Hum. Reprod. Advance Access published August 17, 2010
Human Reproduction, Vol.00, No.0 pp. 1–8, 2010
doi:10.1093/humrep/deq211

human reproduction ORIGINAL ARTICLE *Reproductive Genetics*

# Genetic variation within the hypothalamus-pituitary-ovarian axis in women with recurrent miscarriage

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*Submitted on March 15, 2010; resubmitted on July 16, 2010; accepted on July 21, 2010*

**BACKGROUND:** Recurrent miscarriage affects 1–2% of couples trying to conceive, and is idiopathic in nearly half. Female fertility is controlled by the hypothalamus-pituitary-ovarian (HPO) axis and we hypothesize that genetic polymorphisms affecting the function of genes involved in regulating the HPO axis will be associated with recurrent miscarriage.

**METHODS:** Whole peripheral blood DNA from 227 women with recurrent miscarriage and 130 control women was obtained for this study. Using the Sequenom iPlex assay for fragment analysis, 31 single-nucleotide polymorphisms (SNPs) and 4 short tandem repeat (STR) polymorphisms in 20 candidate genes were evaluated for genetic association with recurrent miscarriage.

**RESULTS:** Several candidate associations were identified with an uncorrected *P*-value of 0.05. Genotype distribution at an SNP (rs37389) in the prolactin receptor gene ($P = 0.03$), and allele distributions at an SNP (rs41423247) in the glucocorticoid receptor gene ($P = 0.04$) and an STR polymorphism in the estrogen receptor $\beta$ gene ($P = 0.03$) were associated with recurrent miscarriage. The T allele of an SNP (rs2033962) within the activin receptor type 1 gene (*ACVR1*) was associated with increased number of miscarriages in an additive manner ($P = 0.02$). These candidate associations were not statistically significant after correcting for multiple analyses.

**CONCLUSIONS:** Candidate associations were identified between recurrent miscarriage and genetic variation within *ESR2*, *PRLR*, *GCCR* and *ACVR1* genes. Independent confirmation of these results is needed, as limitations of this study include the heterogeneous etiology of recurrent miscarriage, limited sample size, partial availability of reproductive history of the control group and investigation of only a subset of the genetic variation within each gene.

**Key words:** hypothalamus-pituitary-ovarian axis / hormone receptors / recurrent miscarriage / infertility / SNP analysis

## Introduction

Recurrent miscarriage, three or more consecutive miscarriages, occurs at a rate of 1–2% of couples trying to conceive (Stirrat, 1990). There are several known factors associated with risk for recurrent miscarriage, including genetic, endocrine, autoimmune, anatomical, thrombophilic and infectious (Li *et al.*, 2002; Rai and Regan, 2006). These factors vary in frequency, depending on maternal age and population studied, however, these can only be identified in 50–60% of cases (Stephenson, 1996; Li *et al.*, 2002), leaving almost half of women with idiopathic recurrent miscarriage.

The female reproductive system, including sexual differentiation, menstrual cycling and the early stages of pregnancy, is controlled by the hypothalamus-pituitary-ovarian (HPO) axis. This feedback loop begins with gonadotrophin-releasing hormone being released from the hypothalamus, resulting in the secretion of the gonadotrophins (LH and FSH) from the anterior pituitary, which in turn controls estrogen and progesterone production in the ovaries (Djahanbakhch *et al.*, 2007). Estrogen and progesterone play a central role in female fertility by stimulating growth, differentiation and maturation of follicles, preparing the endometrium for implantation (Drummond and Findlay, 1999) and maintaining embryonic development (Schindler, 2005).

Altered levels of hormones and other factors that are involved in maintaining control of the HPO axis can have negative effects on fertility and pregnancy. Elevated levels of gonadotrophins and estradiol have been previously associated with recurrent miscarriage (Li *et al.*,

2000; Gürbüz et al., 2003,2004). Elevated FSH is seen with advancing maternal age and is indicative of reduced ovarian responsiveness (Fitzgerald et al., 1998). Endocrine disorders such as polycystic ovarian syndrome and luteal phase deficiency are associated with increased rates of miscarriage due to altered progesterone and estrogen production in the ovary (Li, 1998). During the first 2 months of pregnancy, the primary source of progesterone and estrogen is the corpus luteum (Schindler, 2005), and if removed, the pregnancy will end in miscarriage (Csapo et al., 1972).

We therefore hypothesized that genetic polymorphisms in genes involved in regulating the HPO axis would be associated with recurrent miscarriage. To investigate this, we compared genotype frequencies of short tandem repeats (STRs) and single-nucleotide polymorphisms (SNPs) in 20 genes involved in the HPO axis (Tables I and II) among women with recurrent miscarriage and controls. Polymorphisms assayed include those that have been previously reported to affect transcription, hormone levels or reproductive outcome.

# Materials and Methods

## Samples

A total of 357 women were recruited from a Western Canadian population at the BC Women's Hospital & Health Centre in Vancouver, British Columbia. The case group consisted of 227 women with recurrent miscarriage (all evaluated by a single physician, M.D.S.), defined as three or more consecutive miscarriages before 20 weeks of gestation. This recurrent miscarriage group had a mean age at time of pregnancy (standard deviation; range) of 31.4 (6.1; 15–40) years with a total of 1379 pregnancies, of which 1027 (75%) ended in miscarriage. The mean number of miscarriages (standard deviation; range) was 4.5 (1.9; 3–13). Chromosome results were obtained in 208 of these miscarriages, of which 110 (53%) were euploid, with a 46,XX/46,XY ratio of 0.80 (49/61). Ninety eight (47%) of the miscarriages were karyotypically abnormal, including 70 autosomal trisomies, 16 polyploidies, 3 polyploidies with trisomies, 4 unbalanced translocations, 3 monosomy X (45,X), 1 monosomy X and trisomy 21 and 1 sex chromosome trisomy (47,XXY). Carriers of a structural chromosome rearrangement were excluded from this study. Forty (18%) of the 227 women with recurrent miscarriage had concomitant infertility.

The control group used in this study consisted of 130 women of reproductive age. Proven fertility and/or regular menstrual cycles were known in 67 of these women with a mean (standard deviation, range) menstrual cycle length of 28.4 (2.1; 23–35) days. Women with a known history of miscarriage, infertility or abnormal cycles were excluded from this study group. Reproductive history was unknown in the remaining 63 women; however, inclusion of these controls will only marginally reduce the power, as few will have irregular cycles and/or recurrent miscarriage. On the basis of the 357 subjects, with a power of 0.80 and an $\alpha$ of 0.05, an effect size of 0.16 can be observed in this study (Faul et al., 2007).

The collection of the samples for this study was approved by the University of British Columbia Clinical Ethics Review Board.

## Variant selection

Candidate genes in this study were identified through a literature search, using the search words 'recurrent miscarriage', 'fertility' and 'female reproduction'. Genes identified to be involved in female fertility through involvement in or modulation of the HPO axis, were further investigated for potential functional polymorphisms (Supplementary Table SI). Polymorphisms chosen are those that have been reported previously to be associated with reduced fertility in women and/or altered HPO axis hormone levels. In some cases, published polymorphisms could not be utilized due to technical constraints on the applied assay design in the current study (Sequenom iPlex) or due to limited available information.

To assess the possibility of population stratification, a difference in ethnic distribution between cases and controls, as a confounding factor in this study, 23 ancestral informative SNPs were chosen to assay in cases and controls, as described by Kosoy et al. (2009).

**Table I Comparison of allele distributions between women with recurrent miscarriage ($n$ = 227) and controls ($n$ = 130) for microsatellite polymorphisms within hormone receptors.**

| Allele | Recurrent miscarriage Obs. (freq.) | Controls Obs. (freq.) | $P$-value[a] |
|---|---|---|---|
| *AR* (androgen receptor) CAG repeat | | | 0.631 |
| ≤20 | 124 (0.27) | 85 (0.33) | |
| 21 | 81 (0.18) | 44 (0.17) | |
| 22 | 53 (0.12) | 26 (0.10) | |
| 23 | 62 (0.14) | 31 (0.12) | |
| ≥24 | 134 (0.30) | 74 (0.28) | |
| *ESR1* (Estrogen receptor α) TA repeat | | | 0.250 |
| ≤13 | 47 (0.10) | 24 (0.10) | |
| 14 | 135 (0.30) | 87 (0.34) | |
| 15 | 54 (0.12) | 19 (0.07) | |
| 16 | 13 (0.03) | 11 (0.04) | |
| 17–20 | 46 (0.10) | 21 (0.08) | |
| 21 | 45 (0.10) | 23 (0.09) | |
| 22 | 28 (0.06) | 26 (0.10) | |
| 23 | 37 (0.08) | 25 (0.10) | |
| ≥24 | 49 (0.11) | 24 (0.09) | |
| *ESR2* (Estrogen receptor β) CA repeat | | | 0.026 |
| ≤18 | 74 (0.16) | 33 (0.13) | |
| 19 | 25 (0.06) | 20 (0.08) | |
| 20 | 11 (0.02) | 15 (0.06) | |
| 21 | 32 (0.07) | 17 (0.07) | |
| 22 | 52 (0.12) | 45 (0.17) | |
| 23 | 164 (0.36) | 76 (0.29) | |
| ≥24 | 96 (0.21) | 54 (0.21) | |
| *SHBG* (Sex hormone-binding globulin) TAAAA repeat | | | 0.511 |
| ≤6 | 121 (0.27) | 61 (0.23) | |
| 7 | 31 (0.07) | 23 (0.09) | |
| 8 | 149 (0.33) | 95 (0.38) | |
| 9 | 111 (0.24) | 63 (0.24) | |
| ≥10 | 42 (0.10) | 18 (0.07) | |

[a]Chi-square analysis.

## Genotyping

DNA was extracted from whole peripheral blood using conventional methods. Thirty-one SNPs and 21 ancestry informative marker SNPs

**Table II Comparison of genotype distributions between women with recurrent miscarriage ($n$ = 227[a]) and controls ($n$ = 130[a]) for hormone pathway gene polymorphisms.**

| SNP | Genotype | Recurrent miscarriage Obs. (freq.) | Controls Obs. (freq.) | P genotypes[b] | P alleles[b] |
|---|---|---|---|---|---|
| *ACVR1* (Activin receptor type 1) | | | | | |
| rs2033962 | GG | 159 (0.70) | 92 (0.71) | 0.896 | 0.920 |
| | GT | 61 (0.27) | 33 (0.25) | | |
| | TT | 7 (0.03) | 5 (0.04) | | |
| *AR* (Androgen receptor) | | | | | |
| rs6152 | GG | 161 (0.71) | 95 (0.73) | 0.752 | 0.920 |
| | GA | 65 (0.29) | 33 (0.25) | | |
| | AA | 1 (0.00) | 2 (0.02) | | |
| *CBG* (Corticosteroid-binding globulin) | | | | | |
| rs2281517 | TT | 141 (0.62) | 79 (0.61) | 0.767 | 0.699 |
| | TC | 76 (0.34) | 43 (0.33) | | |
| | CC | 10 (0.04) | 8 (0.06) | | |
| *CGB5* (Chorionic gonadotrophin β polypeptide 5) | | | | | |
| rs4801789 | CC | 123 (0.55) | 69 (0.53) | 0.803 | 1.000 |
| | CT | 72 (0.32) | 46 (0.35) | | |
| | TT | 29 (0.13) | 15 (0.12) | | |
| *CYP17* (Steroid 17-hydrolase) | | | | | |
| rs743572 | AA | 77 (0.34) | 54 (0.42) | 0.323 | 0.320 |
| | AG | 105 (0.46) | 51 (0.39) | | |
| | GG | 45 (0.20) | 25 (0.19) | | |
| *CYP19* (Aromatase) | | | | | |
| rs10046 | TT | 62 (0.27) | 33 (0.25) | 0.307 | 0.663 |
| | TC | 108 (0.48) | 72 (0.55) | | |
| | CC | 57 (0.25) | 25 (0.19) | | |
| *ESR1* (Estrogen receptor α) | | | | | |
| rs2234693 | TT | 70 (0.31) | 43 (0.33) | 0.231 | 0.230 |
| | TC | 103 (0.45) | 66 (0.51) | | |
| | CC | 54 (0.24) | 21 (0.16) | | |
| rs9340799 | AA | 101 (0.45) | 57(0.44) | 0.113 | 0.450 |
| | AG | 90 (0.40) | 62 (0.48) | | |
| | GG | 35 (0.15) | 11 (0.09) | | |
| *ESR2* (Estrogen receptor β) | | | | | |
| rs1256049 | GG | 199 (0.88) | 115 (0.88) | 1.000 | 0.764 |
| | GA | 24 (0.11) | 14 (0.11) | | |
| | AA | 4 (0.02) | 1 (0.01) | | |
| *FBLN1* (Fibulin 1) | | | | | |
| rs9682 | CC | 91 (0.40) | 40 (0.31) | 0.208 | 0.130 |
| | CT | 109 (0.48) | 71 (0.55) | | |
| | TT | 27 (0.12) | 19 (0.15) | | |
| *FSHR* (Follicle-stimulating hormone receptor) | | | | | |
| rs1394205 | GG | 112 (0.50) | 69 (0.53) | 0.677 | 0.454 |
| | GA | 93 (0.41) | 52 (0.40) | | |
| | AA | 21 (0.09) | 9 (0.07) | | |
| rs6166 | AA | 67 (0.30) | 41 (0.32) | 0.831 | 0.624 |
| | AG | 118 (0.52) | 68 (0.52) | | |
| | GG | 42 (0.19) | 21 (0.16) | | |

*Continued*

**Table II** *Continued*

| SNP | Genotype | Recurrent miscarriage Obs. (freq.) | Controls Obs. (freq.) | P genotypes[b] | P alleles[b] |
|---|---|---|---|---|---|
| *GCCR* (Glucocorticoid receptor) | | | | | |
| rs41423247 | GG | 102 (0.45) | 47 (0.36) | 0.120 | **0.044** |
| | GC | 97 (0.43) | 58 (0.45) | | |
| | CC | 28 (0.12) | 25 (0.19) | | |
| rs6198 | AA | 164 (0.73) | 88 (0.69) | 0.381 | 0.269 |
| | AG | 55 (0.25) | 34 (0.27) | | |
| | GG | 5 (0.02) | 6 (0.05) | | |
| *INHA* (Inhibin α) | | | | | |
| rs35118453 | CC | 147 (0.65) | 81 (0.62) | 0.878 | 0.671 |
| | CT | 68 (0.30) | 41 (0.32) | | |
| | TT | 12 (0.05) | 8 (0.06) | | |
| *LHR* (Luteinizing hormone receptor) | | | | | |
| rs2293275 | GG | 92 (0.41) | 41 (0.32) | 0.148 | 0.279 |
| | GA | 90 (0.40) | 65 (0.50) | | |
| | AA | 41 (0.18) | 23 (0.18) | | |
| rs12470652 | TT | 200 (0.88) | 115 (0.88) | 1.000 | 1.000 |
| | TC | 27 (0.12) | 15 (0.12) | | |
| | CC | 0 (0.00) | 0 (0.00) | | |
| *PAPPA* (Pregnancy-associated plasma protein A) | | | | | |
| rs7020782 | AA | 100 (0.44) | 56 (0.43) | 0.947 | 0.842 |
| | AC | 105 (0.46) | 60 (0.46) | | |
| | CC | 22 (0.10) | 14 (0.11) | | |
| *PGR* (Progesterone receptor) | | | | | |
| rs518162 | CC | 190 (0.84) | 114 (0.88) | 0.387 | 0.584 |
| | CT | 36 (0.16) | 14 (0.11) | | |
| | TT | 1 (0.00) | 2 (0.02) | | |
| rs1042838 | GG | 176 (0.78) | 92 (0.71) | 0.409 | 0.282 |
| | GT | 46 (0.20) | 34 (0.26) | | |
| | TT | 5 (0.02) | 3 (0.02) | | |
| *PRL* (Prolactin) | | | | | |
| rs1341239 | GG | 94 (0.42) | 52 (0.40) | 0.923 | 0.752 |
| | GT | 102 (0.45) | 59 (0.45) | | |
| | TT | 30 (0.13) | 19 (0.15) | | |
| rs2244502 | AA | 105 (0.47) | 69 (0.53) | 0.340 | 0.446 |
| | AT | 104 (0.46) | 49 (0.38) | | |
| | TT | 17 (0.08) | 11 (0.09) | | |
| *PRLR* (Prolactin receptor) | | | | | |
| rs9292573 | TT | 100 (0.44) | 54 (0.40) | 0.304 | 0.842 |
| | TC | 94 (0.41) | 63 (0.48) | | |
| | CC | 33 (0.15) | 13 (0.12) | | |
| rs37389 | CC | 178 (0.78) | 108 (0.83) | **0.028** | 0.920 |
| | CT | 45 (0.20) | 15 (0.12) | | |
| | TT | 4 (0.02) | 7 (0.05) | | |
| rs13354826 | TT | 105 (0.47) | 57 (0.44) | 0.807 | 0.572 |
| | TC | 92 (0.41) | 53 (0.41) | | |
| | CC | 28 (0.12) | 19 (0.15) | | |
| *SHBG* (Sex hormone-binding globulin) | | | | | |

*Continued*

**Table II** *Continued*

| SNP | Genotype | Recurrent miscarriage Obs. (freq.) | Controls Obs. (freq.) | P genotypes[b] | P alleles[b] |
|---|---|---|---|---|---|
| rs6259 | GG | 171 (0.75) | 96 (0.74) | 0.842 | 0.823 |
| | GA | 51 (0.23) | 31 (0.24) | | |
| | AA | 3 (0.01) | 2 (0.02) | | |
| rs1799941 | GG | 138 (0.61) | 77 (0.59) | 0.733 | 0.920 |
| | GA | 75 (0.33) | 47 (0.36) | | |
| | AA | 14 (0.06) | 6 (0.05) | | |
| rs6257 | TT | 194 (0.85) | 109 (0.84) | 0.791 | 0.617 |
| | TC | 32 (0.14) | 19 (0.15) | | |
| | CC | 1 (0.00) | 2 (0.02) | | |
| *THRB* (Thyroid hormone receptor β) | | | | | |
| rs3752874 | CC | 172 (0.76) | 91 (0.70) | 0.425 | 0.377 |
| | CT | 49 (0.22) | 36 (0.28) | | |
| | TT | 6 (0.03) | 3 (0.02) | | |
| *TSHR* (Thyroid stimulating hormone receptor) | | | | | |
| rs2234919 | CC | 196 (0.86) | 118 (0.91) | 0.286 | 0.357 |
| | CA | 30 (0.13) | 11 (0.08) | | |
| | AA | 1 (0.00) | 1 (0.01) | | |
| rs1991517 | CC | 184 (0.81) | 112 (0.86) | 0.277 | 0.224 |
| | CG | 39 (0.17) | 17 (0.13) | | |
| | GG | 4 (0.02) | 1 (0.01) | | |

[a]*N* is the total number of samples run on the platform, the number of successful genotype calls may be less for some SNPs.
[b]Chi-square analysis.

were successfully assayed using the Sequenom iPlex Assay (Sequenom Inc., San Diego, CA, USA) by the Génome Québec Innovation Centre at McGill University, Montreal, Canada. STRs near the promoters of, or within, *ESR1*, *ESR2*, *AR* and *SHBG* genes were assessed by PCR as previously reported (Bretherick *et al.*, 2008).

## Statistical analysis

Hardy–Weinberg Equilibrium (HWE) was tested for each of the polymorphisms in controls (Supplementary Table SII). Chi-squared analysis was used for comparisons of allele and genotype frequencies for the 35 polymorphisms (31 SNPs and 4 STRs) between the recurrent miscarriage cases and controls. Within the recurrent miscarriage cases, the comparison of mean number of miscarriages grouped by genotype for each SNP individually was completed using analysis of covariance, which also corrected for differences in maternal age between groups (Pineda *et al.*, 2009). The Benjamini–Hochberg False Discovery Rate model was used to correct for multiple analyses (Benjamini and Hochberg, 1995).

## Population stratification

Twenty-one of the 23 SNPs were genotyped successfully, 1 was excluded as it was not in HWE, suggesting possible genotyping error and the remaining 20 were analyzed for allele frequencies. There was no significant difference in genotype distribution of the ancestral informative SNPs between control and recurrent miscarriage groups (Supplementary Table SIII), suggesting that population stratification is unlikely to be a confounding factor in this study.

# Results

The allele distributions for *AR* $CAG_{(n)}$, *ESR1* $TA_{(n)}$, *ESR2* $CA_{(n)}$ and *SHBG* $TAAAA_{(n)}$ STRs were compared between women with recurrent miscarriage ($n = 227$) and controls ($n = 130$) (Table I). The *ESR2* $CA_{(n)}$ allele distribution varied between recurrent miscarriage women and controls ($P = 0.03$), however there is no apparent trend based on allele size.

The allele and genotype distributions were compared between the recurrent miscarriage group and controls for the 31 SNPs assayed in 20 genes (Table II). The genotypes at a C/T SNP (rs37 389) within intron 4 of the prolactin receptor (*PRLR*) gene differed between the recurrent miscarriage group and controls with an excess of heterozygotes and deficiency of homozygotes in the recurrent miscarriage group ($P = 0.03$). The alleles at a G/C SNP (rs41423247) within intron 2 of the glucocorticoid receptor (*GCCR*) gene also differed ($P = 0.04$), with a minor allele frequency of 33.7% in recurrent miscarriage women compared with 41.5% in controls. The odds ratio (OR) for the GG genotype in the recurrent miscarriage group was 1.44 (95% CI, 0.93–2.24).

As some effects may be more pronounced among women with multiple miscarriages, we grouped the recurrent miscarriage cases by genotype and compared the mean number of miscarriages within these groups, correcting for maternal age as a covariant (Supplementary Table SIV). For a G/T SNP (rs2033962) within the activin receptor type 1 gene (*ACVR1*), the presence of the minor T allele was associated with increased number of miscarriages in an additive

fashion ($P = 0.02$), with GG genotypes ($n = 160$) having a mean number of miscarriages (standard deviation) of 4.3 (1.6), GT genotypes ($n = 61$) with 5.0 (2.3) and TT genotypes ($n = 7$) with 5.3 (2.7); however, the OR for the presence of the T allele was not higher (1.04, 95% CI 0.65–1.66).

The minor G allele for the -351A/G SNP (rs9340799) within the promoter region of the estrogen receptor α gene (*ESR1*) was not associated with recurrent miscarriage. Although, there is a non-significant increased frequency in the GG genotype in the recurrent miscarriage group (15%) compared with controls (9%) ($P = 0.11$), as well as an increasing number of miscarriages observed with the number of G alleles present ($P = 0.08$) (Supplementary Table SIV). No difference was observed for the *ESR1* -397C/T (rs2234693) polymorphisms with recurrent miscarriage or number of miscarriages ($P = 0.23$ and $P = 0.25$, respectively), which is in strong linkage disequilibrium (LD) with the -351A/G SNP (van Meurs *et al.*, 2003).

After using the Benjamini–Hochberg False Discovery Rate model to correct for multiple comparisons, none of the associations was found to be statistically significant.

## Discussion

Our study examined 35 polymorphisms within 20 genes that influence the HPO axis. We identified several candidate associations; polymorphisms within three genes (*ESR2*, *PRLR* and *GCCR*) were associated with recurrent miscarriage and *ACVR1* showed an additive trend of increased number of miscarriages with the minor allele. However, after correction for multiple analyses, these associations were not statistically significant.

These candidate genes have previously been suggested to have a role in female fertility; therefore, a potential role in recurrent miscarriage required investigation. Two independent studies reported that prolactin may play a role in miscarriage, with a reduction in prolactin expression in the endometrium in women with recurrent miscarriage (Garzia *et al.*, 2004) and the down-regulation of the *PRLR* in women who underwent *in vitro* fertilization and miscarried compared with those with ongoing pregnancies (Bersinger *et al.*, 2008). Mouse models have also shown that a lack of *PRLR* is associated with female infertility due to failure of embryo implantation (Ormandy *et al.*, 1997), suggesting that the *PRLR* is an essential component for endometrial receptivity.

Elevated activin levels have been associated with miscarriage (Prakash *et al.*, 2005). In addition, the G/T SNP (rs2033962) in the *ACVR1* gene has been associated with levels of anti-Mullerian hormone and follicle numbers in women with polycystic ovarian syndrome (Kevenaar *et al.*, 2009), which in turn has been linked to recurrent miscarriage (Rai and Regan, 2006).

The *GCCR* mediates the activity of cortisol, a marker of elevated stress. The GG genotype of the Bc/I (rs41423247) polymorphism within the *GCCR* gene has been associated with increased cortisol levels in women on oral contraceptives who underwent psychological stress testing (Kumsta *et al.*, 2007). Elevated levels of maternal urinary cortisol prior to 6 weeks of gestation were associated with a higher risk of miscarriage (Nepomnaschy *et al.*, 2006). Lastly, women with self-reported high levels of distress and long menstrual cycles were found to have a higher risk of miscarriage (Hjollund *et al.*, 1999). This is consistent with our finding of a tendency towards an increased frequency of the G allele of the rs41423247 polymorphism within the *GCCR* gene in women with recurrent miscarriage, with an OR of the GG genotype of 1.44 (95% CI 0.65–1.66).

Estrogen plays an essential role in follicular development and maintenance of early pregnancy. *ESR1* null female mice are infertile, with no corpus luteum formation and altered gonadotrophin levels, whereas *ESR2* null female mice have a subfertile phenotype with fewer number of oocytes, which may be due to decreased ovarian responsiveness to gonadotrophins (Emmen and Korach, 2003). There have been several studies investigating a potential association between the -397T/C and -351A/G SNPs in *ESR1* and recurrent miscarriage. The -397C allele has been associated with increased expression of the *ESR1* gene (Zhai *et al.*, 2006)—an effect that may be explained by the creation of a transcription factor binding site or by the LD with shorter $TA_{(n)}$ alleles in the promoter that may influence expression (Herrington *et al.*, 2002). Aléssio *et al.* (2008) assessed both these *ESR1* SNPs and the *ESR2* STR in 75 Brazilian women with recurrent miscarriage and found no association. However, a recent study found an association with an increased number of miscarriages and the *ESR1* haplotype composed of the -397T and -351A alleles (Pineda *et al.*, 2009). We did not find such an association, although our data suggest that the role of *ESR* polymorphisms in recurrent miscarriage may be of interest to investigate further in a larger study.

Contradictory results from these different studies may be due to the differences in ascertainment of women with sporadic and recurrent miscarriage. Historically, miscarriage risks were estimated at 15%, because only clinical pregnancies of 6 weeks or greater were included (Jacobs and Hassold, 1987). With the inclusion of preclinical pregnancies, miscarriage risks approach 30–50% (Edmonds *et al.*, 1982; Wilcox *et al.*, 1988). Many cases of a single preclinical miscarriage may be due to chance rather than an increased susceptibility. This is supported by the finding that rates of chromosome errors, such as trisomy, monosomy and polyploidy, are inversely associated with the number of miscarriages (Ogasawara *et al.*, 2000). Therefore, susceptibility due to genetic variability in hormone regulation may be more likely to play a role in women with strictly defined recurrent miscarriage. In this study, the mean number of miscarriages within the recurrent miscarriage group is higher than most other studies, increasing the likelihood of ascertaining women at an increased risk of miscarriage.

Recurrent miscarriage is known to be heterogeneous in etiology. We did not stratify our sample population for primary (no prior live birth) or secondary (prior live birth) recurrent miscarriage, or for clinical risk factors identified. In addition, many of the miscarriages were not karyotyped, therefore, we could not compare results stratified for euploid and aneuploid miscarriages. We were unable to obtain information on menstrual cycle length or regularity for a subset of the controls and the women with recurrent miscarriage. Ensuring all women in the control group had regular cycles would strengthen the study, possibly increasing the significance of true associations. In addition, the role of genetic variation in the HPO axis may be augmented in recurrent miscarriage women with irregular menstrual cycles.

The selection of only a few polymorphisms for each gene studied in this investigation allows only the assessment of that given site and those in LD with it. It does not, however, capture all of the genetic variation within these genes; therefore, the potential role of other

SNPs and rare mutations in the risk for recurrent miscarriage cannot be excluded. Furthermore, the synergistic effect of combinations of SNPs, particularly in extremely polymorphic genes, and gene-environmental interactions is difficult to appropriately address in association studies. A more extensive analysis of the genetic variation within these genes is needed in future studies to entirely evaluate the role of the HPO axis in the risk for recurrent miscarriage.

In conclusion, in this study we investigated the association between genetic polymorphisms affecting the function of genes involved in regulating the HPO axis and recurrent miscarriage. We identified candidate associations between recurrent miscarriage and genetic variants in *ESR2*, *PRLR*, *GCCR* and *ACVR1*. However, these associations were not significant after correcting for multiple comparisons. These findings may suggest that these gene variants have little or no effect on folliculogenesis and/or early maintenance of pregnancy. However, due to the limitation of sample size in this analysis, future studies in a larger, well-characterized group of women with recurrent miscarriage are needed to determine whether these candidate genes are associated with recurrent miscarriage.

## Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

## Acknowledgements

We thank the recurrent miscarriage patients and control women for their voluntary participation in this research. Thank you to our research assistant, Ruby Jiang, for all her assistance in DNA extractions, sample preparation and completing data sets.

## Funding

This research was supported by an operating grant from the Canadian Institutes for Health Research (to W.P.R.) and a Graduate Studentship from the Canadian Institutes for Health Researchstrategic training programme: Interdisciplinary Women's Reproductive Health Training Programme (to C.W.H.).

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