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ORIGINAL RESEARCH

Genetic association of the activin A receptor gene (*ACVR2A*) and pre-eclampsia

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Pre-eclampsia is a common serious disorder of human pregnancy, which is associated with significant maternal and perinatal morbidity and mortality. The suspected aetiology of pre-eclampsia is complex, with susceptibility being attributable to multiple environmental factors and a large genetic component. Recently, we reported significant linkage to chromosome 2q22 in 34 Australian/New Zealand (Aust/NZ) pre-eclampsia/eclampsia families, and activin A receptor type IIA (*ACVR2A*) was identified as a strong positional candidate gene at this locus. In an attempt to identify the putative risk variants, we have now comprehensively re-sequenced the entire coding region of the *ACVR2A* gene and the conserved non-coding sequences in a subset of 16 individuals from these families. We identified 45 single nucleotide polymorphisms (SNPs), with 9 being novel. These SNPs were genotyped in our total family sample of 480 individuals from 74 Aust/NZ pre-eclampsia families (including the original 34 genome-scanned families). Our best associations between *ACVR2A* polymorphisms and pre-eclampsia were for rs10497025 ($P = 0.025$), rs13430086 ($P = 0.010$) and three novel SNPs: LF004, LF013 and LF020 (all with $P = 0.018$). After correction for multiple hypothesis testing, none of these associations reached significance ($P > 0.05$). Based on these data, it remains unclear what role, if any, *ACVR2A* polymorphisms play in pre-eclampsia risk, at least in these Australian families. However, it would be premature to rule out this gene as significant associations between *ACVR2A* SNPs and pre-eclampsia have recently been reported in a large Norwegian (HUNT) population sample.

Key words: activin / *ACVR2A* / genetic association / pre-eclampsia / SNP

Introduction

Pre-eclampsia is a complex and serious disorder of human pregnancy diagnosed by the new onset of hypertension and proteinuria in the latter half of pregnancy (Witlin and Sibai, 1997; Roberts *et al*., 2003; Roberts and Gammill, 2005). The disorder is pregnancy specific and reverses after delivery. The heritable nature of pre-eclampsia has long been recognized (Chesley *et al*., 1968) with susceptibility to the pre-eclampsia/eclampsia syndrome being attributable to a large genetic component (Salonen Ros *et al*., 2000; Nilsson *et al*., 2004; Carr *et al*., 2005) that involves both maternal and fetal contributions (Lie *et al*., 1998). Pre-eclampsia occurs in all major ethnic groups and affects ~5% of all pregnancies. It is a major cause of maternal mortality, accounting for 50 000 maternal deaths yearly in developing countries (Duley, 1992). Although diagnosed by hypertension and proteinuria, it is a multisystemic disorder affecting virtually all organ systems. There is no reliable predictive test, nor is there an effective therapy other than delivery, to cure this serious condition. The identification of the genetic determinants that underlie susceptibility to pre-eclampsia is likely to be crucial to understanding the disease process and to the development of effective screening tests and treatments. Defining the allelic architecture of pre-eclampsia, therefore, remains a priority in obstetric research.

Certain pathogenic features of pre-eclampsia such as endothelial dysfunction and inflammation, and the risk factors for pre-eclampsia are very similar to those of later life cardiovascular disease. Not surprisingly, pre-eclampsia is associated with an increased risk of later life cardiovascular disease (Chesley, 1975; Jonsdottir *et al*., 1995;

Irgens et al., 2001; Funai et al., 2005). This risk is highest for certain subsets [pre-eclampsia occurring before 34 weeks (Irgens et al., 2001) and recurrent pre-eclampsia (Chesley, 1975; Funai et al., 2005)] but even pre-eclampsia that occurs in the first pregnancy at term and never recurs is associated with increased later life cardiovascular disease (Funai et al., 2005). Furthermore, women who have had pre-eclampsia, when examined several years later, manifest changes which were present when they were pre-eclamptic and which indicate increased risk for cardiovascular disease. Thus, previously pre-eclamptic women, even those with normal blood pressure, have higher blood pressures than women who have had only normal pregnancies (Agatisa et al., 2004). Similarly, endothelial dysfunction (Chambers et al., 2001; Agatisa et al., 2004), abnormal lipids (Hubel et al., 2000) and insulin resistance (Laivuori et al., 2000) are more common in previously pre-eclamptic women. It is likely that identification of genetic factors important in pre-eclampsia will also provide insight into genetic factors relevant to cardiovascular disease in women.

Linkage analysis in affected families has been the strategy employed in several large international efforts, including our own, to identify the maternal genetic contributions to susceptibility (Arngrímsson et al., 1999; Moses et al., 2000; Lachmeijer et al., 2001; Laivuori et al., 2003). In our previous genome-wide linkage scan of 34 Australian/New Zealand (Aust/NZ) pre-eclampsia families, we detected significant linkage to chromosome 2q22 (Moses et al., 2000; Fitzpatrick et al., 2004). Fine mapping of the 2q locus in combination with an objective positional candidate gene prioritization strategy combining bioinformatics and gene expression profiling subsequently identified the activin A receptor type IIA (*ACVR2A*) gene as a high priority positional candidate (Fitzpatrick et al., 2004; Moses et al., 2006). ACVR2A is a key receptor for the cell signalling protein, activin A, which has long been recognized as an important regulator of human pregnancy (Peng et al., 1993). Activin A is also a recognized biological marker of pre-eclampsia and is known to be elevated in the maternal serum of women with pre-eclampsia (Grobman and Wang, 2000; Muttukrishna et al., 2000; Zwahlen et al., 2007). Preliminary association analysis using five known and validated single nucleotide polymorphisms (SNPs) at approximately equidistant intervals spanning the *ACVR2A* gene provided strong evidence of association for one SNP with pre-eclampsia (Moses et al., 2006).

In this study, we have extended our characterization of *ACVR2A* as a potential genetic risk factor for pre-eclampsia. First, we comprehensively re-sequenced the *ACVR2A* gene in affected individuals from our originally genome-scanned 34 Aust/NZ families to identify the complement of polymorphisms most likely to be causal. We then performed a series of association tests with these polymorphisms in our now larger family material of 74 Aust/NZ pre-eclampsia families. A high-density SNP map of 35 SNPs was also used to identify linkage disequilibrium (LD) patterns across the *ACVR2A* locus.

Materials and Methods

Subjects

All DNA samples were obtained from families recruited over a 15-year period through the Royal Women's Hospital and the Monash Medical Centre in Melbourne, Australia, through print and electronic advertisements in Sydney, Australia, and through the National Women's Hospital in Auckland, New Zealand. The extended cohort consisted of 480 individuals from 74 pre-eclampsia families, including the 34 families whom we have studied to date (Moses et al., 2000; Fitzpatrick et al., 2004; Moses et al., 2006). For the purposes of this manuscript, we shall hereafter refer to the original 34 pre-eclampsia families used to identify the 2q22 locus (Moses et al., 2000) as 'The 34 Family Cohort' and the now extended 74 pre-eclampsia families as 'The 74 Family Cohort'. The 74 Family Cohort included 20 women with eclampsia, 120 women with severe pre-eclampsia, 56 women who had hypertensive first pregnancies without proteinuria (mild pre-eclampsia) and 90 women who had normotensive first pregnancies. The composition of the familial material according to the severe and mild diagnostic criteria is presented in Table I.

Diagnosis was based on clinical assessment, using the criteria of the Australasian Society for the Study of Hypertension in Pregnancy (Brown et al., 1993). Women were considered to have severe pre-eclampsia if they had either (i) an increase from baseline systolic blood pressure of $\geq$25 mmHg, and/or diastolic pressure of $\geq$15 mmHg; or (ii) the presence of systolic pressure of $\geq$140 mmHg, and/or diastolic pressure of $\geq$90 mmHg. These levels had to occur on at least two occasions 6 h or more apart. Proteinuric levels had to exceed 0.3 g/l in a 24 h specimen, or the dipstick proteinuria score was $\geq$2+ in random urine collection. Women who met these criteria and experienced convulsions or unconsciousness in the prenatal period were classified as having eclampsia. Women with the pattern of hypertension outlined above but with no proteinuria were classified as mild pre-eclampsia. Because pre-eclampsia is considered a disease of first pregnancies (Chesley et al., 1968; Fisher et al., 1981), only those women with the above-mentioned features in their first pregnancies were included. Women with pre-existing hypertension or other medical conditions known to predispose them to pre-eclampsia (e.g. renal disease, diabetes, twin pregnancies or fetal chromosomal abnormalities) were excluded. Ethics approval was obtained from the Research and Ethics committees of The Royal Women's Hospital, Melbourne, Australia, and informed written consent was obtained from family members. Ethical approval for genotyping and statistical analysis of The 74 Family Cohort was granted from the Institutional Review Board, The University of Texas Health Science Center at San Antonio, TX, USA.

Subject selection for re-sequencing

For the re-sequencing of the *ACVR2A* gene, we selected 16 unrelated pre-eclamptic individuals from a subset of 16 families from The 34 Family Cohort that were the main contributors to the 2q22 linkage signal.

Table I Composition of the familial material according to the severe and mild diagnostic criteria.

No. of affected members	Severe pre-eclampsia	Mild pre-eclampsia
1	28	10
2	27	29
3	15	21
4	2	9
5	1	3
6	0	1
7	0	0
8	0	1

Entries are the number of families containing the indicated number of affected members.

Hence, by using affected individuals from these families, we increased the chances of identifying the majority of relevant functional variation.

Genomic DNA reference sequence

Sequence information for use as a reference template and primer design for *ACVR2A* re-sequencing was obtained from The NCBI Human Genome Database Build 34 (http://www.ncbi.nlm.nih.gov). We completely re-sequenced 1.5 kb of the proximal promoter, all coding regions, the 3′UTR and evolutionarily conserved non-coding regions considered most likely to harbour regulatory variation. Conserved regions were identified using a comparative genomics strategy that involved BLAST sequence comparison of human genomic DNA sequence against mouse genomic DNA sequence (http://www.ncbi.nlm.nih.gov/BLAST). Genomic sequence showing greater than ~70% homology was considered to be conserved between the two species (Prabhakar *et al*., 2006).

Primer design

All gene sequences were analysed for repetitive DNA elements using Repeat Masker (http://www.repeatmasker.org) to facilitate primer design. Using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), primers were designed to between 20 and 30 bp in length, with an annealing temperature between 55 and 60°C and within 1°C of each other.

Gene re-sequencing

PCRs were performed with 20 ng genomic DNA in a 20 μl reaction containing 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 1× GeneAMP PCR buffer (Amersham Biosciences, Piscataway, NJ, USA), 0.25 mM dNTP, 2.5 mM $MgCl_2$ and 100 ng each of sense and anti-sense primers. PCR amplification was carried out on a Perkin Elmer GeneAmp 9700 thermal cycler (Applied Biosystems). Amplification was performed with an initial denaturation step at 95°C for 1 min, then 35 cycles of 95°C for 30 s, a primer pair specific annealing temperature (55–60°C) for 30 s and 74°C for 45 s, then a final extension step of 74°C for 2 min.

PCR products were purified using ExoSAP-IT (Amersham Biosciences) according to the manufacturers' instructions and purified PCR products were used as a template for sequencing reactions.

Cycle sequencing was performed on both sense and anti-sense DNA strands in a 20 μl reaction. The sequencing reactions contained 2.5 μl of purified PCR product, 2 μl ABI Prism Big Dye Terminators version 3.1 (Applied Biosystems), 1× ABI Prism Big Dye Terminator Buffer (Applied Biosystems) and 60 ng of either sense or anti-sense primer. Sequence template amplification was performed on a Perkin Elmer GeneAmp 9700 thermal cycler (Applied Biosystems). The cycling conditions were standard for all purified PCR products and involved an initial denaturation step of 95°C for 5 min, then 30 cycles of 95°C for 10 s and 50°C for 5 s, then a final extension at 60°C for 4 min. Sequencing products were precipitated by the addition of four volumes of 75% isopropanol. Excess isopropanol was removed and the precipitate was allowed to dry at 80°C for several minutes. Samples were analysed using the commercial DNA sequencing service of the Australian Genome Research Facility, Melbourne, Australia. DNA sequence polymorphisms were then identified visually using the chromatogram viewing program Chromas 1.44 (http://www.technelysium.com.au/chromas14x.html).

SNP genotyping

The Illumina GoldenGate® SNP Genotyping Assay (Illumina Inc., San Diego, CA, USA) was employed for all genotyping. This assay does not accommodate the genotyping of insertion/deletion variants and consequently only the 45 SNPs that were identified by re-sequencing were subjected to Illumina's SNP Assay Design Tool. An assay was unable to be designed for one SNP (rs13034494). In total, 44 SNPs were successfully designed for inclusion into an Illumina custom SNP panel for genotyping on an Illumina 8 × 12 Sentrix Array Matrix (SAM) (Table II). Each SAM was imaged on the Illumina BeadStation 500GX BeadArray Reader using Illumina BeadScan image data acquisition software (version 2.3.0.13). SNP genotype clustering and individual sample genotype calls were interrogated using the Illumina BeadStudio software, Genotyping Module (version 2.3.41).

Genotype cleaning

Mistyping analysis was conducted using SimWalk2 (Sobel and Lange, 1996). Mendelian discrepancies and spurious recombinations were removed by blanking genotypes identified by SimWalk2 as having a high probability of being in error.

Statistical analyses

Allele frequency calculations, tests for deviations from Hardy–Weinberg and pattern assessment of LD among genotyped *ACVR2A* SNPs were performed using the statistical computer software SOLAR (Almasy and Blangero, 1998). SNP association analysis was conducted with SOLAR's quantitative trait linkage disequilibrium (QTLD) procedure. This procedure performs a test for population stratification and two commonly used association tests: measured genotype analysis (Boerwinkle *et al*., 1986) and the quantitative transmission disequilibrium test (QTDT) (Abecasis *et al*., 2000). It also performs a variant of the QTDT, the QTLD test (Havill *et al*., 2005), which achieves greater power by utilizing genotype information from founders but is not applicable if population stratification is present. In this study, the statistical significance of SNP association was determined by the QTLD test, unless there was evidence of stratification, in which case the QTDT was used. The imputation of missing genotypes was performed from the haplotype estimation procedure in SimWalk2. SNP association analyses were performed using both a general diagnostic criteria (where women with mild pre-eclampsia, severe pre-eclampsia or eclampsia were coded as affected) and a strict diagnostic criteria (where only women with severe pre-eclampsia or eclampsia were coded as affected).

Results

ACVR2A re-sequencing

The genomic sequence of *ACVR2A* spans 83.3 kb, of which we have re-sequenced ~40 kb that incorporates 1.5 kb of the proximal promoter, all coding regions, the 3′UTR and non-coding intragenic DNA conserved between human and mouse. We identified a total of 49 gene polymorphisms (Fig. 1) and at the time of discovery, 20 of these variants were identified as being novel. Subsequently, 7 of these have been deposited on the public databases (i.e. NCBI's dbSNP) leaving 13 novel variants. All but four variants identified were SNPs and the remainder were small (1–8 bp) insertion/deletion variants (Table II).

ACVR2A SNP genotyping

We used the Illumina GoldenGate® SNP Genotyping Assay to genotype the 44 successfully designed SNPs in all 480 members of The 74 Family Cohort. One SNP (rs13034494) failed assay design and the four insertion deletion variants identified were not designed due to

Table II Location and description of polymorphisms identified by the re-sequencing of *ACVR2A*

Variant	Chr locn (bp)	Function	Polymorphism	MAF
rs1424954	148317264	5′UTR	G→A	0.330
rs13224	148319154	5′UTR	NP	0
rs7572676	148320092	Intron	NP	0
LF001	148323680	Intron	C→A	0.224
rs1364658	148324278	Intron	C→G	0.328
LF021	148324711	Intron	T indel	No assay design
LF005	148327708	Intron	G indel	No assay design
rs6747792	148327998	Intron	A→C	0.224
LF002	148328072	Intron	NP	0
rs1014064	148328624	Intron	A→G	0.332
LF003	148330129	Intron	A→G	0.014
rs13034494	148332000	Intron	C→T	Failed assay design
rs12998729	148333762	Intron	G→A	0.328
rs17741978	148333850	Intron	C→G	0.224
rs1364657	148334737	Intron	G→A	0.055
rs7582403	148335224	Intron	A→G	0.354
rs1895694	148336216	Intron	A→G	0.369
rs2113794	148339447	Intron	A→C	0.369
LF004	148340383	Intron	G→A	0.012
LF008	148343404	Intron	GCTTATTC indel	No assay design
rs17742134	148343866	Intron	G→A	0.224
rs929939	148343998	Intron	C→A	0.342
rs1424943	148344632	Intron	NP	0
rs6713811	148348005	Intron	G→A	0.369
rs1424941	148359588	Intron	G→A	0.224
rs2161983	148365856	Intron	G→A	0.342
rs1128919	148373587	Synonymous	G→A	0.331
rs2288190	148374059	Intron	G→A	0.415
LF016	148374101	Intron	T→C	Genotyping failed
rs3754541	148375065	Intron	G→A	0.224
rs10497025	148378672	Intron	G→C	0.267
rs1227307	148378775	Intron	NP	0
rs13008497	148383231	Intron	G→A	0.328
rs1469211	148383305	Intron	G→A	0.224
LF012	148385604	Intron	G→A	0.012
LF013	148388204	Intron	A→G	0.012
rs3768687	148388490	Intron	G→A	0.332
LF020	148390323	Intron	A→G	0.012
rs13026650	148390671	Intron	G→A	0.332
LF014	148390982	Intron	NP	0
LF015	148391000	Intron	AAG indel	No assay design
rs3764955	148391267	Intron	G→C	0.328
rs7601098	148393084	Intron	G→C	0.055
rs3820716	148396730	Intron	A→G	0.425
rs2303392	148396897	Intron	C→G	0.342
rs1803029	148401443	3′UTR	NP	0
rs13430086	148403531	3′UTR	T→A	0.310

Continued

Table II *Continued*

Variant	Chr locn (bp)	Function	Polymorphism	MAF
rs17692648	148404201	3′UTR	A→C	0.214
rs1047081	148404773	3′UTR	NP	0

Chr, chromosome; locn, location; bp, base pair; MAF, minor allele frequency; NP, non-polymorphic; indel, insertion/deletion.

the constraints of the chosen genotyping assay. All but one (LF016) of the designed SNPs were successfully genotyped and of these, eight SNPs were non-polymorphic (Table II). One of the eight non-polymorphic SNPs, LF014, was subsequently determined to be non-polymorphic after genotypes were blanked due to Mendelian inheritance discrepancies. We observed a very high individual genotyping success rate (97.9–99.4%) and all polymorphic SNPs conformed to Hardy–Weinberg expectations ($P > 0.05$). All non-polymorphic SNPs were omitted from subsequent SNP association and LD analyses.

LD (association) analysis

Under the mild diagnostic criteria for pre-eclampsia, nominal association was seen with two SNPs, rs10497025 (QTLD $P = 0.025$) and rs13430086 (QTDT $P = 0.010$) (Table III). Under the strict diagnostic criteria for pre-eclampsia, we observed nominal associations with three novel SNPs, LF004, LF013 and LF020 (all QTLD *P*-values = 0.018) (Table IV). To correct for multiple hypothesis testing, we used SOLAR to simulate a quantitative trait with a heritability of 0.51 (Moses *et al.*, 2006) and assigned affection status to a set number of affected women with the highest simulated trait values. The number of affected women was determined from our 74 Family Cohort ($n = 140$ women denoted as having severe pre-eclampsia). The QTLD analysis procedure was repeated 10 000 times to our simulated trait and an experiment-wide *P*-value for each SNP was determined at an α of 0.05. The experiment-wide *P*-values for our nominally associated SNPs failed to satisfy the statistical significance threshold ($P > 0.05$).

Figure 2 depicts a variable pattern of LD across *ACVR2A* as measured by the squared value of the pair-wise correlation among intragenic genotypes (ρ^2). From this plot, a very strong correlation ($\rho^2 > 0.9$) is observed among the three novel SNPs (LF004, LF013 and LF020) displaying a nominal association with severe pre-eclampsia. These SNPs are not correlated with any other variant tested in *ACVR2A* suggesting that one, or a combination of all three of these SNPs, may have a functional influence on the gene. Given the intronic position of these SNPs and the relatively small re-sequencing sample data set ($n = 16$), we cannot exclude, however, the possibility of these SNPs being in LD with another as yet unidentified putative functional SNP. The imputation of missing genotypes using haplotype estimation procedures did not reveal any additional association information (data not shown).

Discussion

In this study, we have identified, by re-sequencing, novel intronic SNPs in the *ACVR2A* gene which are in LD with each other and which are associated with pre-eclampsia. These results are consistent with our previous preliminary association analysis of the *ACVR2A* gene (Moses *et al.*, 2006), where known SNPs in *ACVR2A* displayed association in a subset of families (The 34 Family Cohort) of the larger family cohort used in this current study (The 74 Family Cohort). The fact that these three SNPs have no correlation with any other SNP tested suggests that this association would not have been detected using commercial SNP arrays or experiments designed using SNPs from the public databases. At present, it is not clear how these variants influence the expression or function of *ACVR2A*. Even though no correlation is seen with this haplotype and any other SNP tested, it is still not possible to determine whether they are in LD with more distant variants without deeper re-sequencing efforts.

We were not able to replicate the rs1424954 SNP association previously reported by Moses *et al.* (2006). This association was detected in the 34 Family Cohort used for the original chromosome 2 localization (Moses *et al.*, 2000). These families were chosen for the

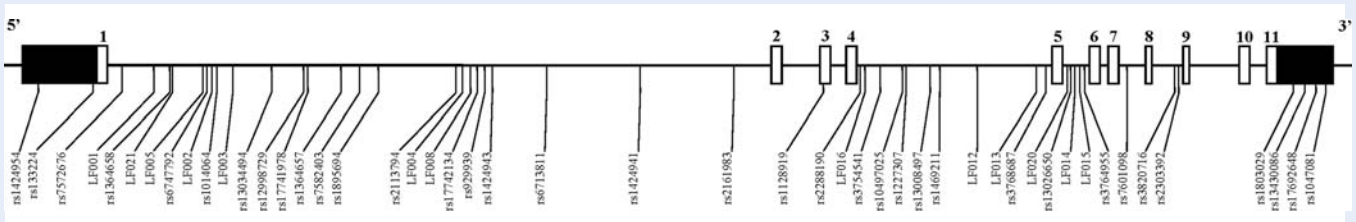

Figure 1 Schematic representation of the *ACVR2A* gene.The open boxes represent the 11 numbered exons and the solid boxes represent the 5′ and 3′ untranslated regions, respectively. The location of the 49 polymorphisms identified by re-sequencing are positioned below. The polymorphisms are labelled with their unique identifiers [rs-numbered SNPs deposited in NCBI's dbSNP or LF-numbered variants (SNPs or insertion deletions) assigned by the investigators to novel polymorphisms].

Table III Association analyses of SNPs identified by re-sequencing of *ACVR2A* using the 'Mild diagnostic criteria'.

SNP	Population stratification	QTDT	QTLD
	P-values		
rs1424954	0.477	0.166	0.128
LF001	1.000	1.000	0.217
rs1364658	0.381	0.172	0.134
rs6747792	0.829	0.784	0.217
rs1014064	0.478	0.166	0.128
LF003	0.847	0.542	0.278
rs12998729	0.381	0.172	0.134
rs17741978	1.000	1.000	0.217
rs1364657	0.477	0.210	0.625
rs7582403	0.128	0.116	0.240
rs1895694	0.087	0.078	0.128
rs2113794	0.087	0.078	0.128
LF004	0.293	0.757	0.149
rs17742134	1.000	1.000	0.217
rs929939	1.000	0.452	0.156
rs6713811	0.060	0.079	0.132
rs1424941	1.000	1.000	0.217
rs2161983	0.339	0.169	0.136
rs1128919	0.478	0.166	0.128
rs2288190	0.253	0.101	0.133
rs3754541	1.000	1.000	0.217
rs10497025	**0.157**	**0.054**	**0.025**
rs13008497	0.381	0.172	0.134
rs1469211	1.000	1.000	0.217
LF012	0.602	0.978	0.284
LF013	0.293	0.757	0.149
rs3768687	0.477	0.166	0.128
LF020	0.960	1.000	0.150
rs13026650	0.477	0.166	0.128
rs3764955	0.381	0.172	0.134
rs7601098	0.477	0.210	0.625
rs3820716	0.183	0.221	0.216
rs2303392	0.323	0.168	0.135
rs13430086	**0.023**	**0.010**	**0.035**
rs17692648	0.959	0.662	0.141

Table IV Association analyses of SNPs identified by re-sequencing of *ACVR2A* using the 'Strict diagnostic criteria'.

SNP	Population stratification	QTDT	QTLD
	P-values		
rs1424954	0.406	0.427	0.412
LF001	0.054	0.129	0.642
rs1364658	0.337	0.428	0.423
rs6747792	0.007	0.074	0.670
rs1014064	0.406	0.427	0.412
LF003	0.983	0.425	0.127
rs12998729	0.337	0.428	0.423
rs17741978	0.054	0.129	0.642
rs1364657	0.729	1.000	0.818
rs7582403	0.071	0.286	0.671
rs1895694	0.041	0.175	0.344
rs2113794	0.041	0.175	0.344
LF004	**0.941**	**0.226**	**0.018**
rs17742134	0.054	0.129	0.642
rs929939	0.476	0.727	0.674
rs6713811	0.115	0.329	0.346
rs1424941	0.054	0.129	0.642
rs2161983	0.387	0.614	0.578
rs1128919	0.406	0.427	0.412
rs2288190	0.870	0.852	0.886
rs3754541	0.054	0.129	0.642
rs10497025	0.318	0.471	0.504
rs13008497	0.337	0.428	0.423
rs1469211	0.054	0.129	0.642
LF012	0.656	0.696	0.132
LF013	**0.941**	**0.226**	**0.018**
rs3768687	0.406	0.427	0.412
LF020	**0.944**	**0.226**	**0.018**
rs13026650	0.406	0.427	0.412
rs3764955	0.337	0.428	0.422
rs7601098	0.729	1.000	0.818
rs3820716	0.017	0.070	0.185
rs2303392	0.277	0.683	0.573
rs13430086	0.010	0.051	0.273
rs17692648	0.068	0.109	0.722

genome-wide linkage scan as they were mostly large three-generation pedigrees with multiple members affected. Increasing sample size by the addition of extra families has the potential to increase power to detect variants with small genetic effects (Terwilliger and Goring, 2000). However, this also introduces the potential to dilute genetic effects by introducing greater genetic heterogeneity. The additional 40 families included in this study were smaller pedigrees with fewer affected and these may have diluted the effect of the rs1424954 polymorphism and introduced additional functional variation detected here. In addition, the five SNPs chosen from the previous pilot study were tag SNPs not chosen for any potential functional contribution. In this current study, the re-sequencing of conserved gene regions was more likely to identify polymorphisms to be functional.

Most studies looking for genetic variation affecting common disease focus on alleles altering the coding sequence of genes. However, studies now often identify non-coding variation influencing human disease [e.g. Type II diabetes and serum triglycerides (Saxena *et al.*, 2007), schizophrenia (Law *et al.*, 2006) and psoriasis (Capon *et al.*, 2004)]. This is also consistent with what is known of the genetics that underlie similar phenotypes in other organisms (Hendrich and

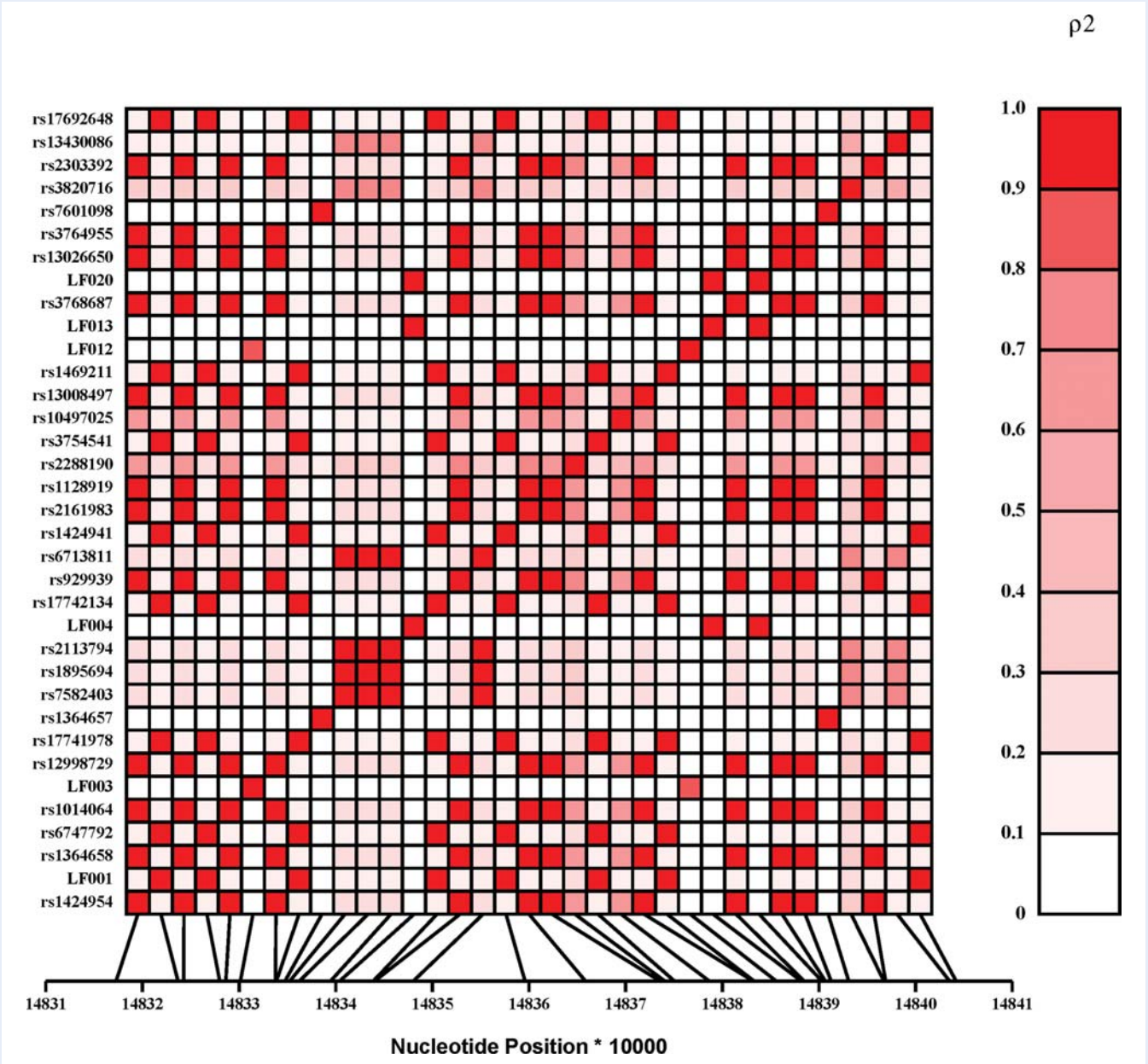

Figure 2 Linkage disequilibrium (LD) plot of all genotyped *ACVR2A* SNPs. The metric to the right of the plot (ρ^2) indicates the strength of the correlation among genotypes at each SNP locus with 0.0 indicating no correlation (i.e. LD) and 1.0 indicating a perfect correlation (i.e. complete LD).

Willard, 1995; Mackay, 1996). The idea that common non-coding SNPs are responsible for many complex human disorders fits well in explaining traits that typically arise after decades of life, such as diabetes or cardiovascular disease, or, in the case of pre-eclampsia, only during pregnancy. Regulatory regions may be particularly relevant to chronic diseases where response to homeostatic epistasis or environmental variation results in gradual accumulation of damage over many years before reaching a critical threshold (Weiss and Terwilliger, 2000). In the case of pre-eclampsia, the effects could be brought about due to the increased physiological demands of pregnancy.

The three novel intronic SNPs that initially indicated association with pre-eclampsia in this present study are all in regions not previously recognized as having regulatory function. If these conserved regions are unidentified regulatory sequences, then these polymorphisms could have the potential to affect the transcription of *ACVR2A*. This would be consistent with the perturbations seen in the circulating levels of activin A during pre-eclamptic pregnancies (described below) and with emerging knowledge of the complexity of transcriptional regulation. For example, a recent study by Carninci *et al*. (2005) identified hundreds of transcription sites, many located within internal exons and introns of protein coding genes, indicating that promoter sites are common and that transcriptional organization is complex. This transcriptional architecture implies that most genomic regions serve multiple functions. This is the reason behind our re-sequencing strategy in this current study, whereby in an effort to capture all

potentially functional genetic variation in the *ACVR2A* gene, all intragenic DNA regions showing evolutionary conservation in addition to the coding, promoter and 3′UTR sequences were re-sequenced, based on the hypothesis that those regions showing evolutionary conservation are more likely to harbour regulatory sequences.

ACVR2A is a receptor for the cell signalling protein activin A that is known to be an important regulator of reproductive function. Activin A belongs to the TGF-β super family of genes and is known to regulate cell differentiation, proliferation and apoptosis and is found in a variety of cells including the endometrium, placenta and vascular endothelium. Activin A has key roles in promoting decidualization of endometrial stromal cells (Jones *et al*., 2002) and in the regulation of trophoblast differentiation and invasion into the decidua (Caniggia *et al*., 1997). This means the normal physiological remodelling of the spiral arteries that is reduced in pre-eclampsia is highly dependent on activin A signalling through ACVR2A. Activin A also has an established role in endothelial functioning and vascular homeostasis, where altered ACVR2A binding could result in endothelial dysfunction triggering systemic inflammation, hypertension, proteinuria and oedema.

There have been several studies, looking at maternal serum in pre-eclampsia patients, which have shown increased circulating activin A concentrations compared with gestational matched controls (Petraglia *et al*., 1995a, b; Muttukrishna *et al*., 1997; Fraser *et al*., 1998; Laivuori *et al*., 1999; Silver *et al*., 1999; D'Antona *et al*., 2000). The main source of this increased activin A in maternal blood during pregnancy is thought to originate from the placenta (Petraglia *et al*., 1987). It is possible that in a proportion of cases, altered *ACVR2A* expression would have a similar effect as altered activin A expression, resulting in an unbalancing event that in combination with an ischaemic placenta could initiate the pathophysiological cascade leading to the maternal syndrome. Changes in the expression or action of the activin A receptors, specifically ACVR2A, could increase the activity of already increased levels of activin A, exacerbating the endothelial activation. This extends further, explaining why in some women the insufficient modification of spiral arteries and subsequent placental hypoxia and release of activin A are not enough to trigger the maternal syndrome of pre-eclampsia.

Our data were analysed under two different diagnostic criteria. Pre-eclampsia/eclampsia is one of a number of hypertensive disorders that complicate human pregnancy. A particular confounding problem associated with a working definition of pre-eclampsia is whether mild pre-eclampsia (pregnancy-induced hypertension without proteinuria), severe pre-eclampsia (pregnancy-induced hypertension with proteinuria) and eclampsia (severe pre-eclampsia with epileptic seizures) are a continuum of degrees of severity or whether each represents a distinct group.

The severe pre-eclampsia/eclampsia syndrome represents the greatest danger to the health and welfare of the mother and the fetus, whereas pregnancy-induced hypertension is a relatively benign disorder characterized by mild to moderate elevations of blood pressure late in pregnancy that return to normal post-partum (Roberts and Lain, 2002). It has been suggested that women who develop mild pre-eclampsia have a greater future risk of developing essential hypertension later in life than women with severe pre-eclampsia and eclampsia (Adams and Finlayson, 1961; Adams and Macgillivray, 1961). On the other hand, an increased risk of myocardial infarction (Mann *et al*., 1976) and ischaemic heart disease (Jonsdottir *et al*., 1995) has been demonstrated in women with severe pre-eclampsia or eclampsia but not in pregnancy-induced hypertension. The subgroups clearly share several pathological features, but differing future risk profiles suggest that the severe and mild phenotypes may have divergent aetiologies. This may explain the differing genetic associations identified between these two diagnostic groups in this study.

It has been suggested that pregnancy can unmask a woman's potential for disease, providing a window to her long-term health and presenting opportunities for primary prevention (Sattar and Greer, 2002). A major problem in the prevention of vascular disease has been the difficulty in identifying individuals at risk at an early enough stage for them to benefit from intervention such as modification of their lifestyle. If pre-eclampsia is confirmed as an indicator of increased vascular risk in mothers, the identification of genetic factors underlying susceptibility provides a great opportunity for screening and early intervention strategies for cardiovascular disease.

In this study, we identified, by re-sequencing, three novel, rare intronic SNPs in *ACVR2A* which are in strong LD with each other and which, on first assessment, showed significant evidence of association with pre-eclampsia, although this was not confirmed after correction for multiple testing. There are, however, compelling reasons not to rule out a role for this gene in genetic susceptibility to pre-eclampsia, including the biological role that activin A is known to play in the establishment of pregnancy (Petraglia *et al*., 1993; Caniggia *et al*., 1997; Jones *et al*., 2002), its apparent utility as a biological marker of pre-eclampsia (Petraglia *et al*., 1995a, b; Muttukrishna *et al*., 1997; Fraser *et al*., 1998; Laivuori *et al*., 1999; Silver *et al*., 1999; D'Antona *et al*., 2000) and the recent report of association between *ACVR2A* SNPs and pre-eclampsia in an independent Norwegian population (Roten *et al*., 2008). Taken together, this body of data should encourage further sufficiently powered studies to confirm the existence of risk polymorphisms in this gene, which may help to identify high-risk individuals and guide new diagnostic procedures or novel therapies for the treatment of pre-eclampsia.

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References

Abecasis GR, Cookson WO, Cardon LR. Pedigree tests of transmission disequilibrium. *Eur J Hum Genet* 2000;**8**:545–551.

Adams EM, Finlayson A. Familial aspects of pre-eclampsia and hypertension in pregnancy. *Lancet* 1961;**2**:1375–1378.

Adams EM, Macgillivray I. Long-term effect of preeclampsia on blood-pressure. *Lancet* 1961;**2**:1373–1375.

Agatisa PK, Ness RB, Roberts JM, Costantino JP, Kuller LH, McLaughlin MK. Impairment of endothelial function in women with a history of preeclampsia: an indicator of cardiovascular risk. *Am J Physiol Heart Circ Physiol* 2004;**286**:H1389–H1393.

Almasy L, Blangero J. Multipoint quantitative-trait linkage analysis in general pedigrees. *Am J Hum Genet* 1998;**62**:1198–1211.

Arngrímsson R, Sigurard ttir S, Frigge ML, Bjarnad ttir RI, Jonsson T, Stefansson H, Baldursdottir A, Einarsdottir AS, Palsson B, Snorradottir S *et al*. A genome-wide scan reveals a maternal susceptibility locus for pre-eclampsia on chromosome 2p13. *Hum Mol Genet* 1999;**8**:1799–1805.

Boerwinkle E, Chakraborty R, Sing CF. The use of measured genotype information in the analysis of quantitative phenotypes in man. I. Models and analytical methods. *Ann Hum Genet* 1986; **50**:181–194.

Brown MA, Gallery EDM, Gatt SP, Leslie G, Robinson J. Management of hypertension in pregnancy: executive summary. *Med J Aust* 1993; **158**:700–702.

Caniggia I, Lye SJ, Cross JC. Activin is a local regulator of human cytotrophoblast cell differentiation. *Endocrinology* 1997;**138**:3976–3986.

Capon F, Allen MH, Ameen M, Burden AD, Tillman D, Barker JN, Trembath RC. A synonymous SNP of the corneodesmosin gene leads to increased mRNA stability and demonstrates association with psoriasis across diverse ethnic groups. *Hum Mol Genet* 2004; **13**:2361–2368.

Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, Oyama R, Ravasi T, Lenhard B, Wells C *et al*. The transcriptional landscape of the mammalian genome. *Science* 2005;**309**:1559–1563.

Carr DB, Epplein M, Johnson CO, Easterling TR, Critchlow CW. A sister's risk: family history as a predictor of preeclampsia. *Am J Obstet Gynecol* 2005;**193**:965–972.

Chambers JC, Fusi L, Malik IS, Haskard DO, De Swiet M, Kooner JS. Association of maternal endothelial dysfunction with preeclampsia. *JAMA* 2001;**285**:1607–1612.

Chesley LC. Cardiovascular changes in pregnancy. *Obstet Gynecol Annu* 1975;**4**:71–97.

Chesley LC, Annitto JE, Cosgrove RA. The familial factor in toxemia of pregnancy. *Obstet Gynecol* 1968;**32**:303–311.

D'Antona D, Reis FM, Benedetto C, Evans LW, Groome NP, de Kretser DM, Wallace EM, Petraglia F. Increased maternal serum activin A but not follistatin levels in pregnant women with hypertensive disorders. *J Endocrinol* 2000;**165**:157–162.

Duley L. Maternal mortality associated with hypertensive disorders of pregnancy in Africa, Asia, Latin America and the Caribbean. *Br J Obstet Gynaecol* 1992;**99**:547–553.

Fisher KA, Luger A, Spargo BH, Lindheimer MD. Hypertension in pregnancy: clinical-pathological correlations and remote prognosis. *Medicine (Baltimore)* 1981;**60**:267–276.

Fitzpatrick E, Goring HH, Liu H, Borg A, Forrest S, Cooper DW, Brennecke SP, Moses EK. Fine mapping and SNP analysis of positional candidates at the preeclampsia susceptibility locus (PREG1) on chromosome 2. *Hum Biol* 2004;**76**:849–862.

Fraser RF 2nd, McAsey ME, Coney P. Inhibin-A and pro-alpha C are elevated in preeclamptic pregnancy and correlate with human chorionic gonadotropin. *Am J Reprod Immunol* 1998;**40**:37–42.

Funai EF, Friedlander Y, Paltiel O, Tiram E, Xue X, Deutsch L, Harlap S. Long-term mortality after preeclampsia. *Epidemiology* 2005;**16**:206–215.

Grobman WA, Wang EY. Serum levels of activin A and inhibin A and the subsequent development of preeclampsia. *Obstet Gynecol* 2000; **96**:390–394.

Havill LM, Dyer TD, Richardson DK, Mahaney MC, Blangero J. The quantitative trait linkage disequilibrium test: a more powerful alternative to the quantitative transmission disequilibrium test for use in the absence of population stratification. *BMC Genet* 2005; **6**(Suppl 1):S91.

Hendrich BD, Willard HF. Epigenetic regulation of gene expression: the effect of altered chromatin structure from yeast to mammals. *Hum Mol Genet* 1995;**4**:1765–1777.

Hubel CA, Snaedal S, Ness RB, Weissfeld LA, Geirsson RT, Roberts JM, Arngrimsson R. Dyslipoproteinaemia in postmenopausal women with a history of eclampsia. *BJOG* 2000;**107**:776–784.

Irgens HU, Reisaeter L, Irgens LM, Lie RT. Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study. *Br Med J* 2001;**323**:1213–1217.

Jones RL, Salamonsen LA, Findlay JK. Activin A promotes human endometrial stromal cell decidualization in vitro. *J Clin Endocrinol Metab* 2002;**87**:4001–4004.

Jonsdottir LS, Arngrimsson R, Geirsson RT, Sigvaldason H, Sigfusson N. Death rates from ischemic heart disease in women with a history of hypertension in pregnancy. *Acta Obstet Gynecol Scand* 1995;**74**:772–776.

Lachmeijer AM, Arngrimsson R, Bastiaans EJ, Frigge ML, Pals G, Sigurdardottir S, Stefansson H, Palsson B, Nicolae D, Kong A *et al*. A genome-wide scan for preeclampsia in the Netherlands. *Eur J Hum Genet* 2001;**9**:758–764.

Laivuori H, Kaaja R, Turpeinen U, Stenman UH, Ylikorkala O. Serum activin A and inhibin A elevated in pre-eclampsia: no relation to insulin sensitivity. *Br J Obstet Gynaecol* 1999;**106**:1298–1303.

Laivuori H, Kaaja R, Koistinen H, Karonen SL, Andersson S, Koivisto V, Ylikorkala O. Leptin during and after preeclamptic or normal pregnancy: its relation to serum insulin and insulin sensitivity. *Metabolism* 2000;**49**:259–263.

Laivuori H, Lahermo P, Ollikainen V, Widen E, Haiva-Mallinen L, Sundstrom H, Laitinen T, Kaaja R, Ylikorkala O, Kere J. Susceptibility loci for preeclampsia on chromosomes 2p25 and 9p13 in Finnish families. *Am J Hum Genet* 2003;**72**:168–177.

Law AJ, Lipska BK, Weickert CS, Hyde TM, Straub RE, Hashimoto R, Harrison PJ, Kleinman JE, Weinberger DR. Neuregulin 1 transcripts are differentially expressed in schizophrenia and regulated by 5′ SNPs associated with the disease. *Proc Natl Acad Sci USA* 2006; **103**:6747–6752.

Lie RT, Rasmussen S, Brunborg H, Gjessing HK, Lie-Nielsen E, Irgens LM. Fetal and maternal contributions to risk of pre-eclampsia: population based study. *Br Med J* 1998;**316**:1343–1347.

Mackay TF. The nature of quantitative genetic variation revisited: lessons from Drosophila bristles. *Bioessays* 1996;**18**:113–121.

Mann JI, Doll R, Thorogood M, Vessey MP, Waters WE. Risk factors for myocardial infarction in young women. *Br J Prev Soc Med* 1976; **30**:94–100.

Moses EK, Lade JA, Guo G, Wilton AN, Grehan M, Freed K, Borg A, Terwilliger JD, North R, Cooper DW *et al*. A genome scan in families from Australia and New Zealand confirms the presence of a maternal susceptibility locus for pre-eclampsia, on chromosome 2. *Am J Hum Genet* 2000;**67**:1581–1585.

Moses EK, Fitzpatrick E, Freed KA, Dyer TD, Forrest S, Elliott K, Johnson MP, Blangero J, Brennecke SP. Objective prioritization of positional candidate genes at a quantitative trait locus for pre-eclampsia on 2q22. *Mol Hum Reprod* 2006;**12**:505–512.

Muttukrishna S, Child TJ, Groome NP, Ledger WL. Source of circulating levels of inhibin A, pro alpha C-containing inhibins and activin A in early pregnancy. *Hum Reprod* 1997;**12**:1089–1093.

Muttukrishna S, North RA, Morris J, Schellenberg JC, Taylor RS, Asselin J, Ledger W, Groome N, Redman CW. Serum inhibin A and activin A are

elevated prior to the onset of pre-eclampsia. *Hum Reprod* 2000; **15**:1640–1645.

Nilsson E, Salonen Ros H, Cnattingius S, Lichtenstein P. The importance of genetic and environmental effects for pre-eclampsia and gestational hypertension: a family study. *BJOG* 2004;**111**:200–206.

Peng C, Huang TH, Jeung EB, Donaldson CJ, Vale WW, Leung PC. Expression of the type II activin receptor gene in the human placenta. *Endocrinology* 1993;**133**:3046–3049.

Petraglia F, Sawchenko P, Lim AT, Rivier J, Vale W. Localization, secretion, and action of inhibin in human placenta. *Science* 1987; **237**:187–189.

Petraglia F, Anceschi MM, Calza L, Garuti GC, Fusaro P, Giardino L, Genazzani AR, Vale W. Inhibin and activin in human fetal membranes: evidence for a local effect on prostaglandin release. *J Clin Endocrinol Metab* 1993;**77**:542–548.

Petraglia F, Aguzzoli L, Gallinelli A, Florio P, Zonca M, Benedetto C, Woodruff K. Hypertension in pregnancy: changes in activin A maternal serum concentration. *Placenta* 1995a;**16**:447–454.

Petraglia F, De Vita D, Gallinelli A, Aguzzoli L, Genazzani AR, Romero R, Woodruff TK. Abnormal concentration of maternal serum activin-A in gestational diseases. *J Clin Endocrinol Metab* 1995b;**80**:558–561.

Prabhakar S, Poulin F, Shoukry M, Afzal V, Rubin EM, Couronne O, Pennacchio LA. Close sequence comparisons are sufficient to identify human cis-regulatory elements. *Genome Res* 2006;**16**:855–863.

Roberts JM, Gammill HS. Preeclampsia: recent insights. *Hypertension* 2005; **46**:1243–1249.

Roberts JM, Lain KY. Recent Insights into the pathogenesis of pre-eclampsia. *Placenta* 2002;**23**:359–372.

Roberts JM, Pearson G, Cutler J, Lindheimer M. Summary of the NHLBI Working Group on Research on Hypertension During Pregnancy. *Hypertension* 2003;**41**:437–445.

Roten LT, Johnson MP, Forsmo S, Fitzpatrick E, Dyer TD, Brennecke SP, Blangero J, Moses EK, Austgulen R. Association between the candidate susceptibility gene ACVR2A on chromosome 2q22 and pre-eclampsia in a large Norwegian population-based study (the HUNT study). *Eur J Hum Genet.* 2008;**17**:250–257.

Salonen Ros H, Lichtenstein P, Lipworth L, Cnattingius S. Genetic effects on the liability of developing pre-eclampsia and gestational hypertension. *Am J Med Genet* 2000;**91**:256–260.

Sattar N, Greer IA. Pregnancy complications and maternal cardiovascular risk: opportunities for intervention and screening? *Br Med J* 2002; **325**:157–160.

Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, Roix JJ, Kathiresan S, Hirschhorn JN, Daly MJ *et al.* Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* 2007;**316**:1331–1336.

Silver HM, Lambert-Messerlian GM, Star JA, Hogan J, Canick JA. Comparison of maternal serum total activin A and inhibin A in normal, preeclamptic, and nonproteinuric gestationally hypertensive pregnancies. *Am J Obstet Gynecol* 1999;**180**:1131–1137.

Sobel E, Lange K. Descent graphs in pedigree analysis: applications to haplotyping, location scores, and marker-sharing statistics. *Am J Hum Genet* 1996;**58**:1323–1337.

Terwilliger JD, Goring HH. Gene mapping in the 20th and 21st centuries: statistical methods, data analysis, and experimental design. *Hum Biol* 2000;**72**:63–132.

Weiss KM, Terwilliger JD. How many diseases does it take to map a gene with SNPs? *Nat Genet* 2000;**26**:151–157.

Witlin AG, Sibai BM. Hypertension in pregnancy: current concepts of preeclampsia. *Annu Rev Med* 1997;**48**:115–127.

Zwahlen M, Gerber S, Bersinger NA. First Trimester Markers for Pre-Eclampsia: Placental vs. Non-Placental Protein Serum Levels. *Gynecol Obstet Invest* 2007;**63**:15–21.

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