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ORIGINAL RESEARCH

Effect of the FSH receptor single nucleotide polymorphisms (FSHR 307/680) on the follicular fluid hormone profile and the granulosa cell gene expression in human small antral follicles

T. Borgbo[1,2,*], J.V. Jeppesen[1], I. Lindgren[3], Y. Lundberg Giwercman[3], L.L. Hansen[2], and C. Yding Andersen[1]

[1]Laboratory of Reproductive Biology, Rigshospitalet, Copenhagen, Denmark [2]Department of Biomedicine, University of Aarhus, Aarhus, Denmark [3]Molecular Genetic Reproductive Medicine, Department of Clinical Sciences, Lund University, Malmö, Sweden

*Correspondence address. Laboratory of Reproductive Biology, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. Tel: +45-3545-5826; E-mail: tanni.kjaer.borgbo@regionh.dk

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ABSTRACT: The most pronounced effects of FSH signalling are potentially displayed in the follicle fluid, which acts as a reservoir for FSH-induced granulosa cell (GC) secreted hormones. This study investigates the effects of two common polymorphisms of *FSHR*, FSHR 307 (rs6165) and FSHR 680 (rs6166), by evaluating the hormone and gene expression profiles of human small antral follicles collected under physiological conditions in connection with fertility preservation. In total 69 women at various time during the menstrual cycle were included in this study. The intrafollicular hormone content of 179 follicular fluid samples and the gene expression levels of 85 GC samples were correlated to the genotype of both *FSHR* polymorphisms. The following parameters were evaluated: follicle diameter, levels of Anti-Müllerian hormone (AMH), progesterone, estradiol, testosterone and androstenedione and gene expression levels of *FSHR,* luteinizing hormone receptor (*LHR*), androgen receptor, aromatase cytochrome p450 (*CYP19A1*), *AMH* and *AMH receptor II* (*AMHR2*). There was 100% concordance between the FSHR 307 and the FSHR 680 genotypes: A/A (p.307Thr/Thr and p.680Asn/Asn), A/G (p.307Thr/Ala and p.680Asn/Ser) and G/G (p.307Ala/Ala and p.680Ser/Ser). Considering all follicles, compared with the other genotypes the G/G genotype was associated with significantly elevated gene expression levels for *LHR,* while *AMHR2* gene expression levels were significantly reduced. In follicles 3–6 mm in diameter *LHR* gene expression was significantly increased, whereas *AMH* gene expression was significantly reduced for the G/G genotype. In follicles >6 mm, estradiol and *CYP19A1* gene expression levels were significantly higher for the G/G genotype. In conclusion, significant changes were observed between the FSHR 307/680 polymorphisms in human small antral follicles collected under physiological FSH conditions.

Key words: follicular fluid / FSHR polymorphisms / FSHR 307/680 / granulosa cell gene expression / sex steroids

Introduction

FSH is one of the key hormones of mammalian reproduction and is involved in the regulation of the ovarian function, such as follicle development, ovarian steroidogenesis and oocyte maturation (Richards and Pangas, 2010; Miller and Auchus, 2011). FSH acts through the FSH receptor (FSHR) which in healthy women is mainly expressed on granulosa cells (GC), already from the early stages of folliculogenesis (Oktay *et al.*, 1997). Although the FSHR is a classical G protein-coupled receptor, activation of this receptor exerts a myriad of effects on GC function. The FSH-induced response is, however, dependent on the specific FSH isoform composition, as well as the genetic constitution of *FSHR*.

Although the FSH isoform composition has been shown to exert different effects in GCs upon stimulation (Barrios-De-Tomasi *et al.*, 2002; Yding Andersen, 2002; Arey and López, 2011), recent studies have suggested that common genetic variations of *FSHR* may also influence the FSH signalling (Casarini *et al.*, 2014). Until now, the *FSHR* gene is the most studied genetic factor associated with controlled ovarian stimulation (COS) (La Marca *et al.*, 2013), and two of its common single nucleotide polymorphisms (SNPs), FSHR 307 (rs6165, c.919A>G,

p.Thr307Ala) and FSHR 680 (rs6166, c.2039A>G, p.Asn680Ser), have been investigated in a number of studies regarding their impact on the FSH-induced response during COS (Perez Mayorga et al., 2000; Sudo et al., 2002; Laven et al., 2003; Daelemans et al., 2004; Behre et al., 2005; Klinkert et al., 2006; Achrekar et al., 2009; Boudjenah et al., 2012). Both SNPs reside in exon 10 in the protein-coding region of *FSHR*, and due to their close proximity, FSHR 307 and FSHR 680 are observed in strong linkage disequilibrium (Simoni, 1997; Gromoll and Simoni, 2005; Casarini et al., 2011; Desai et al., 2013b).

Both SNPs cause amino acid substitutions due to single nucleotide substitutions from adenine (A) to guanine (G). The A/A genotype constitutes the amino acid combination p.307Thr/Thr and p.680Asn/Asn, which is present in ~30% of the European population (Simoni and Casarini, 2014). The A/G genotype constitutes the amino acid combination p.307Thr/Ala and p.680Asn/Ser, present in ~48% of Europeans, whereas the G/G genotype (p.307Ala/Ala and p.680Ser/Ser) is observed in ~22% of the European population (Database of Single Nucleotide Polymorphisms (dbSNP); Simoni and Casarini, 2014).

As both SNPs cause amino acid substitutions, it is likely that the changes in the amino acid composition of the FSHR protein may influence the signal transduction. However, it is still unclear how the two polymorphisms affect the downstream signalling pathways within the follicle (Simoni, 1997; Gromoll and Simoni, 2005; Casarini et al., 2011; Desai et al., 2013b).

The FSHR is significantly higher expressed in small antral follicles with a diameter of around 6 mm, as compared with follicles in the later stages of folliculogenesis (Jeppesen et al., 2013). The follicle fluid acts as a reservoir for GC secreted substances, with concentrations of sex- and peptide hormones several orders of magnitude higher than in the circulation (Andersen et al., 1992, 2010). Thus, changes in GC function caused by *FSHR* polymorphisms are most likely to be expressed in the follicular fluid or in the mRNA profiles of the GC from small human antral follicles.

The aim of this study was to associate intrafollicular hormone concentrations and GC-specific gene expression of normal small antral follicles of 3–13 mm in diameter, with the FSHR 307/680 polymorphisms.

Materials and Methods

Sample material

In total, 69 women were included in the study; 57 women donated follicular fluid (mean age of 28, range: 15–38 years), and 30 donated GC (mean age of 27, range: 15–38 years), with 20 women donating both follicular fluid and GC. Thus, the follicular fluid and GC samples were evaluated separately.

The follicles analysed per woman ranged from 1 to 8 (mean 3.9 follicles per woman). In total, 179 follicular fluid samples were obtained from human small antral follicles with diameters ranging from 3 to 13 mm, as judged from the aspirated volume. In total, 85 GC samples were obtained from follicles with diameters ranging from 3 to 11 mm. Two subgroups of follicles were created according to diameter, with a cut-off at 6.5 mm in diameter: group 1 (3–6 mm) and group 2 (>6 mm).

The follicles were collected at various times during the menstrual cycle. Blood samples were not collected. All women included in this study underwent fertility preservation by cryopreservation of ovarian tissue. The patients were diagnosed with various cancer diseases. None of the patients had polycystic ovary syndrome (PCOS) or other ovarian pathologies and in all cases, the ovary had an overall normal gross appearance. The fertility preservation procedure involved excision of one entire ovary from which individual visible antral follicles were aspirated with a 23G needle attached to a syringe prior to preparation of the ovarian cortex for freezing. The study was approved by the ethical committee of the municipalities of Copenhagen and Frederiksberg (H-2-2011-044) and informed consent was obtained from all subjects.

A minor part of the sample material has been used in previously published studies (Jeppesen et al., 2012, 2013; Borgbo et al., 2014).

DNA extraction and genotyping by high resolution melting analysis

In total, 41 patients were genotyped for both polymorphisms using DNA extracted from ~25 mg ovarian tissue, using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The DNA samples were subsequently stored at −20°C.

The remaining 28 patients were genotyped for both polymorphisms from DNA extracted from the GC of small antral follicles, as no ovarian tissue was available from these samples. The GC were isolated from the follicular fluid after a brief centrifugation (400*g*, 10 min), snap frozen in liquid nitrogen and stored at −80°C. DNA was extracted from the GC using Tri Reagent (Sigma-Aldrich, St Louis, MO, USA), with slight modifications as published by Borgbo et al. (2014).

Genotyping of both *FSHR* polymorphisms was achieved by PCR and high resolution melting (HRM) analysis, using a new genotyping assay based on competitive amplification of differentially melting amplicons (CADMA), as described by Borgbo et al. (2014).The CADMA-based genotyping was performed on the LightCycler (R) 480 Instrument II (Roche Diagnostics, Ropkreuz, Switzerland).

For data analysis, the LightCycler® 480 series software version 1.5.0.39 was applied. The melting profiles were visualized using the software function 'Melt Curve Genotyping'.

Hormone measurements

The follicular fluid hormone levels were measured for Anti-Müllerian hormone (AMH), estradiol, progesterone, androstenedione and testosterone, using commercially available radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) kits.

AMH was measured using the UltraSensitive AMH/MIS Elisa kit (AI-105-i, AnshLabs, Webster, TX, USA), according to the manufacturer's instructions. To obtain AMH levels within the standard curve, the follicular fluids were diluted 1:300 in the supplied assay buffer.

Estradiol, progesterone, androstenedione and testosterone were initially measured using commercially available RIA kits (DSL-43100, DSL-3400, DSL-3800, DSL-4000; DSL, Webster, TX). However, during the course of sample collection, the RIA assays became unavailable and it was necessary to switch to alternative commercially available ELISA assays (NovaTec Immundiagnostica, Dietzenbach, Germany; DNOV 002, 003, 006 and 008, respectively).

Due to limited sample material, it was not possible to re-analyse the follicular fluid analysed with RIA. However, based on highly significant linear correlations between the two types of assays (correlation coefficients: estradiol, $r = 0.99$; progesterone, $r = 0.93$; testosterone, $r = 0.91$ and androstenedione, $r = 0.91$), it was possible to create mathematical equations for transforming data from RIA into ELISA as previously published by Jeppesen et al. (2013).

The RIA kit was used to analyse ~1/6 of the follicles fluids, estradiol ($n = 27$), progesterone ($n = 25$), androstenedione ($n = 33$) and testosterone ($n = 33$), according to the manufacturers' protocol, using in-house prepared steroid-free serum for follicular fluid dilution.

The remaining follicular fluids were analysed using the NovaTech ELISA assays, estradiol ($n = 152$), progesterone ($n = 154$), androstenedione ($n = 146$) and testosterone ($n = 146$), according to the manufacturers'

protocol, also using in-house prepared steroid-free serum for follicular fluid dilution.

Gene expression analysis

The gene expression levels of the 85 GC samples were analysed for *FSHR*, luteinizing hormone receptor (*LHR*), Androgen receptor (*AR*), the aromatase Cytochrome P450 family 19 subfamily A polypeptide 1 (*CYP19A1*), *AMH* and Anti-Müllerian hormone Receptor II (*AMHR2*), with gene expression levels normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) housekeeping gene expression. RNA purification, cDNA and qPCR analysis were performed as previously published (Jeppesen *et al.*, 2013). As the amount of isolated RNA was limited, not all GC samples were analysed for all genes.

Statistical analysis

The statistical analyses were performed using STATA version 13.1 (STATA-Corp LP, USA). Hormone and gene expression levels were log- or square root-transformed in order to approximate normal distributions. A one-level mixed effects model were used for the statistical analysis, including follicle size as co-variate and women as random effect, in order to take into account that GC function varies according to size and that follicles from one woman may be more similar than follicles from different women. *P*-values ≤ 0.05 were considered statistically significant.

Results

There was a 100% concordance between the FSHR 307 genotype and the FSHR 680 genotype. The genotype frequencies among the 69 women were 35% (A/A), 42% (A/G) and 23% (G/G) for both polymorphisms.

Association between *FSHR* genotype and intrafollicular hormone levels

The follicular fluid hormone levels grouped according to the *FSHR* polymorphisms are summarized in Table I and visualized in Supplementary data, Fig. S1.

Overall

No significant genotype-specific differences were observed for follicular fluid hormone levels. However, levels of AMH and progesterone displayed a similar pattern of hormone profiles, with highest hormone levels in the homozygote genotypes (A/A and G/G/) in contrast to estradiol levels which showed an inverse pattern with highest hormone levels for the heterozygous genotype (A/G). As the follicle hormone production depends on the developmental stage and size of the follicles, the follicles were divided into two subgroups with a cut-off at 6.5 mm in diameter, which approximately designates the size at which follicle selection takes place (Grøndahl *et al.*, 2011; Jeppesen *et al.*, 2013).

Follicles 3–6 mm in diameters

This group constitutes >75% of all of the follicles included in this study. As a consequence, the hormone profile of these follicles resembles those of all the follicles combined. No statistically significant differences in hormone levels between the genotypes were observed. The hormone profile of AMH resembled that of progesterone, with higher hormone levels observed for the homozygous genotypes A/A and G/G, while levels of estradiol showed an inverse pattern. No genotype-specific differences were observed for the androgens (testosterone and androstenedione).

Follicles >6 mm in diameter

In the larger follicles, >6 mm in diameter, genotype-specific differences were observed for estradiol, in which women carrying the G/G genotype displayed significantly higher estradiol levels as compared with the A/A genotype ($P = 0.04$). Again, estradiol displayed an inverse pattern compared with AMH and progesterone.

The intrafollicular hormone levels according to the follicular diameter (i.e. 3–4, 5–6, 7–8 and >8 mm) are summarized in Supplementary data, Table S1 and Fig. S3, grouped according to *FSHR* genotype.

Association between *FSHR* genotype and GC gene expression levels

The results of the gene expression analysis is summarized in Table II and visualized in Supplementary data, Fig. S2.

Overall

The pattern of gene expression profiles of the *LHR* and the *CYP19A* were similar, with significantly higher *LHR* expression for the G/G genotype as compared with A/A ($P = 0.01$). The gene expression profiles of *AMHR2* and *AMH*, seemed to be inversely related to *LHR* and *CYP19A*, with significant lower *AMHR2* gene expression for the G/G genotype as compared with A/A ($P = 0.05$). No statistical significant genotype differences were observed in the gene expression profiles of *AR* and *FSHR*.

Follicles 3–6 mm in diameters

Significant higher gene expression levels were observed for *LHR* for the G/G genotype compared with the A/A genotype ($P = 0.05$), similar to the overall results. In addition, significant higher gene expression levels were observed for *AMH* for the A/A genotype as compared with the G/G genotype ($P = 0.02$).

Follicles >6 mm in diameter

Genotype-specific differences were observed for *CYP19A1*, with significant higher gene expression for G/G as compared with A/A ($P = 0.02$) and A/G ($P < 0.001$). The gene expression levels according to follicle diameter and genotype are summarized in Supplementary data, Table S2 and Fig. S4.

Discussion

This study demonstrates that the two common *FSHR* polymorphisms FSHR 307 and FSHR 680 exert significant effects in human small antral follicles. Overall, significant genotype-dependent differences were observed for *LHR* and *AMHR2* gene expression levels with an inverse relationship, in which *LHR* expression for the G/G genotype was significantly higher than for the A/A genotype, whereas the *AMHR* expression for the G/G genotype was significantly lower than the A/A genotype.

An inverse relationship between AMH and estradiol was observed, which was in line with other studies in human small human antral follicles (Eilsø Nielsen *et al.*, 2010; Jeppesen *et al.*, 2013). In the follicles 3–6 mm in diameter, significantly increased gene expression levels of *LHR* were observed for the G/G genotype. In contrast, *AMH* gene expression levels were significantly lower in the G/G genotype, as compared with

Table I Intrafollicular hormone levels grouped according to genotype of the FSHR 307/680 polymorphisms.

Genotype	All follicles			3–6 mm			>6 mm		
	A/A	A/G	G/G	A/A	A/G	G/G	A/A	A/G	G/G
Number of follicles	64	83	32	53	61	25	11	22	7
Number of Women†	19	26	11	14	14	9	5	12	2
AMH (pmol/l)	5739 (4519;7288)	4415 (3508;5557)	6541 (4165;10272)	6287 (4835;8176)	4694 (3666;6010)	7945 (5108;12353)	3698 (2041;6701)	3726 (2114;6564)	3268 (696;15358)
Estradiol (nmol/l)	12 (7;19)	27 (16;45)	18 (8;41)	13 (7;22)	27 (14;50)	13 (5;35)	8*1,2 (3;20)	27*1 (11;67)	50*2 (6;430)
Progesterone (nmol/l)	28 (19;41)	22 (16;32)	29 (15;56)	26 (17;39)	19 (13;28)	31 (15;64)	45 (15;135)	36 (16;83)	23 (3;171)
Testosterone (nmol/l)	180 (151; 214)	158 (134;187)	189 (153;235)	180 (147;220)	165 (135;202)	177 (136;230)	180 (127;253)	142 (106;190)	244 (171;347)
Androstenedione (nmol/l)	1130 (992;1288)	1073 (949;1212)	1070 (813;1411)	1078 (931;1247)	1066 (929;1222)	984 (701;1381)	1422 (1063;1902)	1091 (822;1450)	1449 (977;2151)
Follicle size (mm)	5.4 (5.0;5.7)	5.4 (5.1;5.8)	5.5 (5.1;6.1)	5.0 (4.7;5.3)	4.8 (4.5;5.1)	5.0 (4.7;5.4)	7.8 (7.2;8.4)	7.8 (7.2;8.3)	8.0 (6.2;9.7)

Data are geometric mean (95% CI) unless stated otherwise.
The genotypes are representative for both FSHR307 and FSHR680. Both polymorphisms have been tested for all samples with matching results, with the following genotype-to-protein composition. **A/A**: p.307Thr/Thr and p.680Asn/Asn, **A/G**: p.307Thr/Ala and p.680Asn/Ser and **G/G**: p.307Ala/Ala and p.680Ser/Ser.
*Statistical significance: >6 mm in diameter: *Estradiol*: $^{1}P = 0.05$, $^{2}P = 0.04$.
†Women may have follicles allocated to both size groups. The number of women in the two size groups does therefore not correspond to the total number in the 'all follicles' group.

Table II Granulosa cell gene expression × 1000 normalized to GAPDH and grouped according to genotype of the FSHR 307/680 polymorphisms.

Genotype	All follicles			3–6 mm in diameter			>6 mm in diameter		
	A/A	A/G	G/G	A/A	A/G	G/G	A/A	A/G	G/G
FSHR ($n = 85;30$)	96 (49;190)	146 (86;246)	81 (33;200)	134 (75;237)	116 (65;207)	71 (25;202)	15 (0;756)	312 (73;1332)	189 (62;573)
LHR ($n = 71;29$)	0.7*1,2 (0.4;1.6)	1.8*1 (1.0;3.2)	3.1*1,2 (1.1;8.7)	0.9*4 (0.4;1.9)	1.6 (0.7;3.5)	3.6*4 (1.0;12.9)	0.1 (0.0000002; 52081)	2.4 (1.0;6.0)	1.7 (0.1;33.5)
CYP19A1 ($n = 68;22$)	18*8 (13;25)	26 (13;51)	39*8 (15;101)	18 (13;26)	34 (16;76)	31 (10;93)	16*6 (1;287)	9*7 (4;25)	117*6,7 (3;3763)
AR ($n = 83;30$)	38 (26;55)	48 (31;75)	33 (22;50)	43 (28;65)	40 (26;64)	33 (20;53)	19 (6;61)	84 (20;363)	35 (22;57)
AMH ($n = 85;30$)	283 (128;624)	209 (96;456)	45 (19;294)	448*5 (239;840)	148 (64;344)	79*5 (3;12)	22 (0;1683)	654 (82;5245)	57 (0;106774)
AMHR2 ($n = 64;22$)	14.0*3 (9.7;20.2)	8.6 (5.9;12.7)	6.9*3 (4.1;11.5)	14.5*9 (9.8;21.3)	8.5 (5.7;12.7)	6.3*9 (3.3;12.1)	10.8 (0.7;172.6)	9.0 (1.8;44.5)	9.7 (4.3–21.8)
Follicle size (mm)	5.2 (4.8;5.7)	5.3 (4.8;5.9)	5 (4.4;5.6)	5.0 (4.7;5.4)	5.2 (4.9;5.5)	4.7 (4.2;5.2)	7.5 (6.4;8.8)	7.5 (6.5;8.7)	7.4 (6.1;8.9)

Geometric mean (95% CI).
Not all follicles yielded sufficient mRNA for the gene expression analysis. As a consequence, not all samples are analysed for all six genes. Below each gene is listed the number of follicles and women analysed (n = follicles; women). The exact number of follicles and women in each category is given in Supplementary data, Table S2.
*Statistical significances. All follicles: *LHR*: $^{1}P = 0.04$, $^{2}P = 0.01$; *AMHR2*: $^{3}P = 0.05$; *CYP19A1*: $^{8}P = 0.06$. 3–6 mm in diameter: *LHR*: $^{4}P = 0.05$; *AMH*: $^{5}P = 0.02$; *AMHR2*: $^{9}P = 0.06$. >6 mm in diameter: *CYP19A1*: $^{6}P = 0.02$, $^{7}P < 0.001$.

the A/A genotype. In the follicles >6 mm in diameter, significantly elevated gene expression levels of *CYP19A1* were observed for the G/G genotype. Likewise, significant higher levels of estradiol were observed in carriers of the G/G genotype as compared with the A/A genotype. This increased capacity to undertake estradiol synthesis by the group of larger follicles probably reflects that selection takes place around this follicular diameter and the selected follicle accelerate its estradiol output in response to circulating levels of gonadotrophins. In line with an augmented estradiol production for the G/G genotype, increased gene expression levels were observed in both *LHR* and *CYP19A1*, whereas the *AMH* gene expression levels were significantly reduced. Therefore, a possible effect of the G/G genotype in human follicles reaching follicular selection may be to induce *LHR* and *CYP19A1* expression more efficiently, and in this way augment the synthesis of steroid hormones. This observation corresponds to the results of two other studies, in which higher FSH responsiveness has been observed for the G/G genotype of FSHR 307/680 in women undergoing COS (Achrekar *et al.*, 2009; Boudjenah *et al.*, 2012). However, it cannot be excluded that the augmented estradiol production in the group of larger follicles reflects collection of the relatively few follicles at a specific stage of the menstrual cycle or influence from exposure to for instance LH.

It is interesting to notice that a number of studies have found that the A/A genotype resulted in a higher FSH response than the G/G genotype, which is in contrast to this study (Simoni *et al.*, 1999; Sudo *et al.*, 2002; Nordhoff *et al.*, 2011; Zalewski *et al.*, 2013; Casarini *et al.*, 2014). However, several differences exist between the studies: (i) Most of the above studies used granulosa lutein cells collected in connection with COS and IVF, and the cells have subsequently been cultured for several days. These GC are very different from the immature GCs investigated in our study (Jeppesen *et al.*, 2012; Ophir *et al.*, 2014). For example, the FSHR density is different and may affect the signal transduction of FSH. (ii) Most of the above studies have used GC that have either been exposed to supra physiological concentrations of FSH either during COS and/or during the culture period. It may be speculated that high levels of FSH will overshadow potential differences in the response to FSH stimulation to the different *FSHR* polymorphisms. (iii) In some studies, cells have been transfected with *FSHR* genes and their functionality may not reflect the normal physiological situation. (iv) It could be speculated that the significant lower gene expression of *AMH* and *AMHR2* observed for the G/G genotype as compared with the A/A genotype, exerted an effect on steroidogenesis in small antral follicles, in which AMH production is very high. These effects are unlikely to occur in luteinized GCs, in which AMH production is minimal. (v) Although this study included follicular fluid from almost 200 follicles, the observed differences may reflect that the FSHR genotypes only to a limited extend affect the intrafollicular conditions and that an even larger group is required to detect differences robustly.

Supporting the above discussion, a number of contradicting findings have been published investigating the effects of the 307/680 polymorphisms. Several studies suggests that the A/A genotype produces a higher response to rFSH as compared with the G/G genotype (Perez Mayorga *et al.*, 2000; Sudo *et al.*, 2002; Behre *et al.*, 2005; Huang *et al.*, 2014), whereas other studies did not observe any significant association between *FSHR* genotype and the outcome of COS (Laven *et al.*, 2003; Klinkert *et al.*, 2006). Some studies have reported an association between the G/G genotype and ovarian hyperstimulation syndrome, presumably because the G/G genotype constitutes a more responsive FSHR, which is in line with our findings (Daelemans *et al.*, 2004; Achrekar *et al.*, 2009; Boudjenah *et al.*, 2012). In contrast to our results, a recent study by Casarini *et al.* showed significant differences in cAMP kinetics and in the downstream PKA pathway (STAR1 expression, CREB phosphorylation and progesterone production) in luteinized GC, with a more effective activation found for the A/A genotype as compared with the G/G genotype (A/G genotype was not investigated) (Casarini *et al.*, 2014). To get a more detailed understanding of how the *FSHR* polymorphisms affect GC function in small antral follicles *in vivo*, it would be interesting to test for possible differences in the FSH response in relation to the *FSHR* polymorphisms as observed during the natural environment, for instance during culture experiments using human immature GC stimulated with physiological concentrations of FSH and include some of the methods used by Casarini *et al.*, (2014).

In a study evaluating the effects of FSHR 307/680 on GC function, Zalewski *et al.* (2013) noted that the steroid levels showed high variability even within each genotype group, indicating that additional factors might be involved. One such factor may be a recently discovered polymorphism located in the promoter region of the *FSHR*, rs1394205 (c.-29G>A, FSHR-29). Functional studies of the promoter variant have demonstrated that the transcriptional activity of the A-allele was significantly reduced compared with the G-allele, resulting in lower mRNA FSHR levels and a reduced response to COS (Nakayama *et al.*, 2006; Desai *et al.*, 2011, 2013a). Furthermore, the combination of the *FSHR* polymorphism FSHR 307/680 and FSHR-29 have been evaluated in a study by Desai *et al.* (2013a). The results of this study suggested that FSHR-29 affects the GC function to a much higher extend than FSHR 307/680.

Two concerns may be raised regarding the validity of this study: (i) the small human antral follicles used in this study were collected at various times during the menstrual cycle, and (ii) no blood samples were collected to evaluate serum hormone levels. Since FSH levels vary across the menstrual cycle, it could be questioned whether the intrafollicular hormone milieu of small antral follicles also varied. The majority of follicles in this study had a diameter corresponding to stages prior to follicular selection and is therefore considered to be at the recruitable stage. At this stage, follicles are dependent on tonic levels of gonadotrophins, and will only become dependent on circulating levels upon selection. This has been addressed in a study by Westergaard *et al.* (1986), which concluded that the hormonal content of human small antral follicles developed independently of the cyclic fluctuations in FSH and LH secretion, during the menstrual cycle. Additional studies in the 1980s furthermore showed that the hormonal content of small antral follicles from pregnant women at the time of childbirth (who had been without menstrual cycles and deprived of FSH for nine months) was similar to normal women (Westergaard *et al.*, 1985).

It is now accepted that CRASH protocols for ovarian stimulation can be started anywhere in the cycle without having a (major) impact on outcome (Kuang *et al.*, 2014), which suggests that the dynamics of the recruited small antral follicles are similar throughout the menstrual cycle.

Regarding the absence of serum hormone levels in this study, it should be noted that the small antral follicles do not have a major impact on the hormone conditions in circulation, and it is thus unlikely that a blood

sample would reflect the intrafollicular hormone milieu of the small antral follicles. Indeed, circulating levels of estradiol is unlikely to show any difference, as >90% of all estradiol in circulation derives from the selected follicle already on Day 7 of the cycle.

AMH in circulation is essentially a spillover from the small antral follicles (in which the concentration is two-three orders of magnitude higher than in circulation) and also unlikely to show any difference in the serum (Andersen *et al.*, 2010). Furthermore serum AMH has been shown to be almost constant throughout the menstrual cycle (Hehenkamp *et al.*, 2006).

Because the two polymorphisms are located on the same exon of FSHR, 307 threonine is usually on the same allele with 680 asparagine. No recombination was found in this study and there was 100% concordance between the FSHR 307 and the FSHR 680 genotypes. The genotype frequencies found for this study, 35% (A/A), 42% (A/G) and 23% (G/G) for both polymorphisms, were in accordance with previously published studies on European populations (Simoni and Casarini, 2014).

Collectively, the results presented in this study demonstrate that the the two common FSHR polymorphisms FSHR 307 and FSHR 680 affect FSH-induced GC responses in human small antral follicles. The G/G genotype of both polymorphisms was associated with significantly higher gene expression levels of *LHR* and significant lower *AMHR2* levels across all follicle sizes. Furthermore, significantly higher estradiol levels were observed for the G/G genotype for the larger follicles, suggesting an increased FSH responsiveness in women homozygous for the G/G genotype under normal physiological FSH levels.

Supplementary data

Supplementary data are available at *http://molehr.oxfordjournals.org/* online.

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Authors' roles

T.B. and C.Y.A. designed the study and wrote the manuscript. T.B. performed genotype analysis and ELISA analysis and J.V.J. performed gene expression analysis and ELISA. T.B. and C.Y.A. performed the interpretation of the data and T.B. prepared figures and tables for the manuscript. I.L and Y.L.G provided reference DNA for the genotyping assays. J.V.J., I.L., L.L.H. and Y.L.G. helped finalizing the manuscript.

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Conflict of interest

None declared.

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