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Genome-wide analysis shows no genomic predictors of ovarian response to stimulation by exogenous FSH for IVF


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Abstract The current proof of principle study explores the possibility that a genetic single-nucleotide polymorphism (SNP) profile is associated with ovarian response to standardized stimulation for IVF using exogenous FSH. Such a pharmacogenomic approach could aid in rendering ovarian stimulation for IVF more tailored to the patient, potentially improving the delicate balance between efficacy, side effects and chances for complications. Genome-wide association (GWA) analysis using Illumina Human 610-Quad Bead-Chips was used in a homogeneous group of 102 healthy, Caucasian, regularly cycling, non-smoking women aged 38 years or less with a body mass index $<30 \text{ kg/m}^2$ with a regular indication for IVF in a tertiary referral University Hospital. Genetic profiles were associated with the number of oocytes obtained. Ovarian response varied widely, ranging from cancellation (less than three follicles) to more than 20 oocytes. After correction for multiple testing, no SNPs were observed to be significantly correlated to ovarian response, embryo quality or pregnancy. Restricting the information to SNPs involved in granulosa cell function, cell cycle regulation or apoptosis also did not yield significant associations for ovarian response. A study in a larger cohort is warranted, aiming to further explore subtle genetic variants with greater power. 

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KEYWORDS: genes, genome-wide association study, IVF, ovarian response, ovarian stimulation

Introduction

Currently employed clinical screening parameters provide only limited information to predict the individual response to ovarian stimulation for IVF (Broekmans et al., 2009; Fauser et al., 2008). Chronological age of the woman represents a reasonable ovarian response predictor (Verhagen et al., 2008), and other patient characteristics associated with ovarian response, such as body weight and smoking, have also been identified (Bellver et al., 2010; Popovic-Todorovic et al., 2003). Recently, a prospective multicentre study demonstrated the limited effectiveness of a dosing algorithm involving age, body mass index (BMI), antral follicle count (AFC) and FSH concentrations as compared with a standard FSH dose (Olivennes et al., 2009). On the contrary, polymorphisms in the *STK11* gene (rs8111699) and FSH receptor (rs6166) and FSHR splice variants have been shown to be related to the individual ovarian response to gonadotrophin stimulation (Gerasimova et al., 2010; Legro, 2008; Valkenburg et al., 2009).

Although a shift towards the individualization of stimulation protocols has been observed in recent years, applying current clinical and endocrine parameters has not yet resulted in the successful implementation of a more patient-tailored approach. Extreme responses, both in terms of hyporesponse (often resulting in cycle cancellation without the possibility to become pregnant) or hyperresponse (resulting in decreased pregnancy chances; van der Gaast et al., 2006), along with increased patient discomfort and higher chances for ovarian hyperstimulation syndrome may occur following standard stimulation regimens (Fauser et al., 2008).

Even following mild ovarian stimulation, a wide range of ovarian responses can be observed ranging from no response (i.e. cancellation; Verberg et al., 2007) to over 20 oocytes being retrieved (Verberg et al., 2009). Moreover, a recent meta-analysis involving all published randomized controlled trials comparing different gonadotrophin doses demonstrated no clinical benefit of higher doses even in women of more advanced reproductive age (Sterrenburg et al., 2009). The development of more individualized ovarian stimulation protocols seems the way forward towards improving the balance between success and risks of IVF treatment.

Pharmacogenomics, leading to more patient-tailored drug dosing on the basis of an individual's genetic make-up, may provide a powerful novel tool to tailor treatments (Bertrand et al., 2009; Byun et al., 2008). For instance, a genome-wide pharmacogenomic approach has recently been shown useful to predict response of multiple sclerosis patients to interferon β treatment (Byun et al., 2008). Furthermore, it has already been suggested that pharmacogenomics may aid in the tailoring of IVF treatments to the individual patient (Greb et al., 2005a; Legro, 2008; Simoni et al., 2008). The FSH receptor in particular has been studied extensively in this respect, both in terms of inactivating and activating mutations, polymorphisms and splice variants (Jun et al., 2006; Loutradis et al., 2006; Lussiana et al., 2008; Simoni et al., 1999).

This proof-of-principle study in a relatively small, but homogenous IVF population applied a genome-wide

pharmacogenomic approach aiming to identify a correlation between single-nucleotide polymorphisms (SNP) and ovarian response to standard stimulation with recombinant FSH.

Materials and methods

Subjects and sample collection

Between October 2006 and July 2008, 102 patients were prospectively recruited from the study centre's standardized preconceptional screening programme prior to starting IVF treatment. Approval was obtained from the local ethics review board, and written informed consent was provided by all participants. In order to limit as much as possible the influence of non-genetic, clinical characteristics on ovarian response, inclusion criteria were: (i) healthy Caucasian; (ii) non-smoking; (iii) regularly cycling; (iv) aged 38 years or less; and (v) BMI <30 kg/m². Blood serum and lymphocytes for DNA analysis were obtained from all individuals and analysed after all samples were obtained. For most individuals, FSH and anti-Müllerian hormone (AMH) measurements were available from their fertility work-up.

All women were treated with a standard ovarian stimulation protocol applying 150 IU/day recombinant FSH (Puregon; Organon, Oss, The Netherlands; or Gonal-F; Merck Serono, The Hague, The Netherlands), starting on cycle day 2 using gonadotrophin-releasing hormone (GnRH) antagonist co-treatment (25 µg/day, Orgalutran; Schering Plough; or Cetrotide; Merck Serono), starting on cycle day 6. Dose adjustments were allowed after cycle day 6. In the case of three or more follicles \geq 17 mm diameter being observed by ultrasound, 10,000 IU of human chorionic gonadotrophin (Pregnyl; Schering Plough) was administered and 36 h later transvaginal oocyte retrieval was performed. After fertilization *in vitro* and 4 days after aspiration of the follicles, one or two good-quality embryos were transferred. Remaining embryos were cryopreserved for transfer in a subsequent natural cycle. Embryo quality was determined by taking compaction, cavitation and expansion into consideration (Verberg et al., 2008). The luteal phase was supported with progesterone vaginal pessaries for 12 days starting on the evening of oocyte pick up (Utrogestan; Good-life, The Hague, The Netherlands). A pregnancy test was performed 18 days after oocyte retrieval. If this test was positive, a subsequent ultrasound examination was scheduled at a gestational age of 9 weeks.

Data analysis

Genome-wide technology (Illumina, San Diego, CA, USA) was used, as previously described (Knauff et al., 2009; van der Zwaag et al., 2009). All cases were genotyped using Infinium II Human610-Quad BeadChips v.1_B October/November 2008 (Illumina). All experiments were carried out at the Division of Medical Genetics in the UMC Groningen, according to the manufacturer's protocol. In short, 750 ng of DNA per sample was whole-genome amplified, fragmented, precipitated and resuspended in the appropriate hybridization buffer. Denatured samples were then hybridized on BeadChips (Illumina) at 48°C for a minimum of 16 h. After hybridization, the BeadChips were processed

for single base extension reaction and stained. Chips were then imaged using the Bead Array Reader (Illumina).

After genotyping all samples, the following quality control procedures were employed. Beadstudio version 3.0 was used to call genotypes for each sample using normalized bead intensity data. Samples with overall call rates below 95% were removed. Furthermore, SNP with call rates less than 95%, minor allele frequencies (MAF) below 5% and deviations from Hardy–Weinberg equilibrium (HWE) (Exact HWE P -value <0.001) were removed from subsequent analyses.

To test for a quantitative association, the open-access software PLINK (version 6; <http://pngu.mgh.harvard.edu/purcell/plink/>; Purcell et al., 2007) was used. Significance of association was determined by using likelihood ratio tests and Wald tests. As over 600,000 tests were performed, correction for multiple testing was done by determining what nominal single SNP P -value would correspond to $P = 0.05$. A commonly used threshold for deeming a SNP association genome-wide significant is $P < 5 \times 10^{-7}$ (Wellcome Trust Case Control Consortium, 2007), corresponding to a genome-wide significance of $P = 0.05$ on the Human610-Quad BeadChip. Since this is a preliminary study which is only able to investigate large effects of individual SNPs, the data were analysed using both naïve and intelligent approaches.

In the naïve approach, likelihood ratio tests were performed with PLINK software, including adaptive permutation analysis and analysis with adjustment for female age, FSH and AMH. Because of a relatively small sample size and consequent ability to detect relatively large contributions of individual SNPs only, additional analysis was also performed after a dichotomization of the primary outcome at the median of eight oocytes and by comparing the lower

tertile (six or fewer oocytes obtained) with the upper tertile (11 or more oocytes obtained). Other ovarian stimulation outcomes, such as total dose of FSH administered and FSH/oocyte were also analysed. Furthermore, analyses investigating the qualitative aspect of COH, including embryo quality and pregnancy were analysed separately.

In the intelligent approach, the influence of gene–gene interactions of known polymorphisms on the associations found were assessed for the FSHR polymorphism Asn680Ser (rs6166) and AMH polymorphism (rs10407022). Since signal intensities in the process of correction for multiple testing might be lost in this small sample size, only the influence of certain pathways described on the ovarian kaleidoscope database was assessed (Ben-Shlomo et al., 2002). To study pathways involved in granulosa cell function, cell cycle regulation and apoptosis, only SNP relevant to these pathways within 25 kB of genes of interest were included (for a summary of genes, see the Supplementary Table which is available online only).

Results

In total, 102 IVF patients were genotyped using Illumina Human610-Quad BeadChips. This population was selected from a total of about 800 women with an indication for IVF. Patient characteristics from the study population are summarized in Table 1. Patients with idiopathic subfertility and mild male factor subfertility received intrauterine insemination before the start of their IVF treatment.

In this homogeneous population, ovarian response ranged from cancelled cycles due to monofollicular growth or no oocytes being obtained on one end of the spectrum to 24

Table 1 Patient characteristics.

Characteristic	Population	<i>P</i> -value for relation to no. of oocytes
Age at screening (years)	33.8 ± 2.7	NS ^a
Primary subfertility (%)	57	NS ^b
Cause of subfertility		
Idiopathic	57	NS ^b
Mild male factor	23	
Tubal pathology	13	
Other	7	
BMI (kg/m ²)	22.4 ± 2.7	NS ^a
FSH	7.99 ± 2.9	0.02 ^a
AMH	2.68 ± 1.9	<0.001 ^a
Duration of stimulation (days)	8.8 ± 1.5	NS ^a
Dose adjustment (n)		NA
Total dose (IU)	1326 ± 219	NS ^a
Oocytes	8.9 ± 5.4	
Competent embryos ^c	3.9 ± 3.2	
Pregnancy rate	29	

Values are mean ± SD or %, unless otherwise stated.

AMH = anti-Müllerian hormone; BMI = body mass index; NA = not applicable; NS = not statistically significant.

^aSpearman correlation. ^bChi-squared test.

^cExcluding monpronucleate and tripronucleate embryos as well as arrested embryos.

oocytes being retrieved or cancelled cycles due to hyperresponse (>30 follicles) on the other end, with a median of eight mature oocytes (Figure 1). As expected, the variables used for selecting this homogenous population were not significantly associated with the number of oocytes obtained (Table 1). Since oocyte yield is known to be influenced by female age, the analysis corrected for this variable in subsequent analyses. Since FSH and AMH were related to oocyte yield, the analysis also corrected for AMH and FSH.

Sample quality control resulted in the exclusion of 10 cases from analysis because their call rates fell below 95%. These 10 cases did not significantly differ from the total group. After comparing the remaining 92 cases, no related

individuals were identified. A total of 454,102 SNP passed quality control (Exact HWE P -value >0.001, MAF >0.05, call rates >95%).

After correction for multiple testing, no SNPs were observed to be significantly correlated to ovarian response in the naïve approach, as shown in a quantile–quantile plot (Figure 2). Permutation analysis, dichotomization of the outcome and adjustment for age, FSH and AMH did not change the results (data not shown). The few SNP showing a possible trend towards significance are shown in Table 2. The two SNP for RAB 32 (rs1885678 and rs9403799) are almost in perfect linkage disequilibrium (LD) ($r^2 = 0.965$, $D' = 1$). Haplotype analysis did not show significant associations.

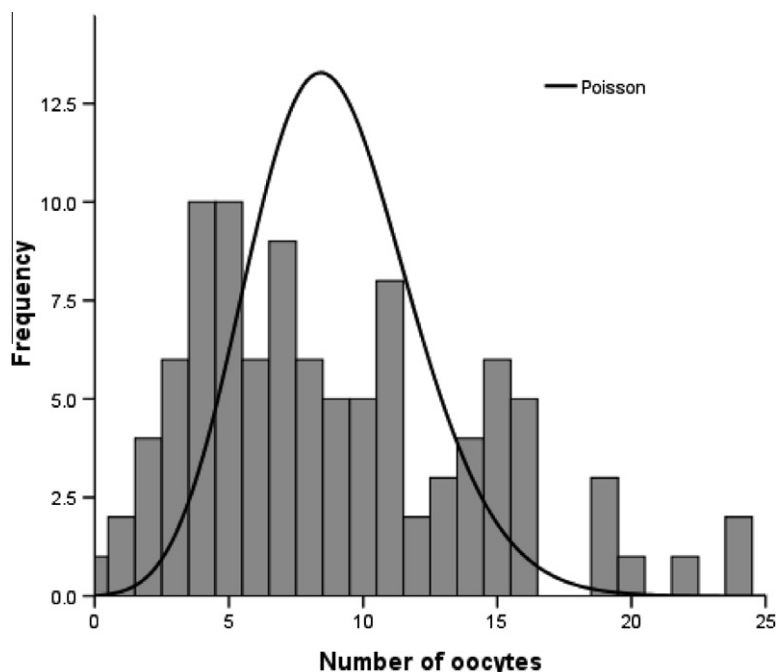


Figure 1 Poisson distribution of the number of oocytes retrieved with normal curve.

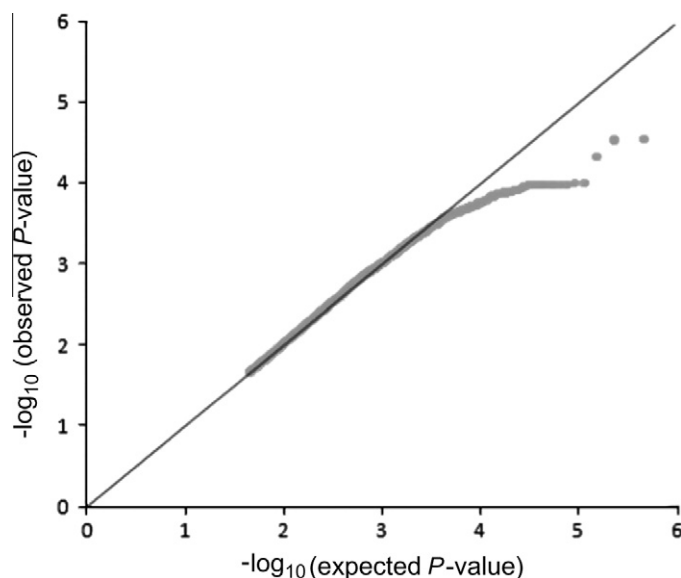


Figure 2 Quantile–quantile plot of observed versus expected P -values of the 10,000 most significant SNPs, with reference line.

Table 2 Possible single-nucleotide polymorphisms (SNP) involved in ovarian response.

SNP	P-value	OR	Nearest gene
rs1885678	1.0×10^{-6}	17.93	<i>RAB32</i> : GTPase: mitochondrial dynamics ^a
rs9403799	2.0×10^{-6}	17.76	<i>RAB32</i> ^a
rs4499783	2.6×10^{-6}	3.724	<i>MAST4</i> : protein phosphorylation
rs8025763	2.0×10^{-6}	25.62	<i>ARRDC4</i> : unknown
rs2271463	7.0×10^{-6}	24.3	<i>CUBN</i> : intrinsic factor-cobalamin receptor

P-values $>5 \times 10^{-7}$ are not statistically significant.

^aBoth SNPs for *RAB32* (rs1885678 and rs9403799) are almost in perfect linkage disequilibrium (LD) ($r^2 = 0.965$, $D' = 1$).

Genetic polymorphisms that have previously been shown to be associated with response were also checked in the current dataset (Table 3). If a particular SNP was not available on the Human610-Quad BeadChip, a proxy SNP approach was adopted with a SNP in perfect LD with the missing SNP.

Analyses with different outcomes, such as total dose of FSH administered, average FSH per oocyte, embryo quality and pregnancy, yielded no SNP that were significantly correlated (data not shown). Again adjustment for age, FSH and AMH did not change these results.

In the intelligent approach, the influence of gene–gene interactions and selection of SNP involved in pathways regulating granulosa cell function, cell cycle regulation or apoptosis did not yield statistically significant associations with the number of oocytes obtained.

Discussion

This preliminary study in a relatively small, homogeneous, Caucasian population was unable to identify SNPs significantly associated with parameters reflecting oocyte yield, ovarian sensitivity to stimulation or oocyte quality after standardized ovarian stimulation for IVF. Parameters of

oocyte yield, ovarian sensitivity to stimulation and oocyte quality were tested through different outcomes, such as number of oocytes obtained, total FSH dose needed for this response, average FSH needed per oocyte obtained, embryo quality and ongoing pregnancy. The study confirmed that ovarian response to stimulation varies widely, even in this relatively homogenous and young patient group. The finding that no single SNP is clearly associated with oocyte yield and quality parameters in the current preliminary study suggests that individual genetic variation may not represent a major determinant for the variability in ovarian response to stimulation. Because of the small sample size, copy number variation analysis could not be performed, since copy number variations were too infrequent for a reliable analysis.

As far as is known, this is the first genome-wide approach to study pharmacogenomic influences in ovarian stimulation. Current results might suggest that a combination of smaller gene effects (regulating uptake, metabolism and response to the various hormones) could be involved. The limited sample size only permits the identification of genes with strong effects. Moreover, an individual's ovarian reserve status may also be affected by impaired gametogenesis during early fetal development leading to fewer primordial follicles, inappropriate follicular atresia or dysfunctional follicular recruitment and maturation (ESHRE Capri Workshop Group, 2008). Also, gene–gene interactions may further complicate the search for SNPs possibly associated with ovarian response to stimulation. The current study should be repeated in a (much) larger cohort of IVF patients. A future study could have a similar design and it is calculated that, when assuming a common SNP variant explains 3.4% of the total ovarian response variation, 1000 samples are required in order to have an 80% power to detect such a common variant with a P -value $<5 \times 10^{-7}$ (assuming the SNP has a MAF of 26%, which is the average for the platform used here).

Another possibility could be to be even more restrictive in the applied inclusion criteria of patients studied. However, it took almost 2 years to recruit a mere 100 patients for this study, representing only around 13% of all patients starting IVF treatment during the recruitment phase (data not shown). Hence, even more restrictive inclusion criteria may render the study population more homogeneous, but also even less representative for women currently undergoing IVF.

Previous studies have focused on one or a few polymorphisms in genes known to be involved in ovarian stimulation

Table 3 Candidate single-nucleotide polymorphisms (SNP) involved in ovarian response, as published previously.

SNP	P-value	Beta	Gene
rs6166	0.8	−0.3	<i>FSHR</i>
rs2234693	NA		<i>ESR1</i>
rs9479130	0.09	−1.3	In LD with rs2234693
rs928554	0.8	−0.3	<i>ESR2</i>
rs10407022	0.007	−3.3	<i>AMH</i>
rs2002555	NA		<i>AMHR</i>
rs2683525	0.05	1.9	In LD with rs2002555
rs11170555	0.08	1.7	In LD with rs2002555
rs7300593	0.02	2.2	In LD with rs2002555

P-values $>5 \times 10^{-7}$ are not statistically significant.

Data were taken from Aittomaki et al. (1995), Altmae et al. (2009), Behre et al. (2005), de Castro et al. (2003, 2004), Falconer et al. (2005), Greb et al., 2005b, Jun et al. (2006), Kevenaar et al. (2007), Laven et al. (2003), Loutradis et al. (2006), Perez Mayorga et al. (2000), and Sudo et al. (2002).

LD = linkage disequilibrium; NA = not available on the Illumina Infinium II Human610-Quad BeadChip, therefore proxy SNP analysis was performed.

for ovulation induction or IVF (Table 3), including *FSHR*, *ESR1* and *ESR2*, *CYP19* aromatase, *AMH* and *AMHR* (Aittomaki et al., 1995; Altmae et al., 2009; Behre et al., 2005; de Castro et al., 2003, 2004; Falconer et al., 2005; Greb et al., 2005b; Jun et al., 2006; Kevenaar et al., 2007; Laven et al., 2003; Loutradis et al., 2006; Perez Mayorga et al., 2000; Sudo et al., 2002). However, the current study does not support a major role for these biological candidate genes even if a less stringent *P*-value cut-off was applied. It should be realized that, due to correction for multiple testing in a genome-wide approach, a distinctly higher significance level compared with single-gene studies is required (Elbers et al., 2009).

The role of genetic factors in ovarian response may be questioned. No data concerning ovarian response are available in twin studies and heritability measurements are unknown. Yet, genetic factors in ovarian response have been extensively studied. The FSH receptor gene is by far the most studied gene in relation to ovarian stimulation (Greb et al., 2005b; Perez Mayorga et al., 2000). Particular interest has been given to two polymorphisms, at codons 307 and 680. Although various studies show significant differences in hormonal markers of ovarian response, a direct relation between the three genotypes and response in terms of oocyte yield has not been established (Loutradis et al., 2008). The distribution of FSH receptor polymorphisms has been suggested to be different in women with WHO type-II anovulatory infertility and a general infertility population, when compared with normal fertile controls (Falconer et al., 2005; Laven et al., 2003). Recent studies have shown that ovulatory response to treatment in PCOS women is associated with polymorphisms in both the *FSH receptor* and *STK11* genes (Legro, 2008; Overbeek et al., 2009). Furthermore, the oestrogen receptor genes *ESR1* and *ESR2* may interact with the *FSH receptor* gene and when combined in a model, these three genes may partly predict poor response in IVF (de Castro et al., 2004). Next to FSH receptor variants, AMH and AMH type-II receptor polymorphisms, which are thought to be involved in regulating FSH sensitivity (Dumesic et al., 2008), were shown to influence follicular-phase oestradiol concentrations in normo-ovulatory women (Kevenaar et al., 2007). These findings, however, need to be validated in other studies.

In conclusion, although evidence suggests that ovarian response is mediated by various polymorphisms, it is unlikely that gene effects represent a significant factor underlying the observed individual variability in ovarian response to stimulation for IVF. Since a genome-wide approach requires correction for multiple testing, it may obscure significant findings of genes of smaller effects that would be more readily discriminated through, for example, pathway analyses. To assess more subtle genetic effects, a larger sample size study is warranted to further investigate which genes and pathways could be involved.

Acknowledgements

This study was sponsored by unconditional research grants obtained from Merck Serono and Schering Plough. We would like to thank Carolien Boomsma and all other collaborators involved in the preconceptional screening of the

IVF population at the University Medical Center Utrecht and the Genomics facility of the University Medical Center Groningen for their work in preparing and analysing the Illumina chips.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.rbmo.2010.12.006](https://doi.org/10.1016/j.rbmo.2010.12.006).

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Declaration: JvD, LF, CW and MJE have nothing to disclose. FJB is a member of the external advisory board for Ferring Pharmaceuticals, Hoofddorp, The Netherlands. He receives no monetary compensation. NSM has received fees and grant support from the following companies (in alphabetic order): Ferring, Merck Serono, Organon, Schering Plough and Serono. BCF has received fees and grant support from the following companies (in alphabetic order): Andromed, Ardana, Ferring, Genovum, Glycotope, Merck Serono, Organon, Pantharei Bioscience, Philips, PregLem, Schering, Schering Plough, Serono and Wyeth.

Received 5 August 2010; refereed 11 December 2010; accepted 14 December 2010.