Genetic Polymorphisms Influence the Ovarian Response to rFSH Stimulation in Patients Undergoing In Vitro Fertilization Programs with ICSI

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Abstract

Introduction: Obtaining an adequate number of high-quality oocytes is a major challenge in controlled ovarian hyperstimulation (COH). To date, a range of hormonal and clinical parameters have been used to optimize COH but none have significant predictive value. This variability could be due to the genetic predispositions of single-nucleotide polymorphisms (SNPs). Here, we assessed the individual and combined impacts of thirteen SNPs that reportedly influence the outcome of in vitro fertilisation (IVF) on the ovarian response to rFSH stimulation for patients undergoing intracytoplasmic sperm injection program (ICSI).

Results: Univariate analysis revealed that only FSHR, ESR2 and p53 SNPs influenced the number of mature oocytes. The association was statistically significant for FSHR (p = 0.0047) and ESR2 (0.0017) in the overall study population and for FSHR (p = 0.0009) and p53 (p = 0.0048) in subgroup that was more homogeneous in terms of clinical variables. After Bonferroni correction and a multivariate analysis, only the differences for FSHR and ESR2 polymorphisms were still statistically significant. In a multic locus analysis, only the FSHR and AMH SNP combination significantly influenced oocyte numbers in both population (p<0.01).

Discussion: We confirmed the impact of FSHR and ESR2 polymorphisms on the IVF outcome. Furthermore, we showed for the first time that a p53 polymorphism (which is already known to impact embryo implantation) could influence the ovarian response. However, given that this result lost its statistical significance after multivariate analysis, more data are needed to draw firm conclusions. Only the FSHR and AMH polymorphism combination appears to influence mature oocyte numbers but this finding also needs to be confirmed.

Materials and Methods: A 13 gene polymorphisms: FSHR(Asn680Ser), p53(Arg72Pro), AMH(Ile49Ser), ESR2((+1730G>A), ESR1(−397T>C), BMP15(−9C>G), MTHFR1(677C>G), MTHFR2(1298A>C), HLA-G(−725C>G), VEGF(+405G>C), TNFα(−308A>G), AMHR(−482 A>G), PAI-1 (4 G/S G), multiplex PCR assay was designed to genotype women undergoing ICSI program. We analyzed the overall study population (n=427) and a subgroup with homogeneous characteristics (n=112).

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Introduction

In vitro fertilization (IVF) is a complex, multistep process. Oocytes-containing follicles are collected after controlled ovarian hyperstimulation (COH) with follicle stimulating hormone (FSH). Some of the subsequently fertilized oocytes will be transferred to the uterus for implantation, whereas others may be cryopreserved for future implantation attempts (or destroyed if they are unlikely to survive cryopreservation). All these steps are critical for successful IVF.

The aim of COH is to safely obtain a high number of mature oocytes so that the most viable embryo can be selected for transfer. Both quantitative and qualitative factors in oocyte production have a high influence on the IVF outcome. The goal is to transfer a single embryo and thus reduce the risk of multiple pregnancies - the main complication of IVF [1].

The significant inter-individual variability to COH with FSH is one of the most challenging issues in IVF treatment. Although low responses are troublesome, high responses can trigger a serious
medical condition - ovarian hyperstimulation syndrome (OHSS). Hence, the ability to predict an individual's responses to COH would constitute a major advance in patient care. Although many hormonal and clinical parameters (such as baseline FSH [2], oestradiol [3], inhibin B [4] and anti-Mullerian hormone (AMH) levels [5], patient age [6] and the antral follicle count [7]) have been used to optimize COH, none of these markers have significant predictive value when considered alone [8,9]. However, predictive performance levels can be improved by considering combinations of these parameters [10]. Despite these advances in patient management, there is still a need to individualise and optimise stimulation protocols, reduce the likelihood of an extreme response and thus increase the probability of a live birth. A complementary strategy involves studying the pharmacogenetics of the COH response. Candidate genes should have a specific effect on the reproductive system and present single-nucleotide polymorphisms (SNPs) that affect gene expression or function.

Gene association studies have identified a number of SNPs (affecting gonadotrophin, steroid and TGFβ pathways, etc.) involved in the ovarian response. Most of them affect mRNA levels or the protein sequence and thus lead to quantitative or functional protein variations that may account for the observed inter-individual variability in the COH. The first SNP to be studied was the FSH receptor polymorphism Asn680Ser, which affects baseline FSH level and increases gonadotrophin requirements during COH [11,12]. The ESR1 (−397 T>C) polymorphism was positively correlated with low oocyte retrieval after COH [13]. AMH (Ile49Ser) and AMHR polymorphisms have been used to optimize COH, none of these markers have been used to optimize COH, none of these markers have significant predictive value when considered alone [8,9]. However, predictive performance levels can be improved by considering combinations of these parameters [10].

Candidate SNP Allele frequencies of the studied SNPs in our population and in the NCBI database.

| Candidate SNP Allele frequencies | Allele overall study population | Homogeneous subgroup | NCBI |
|---------------------------------|---------------------------------|----------------------|------|
| AMH A/C                         | 654 (76%)/206 (24%)             | 159 (71%)/65 (29%)   | 82%  |
| AMHR A                          | 700 (82%)                       | 176 (79%)            | 83%  |
| BMP15 C                        | 193 (23%)                       | 65 (29%)             | 23%  |
| ESR1 A                         | 428 (52%)                       | 112 (50%)            | 58%  |
| ESR2 G                         | 520 (61%)                       | 136 (61%)            | 62%  |
| FSHR A                         | 475 (56%)                       | 136 (61%)            | 60%  |
| HLA-G G                        | 765 (90%)                       | 197 (88%)            | 84%  |
| MTHFR1 T                       | 216 (28%)                       | 81 (36%)             | 31%  |
| p53 C                          | 623 (73%)                       | 160 (73%)            | 70%  |
| PAI 4 G                        | 445 (52%)                       | 119 (53%)            | 50%  |
| VEGF G                         | 616 (78%)                       | 156 (75%)            | 80%  |

Results

In the overall study population, we observed a significant difference in the distribution of the oocyte number with age (p<0.0001) and FSH level (p=0.0044). However, in the homogeneous subgroup, only a slight difference in the distribution of the oocyte number with age (p=0.0289) was noted. The allele frequencies in the homogeneous subgroup and in the overall study population are listed in Table 1. The observed combinations of genetic and environmental factors [15]. In support of this hypothesis, an oligo-SNP model (including FSHR: Asn680Ser, ESR1: −397 T>C, and ESR2: Ile49Ser) was reportedly associated with a low response to FSH during COH [13]. However, due to the small sample sizes and the heterogeneity of the studied populations, the impact of these polymorphisms requires further investigations.
frequencies were similar to those quoted on the NCBI website (http://www.ncbi.nlm.nih.gov/snp).

After a univariate analysis, only three of the thirteen SNPs (FSHR p.Asn680Ser, +2039 A>G; p53 (p.Arg72Pro, +215 C>G), and oestradiol receptor 2 (p1730 G>A) polymorphisms) appeared to be significantly associated with baseline characteristics and/or the number of mature oocytes. These polymorphisms were in Hardy-Weinberg equilibrium, with a 1% error interval.

The other polymorphisms (AMH, AMHR, BMP15, VEGF, MTHFR1, MTHFR2, ESR1, TFN, HLA-G and PAI) did not appear to influence the number of mature oocytes collected (Table 2).

After applying Holm’s correction and a multivariate analysis (Table 3), the influence of p53 (p.Arg72Pro) was no longer statistically significant.

In a multilocus analysis of ESR2, p53, FSHR680 and AMH polymorphisms, only the FSHR Asn680Ser/AMH Ile49Ser combination was found to be associated with the number of mature oocytes (Table 2). After applying Holm’s correction and a multivariate analysis, the FSHR Asn680Ser polymorphism was no longer statistically significant in the overall study population. However, it was still significantly correlated (p=0.0225) with the oocyte number in the homogeneous subgroup.

Moreover, in the overall study population, we observed that women who were homozygous for the Ser680 variant were less likely to have had a low response than women who were homozygous for Asn680 (18% vs. 27%, respectively). Likewise, women who were homozygous for the Ser680 variant were more likely to have had a high response than women who were homozygous for Asn680 (24% vs. 12%, respectively) (p=0.0131). These observations were also confirmed in the homogeneous subgroup (p=0.046) (Figure 1).

The FSH Receptor Polymorphism (FSHR p.Asn680Ser, +2039 A>G) (Table 4)

In the overall study population, women with the Ser680 variant had significantly higher day-3 FSH and LH levels than women who were homozygous for the Asn680 variant (7.6±3.7 IU/l vs. 6.6±3.1 IU/l (p=0.0126) for FSH and 4.8±2.3 IU/l vs. 4.2±2.0 IU/l (p=0.0207) for LH, respectively). There was no such association with day-3 FSH or LH levels in the homogeneous subgroup (women under the age of 38 years old and with FSH levels <10 IU/l). The amount of FSH units administered and the oestradiol level on the day of HCG administration were similar for all genotypes in both the overall study population and the homogeneous subgroup.

Surprisingly, women who were homozygous for the Ser680 variant had a greater number of mature oocytes than women who were homozygous for the Asn680 variant, with averages of 8.1±4.3 vs. 7.1±3.9 oocytes (p=0.0047) in the overall study population and 9.8±4.6 vs. 7.2±4.0 oocytes (p=0.0009) in the homogeneous subgroup, respectively.

After applying Holm’s correction and a multivariate analysis, the FSHR Ser680 polymorphism was no longer statistically significant in the overall study population. However, it was still significantly correlated (p=0.0225) with the oocyte number in the homogeneous subgroup.

Moreover, in the overall study population, we observed that women who were homozygous for the Ser680 variant were less likely to have had a low response than women who were homozygous for Asn680 (18% vs. 27%, respectively). Likewise, women who were homozygous for the Ser680 variant were more likely to have had a high response than women who were homozygous for Asn680 (24% vs. 12%, respectively) (p=0.0131). These observations were also confirmed in the homogeneous subgroup (p=0.046) (Figure 1).

The Combination of FSHR and AMH Polymorphisms (Table 5)

Within both the overall study population and the homogeneous subgroup, the AMH Ile49 Ser polymorphism was not associated with any clinical or hormonal parameters or the number of oocytes retrieved. However, in both populations, women who were homozygous for both the FSHR Ser680 variant and AMH Ser49 variant yielded more mature oocytes than women who were homozygous for FSHR Asn680 and/or homozygous for AMH Ile49 with 10.3±5.5 and 7.3±3.9 mature oocytes (p=0.0068) in overall study population and 12.8±3.7 vs. 7.9±3.9 mature oocytes (p=0.009) in the homogeneous subgroup.

In the overall study population, we observed that women who were homozygous for the FSHR Ser680 and AMH Ser49 variants were less likely to have had a low response than women who were homozygous for FSHR Asn680 and/or homozygous for AMH Ile49 (14% vs. 24%, respectively). Similarly, women who were homozygous for the FSHR Ser680 and AMH Ser49 variants were

| Gene          | Overall studied n=427 | Homogenous n=112 |
|---------------|-----------------------|------------------|
| ESR2          | 0.0511                | Not included     |
| p53           | 0.1685                | Not included     |
| FSHR680       | 0.1969                | 0.0002           |
| Age           | 0.9017                | 0.3842           |
| FSH level at J3| 0.1409               | Not included     |

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### Table 4. The FSHR (Asn<sup>680</sup>Ser) polymorphism: baseline population characteristics, treatment parameters and ovarian response.

| Candidate SNP (rs) | Population | Genotype       | Number of women | Age (years) | Day-3 FSH level (IU/l) | Day-3 LH level (IU/l) | Amount of exogenous FSH required for ovulation induction (IU) | Oestradiol level on the day of hCG administration (pg/ml) | Number of mature oocytes |
|--------------------|------------|----------------|-----------------|-------------|------------------------|-----------------------|----------------------------------------------------------------|----------------------------------------------------------|--------------------------|
| **FSHR (rs6166)**  | Overall study population | Asn/Asn | 142 | 30.1 ± 12.6 | 6.6 ± 3.1 | 4.2 ± 2.0 | 2384 ± 947 | 2197 ± 941 | 7.1 ± 3.9 |
|                    |            | Asn/Ser | 191 | 32.0 ± 4.6<sup>a</sup> | 7.7 ± 4.0<sup>b</sup> | 4.8 ± 2.4<sup>b</sup> | 2386 ± 976 | 2184 ± 908 | 7.6 ± 3.9 |
|                    |            | Ser/Ser | 94  | 32.0 ± 5.3 | 7.3 ± 2.8 | 4.8 ± 2.2 | 2386 ± 899 | 2172 ± 1005 | 8.1 ± 4.3<sup>c</sup> |
|                    |            | Asn/Ser + Ser/Ser | 285 | 32.0 ± 4.8<sup>c</sup> | 7.6 ± 3.7<sup>d</sup> | 4.8 ± 2.3<sup>c</sup> | 2207 ± 951 | 2207 ± 951 | 7.7 ± 4.0 |
| **Homogeneous subgroup** |            | Asn/Asn | 45  | 30.3 ± 3.5 | 6.5 ± 1.7 | 4.0 ± 1.5 | 2353 ± 819 | 2128 ± 802 | 7.2 ± 4.0 |
|                    |            | Asn/Ser | 46  | 30.7 ± 3.7 | 6.0 ± 1.7 | 4.5 ± 2.3 | 2287 ± 748 | 2246 ± 844 | 8.1 ± 3.3 |
|                    |            | Ser/Ser | 21  | 30.7 ± 3.0 | 6.8 ± 1.5 | 4.2 ± 2.2 | 2278 ± 815 | 2271 ± 962 | 9.8 ± 4.6<sup>g,h</sup> |
|                    |            | Asn/Ser + Ser/Ser | 67  | 30.7 ± 3.5 | 6.3 ± 1.7 | 4.4 ± 2.2 | 2248 ± 764 | 2253 ± 875 | 8.6 ± 3.8 |

The major allele was used as a reference.

<sup>a</sup>p value = 0.0441, <sup>b</sup>p value = 0.0341.
<sup>c</sup>p value = 0.0071, <sup>d</sup>p value = 0.0126.
<sup>e</sup>p value c = 0.0325, <sup>f</sup>p value = 0.0207.
<sup>g,h</sup>Univariate p value = 0.0047, Univariate p after Holm's correction = NS, multivariate p value = NS.

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more likely to have a high response than women who were homozygous for FSHR Asn680 and/or homozygous for AMH Ile49 (43% vs. 14%, respectively) (p = 0.0188). These observations were confirmed in the homogeneous subgroup (p = 0.004) (Figure 2).

The Oestradiol Receptor 2 Polymorphism (ESR2 +1730 G>A) (Table 6)

In the overall study population, there was no association between this polymorphism and the day-3 serum levels of LH and FSH. However, in the homogeneous subgroup, an elevated LH serum level was observed for heterozygous women, with mean LH levels of 3.7±1.4, 4.8±2.3 and 3.7±1.6 IU/l for the GG, GA and AA genotypes, respectively. Day-3 E2 and FSH levels did not vary with genotype in the homogeneous subgroup.

The amount of FSH administered was not associated with the polymorphism in the overall study population. However, the mean E2 level on the day of hCG administration was higher in women who were homozygous for the G allele (2396±1015 pg/ml) than in heterozygous women and women who were homozygous for the A allele (2067±726, p = 0.0016 and 2049±915 pg/ml, p = 0.0155 respectively).

In the overall study population, women who were homozygous for the G allele had a greater mean number of mature oocytes than (i) women who were heterozygous for the A allele (8.1±4.2 vs. 7.2±4.0, respectively; p = 0.0017) or (ii) group of women who were homozygous or heterozygous for the A allele (8.1±4.2 vs. 7.2±3.9, respectively; p = 0.0314).

The results differed in the homogeneous subgroup, where the number of mature oocytes was not associated with the polymorphism. However, women who were homozygous for the A allele required more exogenous FSH (2706±879 IU) than women who were homozygous or heterozygous for the G allele (2375±752 IU and 2145±744 IU respectively) to produce a similar number of oocytes.

In the overall study population, the ESR2 polymorphism was still significantly correlated with the oocyte number after applying Holm’s correction (p = 0.0425) but not after a multivariate analysis (p = 0.0511). In both populations, there were no relationships between this polymorphism and the likelihood of a low or high response.

The p53 Gene Polymorphism (p.Arg72Pro, +215 C>G) (Table 7)

In both populations, there were no genotype-related differences in terms of age, day-3 hormone levels, the amount of FSH required for ovulation induction and the E2 level on the day of HCG administration.

In the overall study population, the number of mature oocytes obtained was not significantly associated with the genotype.

In the homogeneous subgroup, women who were homozygous for the Arg72 variant had a greater number of oocytes than (i) women who were heterozygous for the Pro72 variant (8.8±3.9 vs. 7.0±3.3, respectively; p = 0.0048) and (ii) group of women who were homozygous and heterozygous for Pro72 variant (8.8±3.9 vs. 7.2±3.3, respectively; p = 0.0451). However, this difference was no longer significant after applying Holm’s correction and a multivariate analysis.
Moreover, in the homogenous subgroup, we observed that women who were homozygous for Arg^72 were less likely to have a low response than women who were heterozygous or homozygous for the Pro^72 allele (15% vs. 28%, respectively). Accordingly, the women who were homozygous for Arg^72 were more likely to show a high response than those who were heterozygous or homozygous for Pro^72 allele (23% vs. 12%, respectively; p = 0.0429) (Figure 3).

Discussion

Of the thirteen polymorphisms studied here, only three SNPs (in the genes coding for FSHR, ESR2 and p53) and one SNP combination (FSHR Asn^680Ser/AMH Ile^49Ser) appeared to be significantly associated with the number of mature oocytes retrieved after COH. To improve our analysis, we applied Holm’s correction for p-values and performed a multivariate analysis to evaluate the polymorphisms’ respective impacts on the women’s IVF results.

The polymorphisms studied here have been previously shown to affect the response in some but not all studies.

We did not genotype FSHr307 in the current study because it is in near-complete linkage disequilibrium with FSHr680 [16]. In the present study, we investigated gene-environment interactions. Both genetic variants and environmental factors (as age, ovulation and length of cycle) have a significant influence on the ovarian response to gonadotrophins.

We showed that some effects are not apparent in the unselected ICSI population and clearly highlights the care that must be taken when comparing these studies. For example, FSH and LH levels on day 3 were not dependent on the FSH receptor polymorphism but a difference was found in the total unselected ICSI population. Thus, differences between studies might reflect the heterogeneity of included patients. In the homogeneous subgroup, the woman’s age and FSH level had less impact on the number of oocytes retrieved and so clearer conclusion could be drawn in this respect - even though the sample size was very small.

The FSH Receptor Polymorphism (FSHR p.Asn^680Ser, FSHR 2039 A>G)

In the homogeneous subgroup, there was no relationship between the genotype on one hand and the day-3 FSH level or age on the other - mainly because an FSH level below to 10 IU/l and age under 38 were criteria for inclusion in this subgroup.

In contrast, in the overall study population, we found that a significantly higher day-3 serum FSH level was associated with the FSHR Ser^680 variant. The increased age might explain the increased day 3 FSH. These data indicate that the polymorphism has no effect on young patients but might interfere when the patients are getting older.

The genotype did not appear to be associated with the amount of exogenous FSH. The attending gynaecologists were blinded to the genotype at the time of prescription. Similarly, there was no FSHR genotype-related difference in the E2 level on the day of hCG administration.

As described in the Results section, the women who were homozygous for the FSHR Ser^680 variant were less likely to have been low responders and more likely to have been high responders. These results were confirmed in the overall study population. Furthermore, the statistical significance after Holm’s correction and a multivariate analysis confirmed the impact of the FSHr680 genotype on the ovarian response to rFSH in IVF cycles.

Several previous studies have sought a correlation between the FSHR polymorphism and the outcome of the ovarian response but yielded discordant results.

### Table 5.
The FSHR (Asn680Ser) polymorphism combined with the AMH (Ile^49Ser) polymorphism: baseline population characteristics, treatment parameters and ovarian response.

| Genotype FSHR/AMH | Population | Number of women | Age (years) | Number of mature oocytes |
|-------------------|------------|----------------|-------------|------------------------|
| FSHR/AMH | Overall study population | 14 | 311±9.2 | 320±3.3 | 16 | 305±3.5 | 313±1.5 |
| Homogenous subgroup | 82 | 306±3.5 | 313±1.5 |
| NN: whatever the genotype, the major allele was used as a reference. | 20 | 70 | 12 | 17 |
| Candidate SNP (rs) | Population | Number of women | Age (years) | Day-3 FSH level (IU/ml) |
|-------------------|------------|----------------|-------------|------------------------|
| FSHR Ser680 variant | Overall study population | 114 | 311±9.2 | 738±1.8 |
| Homogenous subgroup | 82 | 306±3.5 |
| NN: whatever the genotype, the major allele was used as a reference. | 20 | 70 |

| Amount of exogenous FSH required for the day of hCG administration (IU) | Number of mature oocytes |
|--------------------------------------------------|------------------------|
| Day-3 FSH level (IU/ml) | Number of mature oocytes |
|--------------------------------------------------|------------------------|
| 20 | 70 |

| Day-3 LH level (Pg/ml) | Number of mature oocytes |
|--------------------------------------------------|------------------------|
| Day-3 FSH level (IU/ml) | Number of mature oocytes |
|--------------------------------------------------|------------------------|
| 20 | 70 |

| Genotype FSHR (Asn680Ser)/AMH (Ile49Ser) | Population |
|-----------------------------------------|------------|
| Number of women | Age (years) |
|--------------------------------------------------|----------------|
| 82 | 306±3.5 |

| Candidate SNP (rs) | Population | Number of women | Age (years) | Day-3 FSH level (IU/ml) |
|-------------------|------------|----------------|-------------|------------------------|
| FSHR Ser680 variant | Overall study population | 114 | 311±9.2 | 738±1.8 |
| Homogenous subgroup | 82 | 306±3.5 |
| NN: whatever the genotype, the major allele was used as a reference. | 20 | 70 |
In contrast with our present results, it has been reported that FSHR Asn<sup>680</sup>Ser is associated with a low E2 level during ovarian stimulation. To achieve similar oestradiol peak levels, homozygous FSHR Ser<sup>680</sup> women were found to need more exogenous FSH than women with FSHR Asn<sup>680</sup> [11,12,17]. Indeed, other researchers have suggested that women with the FSHR Ser<sup>680</sup> polymorphism have a higher ovarian threshold for FSH and thus a longer follicular cycle. The FSHR Ser<sup>680</sup> polymorphism has also been linked to lower sensitivity to the action of FSH [18]. Other studies have not found any association between FSHR Ser<sup>680</sup> polymorphism and various baseline hormone levels or the amount of exogenous FSH required for ovarian stimulation [19–22]. More recently, it was reported that women who were homozygous for the FSHR Asn<sup>680</sup> polymorphism needed higher amounts of exogenous FSH and tended to need more stimulation days [23]. This observation is concordant with our present study, in which women who were homozygous for FSHR Asn<sup>680</sup> had fewer mature oocytes than women homozygous for FSHR Ser<sup>680</sup> (despite the administration of similar amounts of exogenous FSH).

In other studies, women who were homozygous for the FSHR Ser<sup>680</sup> polymorphism were more at risk of a high response and iatrogenic OHSS after similar ovarian stimulation [22,24]. Only the occurrence of moderately intense OHSS was correlated with the FSHR Ser<sup>680</sup> polymorphism [24]. Our results were similar, with an increased likelihood of a high response (≥12 mature oocytes) for women who were homozygous for the FSHR Ser<sup>680</sup> polymorphism. The occurrence of iatrogenic hyperstimulation is probably linked to the hypersensitivity to FSH; this is quite unexpected because the Ser<sup>680</sup> polymorphism has previously been correlated with low sensitivity to FSH [11].

There is currently no evidence of an effect of the FSHR genotype on hormone binding characteristics or cAMP or inositol phosphate production following FSH stimulation. It is also possible that the FSHR Asn<sup>680</sup>Ser polymorphism only has a direct impact on the ovarian gonadotrophin response and oocyte recruitment when it is combined with other polymorphisms.

This hypothesis was strengthened by the results of our study, since the association between the FSHR polymorphism and the number of mature oocytes appeared to be higher when combined...
Table 6. The ESR2 (+1730 G>A) polymorphism: baseline population characteristics, treatment c parameters and ovarian response.

| Candidate SNP (rs)     | Population        | Genotypes | Women number | Age (years) | Day-3 FSH level (IU/ml) | Day-3 LH level (IU/ml) | Amount of exogenous FSH required for ovulation induction (IU) | Oestradiol level on the day of hCG administration (Pg/ml) | Number of mature oocytes |
|------------------------|-------------------|-----------|--------------|-------------|--------------------------|------------------------|---------------------------------------------------------------|----------------------------------------------------------------|--------------------------|
| ESR2 (rs4986938)       | Overall study Population | GG        | 158          | 30.9 ± 0.93 | 7.2 ± 3.7                | 4.7 ± 2.3              | 2282 ± 847                                                    | 2396 ± 1015                                                    | 8.1 ± 4.2                |
|                        |                   | GA        | 204          | 30.8 ± 0.61 | 7.4 ± 3.3                | 4.5 ± 2.1              | 2457 ± 999                                                    | 2067 ± 726           | 7.2 ± 4.0             |
|                        |                   | AA        | 66           | 31.1 ± 0.52 | 7.2 ± 3.9                | 4.7 ± 2.3              | 2390 ± 1000                                                   | 2049 ± 915           | 7.3 ± 3.6             |
|                        |                   | AA+AG     | 270          | 31.6 ± 0.76 | 7.4 ± 3.3                | 4.6 ± 2.2              | 2440 ± 847                                                    | 2063 ± 872           | 7.2 ± 3.9             |
|                        | Homogeneous subgroup | GG        | 40           | 30.6 ± 0.32 | 6.8 ± 1.4                | 3.7 ± 1.4              | 2375 ± 752                                                    | 2299 ± 936           | 7.9 ± 3.6             |
|                        |                   | GA        | 56           | 30.8 ± 0.31 | 6.3 ± 1.7                | 4.8 ± 2.3             | 2145 ± 744                                                    | 2238 ± 844           | 8.2 ± 4.4             |
|                        |                   | AA        | 16           | 30.3 ± 0.32 | 6.4 ± 1.8                | 3.7 ± 1.6             | 2706 ± 879                                                    | 1849 ± 494           | 8.0 ± 3.1             |
|                        |                   | AA+AG     | 72           | 30.6 ± 0.36 | 6.4 ± 1.6                | 4.6 ± 2.2             | 2276 ± 807                                                    | 2148 ± 791           | 8.1 ± 4.1             |

The major allele was used as a reference.

1p value = 0.0016,
2p value = 0.0155,
3p value 0.0007.
4p value = 0.0017, Univariate p after Holm’s correction = 0.0425, multivariate p value = NS.
5p value = 0.0314.
6p value = 0.0095, 6p value = 0.0346.
7p value AG compared to AA = 0.0150.
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Table 7. The p53 (Arg<sup>72</sup>Pro) polymorphism: baseline population characteristics, treatment parameters and ovarian response.

| Candidate SNP (rs) | Population                  | Genotype | Women number | Age (years) | Day-3 FSH level (IU/ml) | Day-3 LH level (IU/ml) | Amount of exogenous FSH required for ovulation induction (IU) | Oestradiol level on the day of hCG administration (Pg/ml) | Number of mature oocytes |
|-------------------|-----------------------------|----------|--------------|-------------|-------------------------|------------------------|-----------------------------------------------------------|----------------------------------------------------------|-------------------------|
| p53 (rs1045222)   | Overall study population    | Arg/Arg  | 214          | 31.5±8.0    | 7.4±3.1                 | 4.8±2.4                | 2361±978                                                   | 2162±885                                                   | 7.8±4.3                 |
|                   |                             | Arg/Pro  | 154          | 31.6±5.1    | 7.1±3.5                 | 4.4±1.9                | 2387±960                                                   | 2169±1006                                                  | 7.2±3.9                 |
|                   |                             | Pro/Pro  | 59           | 30.3±3.9    | 7.4±4.8                 | 4.3±2.2                | 2416±781                                                   | 2322±952                                                   | 7.0±3.3                 |
|                   |                             | Arg/Pro + Pro/Pro | 213      | 31.5±8.0    | 7.2±3.9                 | 4.4±2.0                | 2248±997                                                   | 2401±916                                                   | 7.1±3.7                 |
|                   | Homogeneous subgroup        | Arg/Arg  | 60           | 30.1±3.0    | 6.6±1.8                 | 4.4±2.1                | 2175±702                                                   | 2204±760                                                   | 8.8±3.9                 |
|                   |                             | Arg/Pro  | 34           | 31.0±4.1    | 5.9±1.6                 | 4.5±1.9                | 2446±862                                                   | 2132±984                                                   | 7.0±4.1<sup>abc</sup>   |
|                   |                             | Pro/Pro  | 16           | 31.1±3.7    | 6.3±1.6                 | 3.3±1.7                | 2476±836                                                   | 2335±862                                                   | 7.7±3.0                 |
|                   |                             | Arg/Pro + Pro/Pro | 50        | 31.1±3.9    | 6.1±1.6                 | 4.1±1.9                | 2198±942                                                   | 2457±845                                                   | 7.2±3.8<sup>d</sup>     |

The major allele was used as a reference.
<sup>a,b,c</sup>Univariate p value = 0.0048, Univariate p after Holm’s correction = NS, multivariate p value = NS.
<sup>d</sup>p value = 0.0451.
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with the AMH Ile\(^{49}\)Ser polymorphism (which is thought to slightly alter the biological activity of AMH [25]).

In both the overall study population and the homogeneous subgroup, the AMH Ile\(^{49}\)Ser polymorphism alone was not significantly associated with baseline day-3 hormone levels or the number of mature oocytes recovered after COH. Our results agree with previous data in this respect (Hanevik et al. 2010).

In both the overall study population and the homogeneous subgroup, we found that women who were homozygous for both the FSHR Ser\(^{680}\) and the AMH Ser\(^{49}\) alleles had a significantly greater mean number of mature oocytes than women who were homozygous for FSHR Asn\(^{680}\) and/or homozygous for AMH Ile\(^{49}\)—even though the various groups received similar amounts of exogenous FSH. A genotype-related difference was also present in the overall study population and the homogeneous subgroup when we compared the likelihood of belonging to the subgroups formed according to the number of oocytes. An increased likelihood of a high response was observed for women who were homozygous for both the FSHR Ser\(^{680}\) and the AMH Ser\(^{49}\) alleles, when compared with women who were homozygous for FSHR Asn\(^{680}\) and/or homozygous for AMH Ile\(^{49}\).

It has been shown that AMH-knockout mice display fast, high-quality primordial follicle recruitment and have a more pronounced response than the wild type in the presence of high serum FSH concentrations. In view of these data, AMH inhibits primordial follicle growth in vitro [26–28] and attenuates sensitivity to FSH. Conversely, follicles are more responsive to FSH in the absence of AMH [29,30].

These data are in agreement with our results, which suggest that AMH and FSH polymorphisms might improve the recruitment of primordial follicles. Our findings also suggest that the combination of AMH and FSHR polymorphisms may have potential value as a
marker for the ovarian response in women undergoing IVF treatment but due to sample size this finding needs to be confirmed.

The Oestrogen Receptor β Gene Polymorphism (ESR2+1730 G>A)

In the overall study population, women who were heterozygous or homozygous for the A allele had a significantly greater number of mature oocytes than those who were homozygous for the G allele, despite receiving similar amounts of exogenous FSH. Statistical impact of ESR2+1730 genotype on ovarian response to rFSH in IVF cycle was confirmed using Holm’s correction but not using multivariate analysis. This discrepancy is probably due to sample size, limited for multivariate analysis.

No difference was observed in the homogeneous subgroup; women who were homozygous for the A allele needed to receive more exogenous FSH than women who were heterozygous for the A allele (2706±879 IU and 2145±744 IU, respectively; p = 0.0150) to achieve adequate oocyte maturation and obtain a similar number of oocytes. Heterozygous women received similar amounts of exogenous FSH when compared with women who were homozygous for the G allele but had a higher day-3 LH level; which may contribute to best final adequate oocytes maturation. There were no genotype-related differences in the likelihood of belonging to the low-response or high-response groups.

In view of previous reports [31] associating ESR2 polymorphism with ovulatory dysfunction, we believe that our fertility-based inclusion criteria for the homogeneous subgroup (i.e. excluding women with known causes of infertility) can explain why the ESR2 polymorphism was not associated with the nature of the ovarian response.

Although oestrogen’s action is mediated by the ESR1 and ESR2 receptors, the latter predominates in the ovary [32,33]. ESR2 stimulates early folliculogenesis, decreases follicular atresia and stimulates late follicular growth [34] by inducing the action of FSH. In turn, FSH promotes granulosa cell proliferation. ESR2 action may thus explain the synergistic effect of oestrogen and FSH on the number of FSH receptors in granulosa cells (resulting in follicular growth and maturation [35]). However, our data did not reveal an association between the FSHR p.Asn650Ser and ESR2+1730 G>A polymorphisms on ovarian response.

ERS2 knockout mice have inefficient ovulation efficiency and produce few oocytes. This is mainly due to a defect within ovarian tissue in general [36, 37] and inability of E2 to exert its effect on the granulosa cells in maturing follicles in particular. Furthermore, it has been shown that ESR2 had no effect on the serum concentration of pituitary reproductive hormones [38]. Our results are in agreement with ESR2-knockout mouse data, since the mRNA of the ESR2+1730 A variant folds differently and is expressed less [39]. Furthermore, it has also been shown that follicles with low oestrogen level had low-quality, apoptotic oocytes [40,41], which could reduce the number of mature oocytes.

Hence, our results suggest that the ESR2+1730 G>A polymorphism modulates the IVF outcome by affecting the number of mature oocytes.

The p53 Gene Polymorphism (p. Arg72Pro, +215 G>C)

In the overall study population and in the homogeneous subgroup, the FSH treatment did not vary as a function of the p53 polymorphism. However, for the homogeneous subgroup, we observed that women who were homozygous for the Arg72 allele had a significantly higher mean number of retrieved oocytes than women who were heterozygous or homozygous for the Pro72 polymorphism. As this result was no longer statistically significant after Holm’s correction and a multivariate analysis, these data should be considered with a degree of caution. Further analyses are required to confirm or repudiate this finding.

Moreover, the genotype p53 Arg72 appeared to increase the likelihood of a high response and decrease the likelihood of a low response in the homogeneous subgroup.

The p53 tumour suppressor protein plays a crucial role in maintaining genomic stability in somatic cells. It has been shown that small changes in the level and/or activity of p53 can alter its functional efficiency. In Drosophila and C. elegans, p53 protein is most commonly expressed in germline cells, where it eliminates defective gametes and, consequently, defective offspring from the population [42,43].

The first report of an impact of p53 on fertility found a high association between the Pro72 polymorphism and recurrent implantation failure [44]. It has been further suggested that p53

Table 8. Women characteristics.

| Women characteristics | Overall study population | Homogeneous subgroup |
|-----------------------|--------------------------|----------------------|
| Patients number (n=)  | 427                      | 112                  |
| Age (years)           | 31.2± 9.55               | 30.6±3.53            |
| Hormonal profile      |                          |                      |
| Day-3 E2 level (IU/ml)| 46.27±26.15              | 46.89±28.17          |
| Day-3 LH level (IU/ml)| 4.64±2.77                | 4.32±2.02            |
| Day-3 FSH level (IU/ml)| 7.02±2.44               | 6.40±1.74            |
| ICSI indication       |                          |                      |
| IVF failure           | 28.6%                    | 0%                   |
| Male infertility      | 71.4%                    | 100%                 |
| Ethnic origin         |                          |                      |
| Caucasian             | 77.6%                    | 100%                 |
| Others                | 22.4%                    | 0%                   |
| Menstrual cycle       |                          |                      |
| Normal                | 71.7%                    | 100%                 |
| Abnormal              | 28.3%                    | 0%                   |
| Ovulation             |                          |                      |
| Normal                | 77.6%                    | 100%                 |
| Abnormal              | 22.4%                    | 0%                   |
| Ovulation             |                          |                      |
| Normal                | 71.4%                    | 100%                 |
| Abnormal              | 28.6%                    | 0%                   |
| Ethnic origin         |                          |                      |
| Caucasian             | 80%                      | 100%                 |
| Others                | 20%                      | 0%                   |

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| Gene name | Protein name                  | Protein function                  | Variant Name          | Association with ovarian response | Association with embryo implantation |
|-----------|-------------------------------|-----------------------------------|-----------------------|------------------------------------|--------------------------------------|
|           |                               |                                   | Positive association  | Negative association               | Positive association  | Negative association |
| AMH       | Anti-Mullerian hormone        | Hormone                           | +146 G/T (p.Ile49Ser) | [14,25]                            | [50]                   |
| AMHR2     | Anti-Mullerian hormone type II receptor | Hormone receptor                   | −482 A/G              | [14,25]                            | [50]                   |
| BMP15     | Bone morphogenic protein 15   | Oocyte and follicle development    | −9 C/G                | [51]                               |                        |
| ESR1      | Oestrogen receptor 1          | Hormone receptor                   | −397 T/C              | [13,52,53]                         |                        |
| ESR2      | Oestrogen receptor 2          | Hormone receptor                   | +1730 A/G             | [13,31,52,53]                      |                        |
| FSHR      | Follicle-stimulating hormone receptor | Hormone receptor                   | +2039 A→G (p.Asn680Ser) | [11–13,17–22,24,54–56]; [57]       | [54,57]               |
| HLA-G     | Major histocompatibility complex class I, G | Antigen processing and presentation | −725 C/G              | [58]                               |                        |
| MTHFR1    | Methylenetetrahydrofolate reductase | Folate metabolism                 | +677 C/T (p.Ala222Val) | [59]                               | [60]                   | [61] | [62] |
| MTHFR2    | Methylenetetrahydrofolate reductase | Folate metabolism                 | +1298 A/C (p.Glu429Ala) | [60]                               |                        | [63]                     |
| P 53      | Tumour protein p53            | Apoptosis and DNA reparation      | +215 C/G (p.Arg194Pro) | [48]                               |                        | [44]                     |
| PAI-1     | Plasminogen activator inhibitor-1 | Tissue development and coagulation | −675 (4 G/5 G)        | [64]                               |                        |                          |
| TNF       | Tumour necrosis factor alpha  | Pro-inflammatory cytokine          | −308 A/G              | [65]                               |                        |                          |
| VEGF      | Vascular endothelial growth factor | Vascular permeability and angiogenesis | +405 G/C              | [66]                               |                        |                          |
regulates female reproduction and blastocyst implantation through transcriptional up-regulation of uterine leukocyte inhibitory factor (LIF), which is an important factor in implantation. Elevated endometrial LIF levels are observed at the time of implantation in fertile women [45]. Women with unexplained infertility have lower LIF levels than fertile women do [46,47].

In conclusion, our objective was to determine a genetic profile suitable for use prior to the IVF protocol, in order to adjust the amount of prescribed FSH. We genotyped women after participation in an IVF protocol and investigated the potential usefulness of genetic testing for predicting the COH response. On the basis of literature reports, we identified thirteen polymorphisms that may impact ovarian function. Using a univariate statistical analysis, we found that three of the latter polymorphisms (in the genes coding for the FSHR, ESR2 and p53) and one combination of polymorphisms (FSHR680/AMH) were significantly associated with the number of mature oocytes retrieved after COH. After Holm’s correction and a multivariate statistical analysis, p53 was no longer statistically significant.

There is a need for clinical studies in which the amount of FSH given to patients is modulated according to the genotype - especially for women who are genetically predisposed to low or high responses to COH.

Materials and Methods

Subject Population

The study was approved by an independent ethics committee (the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale; project reference: 01032) and was performed in accordance with the tenets of the Declaration of Helsinki. All the women provided their prior, written, informed consent to participation.

We included a total of 427 women undergoing an initial ICSI procedure with oocyte retrieval and embryo transfer for severe male infertility. Before the ICSI procedure, the patients had been extensively evaluated in terms of their personal and family medical history, clinical and serological status, hysterosalpingography, extensive evaluated in terms of their personal and family medical history, clinical and serological status, hysterosalpingography, and karyotype. The exclusion criteria included, prior chemotherapy, unilateral ovariectomy, maternal diethylstilbene estrogen) and karyotype. The exclusion criteria included, prior chemotherapy, unilateral ovariectomy, maternal diethylstilbene estrogen (the

Table 10. Primer design for the selected SNPs.

| Gene name | Reference sequence | Primer (5' - 3') | PCR product size |
|-----------|-------------------|-----------------|-----------------|
| AMH       | rs10407022        | F1 F2 R         | 247             |
| AMHR      | rs2002555         | F1 F2 R         | 207             |
| BMP15     | rs3810682         | F1 F2 R         | 197             |
| ESR1      | rs2234693         | F1 F2 R         | 234             |
| ESR2      | rs4986938         | F1 F2 R         | 157             |
| FSHR      | rs6166            | F1 F2 R         | 224             |
| HLA-G     | rs1801133         | F1 F2 R         | 191             |
| MTHFR1    | rs1801133         | F1 F2 R         | 238             |
| MTHFR2    | rs1801131         | F1 F2 R         | 178             |
| p53       | rs10425222        | F1 F2 R         | 163             |
| PAI       | rs1799889         | F1 F2 R         | 148             |
| TNR       | rs1800629         | F1 F2 R         | 149             |
| VEGF      | rs2010963         | F1 F2 R         | 351             |

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GnRH agonist desensitization protocol and treatment with recombinant FSH for COH. The exclusion criteria included uterine malformation, grade 3 or 4 endometriosis, polycystic ovary syndrome (PCOS). Clinical and demographic characteristics were described in Table 8.

Ovarian follicle stimulation was performed with recombinant FSH (GonalF from Merck Serono or Puregon® from Organon-Schering Plough) and monitored by estrogen measurements and transvaginal ultrasound from day 5 onwards. Ovulation was induced with 10,000 IU of hCG or recombinant hCG. Transvaginal, ultrasound-guided follicle aspiration was performed 35 hours later and maturity of oocytes with a single polar body was evaluated after hyaluronidase treatment.

The number of mature oocytes obtained after ovarian hyperstimulation was analysed by genotype. In order to evaluate the association between the SNPs and the average number of oocytes in response to COH, we divided the population into three categories: low responders (4 oocytes or less), normal responders (between 5 and 11 oocytes) and high responders (12 oocytes or more).

DNA Preparation

For each patient, a blood sample was collected for DNA analysis. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Southampton, UK), according to the manufacturer’s protocol. Genotyping was performed after the IVF procedures had been completed.

SNPs Selected for Genotyping

We selected thirteen SNPs that reportedly impact the ovarian response and/or embryo implantation in women in IVF programmes (Table 9):
and 0.5 µl RO×500 size standard (Applied Biosystems) in 96-well PCR plates. The samples were denatured for 3 min at 95°C. Multiplex PCR products were separated by capillary electrophoresis using an ABI Prism 3100 genetic analyzer (Applied Biosystems). Allelic call was performed using the Genemapper® ID v.3.1 software (Applied Biosystem) (Figure 4).

Statistics
Statistical analysis was performed in three steps:

1. Univariate analysis. For age, the day-3 serum levels of LH and FSH, the amounts of exogenous FSH and the oestradiol level on the day of HCG administration, means were compared in an analysis of variance. The threshold for statistical significance was set to 5%.

For the oocyte number, the means were compared using generalized linear models (GLMs) supported by the GENMOD procedure (SAS Institute Inc., Cary, NC, USA), with the hypothesis of a Poisson distribution for the response variable.

For each genotype, the respective proportions with a high response (≥12 mature oocytes) and a low response (≤4 mature oocytes) were compared in a Stat view program (SAS institute) using Chi-2 and Fisher tests.

To take into account multiple testing for the polymorphisms, the familywise error rate was adjusted with the sequential Bonferroni correction.

Lastly, in order to better identify polymorphism interactions, a multilocus analysis was performed with GLM models. All polymorphisms found to be significant at p≤0.075 in the univariate analysis (without Holm correction) were introduced into the multilocus analysis; this enabled us to decrease the number of independent covariates in the models. Backward selection of variables was performed as above, and variables with p>0.05 and <0.10 were kept in the final models.

3. Multilocus analysis. We performed a multivariate analysis with all polymorphisms and putative confounding clinical factors (age, FSH level, cycle length and case of ovulation). All polymorphisms found to be significant at p≤0.30 after Holm’s correction were introduced as covariates into GLM models. Any suitable, first-order interactions derived from these factors were also added as covariates. Final models were obtained using backward selection of variables, with the likelihood ratio as the selection criterion. Variables with p>0.05 and <0.10 were kept in the final models.

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Author Contributions
Conceived and designed the experiments: PM JS FV. Performed the experiments: RB FV. Analyzed the data: RB FV. Contributed reagents/materials/analysis tools: RB ST FV. Wrote the paper: RB FV. Revised the manuscript for intellectual content: M. Benahmed PM JS. Included patients: DMG JS FV. Performed IVF program: AT M. Bailly M. Bergere FB RW.

References
1. Grady R, Alavi N, Vale R, Khandwala M, McDonald SD (2012) Elective single embryo transfer and perinatal outcomes: a systematic review and meta-analysis. Fertil Steril 97: 324–331.
2. Scott RT, Toner JP, Muasher SJ, Oehninger S, Robinson S, et al. (1989) Folic acid-stimulating hormone levels on cycle day 3 are predictive of in vitro fertilization outcome. Fertil Steril 53: 631–635.
3. Licciardi FL, Liu HC, Rosenwaks Z (1995) Day 3 estradiol serum levels are suitable, first-order interactions derived from these factors were also added as covariates. Final models were obtained using backward selection of variables, with the likelihood ratio as the selection criterion. Variables with p>0.05 and <0.10 were kept in the final models.

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25. Kevenaar MF, Laven JS, Fong SL, Unterlindein AG, de Jong FH, et al. (2008) A functional anti-mullerian hormone gene polymorphism is associated with follicle number and androgen levels in polycystic ovary syndrome patients. J Clin Endocrinol Metab 93: 1310-1316.

26. Durlinger AL, Visser JA, Themmen AP (2002) Regulation of ovarian function: the role of anti-mullerian hormone. Reproduction 124: 601-609.

27. Gigli I, Cashman RA, Wahl CM, Fortune JE (2005) Evidence for a role for anti-mullerian hormone in the suppression of follicle activation in mouse ovaries and bovine ovarian cortex glands beneath the chick chorioallantoic membrane. Mol Reprod Dev 71: 480-488.

28. Carlsson IB, Scott JE, Visser JA, Ritos O, Themmen AP, et al. (2006) Anti-mullerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. Hum Reprod 21: 2223-2237.

29. Durlinger AL, Grujich MJ, Kramer P, Karels B, Kumar TR, et al. (2001) Anti-mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142: 4891-4899.

30. Visser JA, Durlinger AL, Peters O, Jansen LJ, Kramer P, et al. (2007) Increased oocyte degeneration and follicular atresia during the estrous cycle in anti-mullerian hormone null mice. Endocrinology 148: 2301-2308.

31. Sundarrajan C, Liao WY, Boy C, Ng SC (2001) Association between estrogen receptor-beta gene polymorphisms and ovariolytic dysfunctions in patients with menstrual disorders. J Clin Endocrinol Metab 86: 135-139.

32. Byers M, Kuiper GG, Gustafsson JA, Park-Sarge OK (1997) Estrogen receptor-beta mRNA expression in rat ovary: down-regulation by gonadotropins. Mol Endocrinol 11: 172-182.

33. Drummond AE, Findlay JK (1999) The role of estrogen in folliculogenesis. Mol Cell Endocrinol 151: 57-64.

34. Hegele-Hartung C, Siebel P, Peters O, Kosemund D, Muller G, et al. (2004) The role of anti-mullerian hormone in the suppression of follicle activation in mouse ovaries. J Assist Reprod Genet 21: 23-29.

35. Ireland JJ, Richards JS (1978) Acute effects of estradiol and follicle-stimulating hormone on oocytes in the rat. Cell Endocrinol 151: 57-64.

36. Visser JA, Durlinger AL, Peters O, van den Heuvel ER, Rose UM, et al. (2007) Increased oocyte degeneration and follicular atresia during the estrous cycle in anti-mullerian hormone null mice. Endocrinology 148: 2301-2308.

37. Windahl SH, Andersson G, Gustafsson JA (2002) Activation of estrogen receptor function in bone with the use of mouse models. Trends Endocrinol Metab 13: 195-200.

38. Keene CH, Hodgkin JB, Goude JF, Enmark E, Warner M, et al. (1998) Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A 101: 5129-5134.

39. Ireland JJ, Richards JS (1978) Acute effects of estradiol and follicle-stimulating hormone on oocytes in the rat. Cell Endocrinol 151: 57-64.

40. Windahl SH, Andersson G, Gustafsson JA (2002) Activation of estrogen receptor function in bone with the use of mouse models. Trends Endocrinol Metab 13: 195-200.

41. Moron FJ, de Castro F, Rojo JL, Mostorero L, Mira E, et al. (2006) Bone morphogenetic protein 15 (BMP15) alleles predict over-response to recombinant follicle-stimulating hormone stimulation and iatrogenic ovarian hyperstimulation syndrome (OHSS). Pharmacogenet Genomics 16: 485-495.

42. Almnaa S, Haller K, Peters M, Hovatta O, Stavee-Evers A, et al. (2007) Allelic estrogen receptor 1 (ESR1) gene variants predict the outcome of ovarian stimulation in vitro fertilization. Mol Hum Reprod 13: 521-526.

43. Sundarrajan C, Liao WY, Boy C, Ng SC (1999) Association of oestrogen receptor gene polymorphisms with outcome of ovarian stimulation in patients undergoing IVF. Mol Hum Reprod 5: 797-802.

44. Jun JK, Youn JS, Ku SY, Chen YM, Hwang KR, et al. (2006) Follicle-stimulating hormone receptor gene polymorphism and ovarian responses to controlled ovarian hyperstimulation for IVF-ET. J Hum Genet 51: 663-670.

45. Conway GS, Conway E, Walker C, Hopper WN, Gromoll J, et al. (1999) Mutation screening and isoform prevalence of the follicle stimulating hormone receptor gene in women with premature ovarian failure, resistant ovary syndrome and polycystic ovary syndrome. Clin Endocrinol (Oxf) 51: 97-99.

46. Greb RR, Griesshaber K, Gromoll J, Sonntag B, Nieschlag E, et al. (2005) A common single nucleotide polymorphism in exon 10 of the human follicle stimulating hormone receptor is a major determinant of length and hormonal dynamics of the menstrual cycle. J Clin Endocrinol Metab 90: 4866-4872.

47. Klinkert ER, te Velde ER, Weima S, van Randvoort PM, Hansen RG, et al. (2006) FSH receptor genotype is associated with pregnancy but not with ovarian response in IVF. Reprod Biomed Online 13: 687-695.

48. Roussey RG, Coulam CB (2007) HLA-G and its role in implantation (review). J Assist Reprod Genet 24: 288-295.

49. Thaler CJ, Budman H, Rueschmann H, Nagel D, Lohse P (2006) Effects of the common 677C>T mutation of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene on ovarian responsiveness to recombinant follicle-stimulating hormone. Am J Reprod Immunol 55: 231-238.

50. Rosen MP, Shen S, McCulloch CE, Renaudo PF, Cedars ML, et al. (2007) Methylene tetrahydrofolate reductase [MTHFR] gene is associated with ovarian follicular activity. Fertil Steril 88: 632-638.

51. Azem F, Many A, Ben Ami I, Yovel I, Amit A, et al. (2004) Increased rates of thrombophilia in women with repeated IVF failures. Hum Reprod 19: 360-370.

52. Martinelli I, Taisa E, Rogni G, Levi-Serti F, Passamonti SM, et al. (2003) Embryo implantation after assisted reproductive procedures and maternal thrombophilia. Haematologica 88: 789-793.

53. Hagerty P, McCallum H, Bainham A, Andrews K, Duthie S, et al. (2003) Effect of B-vitamins and genetics on success of in-vitro fertilization: prospective cohort study. Lancet 367: 1513-1519.

54. Coulam CB, Jeyendran RS, Fishe LA, Roussey R (2006) Multiple thrombophilic gene mutations rather than specific gene mutations are risk factors for recurrent miscarriage. Am J Reprod Immunol 55: 360-368.

55. Vialard F, El Sirkasi M, Trouchaud V, Bouzahlah R, Molina-Gomes D, et al. (2005) Functional anti-mullerian hormone gene polymorphism is associated with recurrent implantation failure. Mol Hum Reprod 11: 110-114.

56. Martinelli I, Taioli E, Ragni G, Levi-Setti P, Passamonti SM, et al. (2003) Effect of B-vitamins and genetics on success of in-vitro fertilization: prospective cohort study. Lancet 367: 1513-1519.

57. Coulam CB, Jeyendran RS, Fishe LA, Roussey R (2006) Multiple thrombophilic gene mutations rather than specific gene mutations are risk factors for recurrent miscarriage. Am J Reprod Immunol 55: 360-368.

58. Vialard F, El Sirkasi M, Trouchaud V, Bouzahlah R, Molina-Gomes D, et al. (2005) Functional anti-mullerian hormone gene polymorphism is associated with recurrent implantation failure. Mol Hum Reprod 11: 110-114.

59. Coulam CB, Jeyendran RS, Fishe LA, Roussey R (2006) Multiple thrombophilic gene mutations rather than specific gene mutations are risk factors for recurrent miscarriage. Am J Reprod Immunol 55: 360-368.

60. Vialard F, El Sirkasi M, Trouchaud V, Bouzahlah R, Molina-Gomes D, et al. (2005) Functional anti-mullerian hormone gene polymorphism is associated with recurrent implantation failure. Mol Hum Reprod 11: 110-114.

61. Kohler RA, Laven JS, Fong SL, Unterlindein AG, de Jong FH, et al. (2008) A functional anti-mullerian hormone gene polymorphism is associated with follicle number and androgen levels in polycystic ovary syndrome patients. J Clin Endocrinol Metab 93: 1310-1316.