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ARTICLE

Genetic variants associated with female reproductive ageing – potential markers for assessing ovarian function and ovarian stimulation outcome

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Abstract This study searched for genetic markers of ovarian function, ovarian stimulation and IVF treatment outcome among genetic variants related to female reproductive ageing. It included 471 treatment cycles from 306 women undergoing IVF treatment. Genotypes for 36 single nucleotide polymorphisms (SNPs) were evaluated for their association with early follicular phase parameters together with ovarian stimulation and IVF outcome parameters. Results show that genetic variation related to menopause timing also affects ovarian function, as several selected genetic markers were associated with studied traits. For example, rs2153157 (*SYCP2L*) was associated with amount of recombinant FSH (rFSH) necessary for obtaining one oocyte ($P = 0.049$) and the chances of biochemical and clinical pregnancy ($P = 0.024$ and $P = 0.011$, respectively), while rs4886238 (*TDRD3*) showed association with both the number of punctured ovarian follicles and oocytes obtained ($P = 0.008$ and $P = 0.037$, respectively). Furthermore, *FSHB* polymorphisms influence early follicular phase FSH concentrations and IVF treatment outcome, whereas SNPs in *FSHR* affect early antral follicle count and follicle numbers obtained during ovarian stimulation. This study suggests that genetic markers of female reproductive ageing are potential new biomarker candidates that could be considered in clinical ovarian reserve and function assessment in assisted conception.

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Introduction

Female reproductive ageing is a term describing the age-related decline in reproductive success that ultimately leads to cessation of reproductive function – the menopause. Reproductive ageing is closely related to ovarian function, number and quality of oocytes, which also decrease with age. The heritability for reproductive ageing is estimated to be as high as 90% (de Bruin et al., 2001), therefore quite a lot of research has recently focused on genetic predictors of reproductive ageing and ovarian function. These markers are believed to be of use in clinical ovarian reserve and function assessment in natural and assisted conception, where reliable genetic markers are extensively sought after in order to increase treatment effectiveness and reduce harmful side effects (reviewed by Altmae et al., 2011). The majority of research has concentrated on obvious candidates, such as FSH and its receptor (FSHR) (reviewed in La Marca et al., 2013b), since FSH plays a central role in both natural follicle maturation and controlled ovarian stimulation (COS). Several papers have also considered genetic variation in certain pathways, such as oestrogen synthesis/metabolism (Altmae et al., 2007, 2009) and the anti-Müllerian hormone signalling (Hanevik et al., 2010) pathway, but overall the number of considered and tested variants is small.

Advancements in genotyping technologies and the formation of large international consortiums have enabled scientists to use a genome-wide hypothesis-free approach in the search for genetic markers of reproductive ageing and the ovarian reserve (He et al., 2009; Schuh-Huerta et al., 2012; Stolk et al., 2012). Unfortunately, this method has been unsuccessful for identifying genetic predictors for IVF success or IVF-COS (van Disseldorp et al., 2011). To the best of our knowledge, the study by van Disseldorp et al. (2011) is so far the only IVF-COS genome-wide association study (GWAS) conducted. Their study involved 102 women undergoing IVF treatment but unfortunately no genetic variant was significantly correlated with parameters of IVF-COS and the authors concluded that it is possible that the GWAS methodology may dismiss variants with smaller effects and due to GWAS stringent analysis criteria, the study would need to be repeated in a much larger study cohort (van Disseldorp et al., 2011). However, since the very large and rather homogenous study cohorts suitable for GWAS are difficult to achieve in a single-centre IVF setting, an (international) multi-centre effort is needed to achieve the necessary sample size for conducting a successful GWAS. Until then, other approaches have to be used for detecting potential biomarkers for IVF-COS traits. The identified genetic variants associated with reproductive ageing are good candidates for markers of ovarian function and IVF-COS, because reproductive ageing is closely related to ovarian function and pre-selection would help to narrow down the number of tested markers and thus to bypass the analysis bottleneck associated with GWAS.

The most popular study design for evaluating genotype effects on ovarian stimulation outcome includes the use of data from women undergoing IVF-COS for infertility treatment. The use of assisted reproduction treatment data presents a number of statistical challenges, as the process involves several interdependent steps and often women undergo more than one treatment cycle. For data analysis usually all but one cycle are discarded, resulting in decreased statistical power, and thus several sophisticated statistical models have been proposed to make full use of the collected multicycle data (Missmer et al., 2011).

The aim of this retrospective cohort study was to analyse the effect of several single nucleotide polymorphisms (SNPs) in genes associated with female reproductive ageing (Stolk et al., 2012) or ovarian biology (*FSH and FSHR*) on ovarian stimulation parameters using a complex statistical approach in order to increase statistical power to detect possible associations. The same genetic markers were also tested for association with markers of ovarian function and reserve – ovarian volume together with early follicular phase follicle count and FSH concentrations.

Materials and methods

Participants

The current retrospective cohort study included female patients undergoing IVF or intracytoplasmic sperm injection (ICSI) at the Nova Vita Clinic (Estonia) from 2004 to 2007. Patients were considered ineligible to participate in the study if they: (i) were diagnosed with polycystic ovary syndrome; (ii) had only one ovary; and/or (iii) were undergoing treatment cycles in which donor oocytes or frozen embryo transfer were used. All patients had been infertile for at least 1 year before entering the study. After initial filtering, a total of 306 women with 471 treatment cycles remained in the study group, but the number of participants/observations in different analyses may vary slightly due to missing phenotype/genotype data. Of the 306 patients included in this study, 123 (40%) underwent two or more treatment cycles during the study period. Main characteristics of the patients are presented in **Table 1**. A peripheral venous blood sample was taken from all women for DNA extraction and genotyping.

The study was approved by the Research Ethics Committee of the University of Tartu on 23 August 2004 (protocol number 129/17), and informed consent was obtained from all participants.

Early follicular phase parameters

Ultrasound data for assessing ovarian parameters (ovarian volume and antral follicle count) were available for 135 participants, while a blood sample for measuring early follicular phase FSH concentrations was obtained from 128 women. Values for analysed early follicular phase parameters, and for characteristics used as cofactors in statistical analysis, are presented in **Table 1**. Transvaginal ultrasound to assess the ovarian volume and early follicle count were performed during

Table 1 Main characteristics of study participants and analysed parameters.

Characteristic	*Mean ± SD (median; range)*
Age	
First treatment cycle	33.8 ± 4.4 (33.9; 23.8–46.2)
Across all treatment cycles	34.2 ± 4.4 (34.1; 23.8–47.7)
BMI	22.6 ± 3.2 (21.9; 16.1–35.7)
Cause of infertility	
Male, *n* (%)	91 (29.7)
Tubal, *n* (%)	130 (42.5)
Endometriosis, *n* (%)	31 (10.1)
Unexplained, *n* (%)	21 (6.9)
Other, *n* (%)	32 (10.5)
Unknown, *n* (%)	1 (0.3)
Previous IVF treatment cycles	0.9 ± 1.2 (1; 0–10)
Ovarian surgery[a]	
Yes, *n* (%)	19 (14.1)
No, *n* (%)	115 (85.2)
Unknown, *n* (%)	1 (0.7)
Early follicular phase parameters	
Ovarian volume (mL)	4.7 ± 1.9 (4.4; 1.6–10.9)
Follicle count	4.8 ± 1.9 (4.5; 2.0–17.5)
FSH (IU/L)	8.1 ± 3.0 (7.7; 2.7–21.8)
Estradiol (pmol/L)	132.9 ± 73.6 (124.0; 20.0–510.0)
Cycle day sample taken at	3.3 ± 0.9 (3; 1–5)
Ovarian stimulation	
Agonist, *n* (%)	19 (4.0)
Antagonist, *n* (%)	384 (81.5)
Unknown, *n* (%)	68 (14.4)
IVF procedure	
IVF, *n* (%)	197 (41.8)
ICSI, *n* (%)	274 (58.2)
Endometrial thickness (mm)	10.7 ± 2.1 (10; 6.5–17.7)
Ovarian stimulation outcome	
rFSH total (IU)	1980.7 ± 585.3 (1950.0; 900.0–4725.0)
Ovarian follicles punctured	13.5 ± 6.7 (12.0; 1.0–40.0)
Oocytes retrieved	11.8 ± 7.0 (10.0; 1.0–40.0)
rFSH per oocyte (IU)	271.6 ± 298.9 (168.8; 35.1–2625.0)
Fertilised oocytes	7.0 ± 4.6 (6.0; 0–30.0)
Good quality embryos	2.9 ± 2.9 (2.0; 0–21.0)
Number of embryos transferred	2.0 ± 0.5 (2; 0–3)
IVF outcome	
Biochemical pregnancy	
Yes, *n* (%)	163 (34.6)
No, *n* (%)	277 (58.8)
Unknown, *n* (%)	31 (6.6)
Clinical pregnancy	119 (25.3)

BMI = body mass index; ICSI = intracytoplasmic sperm injection; rFSH = recombinant FSH; SD = standard deviation.
[a]Subset of women with data on early follicular phase parameters.

a spontaneous menstrual cycle. Early antral follicles were counted and ovarian volume was calculated as described previously (Altmae et al., 2009). Mean ovarian volume and mean follicle count were calculated as the sum of values for each ovary divided by 2.

Serum samples for measuring FSH concentrations were collected during the first 5 days of a spontaneous menstrual cycle and measured using chemiluminescence immunoassay (Immulite 2000; Siemens Healthcare Diagnostics, Deerfield, IL, USA).

IVF treatment protocol

Either the gonadotrophin-releasing hormone (GnRH) antagonist or agonist protocol with recombinant FSH (rFSH) was used

for ovarian stimulation, as described previously (Altmae et al., 2007). In the majority of patients who underwent two or more treatment cycles, adjustments were made in rFSH dosing (both up [52%] and down [27%]). IVF and ICSI were performed as described by Salumets et al. (2003). Vaginal progesterone (Lugesteron, Leiras, Turku, Finland) was used for luteal support. The ovarian stimulation parameters analysed included: (i) total amount of rFSH used (IU); (ii) amount of FSH administered per retrieved oocyte (IU/oocyte); (iii) number of ovarian follicles punctured (OPU follicles); and (iv) number of oocytes retrieved at puncture. The IVF outcome parameters analysed included: (i) biochemical pregnancy (positive serum HCG test conducted 14 days after embryo transfer); and (ii) clinical pregnancy (presence of gestational sac(s) in the uterus on transvaginal ultrasound at 6–7 weeks of gestation). Values for analysed parameters, and for all other parameters used in statistical analysis, are presented in **Table 1**.

SNP selection and genotyping

In addition to the 20 SNPs from the menopause timing meta-analysis (Stolk et al., 2012), another 31 genetic variants in the *FSHR and FSHB* genes (including SNP tagging the *FSHB* core haplotype; Grigorova et al., 2007) were selected based on a literature search and from the NCBI database (http://www.ncbi.nlm.nih.gov).

Genotyping assays were designed using the Sequenom MassARRAY Assay Design Suite (Sequenom, San Diego, CA, USA), with an aim to multiplex the SNPs to a maximum of two assays. During the assay design, four SNPs were excluded due to problems with assay design and one because it was multiplexed to a third 1-plex assay. The remaining 46 SNPs were multiplexed to 29-plex and 17-plex assays and genotyped using the Sequenom MassARRAY iPLEX Platform according to the manufacturer's instructions. Genotypes were called automatically and confirmed manually using the MassARRAY Typer 4.0.22 software (Sequenom). Both assays were performed in 384-well plates, with random duplicates for quality control (concordance rate 99.9%). SNPs were excluded from further analysis due to following reasons: (i) genotype call rate <90% (six SNPs); (ii) minor allele frequency <1% (two SNPs); and/or (iii) deviations from the Hardy–Weinberg equilibrium ($P < 0.05$; two SNPs). A total of 36 SNPs remained in the final analysis. For markers with strong linkage disequilibrium (LD, $r^2 > 0.9$), final results are presented for the SNP with the best call rate to avoid duplication of data. The 36 analysed SNPs together with genotype distribution are listed in **Supplementary Table S1**.

Statistical analysis

To test the statistical significance of effects of preselected genotypes on repeatedly measured ovarian stimulation and IVF treatment parameters (total amount of rFSH administered, number of follicles and oocytes obtained on ovarian puncture, the amount of rFSH administered per retrieved oocyte, and biochemical and clinical pregnancies), generalized linear mixed models with logit (for binary traits) or logarithm (for count and right-skewed numerical traits) link function were fitted. All tested models were adjusted for appropriate confounding factors (**Supplementary Table S2**) and the effect of repeated measurements from the same woman was considered as a random effect. The early follicular phase parameters (mean ovarian volume, mean follicle count and early follicular phase FSH concentrations) measured only once per woman were analysed with generalized linear models with logarithm link function considering the genotype effects and appropriate confounding factors (**Supplementary Table S2**). For all tested SNPs, comparisons were made across all three genotype groups, and dominant (minor homozygote + heterozygote versus major homozygote) and recessive (major homozygote + heterozygote versus minor homozygote) models were also used. The results are presented as least square means (model based means) in inverse link scale (parameters' initial scale). As SNP selection was based on an *a priori* hypothesis, correction for multiple testing was not used and a *P*-value < 0.05 was considered statistically significant. When a clear dominant/recessive effect was present, *P*-values for corresponding models are shown (indicated by P_{dom} and P_{rec}, respectively), whereas in the case of unclear genotype effect or suspected allele dosage effect, a *P*-value for comparison across all three genotypes was used. For evaluating model prediction accuracy, the squared correlation between observed and predicted values (R^2) or area under the ROC-curve (AUC) were calculated for continuous or binary variables, respectively, based on the statistical models used. In addition, the leave-one-out cross-validation (CV) algorithm was applied and the same characteristics (R^2 and AUC) were calculated and compared with the values obtained for original models. The general idea of cross-validation is that some of the data are removed before modelling, and the model parameters are then estimated based on the remaining data and the model prediction accuracy is calculated based on the initially removed data. Performing this procedure repeatedly allows a more conservative estimation of model prediction ability. Leave-one-out CV algorithms were applied on models considering all statistically significant genetic markers plus confounding factors, and also on models considering simultaneously all studied genetic markers. The last was done to check the overall prediction ability of all analysed genotypes and to better appreciate the effect of only a small number of selected (significant) genetic markers. Similar analyses were performed on models considering genetic factors without confounding factors (clinical data). For R^2 values, zero corresponds to minimum (0%) prediction accuracy and one corresponds to maximum (100%) prediction accuracy. For AUC, 0.5 indicates minimal prediction accuracy and 1.0 maximal discrimination power. All statistical analyses were performed with SAS 9.1 software (SAS Institute Inc., USA).

Results

Early follicular phase parameters

The statistical significance of genotype effects and the least square means of tested characteristics are shown in **Supplementary Table S3**. rs611246 (*FSHB*) tagging the *FSHB* core haplotype was significantly associated with measured early follicular phase FSH values ($P_{rec} = 0.008$) as individuals with the T-allele had higher mean FSH values (**Figure 1A**). Also, individuals with the T-allele of rs2303369 (*FNDC4*) had

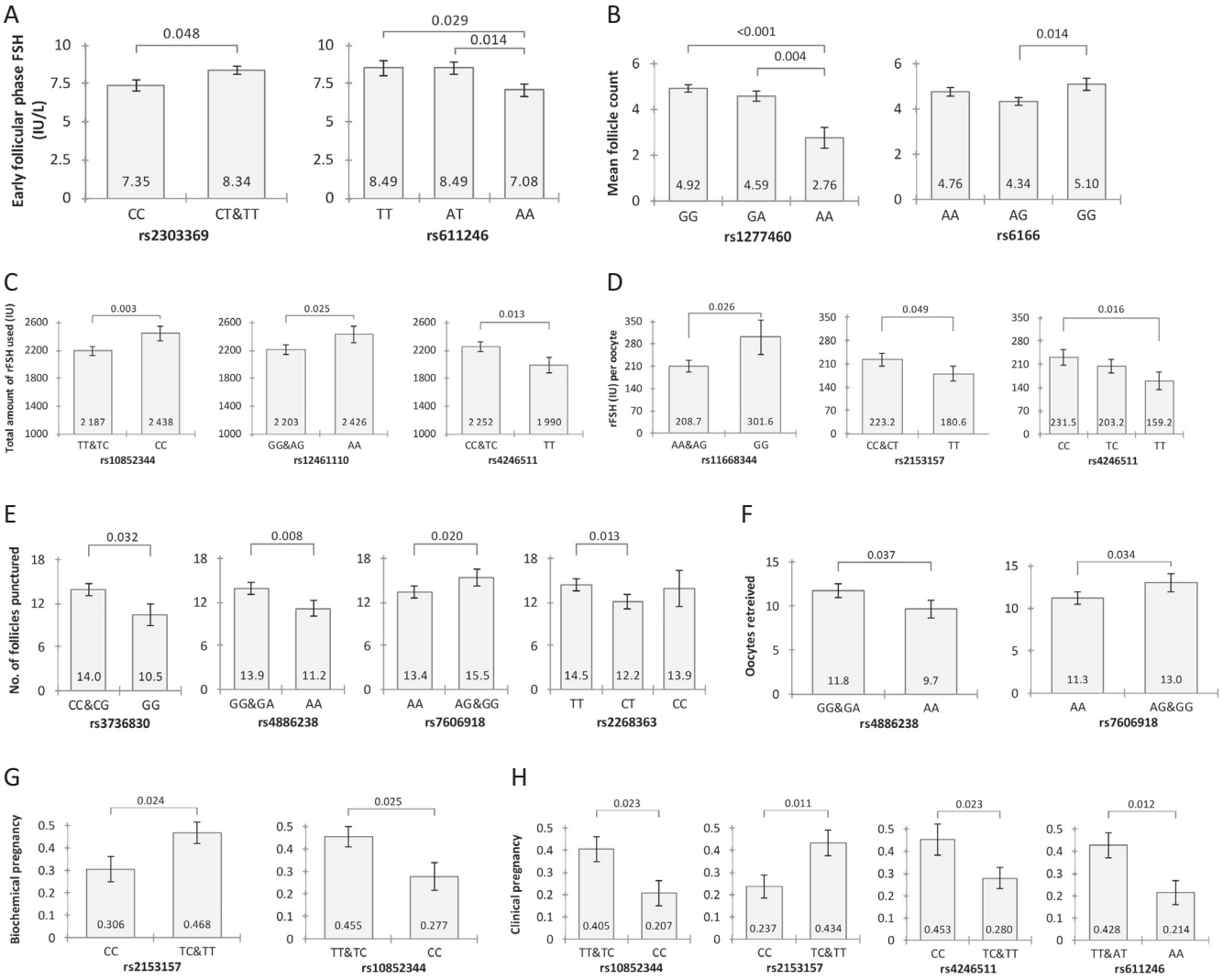

Figure 1 Effect of studied genotypes on analysed traits. Least square means (±standard error) or estimated probabilities (± standard error) of studied parameters for SNP genotypes with statistically significant effect (model details in **Supplementary Table S2** and least square means/estimated probabilities for all genotypes in **Supplementary Table S3**). (A) Early follicular phase follicle stimulating hormone (FSH) concentrations according to rs2303369 (*FNDC4*) and rs611246 (*FSHB*) genotypes; (B) mean early antral follicle count according to rs1277460 (*FSHR*) and rs6166 (*FSHR*) genotypes; (C) total amount of rFSH used according to rs10852344 (intergenic), rs12461110 (*NLRP11*) and rs4246511 (*RHBDL2*) genotypes; (D) amount of recombinant FSH (rFSH) administered per retrieved oocyte according to rs11668344 (*TMEM150B*), rs2153157 (*SYCP2L*) and rs4246511 (*RHBDL2*) genotypes; (E) number of ovarian follicles punctured according to rs3736830 (*KPNA3*), rs4886238 (*TDRD3*), rs7606918 (*METAP1D*) and rs2268363 (*FSHR*) genotypes; (F) number of obtained oocytes according to rs4886238 (*TDRD3*) and rs7606918 (*METAP1D*) genotypes; (G) probability of biochemical pregnancy according to rs2153157 (*SYCP2L*) and rs10852344 (intergenic) genotypes; and (H) probability of clinical pregnancy according to rs10852344 (intergenic), rs2153157 (*SYCP2L*), rs4246511 (*RHBDL2*) and rs611246 (*FSHB*) genotypes.

significantly higher FSH values ($P_{dom} = 0.048$). Finally, two SNPs in *FSHR* (rs1277460 and rs6166) influenced mean early antral follicle count ($P_{rec} = 0.001$ and $P = 0.038$, respectively; **Figure 1B**). None of the tested SNPs showed any significant association with mean ovarian volume.

Ovarian stimulation parameters

Three SNPs influencing the age at natural menopause (rs10852344 [intergenic], rs12461110 [*NLRP11*] and rs4246511 [*RHBDL2*]) also appear to modulate the amount of rFSH necessary for ovarian stimulation. Individuals with the minor homozygotic genotype at rs10852344 and rs12461110 received significantly larger amounts of rFSH during stimulation ($P_{rec} = 0.003$ and $P_{rec} = 0.025$, respectively), whereas minor homozygotic genotype at rs4246511 seems to be associated with smaller doses of rFSH ($P_{rec} = 0.013$; **Figure 1C**). The same SNP also has an impact on the amount of rFSH necessary for retrieving one oocyte during stimulation ($P = 0.043$), as do rs11668344 (*TMEM150B*) and rs2153157 (*SYCP2L*). While individuals with the minor homozygotic genotype at rs11668344

need more rFSH to retrieve one oocyte ($P_{rec} = 0.026$), the opposite applies for those carrying the minor homozygotic genotype at rs2153157 ($P_{rec} = 0.049$).

Both the numbers of ovarian follicles punctured and oocytes retrieved were associated with two SNPs – rs4886238 (*TDRD3*) and rs7606918 (*METAP1D*) (**Figure 1E-F**). The minor homozygotic genotype at rs4886238 and the major homozygotic genotype at rs7606918 result in smaller numbers of ovarian follicles punctured ($P_{rec} = 0.008$ and $P_{dom} = 0.020$, respectively) and retrieved oocytes ($P_{rec} = 0.037$ and $P_{dom} = 0.034$, respectively). In addition, rs3736830 (*KPNA3*) and rs2268363 (*FSHR*) were associated with the number of ovarian follicles punctured ($P_{rec} = 0.032$ and $P_{dom} = 0.018$, respectively), but not with the number of retrieved oocytes.

IVF treatment outcome

rs10852344 (intergenic) and rs2153157 (*SYCP2L*) from the menopause study were associated with both biochemical and clinical pregnancies. rs10852344 minor homozygotes were significantly less likely to achieve biochemical or clinical pregnancy ($P_{rec} = 0.025$ and $P_{rec} = 0.023$, respectively), whereas in the case of rs2153157, individuals carrying the minor allele were more likely to get a positive treatment outcome ($P_{dom} = 0.024$ for biochemical pregnancies, $P_{dom} = 0.011$ for clinical pregnancies; **Figure 1G-H**). In addition, rs4246511 (*RHBDL2*) also seems to be linked with the probability of clinical pregnancy, as the presence of the minor allele decreased the likelihood of clinical pregnancy ($P_{dom} = 0.023$), while individuals carrying the *FSHB* rs611246 T-allele were more likely to achieve clinical pregnancy ($P_{rec} = 0.012$).

Predictive value of selected markers

In order to evaluate the predictive ability of selected genetic variants and used statistical models, leave-one-out CV modelling was applied and the calculated R^2 and AUC values compared with those obtained for original models (specific values given in **Table 2**). This approach was used both for models including cofactors and for models considering genetic factors without cofactors (clinical data).

A model combining cofactors and all statistically significant genotypes explains 19% and 10% of the variation seen in mean antral follicle count ($R^2 = 0.194$) and early follicular phase FSH concentrations ($R^2 = 0.108$), respectively (**Table 2**). If CV modelling is applied, the numbers decrease to 9% and 2%, respectively for mean follicle count (CV $R^2 = 0.089$) and early follicular phase FSH concentrations (CV $R^2 = 0.019$). However, without additional clinical data, the predictive power of statistically significant genotypes decreases remarkably for mean antral follicle count, but remains at ~2–5% for early follicular phase FSH ($R^2 = 0.050$, CV $R^2 = 0.017$). The prediction ability of significant genotypes together with selected clinical data for ovarian stimulation parameters is approximately 22–27% (~20% for CV modelling), and without clinical data 4–13% (2–5% for CV modelling) for all studied characteristics. In the case of IVF treatment outcome parameters, a CV model combining cofactors and selected (significant) genotypes yielded AUC < 0.7 (CV AUC = 0.63) for

Table 2 Results of cross-validation (CV) studies for all studied genotypes and for selected genotypes with a statistically significant effect on studied traits.

Analysed characteristic	*Selected genotypes with cofactors*		*All genotypes with cofactors*		*Selected genotypes without cofactors*		*All genotypes without cofactors*	
	R^2 / *AUC*	CV R^2/*AUC*	R^2/*AUC*	CV R^2/*AUC*	R^2/*AUC*	CV R^2/*AUC*	R^2/*AUC*	CV R^2/*AUC*
Early follicular phase parameters[a]								
Mean ovarian volume[b]	-	-	0.020	0.001	-	-	0.024	0.000
Mean antral follicle count	0.194	0.089	0.428	0.020	0.011	0.000	0.291	0.000
Early follicular phase FSH	0.108	0.019	0.337	0.006	0.050	0.017	0.288	0.005
Ovarian stimulation and outcome parameters[a]								
Total FSH	0.274	0.208	0.488	0.123	0.132	0.050	0.400	0.062
Number of ovarian follicles punctured	0.248	0.194	0.305	0.145	0.071	0.028	0.147	0.014
Number of retrieved oocytes	0.220	0.167	0.266	0.116	0.050	0.016	0.117	0.009
Amount of FSH necessary for retrieving one oocyte	0.242	0.199	0.293	0.156	0.043	0.015	0.143	0.021
IVF treatment outcome[c]								
Biochemical pregnancies	0.652	0.626	0.747	0.608	0.574	0.486	0.695	0.474
Clinical pregnancies	0.694	0.633	0.788	0.551	0.598	0.523	0.700	0.529

[a]The squared correlation (R^2) between observed and predicted values is reported.
[b]No genotypes with statistically significant effect.
[c]The area under the ROC-curve (AUC) is reported.

both biochemical and clinical pregnancies (**Table 2**). If clinical data were excluded from the model, the predictive value of only significant genotypes was modest (AUC < 0.6, CV AUC approximately 0.5).

Discussion

We believe that the search for genetic markers of ovarian reserve and IVF-COS among the genes related to ovarian ageing is the best way to identify the variants with the strongest impact, and therefore decided to test the SNPs highlighted in a recent menopause timing meta-analysis (Stolk et al., 2012). As expected, SNPs in several genes previously associated with the timing of natural menopause showed significant associations with a number of studied parameters (**Figure 2**). For example, the C-allele of rs4246511 in *RHBDL2* (rhomboid, veinlet-like 2; *Drosophila*) increased the amount of rFSH used during stimulation and necessary for retrieving one oocyte, whereas the T-allele was associated with a decreased chance of clinical pregnancy. RHBDL2 is an intramembrane serine protease from the rhomboid family that releases soluble growth factors by proteolytic cleavage of membrane-bound substrates, including epidermal growth factor (EGF), ephrins and thrombomodulin (reviewed by Etheridge et al., 2013). While the EGF network is involved in many aspects of human reproduction, including ovarian steroidogenesis and oocyte maturation (Jamnongjit et al., 2005), thrombomodulin has been associated with embryo implantation (Dassen et al., 2007; Singh et al., 2011). The fact that the effects of rs4246511 detected in this study are somewhat conflicting – women with the T-allele respond better to ovarian stimulation (and enter menopause later; Stolk et al., 2012) yet have reduced chances of pregnancy – suggests that the observed results are manifested via different pathways in the ovary and in the endometrium. However, since the rs4246511 has been scarcely studied in humans, further studies are needed to clarify the role of rs4246511 and RHBDL2 in human reproduction.

Apart from rs4246511 in *RHBDL2*, two other SNPs – rs2153157 (*SYCP2L*) and rs10852344 (intergenic) – associated with the chances for a biochemical or clinical pregnancy. rs2153157 (*SYCP2L*; synaptonemal complex protein 2-like) has repeatedly been associated with age at natural menopause (Carty et al., 2013; He et al., 2009; Stolk et al., 2012) and studies from mice show that SYCP2 is necessary for synaptonemal complex assembly and chromosomal synapsis during male meiosis, and furthermore, female SYCP2 knockout mice show signs of subfertility and reduced litter size (Yang et al., 2006). rs2153157 minor homozygotes also need significantly smaller amounts of rFSH/oocyte, which combined with the fact that the minor allele increases age at menopause (Stolk et al., 2012), indicates that the minor allele carriers have a better ovarian reserve. The positive effect of the same T-allele on clinical pregnancy rates can stem directly from improved ovarian reserve or be associated with the role the synaptonemal complex plays in preventing chromosome segregation errors and embryo aneuploidy – a major cause of implantation failure and early miscarriage. A similar mechanism of action can be suspected for rs10852344, as the T-allele carriers have increased chances for both biochemical and clinical pregnancy and also need less rFSH during stimulation. rs10852344 is located in an intergenic region and is surrounded by three genes (*GSPT1*, *SNX29* and *TNFRSF17*) that harbour many SNPs that are in LD with rs10852344. The nearest gene is *GSPT1* (G1 to S phase transition 1) that encodes the eRF3a protein involved in chromosomal segregation and cytokinesis during mitosis, and highly expressed in blastocysts (Adjaye et al., 2007), suggesting that the protein might be important for early embryonic development, which supports the present results.

Several SNPs – rs4886238 (*TDRD3*), rs3736830 (*KPNA3*) and rs7606918 (*METAP1D*) – showed direct associations with ovarian

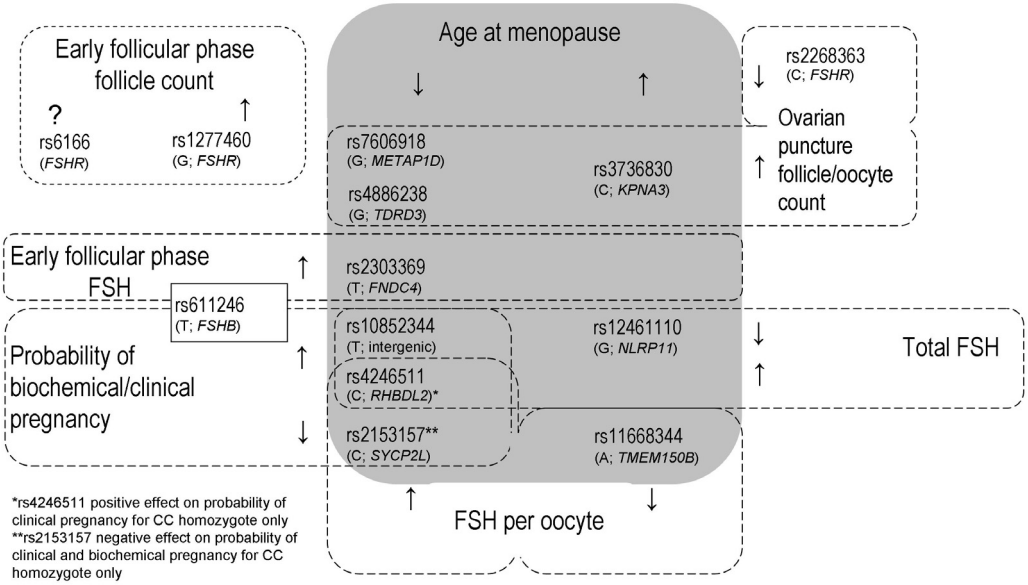

Figure 2 Schematic overview of the current results. Effect estimates given for allele shown in brackets. For SNP from the menopause meta-analysis, the influence on the timing of natural menopause is also shown (as derived from **Table 1** of the paper by Stolk et al., 2012).

biology as they were linked with the number of ovarian follicles punctured and/or oocytes retrieved. Of these three SNPs, rs4886238 (*TDRD3*; tudor domain containing 3) is probably the most interesting in this framework because TDRD3 interacts with the FMR1 protein (Linder et al., 2008) implicated in the development of primary ovarian insufficiency (Sullivan et al., 2011). In this study the ovarian stimulation in rs4886238 G-allele carriers resulted in more follicles and oocytes, whereas previously the same allele was associated with earlier age at menopause (Stolk et al., 2012). These two results indicate that the ovarian pool is depleted quicker in women carrying the G-allele. Not much is known about rs3736830 in *KPNA3* (karyopherin alpha 3, also known as importin α4), which belongs to importin alpha family and mediates nuclear protein import. Importin α4 mRNA is expressed in rodent testis (Hogarth et al., 2006) and importin α4 protects against oxidative stress in murine spermatids (Young et al., 2013), further connecting the protein with germ cell development. However, until now the association with menopause timing was the only link with female reproductive biology, as is the case with rs7606918 in *METAP1D* (methionyl aminopeptidase type 1D), a methionyl aminopeptidase found in mitochondria and often overexpressed in different cancers (Randhawa et al., 2013).

The total amount of rFSH administered or the amount of rFSH necessary for obtaining one oocyte were influenced by several of the studied polymorphisms. In addition to the already mentioned rs10852344 (intergenic), rs2153157 (*SYCP2L*) and rs4246511 (*RHBDL2*), two other SNPs – rs11668344 (*TMEM150B*, transmembrane protein 150B) and rs12461110 (*NLRP11*, NLR family, pyrin domain containing 11) – were associated with these traits. TMEM150B is a member of the damage-regulated autophagy modulator family of membrane-spanning proteins, while NLRP11 is a cytoplasmic protein implicated in the activation of pro-inflammatory caspases. Both of these genes have repeatedly come up in studies of menopause timing (Chen et al., 2012; Chen et al., 2014; Perry et al., 2013; Shen et al., 2013; Stolk et al., 2009; Stolk et al., 2012), indicating their influence on the trait is similar and conserved among women of different ethnicities; however, the exact mechanism of effect remains unclear. The major alleles of these SNPs were associated with decreased amount of total rFSH/rFSH per oocyte, and also with increased age at menopause (Stolk et al., 2012), suggesting the proteins might somehow mediate the action of FSH on follicular maturation, which is supported by the fact that the NLRP proteins are important for oogenesis (McDaniel and Wu, 2009).

The FSH plays a central role in ovarian biology; hence it is natural to assume that polymorphisms affecting the protein might have some effect on ovarian function. In this study, the four SNPs representing the *FSHB* core haplotypes (as these SNPs were in complete LD, data is shown only for rs611246 with the best call rate) were significantly associated with early follicular phase FSH concentrations and also with the probability of clinical pregnancy. The core haplotypes identified in the *FSHB* gene region have been associated with conception success in healthy females (Grigorova et al., 2007). Interestingly, in this study the SNPs forming the haplotypes had an effect on the probability of successful (IVF) clinical pregnancy. Grigorova *et al*. speculated that *FSHB* gene variants may influence female conception efficiency, and the results from this study seem to support this notion, although contrary to Grigorova *et al.*, included only infertile couples. As far as we know, the effect of *FSHB* core haplotypes on hormone concentrations is unknown. This study showed women with the T-allele had slightly higher FSH values (8.5 IU/L), which still remained in the normal range for follicular phase (3.5–12.5 IU/L), and at the same time had an increased chance of clinical pregnancy compared with AA homozygotes. Whether the positive influence on clinical pregnancy rates is achieved through relatively higher yet normal FSH concentrations or some other mechanism is currently unclear. Also, further studies are needed to clarify if the effects seen in this study also apply to healthy women. The polymorphisms forming the haplotype, rs611246 among them, are most likely regulatory SNPs (Grigorova et al., 2007), which may affect transcription levels or stability and thereby also protein concentrations, but their exact functional significance is yet to be elucidated. Although there is some evidence that the rs10835638 in *FSHB* promoter may also have an impact on FSH concentrations in women (reviewed in Simoni and Casarini, 2014), we did not observe a similar trend in our study subjects and this may be caused by several factors. First, our study group included infertile women of different age, and second, the study group was not big enough to stratify by *FSHR* genotypes, and ignoring the influence of *FSHR* genotypes may mask the *FSHB* polymorphism effect (La Marca et al., 2013a). A finding that deserves special attention, especially when planning future studies aimed at revealing the genetic regulation of FSH action, is the association between rs2303369 (*FNDC4*; fibronectin type III domain-containing 4) and early follicular phase FSH concentrations. The SNP is in LD with others in the adjacent *GCKR* (glucokinase regulator) gene, and the *FNDC-GCKR* locus has been associated with concentrations of albumin (Franceschini et al., 2012) and C-reactive protein (Ridker et al., 2008), indicating that the locus may have a more general effect on the concentrations of different serum components.

Polymorphisms in the *FSHR* gene, especially rs6165 and rs6166, have been thoroughly studied in the context of ovarian reserve and ovarian stimulation, but the results are somewhat conflicting probably due to heterogeneity of study groups (reviewed in Simoni and Casarini, 2014). In this study, the two SNPs showed complete LD; therefore data are presented only for rs6166, which was associated with mean antral follicle count but contrary to previous studies, not with ovarian response to gonadotrophin stimulation or basal FSH concentrations. Although the rs6166 was statistically associated with antral follicle count, the individual genotype effects were unclear. Unfortunately the genotyping of *FSHR* promoter polymorphism rs1394205 (-29G>A) failed (call-rate <90%), thus we were unable to use the analysis methodology recommended by Simoni and Casarini (2014), that is the combined analysis of rs6166 and rs1394205 in *FSHR*, and rs10835638 in *FSHB* promoter. The grouping of these three SNPs results in 27 combinations that associate with different FSH concentrations and FSHR activity, and failure to consider the interactions of these SNPs may cause incorrect results (Simoni and Casarini, 2014), which we believe might be the case in this study as well. The other two *FSHR* SNP that associated with early follicular phase or punctured ovarian follicle count – rs1277460 and rs2268363 – represent some of the less-studied *FSHR* polymorphisms. rs1277460 may play a role in female fertility (Kuningas et al., 2011), while rs2268363 is a susceptibility locus for erectile dysfunction (Kerns et al., 2010).

To assess the predictive power of the selected genotypes and models, the CV approach was applied to check the predictive ability of fitted models. The analyses showed that the prediction ability of selected (significant) genotypes together with clinical data for ovarian stimulation and outcome parameters is between 20% and 30%, and decreases only by about 5% if CV is applied. The latter indicates that the detected markers should also have a predictive effect in prospective studies, if combined with clinical data. The predictive power of selected genotypes without additional clinical data (4–13%, 2–5% if CV is applied) is similar to that reported for other complex traits, such as age at menopause (Stolk et al., 2012) and sex hormone binding globulin concentrations (Coviello et al., 2012). This highlights the fact that if in the future a genetic test is used for assessing ovarian function, it is most likely a panel test including tens of SNPs, because the variability seen in complex traits (such as those included in this study) is determined by a large number of genetic variants with a relatively small individual contribution, meaning that even variants that show very strong associations actually explain a very small part of the phenotypic variation. Furthermore, as suggested previously, no single biomarker can be sufficient to determine the best treatment approach and various clinical and genetic biomarkers need to be combined to personalize IVF-ovarian stimulation (Alviggi et al., 2012).

The relatively poor predictive ability for early follicular phase parameters and IVF treatment outcome parameters can be explained by: (i) the limited number of participants with early follicular phase data; and (ii) the fact that a successful pregnancy requires a complex dialogue between the embryo and the mother, and the used models are probably insufficient to cover the intricate network of interactions. The higher prediction accuracy values for models considering all studied genetic markers compared with models including only selected markers (**Table 2**) is logical considering the limited number of patients and the relatively high number of genetic variants studied. However, the CV prediction accuracy of full models is lower than for models with only selected genetic markers, indicating that the additional markers did not actually add anything to prediction accuracy of the models, once again underlining the predictive ability of selected (significant) markers.

When considering the results of the current study, some limitations should also be taken into account. First, the study group was quite diverse and included patients with various diagnoses and of different age. Nevertheless, patients with apparent ovarian dysfunction (polycystic ovary syndrome and patients with only one ovary) were excluded from the study. Also, the potential markers of IVF-ovarian stimulation should be usable in everyday practice, which deals with very heterogeneous patients, thus searching for markers in a very homogeneous population of patients might not be very practical in the long run. The authors would also like to stress that all statistical analyses were adjusted for potential confounding factors (for a full list of used cofactors, please see **Supplementary Table S2**), which means the reported results are valid regardless of, for example, the diagnosis for infertility, the age of the patient or the stimulation protocol used. Second, since this is a retrospective study, we were not able to assess whether the identified markers hold any actual predictive power in the clinical setting; therefore further studies are warranted for replicating and confirming the current results. At this stage, a prospective clinical study would mean that very important treatment decisions would have to be based on findings reported only once. Since differential treatment (such as FSH dosing) would be applied according to the genotype, a prospective study without previous replication studies could pose serious ethical concerns. Only if results from future studies are consistent with ours, a prospective clinical study can be conducted to determine the clinical validity of these marker candidates. However, results of statistical modelling demonstrated that the detected markers most likely have an actual predictive effect that is more pronounced when combined with relevant clinical data.

In conclusion, this study sheds some light on how the genes associated with female reproductive ageing exert their effect, providing new directions for future research. Furthermore, it was showed that genetic variants associated with reproductive ageing have the potential to be used as markers of ovarian function and IVF-ovarian stimulation, and identified several new biomarker candidates. However, the limited predictive ability of only genetic variants emphasizes the need to combine clinical and genetic data, and additional well-designed studies are needed to confirm these results and assess the actual clinical validity of the proposed markers and models.

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Appendix: Supplementary material

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