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Genome-wide association study in premature ovarian failure patients suggests **ADAMTS19** as a possible candidate gene

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BACKGROUND: Spontaneous premature ovarian failure (POF) occurs in 1% of women and has major implications for their fertility and health. Besides X chromosomal aberrations and fragile X premutations, no common genetic risk factor has so far been discovered in POF. Using high-density single nucleotide polymorphism (SNP) arrays, we set out to identify new genetic variants involved in this condition.

METHODS: A genome-wide association study involving 309 158 SNPs was performed in 99 unrelated idiopathic Caucasian POF patients and 235 unrelated Caucasian female controls. A replication study on the most significant finding was performed. We specifically focused on chromosomal areas and candidate genes previously implicated in POF.

RESULTS: Suggestive genome-wide significant association was observed for rs246246 (allele frequency $P = 6.0 \times 10^{-7}$) which mapped to an intron of *ADAMTS19*, a gene known to be up-regulated in the female mouse gonads during sexual differentiation. However, replication in an independent Dutch cohort (60 POF patients and 90 controls) could not confirm a clear association ($P = 4.1 \times 10^{-5}$ in a joint analysis). We did not observe strong evidence for any of 74 selected POF candidate genes or linkage regions being associated with idiopathic POF in Caucasian females, although suggestive association ($P < 0.005$) was observed for SNPs that mapped in *BDNF*, *CXCL12*, *LHR*, *USP9X* and *TAF4B*.

CONCLUSION: We observed a possible association between POF and a SNP in a biologically plausible candidate gene. Although limited by sample size, this proof-of-principle study's findings reveal *ADAMTS19* as a possible candidate gene for POF and thus a larger follow-up study is warranted.

Key words: premature ovarian failure / genome-wide association study / *ADAMTS19* / candidate gene / single nucleotide polymorphism

[†]These authors contributed equally to this manuscript.

Introduction

Spontaneous premature ovarian failure (POF) is a common disorder in women, with a prevalence of 1% (Coulam *et al.*, 1986). POF is characterized by secondary amenorrhea before the age of 40 years along with post-menopausal gonadotrophin levels (FSH > 40 IU/l) and very low or undetectable anti-Müllerian hormone (AMH) levels (Knauff *et al.*, 2009). POF not only truncates the patients' fertile lifespan, but also has major implications for their long-term health (i.e. osteoporosis, cardiovascular health and cognition). POF is usually due to premature exhaustion of the primordial follicle pool. Although an association with auto-immunity and macroscopic genetic aberrations has been demonstrated, the aetiology of the great majority of spontaneous POF cases remains unknown (Goswami and Conway, 2005).

Since family history has been shown to be the best predictor for early menopause and strong associations have been disclosed between the menopausal ages of mothers and daughters, sisters and twin pairs, idiopathic POF is most likely due to genetic factors (Cramer *et al.*, 1995; Torgerson *et al.*, 1997; Snieder *et al.*, 1998; Treloar *et al.*, 1998; de Bruin *et al.*, 2001; van Asselt *et al.*, 2004a). The incidence of familial POF is reported to be between 4 and 31% (van Kasteren *et al.*, 1999; Vegetti *et al.*, 2000).

Cytogenetic abnormalities involving the X chromosome have been identified in some POF patients, in particular XO mosaicism and X chromosomal re-arrangements (macrodeletions and translocations) (Schlessinger *et al.*, 2002). The only common genetic risk factor (prevalence > 1%) described in POF is being a carrier of a Fragile X premutation (FMR1). Sixteen percent of these carriers suffer from POF, whereas in POF patients the prevalence of Fragile X carriership is reported to lie between 3 and 15%, depending on familial distribution (Conway *et al.*, 1998; Allingham-Hawkins *et al.*, 1999; Wittenberger *et al.*, 2007).

Recently two familial linkage studies identified POF loci on Xq21.1–Xq21.3.3 (in a gene named POF1B) and on 5q14.1–5q15 (Lacombe *et al.*, 2006; Oldenburg *et al.*, 2008). Linkage analysis in sibling pairs discordant for menopausal age previously performed by our group resulted in two suggestive linkage regions on 9q21.3 and again on Xp21.3 (van Asselt *et al.*, 2004b). Many other POF candidate genes have been suggested. These were mostly identified in single patients or families, small patient groups, isolated populations or through animal knock-out models (see Supplementary Table S1).

Using high-density oligonucleotide genotyping platforms, it is now possible to screen millions of single nucleotide polymorphisms (SNPs) throughout the genome of a single individual or cohort; the so-called genome-wide association studies (GWAS). Via GWAS it is possible to identify common genetic variants contributing to susceptibility to genetically complex (or polygenic) diseases such as diabetes, hypertension, Crohn's disease, neurological and psychiatric disorders (Manolio *et al.*, 2008). We designed a GWAS using SNP arrays to identify predisposing genetic risk factors in a well-phenotyped set of POF patients and controls.

Materials and Methods

Study population and sample collection

From October 2004 onwards, a nationwide, standardized, systematic screening protocol has been applied to women with suspected POF visiting

the outpatient clinics of 10 Dutch hospitals. This protocol was approved by all the local institutional review boards and written informed consent was obtained from all participants. Screening included a questionnaire regarding fertility, family history and climacteric complaints, a transvaginal ultrasonography, and blood withdrawal. Blood samples were also collected in 10 ml EDTA tubes. DNA was isolated using a salting-out procedure and frozen at -80°C until genotyping experiments were conducted.

POF was defined as at least one episode of spontaneous secondary amenorrhea for more than 120 days (4 months), along with two measured serum FSH levels >40 IU/L, before age 40 years, and AMH levels below the menopause threshold of 0.086 $\mu\text{g/L}$ (van Disseldorp *et al.*, 2008). All patients presented with spontaneous menarche, with no history of chemotherapy, pelvic radiotherapy/surgery, or other medical conditions known to be associated with POF. All patients were Caucasian. Their karyotypes were obtained and they all underwent FMR1 premutation screening. Those with an abnormal karyotype (also including low 45,X/46,XX mosaicism) were excluded from the current analysis. Patients with more than 40 CGG repeats in the promotor region of the FMR1 gene were also excluded.

DNA samples from population control women with an age at menopause above 53 years were selected from the GOAL (Genetics of ovarian ageing by linkage-analysis) study cohort, further referred to as OldMP (van Asselt *et al.*, 2004b). These Caucasian women had at least 12 consecutive months of spontaneous secondary amenorrhea. Genotyping data from 181 healthy Dutch female controls (mean age 60.8 ± 10.3 years) were added from a GWAS in amyotrophic lateral sclerosis to increase study power, further referred to as FemC (van Es *et al.*, 2008). No menstruation history of these women was obtained. These two groups formed the control cohort for the current study in Phase I.

For the replication study (Phase II), we genotyped the SNP identified in Phase I in 60 additional POF samples and 90 OldMP females. Of the POF cases, 19 were recruited from the ongoing research protocol in the UMC Utrecht and nine from Phase I samples with low genome-wide call rates (<95%). An additional 32 POF samples were recruited from the GOAL cohort, in which women with a very early age at menopause had also been genotyped.

Genotyping methods

In Phase I, POF cases and OldMP women were genotyped using Illumina Infinium II Hapmap370 SNP duochips v.1.1 April 2007 (Illumina, San Diego, CA, USA). All experiments in Phase I were carried out at the Complex Genetics Group in the UMC Utrecht according to the manufacturer's protocol. In short, 750 ng of DNA per sample was whole-genome amplified, fragmented, precipitated and resuspended in the appropriate hybridization buffer. Denaturated samples were then hybridized on Illumina BeadChips at 48°C for a minimum of 16 h. After hybridization, the BeadChips were processed for single base extension reaction and stained. Chips were then imaged using the Illumina Bead Array Reader.

For Phase II (replication) the most significant SNP from Phase I was genotyped in the Genetic Department, UMC Groningen, using Taqman allelic discrimination assays. PCR was carried out with mixes consisting of 15 ng of genomic DNA, 1 \times AbSolute QPCR ROX mix (AbGene Mix, Thermo Scientific) and 1 \times assay mix (Applied Biosystems, Foster City, CA, USA) and ultraPURE distilled water (Dnase, Rnase Free, Gibco) in a 5 μl reaction volume in 384-well plates (Applied Biosystems). PCR conditions were as follows: denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Allelic PCR products were analysed on the ABI Prism 7900HT sequence detecting system using SDS 2.3 software (Applied Biosystems). Primer sequences for ADAMTS19 was TCTCTTGCTCTCTTTGGGCACTTTA[G/T]AAATTTGTGGATGGCTATTATTGG.

Statistical analyses and quality control

Once all samples for the GWAS had been genotyped, various quality control procedures were employed. For each sample, normalized bead intensity data was used in BeadStudio v3.0. to call genotypes. Samples that had an overall call rate <95% were removed. SNPs that had a call rate <95%, a minor allele frequency (MAF) in the controls <1%, or showed deviations from Hardy–Weinberg equilibrium (HWE) in the controls (Exact HWE P -Value <0.001) were removed from subsequent analyses.

To test for association we used Prioritizer GWA (Franke et al., 2006), and employed a single marker test, comparing allele count frequencies between cases and controls. Significance of association was determined by using an allele count χ^2 test (1 df). As over 300 000 tests were performed, we corrected for multiple testing. First, Type I errors were ascertained by a quantile–quantile (Q-Q) plot, generated by plotting the observed ordered null-allele associations against the ordered expected associations (see Fig. 1). Then we fitted a line to the lower 90% of the distribution, of which the slope ($\lambda_{\text{inflation}}$) denotes either the inflation or deflation of the test statistic.

Subsequently we determined what nominal single SNP P -Value corresponded to a $P = 0.05$, after correction for multiple testing. A commonly used threshold for deeming a SNP association genome-wide significant is $P = 5 \times 10^{-7}$ (Wellcome Trust Case Control Consortium, 2007). We established through 200 permutations of the affection status labels of our samples that a nominal $P = 2.0 \times 10^{-7}$ corresponds to a genome-wide significance of $P = 0.05$ on the Infinium II Hap370 platform.

For the replication analyses, we carried out Cochran–Mantel–Haenszel allele count χ^2 association tests using SPSS 16.0.1 with two clusters: Phase I (Infinium assay), Phase II (TaqMan assay). All P -values are two-tailed.

As we observed no evidence for bias in the test statistics as the Type I error rate ($\lambda_{\text{inflation}} = 1.017$) was not inflated, we present uncorrected statistics throughout this paper.

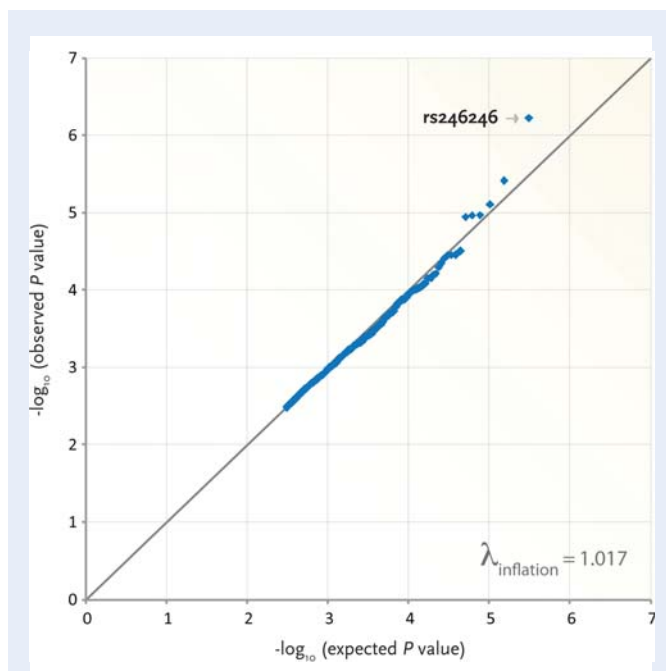


Figure 1 Q-Q plot of observed versus expected P -values. $\lambda_{\text{inflation}} = 1.017$, suggesting no inflation of the test statistic.

Power calculations

Power calculations for the GWAS were performed using the Genetic Power Calculator (<http://pngu.mgh.harvard.edu/~purcell/gpc/>) based on our sample size, the average observed MAF for SNPs present on the Illumina HumanHap370 (26%), under the assumption of a multiplicative model and a POF prevalence of 1 per 100 (Coulam et al., 1986). Using these parameters the genome-wide scan was 80% powered to detect an allelic association with $P < 2.0 \times 10^{-7}$ (which corresponds to a genome-wide significance of $P = 0.05$ when taking linkage disequilibrium (LD) into account, determined using permutations) and an odds ratio (OR) of 2.85 (see also Table I).

Linkage regions analysis

Similar to the candidate gene analysis, we first determined the most significant SNP (allele count 1 df χ^2 P -value) for each of these loci and then performed a permutation analysis, assuming that only one variant was responsible for the observed linkage signal. By permuting affection status labels 500 times (which leaves the LD structure intact), we could empirically determine the significance of association for each of these SNPs, correcting for the fact that linkage regions can differ in size and in the number of SNPs that map in them.

We also employed another procedure (allelic heterogeneity), in which we assumed that multiple independent, but common, variants within each of these loci might have contributed to the observed linkage signal. For each of the loci we determined the product of the individual allele frequency P -values for all of the SNPs that mapped in these loci. Subsequently we permuted the affection status labels 500 times, and in each permutation we compared the permuted product of allele frequency P -values against the observed product P -value, enabling us to determine a significance of P -values, although assuming allelic heterogeneity.

Functional candidate gene analysis

We selected 74 candidate genes based on one of the three following criteria: (1) incidental finding in POF patients, (2) previously tested in POF patients or (3) animal knock-out model showed a POF-like phenotype and the gene involved had a human homolog (see Supplementary Table S1). For each of these candidate genes, we determined whether associated SNPs were present that either mapped within these genes or were in strong LD with SNPs within these genes ($R^2 > 0.25$). SNPs which are in LD with SNPs within these genes were included, because these SNPs might tag for a causal variant that maps within these genes.

We then determined the most significant SNP for each gene (through an allele frequency P -value) and performed a permutation analysis, enabling us to empirically determine the significance of each candidate gene. The reason for this procedure was that the number of SNPs can differ considerably per

Table I Overall statistical power of study

MAF	OR	Power % ($P = 0.01$)	Power % ($P = 2.0 \times 10^{-7}$)
0.26	3	100	89
0.26	2.83	100	80
0.26	2.5	100	54
0.26	2	92	11

Power shown was calculated for 99 cases and 235 controls, assuming a MAF of 0.26, which corresponds to the mean MAF of all SNPs present on the oligonucleotide array. Results are shown for four different ORs with the expected power: $P = 0.01$ or $P = 2.0 \times 10^{-7}$ (genome-wide significance).

Table II POF patient phenotype characteristics (n = 99)

	Mean ± SD	%
Age at screening	36.5 ± 7.3	
1st FSH (IU/l)	82.4 ± 29.5	
2nd FSH (IU/l)	79.7 ± 38.1	
Age at menarche (years)	13.2 ± 1.6	
Age at amenorrhea (years)	31 ± 8.1	
Familial clustering ^a (%)		19
46 XX karyotype (%)		100
FMRI repeats <i>n</i> < 40 (%)		100
Caucasian (%)		100
AMH below menopause threshold ^b (%)		100
Undetectable AMH (%)		93
Positive anti-TPO antibodies (%)		25
Adrenal antibodies (%)		2

POF = premature ovarian failure; FSH = follicle stimulating hormone; FMRI = Fragile X mental retardation I; AMH = anti-Müllerian hormone; anti-TPO = anti-thyroid peroxidase.

^aDefined as at least two first- or second-degree female family members with POF (including the index patient).

^bMenopause threshold for AMH is <0.086 µg/L (van Disseldorp *et al.*, 2008).

gene, because genes vary in size and LD patterns differ. This is particularly true for SNPs that map within or very close to the major histocompatibility locus on chromosome 6, where LD patterns are very extensive and many SNPs might tag the same causal variant. As such, by permuting affection status labels 500 times (leaving the LD structure intact), we empirically determined the significance of association for each of these candidate genes while controlling for LD and the number of SNPs.

Results

In total, 108 POF samples and 60 OldMP samples were genotyped using the Illumina HumanHap370 BeadChip. Nine POF samples and 6 OldMP samples were excluded from analysis because their call rates fell below 95%. Ninety-nine POF cases (Table II) and 235 controls (54 OldMP and 181 FemC) were included for further analysis. All the POF patients had AMH values below the menopause threshold, and most had undetectable AMH levels. No related individuals were identified after comparing all the samples. In total 309 158 SNPs passed our quality control (Exact HWE *P*-value >0.001 in the controls, MAF in the controls >0.01, call rates for controls >95% and for cases >95%). Three SNPs were not genotyped correctly upon visual inspection, and were excluded from further analysis. A Q-Q plot analysis indicated no inflation of the test statistics (Fig. 1) as the $\lambda_{\text{inflation}} = 1.017$.

One SNP achieved near genome-wide significance after correction for multiple testing (A *P*-value <5 × 10⁷ is considered to reflect genome-wide significance (Wellcome Trust Case Control Consortium, 2007), permutation analysis of our data indicated a genome-wide significance threshold of *P*-value <2 × 10⁻⁷). SNP rs246246 was associated with an allele frequency *P*-value of 5.98 × 10⁻⁷. rs246246 SNP maps to an intronic region of a gene named *ADAMTS19*, in a 200 kb block of LD on chromosome 5q31, which also includes the small *KIAA1024L* gene on 5q23.3 with unknown function (Fig. 2).

Since thyroid peroxidase auto-antibodies (anti-TPO) were present in 25% of the POF samples we stratified for anti-TPO in relation to rs246246 genotype. No relation could be identified using a two tailed Fisher's exact test (*P* = 0.283).

We performed a small-scale replication study in Phase II covering 60 additional POF cases and 90 additional controls. Apart from genotyping rs246246 in the replication cohort, we also genotyped six random individuals from Phase I to ensure both SNP genotyping platforms generated the same genotype for each individual (concordance rate = 100%). No significant difference in MAF between cases and controls from the replication cohort was observed (MAF_{cases} = 0.05 and MAF_{controls} = 0.0556), resulting in *P* = 0.83. A joint analysis (Skol *et al.*, 2006) of Phases I and II using a Maentel–Haenszel test resulted in *P* = 4.05 × 10⁻⁵. Table III presents an overview of the descriptive statistics and results for SNP rs246246 in Phase I/Phase II and the joint analysis. An overview of all SNPs from Phase I with a *P* < 0.05 can be found in Supplementary Table S2.

In each of the three POF associated linkage regions (Xq21.1–Xq21.3.3, 5q14.1–5q15 and 9q21.3), SNPs were identified that had a nominal *P*-value <0.01. However, none of these findings remained significant after the permutation analysis, irrespective of the model assumed.

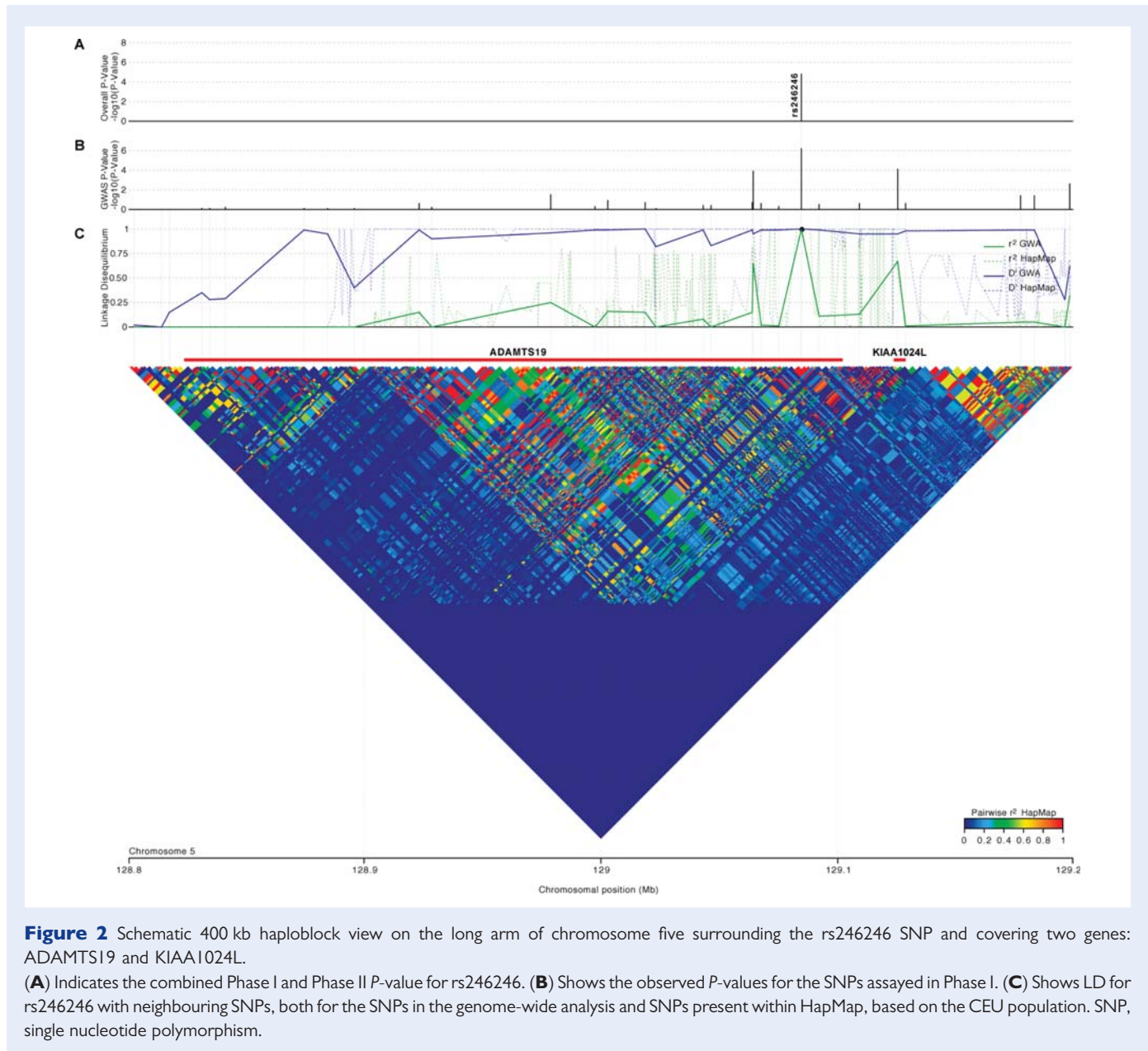
Candidate analysis of the most significant SNP revealed 29 candidate genes with a nominal *P*-value <0.05. After permutation, five genes (*BDNF*, *CXCL12*, *LHR*, *USP9X* and *TAF4B*) showed a nominal *P*-value <0.05 on gene level, which is more than expected by chance (0.05 × 74 = 3.7).

Discussion

To our knowledge, this is the first reported GWAS in POF using SNP arrays. Our results suggest that the gene *ADAMTS19*, located on chromosome 5q31, may be involved in POF. This finding will need to be replicated in a larger and independent study population.

ADAMTS19 is a member of the large ADAMTS (a desintegrin-like and metalloprotease with thrombospondin type I motif) family of metalloproteases (metal-binding enzymes). ADAM proteins are responsible for the proteolytic cleavage of many transmembrane proteins and the release of their extracellular domain, and seem to play an important role in gonad formation and function (Toussey *et al.*, 2006; Tamai and Nishiwaki, 2007). In a previous study on gene expression differences between embryonic XX and XY mouse gonads using cDNA subtraction, *ADAMTS19* was shown to be significantly up-regulated in XX gonads at the moment of sex differentiation. Using whole-mount *in situ* hybridization abundant expression of *ADAMTS19* was noted during the embryonic phase of gonadal development (Menke and Page, 2002). These findings provide a biological plausibility to *ADAMTS19* as a possible candidate gene for POF. Other ADAMTS proteases are also widely involved in female reproduction; gonad formation is disrupted in *Caenorhabditis elegans* when the ortholog of *ADAMTS-9* and 20 is mutated, while female homozygous *ADAMTS1* knock-out mice had a reduced number of ovarian follicles (Shindo *et al.*, 2000). Furthermore, ADAMTS proteins seem to play a role in ovulation processes as well as in folliculogenesis (Richards *et al.*, 2005; Brown *et al.*, 2006).

The near genome-wide significance of a SNP in a study with a relatively small number of patients and that it mapped to a plausible candidate gene like *ADAMTS19* (Phase I) were the reasons to perform a



validation study in an independent subset of patients and controls (Phase II).

A weakness of our study is that for Phase II, the cases were less extensively phenotyped than in Phase I, since these samples were taken from the GOAL cohort (van Asselt et al., 2004b). This group was only phenotyped as POF via their last recorded, spontaneous, menstruation date. It is possible that this phenotypic heterogeneity interferes with the results, although there was no statistically significant difference between the heterozygosity incidence. The 90 control samples in Phase II were again selected from the OldMP cohort and all had a history of spontaneous menstruations beyond the age of 52 years. Despite these limitations, we feel encouraged by the initial robust Phase I results and think the novelty of these preliminary findings are of interest for the scientific community.

Since POF is considered a complex genetic condition involving multiple genes, our genome-wide SNP data allowed us to investigate

associated SNPs in POF candidate genes identified in previous studies (see Supplementary Table S1). After permutation analysis five candidate genes showed higher *P*-values than expected. All five have been labelled as possible candidate genes via animal models showing a POF or POF-like phenotype. Brain-derived neurotrophic factor (BDNF) maps on chromosome 11p13. Ovaries of BDNF knock-out mice show loss of follicular organization, preceded by massive oocyte death (Paredes et al., 2004). Chemokine (C-X-C motif) ligand 12 (CXCL12) maps on chromosome 10q11.1 and is involved in guiding primordial germ cell migration (Doitsidou et al., 2002). Luteinizing hormone receptor (LHR) knock-out mice show a block in pre-antral folliculogenesis in combination with underdeveloped sex organs (Lei et al., 2001; Zhang et al., 2001). Ubiquitin-specific protease 9 (USP9X) is a gene required for oogenesis in *Drosophila* and maps in humans in a highly POF susceptible region on the short arm of the X chromosome (Xp11) (Jones et al., 1996).

Table III Descriptive statistics and results for SNP rs246246 (G/T) in Phase I/Phase II and joint analysis

	Number of cases	Number of controls	MAF ^a cases	MAF ^a controls	HWE ^b cases	HWE ^b controls	P-value ^c	OR ^d	95% CI
Phase I	99	235	0.15	0.04	0.09	0.25	5.98×10^{-7}	4.31	(2.33–7.94)
Phase II	60	90	0.05	0.05	0.68	0.15	0.83	0.89	(0.32–2.53)
Combined ^e	159	325	0.10	0.04	0.11	0.06	4.05×10^{-5}	2.75	(1.65–4.59)

Allele G is the susceptibility allele.

^aMAF = minor allele G frequency.

^bHardy–Weinberg equilibrium P-values.

^cP-values were calculated for each individual population using χ^2 test on allele counts.

^dORs were calculated for the minor allele.

^eP-values and ORs were calculated using the Mantel–Haenszel method.

TATA box binding protein (TBP)-associated factor 4B (TAF4B) maps on chromosome 18q11.2. Heterozygous TAF4B mice have a reduced number of ovarian follicles (Falender *et al.*, 2005). Although SNPs in these genes did not show strong significance on a genome-wide level in our study, their biological relevance might warrant attention in future genetic studies.

In conclusion, this first-stage, GWAS in a relatively small, homogeneous cohort of well-phenotyped POF patients did not reveal any common variants with genome-wide significance that confers risk to POF. However, *ADAMTS19* was identified as a potential candidate gene for POF. Assuming that the Illumina HumanHap370 tags human genetic variation well (Barrett and Cardon, 2006), and taking into account the power calculations that we performed, we conclude that the contribution of common genetic variants to POF is modest (OR < 2.83). However, in the current study, we only assessed common SNP variants; rare variants or common structural variants were not investigated. As such, additional, systematic, genome-wide analyses of POF patients using more extensive arrays, copy number variation (CNV) detection algorithms, and larger sample sizes are warranted in the search for POF-associated genetic variants.

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Supplementary data

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

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