Human Reproduction, Vol.31, No.3 pp. 672–683, 2016
Advanced Access publication on January 14, 2016 doi:10.1093/humrep/dev342

human reproduction

ORIGINAL ARTICLE ***Reproductive genetics***

Combined assessment of polymorphisms in the *LHCGR* and *FSHR* genes predict chance of pregnancy after *in vitro* fertilization

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Submitted on June 29, 2015; resubmitted on December 10, 2015; accepted on December 17, 2015

STUDY QUESTION: Can gonadotrophin receptor variants separately or in combination, be used for the prediction of pregnancy chances in *in vitro* fertilization (IVF) trials?

SUMMARY ANSWER: The luteinizing hormone/human chorionic gonadotrophin receptor (*LHCGR)* variant N312S and the follicle-stimulating hormone receptor (*FSHR)* variant N680S can be utilized for the prediction of pregnancy chances in women undergoing IVF.

WHAT IS KNOWN ALREADY: The *FSHR* N680S polymorphism has been shown to affect the ovarian response in response to gonadotrophin treatment, while no information is currently available regarding variants of the *LHCGR* in this context.

STUDY DESIGN, SIZE, DURATION: Cross-sectional study, duration from September 2010 to February 2015. Women undergoing IVF were consecutively enrolled and genetic variants compared between those who became pregnant and those who did not. The study was subsequently replicated in an independent sample. Granulosa cells from a subset of women were investigated regarding functionality of the genetic variants.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Women undergoing IVF ($n = 384$) were enrolled in the study and genotyped. Clinical variables were retrieved from medical records. For replication, an additional group of $n = 233$ women was utilized. Granulosa cells from $n = 135$ women were isolated by flow cytometry, stimulated with Follitropin alpha or Menotropin, and the downstream targets 3′,5′-cyclic adenosine monophosphate (cAMP) and inositol 1,4,5-trisphosphate (IP_3) measured with enzyme-linked immunosorbent assay.

MAIN RESULTS AND THE ROLE OF CHANCE: Women homozygous for serine (S) in both polymorphisms displayed higher pregnancy rates than women homozygous asparagine (N) (OR = 14.4, 95% CI: [1.65, 126], $P = 0.016$). Higher pregnancy rates were also evident for women carrying *LHCGR* S312, regardless of *FSHR* variant (OR = 1.61, 95% CI: [1.13, 2.29], $P = 0.008$). These women required higher doses of FSH for follicle recruitment than women homozygous N (161 versus 148 IU, $P = 0.030$). When combining the study cohort with the replication cohort ($n = 606$), even stronger associations with pregnancy rates were noted for the combined genotypes (OR = 11.5, 95% CI: [1.86, 71.0], $P = 0.009$) and for women carrying *LHCGR* S312 (OR = 1.49, 95% CI: [1.14, 1.96], $P = 0.004$). A linear significant trend with pregnancy rate and increasing number of G alleles was also evident in the merged study population (OR = 1.34, 95% CI: [1.10, 1.64], $P = 0.004$). A lower cAMP response in granulosa cells was noted following Follitropin alpha stimulation for women homozygous N in both polymorphisms, compared with women with other genotypes (0.901 pmol cAMP/mg total protein versus 2.19 pmol cAMP/mg total protein, $P = 0.035$).

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LIMITATIONS, REASONS FOR CAUTION: Due to racial differences in *LHCGR* genotype distribution, these results may not be applicable for all populations.

WIDER IMPLICATIONS OF THE FINDINGS: Despite that >250 000 cycles of gonadotrophin stimulations are performed annually worldwide prior to IVF, it has not been possible to predict neither the pregnancy outcome, nor the response to the hormone with accuracy. If *LHCGR* and *FSHR* variants are recognized as biomarkers for chance of pregnancy, more individualized and thereby more efficient treatment modalities can be developed.

STUDY FUNDING, COMPETING INTEREST(S): This work was supported by Interreg IVA, EU (grant 167158) and ALF governments grant (F2014/354). Merck-Serono (Darmstadt, Germany) supported the enrollment of the subjects. The authors declare no conflict of interest.

Key words: LHCG receptor / FSH receptor / *in vitro* fertilization / polymorphism / female fertility

Introduction

About 15% of all couples experience involuntary childlessness (World Health Organization, 2000). This number is expected to increase as a combination of socially related issues with prolonged time to start a family and increased access to assisted reproductive techniques also in rapidly growing economies as in Asia.

It has for a long time been known that follicle-stimulating hormone (FSH) plays a central role in the endocrine regulation of female as well as male gametogenesis. Follicle-stimulating hormone mediates its effect via the FSH receptor (FSHR) located on the cell membrane of ovarian granulosa cells (Camp *et al.*, 1991) from where FSH drives follicular maturation (McNeilly *et al.*, 1991) and estrogen production (Nordhoff *et al.*, 2011). The FSHR belongs to the G protein-coupled receptor family and hence signals through the classical $G\alpha_s$/3′,5′-cyclic adenosine monophosphate (cAMP)/protein kinase A pathway (Means *et al.*, 1974), but also through for example the adapter protein containing pleckstrin homology domain, phosphotyrosine-binding domain and leucine zipper motif (APPL11)/inositol 1,4,5-triphosphate (IP_3) signaling pathway (Thomas *et al.*, 2011).

The *FSHR* gene is located on chromosome 2 and consists of 10 exons. Exon 10 holds 5 single nucleotide polymorphisms (SNPs) in the coding region, of which the one in amino acid position 680 (N680S; rs6166), in the intracellular domain, is the most thoroughly studied (Simoni *et al.*, 1999; Gromoll and Simoni, 2005). In Caucasian populations, ~30% are homozygous for asparagine (N), 50% are heterozygous and 20% are homozygous for serine (S) (Kuijper *et al.*, 2010; Lindgren *et al.*, 2012). It has been proposed that women homozygous for *FSHR* S680 require a higher dose exogenous FSH prior to IVF than those with *FSHR* N680, in order to achieve successful ovarian stimulation (Perez Mayorga *et al.*, 2000; Sudo *et al.*, 2002). These women also seem to have longer menstrual cycles and have a higher risk for severe ovarian hyperstimulation syndrome compared with women with other genotypes (Daelemans *et al.*, 2004). In a previous study, it was also evident that women homozygous for *FSHR* N680 more often became pregnant after IVF, compared with women with other genotypes (Jun *et al.*, 2006). Altogether, these observations indicate that individuals who are carriers of *FSHR* S680 may have decreased FSHR response compared with carriers of one or two copies of *FSHR* N680. Furthermore, it was recently suggested that intracellular cAMP production was faster in human granulosa cells from homozygous carriers of *FSHR* N680 than in women homozygous for *FSHR* S680 when stimulated with FSH *in vitro* (Casarini *et al.*, 2014). However, this study only comprised four women.

Luteinizing hormone (LH) mediates its effect through the LH/human chorionic gonadotrophin (hCG) receptor (LHCGR) located on cell membranes of granulosa and theca cells (Camp *et al.*, 1991). When LH binds to LHCGR on theca cells, androstenedione and subsequently estradiol production is triggered (Short, 1962). Like the FSHR, the LHCGR also belongs to the G protein-coupled receptor family and holds 7 transmembrane helices. The *LHCGR* gene is located on chromosome 2, close to the *FSHR* gene and contains 11 exons. Of the polymorphisms in the *LHCGR* gene, the N312S polymorphism (rs2293275) in exon 10 is one of the most studied. Approximately 18% of Caucasian populations are homozygous for the A allele of the *LHCGR* N312S polymorphism encoding N, 49% are heterozygous and 33% are homozygous for the G allele, encoding S (Valkenburg *et al.*, 2009). The *LHCGR* N312S polymorphism is located near a glycosylation site which indicates that variations in the sequence could affect sensitivity. A few cohort studies have proposed that the N variant may render the LHCGR more sensitive (Piersma *et al.*, 2007; Simoni *et al.*, 2008). The *LHCGR* N312S polymorphism has also been linked to polycystic ovary syndrome (PCOS), where heterozygous women had 2-fold increased risk of PCOS and those homozygous for N had a 3-fold increased risk in a Sardinian population (Capalbo *et al.*, 2012).

Since the endocrine regulation of the process leading to the creation of a competent oocyte and thereby female fertility is dependent on both FSH and LH, the objective of the current study was to investigate if described polymorphisms in the *FSHR* and *LHCGR* genes, separately and in combination, impact IVF outcomes and clinical parameters in IVF trials. In addition, since FSHR and LHCGR are expressed on granulosa cells, the downstream hormonal effects were investigated by culturing and stimulating these cells *in vitro*.

Materials and Methods

Subjects

Women undergoing IVF at the Reproductive Medicine Centre, Skåne University Hospital, Malmö, Sweden ($n = 384$) were consecutively enrolled in the study from September 2010 to February 2015. Inclusion criteria were regular menstruation cycle of 21–35 days, bilateral ovaries, body mass index (BMI) <30 kg/m^2, younger than 40 years of age and nonsmokers. The women were between 22 and 39 years of age (mean: 32.0 ± 3.82) on the day of follicular fluid aspiration (Table I). A venous blood sample was drawn for DNA extraction with subsequent *FSHR* and *LHCGR* genotyping. Follicular fluid was collected for subsequent granulosa cell isolation from $n = 135$ women while undergoing oocyte retrieval. Clinical data for the

Table I Clinical parameters and IVF outcome.

	LHCGR N312S						*FSHR* N680S				
	All	**N/N**	**N/S**	**S/S**	***P***	***P*[§]**	**N/N**	**N/S**	**S/S**	***P***	***P*[§]**
First cohort *n* (%)	373 (100)[¶]	67 (18)[#]	175 (47)[‖]	131 (35)[**]	ref	–	102 (28)[††]	206 (55)[‡‡]	65 (17)[§§]	ref	–
Validation cohort *n* (%)	233 (100)	34 (15)	101 (43)	98 (42)	0.080	–	79 (34)	116 (50)	38 (16)	0.191	–
Merged	606 (100)	101 (17)	276 (45)	229 (38)	0.393	–	181 (30)	322 (53)	103 (17)	0.542	–
Age (years)[†]	32.0 ± 3.8	31.2 ± 3.6	31.9 ± 3.9	32.6 ± 3.7	0.029*	–	32.7 ± 4.0	31.8 ± 3.7	31.9 ± 3.7	0.140	–
Age (years)[†,1]	32.3 ± 3.9	33.2 ± 3.9	32.2 ± 4.0	32.1 ± 3.8	0.395	–	32.9 ± 3.6	31.7 ± 4.1	33.0 ± 3.5	0.079	–
Age (years)[†,2]	32.1 ± 3.8	31.8 ± 3.8	32.0 ± 4.0	32.4 ± 3.7	0.328	–	32.7 ± 3.8	31.8 ± 3.9	32.3 ± 3.6	0.020*	–
BMI (kg/m^2)[†]	23.6 ± 3.0	23.8 ± 3.0	23.7 ± 3.0	23.5 ± 3.0	0.673	–	23.7 ± 3.0	23.6 ± 3.0	23.6 ± 2.9	0.847	–
Endometriosis[‡]	26 (6.9)	4 (6.0)	16 (9.0)	6 (4.6)	0.208	–	8 (7.8)	15 (7.3)	3 (4.6)	0.668	–
PCOS[‡]	13 (3.5)	2 (3.0)	8 (4.6)	3 (2.3)	0.402	–	4 (3.9)	9 (4.3)	0 (0)	0.198	–
FSH baseline[†]	6.34 (2.7)	6.26 (2.3)	6.54 (2.5)	6.12 (3.0)	0.500	0.522	6.46 (2.4)	6.28 (2.7)	6.38 (2.7)	0.682	0.668
LH baseline[†]	9.56 (11)	9.32 (11)	9.66 (12)	9.58 (10)	0.428	0.428	11.6 (14)	8.19 (8.6)	10.7 (12)	0.051	0.051
E^2 baseline[†]	419 (318)	415 (342)	390 (274)	450 (342)	0.319	0.422	417 (300)	399 (310)	467 (335)	0.228	0.227
Cycle length (days)[†]	28.9 ± 2.9	29.4 ± 2.7	28.7 ± 2.9	29.0 ± 3.0	0.340	0.324	28.9 ± 3.0	29.2 ± 2.9	28.3 ± 2.5	0.110	0.117
Total dose FSH (IU)[†]	1699 ± 781	1546 ± 827	1762 ± 781	1694 ± 753	0.064	0.037*	1707 ± 696	1705 ± 824	1673 ± 778	0.840	0.610
Total dose FSH (IU)[†,1]	2059 ± 884	2342 ± 873	1967 ± 726	2056 ± 1015	0.091	0.169	2090 ± 758	1972 ± 806	2259 ± 1260	0.191	0.498
Total dose FSH (IU)[†,2]	1837 ± 840	1814 ± 920	1837 ± 767	1847 ± 890	0.873	0.782	1873 ± 746	1800 ± 826	1886 ± 1021	0.291	0.918
Daily FSH dose (IU)[†]	161 ± 51	148 ± 47	165 ± 53	161 ± 50	0.031	0.030*	161 ± 43	161 ± 54	159 ± 52	0.793	0.500
Number of follicles[†]	11.7 ± 6.1	12.0 ± 5.3	11.3 ± 6.5	12.1 ± 5.9	0.168	0.144	12.2 ± 6.8	11.5 ± 5.8	11.5 ± 5.7	0.942	0.875
Number of follicles[†,1]	12.8 ± 7.3	11.6 ± 6.9	12.3 ± 6.5	12.7 ± 8.1	0.598	0.675	12.6 ± 7.5	12.7 ± 7.5	11.0 ± 5.9	0.494	0.627
Number of follicles[†,2]	12.0 ± 6.6	11.8 ± 5.8	11.6 ± 6.5	12.4 ± 6.9	0.633	0.557	12.4 ± 7.1	12.0 ± 6.5	11.3 ± 5.8	0.713	0.703
Mature oocytes[†]	9.00 ± 5.6	9.09 ± 5.2	8.71 ± 5.8	9.21 ± 5.5	0.457	0.397	9.25 ± 6.0	8.85 ± 5.3	9.03 ± 5.6	0.734	0.690
Mature oocytes[†,1]	10.5 ± 6.8	10.0 ± 6.4	10.5 ± 6.6	10.8 ± 7.2	0.947	0.935	11.3 ± 7.1	10.5 ± 7.1	9.18 ± 5.2	0.497	0.489
Mature oocytes[†,2]	9.57 ± 6.1	9.34 ± 6.2	9.93 ± 6.3	9.93 ± 6.3	0.740	0.717	10.1 ± 6.5	9.43 ± 6.1	9.05 ± 5.4	0.405	0.355
GQE/oocyte[†]	0.21 ± 0.18	0.19 ± 0.18	0.21 ± 0.19	0.20 ± 0.17	0.997	0.990	0.20 ± 0.18	0.21 ± 0.18	0.20 ± 0.17	0.404	0.433
GQE/oocyte[†,1]	0.25 ± 0.22	0.20 ± 0.14	0.26 ± 0.24	0.26 ± 0.22	0.358	0.348	0.26 ± 0.20	0.25 ± 0.23	0.25 ± 0.23	0.399	0.410
GQE/oocyte[†,2]	0.22 ± 0.20	0.20 ± 0.16	0.23 ± 0.21	0.22 ± 0.19	0.559	0.549	0.22 ± 0.19	0.22 ± 0.20	0.21 ± 0.19	0.930	0.929
Embryo transfer[‡]	310 (83)	54 (81)	147 (84)	109 (83)	0.818	–	84 (82)	171 (83)	55 (85)	0.929	–
Embryo transfer[‡,1]	212 (91)	31 (91)	91 (90)	90 (92)	0.912	–	74 (94)	102 (88)	36 (95)	0.264	–
Embryo transfer[‡,2]	522 (86)	85 (84)	238 (86)	199 (87)	0.801	–	158 (87)	273 (85)	91 (88)	0.571	–
Clinical pregnancy[‡]	104 (28)	12 (18)	49 (28)	43 (33)	0.086	–	26 (25)	58 (28)	20 (31)	0.753	–
Clinical pregnancy[‡,1]	60 (26)	6 (18)	25 (25)	29 (30)	0.372	–	17 (22)	32 (28)	11 (29)	0.563	–
Clinical pregnancy[‡,2]	164 (27)	18 (18)	74 (27)	72 (31)	0.037*	–	43 (24)	90 (28)	31 (30)	0.447	–

Clinical pregnancy/embryo transfer	0.34	0.22	0.33	0.39	0.090	–	0.31	0.34	0.36	0.795	–	
Clinical pregnancy/ET[1]	0.28	0.19	0.27	0.32	0.380	–	0.23	0.31	0.31	0.449	–	
Clinical pregnancy/ET[2]	0.31	0.21	0.31	0.36	0.044*	–	0.27	0.33	0.34	0.388	–	

[1]Validation cohort; [2]merged cohort; *$P < 0.05$; [†]mean ± SD; [§]adjusted values; [‡]*n* (%); [¶]FSH baseline data *n* = 343 and cycle length data *n* = 336; [#]FSH baseline data *n* = 58 and cycle length data *n* = 60; [‖]FSH baseline data *n* = 162 and cycle length data *n* = 157; [**]FSH baseline data *n* = 123 and cycle length data *n* = 119; [††]FSH baseline data *n* = 94 and cycle length data *n* = 88; [‡‡]FSH baseline data *n* = 190 and cycle length data *n* = 190; [§§]FSH baseline data *n* = 59 and cycle length data *n* = 58.

women were retrieved from medical records. In $n = 11$ women, BMI was missing and these women were hence excluded when associating genotype with clinical parameters, resulting in a total of $n = 373$. In $n = 30$ women, data regarding baseline FSH values were missing, and these women were therefore also excluded when associating genotype with baseline FSH values. Additionally, in $n = 37$ women, data regarding cycle length were missing (although the cycle length of these women were in the normal range) and hence these women were excluded when associating cycle length with genotype.

An independent population of $n = 233$ women was enrolled *a posteriori* in order to validate pregnancy associations in the study cohort. These women underwent IVF at the same medical unit as the first cohort from the start of year 2007 until June 2015, and the inclusion criteria were the same as for the first study cohort. The women were between 20 and 40 years of age (mean: 32.5 ± 3.93) on the day of follicular fluid aspiration (Table I). The BMI values for these women were not recorded, and therefore this parameter was missing for this population. However, in order to undergo an IVF treatment at this clinical unit, BMI has to be <30 kg/m^2. Clinical data regarding age and IVF parameters were retrieved from medical records.

Patient treatment

Ovarian stimulation was performed according to either a short antagonist protocol using the gonadotrophin-releasing hormone (GnRH) antagonist Ganirelix (Orgalutran, Organon [Ireland] Ltd, Dublin, Ireland) or a standard long protocol using the GnRH agonist Nafarelin (Synarela, Pfizer Ab, Sollentuna, Sweden) (Table II). Ovarian hyperstimulation was carried out using individually set, flexible doses of either Follitropin alpha (GONAL-f, Merck-Serono, Darmstadt, Germany), Follitropin beta (Puregon, Organon [Ireland] Ltd), Urofollitropin (Fostimon, Institut Biochimique SA [IBSA], Lugano, Switzerland), or Menotropin (Menopur, Ferring GmbH, Kiel, Germany). The progression of follicle development was monitored by vaginal ultrasound on Days 6–8 of stimulation, and if needed the individual doses were adjusted. When three or more follicles were confirmed by ultrasound, hCG was administered and 35 h later oocyte retrieval was performed.

Genotyping of FSHR and LHCGR

Genomic DNA was extracted from peripheral leukocytes using standard procedures, and the SNP at amino acid position 680 in the FSHR was analyzed by allele-specific PCR as previously described (Lindgren *et al.*, 2012). The PCR results were confirmed by direct sequencing of 20 samples on an eight-capillary Applied Biosystems sequencing gear (Applied Biosystems, Stockholm, Sweden). The N312S polymorphism in the LHCGR was analyzed by PCR amplification followed by direct sequencing. Polymerase chain reactions were performed in a total volume of 50 µl containing 0.4 µM of the forward primer 5′-TGTTGACCATGTGACTAGGGA and 0.4 µM of the reverse primer 5′-ACTCTCTCCTCAGGAAGCAT (Invitrogen, Stockholm, Sweden), 10 mM Tris–HCl (AppliChem GmbH, Gatersleben, Germany) pH 9.1, 45 mM KCl (ICN Biomedicals, Inc., Aurora, OH, USA), 0.01% w/v Tween 20 (Scharlau Chemie S.A., Barcelona, Spain), 1.5 mM $MgCl_2$ (Sigma–Aldrich Sweden AB, Stockholm, Sweden), 200 µM of each dNTP (Fermentas, Sankt Leon-Rot, Germany), 1 U Dynazyme^{TM} II DNA polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 200 ng template DNA. The amplification program was initiated by a denaturation step at 96°C for 10 min, followed by 37 amplification cycles, each consisting of denaturation at 96°C for 1 min, annealing at 61°C for 30 s and elongation at 72°C for 3 min. A final elongation at 72°C for 7 min was applied. The PCR product was purified and directly sequenced on an eight-capillary Applied Biosystems sequencing gear (Applied Biosystems).

Table II Stimulation protocols.

	Stimulated patients, *n* (%)	Short protocol, *n* (%)	Mean total dose ± SD (IU)	Mean daily dose ± SD (IU)
All patients	384 (100%)	207 (54%)	1695 ± 775	160 ± 51
Follitropin alpha	275 (72%)	156 (57%)	1672 ± 720	158 ± 47
Follitropin beta	76 (20%)	37 (49%)	1605 ± 737	186 ± 72
Urofollitropin	21 (5%)	11 (52%)	1908 ± 1021	152 ± 45
Menotropin	11 (3%)	2 (18%)	2617 ± 1119	224 ± 75

Hormonal analysis

For endocrine serum analysis, blood samples were drawn between 8 and 10 a.m. Estradiol (E2), FSH and LH were measured using an electrochemiluminescence immunoassay (Cobas-Roche, Mannheim, Germany) at the routine clinical chemistry laboratory at Skåne University Hospital (Lund, Sweden). The sensitivities of the assays were 18.4 pmol/l for E2 and 0.10 IU/l for FSH and LH. The coefficients of variances for E2 were 7% at 289 pmol/l and 4% at 2011 pmol/l, for FSH 3% at 5 IU/l and 3% at 41 IU/l, and for LH 3% at 5 IU/l and 2% at 37 IU/l.

Isolation of granulosa cells from follicular fluid

Follicular fluid from the right and left ovary of each subject was aspirated and granulosa cell aggregates were manually identified and placed in phenol-red free Roswell park memorial institute (RPMI) 1640 medium (Gibco Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Biological Industries, Beit HaEmek, Israel) and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Mediatech, Inc., Manassas, VA, USA), and subsequently filtered through a 70 μm cell strainer (Becton Dickinson Biosciences, San José, CA, USA) in order to concentrate the granulosa cells and to discard most of the single cells, e.g. red blood cells, leukocytes, and debris. The aggregated cells were washed with RPMI medium followed by dispersion through the cell strainer using the back of a syringe until there were no visible granulosa cell aggregates. The filtered cells were centrifuged for 10 min at 300 × *g*. The supernatant was discarded and the cell pellet was resuspended in supplemented RPMI medium.

Cell sorting

To isolate granulosa cells from follicular fluid CD99 was used as a marker. In order to avoid nonspecific binding of the antibodies used for granulosa cell sorting, Fc receptor blocking was carried out with TrueStain (BioLegend, London, UK) for 5 min prior to antibody staining with anti-CD45 peridinin-chlorophyll-protein (PerCP) (BioLegend) and anti-CD99 phycoerythrin (PE) (eBioscience, San Diego, CA, USA). The granulosa cells were mixed gently and incubated for 15 min in darkness at room temperature. Stained cells were filtered through a 50 μm filcon with syringe fitting (Biogenetics, Padova, Italy) and washed in supplemented RPMI medium. Filtered cells were centrifuged at 430 × *g* for 5 min and resuspended in 200 μl supplemented RPMI medium. Seven-Amino actinomycin D (7-AAD) (BioLegend) was added 5 min prior to flow cytometry acquisition and cell sorting. The samples were subsequently acquired and sorted in a FACSAria (Becton Dickinson Biosciences). The gating strategy for the definition of viable granulosa cells was an acquisition gate based on side scatter and forward scatter, including cells with high granularity and excluding cell debris, dead cells and leukocytes (7-AAD and CD45 positive cells). Remaining cells were gated on the most granular and CD99 positive cells, which were defined as viable granulosa cells and sorted into supplemented RPMI medium containing 55% FBS. Flow cytometry data were analyzed using FlowJo (TreeStar, Inc., Ashland, OR, USA).

Identification of granulosa cells

Specimens from 3 different, randomly selected patients (unsorted and sorted cells) were morphologically evaluated at the Department of Pathology and Cytology, Skåne University Hospital, Malmö, Sweden. The cells, which were kept in R10 medium, were centrifuged at 760 × *g* for 10 min. The supernatant was decanted and Becton Dickinson Cytorich^{TM} (red) was added. The tubes were left at 4°C for at least 30 min to ensure optimal fixation of the cells, before centrifugation at 760 × *g* for 10 min. The supernatant was decanted and the cell pellet re-suspended in deionized water. The cell suspension was added to pre-prepared cell chambers (Settling chamber 240, TriPath Imaging, Becton Dickinson Diagnostics, Sparks, NV, USA), placed on pre-coated slides (SurePath precoat slides, TriPath Imaging, Becton Dickinson Diagnostics) and left for 15 min to allow the cells to adhere to the slides. The slides were subsequently fixed in 95% ethanol for at least 30 min and stained with hematoxylin–eosin (Histolab Products AB, Gothenburg, Sweden) according to routine cyto-staining procedures before examination with an Olympus BH-2 microscope (Olympus Corporation, Center Valley, PA, USA) and morphological evaluation. Granulosa cells and other cell types were counted in randomly chosen high power (objective lens × 40, ocular lens × 10) microscopic fields until ~300 cells were counted.

Culture and granulosa cell activity

Approximately 75 000 human granulosa cells per well were cultured into 24-well plates in supplemented RPMI medium with 0.02 mg/ml gentamicin (PAA Laboratories, Pasching, Austria) for 2–3 days before incubation in serum-free RPMI medium (containing HEPES and gentamicin) for 1 h at 37°C, 5% CO_2, in the absence or the presence of 150 mIU/ml Follitropin alpha (GONAL-f [Merck-Serono]) or 150 mIU/ml Menotropin (Menopur [Ferring GmbH]). The cell culture medium was aspirated and centrifuged for 20 min, 1000 × *g* at room temperature. Endogenous phosphodiesterases were heat inactivated for 5 min at 95°C. Cells were lysed with Passive lysis buffer (Promega, Stockholm, Sweden). Experiments were carried out in duplicates whenever a sufficient number of granulosa cells were retrieved. The FSHR as well as the LHCGR activity through the $G\alpha_s$/cAMP/protein kinase A pathway was measured in the cell culture medium using a cAMP enzyme-linked immunosorbent assay (ELISA) kit (ENZO Life Sciences, Lausen, Switzerland), whereas activity through the IP_3-signaling pathway was measured in 55 randomly selected women using an IP_3 ELISA kit (Cusabio, Wuhan, China). All results were adjusted for total protein concentrations in the cell lysates using Pierce BCA (bicinchoninic acid) protein assay reagent (Thermo Fisher Scientific, Inc.), as well as adjusted for basal activity in unstimulated cells from each patient.

Statistical analysis

Allele frequencies of the two polymorphisms were analyzed in comparison to control populations using q^2 test. The *LHCGR* N312S polymorphism was tested against a normal population of 2996 Caucasians (Piersma *et al.*, 2007), and the *FSHR* N680S polymorphism against a normal population of 1431 Caucasians (Kuijper *et al.*, 2010). Linkage between the two polymorphisms was investigated by calculation of linkage disequilibrium, with data extracted from Phase 1 data from the 1000 genomes project (ENSEMBL).

All residuals were tested for normal distribution in the Kolmogorov–Smirnov test, and log transformed if lack of Gaussian distribution. Calculations for associations with clinical parameters among genotype groups were carried out by comparing each genotype group separately (i.e. N680N versus N680S versus S680S for the *FSHR*; N312N versus N312S versus S312S for the *LHCGR*). Comparisons of age, BMI, cycle length, baseline hormones, hormonal doses given prior to IVF, and follicle and oocyte outcomes among genotype groups were carried out using a univariate analysis of variance. Comparisons of incidence of endometriosis and PCOS, and occurrence of embryo transfer among genotype groups were carried out using a q^2 test. Comparisons of pregnancy outcomes among genotype groups were carried out using a q^2 test and logistic regression. In order to evaluate a combined effect of the *FSHR* and *LHCGR* on pregnancy outcomes in the merged study group, the G alleles from both polymorphisms were considered (0–4 G alleles) and a trend of the increased number of G alleles for the *FSHR* polymorphism and the *LHCGR* polymorphism analyzed using logistic regression. Age (as a continuous variable) and BMI ($\leq$25 and $>$25) were considered as confounding factors when analyzing differences in cycle length, baseline hormones, hormonal doses, and follicle and oocyte counts between genotypes. Age, BMI, use of intracytoplasmic sperm injection, type of stimulation protocol and type of hormonal agent used (Follitropin alpha or Menotropin) were considered as confounding factors when analyzing differences in pregnancy outcomes. Analysis concerning pregnancy outcome was also performed, using the same covariates, after exclusion of the 13 women who were diagnosed with PCOS.

In order to evaluate a combined effect of the *FSHR* and *LHCGR* polymorphisms on cAMP and IP_3 concentrations in cell culture supernatants from granulosa cells, the G alleles from both polymorphisms were added (0–4 alleles) and a trend of the increased number of G alleles for the *FSHR* polymorphism and the *LHCGR* polymorphism was analyzed using linear regression. Each polymorphism was also analyzed separately. In addition, we used a univariate analysis of variance in order to compare individual groups of combined polymorphisms. Age (as a continuous variable) was considered as a confounding factor when analyzing differences in cAMP and IP_3 concentrations in cell culture supernatants.

Since the study was performed on candidate genes, no correction for mass significance was done (Cordell and Clayton, 2005).

Data were analyzed using SPSS software version 22 (SPSS, Inc., Chicago, IL, USA). A *P*-value of $<$0.05 was considered statistically significant. Minitab 12.21 (Minitab, Inc., State College, PA, USA) was used when calculating exact binomial proportion confidence interval of purity of sorted granulosa cells.

Ethical approval

Written informed consent was obtained from all participants. The study was approved by the ethical committee board at Lund University, Sweden.

Results

Genotyping

Allele frequencies for the *FSHR* N680S polymorphism were 55% for the A allele, encoding asparagine, and 45% for the G allele, encoding serine. Genotype distribution was 28% homozygous N, 55% heterozygous and 17% homozygous S. For the *LHCGR* N312S polymorphism, allele frequencies were 41% A, encoding asparagine, and 59% G, encoding serine. The genotype distribution was 18% homozygous N, 47% heterozygous and 35% homozygous S (Table I). There was no difference in *FSHR* N680S allele frequency between the study population and a population previously reported (Kuijper *et al.*, 2010) ($P = 1$), or in *LHCGR* N312S allele frequency between the study population and the general population (Piersma *et al.*, 2007) ($P = 0.554$). The allele frequencies of both polymorphism were in Hardy–Weinberg equilibrium, $\chi^2 = 0.06$, $P > 0.05$ for *LHCGR* N312S and $\chi^2 = 1.29$, $P > 0.05$ for *FSHR* N680S. The two polymorphisms are in linkage equilibrium, $D' = 0.042$, $r^2 = 0.0015$.

In the second study population, the allele frequencies for the *FSHR* N680S polymorphism were 59% for the A allele and 41% for the G allele. Genotype distribution was 34% homozygous N, 50% heterozygous and 16% homozygous S. For the *LHCGR* N312S polymorphism, allele frequencies were 36% for the A allele and 64% for the G allele. Genotype distribution was 15% homozygous N, 43% heterozygous and 42% homozygous S (Table I). There were no differences in the allele frequencies between the replication population and the first study population ($P = 0.191$ for *FSHR* S680N and $P = 0.080$ for *LHCGR* N312S) and altogether, the allele frequencies of both polymorphisms were in Hardy–Weinberg equilibrium, $\chi^2 = 0.52$, $P > 0.05$ for *LHCGR* N312S and $\chi^2 = 0.11$, $P > 0.05$ for *FSHR* N680S.

Clinical parameters and IVF outcome

LHCGR *N312S*

For the *LHCGR* N312S polymorphism, no differences regarding background characteristics were found between women with N312 or S312 in the first cohort of 373 women, except for a marginal difference in age (Table I). Women homozygous for N received lower mean daily as well as total dose of FSH during IVF treatment. A tendency towards higher pregnancy rates for women carrying *LHCGR* S312 was found (Table I; Fig. 1A and B), and using a logistic regression model, a statistically significant association was evident (unadjusted: OR = 1.43, 95% CI: [1.03, 1.99], $P = 0.033$; adjusted: OR = 1.57, 95% CI: [1.11, 2.22], $P = 0.011$; Fig. 1A). In women who received embryo transfer, the same difference was found (unadjusted: OR = 1.46, 95% CI: [1.03, 2.06], $P = 0.033$; adjusted: OR = 1.59, 95% CI: [1.10, 2.29], $P = 0.013$, Fig. 1B).

Analysis performed after exclusion of PCOS patients resulted in slightly stronger associations: (unadjusted: OR = 1.44, 95% CI: [1.03, 2.01], $P = 0.032$; adjusted: OR = 1.61, 95% CI: [1.13, 2.29], $P = 0.008$). In women who received embryo transfer, the same difference was found (unadjusted: OR = 1.46, 95% CI: [1.04, 2.07], $P = 0.031$; adjusted: OR = 1.60, 95% CI: [1.11, 2.31], $P = 0.012$).

No significant difference in pregnancy rates for carriers of *LHCGR* S312 was found in the replication population of $n = 233$ women (unadjusted: OR = 1.36, 95% CI: [0.881, 2.10], $P = 0.165$; adjusted: OR = 1.27, 95% CI: [0.798, 2.00], $P = 0.316$). The same was true among women receiving embryo transfer (unadjusted: OR = 1.36, 95% CI: [0.878, 2.11], $P = 0.168$; adjusted: OR = 1.27, 95% CI: [0.795, 2.03], $P = 0.316$).

When merging the first study population with the second validation group, in total $n = 606$ women, a higher pregnancy rate was evident for *LHCGR* S312 carriers (Table I; unadjusted: OR = 1.40, 95% CI: [1.07, 1.81], $P = 0.013$; adjusted: OR = 1.49, 95% CI: [1.14, 1.96],

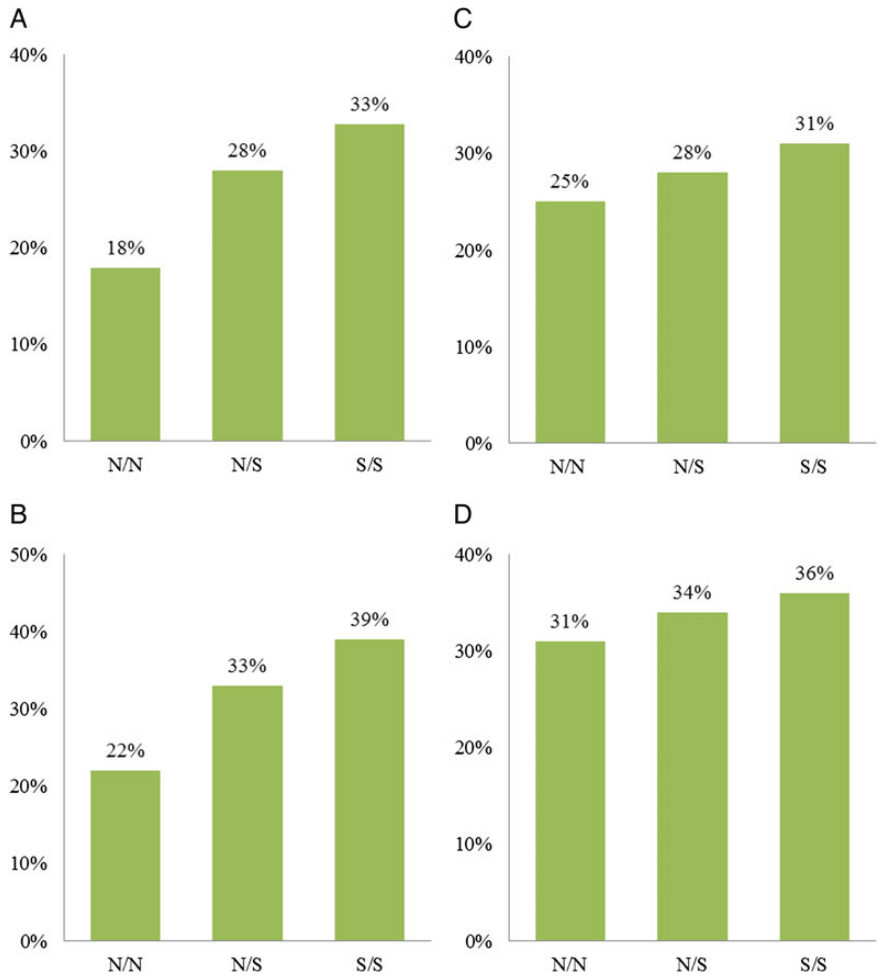

Figure 1 Pregnancy frequencies for the *LHCGR* N312S and *FSHR* N680S polymorphisms. (**A**) *LHCGR* N312S, all women in the study: N/N ($n = 67$), N/S ($n = 175$), S/S ($n = 131$); (**B**) *LHCGR* N312S, only women receiving embryo transfer: N/N ($n = 54$), N/S ($n = 147$), S/S ($n = 109$); (**C**) *FSHR* N680S, all women: N/N ($n = 102$), N/S ($n = 206$), S/S ($n = 65$); (**D**) *FSHR* N680S, embryo transfer: N/N ($n = 84$), N/S ($n = 171$), S/S ($n = 55$).

$P = 0.004$). A higher pregnancy rate was also found among those receiving embryo transfer (unadjusted: OR = 1.39, 95% CI: [1.07, 1.83], $P = 0.016$; adjusted: OR = 1.50, 95% CI: [1.13, 1.99], $P = 0.005$).

FSHR *N680S*

No difference among pregnancy rates was noticed for carriers of S in the *FSHR* N680S polymorphism (unadjusted: OR = 1.14, 95% CI: [0.810, 1.61], $P = 0.452$, adjusted: OR = 1.11, 95% CI: [0.746, 1.60], $P = 0.577$, Fig. 1C). Furthermore, there was no significant difference in women who received embryo transfer (unadjusted: OR = 1.13, 95% CI: [0.792, 1.61], $P = 0.499$; adjusted: OR = 1.08, 95% CI: [0.774, 1.60], $P = 0.679$; Fig. 1D) or in other clinical variables analyzed in relation to *FSHR* N680S.

Analysis performed after exclusion of PCOS patients displayed similar results (unadjusted: OR = 1.14, 95% CI: [0.811, 1.61], $P = 0.443$; adjusted: OR = 1.10, 95% CI: [0.769, 1.58], $P = 0.597$). There was no significant difference among those who received embryo transfer (unadjusted: OR = 1.13, 95% CI: [0.786, 1.61], $P = 0.519$; and adjusted: OR = 1.08, 95% CI: [0.741, 1.57], $P = 0.698$) or in other clinical variables analyzed in relation to *FSHR* N680S.

Also in the replication population of $n = 233$ women, no difference in pregnancy rates was noticed for carriers of *FSHR* S680 (unadjusted: OR = 1.24, 95% CI: [0.810, 1.90], $P = 0.321$; adjusted: OR = 1.20, 95% CI: [0.752, 1.91], $P = 0.445$). The same was true for *FSHR* S680 carriers receiving embryo transfer (unadjusted: OR = 1.24, 95% CI: [0.808, 1.91], $P = 0.323$; adjusted: OR = 1.17, 95% CI: [0.729, 1.88], $P = 0.514$).

When combining the first study population with the second group ($n = 606$), no significant difference in pregnancy rates was noticed for *FSHR* S680 (unadjusted: OR = 1.18, 95% CI: [0.906, 1.54], $P = 0.216$; adjusted: OR = 1.17, 95% CI: [0.889, 1.55], $P = 0.260$). The same was observed among *FSHR* S680 carriers receiving embryo transfer (unadjusted: OR = 1.19, 95% CI: [0.903, 1.56], $P = 0.218$; adjusted: OR = 1.17, 95% CI: [0.878, 1.55], $P = 0.288$).

LHCGR *N312S and* FSHR *N680S combined*

When analyzing the two polymorphisms combined, a higher pregnancy rate was found for women homozygous for S in both polymorphisms ($n = 23$) compared with those homozygous for N ($n = 20$) (unadjusted:

OR = 5.79, 95% CI: [1.07, 31.1], $P = 0.041$; adjusted: OR = 14.4, 95% CI: [1.65, 126], $P = 0.016$, Fig. 2A). The same pattern was found in women who received embryo transfer (unadjusted: OR = 6.55, 95% CI: [1.18, 36.3], $P = 0.032$, adjusted: OR = 25.7, 95% CI: [1.95, 340], $P = 0.014$; Fig. 2B).

In the independent sample of $n = 233$ women, no significant difference in pregnancy rates was found among women homozygous for S in both polymorphisms ($n = 12$) compared with women homozygous for N in both polymorphisms ($n = 10$) (unadjusted: OR = 3.00, 95% CI: [0.260, 34.6], $P = 0.378$; adjusted: OR = 3.23, 95% CI: [0.103, 101], $P = 0.505$). A similar association was found in women who received embryo transfer (unadjusted: OR = 3.38, 95% CI: [0.290, 39.3], $P = 0.332$, adjusted: OR = 3.37, 95% CI: [0.110, 103], $P = 0.486$).

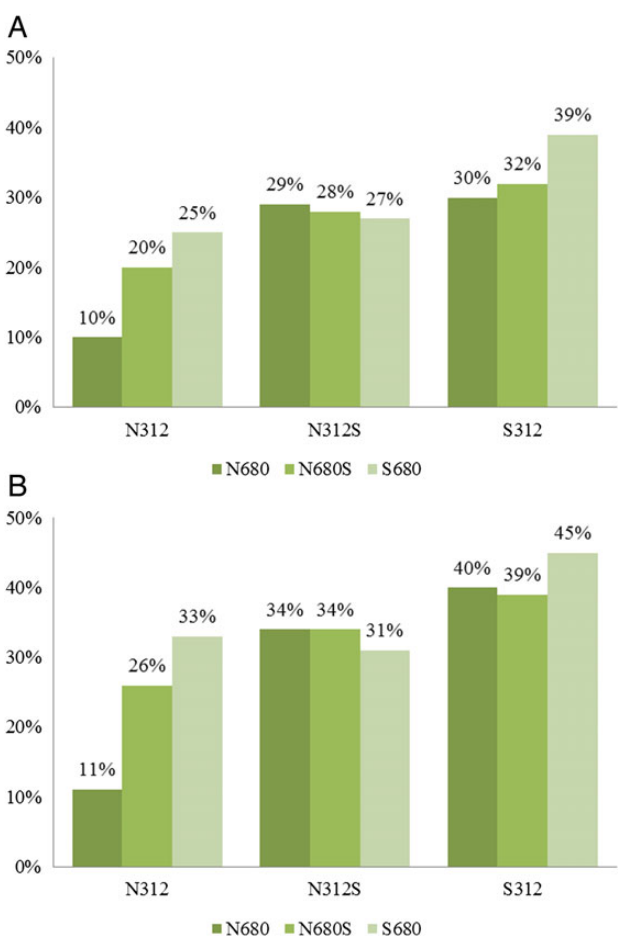

Figure 2 Pregnancy frequencies for combined genotypes. (**A**) All women: *LHCGR* N312 in combination with *FSHR* N680, N680S and S680 comprised $n = 20$, $n = 35$ and $n = 12$ women, respectively. *LHCGR* N312S in combination with *FSHR* N680, N680S and S680 comprised $n = 49$, $n = 96$ and $n = 30$ women, respectively. *LHCGR* S312 in combination with *FSHR* N680, N680S and S680 comprised $n = 33$, $n = 75$ and $n = 23$ women, respectively. (**B**) Only women receiving embryo transfer: *LHCGR* N312 in combination with *FSHR* N680, N680S and S680 comprised $n = 18$, $n = 27$ and $n = 9$ women, respectively. *LHCGR* N312S in combination with *FSHR* N680, N680S and S680 comprised $n = 41$, $n = 80$ and $n = 26$ women, respectively. *LHCGR* S312 in combination with *FSHR* N680, N680S and S680 comprised $n = 25$, $n = 64$ and $n = 20$ women, respectively.

In the merged study population, women homozygous for S ($n = 35$) had higher pregnancy rate than those homozygous for N ($n = 30$) (unadjusted: OR = 4.70, 95% CI: [1.18, 18.7], $P = 0.028$; adjusted: OR = 11.5, 95% CI: [1.86, 71.0], $P = 0.009$). The same pattern was found in women who received embryo transfer (unadjusted: OR = 5.26, 95% CI: [1.30, 21.3], $P = 0.020$; adjusted: OR = 11.5, 95% CI: [1.89, 69.9], $P = 0.008$). A linear significant association with pregnancy rate and increasing number of G alleles was also noted in the merged study population (unadjusted: OR = 1.30, 95% CI: [1.08, 1.58], $P = 0.007$; adjusted: OR = 1.34, 95% CI: [1.10, 1.64], $P = 0.004$, Fig. 3A). The same was also true for women receiving embryo transfer (unadjusted: OR = 1.30, 95% CI: [1.07, 1.58], $P = 0.009$; adjusted: OR = 1.33, 95% CI: [1.09, 1.63], $P = 0.005$, Fig. 3B).

Granulosa cell stimulation

A distinct and viable granulosa cell population was isolated by fluorescence activated cell sorting (FACS), consisting of 97.8% (95% CI: [96.6%, 98.6%]) granulosa cells (Table III and Fig. 4). Regarding the *FSHR* N680S and *LHCGR* N312S polymorphisms, no differences in response to Follitropin alpha or Menotropin were observed in the induction of cAMP or IP_3 production (Table IV). A combination of the two polymorphisms showed no linear association between genotype and induction of cAMP following either Follitropin alpha stimulation (unadjusted: $\beta = 0.247$, 95% CI: [−0.070, 0.563], $P = 0.125$; adjusted: $\beta = 0.247$, 95% CI: [−0.071, 0.565], $P = 0.126$, Fig. 5A) or Menotropin stimulation (unadjusted: $\beta = -0.037$, 95% CI: [−0.312, 0.238], $P = 0.788$; adjusted: $\beta = -0.054$, 95% CI: [−0.334, 0.225], $P = 0.701$, Fig. 5B). Following Follitropin alpha stimulation, the group of women homozygous N in both polymorphism displayed lower mean cAMP levels compared with others (unadjusted: 0.901 pmol cAMP/mg total protein versus 2.19 pmol cAMP/mg total protein, $P = 0.034$; adjusted: 0.901 pmol cAMP/mg total protein versus 2.19 pmol cAMP/mg total protein, $P = 0.035$, Fig. 5A). Furthermore, no linear association between genotype combinations and induction of IP_3 following either Follitropin alpha stimulation (unadjusted: $\beta = -0.024$, 95% CI: [−0.153, 0.106], $P = 0.714$; adjusted: $\beta = -0.025$, 95% CI: [−0.158, 0.109], $P = 0.709$, Fig. 5C) or Menotropin stimulation (unadjusted: $\beta = 0.004$, 95% CI: [−0.109, 0.117], $P = 0.943$; adjusted: $\beta = -0.012$, 95% CI: [−0.128, 0.104], $P = 0.836$, Fig. 5D) was found.

Discussion

The main findings of the present study was that women homozygous for S in both polymorphisms studied had a 4-fold higher chance of pregnancy compared with women homozygous for N in corresponding codons. It was also evident that carriers of S312 in the *LHCGR* more often became pregnant after IVF than those with N in the same position; 56% higher rate for heterozygous women with one S and 83% for homozygous women, compared with women homozygous for N. The same patterns were also present in women who had an embryo transferred. The main finding of the present study was not validated in an independent population of women, as results were not statistically significant, most probably due to a smaller number of women eligible than in the first study population. However, in the merged cohort, consisting of more

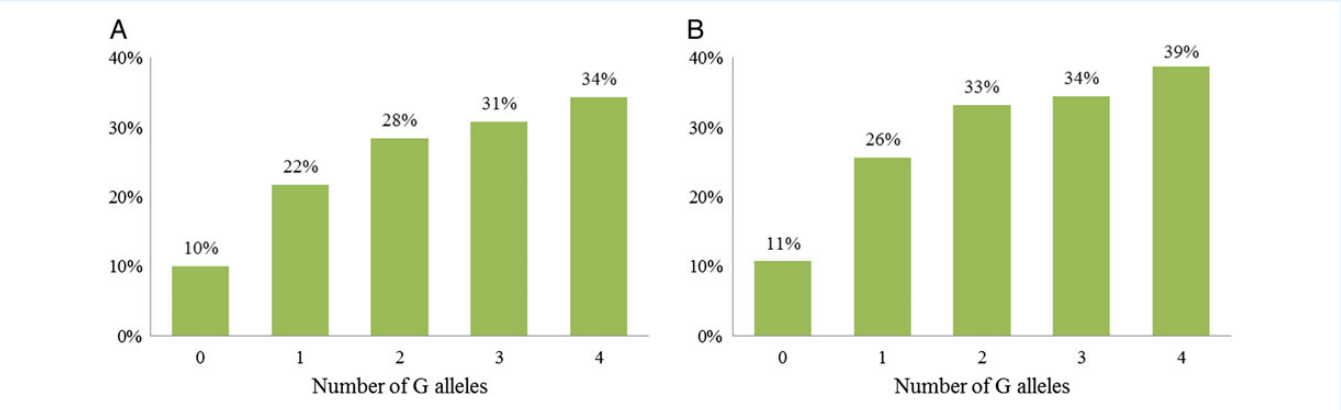

Figure 3 Pregnancy frequencies for combined genotypes in the merged population ($n = 606$). (**A**) All women. 0: $n = 30$ women; 1: $n = 129$ women; 2: $n = 243$ women; 3: $n = 169$ women and 4: $n = 35$ women. (**B**) Only women receiving embryo transfer. 0: $n = 28$ women; 1: $n = 109$ women; 2: $n = 208$ women; 3: $n = 151$ women and 4: $n = 31$ women.

Table III Microscope phenotyping of isolated granulosa cells in unsorted and sorted patient material ($n = 3$).

	Specimen 1		Specimen 2		Specimen 3	
	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted
Granulosa cells	200	291	211	296	220	293
Other cells[¶]	94	9	99	4	86	7
Total	294	300	310	300	306	300
% granulosa cells	66.6	97.0	68.1	98.7	71.9	97.7

[¶]Degenerated cells and stripped nuclei, small lymphocyte like cells and squamous cells against a background of debris.

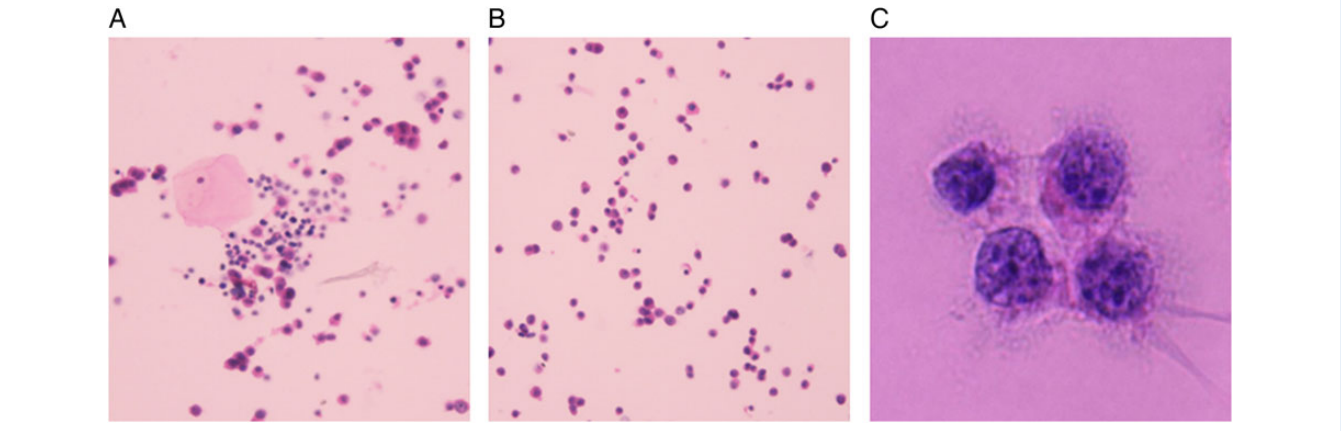

Figure 4 (**A**) Unsorted sample: Granulosa cells with a mixture of squamous cells, small lymphocyte like cells, degenerated nuclei and some debris (hematoxylin–eosin, 10×). (**B**) Sorted sample: virtually pure population of granulosa cells, dispersed or in loose clusters. The cells have pale cytoplasm with indistinct cell borders, round or ovoid, often eccentric nuclei with coarse but uniform chromatin (hematoxylin–eosin, 10×). (**C**) A small cluster of granulosa cells (hematoxylin–eosin, 100×).

than 600 women, relationships with pregnancies were even stronger than in the original cohort, and a linear association with pregnancy rate and increasing number of G alleles was noted.

Both FSH and LH are required for adequate oocyte maturation (Segaloff and Ascoli, 1993), and hence it is not surprising that variants of the *LHCGR* play a role in the outcome of IVF treatments. The mechanism

Table IV Granulosa cell response *in vitro*, presented as mean ± SD.

		Genotype						
	***LHCGR* N312S**	**N/N**	**N/S**	**S/S**	**Slope#**	**95% CI#**	***P***	***P#***
Menotropin	pmol cAMP/mg protein ($n = 102$)	26.0 ± 31.3	21.8 ± 30.3	34.4 ± 73.2	−0.178	[−0.55, 0.19]	0.482	0.343
	pg IP_3/mg protein ($n = 49$)	1.33 ± 0.52	1.26 ± 0.43	1.37 ± 0.53	0.051	[−0.09, 0.20]	0.746	0.479
Follitropin alpha	pmol cAMP/mg protein ($n = 119$)	24.3 ± 56.5	22.4 ± 37.7	52.7 ± 104	0.372	[−0.05, 0.80]	0.089	0.084
	pg IP_3/mg protein ($n = 55$)	1.20 ± 0.38	1.21 ± 0.38	1.30 ± 0.37	0.073	[−0.10, 0.24]	0.411	0.394
	***FSHR* N680S**	**N/N**	**N/S**	**S/S**	**Slope#**	**95% CI#**	***P***	***P#***
Menotropin	pmol cAMP/mg protein ($n = 102$)	22.8 ± 39.3	25.2 ± 32.4	49.5 ± 108	0.095	[−0.31, 0.50]	0.687	0.639
	pg IP_3/mg protein ($n = 49$)	1.34 ± 0.45	1.31 ± 0.52	1.24 ± 0.47	−0.035	[−0.19, 0.12]	0.654	0.643
Follitropin alpha	pmol cAMP/mg protein ($n = 119$)	25.5 ± 55.7	35.4 ± 58.3	44.6 ± 128	0.077	[−0.38, 0.54]	0.727	0.738
	pg IP_3/mg protein ($n = 55$)	1.27 ± 0.32	1.21 ± 0.44	1.25 ± 0.33	−0.031	[−0.20, 0.14]	0.714	0.716

#Adjusted for age.

underlying is unknown, but it has been proposed that G protein-coupled receptors may form homo- and heterodimers (Angers *et al.*, 2002), so that stimulation by one of the hormones could be mediated in part through the other hormone's receptor and that some isoforms of the different receptors may have beneficial function compared with other isoforms. Thus, the *LHCGR* genotype could influence the response to FSH stimulation. Still, many LH effects are considered as indirect since the distribution of LHCGR on granulosa cells is not as dense and stable as the FSHR distribution during the menstrual cycle (Camp *et al.*, 1991). In the current work, an attempt to elucidate the mechanism behind the impact of combinations of FSHR and LHCGR variants on receptor function was made by stimulating granulosa cells *in vitro* with Follitropin alpha and Menotropin, respectively. However, when analyzing a combination of the N680S of the *FSHR* and N312S of the *LHCGR in vitro*, no linear association between combined receptor variants and hormone sensitivity was detected. Nevertheless, granulosa cells from the group of women who were homozygous for N in both polymorphisms, comprising eight women, displayed a lower cAMP activity following Follitropin alpha stimulation when compared with women with other genotypes. Due to the limited sample size, this finding has yet to be regarded as preliminary, due to the fact that the cells used in the *in vitro* experiments were pre-stimulated during the IVF trial in the clinic, which could impact the results. The receptors on the granulosa cells may for example already have been down- or up-regulated to some extent, which could affect further stimulation with hormonal agents.

No differences in the number of follicles or oocytes between those carrying *LHCGR* N312 and S312 were found in the current study population, nor were there any obvious differences regarding embryo quality. Nevertheless, the pregnancy rates differed markedly. This could either be due to small differences in the maturation of the oocyte, not visible through light microscopy, and taking place after the hCG administration; or a problem that occurred at a later developmental stage, after embryo transfer. Since LH regulates the formation of corpus luteum, it could also be due to some insufficiency of this structure, which could affect its ability to produce hCG and progesterone, which is needed to support the hatching embryo. The large difference in pregnancy rates could also partly be an effect of differing stimulation protocols prior to IVF treatment, and we therefore adjusted for this factor in the analyses. Furthermore, when PCOS cases were removed from the analyses, the association with pregnancy rates became stronger, indicating that the interplay between receptors and gonadotrophins in this category of patients may be different than in other women. Women homozygous for *LHCGR* N312 also required lower doses of exogenous FSH for adequate response. Considering the dimerization hypothesis, this could indicate that N renders the receptor more hormone sensitive, which is consistent with earlier hypotheses (Piersma *et al.*, 2007; Simoni *et al.*, 2008). Several studies have also indicated that high LH levels (>10 IU/l) are associated with increased miscarriage rates and lower chances of pregnancy (Regan *et al.*, 1990; Shoham *et al.*, 1990), and it seems likely that a more sensitive LHCGR would have the same effect.

In the current study, the *FSHR* S680 genotype was beneficial for pregnancy outcome only if combined with *LHCGR* S312. There was no sign of influence of *FSHR* variants *per se* regarding receptor sensitivity, neither on clinical outcome in IVF trials nor on *in vitro* stimulation response, which was in contrast to previous reports (Perez Mayorga *et al.*, 2000; Sudo *et al.*, 2002; Jun *et al.*, 2006; Casarini *et al.*, 2014). This could at least partly be explained by differences in study populations, as one of the previously used study population was considerably smaller, only comprising 161 women (Perez Mayorga *et al.*, 2000). Ethnic origin could also account for some differences (Sudo *et al.*, 2002).

The strength of the study was the large cohort of consecutively enrolled patients. These women were hence not selected for the study, but an ordinary cohort of women visiting a fertility clinic. The findings can therefore be generally applied. Another strength was the purity of the granulosa cell material, which made it possible to compare *in vitro* results and clinical results within the same study population. A drawback of the study was that it was not recorded when in the menstrual cycle the baseline reproductive hormones were obtained, which therefore not was possible to adjust for. These proceedings may have masked possible links between receptor genotype and gonadotrophin concentrations.

Another weakness was that due to limited biological material, granulosa cells were not available for *in vitro* stimulation from all women.

In summary, in this large cohort of women, those homozygous for S in both studied polymorphisms had a 4-fold increased chance of pregnancy compared with women homozygous for N; whereas only 10% of women with N in both genes became pregnant, 39% of those with S did. Thus, if

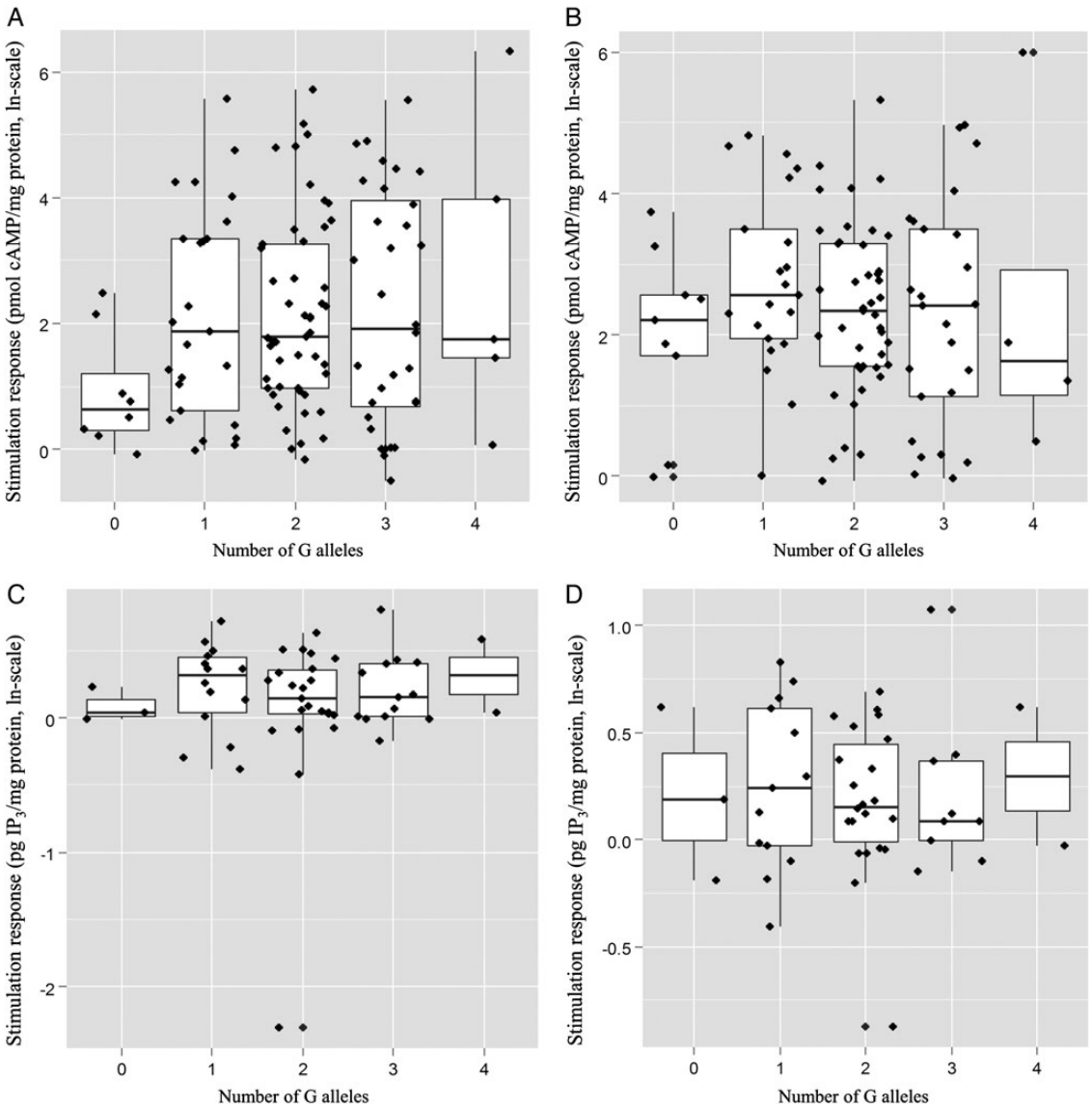

Figure 5 Granulosa cell response measured as cAMP concentration (**A**, **B**) and IP_3 concentration (**C**, **D**), stratified as number of G alleles combined (*FSHR* S680, *LHCGR* S312) stimulated with Follitropin alpha (A, C), Menotropin (B, D).

used in IVF trials, these SNPs could be used as predictors for pregnancy outcome, at least in Caucasian populations.

Acknowledgements

We thank Per-Anders Bertilsson, Department of Clinical Sciences, Cellular Autoimmunity Unit, Lund University, Malmö, Sweden, for excellent technical assistance and handling of the FACSAria. The FACSAria was a donation from the Knut and Alice Wallenberg Foundation.

Authors' roles

Study design: I.L., M.B., L.B., K.U. and Y.L.G. Recruitment of patients and collection of patient data: E.H., M.B., L.B. and I.L. Lab exp: I.L., M.B., K.U., A.D., L.K. and C.C. Statistical analysis: I.L. and M.B. Data interpretation: I.L., M.B., K.U., A.D., E.H., S.S., C.Y.A. and Y.L.G. Writing of manuscript draft: I.L., M.B., A.D. and Y.L.G.. Final manuscript: all co-authors.

Funding

This work was supported by Interreg IV A, EU (grant 167158) and ALF governments grant (F2014/354). Merck-Serono (Darmstadt, Germany) supported the enrollment of the subjects.

Conflict of interest

None declared.

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