

## Loci at chromosomes 13, 19 and 20 influence age at natural menopause

Lisette Stolk<sup>1-3,11</sup>, Guangju Zhai<sup>4,11</sup>, Joyce B J van Meurs<sup>1,3</sup>, Michael M P J Verbiest<sup>1</sup>, Jenny A Visser<sup>1</sup>, Karol Estrada<sup>1</sup>, Fernando Rivadeneira<sup>1,2</sup>, Frances M Williams<sup>4</sup>, Lynn Cherkas<sup>4</sup>, Panos Deloukas<sup>5</sup>, Nicole Soranzo<sup>5</sup>, Jules J de Keyser<sup>6</sup>, Victor J M Pop<sup>7</sup>, Paul Lips<sup>8</sup>, Corinne E I Lebrun<sup>1,9</sup>, Yvonne T van der Schouw<sup>9</sup>, Diederick E Grobbee<sup>9</sup>, Jacqueline Witteman<sup>2,3</sup>, Albert Hofman<sup>2,3</sup>, Huibert A P Pols<sup>1</sup>, Joop S E Laven<sup>10</sup>, Tim D Spector<sup>4,11</sup> & André G Uitterlinden<sup>1-3,11</sup>

**We conducted a genome-wide association study for age at natural menopause in 2,979 European women and identified six SNPs in three loci associated with age at natural menopause: chromosome 19q13.4 (rs1172822; -0.4 year per T allele (39%);  $P = 6.3 \times 10^{-11}$ ), chromosome 20p12.3 (rs236114; +0.5 year per A allele (21%);  $P = 9.7 \times 10^{-11}$ ) and chromosome 13q34 (rs7333181; +0.5 year per A allele (12%);  $P = 2.5 \times 10^{-8}$ ). These common genetic variants regulate timing of ovarian aging, an important risk factor for breast cancer, osteoporosis and cardiovascular disease.**

Menopause, the time of a woman's life when menstrual cycle ceases owing to depletion of the follicle pool, is a key event in reproductive aging. It influences a woman's well-being and is an important risk factor for several major age-related diseases including cardiovascular disease, breast cancer and osteoporosis<sup>1</sup>. Age at menopause averages around 50–51 years and ranges between 40 and 60 years of age<sup>2</sup>; twin studies have shown this variability to be genetically determined with heritabilities of 44–65%<sup>3-5</sup>. Such genetic factors might regulate the size of the follicle pool and the rate of its depletion, and their identification could have biological and clinical applications.

Typical for complex quantitative traits, genome-wide linkage studies of menopause have been unsuccessful, and candidate gene studies have mainly focused on the estrogen pathway<sup>6</sup> and have had conflicting results<sup>7</sup>. This suggests that the apparent effect sizes for genetic variants are small and that the major causative loci have not been identified. Genome-wide association studies (GWAS) have proven successful in identifying common susceptibility genes with

small effect sizes for many complex diseases and traits<sup>8</sup> and might be suitable to identify genetic factors involved in determining age at menopause.

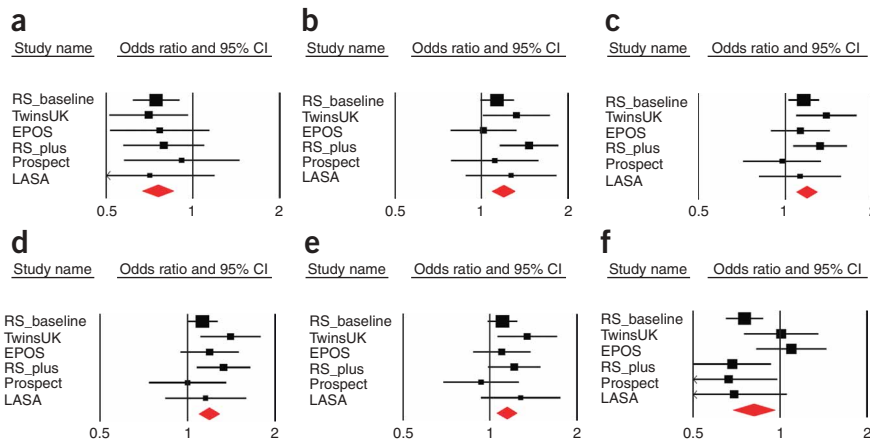
In this GWAS we used a two-stage design to identify previously unknown loci influencing age at menopause. We included women with self-reported natural age at menopause (defined as 12 months without regular periods) between 40 and 60 years, excluding those with hysterectomy, uni- or bilateral ovariectomy, menopause induced by irradiation or occurring after stopping the contraceptive pill, or those currently using hormone replacement therapy.

In stage 1 we genotyped 2,368 women of the Rotterdam Study baseline<sup>9</sup> with the Illumina HumanHap 550v3 Beadarray. After quality control, 535,354 SNPs were left for analysis. Allelic association tests were carried out using PLINKv1.01 software<sup>10</sup> for age at natural menopause. The genomic inflation factor ( $\lambda$ ) was 1.01669 for this analysis, indicating no population stratification, so we based our results on the uncorrected  $P$  values. The strongest association signals were found for rs2151145 ( $P = 5.3 \times 10^{-6}$ ) on chromosome 9, rs236114 ( $P = 5.6 \times 10^{-6}$ ) on chromosome 20 and rs1172822 ( $P = 6.3 \times 10^{-6}$ ) on chromosome 19 (**Supplementary Table 1** and **Supplementary Fig. 1a** online).

We combined the results from the Rotterdam Study baseline with GWA data from the TwinsUK study. A total of 611 women with natural menopause using the same definitions and exclusions as above were genotyped with the Illumina HumanHap 300K beadarray, and after quality control 317,818 SNPs were left for analysis. After adjusting for relatedness and genomic control, we did not observe any genome-wide significant signals in this study (**Supplementary Table 1** and **Supplementary Fig. 1b**). Because of the different study designs we conducted meta-analysis on summary statistics of the two studies using METAL (<http://www.sph.umich.edu/csg/abecasis/metal/>; **Supplementary Methods** online) on 315,418 SNPs common to both cohorts (2,979 women), but we did not observe any genome-wide significant SNPs (**Supplementary Fig. 1c**).

From this meta-analysis, all SNPs with  $P < 1 \times 10^{-4}$ , corresponding to 32 SNPs from 24 loci (with five loci having multiple significant SNPs), were followed up in stage 2 (**Supplementary Table 1**). Twenty-four SNPs were genotyped using Sequenom iPLEX genotyping and seven SNPs using Taqman allelic discrimination (Applied Biosystems) (**Supplementary Methods**) in 2,560 samples of four additional cohorts of postmenopausal females of European ancestry (**Supplementary Methods and Supplementary Table 2** online); one of the SNPs (rs11786333) failed genotyping. For the remaining 31

<sup>1</sup>Departments of Internal Medicine and <sup>2</sup>Epidemiology, Erasmus MC, Rotterdam, The Netherlands. <sup>3</sup>The Netherlands Genomics Initiative-sponsored Netherlands Consortium for Healthy Aging (NGI-NCHA), Rotterdam, The Netherlands. <sup>4</sup>Department of Twin Research and Genetic Epidemiology, King's College London, London, UK. <sup>5</sup>Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, UK. <sup>6</sup>Research Unit, Diagnostic Center Eindhoven, Eindhoven, The Netherlands. <sup>7</sup>Department of Clinical Health Psychology, University of Tilburg, Tilburg, The Netherlands. <sup>8</sup>Department of Internal Medicine, Endocrine Section, and EMGO Institute, VU University Medical Center, Amsterdam, The Netherlands. <sup>9</sup>Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, The Netherlands. <sup>10</sup>Department of Obstetrics and Gynaecology, Erasmus MC, Rotterdam, The Netherlands. <sup>11</sup>These authors contributed equally to this work. Correspondence should be addressed to A.G.U. (a.g.uitterlinden@erasmusmc.nl).



**Figure 1** Meta-analysis of risk for early menopause (<50 years) by genotype for the six genome-wide-significant hits. (a) rs7333181 on chromosome 13. (b) rs1551562 on chromosome 19. (c) rs1172822 on chromosome 19. (d) rs2384687 on chromosome 19. (e) rs897798 on chromosome 19. (f) rs236114 on chromosome 20.

SNPs, we calculated combined  $P$  values, betas and standard errors using inverse variance fixed-effects meta-analysis (**Supplementary Table 1**) and identified six common SNPs that were genome-wide significant in the combined stage 1 and 2 analysis (**Table 1**). Four SNPs on chromosome 19 were significant: rs1172822 (MAF = 0.39),  $P = 6.28 \times 10^{-11}$ , beta =  $-0.391$  year per T allele (s.e.m. = 0.0598); rs2384687 (MAF = 0.40),  $P = 1.39 \times 10^{-10}$ , beta = 0.381 year per C allele (s.e.m. = 0.0594); rs1551562 (MAF = 0.25),  $P = 1.04 \times 10^{-9}$ , beta = 0.4279 year per G allele (s.e.m. = 0.0701); and rs897798 (MAF = 0.48),  $P = 3.91 \times 10^{-8}$ , beta = 0.308 year per G allele (s.e.m. = 0.056). These four SNPs are likely to report the same signal because the linkage is high ( $D' > 0.92$ ,  $r^2 > 0.5$ , **Supplementary Fig. 2b** online).

On chromosome 20, rs236114 (MAF = 0.21) was genome-wide significantly associated with age at natural menopause ( $P = 9.71 \times 10^{-11}$ , beta = 0.4953 year per A allele (s.e.m. = 0.0765)). Furthermore, on chromosome 13 rs7333181 (MAF = 0.12) was genome-wide significant:  $P = 2.50 \times 10^{-8}$ , beta = 0.5201 (s.e.m. = 0.0933). The six genome-wide-significant hits showed no heterogeneity ( $I^2 < 25\%$ ), so fixed effects models were used.

In addition, we estimated the risk for menopause before the age of 50 by allele of the six genome-wide significant SNPs (**Fig. 1**). We conducted fixed-effects meta-analysis for SNPs not showing heterogeneity (rs7333181, rs1551562, rs1172822, rs2384687, rs897798), and random effects meta-analysis for rs236114, for which  $I^2$  was 31%. This meta-analysis showed that the A allele of rs1172822 is associated with a 19% increased risk for natural menopause before 50 years (OR = 1.19,

95% CI = 1.09–1.29,  $P = 6.2 \times 10^{-5}$ ). The other SNPs on chromosome 13, 19 and 20 showed a similar increase or decrease in risk.

The initial analysis was not adjusted for covariates such as age, body mass index, smoking, age at menarche, parity and use of oral contraceptives and female hormones. To rule out an effect of these covariates on the association of the genome-wide-significant hits, we carried out adjusted linear regression of these SNPs in the Rotterdam Study baseline cohort (**Supplementary Table 3** online). None of the previously found associations was affected by the adjustment for these covariates, indicating that the effect of the SNP occurs directly on age at natural menopause and not via one of the covariates. We calculated the total explained variance in age at natural menopause for these SNPs in the combined replication studies to be 1.1% (range 0.1–0.5% per SNP).

We then conducted fine mapping of these signals using meta-analysis of imputed data of the stage 1 studies, and found three SNPs on chromosome 13 with more or equal significance as rs7333181, two SNPs on chromosome 19 and one on chromosome 20 with higher significance compared to the previously reported SNPs (**Supplementary Table 4** and **Supplementary Fig. 2** online). For all three loci, the imputed SNPs are located in the same linkage disequilibrium (LD) block as the genome-wide-significant SNPs.

The four chromosome 19 SNPs are located within an LD block covering almost 20 kb and are located intronic and 3' of the *BRSK1* gene (BR serine threonine kinase 1), in the 3' region and inside the *TMEM224* gene, and 5' of the *SUV420H2* gene (suppressor of variegation 4-20 homolog 2, a lysine methyltransferase) (**Supplementary Fig. 2b**). Literature analysis for these genes did not indicate an immediate functional explanation for the observed association, although for all three genes a possible involvement in ovarian aging was suggested (**Supplementary Note** online).

rs7333181 on chromosome 13 is located >250 kb 3' of the hypothetical gene *LOC121793* and the *ARHGEF7* (rho guanine nucleotide exchange factor 7) gene, also known as *COOL1* (cloned out of library 1). *ARHGEF7* has a role in cell proliferation through phosphorylation of FOXO3a. FOXO3a knockout mice are infertile due to early depletion of the follicle pool, indicating a possible role of this gene in menopause (**Supplementary Note**). The chromosome 20 SNP is located in an intron of the *MCM8* (minichromosome maintenance complex component 8) gene. The more significantly associated SNP

**Table 1** The six genome-wide significant SNPs and association with age at menopause

SNP	Chr	Position	Minor allele	MAF	Stage 1	Overall meta-analysis		
					$P$ value	$P$ value	Effect size	s.e.
rs1172822	19q13.4	60511657	T	0.39	1.94E-07	6.28E-11	0.391	0.060
rs236114	20p12.3	5883385	A	0.21	1.83E-05	9.71E-11	0.495	0.077
rs2384687	19q13.4	60523000	C	0.40	1.57E-06	1.39E-10	-0.381	0.059
rs1551562	19q13.4	60506693	G	0.25	1.02E-06	1.04E-09	-0.428	0.070
rs7333181	13q34	111019298	A	0.12	5.26E-05	2.50E-08	0.520	0.093
rs897798	19q13.4	60525566	G	0.48	7.13E-06	3.91E-08	-0.308	0.056

from the imputed data is a nonsynonymous SNP in exon 9 of this gene (E341K), and could influence the protein structure or function of MCM8. For the gene on chromosome 20 no involvement in ovarian aging or menopause was suggested.

Identification of the causative variant(s) and the responsible gene(s) underlying the observed associations requires further research, which will enhance our molecular understanding of the genetic regulation of the ovarian reserve and aging process. Although the rate of ovarian aging is highly variable among women, identification of women with decreased ovarian reserve is clinically relevant, as timing of menopause is an important risk factor, for example, for breast cancer, cardiovascular disease and osteoporosis.

*Note: Supplementary information is available on the Nature Genetics website.*

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#### AUTHOR CONTRIBUTIONS

L.S., G.Z., T.D.S. and A.G.U. designed the study. L.S. and M.M.P.J.V. did the genotyping of the replication studies. P.D. and N.S. did the genotyping for the TwinsUK study. N.S. did the quality control for the TwinsUK data. L.S., G.Z., J.B.J.v.M. and K.E. did the statistical analyses. L.S., G.Z., J.B.J.v.M., J.A.V., F.R., J.S.E.L., T.D.S. and A.G.U. helped with the interpretation of the results. L.S., G.Z., J.B.J.v.M., J.A.V., F.R., T.D.S. and A.G.U. prepared the manuscript and the revision of the manuscript. F.M.W., A.H. and H.A.P.P. critically revised the manuscript. A.H., J.W., H.A.P.P. and A.G.U. were involved in the sample and phenotype collection of the Rotterdam Study. F.M.W. and L.C. were involved in the phenotype collection of the TwinsUK Study. J.J.d.K., V.J.M.P., P.L., C.E.I.L., Y.T.v.d.S. and D.E.G. were involved in the sample and data collection of the replication studies.

#### COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturegenetics/>.

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1. Burger, H.G. *Baillieres Clin. Obstet. Gynaecol.* **10**, 347–359 (1996).
2. te Velde, E.R. *et al. Maturitas* **30**, 119–125 (1998).
3. Murabito, J.M. *et al. J. Clin. Endocrinol. Metab.* **90**, 3427–3430 (2005).
4. Snieder, H. *et al. J. Clin. Endocrinol. Metab.* **83**, 1875–1880 (1998).
5. van Asselt, K.M. *et al. Fertil. Steril* **82**, 1348–1351 (2004).
6. Weel, A.E. *et al. J. Clin. Endocrinol. Metab.* **84**, 3146–3150 (1999).
7. Kok, H.S. *et al. Hum. Reprod.* **20**, 536–542 (2005).
8. McCarthy, M.I. *et al. Nat. Rev. Genet.* **9**, 356–369 (2008).
9. Hofman, A. *et al. Eur. J. Epidemiol.* **22**, 819–829 (2007).
10. Purcell, S. *et al. Am. J. Hum. Genet.* **81**, 559–575 (2007).