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

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

3 Running title: *LHCGR* and *FSHR* variants predict IVF outcome

4

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29 **Abstract**

30 **Study question:** Can gonadotropin receptor variants separately or in combination, be used for  
31 prediction of pregnancy chances in *in vitro* fertilization (IVF) trials?

32 **Summary answer:** The luteinizing hormone receptor (*LHCGR*) variant N312S and the follicle  
33 stimulating hormone receptor (*FSHR*) variant N680S can be utilized for prediction of pregnancy  
34 chances in women undergoing IVF.

35 **What is known already:** The *FSHR* N680S polymorphism has been shown to affect the ovarian  
36 response in response to gonadotropin treatment, while no information is currently available  
37 regarding variants of the *LHCGR* in this context.

38 **Study design, size, duration:** Cross sectional study, duration from September 2010 to February  
39 2015. Women undergoing IVF were consecutively enrolled and genetic variants compared  
40 between those who became pregnant and those who did not. The study was subsequently  
41 replicated in an independent sample. Granulosa cells from a subset of women were investigated  
42 regarding functionality of the genetic variants.

43 **Participants/materials, setting, methods:** Women undergoing IVF (n=384) were enrolled in  
44 the study and genotyped. Clinical variables were retrieved from medical records. For replication,  
45 an additional group of n=233 women was utilised. Granulosa cells from n=135 women were  
46 isolated by flow cytometry, stimulated with Follitropin alpha or Menotropin, and the downstream  
47 targets cAMP and IP<sub>3</sub> measured with ELISA.

48 **Main results and the role of chance:** Women homozygous for serine (S) in both  
49 polymorphisms displayed higher pregnancy rates than women homozygous asparagine (N)  
50 (OR=14.4, 95% CI: [1.65, 126], p=0.016). Higher pregnancy rates were also evident for women  
51 carrying *LHCGR* S312, regardless of *FSHR* variant (OR=1.61, 95% CI: [1.13, 2.29], p=0.008).

52 These women required higher doses of FSH for follicle recruitment than women homozygous N  
53 (161 IU vs. 148 IU,  $p=0.030$ ). When combining the study cohort with the replication cohort  
54 ( $n=606$ ), even stronger associations with pregnancy rates were noted for the combined genotypes  
55 ( $OR=11.5$ , 95% CI: [1.86, 71.0],  $p=0.009$ ) and for women carrying *LHCGR* S312 ( $OR=1.49$ ,  
56 95% CI: [1.14, 1.96],  $p=0.004$ ). A linear significant trend with pregnancy rate and increasing  
57 number of G alleles was also evident in the merged study population ( $OR=1.34$ , 95% CI: [1.10,  
58 1.64],  $p=0.004$ ). A lower cAMP response in granulosa cells was noted following Follitropin  
59 alpha stimulation for women homozygous N in both polymorphisms, compared to women with  
60 other genotypes (0.901 pmol cAMP/mg total protein vs. 2.19 pmol cAMP/mg total protein,  
61  $p=0.035$ ).

62 **Limitations, reasons for caution:** Due to racial differences in *LHCGR* genotype distribution,  
63 these results may not be applicable for all populations.

64 **Wider implications of the findings:** Despite that more than 250 000 cycles of gonadotropin  
65 stimulations are performed annually worldwide prior to IVF, it has not been possible to predict  
66 neither the pregnancy outcome, nor the response to the hormone with accuracy. If *LHCGR* and  
67 *FSHR* variants are recognized as biomarkers for chance of pregnancy, more individualized and  
68 thereby more efficient treatment modalities can be developed.

69 **Study funding, competing interest(s):** This work was supported by Interreg IV A, EU (grant  
70 167158) and ALF governments grant (F2014/354). Merck-Serono (Darmstadt, Germany)  
71 supported the enrolment of the subjects. The authors declare no conflict of interest.

72

73 **Keywords:** LHCG receptor, FSH receptor, *in vitro* fertilization, polymorphism.

74

## 75 **Introduction**

76 About 15% of all couples experience involuntary childlessness (World Health Organization,  
77 2000). This number is expected to increase as a combination of socially related issues with  
78 prolonged time to start a family and increased access to assisted reproductive techniques also in  
79 rapidly growing economies as in Asia.

80

81 It has for a long time been known that follicle-stimulating hormone (FSH) plays a central role in  
82 the endocrine regulation of female as well as male gametogenesis. Follicle-stimulating hormone  
83 mediates its effect via the FSH receptor (FSHR) located on the cell membrane of ovarian  
84 granulosa cells (Camp *et al.*, 1991) from where FSH drives follicular maturation (McNeilly *et*  
85 *al.*, 1991) and estrogen production (Nordhoff *et al.*, 2011). The FSHR belongs to the G protein-  
86 coupled receptor family and hence signals through the classical  $G\alpha_s/3'$ -5' cyclic adenosine  
87 monophosphate (cAMP)/protein kinase A pathway (Means *et al.*, 1974), but also through for  
88 example the adapter protein containing pleckstrin homology domain, phosphotyrosine binding  
89 domain and leucine zipper motif (APPL1)/inositol 1,4,5,-triphosphate (IP<sub>3</sub>) signaling pathway  
90 (Thomas *et al.*, 2011).

91 The *FSHR* gene is located on chromosome 2 and consists of ten exons. Exon 10 holds five single  
92 nucleotide polymorphisms (SNPs) in the coding region, of which the one in amino acid position  
93 680 (N680S; rs6166), in the intracellular domain, is the most thoroughly studied (Simoni *et al.*,  
94 1999; Gromoll and Simoni, 2005). In Caucasian populations approximately 30% are  
95 homozygous for asparagine (N), 50% are heterozygous, and 20% are homozygous for serine (S)  
96 (Kuijper *et al.*, 2010; Lindgren *et al.*, 2012). It has been proposed that women homozygous for  
97 *FSHR* S680 require a higher dose exogenous FSH prior to IVF than those with *FSHR* N680, in

98 order to achieve successful ovarian stimulation (Perez Mayorga *et al.*, 2000; Sudo *et al.*, 2002).  
99 These women also seem to have longer menstrual cycles and have a higher risk for severe  
100 ovarian hyperstimulation syndrome compared with women with other genotypes (Daelemans *et*  
101 *al.*, 2004). In a previous study it was also evident that women homozygous for *FSHR* N680 more  
102 often became pregnant after IVF, compared to women with other genotypes (Jun *et al.*, 2006).  
103 Altogether, these observations indicate that individuals who are carriers of *FSHR* S680 may have  
104 decreased *FSHR* response compared with carriers of one or two copies of *FSHR* N680.  
105 Furthermore, it was recently suggested that intracellular cAMP production was faster in human  
106 granulosa cells from homozygous carriers of *FSHR* N680 than in women homozygous for *FSHR*  
107 S680 when stimulated with FSH *in vitro* (Casarini *et al.*, 2014). However, this study only  
108 comprised four women.

109  
110 Luteinizing hormone (LH) mediates its effect through the LH/human chorionic gonadotropin  
111 (hCG) receptor (LHCGR) located on cell membranes of granulosa and theca cells (Camp *et al.*,  
112 1991). When LH binds to LHCGR on theca cells, androstenedione and subsequently estradiol  
113 production is triggered (Short, 1962). Like the *FSHR*, the LHCGR also belongs to the G protein-  
114 coupled receptor family and holds seven transmembrane helices. The *LHCGR* gene is located on  
115 chromosome 2, close to the *FSHR* gene, and contains 11 exons. Of the polymorphisms in the  
116 *LHCGR* gene, the N312S polymorphism (rs2293275) in exon 10 is one of the most studied.  
117 Approximately 18% of Caucasian populations are homozygous for the A allele of the *LHCGR*  
118 N312S polymorphism encoding N, 49% are heterozygous, and 33% are homozygous for the G  
119 allele, encoding S (Valkenburg *et al.*, 2009). The *LHCGR* N312S polymorphism is located near a  
120 glycosylation site which indicates that variations in the sequence could affect sensitivity. A few

121 cohort studies have proposed that the N variant may render the LHCGR more sensitive (Piersma  
122 *et al.*, 2007; Simoni *et al.*, 2008). The *LHCGR* N312S polymorphism has also been linked to  
123 polycystic ovary syndrome (PCOS), where heterozygous women had two-fold increased risk of  
124 PCOS and those homozygous for N had a three-fold increased risk in a Sardinian population  
125 (Capalbo *et al.*, 2012).

126

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



## 133 **Materials & Methods**

### 134 *Subjects*

135 Women undergoing IVF at the Reproductive Medicine Centre, Skåne University Hospital,  
136 Malmö, Sweden (n=384) were consecutively enrolled in the study from September 2010 to  
137 February 2015. Inclusion criteria were regular menstruation cycle of 21-35 days, bilateral  
138 ovaries, body mass index (BMI) <30 kg/m<sup>2</sup>, younger than 40 years of age, and non-smokers. The  
139 women were between 22 and 39 years of age (mean: 32.0±3.82) on the day of follicular fluid  
140 aspiration (Table I). A venous blood sample was drawn for DNA extraction with subsequent  
141 *FSHR* and *LHCGR* genotyping. Follicular fluid was collected for subsequent granulosa cell  
142 isolation from n=135 women while undergoing oocyte retrieval. Clinical data for the women  
143 were retrieved from medical records. In n=11 women, BMI was missing and these women were  
144 hence excluded when associating genotype with clinical parameters, resulting in a total of n=373.  
145 In n=30 women, data regarding baseline FSH values were missing, and these women were  
146 therefore also excluded when associating genotype with baseline FSH values. Additionally, in  
147 n=37 women, data regarding cycle length was missing (although the cycle length of these  
148 women were in the normal range) and hence these women were excluded when associating cycle  
149 length with genotype.

150

151 An independent population of n=233 women was enrolled *a posteriori* in order to validate  
152 pregnancy associations in the study cohort. These women underwent IVF at the same medical  
153 unit as the first cohort from the start of year 2007 until June 2015, and the inclusion criteria were  
154 the same as for the first study cohort. The women were between 20 and 40 years of age (mean:  
155 32.5±3.93) on the day of follicular fluid aspiration (Table I). The BMI values for these women

156 were not recorded, and therefore this parameter was missing for this population. However, in  
157 order to undergo an IVF treatment at this clinical unit, BMI has to be  $<30 \text{ kg/m}^2$ . Clinical data  
158 regarding age and IVF parameters were retrieved from medical records.

159

#### 160 *Patient treatment*

161 Ovarian stimulation was performed according to either a short antagonist protocol using the  
162 gonadotropin-releasing hormone (GnRH) antagonist Ganirelix (Orgalutran, Organon [Ireland]  
163 Ltd. Dublin, Ireland) or a standard long protocol using the GnRH agonist Nafarelin (Synarela,  
164 Pfizer Ab, Sollentuna, Sweden) (Table II). Ovarian hyperstimulation was carried out using  
165 individually set, flexible doses of either Follitropin alpha (GONAL-f, Merck-Serono, Darmstadt,  
166 Germany), Follitropin beta (Puregon, Organon [Ireland] Ltd), Urofollitropin (Fostimon, Institut  
167 Biochimique SA [IBSA], Lugano, Switzerland), or Menotropin (Menopur, Ferring GmbH, Kiel,  
168 Germany). The progression of follicle development was monitored by vaginal ultrasound on day  
169 6-8 of stimulation, and if needed the individual doses were adjusted. When three or more  
170 follicles were confirmed by ultrasound, hCH was administered and 35 h later oocyte retrieval  
171 was performed.

172

#### 173 *Genotyping of FSHR and LHCGR*

174 Genomic DNA was extracted from peripheral leukocytes using standard procedures, and the  
175 SNP at amino acid position 680 in the *FSHR* was analyzed by allele-specific PCR as previously  
176 described (Lindgren *et al.*, 2012). The PCR results were confirmed by direct sequencing of 20  
177 samples on an eight-capillary Applied Biosystems sequencing gear (Applied Biosystems,  
178 Stockholm, Sweden). The N312S polymorphism in the *LHCGR* was analyzed by PCR

179 amplification followed by direct sequencing. Polymerase chain reactions were performed in a  
180 total volume of 50  $\mu$ L containing 0.4  $\mu$ M of the forward primer 5'-  
181 TGTTGACCATGTGACTAGGGA and 0.4  $\mu$ M of the reverse primer 5'-  
182 ACTCTCTCCTCAGGAAGCAT (Invitrogen, Stockholm, Sweden), 10 mM Tris-HCl  
183 (AppliChem GmbH, Gatersleben, Germany) pH 9.1, 45 mM KCl (ICN Biomedicals INC.,  
184 Aurora, OH, USA), 0.01% w/v Tween 20 (Scharlau Chemie S.A., Barcelona, Spain), 1.5 mM  
185  $MgCl_2$  (Sigma-Aldrich Sweden AB, Stockholm, Sweden), 200  $\mu$ M of each dNTP (Fermentas,  
186 Sankt Leon-Rot, Germany), 1 U Dynazyme™ II DNA polymerase (Thermo Fisher Scientific  
187 Inc., Waltham, MA, USA) and 200 ng template DNA. The amplification program was initiated  
188 by a denaturation step at 96°C for 10 min, followed by 37 amplification cycles, each consisting  
189 of denaturation at 96°C for 1 min, annealing at 61°C for 30 sec and elongation at 72°C for 3 min.  
190 A final elongation at 72°C for 7 min was applied. The PCR product was purified and directly  
191 sequenced on an eight-capillary Applied Biosystems sequencing gear (Applied Biosystems).

192

### 193 *Hormonal analysis*

194 For endocrine serum analysis, blood samples were drawn between 8 and 10 a.m. Estradiol (E2),  
195 FSH, and LH was measured using an electrochemiluminiscence immunoassay (Cobas-Roche,  
196 Mannheim, Germany) at the routine clinical chemistry laboratory at Skåne University Hospital  
197 (Lund, Sweden). The sensitivities of the assays were 18.4 pmol/L for E2 and 0.10 IU/L for FSH  
198 and LH. The coefficients of variances (CV) for E2 were 7% at 289 pmol/L and 4% at 2011  
199 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.

200

### 201 *Isolation of granulosa cells from follicular fluid*

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

213

#### 214 *Cell sorting*

215 To isolate granulosa cells from follicular fluid CD99 was used as a marker. In order to avoid  
216 non-specific binding of the antibodies used for granulosa cell sorting, Fc receptor blocking was  
217 carried out with TrueStain (BioLegend, London, UK) for 5 min prior to antibody staining with  
218 anti-CD45 PerCP (BioLegend) and anti-CD99 PE (eBioscience, San Diego, CA, USA). The  
219 granulosa cells were mixed gently and incubated for 15 min in darkness at room temperature.  
220 Stained cells were filtered through a 50  $\mu\text{m}$  filcon with syringe fitting (Biogenetics, Padova,  
221 Italy) and washed in supplemented RPMI medium. Filtered cells were centrifuged at 430xg for 5  
222 min and resuspended in 200  $\mu\text{L}$  supplemented RPMI medium. Seven-Amino actinomycin D (7-  
223 AAD) (BioLegend) was added 5 min prior to flow cytometry acquisition and cell sorting. The  
224 samples were subsequently acquired and sorted in a FACSAria (Becton Dickinson Biosciences).

225 The gating strategy for definition of viable granulosa cells was an acquisition gate based on side  
226 scatter (SSC) and forward scatter (FSC), including cells with high granularity and excluding cell  
227 debris, dead cells and leukocytes (7-AAD and CD45 positive cells). Remaining cells were gated  
228 on the most granular and CD99 positive cells, which were defined as viable granulosa cells and  
229 sorted into supplemented RPMI medium containing 55% FBS. Flow cytometry data were  
230 analyzed using FlowJo (TreeStar Inc., Ashland, OR, USA).

231

### 232 *Identification of granulosa cells*

233 Specimens from three different, randomly selected patients (unsorted and sorted cells) were  
234 morphologically evaluated at the Department of Pathology and Cytology, Skåne University  
235 Hospital, Malmö, Sweden. The cells, which were kept in R10 medium, were centrifuged at  
236 760×g for 10 min. The supernatant was decanted and Becton Dickinson Cytorich™ (red) was  
237 added. The tubes were left at 4°C for at least 30 min to ensure optimal fixation of the cells,  
238 before centrifugation at 760×g for 10 min. The supernatant was decanted and the cell pellet re-  
239 suspended in deionized water. The cell suspension was added to pre-prepared cell chambers  
240 (Settling chamber 240, TriPath Imaging, Becton Dickinson Diagnostics, Sparks, NV, USA),  
241 placed on pre-coated slides (SurePath precoat slides, TriPath Imaging, Becton Dickinson  
242 Diagnostics) and left for 15 min to allow the cells to adhere to the slides. The slides were  
243 subsequently fixed in 95% ethanol for at least 30 min and stained with Htx-eosin (Histolab  
244 Products AB, Gothenburg, Sweden) according to routine cyto-staining procedures before  
245 examination with an Olympus BH-2 microscope (Olympus Corporation, Center Valley, Pa,  
246 USA) and morphological evaluation. Granulosa cells and other cell types were counted in

247 randomly chosen high power (objective lens  $\times 40$ , ocular lens  $\times 10$ ) microscopic fields until  
248 approximately 300 cells were counted.

249

#### 250 *Culture and granulosa cell activity*

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

267

#### 268 *Statistical analysis*

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

275  
276 All residuals were tested for normal distribution in the Kolmogorov–Smirnov test, and log  
277 transformed if lack of Gaussian distribution. Calculations for associations with clinical  
278 parameters among genotype groups were carried out by comparing each genotype group  
279 separately (i.e. N680N vs. N680S vs. S680S for the *FSHR*; N312N vs. N312S vs. S312S for the  
280 *LHCGR*). Comparisons of age, BMI, cycle length, baseline hormones, hormonal doses given  
281 prior to IVF, and follicle and oocyte outcomes among genotype groups were carried out using a  
282 univariate analysis of variance. Comparisons of incidence of endometriosis and polycystic ovary  
283 syndrome, and occurrence of embryo transfer among genotype groups were carried out using a  
284  $q^2$  test. Comparisons of pregnancy outcomes among genotype groups were carried out using a  $q^2$   
285 test and logistic regression. In order to evaluate a combined effect of the *FSHR* and *LHCGR* on  
286 pregnancy outcomes in the merged study group, the G alleles from both polymorphisms were  
287 considered (0-4 G alleles) and a trend of the increased number of G alleles for the *FSHR*  
288 polymorphism and the *LHCGR* polymorphism analyzed using logistic regression. Age (as a  
289 continuous variable), and BMI ( $\leq 25$  and  $>25$ ) were considered as confounding factors when  
290 analyzing differences in cycle length, baseline hormones, hormonal doses, and follicle and  
291 oocyte counts between genotypes. Age, BMI, use of intracytoplasmic sperm injection (ICSI),

292 type of stimulation protocol, and type of hormonal agent used (Follitropin or Menotropin) were  
293 considered as confounding factors when analyzing differences in pregnancy outcomes. Analysis  
294 concerning pregnancy outcome was also performed, using the same covariates, after exclusion of  
295 the 13 women who were diagnosed with PCOS.

296

297 In order to evaluate a combined effect of the *FSHR* and *LHCGR* polymorphisms on cAMP and  
298 IP<sub>3</sub> concentrations in cell culture supernatants from granulosa cells, the G alleles from both  
299 polymorphisms were added (0-4 alleles) and a trend of the increased number of G alleles for the  
300 *FSHR* polymorphism and the *LHCGR* polymorphism was analyzed using linear regression. Each  
301 polymorphism was also analyzed separately. In addition, we used a univariate analysis of  
302 variance in order to compare individual groups of combined polymorphisms. Age (as a  
303 continuous variable) was considered as a confounding factor when analyzing differences in  
304 cAMP and IP<sub>3</sub> concentrations in cell culture supernatants.

305

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

308

309 Data was analyzed using SPSS software version 22 (SPSS Inc., Chicago, IL, USA). A p-value of  
310 <0.05 was considered statistically significant. Minitab 12.21 (Minitab Inc., State College, PA,  
311 USA) was used when calculating exact binomial proportion confidence interval of purity of  
312 sorted granulosa cells.

313

314 *Ethical approval*



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

## 317 **Results**

### 318 *Genotyping*

319 Allele frequencies for the *FSHR* N680S polymorphism were 55% for the A allele, encoding  
320 asparagine, and 45% for the G allele, encoding serine. Genotype distribution was 28%  
321 homozygous N, 55% heterozygous, and 17% homozygous S. For the *LHCGR* N312S  
322 polymorphism, allele frequencies were 41% A, encoding asparagine, and 59% G, encoding  
323 serine. The genotype distribution was 17% homozygous N, 47% heterozygous, and 36%  
324 homozygous S (Table I). There was no difference in *FSHR* N680S allele frequency between the  
325 study population and a population previously reported (Kuijper *et al.*, 2010) ( $p=1$ ), or in *LHCGR*  
326 N312S allele frequency between the study and the general population (Piersma *et al.*, 2007),  
327 ( $p=0.554$ ). The allele frequencies of both polymorphism were in Hardy-Weinberg equilibrium,  
328  $\chi^2=0.06$ ,  $p>0.05$  for *LHCGR* N312S and  $\chi^2=1.29$ ,  $p>0.05$  for *FSHR* N680S. The two  
329 polymorphisms are in linkage equilibrium,  $D'=0.042$ ,  $r^2=0.0015$ .

330

331 In the second study population the allele frequencies for the *FSHR* N680S polymorphism were  
332 59% for the A allele and 41% for the G allele. Genotype distribution was 34% homozygous N,  
333 50% heterozygous, and 16% homozygous S. For the *LHCGR* N312S polymorphism, allele  
334 frequencies were 36% for the A allele and 64% for the G allele. Genotype distribution was 15%  
335 homozygous N, 43% heterozygous, and 42% homozygous S (Table I). There were no differences  
336 in the allele frequencies between the replication population and the first study population  
337 ( $p=0.191$  for *FSHR* S680N and  $p=0.080$  for *LHCGR* N312S) and altogether, the allele  
338 frequencies of both polymorphisms were in Hardy-Weinberg equilibrium,  $\chi^2=0.52$ ,  $p>0.05$  for  
339 *LHCGR* N312S and  $\chi^2=0.11$ ,  $p>0.05$  for *FSHR* N680S.

340 *Clinical parameters and IVF outcome*

341 *LHCGR N312S*

342 For the *LHCGR N312S* polymorphism, no differences regarding background characteristics were  
343 found between women with N312 or S312, except for a marginal difference in age (Table I).

344 Women homozygous for N received lower mean daily as well as total dose of FSH during IVF  
345 treatment. A trend towards higher pregnancy rates for women carrying *LHCGR S312* was found  
346 (Table I, Fig IA and B), and using a logistic regression model, a statistically significant  
347 association was evident (unadjusted: OR= 1.43, 95% CI: [1.03, 1.99], p=0.033; adjusted:  
348 OR=1.57, 95% CI: [1.11, 2.22], p=0.011; Fig IA). In women who received embryo transfer, the  
349 same difference was found (unadjusted: OR=1.46, 95% CI: [1.03, 2.06], p=0.033; adjusted:  
350 OR=1.59, 95% CI: [1.10, 2.29], p=0.013, Fig IB).

351  
352 Analysis performed after exclusion of PCOS patients resulted in slightly stronger associations:  
353 (unadjusted: OR= 1.44, 95% CI: [1.03, 2.01], p=0.032; adjusted: OR=1.61, 95% CI: [1.13, 2.29],  
354 p=0.008). In women who received embryo transfer, the same difference was found (unadjusted:  
355 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).

356  
357 A trend, however not significant, towards higher pregnancy rates for carriers of *LHCGR S312*  
358 was also found in the replication population of n=233 women (unadjusted: OR=1.36, 95% CI:  
359 [0.881, 2.10], p=0.165; adjusted: OR=1.27, 95% CI: [0.798, 2.00], p=0.316). A weak trend was  
360 also found among women receiving embryo transfer (unadjusted: OR=1.36, 95% CI: [0.878,  
361 2.11], p=0.168; adjusted: OR=1.27, 95% CI: [0.795, 2.03], p=0.316).

362

363 When merging the first study population with the second validation group, in total n=606  
364 women, a higher pregnancy rate was evident for *LHCGR* S312 carriers (Table I; unadjusted:  
365 OR=1.40, 95% CI: [1.07, 1.81], p=0.013; adjusted: OR=1.49, 95% CI: [1.14, 1.96], p=0.004). A  
366 higher pregnancy rate was also found among those receiving embryo transfer (unadjusted:  
367 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).

368

### 369 *FSHR* N680S

370 A weak, however not significant, trend towards higher pregnancy rates was also noticed for  
371 carriers of S in the *FSHR* N680S polymorphism (unadjusted: OR=1.14, 95% CI: [0.810, 1.61],  
372 p=0.452, adjusted: OR=1.11, 95% CI: [0.746, 1.60], p=0.577, Fig IC). There was no significant  
373 difference in women who received embryo transfer (unadjusted: OR=1.13, 95% CI: [0.792,  
374 1.61], p=0.499; adjusted: OR=1.08, 95% CI: [0.774, 1.60], p=0.679; Fig ID) or in other clinical  
375 variables analyzed in relation to *FSHR* N680S.

376

377 Analysis performed after exclusion of PCOS patients displayed very similar results (unadjusted:  
378 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).  
379 There was no significant difference among those who received embryo transfer (unadjusted:  
380 OR=1.13, 95% CI: [0.786, 1.61], p=0.519; and adjusted: OR=1.08, 95% CI: [0.741, 1.57],  
381 p=0.698) or in other clinical variables analyzed in relation to *FSHR* N680S.

382

383 Also in the replication population of n=233 women, a weak, but not significant trend towards  
384 higher pregnancy rates was found for carriers of *FSHR* S680 (unadjusted: OR=1.24, 95% CI:  
385 [0.810, 1.90], p=0.321; adjusted: OR=1.20, 95% CI: [0.752, 1.91], p=0.445). A weak trend

386 towards higher pregnancy rates among *FSHR* S680 carriers was also present for those receiving  
387 embryo transfer (unadjusted: OR=1.24, 95% CI: [0.808, 1.91], p=0.323; adjusted: OR=1.17,  
388 95% CI: [0.729, 1.88], p=0.514).

389

390 When combining the first study population with the second group (n=606), a weak trend,  
391 however not significant, towards higher pregnancy rates for *FSHR* S680 was evident  
392 (unadjusted: OR=1.18, 95% CI: [0.906, 1.54], p=0.216; adjusted: OR=1.17, 95% CI: [0.889,  
393 1.55], p=0.260). A non-significant trend towards higher pregnancy rates among *FSHR* S680  
394 carriers was also observed for women receiving embryo transfer (unadjusted: OR=1.19, 95% CI:  
395 [0.903, 1.56], p=0.218; adjusted: OR=1.17, 95% CI: [0.878, 1.55], p=0.288).

396

#### 397 *LHCGR* N312S and *FSHR* N680S combined

398 When analyzing the two polymorphisms combined, a higher pregnancy rate was found for  
399 women homozygous for S in both polymorphisms (n=23) compared to those homozygous for N  
400 (n=20) (unadjusted: OR=5.79, 95% CI: [1.07, 31.1], p=0.041; adjusted: OR=14.4, 95% CI:  
401 [1.65, 126], p=0.016, Fig IIA). The same pattern was found in women who received embryo  
402 transfer (unadjusted: OR=6.55, 95% CI: [1.18, 36.3], p=0.032, adjusted: OR=25.7, 95% CI:  
403 [1.95, 340], p= 0.014; Fig IIB).

404

405 In the independent sample of n=233 women, a tendency towards an association was found for  
406 higher pregnancy rates among women homozygous for S in both polymorphisms (n=12)  
407 compared to women homozygous for N in both polymorphisms (n=10) (unadjusted: OR=3.00,  
408 95% CI: [0.260, 34.6], p=0.378; adjusted: OR=3.23, 95% CI: [0.103, 101], p=0.505). A similar

409 association was found in women who received embryo transfer (unadjusted: OR=3.38, 95% CI:  
410 [0.290, 39.3], p=0.332, adjusted: OR=3.37, 95% CI: [0.110, 103], p=0.486).

411

412 In the merged study population, women homozygous for S (n=35) had higher pregnancy rate  
413 than those homozygous for N (n=30) (unadjusted: OR=4.70, 95% CI: [1.18, 18.7], p=0.028;  
414 adjusted: OR=11.5, 95% CI: [1.86, 71.0], p=0.009). The same pattern was found in women who  
415 received embryo transfer (unadjusted: OR=5.26, 95% CI: [1.30, 21.3], p=0.020; adjusted:  
416 OR=11.5, 95% CI: [1.89, 69.9], p=0.008). A linear significant trend with pregnancy rate and  
417 increasing number of G alleles was also noted in the merged study population (unadjusted:  
418 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  
419 IIIA). The same was also true for women receiving embryo transfer (unadjusted: OR=1.30, 95%  
420 CI: [1.07, 1.58], p=0.009; adjusted: OR=1.33, 95% CI: [1.09, 1.63], p=0.005, Fig IIIB).

421

#### 422 *Granulosa cell stimulation*

423 A distinct and viable granulosa cell population was isolated by fluorescence activated cell sorting  
424 (FACS), consisting of 97.8% (95% CI: [96.6%, 98.6%]) granulosa cells (Table III, Fig IV).

425 Regarding the *FSHR* N680S and *LHCGR* N312S polymorphisms, no differences in response to  
426 Follitropin alpha or Menotropin were observed in the induction of cAMP or IP<sub>3</sub> production  
427 (Table IV). A combination of the two polymorphisms showed no linear association between  
428 genotype and induction of cAMP following either Follitropin alpha stimulation (unadjusted:  
429  $\beta=0.247$ , 95% CI: [-0.070, 0.563], p=0.125; adjusted:  $\beta=0.247$ , 95% CI: [-0.071, 0.565],  
430 p=0.126, Fig VA) or Menotropin stimulation (unadjusted:  $\beta=-0.037$ , 95% CI: [-0.312, 0.238],  
431 p=0.788; adjusted:  $\beta=-0.054$ , 95% CI: [-0.334, 0.225], p=0.701, Fig VB). Following Follitropin

432 alpha stimulation, the group of women homozygous N in both polymorphism displayed lower  
433 mean cAMP levels compared to others (unadjusted: 0.901 pmol cAMP/mg total protein vs. 2.19  
434 pmol cAMP/mg total protein,  $p=0.034$ ; adjusted: 0.901 pmol cAMP/mg total protein vs. 2.19  
435 pmol cAMP/mg total protein,  $p=0.035$ , Fig VA). Furthermore, no linear association between  
436 genotype combinations and induction of IP<sub>3</sub> following either Follitropin alpha stimulation  
437 (unadjusted:  $\beta=-0.024$ , 95% CI: [-0.153, 0.106],  $p=0.714$ ; adjusted:  $\beta=-0.025$ , 95% CI: [-0.158,  
438 0.109],  $p=0.709$ , Fig VC) or Menotropin stimulation (unadjusted:  $\beta=0.004$ , 95% CI: [-0.109,  
439 0.117],  $p=0.943$ ; adjusted:  $\beta=-0.012$ , 95% CI: [-0.128, 0.104],  $p=0.836$ , Fig VD) was found.

## 440 **Discussion**

441 The main findings of the present study was that women homozygous for S in both  
442 polymorphisms studied had a four-fold higher chance of pregnancy compared to women  
443 homozygous for N in corresponding codons. It was also evident that carriers of S312 in the  
444 *LHCGR* more often became pregnant after IVF than those with N in the same position; 56%  
445 higher rate for heterozygous women with one S and 83% for homozygous women, compared to  
446 women homozygous for N. The same patterns were also present in women who had an embryo  
447 transferred. The main finding of the present study was also validated in an independent  
448 population of women, though not statistically significant, most probably due to a smaller number  
449 of women eligible than in the first study population. In the merged cohort, consisting of more  
450 than 600 women, relationships with pregnancies were even stronger than in the original cohort,  
451 and a linear association with pregnancy rate and increasing number of G alleles was noted.

452

453 Both FSH and LH are required for adequate oocyte maturation (Segaloff and Ascoli, 1993), and  
454 hence it is not surprising that variants of the *LHCGR* play a role in the outcome of IVF  
455 treatments. The mechanism underlying is unknown, but it has been proposed that G protein-  
456 coupled receptors may form homo- and heterodimers (Angers *et al.*, 2002), so that stimulation by  
457 one of the hormones could be mediated in part through the other hormone's receptor and that  
458 some isoforms of the different receptors may have beneficial function compared to other  
459 isoforms. Thus, the *LHCGR* genotype could influence the response to FSH stimulation. Still,  
460 many LH effects are considered as indirect since the distribution of LHCGR on granulosa cells is  
461 not as dense and stable as the FSHR distribution during the menstrual cycle (Camp *et al.*, 1991).  
462 In the current work, an attempt to elucidate the mechanism behind the impact of combinations of



463 FSHR and LHCGR variants on receptor function was made by stimulating granulosa cells *in*  
464 *vitro* with Follitropin alpha and Menotropin, respectively. However, when analyzing a  
465 combination of the N680S of the *FSHR* and N312S of the *LHCGR in vitro*, no linear association  
466 between combined receptor variants and hormone sensitivity was detected. Nevertheless,  
467 granulosa cells from the group of women who were homozygous for N in both polymorphisms,  
468 comprising eight women, displayed a lower cAMP activity following Follitropin alpha  
469 stimulation when compared to women with other genotypes. Due to the limited sample size, this  
470 finding has yet to be regarded as preliminary, due to the fact that the cells used in the *in vitro*  
471 experiments were pre-stimulated during the IVF trial in the clinic, which could impact the  
472 results. The receptors on the granulosa cells may for example already have been down- or up-  
473 regulated to some extent, which could affect further stimulation with hormonal agents.

474

475 No differences in the number of follicles or oocytes between those carrying *LHCGR* N312 and  
476 S312 were found in the current study population, nor were there any obvious differences  
477 regarding embryo quality. Nevertheless, the pregnancy rates differed markedly. This could either  
478 be due to small differences in the maturation of the oocyte, not visible through light microscopy,  
479 and taking place after the hCG administration; or a problem that occurred at a later  
480 developmental stage, after embryo transfer. Since LH regulates the formation of corpus luteum,  
481 it could also be due to some insufficiency of this structure, which could affect its ability to  
482 produce hCG and progesterone, which is needed to support the hatching embryo. The large  
483 difference in pregnancy rates could also partly be an effect of differing stimulation protocols  
484 prior to IVF treatment, and we therefore adjusted for this factor in the analyses. Furthermore,  
485 when PCOS cases were removed from the analyses, the association with pregnancy rates became

486 stronger, indicating that the interplay between receptors and gonadotropins in this category of  
487 patients may be different than in other women. Women homozygous for *LHCGR* N312 also  
488 required lower doses of exogenous FSH for adequate response. Considering the dimerization  
489 hypothesis, this could indicate that N renders the receptor more hormone sensitive, which is  
490 consistent with earlier hypotheses (Piersma *et al.*, 2007; Simoni *et al.*, 2008). Several studies  
491 have also indicated that high LH levels (>10 IU/L) are associated with increased miscarriage  
492 rates and lower chances of pregnancy (Regan *et al.*, 1990; Shoham *et al.*, 1990), and it seems  
493 likely that a more sensitive *LHCGR* would have the same effect.

494

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

503

504 The strength of the study was the large cohort of consecutively enrolled patients. These women  
505 were hence not selected for the study, but an ordinary cohort of women visiting a fertility clinic.  
506 The findings can therefore be generally applied. Another strength was the purity of the granulosa  
507 cell material, which made it possible to compare *in vitro* results and clinical results within the  
508 same study population. A drawback of the study was that it was not recorded when in the

509 menstrual cycle the baseline reproductive hormones were obtained, which therefore not was  
510 possible to adjust for. These proceedings may have masked possible links between receptor  
511 genotype and gonadotropin concentrations.

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

514

515 In summary, in this large cohort of women, those homozygous for S in both studied  
516 polymorphisms had a four-fold increased chance of pregnancy compared to women homozygous  
517 for N; whereas only 10% of women with N in both genes became pregnant, 39% of those with S  
518 did. Thus, if used in IVF trials, these SNPs could be used as predictors for pregnancy outcome, at  
519 least in Caucasian populations.

520 **Author's roles**

521 Study design: IL, MB, LB, KU, YLG. Recruitment of patients and collection of patient data: EH,  
522 MBu, LB, ILe. Lab exp: IL, MB, KU, AD, LK, CC. Statistical analysis: IL, MB. Data  
523 interpretation: IL, MB, KU, AD, EH, SS, CYA, YLG. Writing of manuscript draft: IL, MB, AD,  
524 YLG. Final manuscript: all co-authors.

525

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530

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536

537 **Conflict of interest**

538 The authors declare no conflict of interest.

539

540

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- 603

604 **Figure legends**

605 **Figure I**

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

610

611 **Figure II**

612 Pregnancy frequencies for combined genotypes. A) All women: *LHCGR* N312 in combination  
613 with *FSHR* N680, N680S, and S680 comprised n=20, n=35, and n=12 women, respectively.  
614 *LHCGR* N312S in combination with *FSHR* N680, N680S, and S680 comprised n=49, n=96, and  
615 n=30 women, respectively. *LHCGR* S312 in combination with *FSHR* N680, N680S, and S680  
616 comprised n=33, n=75, and n=23 women, respectively. B) Only women receiving ET: *LHCGR*  
617 N312 in combination with *FSHR* N680, N680S, and S680 comprised n=18, n=27, and n=9  
618 women, respectively. *LHCGR* N312S in combination with *FSHR* N680, N680S, and S680  
619 comprised n=41, n=80, and n=26 women, respectively. *LHCGR* S312 in combination with *FSHR*  
620 N680, N680S, and S680 comprised n=25, n=64, and n=20 women, respectively.

621

622 **Figure III**

623 Pregnancy frequencies for combined genotypes in the merged population (n=606). A) All  
624 women. 0: n=30 women, 1: n=129 women, 2: n=243 women, 3: n=169 women, and 4: n=35  
625 women. B) Only women receiving ET. 0: n=28 women, 1: n=109 women, 2: n=208 women, 3:  
626 n=151 women, and 4: n=31 women.

627

628 **Figure IV**

629 A) Unsorted sample: Granulosa cells with a mixture of squamous cells, small lymphocyte like  
630 cells, degenerated nuclei, and some debris. (Htx-eosin, 10×). B) Sorted sample: Virtually pure  
631 population of granulosa cells, dispersed or in loose clusters. The cells have pale cytoplasm with  
632 indistinct cell borders, round or ovoid, often eccentric nuclei with coarse but uniform chromatin.  
633 (Htx-eosin, 10×). C) A small cluster of granulosa cells (Htx-eosin, 100×).

634

635 **Figure V**

636 Granulosa cell response measured as cAMP concentration (A-B) and IP<sub>3</sub> concentration (C-D),  
637 stratified as number of G alleles combined (*FSHR* S680, *LHCGR* S312) stimulated with A&C)  
638 Follitropin alpha; B&D) Menotropin.



1 **Table I.** Clinical parameters and IVF outcome.

	<i>LHCGR</i> N312S						<i>FSHR</i> N680S					
	All	N/N	N/S	S/S	p	p <sup>§</sup>	N/N	N/S	S/S	p	p <sup>§</sup>	
First cohort n (%)	373 (100) <sup>¶</sup>	67 (18) <sup>#</sup>	175 (47) <sup>  </sup>	131 (35) <sup>**</sup>	ref	-	102 (28) <sup>††</sup>	206 (55) <sup>‡‡</sup>	65 (17) <sup>§§</sup>	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) <sup>†</sup>	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) <sup>†1</sup>	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) <sup>†2</sup>	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 <sup>2</sup> ) <sup>†</sup>	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 <sup>‡</sup>	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 <sup>‡</sup>	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 <sup>†</sup>	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 <sup>†</sup>	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 <sup>2</sup> baseline <sup>†</sup>	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) <sup>†</sup>	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) <sup>†</sup>	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) <sup>†1</sup>	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) <sup>†2</sup>	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) <sup>†</sup>	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 <sup>†</sup>	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 <sup>†1</sup>	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 <sup>†2</sup>	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 <sup>†</sup>	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 <sup>†1</sup>	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 <sup>†2</sup>	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 <sup>†</sup>	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 <sup>†1</sup>	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 <sup>†2</sup>	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 <sup>‡</sup>	310 (83)	54 (81)	147 (84)	109 (83)	0.818	-	84 (82)	171 (83)	55 (85)	0.929	-
Embryo transfer <sup>‡1</sup>	212 (91)	31 (91)	91 (90)	90 (92)	0.912	-	74 (94)	102 (88)	36 (95)	0.264	-
Embryo transfer <sup>‡2</sup>	522 (86)	85 (84)	238 (86)	199 (87)	0.801	-	158 (87)	273 (85)	91 (88)	0.571	-
Clinical pregnancy <sup>‡</sup>	104 (28)	12 (18)	49 (28)	43 (33)	0.086	-	26 (25)	58 (28)	20 (31)	0.753	-
Clinical pregnancy <sup>‡1</sup>	60 (26)	6 (18)	25 (25)	29 (30)	0.372	-	17 (22)	32 (28)	11 (29)	0.563	-
Clinical pregnancy <sup>‡2</sup>	164 (27)	18 (18)	74 (27)	72 (31)	0.037*	-	43 (24)	90 (28)	31 (30)	0.447	-
Clinical pregnancy/ET	0.34	0.22	0.33	0.39	0.090	-	0.31	0.34	0.36	0.795	-
Clinical pregnancy/ET <sup>1</sup>	0.28	0.19	0.27	0.32	0.380	-	0.23	0.31	0.31	0.449	-
Clinical pregnancy/ET <sup>2</sup>	0.31	0.21	0.31	0.36	0.044*	-	0.27	0.33	0.34	0.388	-

2 <sup>1</sup> Validation cohort; <sup>2</sup> merged cohort; \* p<0.05; † mean±sd; § adjusted values; ‡ n (%); ¶ FSH baseline data n=343 and cycle length data  
3 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

4 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  
5 length data n=190; §§ FSH baseline data n=59 and cycle length data n=58.

6 **Table II.** Stimulation protocols.

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

7

8 **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 <sup>†</sup>	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

9 <sup>†</sup>degenerated cells and stripped nuclei, small lymphocyte like cells, and squamous  
 10 cells against a background of debris

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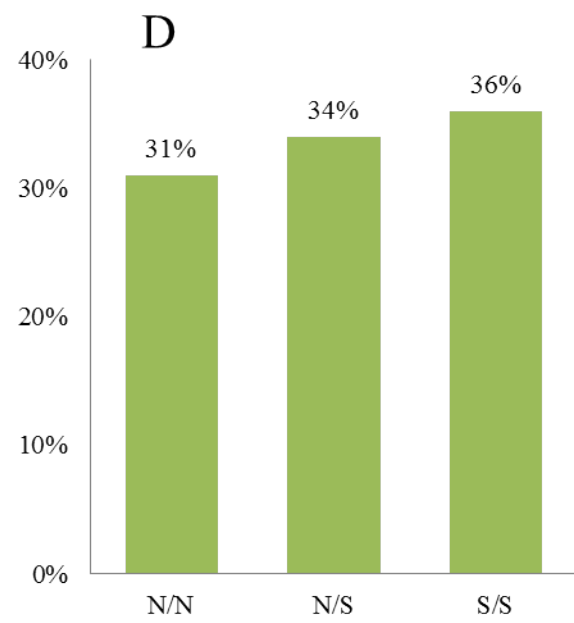
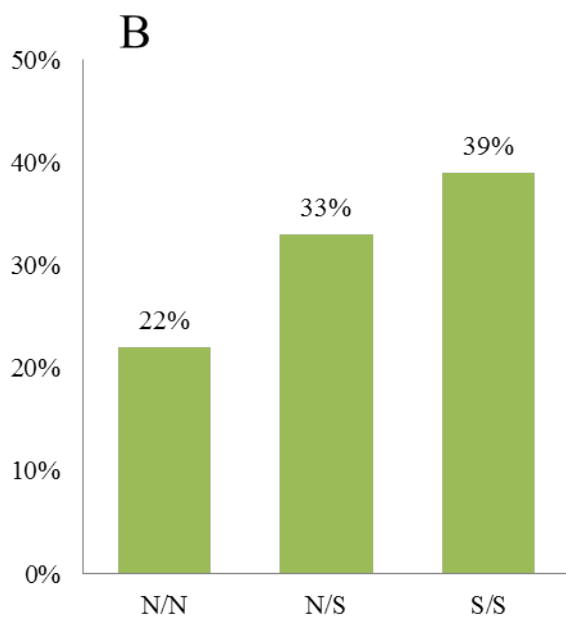
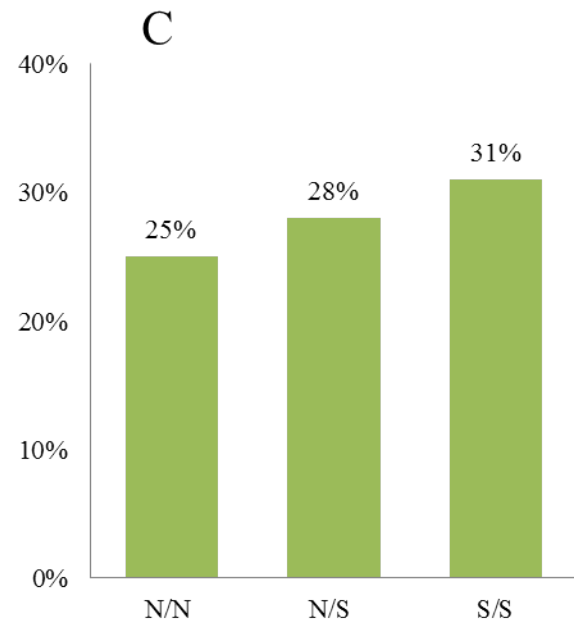
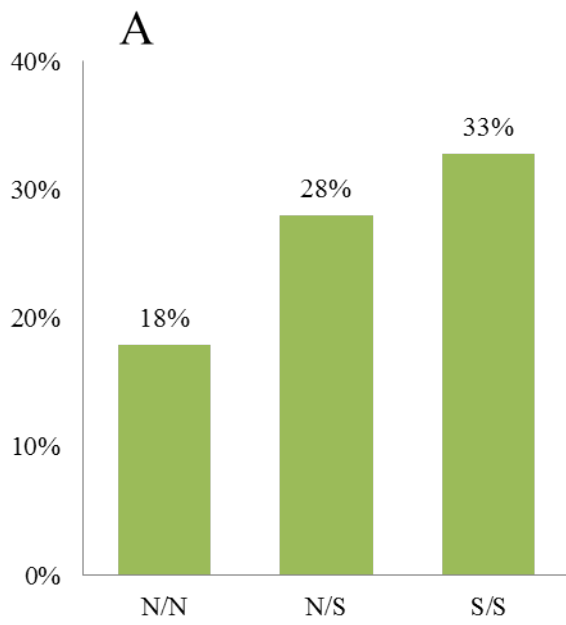
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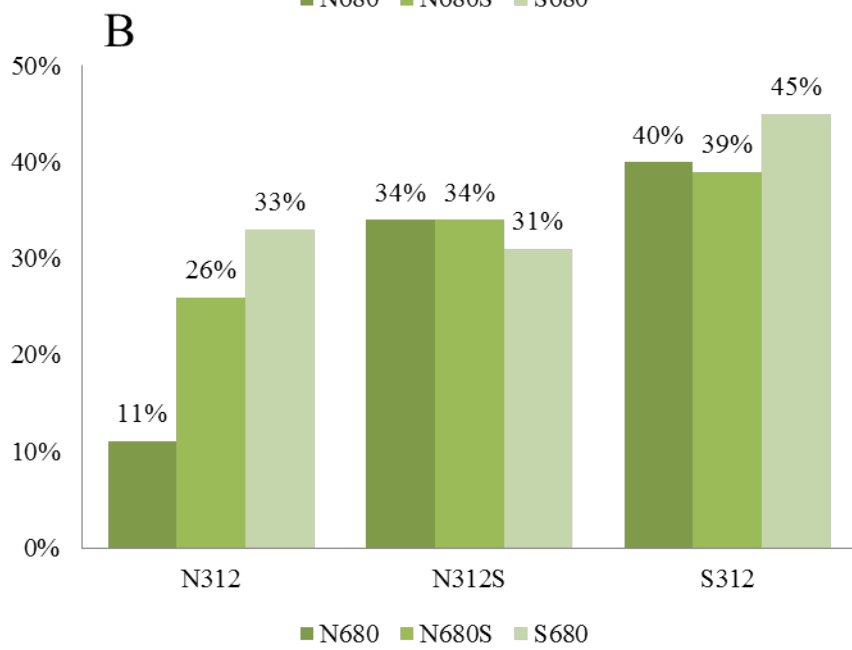
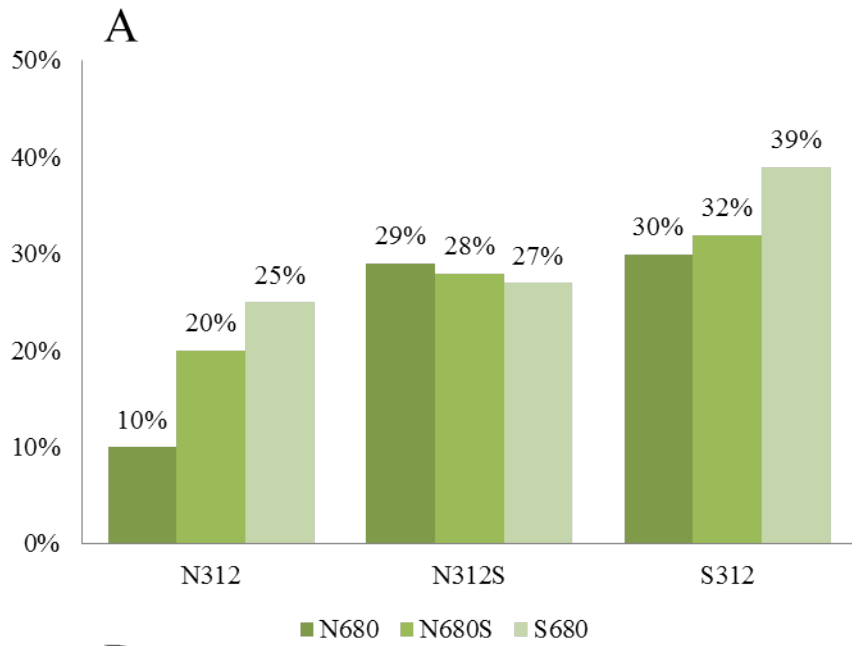
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19 **Table IV.** Granulosa cell response *in vitro*, presented as mean±sd.

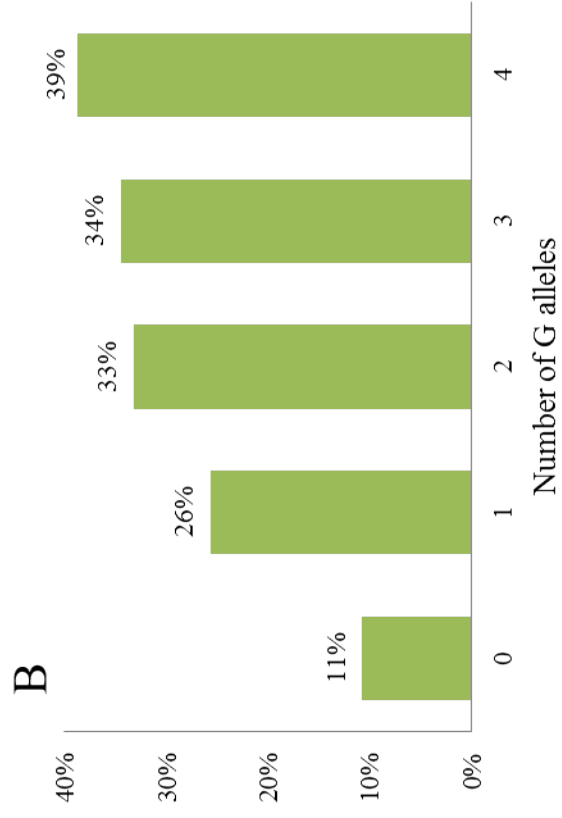
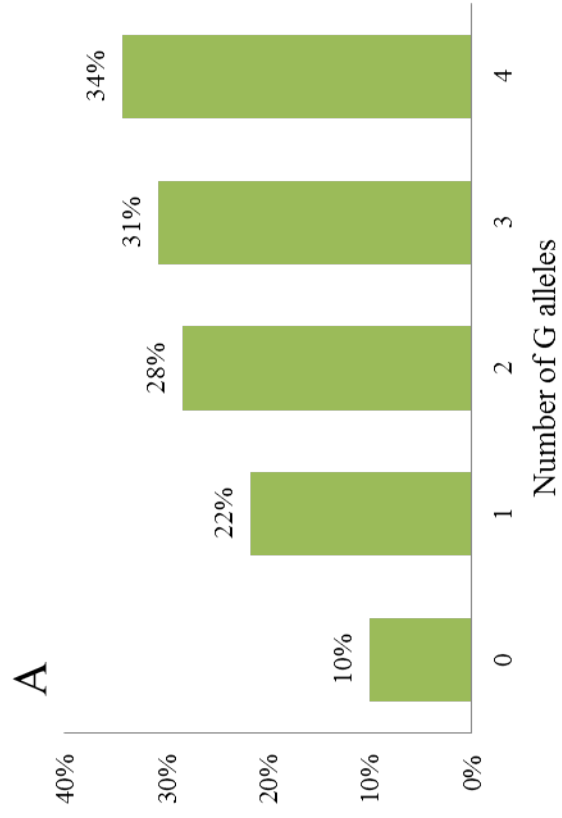
		Genotype						
	<i>LHCGR</i> N312S	N/N	N/S	S/S	Slope <sup>#</sup>	95% CI <sup>#</sup>	p	p <sup>#</sup>
Menotropin	pmol cAMP / mg protein (n=102)	26.0±31.3	21.8±30.3	34.4±73.2	-0.178	[-0.55, 0.19]	0.482	0.343
	pg IP <sub>3</sub> / mg protein (n=49)	1.33±0.52	1.26±0.43	1.37±0.53	0.051	[-0.09, 0.20]	0.746	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 <sub>3</sub> / 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
		<i>FSHR</i> N680S						
		N/N	N/S	S/S	Slope <sup>#</sup>	95% CI <sup>#</sup>	p	p <sup>#</sup>
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 <sub>3</sub> / 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 <sub>3</sub> / 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

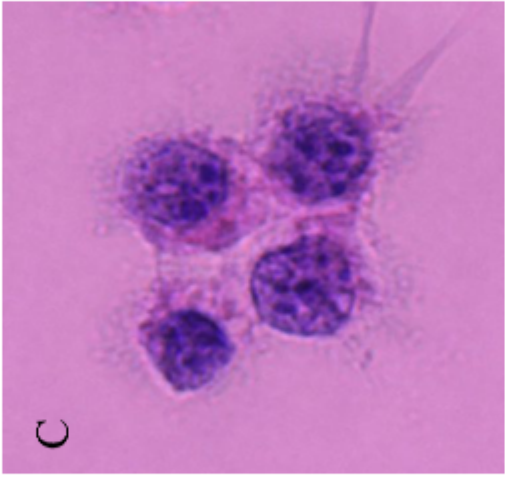
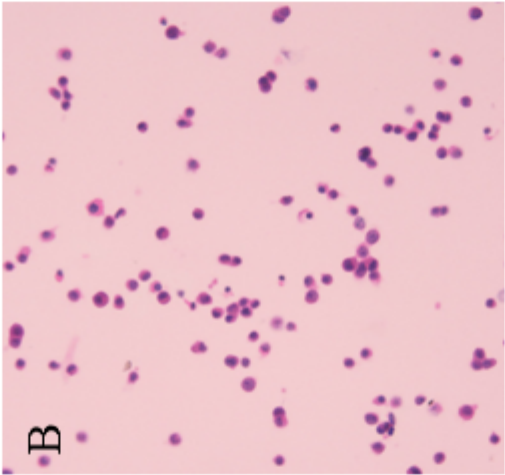
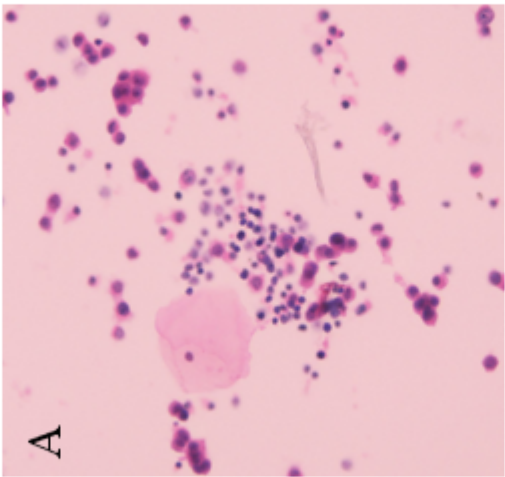
20 <sup>#</sup>= adjusted for age

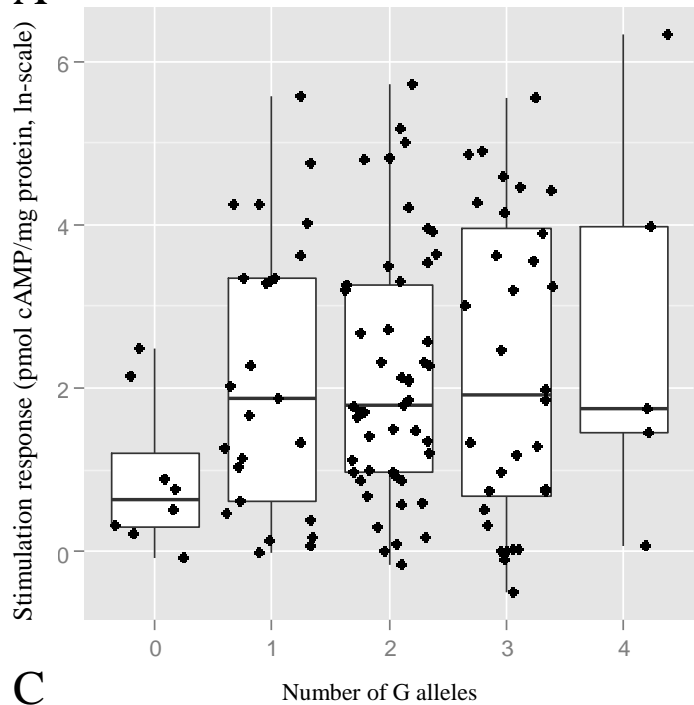
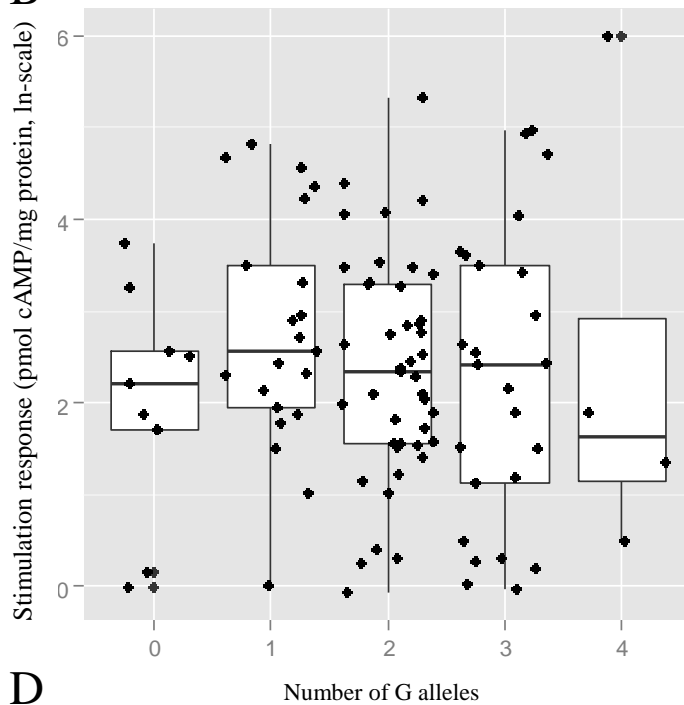
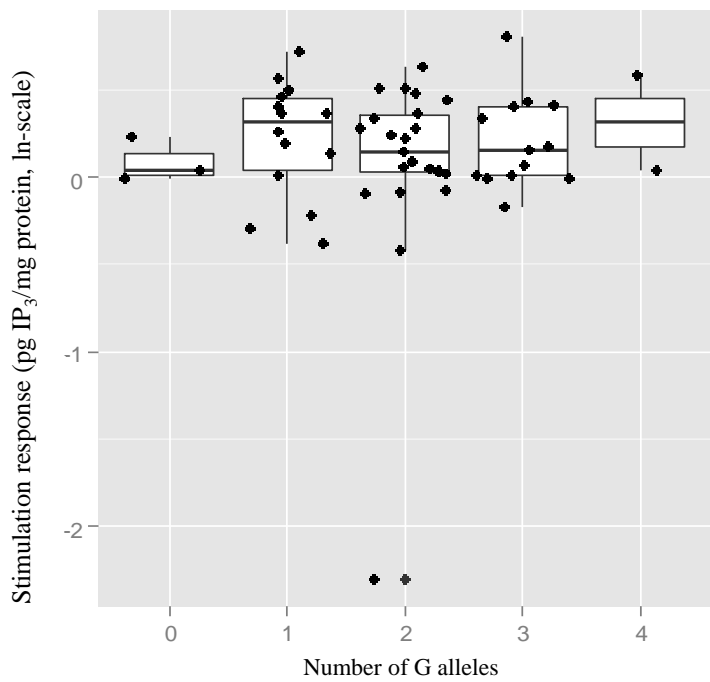










**A****B****C****D**