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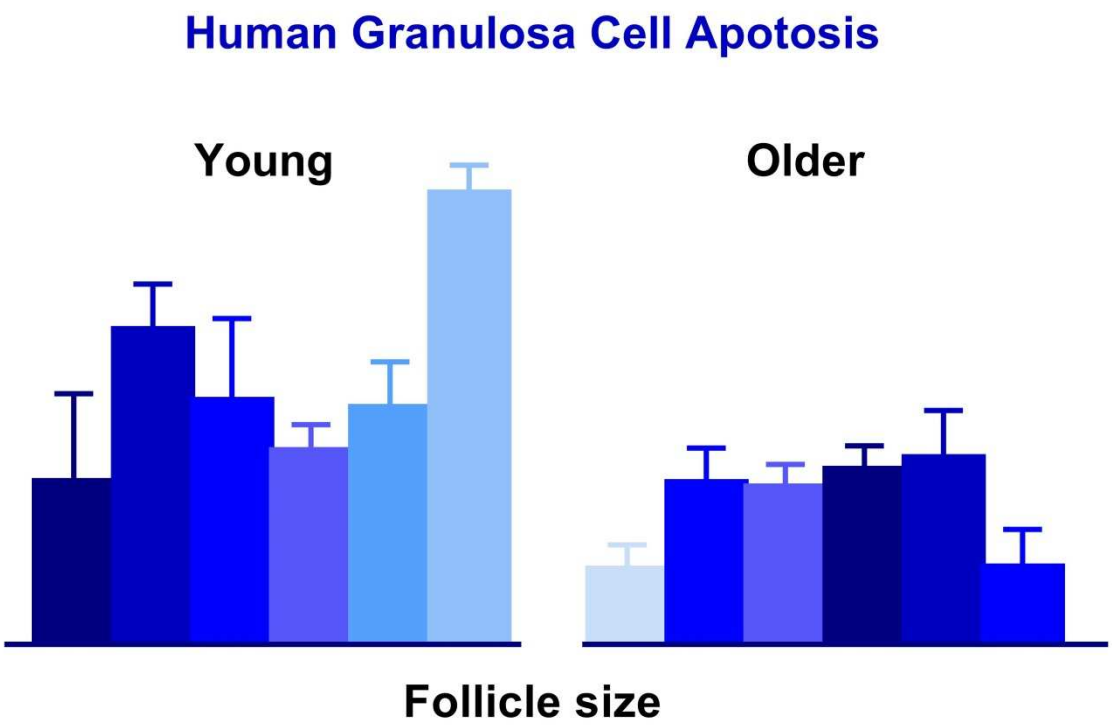

The effect of ovarian reserve and receptor signalling on granulosa cell apoptosis during human follicle development

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Introduction

Ovulation rate is governed by the number of follicles growing in a stage-specific manner. The gonadotrophins follicle stimulating hormone (FSH) and luteinising hormone (LH) govern the growth rates of the follicles during cyclic folliculogenesis, and the receptor density influences the response of the follicles to gonadotrophin stimulation (Hsueh, Kawamura, Cheng et al., 2015). Recent evidence suggests that intraovarian growth factors, such as the bone morphogenetic proteins (BMPs), impact gonadotrophin receptor expression that ultimately controls the growth rate of the follicle (Al-Musawi, Gladwell and Knight, 2007,Di Pasquale, Beck-Peccoz and Persani, 2004).

Reproductive ageing is linked to the decline in capacity of follicular granulosa cells to express receptors, which causes an irreversible change to ovarian cellular dynamics, and ultimately reduces the capacity to reproduce (Cai, Lou, Dong et al., 2007,Nelson, Telfer and Anderson, 2013,Tilly J L, Billig H , Kowalski K I et al., 1992). Older patients typically have increased circulating FSH at the start of the cycle and reduced inhibin B, which gives rise to accelerated early follicle development. However, the growth rate slows towards the terminal stage of cyclic folliculogenesis, resulting in follicles that are smaller and with fewer granulosa cells (Santoro, Isaac, Neal-Perry et al., 2003,Seifer, Scott Jr, Bergh et al., 1999,Robertson, 2009,MacNaughton, Banah, McCloud et al., 1992,Vanden Brink, Robertson, Lim et al., 2015).

Apoptosis is a normal regulatory process that contributes to the maintenance of a healthy complement of follicles and their constituent oocytes (Yuan and Giudice, 1997). The granulosa cells are more susceptible to apoptosis in the follicle than the theca or cumulus cells (Bencomo, Pérez, Arteaga et al., 2006). High levels of granulosa cell death could impact follicle development and suppress oocyte growth (Sasson and Amsterdam, 2002,Irving-Rodgers, Krupa and Rodgers, 2003). In the late 1990s and early 2000s, the levels of apoptosis in follicles were explored as potential markers of oocyte quality and to predict pregnancy outcome. However, its effectiveness as a marker was limited (Jančar, Kopitar, Ihan et

al., 2007,Lee, Joo, Na et al., 2001,Nakahara, Saito, Saito et al., 1997,Oosterhuis, Michgelsen, Lambalk et al., 1998,Moffatt, Drury, Tomlinson et al., 2002). Further investigation then centred on indicators of oxidative stress that induce apoptosis and its impact on oocyte quality (Wiener-Megnazi, Vardi, Lissak et al., 2004). This was followed by research on adjunctive treatments to reduce apoptosis (Hyman, Margalioth, Rabinowitz et al., 2013).

The post-ovulatory fate of granulosa cells is to differentiate into granulosa-lutein cells in the corpus luteum. Alternatively, apoptosis may occur, which results in a systematic degradation of the DNA to low molecular weight fragments extruded from the cytoplasm, and isolated into atretic bodies or entirely engulfed by neighbouring granulosa cells (Van Wezel, Dharmarajan AM, Lavranos TC et al., 1999,van Wezel, Rodgers and Krupa, 1999). Another type of cell death termed necrosis results from a foreign insult to a cell, which subsequently ruptures and causes an inflammatory response. A third type of cell death is referred to as terminal differentiation of the antral granulosa cells, similar to the differentiation that occurs in skin epithelium The terminally differentiated granulosa cells become loosely associated to the granulosa membrana, and are eventually sloughed off into the antrum, similar to skin epithelial cells. The cells coalesce to form coagulated globules ranging in size from 40 μm to 620 μm (van Wezel et al., 1999,Hay, Cran and Moor, 1976). Alternatively, another form of programmed cell death called autophagy may occur, where the cell digests itself (Duerrschmidt, Zabirnyk, Nowicki et al., 2006,Vilser, Hueller, Nowicki et al., 2010).

Earlier studies on apoptosis of follicular cells have employed a range of analyses based on morphological assessment of pyknotic cell counts, TUNEL, and propidium iodide assessment, all with pooled follicle samples of unknown size (Yuan and Giudice, 1997,Nakahara et al., 1997,Oosterhuis et al., 1998,Seifer, 1996 ,Giampietro, Sancilio, Tiboni et al., 2006,Austin, Mihm, Evans et al., 2001,Bomsel-Helmreich, Gougeon, Thebault et al., 1979). Other studies have analysed activated caspase 3 levels, and compared these with TUNEL assay outcomes and with levels of various Bcl2 family

78 members, reporting a wide range of apoptosis levels (D'haeseleer, Cocquyt, Cruchten et al.,
79 2006,Albamonte, Albamonte, Stella et al., 2013).

80

81 Many of these studies suffer from technical limitations because they have relied on pooling follicles of
82 different sizes, counting a small portion of the granulosa cells (~100-1000), and have excluded follicles
83 because of blood contamination, or failed to exclude white blood cells (Nakahara et al., 1997,Oosterhuis
84 et al., 1998,Seifer, 1996). In addition, when propidium iodide and Annexin V-FITC are combined,
85 spectral overlap was not compensated for, and made the incorrect interpretation of the quadrants as being
86 apoptosis-induced necrotic cells (Jančar et al., 2007,Seifer, 1996 ,Giampietro et al., 2006).

87

88 From our previous experience, we determined that Annexin V stain, which indicates early onset of
89 apoptosis, is unreliable because of unintentional damage caused by centrifuging cells at a high speed that
90 induces early apoptosis (Regan, McFarlane, O'Shea et al., 2015).

91 Uniquely, the current study identifies granulosa cells based on positive FSH receptor expression,
92 combined with excluding red and white blood cells. Therefore, the current study aims to further explore
93 the changes in granulosal apoptosis of healthy follicles (not atretic); hence, indicating mitogenic
94 growth/turnover rate rather than follicle death. By using optimized methodologies and experimental
95 techniques, individual follicles ranging in size from 4 mm to 26 mm were analysed to determine the
96 relationship between apoptosis (7AAD +) as the ovarian follicle reserve is depleted with age.

97 **2. Methods**

98 A total of 198 follicles were collected from 31 patients undergoing standard *in vitro* fertilisation
99 treatment (Table 1). Patients were aged between 23 and 45 years, with a range of infertility factors, but
100 limited to exclude unusual medical conditions, endocrine dysfunction, polycystic ovarian syndrome and
101 endometriosis, and were comprised of male factor, low ovarian reserve, donor sperm or unexplained

fertility; and fertilisation was via intracytoplasmic sperm injection (ICSI). Patient treatment consisted of gonadotrophin releasing hormone antagonist suppression of LH (either Orgalutron or Cetrotide) in conjunction with commercially prepared recombinant (r) human FSH stimulation (either Puregon or Gonal F), 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 oocyte retrieval was 36 hours later by transvaginal oocyte aspiration (Regan et al., 2016). Body mass index (BMI) differences were not significant in this study.

Ovarian reserve was measured indirectly by the antral follicle count and was defined as the number of follicles between 2 - 10 mm in size that are present in total on ~ day 2- 5 of a preliminary assessment cycle (Hansen, Hodnett, Knowlton et al., 2011). The patients were divided into groups based on the algorithm, as described previously (Regan et al., 2016), and a well-established clinical practice of patient treatment where IVF gonadotrophin treatment protocols are based on AFC as the main predictor and AMH as a minor modulator when the two measurements conflict (Yovich, Stanger and Hinchliffe, 2012). In the current study, the combined ovary follicle total corresponded to: Group A+ = 30-39 small follicles; group A = 20-29 small follicles; group B = 13-19 small follicles; group C = 9-12 small follicles; group D = 5-8 small follicles; and group E = ≤ 4 small follicles. Body mass index (BMI) differences amongst patient groups A-E were not significant in this study.

The diameter of the follicle was calculated using ultrasonography, as described previously (Regan et al., 2016). Each follicle was measured, punctured, and aspirated to remove only the follicular fluid; this would remove any contamination from other follicles or ovarian or vaginal epithelial cells (Quinn's Advantage with Hepes, Sage Media, Pasadena, California). This fluid is initially collected and checked for an oocyte. While the checking procedure by two embryologists takes place, the clinician flushes the follicle at ~ 180 psi to remove the loosely attached layers of antral granulosa cells until an oocyte is retrieved. When entering an adjacent follicle, a new collection tube is used, and will contain the new

pure follicular fluid; again clearing contamination from other sources. Therefore, the collected follicle flush would only contain the antral granulosa cells that are easily removed during flushing. The cumulus ovarian complex was removed and the follicular flush was then layered onto a ficoll density gradient (555485; BD Biosciences, Perth, Australia) and centrifuged at 1500 rpm (300g) for 30 min at room temp to isolate the granulosa cells and remove red blood cells (Regan et al., 2016).

2.1. Immunolabelling of granulosa cells

Aliquots of suspended granulosa cells ($1x10^6$ cells in 100 μl) were immunolabelled as previously described; analysed for receptor expression and apoptosis fresh on the same day (Regan et al., 2016,Regan, Knight, Yovich et al., 2017). Briefly, the cells were incubated with affinity purified goat polyclonal antibody to goat FSH receptor (sc-7798), and BMPR1B (sc-5679) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then incubated with a secondary antibody, donkey anti-goat conjugated to the fluorochrome Alexa 488 (Life Technologies Australia, Victoria, Australia) (Regan et al., 2016,Al-Samerria and Almahbobi, 2014). 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. In the current study, the 'normal' goat IgG and unstained control cells emitted a similar average mean fluorescent intensity (MFI) and this was subtracted from the receptor measurement.

7-Amino-Actinomycin (7-AAD) is a membrane impermeant dye that is excluded from cells with an intact cell membrane. Granulosa cell membrane integrity breakdown allows 7-AAD to penetrate. It binds to double stranded DNA, excited at 488 nm wavelength, and emitting at a maximum 647 nm (Demchenko, 2013,Amsterdam, Sasson, Keren Tal et al., 2003). Briefly, cells were incubated with 7-AAD (BD Biosciences, Perth, Australia) in the dark for 15 min at room temperature. A combination of unstained cells sample and 7-AAD positive cells from the same follicle, as per manufacturer's recommendation (Demchenko, 2013,Vermes, Haanen, Steffens-Nakken et al., 1995).

2.2. Flow cytometry

Selective gating of the whole sample to identify a pure granulosa cell population was achieved by graphing forward scatter to remove doublets or globules 25-620 μm in size (FSC-H verses FSC-A), as previously described (Regan et al., 2016). The resulting population contained a granulosa cell population that revealed positive staining for the FSH receptor, which is unique to granulosa cells (Fig. 1) (Hermann and Heckert, 2007). Red blood cells were excluded using a Ficol gradient (555485; BD Biosciences, Perth, Australia), and white blood cells excluded since they are FSH receptor negative; monoclonal antibody CD45 was also used to enable the subtraction of the cells positive for the leukocyte common antigen in order to render a homogeneous population of granulosa cells. Atretic bodies formed by budding of the cytoplasm of apoptotic granulosa cells would also not have FSH receptors. Apoptosis would therefore be measured only in antral granulosa cells from the membrana and antral granulosa cells loosley attached to the granulosa membrana undergoing terminal differentiation. Basal granulosa cells would be excluded because of the distance from the atrum and the limited aspiration applied during collection to preserve the follicles with potential to form a corpus luteum. The cumulus cells form clumps and are usually attached to the oocyte forming the cumulus oocyte complex, and would be removed. The cumulus cells require hyloronidase to separate them from the oocyte for oocyte incubation during *in vitro* fertilisation (IVF). If the cells were not attached to the oocyte, they too would gated out during flow cytometry due to the large size of cumulus cell clumps.

FSH receptor and BMPR1B immunostaining was performed in separate tubes, and the Alexa 488 (emmission 519) spectral overlap with 7AAD on the far right of the spectrum was insignificant (emmission 647). Therefore, the proportion of 7AAD positve cells was considered to represent the base rate turnover of apoptosis of healthy (FSH receptor expression) granulosa cells (Fig. 1). The assessment would not account for the phagocytised or autophagocytosed granulosa cells. The data were analysed using FlowJo software (Tree Star Inc., Oregon, USA).

2.3. 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. A two tailed, student t-test was also used.

2.4 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 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

It should be emphasized that the percentage of apoptotic cells amongst the pure population of FSH receptor-expressing granulosa cells was determined here, and not the percentage of the total heterogeneous population of cells contained in the aspirated follicular fluid. In the young patient group, 23-30y with a typically good ovarian reserve that was indirectly measured by day 5 antral follicle count (AFC; groups A+ & A). The level of apoptosis was higher in the granulosa cells from 10 mm follicles of which size, corresponds with the stage of dominant follicle selection ($p<0.01$, Fig. 2). In the largest pre-ovulatory follicles (23-30 mm), the percentage of granulosa cell apoptosis was also significantly greater ($p<0.005$) than at all other stages. A direct comparison between the level of receptor expression and the level of apoptosis can be made using previously published data (Fig. 2) (Regan et al., 2016,Regan et al., 2017). The analysis of apoptosis was performed on the same isolated granulosa cells as that for the receptor expression density. In the older patients, the lower level of apoptosis in the 10 mm follicles corresponded to the significantly reduced granulosal BMPR1B density, whereas the low level of apoptosis in the largest follicles (>23 mm) was associated with the lack of down-regulation of the BMPR1B, FSH receptor, and the LH receptor combined (Fig. 2).

At the stage of dominant follicle selection (10 mm), the level of apoptosis was reduced in the older age group 35–45y, with a typical depleted ovarian reserve of D & E, compared to the youngest patients ($p<0.005$). The level of apoptosis was also greatly reduced (~7-fold) in the largest pre-ovulatory follicles >23 mm in size compared to similar sized follicles in the younger patients ($p<0.0001$, Fig. 2). The level of apoptosis in the old compared to the young females was not significantly different at stages between dominant follicle selection and maturation of the largest follicles (14 mm to 19 mm, $p > 0.1$, Fig. 3). Since most of the comparative studies published have 'pooled' the follicles, in an attempt to compare our results, we combined the follicles of different size for the old compared to the young and this confirms a greater level of apoptosis in the younger patients ($p < 0.0001$, Fig. 4).

When patients of the same age, (40+y) with the same follicle size and ovarian reserve (AFC D) were compared, age alone was not predictive of apoptosis levels based on the finding that patients of the same age had significantly different apoptosis levels. Patients 40+y with a good ovarian reserve for age had levels of apoptosis ~2-fold higher than those with a poorer ovarian reserve ($p < 0.01$, Fig. 5). The dose of rFSH administered to patients did not have a significant effect on the apoptosis of the granulosa cells ($p>0.2$, Fig. 6).

The follicles of the similar size class were combined from patients based on age and ovarian reserve. A strong correlation was observed between the granulosal FSH receptor and BMPR1B density and the corresponding level of apoptosis based on follicle size (Fig. 7). High levels of FSHR and BMPR1B density were significantly associated with reduced apoptosis and necrosis levels in the youngest patients of 23-30 y with an AFC of A+ & A (R square 0.752, p=0.0252 and 0.835, p=0.0108, respectively). The correlation was reversed in the next age group of 31-34 y for both FSHR and BMPR1B, and sequentially reduced in association with increasing age and a reducing ovarian reserve. In the 40+ y patients, the non-significant correlation for apoptosis with FSH receptor and BMPR1B was R square 0.137, p=0.86 and 0.011, p= 0.46, respectively.

4. Discussion

The major findings of this study are that the level of granulosa cell apoptosis increased in follicles of a size corresponding to the stage of dominant follicle selection (10 mm) and of pre-ovulatory maturation (23+ mm) in young IVF patients with an uncompromised ovarian reserve based on the number or antral follicles present on day 5 of a cycle (AFC) (Fig. 2). The granulosal BMPR1B and FSH receptor density were both inversely proportional to the level of granulosal apoptosis in the young patients (Fig. 2). However, as the ovarian reserve declined with age, this relationship was disrupted. The reduction of apoptosis in the older patients was associated with a compromised level of BMPR1B at the time of dominant follicle selection (10 mm), whereas the low level of apoptosis in the largest follicles (23+ mm) was associated with the lack of down-regulation of the BMPR1B, FSH receptor, and the LH receptor combined (Fig. 2).

Unique to this study, only granulosa cells identified by FSH receptors on the cell surface were included in the flow cytometry analyses, providing certainty that the positive events were granulosa cell-specific after removal of both red and white blood cells and other potential confounding signals. In the current study, we do not differentiate between apoptosis or terminally differentiated granulosa cells, as 7AAD would stain all exposed DNA. The follicles analysed in the present study are healthy follicles that would be contributing to the overall serum oestrogen levels (Tilly J L et al., 1992,Amsterdam et al., 2003). The percentage of apoptosis reported would therefore not be comparable to studies that did not identify granulosa cells positively and that did not exclude white blood cells.

In the entire research project there were only four out of 500 follicles that were atretic and did not express any receptors, and were therefore removed from analysis. This is consistent with other studies that reported low levels of TUNEL assay positive granulosa cells in dominant follicles (Yuan and Giudice, 1997,Austin et al., 2001,Albamonte et al., 2013,Poljicanin, Vukusic Pusic, Vukojevic et al., 2013). In healthy dominant follicles, indicated by high oestrogen levels, apoptotic granulosa would not

stain positively with TUNEL, propidium iodide or 7AAD, because they are continuously engulfed by neighbouring granulosa cells via phagocytosis (Van Wezel et al., 1999). Therefore, the level of apoptosis indicated by 7AAD +ve DNA in each follicle is more representative of the mitogenic activity within each follicle. As we age all of our cells multiply at a slower rate, hence the turnover rate is slower (Santoro et al., 2003,Seifer, 1996 ,Acosta, Jernberg, Sanberg et al., 2010). The lower levels of apoptosis in the older patients are reflective of the reduced proliferation occurring (Santoro et al., 2003).

At the time of dominant follicle selection in a natural cycle, the circulating FSH decreases, and the small growing follicles with greater FSH receptor and LH receptor density are stimulated to produce oestrogen and exhibit more cell proliferation at the expense of the subordinate follicles (Mihm, Baker, Ireland et al., 2006). In the young patients, a significant increase in 7AAD + granulosa cell death was evident in follicles around the size at which dominant follicle selection occurs (Fig. 2). FSH has been reported to be anti-apoptotic (Amsterdam, Keren-Tal, Aharoni et al., 2003); therefore, it may be expected that the decline in pituitary FSH initiates and/or contributes to an increase in apoptotic signalling in these granulosa cells (mid-follicular phase; day 7) (Xu, Garverick, Smith et al., 1995,Billig, Chun, Eisenhauer et al., 1996,Billig, Furuta and Hsueh, 1994).

In a gonadotrophin stimulated IVF cycle, high doses of rFSH are administered that override the natural changes in endogenous FSH, but the dose of rFSH did not exert a significant influence on granulosa apoptosis (Fig. 6). We have also previously demonstrated that the FSH receptor and LH receptor are down-regulated, independently of the gonadotrophins administered at the crucial time of dominant follicle selection (Regan et al., 2017), supported by a similar down-regulation mRNA for FSH receptor and BMPR1B in cumulus granulosa cells (Coticchio, Ophir, Yung et al., 2017). Therefore, at this critical time point, the FSH receptor expression is down-regulated (Fig. 2), which may explain the lower apoptosis rate as a consequence of reduced proliferation (Sen, Prizant, Light et al., 2014,Rice, Ojha, Whitehead et al., 2007). Hence in the younger patients, down-regulation of FSH receptors at the time of

dominant follicle selection is consistent with a corresponding increase in apoptosis (Fig 2 and Fig. 5). As the levels of FSH receptor decrease, the granulosa cell would produce less oestrogen and show limited cell division. As the level of receptors increase, again the level of apoptosis reduces, which is consistent with our findings (Fig 2 and, Fig. 5).

When the ovarian reserve declines with age, it is evident that the level of FSH receptor or LH receptor in the small follicles is not compromised (Regan et al., 2017). Whereas, in the same patient cohort, a distinct difference in granulosal BMPR1B density was reported (Regan et al., 2016).Therefore, it is probable that the age-induced effect of reduced BMPR1B density is functionally linked to the level of granulosa cell apoptosis at the time of dominant follicle selection.

Previous research has shown that a reduction in follicular BMP6, BMP15, and BMPR1B coincides with dominant follicle selection (Regan et al., 2015,Regan et al., 2016,Erickson and Shimasaki, 2003,Feary, Juengel, Smith et al., 2007). In addition, BMP4 and 7 are involved at several stages of apoptotic signalling, in particular, the caspase 3 and 9 pathways (Kayamori, Kosaka, Miyamoto et al., 2009). Moreover, BMP2, 6, and 7 have been shown to up-regulate FSH receptor expression (Shi, Yoshino, Osuga et al., 2011,Shi, Yoshino, Osuga et al., 2009). Therefore, it is proposed that down-regulation in BMPR1B signalling would indirectly induce apoptosis, which is consistent with our reported high level of apoptosis in the younger patients (Fig 2).

In young wild type sheep, down-regulation of granulosal BMPR1B during dominant follicle selection was associated with an increased proportion of granulosa cell apoptosis (7AAD + / FSH receptor +) (Regan et al., 2015). Likewise, in the Booroola sheep, higher granulosal BMPR1B density was associated with reduced apoptosis and fewer granulosa cells per follicle (Regan et al., 2015,McNatty, Lun, Heath et al., 1986) . Our finding of a Booroola mutation-induced lowering of granulosa cell

apoptosis levels associated with the high ovulation rate of this breed was recently confirmed (Estienne, Pierre, di Clemente et al., 2015). In the human context, the lower levels, and reversed expression of BMPR1B in the older patients, may directly contribute to low levels of apoptosis associated with poor granulosa cell proliferation (Fig 2).

The extent of granulosa cell apoptosis was maintained at a consistent level in the follicles from 14 to 19 mm in size in the young cohort, which was not significantly different to that seen with the older patients (Fig. 3). The plateau could signify a base rate of continuous removal of atretic granulosa cells via phagocytosis or autophagy and terminal sloughing off of granulosa cells into the antrum. Importantly, the similar levels of apoptosis in the older patients suggest that the general health of the follicle is not compromised.

In contrast, in a study using TUNEL labelling in aspirated follicles from IVF patients, Seifer, et al, 1996, reported that granulosa cell apoptosis was increased as the ovarian reserve declined. These cells were contaminated with white blood cells, and when counterstained with propidium iodide, this quadrant was not included. In another study of IVF stimulated patients (33 year-old), annexin V staining indicated that the level of apoptosis was 7.8 - 9.8 %; however, the propidium iodide stained quadrant was excluded from analysis (Giampietro et al., 2006). In addition, the follicles were centrifuged at 3000 rpm (1200 g) and were pooled; whereas, in the current study, the follicles were individually analysed (~ 8000 granulosa FSH receptor positive cells per follicle) and centrifuged at 1500 rpm (300 g). The same study applied a second method (TUNEL assay) on the same patient group, and the level was found to be much higher, ~ 20 % (Giampietro et al., 2006). The authors acknowledged that the TUNEL assay may also overestimate apoptosis because multiple atretic bodies measured may have originated from a single granulosa cell. The TUNEL assay also estimated apoptosis levels to be higher than that assessed by caspase 3 activity (D'haeseleer et al., 2006). These differences highlight the inaccuracy that may occur

when reporting and comparing results using different methodologies, and raises caution with regard to experimentally induced errors.

Surprisingly, the level of apoptosis in the largest follicles (>23 mm) from the young patients was significantly higher compared to middle sized follicles (Fig. 2). The greater level of apoptosis coincides with the extensive morphological changes that take place in the preovulatory stage to facilitate the rupture of the follicle and expulsion of the oocyte (Fig. 2) (Fan, Liu, Shimada et al., 2009). Even though all the follicles are exposed to the same LH/HCG surge trigger injection, the 'extent of luteinisation' is dependent on the size of the follicle (Regan et al., 2017). Preparation for ovulation begins with a cessation of cell proliferation and early luteinisation. This may cause antral granulosa cells to become apoptotic in the young. For example, the antral granulosa membrana thins out at the surface of the ovary in preparation for rupture(Rodgers and Irving-Rodgers, 2010). This remodelling would increase the apoptosis of antral granulosa cells. In contrast, in the older patients the receptors were not down-regulated which may influence or delay this remodelling process, and result in reduced apoptosis observed in the older patient.

Conflicting with the current study, an increase in DNA fragmentation of granulosa cells (TUNEL assay) has been shown to increase with age, even though errors in methodologies were present, as described above (Oosterhuis et al., 1998,Seifer, 1996 ,Sadraie, Saito, Kaneko et al., 2000). As the granulosa cell differentiates into a progesterone producing granulosa-lutein cell, the oestrogen levels also transiently decline. The decline in oestrogen and other growth factors may account for the increased apoptosis in the largest follicle in the young because this follicle would have the greatest drop in oestrogen (Fig. 2).

Whereas, maturation of the pre-ovulatory follicle requires down-regulation of the BMPR1B, FSH receptor, and LH receptor (Cai et al., 2007,Regan et al., 2015,Regan et al., 2016,Regan et al., 2017,Feary

et al., 2007,LaPolt and Lu, 2001,Ophir, Yung, Maman et al., 2014). The lack of down-regulation of the receptors combined observed in the older patients (as previously described) would limit the maturation of the follicle and maintain a high anti-apoptotic state, consistent with the reduced apoptosis level (Fig 2).

In the current study, we did not find an increase in granulosa cell apoptosis in the large (>23 mm) preovulatory follicles of older patients that had a poor ovarian reserve (antral follicle count-D&E). These patients also had a poor pregnancy and live birth rate (Fig. 2). This is in marked contrast to the finding in the younger patients who showed a ~7-fold higher level of apoptosis in follicles of the same size class (>23 mm) (Fig. 2). Moreover, when the individual results for each follicle size were combined to mimic results from a 'pooled follicle protocol', the younger patients still had a significantly greater level (~2-fold) of 7AAD positive cells (Fig. 4); notwithstanding that, they were uniquely identified as granulosa cells that were FSH receptor positive and free from white blood cell contamination.

Disregarding different methodologies and experimental errors, there are considerable discrepancies in the literature. Increased apoptosis of pooled granulosa cells has been linked to poor oocyte quality and pregnancy rate (Nakahara et al., 1997,Oosterhuis et al., 1998,Clavero, Castilla, Núñez et al., 2003,Suh, Jee, Choi et al., 2002), greater apoptosis in cumulus cells (Lee et al., 2001), and increased oxidative stress (Wiener-Megnazi et al., 2004). However, granulosa cell apoptosis rate has also been reported to have no association with oocyte quality, fertilisation rate or blastocyst development, (Jančar et al., 2007,Moffatt et al., 2002,Clavero et al., 2003).

In support of our findings Nakahara, et al, 1997, reports that when age alone was examined, the 40+y patients had significantly reduced apoptosis of the granulosa cells. Interestingly, when age was removed and the number of oocytes stimulated were the same, apoptosis was related to pregnancy outcome and

not the ovarian reserve (Oosterhuis et al., 1998). Oosterhuis et al, 1998, reported that pregnancy rate was associated with reduced granulosa apoptosis levels (TUNEL assay); conversely, Moffatt et al, 2002, reported that the apoptosis level in cumulus cells from oocytes that were inseminated was higher than in abnormal oocytes, immature or mature oocytes, which indicate that the mechanisms involved in fertilisation induce apoptosis during normal function.

It is noteworthy that despite the initial impetus for apoptosis research as a clinical measure of oocyte quality, this has not been translated into clinical practice in IVF medical centres. The accuracy of apoptosis as a marker for superior oocyte quality and the commercial need for rapid outcome based procedures has limited translational research in this area.

Ovarian reserve and the density of FSH and LH receptors have been linked with reduced fertility and oocyte quality (Cai et al., 2007,Maman, Yung, Kedem et al., 2012). In addition, the dysregulation of gene expression of granulosal BMPR1B, FSH, and LH receptors from older patients has been associated with poor pregnancy rate (Regan et al., 2016,Regan et al., 2017). On the basis of the current findings, dysregulation of receptor expression in the older patient may supress the mitogenic growth rate in healthy follicles indicated by reduced granulosa cell apoptosis. BMPR1B levels were reduced at the critical time of dominant follicle selection and the lack of down-regulation of BMPR1B, FSH and LH receptors involved in preovulatory maturation were associated with lower granulosa apoptosis rates and infertility. Restoring an optimum receptor density and down-regulation of receptors may improve the pregnancy rate in older women.

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, and

obtained informed consent from patients and ethics approval. PK interpretation of data, contributed to the draft of the manuscript, and critically revised the manuscript. JLY supervised, participated in the study design, interpretation of data, and critically revised the manuscript. YL supervised, participated in the study design, obtained informed consent from patients and ethics approval, and revised the manuscript. FA supervised, contributed to the draft of the manuscript, interpretation of data, and critically revised the manuscript. GA supervised, participated in the study design, interpretation of data, and 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.

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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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Figure 1 Schematic diagram of analysis of data

The study design detected 7AAD positive cells (+ve) that expressed FSH receptors localized to the surface of the granulosa cell. FSH receptor positive cells are indicated by the pink halo around the box. White blood cells (FSH receptors –ve), atretic bodies (isolated cytoplasm content e.g. organelles, FSH receptor -ve), and terminally differentiated granulosa cells coalesced into large globules (internalised FSH receptor -ve) would not be represented. To retrieve the oocyte for fertilisation, the cumulus cells (FSH receptor +ve) are removed with the cumulus oocyte complex. Basal granulosa cells (FSH receptor +ve) are unlikely to be included because deep gouging of the granulosa membrana did not occur, as this would compromise subsequent corpus luteum function.

Figure 2 Granulosal apoptosis and receptor levels in the young compared to older IVF patients

Granulosal apoptosis and ovarian reserve depletion collected from different size follicles. Percentage of 7AAD and FSH receptor positive granulosa cells from healthy follicles. The level of apoptosis is defined as; the percentage of cells expressing FSH receptors that are positive for exposed DNA (7AAD+), and not a percentage of the heterogeneous total population of cells in the aspirated follicular fluid. All 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 *$p<0.05$, **$p<0.01$, ***$p<0.005$ and ****$p<0.0001$. The number within the column represents the number of follicles analysed for that group. Patients were grouped according to ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles from 2-10 mm on day 2-5 of a cycle. Follicle count is based on the combined total AFC from both ovaries.

Figure 3 Apoptosis rate of granulosa cells and ovarian reserve depletion in young compared to older IVF patients

Data were subjected to statistical verification using t-test. 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. The percentage of apoptosis is defined as the 7AAD + / FSHR+ cells of the granulosa cell population expressing FSH receptors, and not the heterogeneous total population of cells in the aspirated follicular fluid.

Figure 4 Granulosa apoptosis from follicles when combined.

Individual follicles of different sizes for the young and old with a typical ovarian reserve for age were combined to mimic an experimental protocol of 'pooled' follicles. The percentage of apoptosis is defined as the 7AAD + / FSHR+ cells of granulosa cell population expressing FSH receptors, and not the heterogeneous total population of cells in the aspirated follicular fluid. Data were subjected to statistical verification using t-test. 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.

Figure 5 The effect of ovarian reserve depletion on granulosal apoptosis in 40+year old IVF patients

Percentage of apoptotic granulosa cells and follicle size collected during IVF cycles. 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. The percentage of apoptosis is defined as the 7AAD + / FSHR+ cells of granulosa cell population expressing FSH receptors, and not the heterogeneous total population of cells in the aspirated follicular fluid.

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.

Figure 6 The comparative effect of rFSH dose on FSH receptor and LH receptor expression

The effect of dose of rFSH on granulosal apoptosis in patients matched for aged, ovarian reserve, AMH, and size of follicles: 40+ y, with an ovarian reserve of D, and follicle size of 10-22 mm. The percentage of apoptosis is defined as the 7AAD + / FSHR+ cells of granulosa cell population expressing FSH receptors, and not the heterogeneous total population of cells in the aspirated follicular fluid. Data were subjected to statistical verification using t-test. 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.

Figure 7 Correlations of granulosal FSHR and BMPR1B density with apoptosis, and the influence of declining ovarian reserve

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. Sequential graphs show increasing age and declining ovarian reserve indicated by AFC. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per 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. Linear regression analysis; R squared is indicated for each group. The data points are the average of the receptor

74 expression for the follicle size; patients combined. Values are means ± S.E.M., and

75 differences were considered significant if $p<0.05$.

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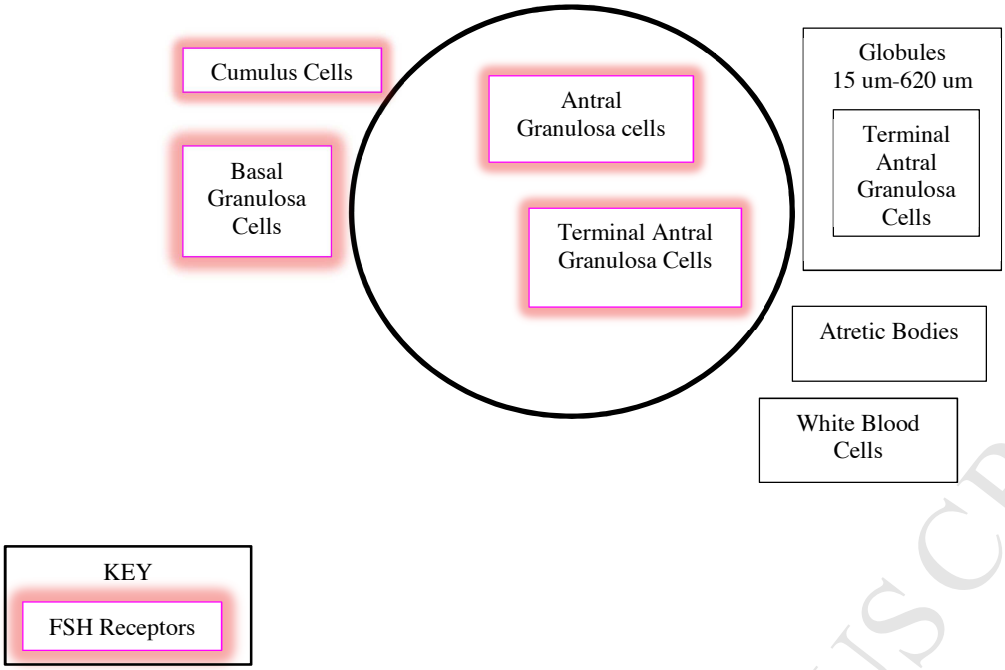
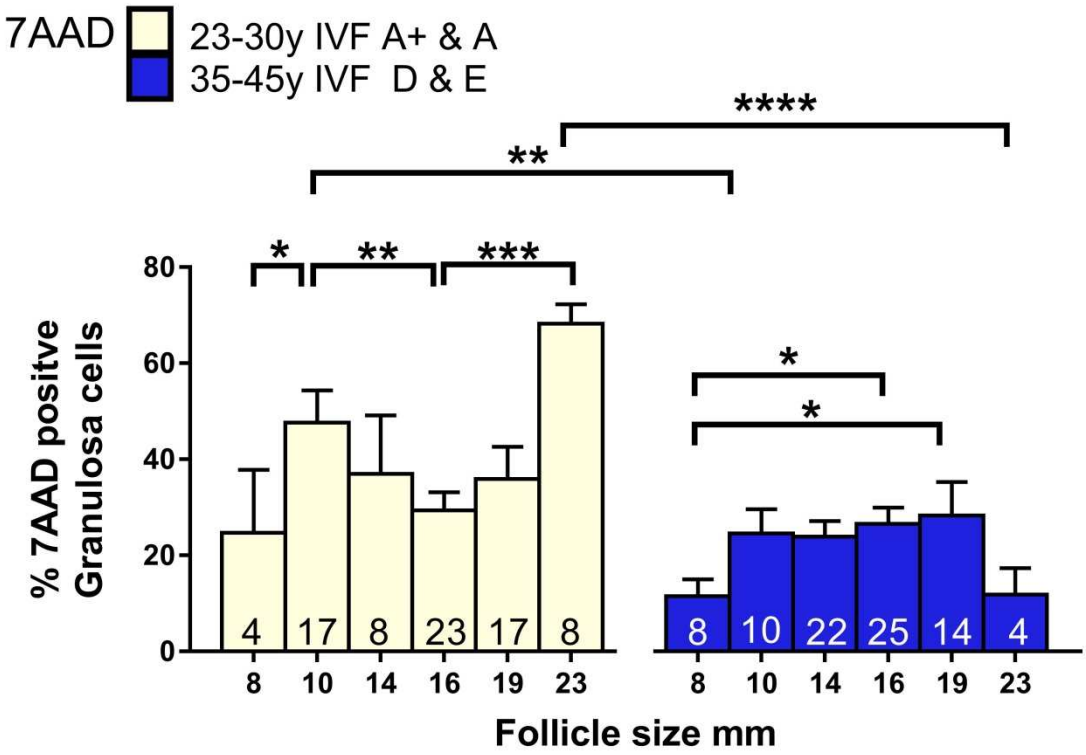
Table 1 Ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

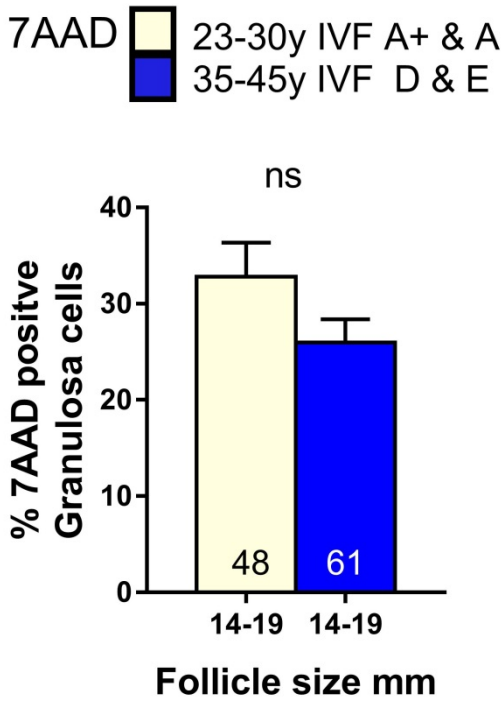
AGE Year	IVF Patient	Total Follicle	BMI	Ovarian Reserve Group Follicles Collected						Fertility Per Embryo Transfer %		
				A+	A	B	C	D	E	Not Pregnant	Pregnant	Live Birth
23-30	**9**	**64**	**24.1±4**	**30**	**46**	**0**	**0**	**0**	**0**	**26**	**73****	**33**
35-45	**18**	**122**	**24.8±5**	**0**	**0**	**34**	**5**	**67**	**16**	**79**	**21**	**7**
***40-45**	**9**	**83**	**23.9±5**	**0**	**0**	**19**	**5**	**54**	**5**	**94**	**6**	**0**

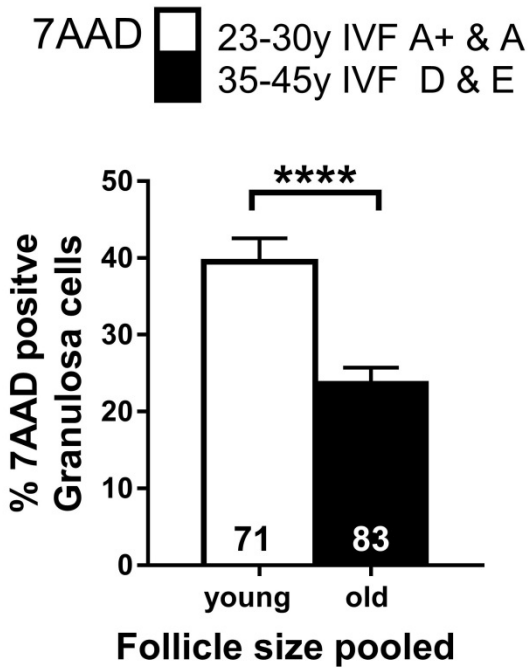
Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: group A+ = 30-39 follicles; group A = 20-29 follicles; group B = 13-19 follicles; group C = 9-12 follicles, group D = 5-8 follicles; group E = ≤4 follicles. Follicle count is based on the combined total from both ovaries to determine AFC. *Subgroup of oldest patients; poorest prognosis cohort. **1 Ectopic pregnancy.

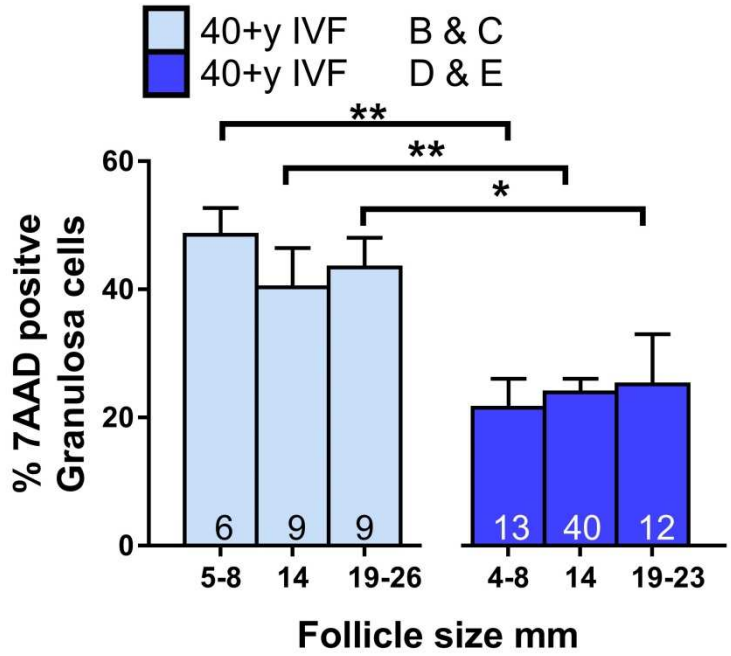




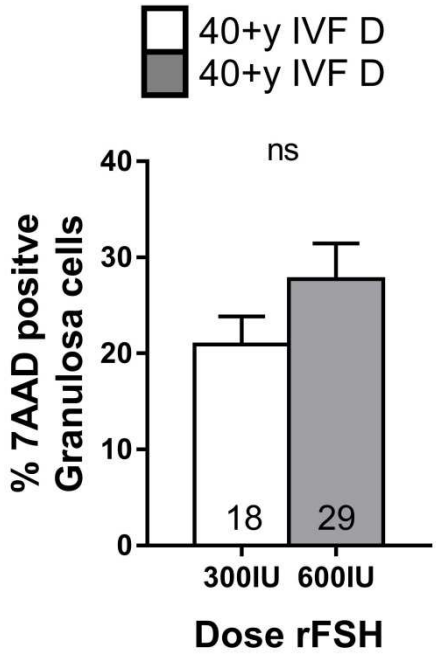
OVARIAN RESERVE	GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR

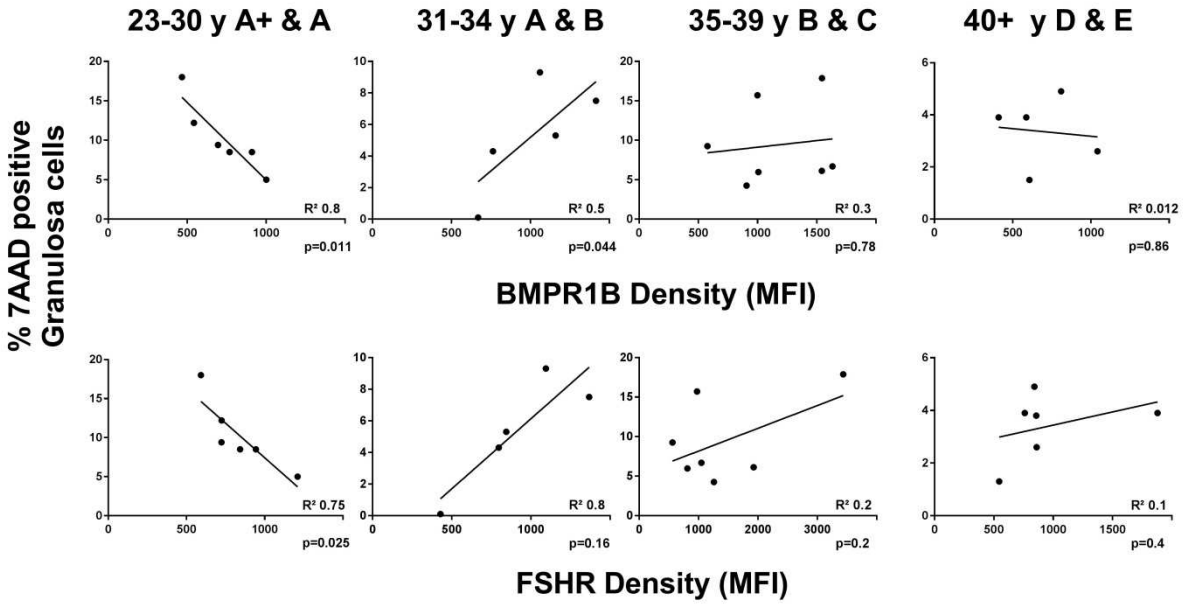






OVARIAN RESERVE	GOOD A+ = 30-39 A = 20-29 B = 13-19 C = 9-12 D = 5-8 E = ≤4 POOR





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

- Apoptosis was higher in follicles in the young compared to older women
- Preovulatory down-regulation of receptors was associated with reduced apoptosis and fertility
- Apoptosis reflects mitogenic turnover rate of granulosa cells in healthy follicles