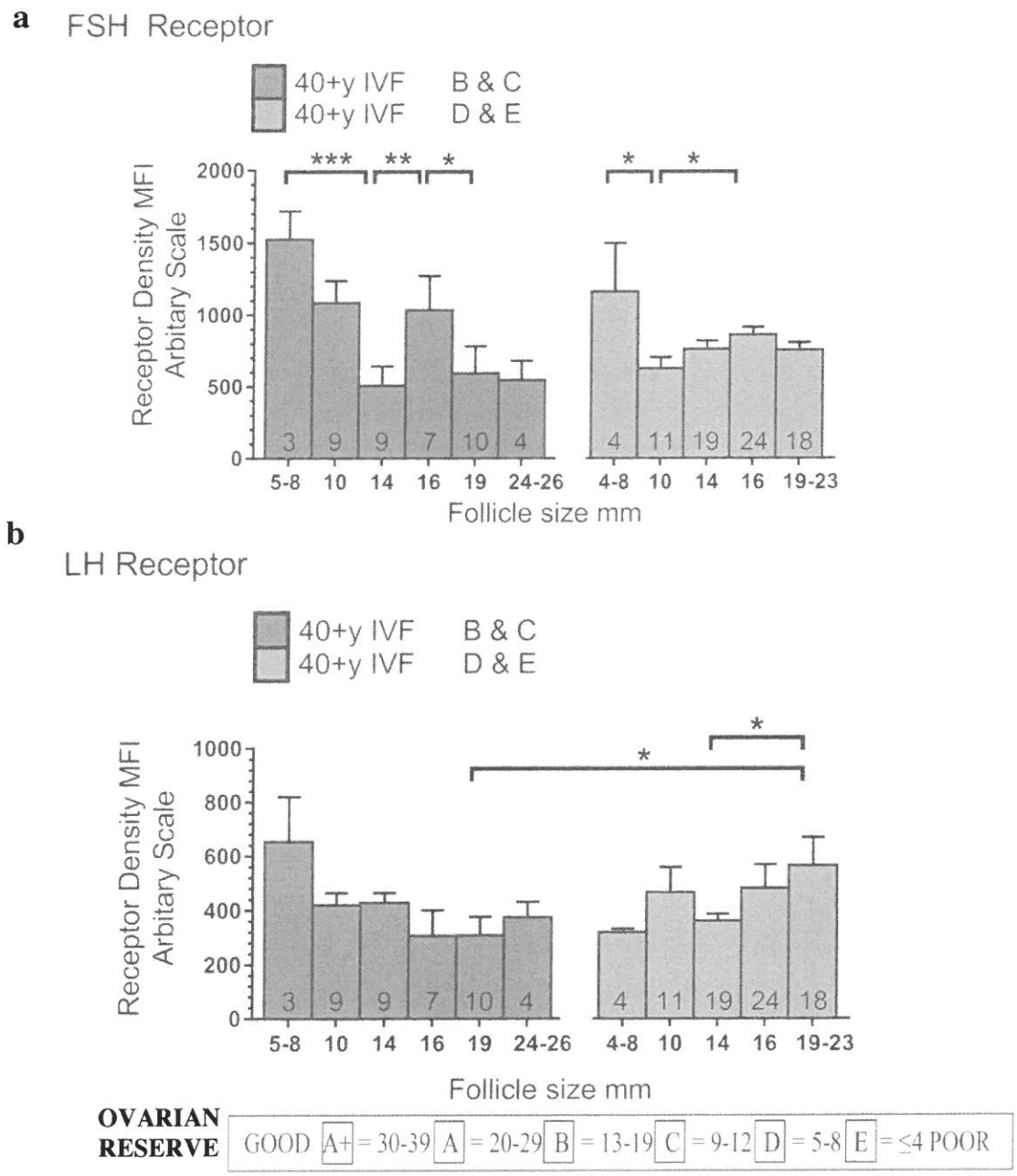
b



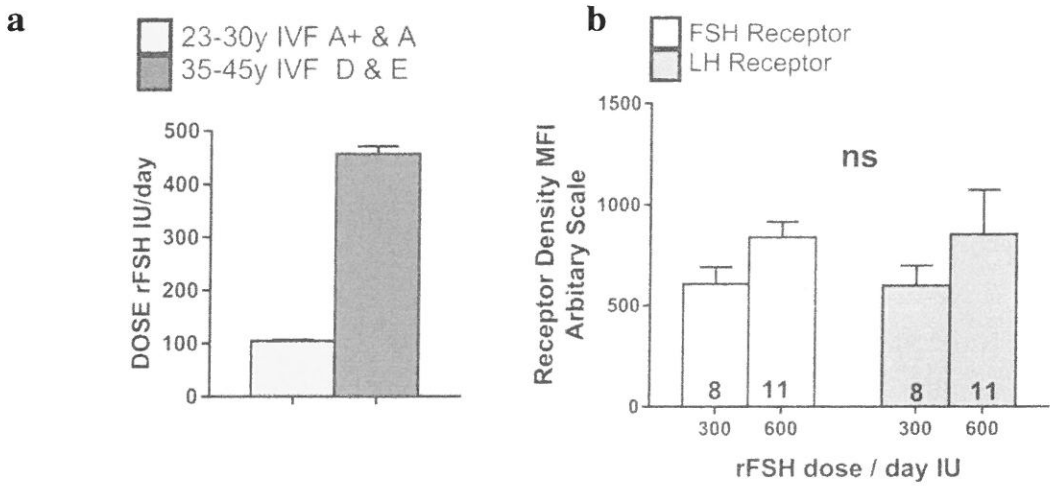
OVARIAN RESERVE

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

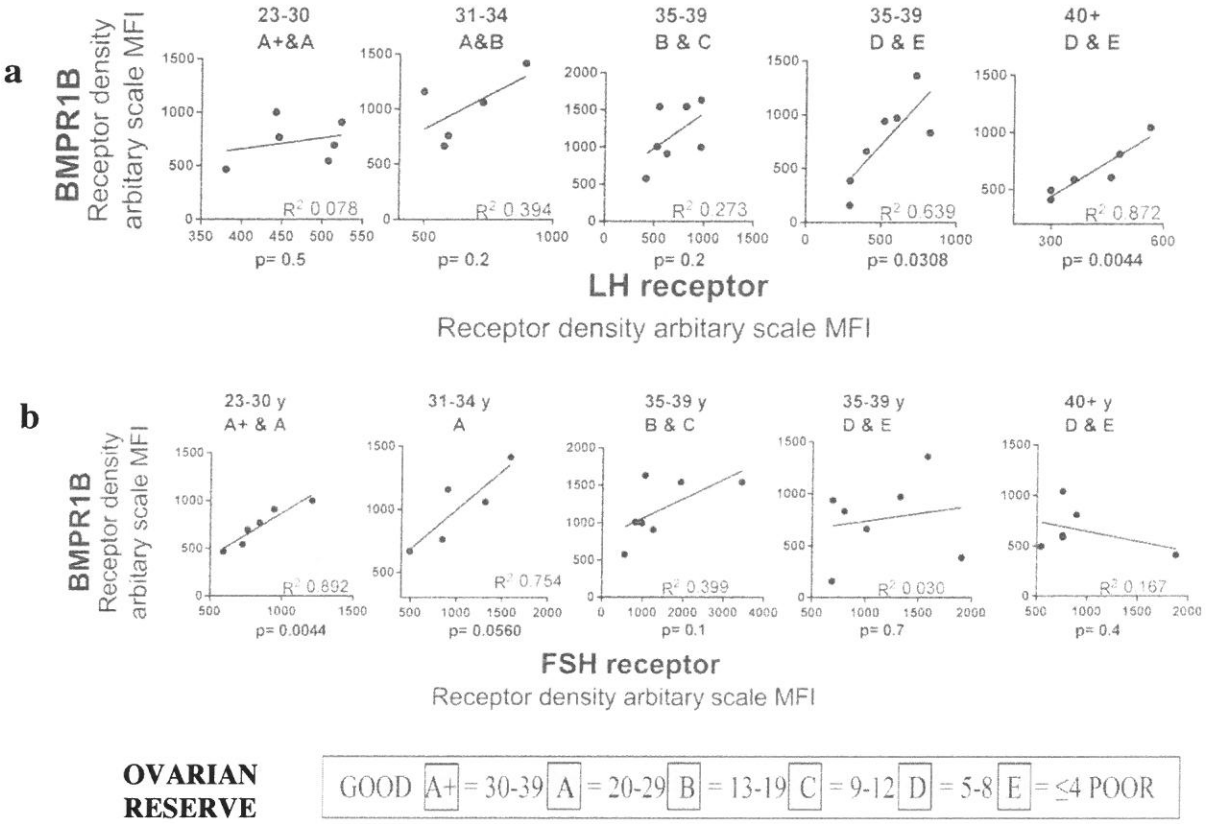
Figure


a
FSH Receptor
40+y IVF B & C
40+y IVF D & E
Receptor Density MFI
Arbitary Scale
2000
1500
1000
500
0
***
**
*
*
*
3 9 9 7 10 4
4 11 19 24 18
5-8 10 14 16 19 24-26
4-8 10 14 16 19-23
Follicle size mm
b
LH Receptor
40+y IVF B & C
40+y IVF D & E
Receptor Density MFI
Arbitary Scale
1000
800
600
400
200
0
*
*
3 9 9 7 10 4
4 11 19 24 18
5-8 10 14 16 19 24-26
4-8 10 14 16 19-23
Follicle size mm
OVARIAN RESERVE
GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

Figure


a
23-30y IVF A+ & A
35-45y IVF D & E
DOSE rFSH IU/day
500
400
300
200
100
0
b
FSH Receptor
LH Receptor
Receptor Density MFI
Arbitary Scale
1500
1000
500
0
ns
8
11
8
11
300
600
300
600
rFSH dose / day IU

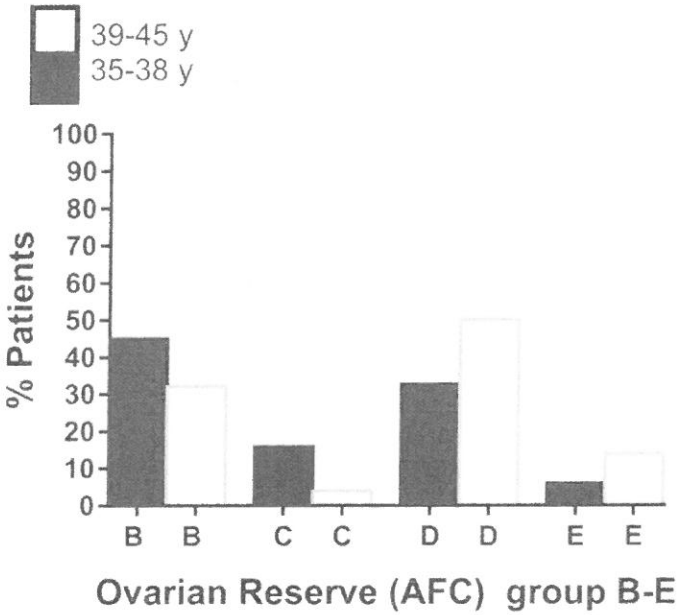
Figure



OVARIAN RESERVE

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

Figure



OVARIAN RESERVE

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

Figure

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 μm. **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 μm.

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 μm. 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 ± 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 ± 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 Granulosal 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 ± 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 Granulosal 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 ± 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 granulosal 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 ± 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 ± 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%.