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Genetic variation may modify ovarian reserve in female childhood cancer survivors

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STUDY QUESTION: Are genetic polymorphisms, previously identified as being associated with age at menopause in the healthy population, associated with ovarian reserve and predicted age at menopause in adult long-term survivors of childhood cancer?

SUMMARY ANSWER: The CT genotype of rs1172822 in the *BRSK1 gene* is associated with lower serum anti-Müllerian hormone (AMH) levels and a younger predicted age at menopause in adult survivors of childhood cancer.

WHAT IS KNOWN ALREADY: Gonadotoxicity is a well-known late side effect of chemotherapy and radiotherapy in adult survivors of childhood cancer. In the healthy population, several genetic polymorphisms are associated with age at natural menopause. Currently, data on the impact of previously identified variants in gene loci associated with ovarian reserve in adult long-term survivors of childhood cancer are lacking.

STUDY DESIGN, SIZE, DURATION: We performed a pilot study in a single-centre cohort of adult female Caucasian childhood cancer survivors ($n = 176$).

PARTICIPANTS/MATERIALS, SETTING, METHODS: We determined serum AMH levels (a marker of ovarian reserve) in adult survivors of childhood cancer ($n = 176$) and studied single nucleotide polymorphisms (SNPs) previously reported to be associated with age at natural menopause: *BRSK1* (rs1172822), *ARHGEF7* (rs7333181), *MCM8* (rs236114), *PCSK1* (rs271924), *IGF2R* (rs9457827) and *TNF* (rs909253). Association analysis was performed using the additive genetic model. Linear regression was conducted to assess the effect of significant polymorphisms in two previously published menopause prediction models.

MAIN RESULTS AND THE ROLE OF CHANCE: The CT genotype of rs1172822 in the *BRSK1* (BR serine/threonine kinase 1) gene was negatively associated with serum AMH levels in our cohort (odds ratio: 3.15, 95% confidence interval: 1.35–7.32, $P = 0.008$) and significantly associated with the predicted age at menopause ($P = 0.04$). The other five SNPs were not associated with serum AMH levels.

LIMITATIONS, REASONS FOR CAUTION: This is a pilot study showing preliminary data which must be confirmed. To confirm our findings and enlarge the project, a nationwide genome-wide association (GWA) project on the ovarian reserve in female survivors of childhood cancer should be performed, including a replication cohort.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings support the hypothesis that previously identified genetic polymorphisms associated with age at menopause in healthy women may have an effect on the onset of menopause in female survivors of childhood cancer. Our study highlights a new aspect of the influences on the ovarian reserve after childhood cancer, which should be investigated further in a nationwide GWA study. Eventually, this information can help us to improve counselling on fertility preservation prior to cancer treatment based on genetic factors in individual patients.

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Key words: childhood cancer survivors / ovarian reserve / polymorphism / *BRSK1* / anti-Müllerian hormone

Introduction

A well-known long-term side effect of cancer treatment in survivors of both adult and childhood cancer is gonadotoxicity (Lie Fong *et al.*, 2008, 2009). The extent of gonadal damage depends on the treatment modality and the total cumulative dose (Sklar *et al.*, 2006; Lie Fong *et al.*, 2009; van Casteren *et al.*, 2009; van Dorp *et al.*, 2012). In addition, the multi-drug approach may have a cumulative toxic effect on reproductive function (Wallace *et al.*, 2005; van Beek *et al.*, 2007).

Along with these iatrogenic variables, the genetic variation of individuals together with environmental influences (e.g. smoking) determines an individual's reproductive health. Hence, fertility can be regarded as a complex trait that is a result of the interplay between a person's genetic make-up and the environmental disruptors to which one was exposed.

In the general population, the age at natural menopause varies between 40 and 60 years, with a mean of 50–51 years, and is largely heritable (te Velde and Pearson, 2002; Murabito *et al.*, 2005). Associations between age at menopause and variants of genes involved in DNA repair, DNA maintenance and immunity have been previously identified in large genome-wide association (GWA) studies (Stolk *et al.*, 2009, 2012; He *et al.*, 2010).

Serum anti-Müllerian hormone (AMH) has been found to be a marker of the ovarian reserve. It is produced by granulosa cells of small, growing follicles in the ovary and is measurable in blood (de Vet *et al.*, 2002). It has been described as a useful serum marker of the ovarian reserve in mice (Kevenaar *et al.*, 2006), healthy women (de Vet *et al.*, 2002) and adult survivors of childhood cancer (Lie Fong *et al.*, 2009). Recently, serum AMH levels were found to be highly predictive for the onset of menopause in healthy women (van Disseldorp *et al.*, 2008; Freeman *et al.*, 2012a,b). Two nomograms to predict the age at menopause have been reported (Broer *et al.*, 2011; Tehrani *et al.*, 2011): in these studies, AMH and age were significantly correlated with the time to menopause, and therefore included as predictors in the prediction models.

Variation of long-term toxicity in childhood cancer survivors (CCSs) who received the same treatment suggests that, besides environmental factors, genetic variation may influence the impact of late effects. In male adult CCSs, genetic polymorphisms in the estrogen receptor were associated with an increased risk of azoospermia (Romerius *et al.*, 2011). Data on the impact of previously identified variants in gene loci associated with the ovarian reserve in adult long-term CCSs are lacking. Since these genes may have an additive influence on the ovarian reserve (together with the effects of gonadotoxic treatment regimens), the aim of this pilot study was to evaluate whether the genetic polymorphisms known to be associated with menopause were associated with serum AMH levels, and together could predict the age at menopause in adult long-term CCSs.

Materials and Methods

Subjects

This retrospective study was performed in a single-centre cohort of female adult CCSs treated at the Erasmus MC-Sophia Children's Hospital from October 1962 to May 2001. Participants were 18 years and younger at the time of diagnosis, 18–50 years old at the time of follow-up and in continuous complete remission. They had completed treatment at least 5 years ago. Participants were recruited during their regular visit at the late effects outpatient clinic. Only Caucasian females were included in order to minimize ethnic influences on the study outcome. Full details of cancer treatment were collected from the late effects clinic database and medical records, i.e. type and total cumulative doses of chemotherapy; site, field and cumulative dose of radiotherapy; extent of surgery; conditioning regimen prior to stem-cell transplantation; complications and relapses. The alkylating agent dose (AAD) score can be used to sum all alkylating agents that are known to be gonadotoxic. We calculated this score by determining the drug dose tertile distribution in our entire cohort of survivors independently and adding the tertile scores (1, 2 and 3) for each of the alkylating agents given to a particular patient, as previously used in CCSs (Tucker *et al.*, 1987; Green *et al.*, 2009) (Supplementary data, Table SI). An AAD score of zero was assigned to patients not exposed to alkylating agents. A general health screening, including extensive history taking and physical examination, was performed. An official written informed consent was obtained from every patient who agreed to participate according to the standards of the Institutional Review Board.

Measurement of ovarian reserve

As most of our patients were of premenopausal age, a marker that is consistent during the menstrual cycle and representative of the ovarian reserve is needed. AMH is strongly correlated with the antral follicle count and is relatively constant during and between menstrual cycles, in contrast to FSH (Hehenkamp *et al.*, 2006; La Marca *et al.*, 2006; Tsepelidis *et al.*, 2007; Streuli *et al.*, 2008). Therefore, AMH is a sensitive serum marker for the ovarian reserve and a proxy for menopause.

Serum samples were taken randomly during the menstrual cycle, in pregnant survivors as well as in survivors taking oral contraceptive pills or hormone replacement therapy. Serum AMH levels were measured with an in-house double-antibody enzyme-linked immunosorbent assay (de Vet *et al.*, 2002; Kevenaar *et al.*, 2006). Intra- and inter-assay coefficients of variation were <10 and $<5\%$, respectively (de Vet *et al.*, 2002; Kevenaar *et al.*, 2006).

Genotyping

Single nucleotide polymorphisms (SNPs) were selected based on a literature search of previous candidate gene studies and more recently published GWA studies [for minor allele frequencies (MAFs), see Table I]. We genotyped seven previously identified polymorphisms associated with age at menopause [*BRSK1* (rs1172822) (Stolk *et al.*, 2009), *ARHGEF7* (rs7333181) (Stolk *et al.*, 2009), *MCM8* (rs236114) (Stolk *et al.*, 2009), *PCSK1* (rs271924) (He *et al.*, 2010), *SRD5A1* (rs494958)

Table I MAFs in previous studies, and in the present study of female Caucasian survivors of childhood cancer.

	Locus	Chr	MAF CCSs AMH ≥1 μg/l, *n* = 115	MAF CCSs AMH <1 μg/l, *n* = 61	MAF previous studies (Stolk *et al.*, 2009; He *et al.*, 2010)
rs7333181 (A)	*ARHGEF7*	13	0.11	0.13	0.12
rs1172822 (T)	*BRSK1*	19	0.35	0.48	0.39
rs236114 (T)	*MCM8*	20	0.19	0.18	0.21
rs494958 (T)	*SRD5A1*	*5*	*0.15*	*0.15*	*0.16*
rs271924 (T)	*PCSK1*	5	0.32	0.34	0.38
rs9457827 (T)	*IGF2R*	6	0.07	0.07	0.06
rs909253 (G)	*TNF*	6	0.33	0.32	0.34

MAF, minor allele frequency; CCSs, childhood cancer survivors; Chr, chromosome; AMH, anti-Mullerian hormone.
All SNPs except rs494958 are in HWE.

(He *et al.*, 2010), *IGF2R* (rs9457827) (He *et al.*, 2010) and *TNF* (rs909253) (He *et al.*, 2010)] in the samples from long-term CCSs.

Genomic DNA was extracted from peripheral blood using standard DNA extraction methods. 1–2 ng of genomic DNA was dispensed into 384-well plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA, USA). Genotypes were determined using Sequenom iPLEX genotyping and Taqman Allelic Discrimination assays. All primers and probes are available on request.

Multiplex PCR assays were designed for the Sequenom iPLEX genotyping using Assay Designer on the website (https://mysequenom.com/tools/genotyping/default.aspx). For this, sequences containing the SNP site and at least 100 bp of flanking sequence on either side of the SNP were used. As previously described (Stolk *et al.*, 2009), 2 ng of genomic DNA was amplified in a 5 μl reaction containing 1× Taq PCR Buffer (Sequenom), 2 mM $MgCl_2$, 500 μM each dNTP, 100 nM each PCR primer and 0.5 U Taq (Sequenom). The reaction was incubated at 94°C for 20 s, 56°C for 30 s, 72°C for 1 min, followed by 3 min at 72°C. Excess dNTPs were then removed from the reaction by incubation with 0.3 U shrimp alkaline phosphatase (Sequenom) at 37°C for 40 min followed by 5 min at 85°C to deactivate the enzyme. Single primer extension over the SNP was carried out using a final concentration of 0.731–1.462 μM for each extension primer (depending on the mass of the probe), iPLEX termination mix (Sequenom), 10× iPLEX Buffer Plus and iPLEX enzyme (Sequenom) and subsequently cycled using the following programme: 94°C for 30 s followed by 94°C for 5 s, five cycles of 52° for 5 s and 80°C for 5 s, the previous three steps being repeated 40 times, then 72°C for 3 min. This reaction was then desalted by the addition of 6 mg clear resin (Sequenom) followed by mixing (15 min) and centrifugation (5 min, 3000 rpm) to settle the contents of the tube. The extension product was then spotted onto a 384-well spectroCHIP using the SEQUENOM MassARRAY Nanodispenser RS1000 before analysis on the MassARRAY Compact System (Sequenom). Data were collected using SpectroACQUIRE 3.3.1.13 and clustering was called using TYPER Analyser 4.0.3.18 (Sequenom). Additionally, to ensure data quality, the genotypes for each subject were also checked manually.

Genotypes for rs236114 and rs909253 were generated using Taqman Allelic Discrimination (Applied Biosystems Inc., Foster City, CA, USA). Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems (for details see www.appliedbiosystems.com). Reactions were performed on the Taqman Prism 7900HT 384-well format.

Statistical analysis

The SNPs were tested for deviation from the Hardy–Weinberg equilibrium (HWE) by comparing the observed and expected genotype frequencies using the χ^2 test. AMH levels were studied as continuous measures and were divided in two groups based on levels considered to be clinically relevant and acting as a proxy for the ovarian reserve: serum AMH levels below 1 μg/l versus AMH levels equal to or above 1 μg/l. A χ^2 test was used to analyse the associations between the SNPs and the two AMH groups.

Association analyses were carried out and the additive genetic model was tested. First, we pooled heterozygous and homozygous carriers of the risk alleles under a dominant inheritance model. Linear regression was performed to calculate the effect of carrying the risk allele on continuous log-transformed AMH levels. Secondly, logistic regression was performed to calculate the odds ratio (OR) and 95% confidence intervals (CI) of the three genotypes (carriers of zero, one or two risk alleles) to assess risk, adjusted for age at AMH assessment, the AAD score and the total dose of radiotherapy to the pelvis. Analyses were not performed when the number of patients per genotype subgroup was below $n = 5$. In view of the multiple comparisons, *P*-values of ≤0.0083 (0.05/6 SNPs) were considered to be significant.

We estimated the age at menopause based on serum AMH levels using two prediction models of the age at menopause based on a Dutch cohort and an Iranian cohort (Broer *et al.*, 2011; Tehrani *et al.*, 2011). These prediction models are based on age and serum AMH levels. Subsequently, the effect of significant SNPs on this predicted age of menopause was evaluated using a linear regression model, adjusted for the AAD score and abdominal radiotherapy dose. *P*-values ≤0.05 were considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences 17.0 software (SPSS, Chicago, IL, USA).

Results

The cohort of CCSs consisted of 238 women, of whom 5 died during follow-up, 6 were non-responders, 6 refused participation, 3 emigrated, 3 were disabled after treatment and 2 patients were >50 years. From the remaining 213 eligible subjects, serum AMH levels and DNA for genotyping were available in 192 survivors. Sixteen non-Caucasian survivors were excluded. Finally, 176 Caucasian women were included. The median age at diagnosis was 5.4 years, (range 0.1–16.8 years) in participants, and was comparable with the median age of survivors who discontinued follow-up and those in whom serum AMH levels or DNA samples were not available (median 6.7 years, range 0.8–14.0 years, $P = 0.98$). Neither the type of malignancy ($P = 0.08$) nor the frequency of relapse (15 versus 10%, $P = 0.48$), or the treatment with alkylating

agents (54 versus 47%, $P = 0.48$) or radiotherapy (34 versus 29%, $P = 0.44$) differed between participants and non-participants, respectively.

The median age at the time of cessation of treatment was 6.9 years (range 0.1–17.8 years). The median age at follow-up was 24.8 years (range 18.0–46.2 years). Fifty-six survivors (32%) received chemotherapy and radiotherapy, 104 (59%) chemotherapy only 4 (2%) radiotherapy only and 12 (7%) surgery only. Twenty-three survivors (13%) were in second remission after relapse and six (3%) were in remission after a second malignancy. Eighty-nine survivors (51%) were treated with alkylating agents. Twelve survivors (7%) were treated with abdominal irradiation or total body irradiation.

At the time of inclusion, 48 of 176 survivors (27.3%) had regular menstrual cycles, whereas 15 survivors (8.5%) had oligo- or amenorrhoea. Three (1.7%) survivors were pregnant. In six survivors (3.4%), data on their menstrual cycle at the time of screening were not available. All other survivors used oral contraceptive pills (55.1%) or hormonal replacement therapy ($n = 7$: 4.0%) at the time of follow-up.

Table II presents the characteristics of the 176 female CCSs. Survivors were divided into two groups on the basis of AMH levels: a level below 1 μg/l (61/176: 35%) was considered to be low; a level equal to or above 1 μg/l (115/176: 65%) was considered to be normal. Age at diagnosis, age at the end of treatment, diagnosis and age at follow-up were not significantly different between the two AMH groups. As expected, significantly more survivors in the low AMH group were treated with abdominal irradiation or total body irradiation, and significantly more survivors in the normal AMH group were not treated with the known gonadotoxic alkylating agents. The median serum AMH level in all survivors was 1.8 μg/l (range <0.1–14.3 μg/l).

All polymorphisms were in HWE except for rs494958 (*SRD5A1*) (Table I). Consequently, this polymorphism was not used for comparison of allele frequencies and haplotype distributions in this study.

After correction for multiple testing, women with the CT genotype of rs1172822 had a significantly increased risk for a low serum AMH level (OR: 3.15, 95% CI: 1.35–7.32, $P = 0.008$), while there was a

Table II Characteristics of female Caucasian patients who survived childhood cancer.

	CCSs all N = 176 Median (range)	CCSs AMH <1 μg/l N = 61 Median (range)	CCSs AMH ≥1 μg/l N = 115 Median (range)	*P*-value*
Age at diagnosis (years)	5.4 (0.1–16.8)	6.1 (0.2–15.4)	5.2 (0.1–16.8)	0.221
Age at discontinuation of treatment (years)	6.9 (0.1–17.8)	7.8 (1.2–17.0)	6.4 (0.1–17.8)	0.130
Age at follow-up (years)	24.8 (18.0–46.2)	26.4 (18.0–42.6)	24.4 (18.0–46.2)	0.061
Follow-up time (years)	17.4 (4.0–41.3)	17.3 (4.0–38.8)	17.5 (5.0–41.3)	0.528
Diagnosis *n* (%)				
Acute lymphoblastic leukaemia	66 (37.5)	25 (41.0)	41 (35.7)	0.488
Acute myeloid leukaemia	4 (2.3)	3 (3.9)	1 (0.9)	0.087
B-cell non-Hodgkin lymphoma	8 (4.5)	4 (6.6)	4 (3.5)	0.352
T-cell non-Hodgkin lymphoma	7 (4.0)	2 (3.3)	5 (4.3)	0.731
Hodgkin lymphoma	13 (7.4)	7 (11.5)	6 (5.2)	0.132
Osteosarcoma	4 (2.3)	2 (3.3)	2 (1.7)	0.515
Renal tumour	24 (13.6)	9 (14.8)	15 (13.0)	0.754
Ewing sarcoma	4 (2.3)	1 (1.6)	3 (2.6)	0.682
Neuroblastoma	17 (9.7)	4 (6.6)	13 (11.3)	0.312
Rhabdomyosarcoma	7 (4.0)	3 (4.9)	4 (3.5)	0.643
Langerhans cell histiocytosis	6 (3.4)	0 (0.0)	6 (5.2)	0.070
Brain tumour	2 (1.1)	0 (0.0)	2 (1.7)	0.302
Other	14 (8.0)	1 (1.6)	13 (11.3)	**0.025**
Therapy *n* (%)				
Abdominal radiotherapy [30 Gy (15–45)]	7 (4.0)	6 (9.8)	1 (0.9)	**0.004**
Total body irradiation [8 Gy 7–12)]	5 (2.8)	5 (8.2)	0 (0.0)	**0.002**
AAD score (%)				
0	87 (49.4)	23 (37.7)	64 (55.7)	**0.027**
1	25 (14.2)	11 (18.0)	14 (12.2)	0.301
2	23 (13.1)	7 (11.5)	16 (13.9)	0.634
3	32 (18.2)	15 (24.6)	17 (14.8)	0.116
4	4 (2.3)	3 (4.9)	1 (0.9)	0.089
5–7	5 (2.8)	2 (3.2)	3 (2.6)	0.807

Bold values significant at $P < 0.05$.
AAD score, alkylating agent dose score; CCSs, childhood cancer survivors.
**P*-values indicate the difference in serum AMH levels in survivors with AMH <1 μg/l versus AMH ≥1 μg/l. Values are absolute numbers (%) or medians (range).

Table III Distribution of genotypes associated with age at menopause and MAFs in 176 Caucasian CCSs.

Genotype	CCSs AMH <1 μg/l, *n* (%)	CCSs AMH ≥1 μg/l, *n* (%)	OR	95% CI	*P*-value
rs7333181					
GG	48 (78.7)	91 (79.8)	Ref.	—	—
GA	10 (16.4)	20 (17.5)	1.14	0.46–2.83	0.777
AA[a]	3 (4.9)	3 (2.6)			
rs1172822					
CC	11 (18.0)	45 (40.5)	Ref.	—	—
CT	41 (67.2)	54 (48.6)	3.15	1.35–7.32	**0.008**
TT	9 (14.8)	12 (10.8)	3.45	1.06–11.27	0.040
rs236114					
CC	38 (69.1)	71 (65.7)	Ref.	—	—
CT	14 (25.5)	33 (30.6)	0.96	0.44–2.11	0.919
TT[a]	3 (5.5)	4 (3.7)	—	—	—
rs271924					
AA	27 (44.3)	49 (43.4)	Ref.	—	—
AT	27 (44.3)	55 (48.7)	0.80	0.39–1.63	0.802
TT	7 (11.5)	9 (8.0)	1.40	0.40–4.91	0.602
rs9457827					
CC	54 (88.5)	99 (86.8)	Ref.	—	—
CT	6 (9.8)	14 (12.3)	0.75	0.24–2.40	0.633
TT[a]	1 (1.6)	1 (0.9)	—	—	—
rs909253					
AA	28 (48.3)	51 (45.5)	Ref.	—	—
AG	23 (39.7)	49 (43.8)	0.80	0.38–1.69	0.563
GG	7 (12.1)	12 (10.7)	1.46	0.47–4.49	0.510

Bold values significant at $P < 0.0083$ (after correction for multiple testing).
OR, odds ratio; CI, confidence interval; RT, radiotherapy; CCSs, childhood cancer survivors.
Multivariate analysis adjusted for age at AMH measurement, AAD score and abdominal radiotherapy.
[a]Analysis was not performed because of the small number of patients (<5 per subgroup).

trend towards a higher risk of a low serum AMH level in women with the TT genotype (OR: 3.45, 95% CI: 1.06–11.27, $P = 0.04$) (Table III) after correction for the age at AMH level, AAD score and abdominal irradiation. None of the other SNPs was associated with an increased risk for a low AMH. Using a carrier model, the T-allele of rs1172822 showed a borderline significant association with continuous serum AMH levels (β: −0.16, 95% CI: −0.62 to −0.06, $P = 0.017$, Table IV). This association may not be as significant as the analysis of the subgroups owing to the small number of patients ($n = 21$) homozygous for the minor allele (T). Therefore, a linear regression of AMH levels and the three genotypes (CC, CT, TT) has been performed. A significant negative association between AMH and the heterozygote CT genotype of rs1172822 has been observed (β: −0.63, 95% CI −1.02 to −0.24, $P = 0.002$), while no significant association between the TT genotype and AMH has been found (β: −0.47, 95% CI −1.06 to 0.13, $P = 0.123$).

We evaluated whether rs1172822 was associated with the predicted age at menopause using two menopause prediction models (Broer *et al.*, 2011; Tehrani *et al.*, 2011). The rs1172822 polymorphism was negatively associated with predicted age at menopause using the Dutch population model (β: −0.17, 95% CI: −1.18 to −0.02, $P = 0.044$) after adjustment for the AAD score and abdominal radiotherapy. However, the effect of treatment with alkylating agents or pelvic radiotherapy seemed to be larger than the effect of the polymorphism rs1172822 on the predicted age at menopause (Table V). When we predicted the age at menopause based on the Iranian population model, we did not find a significant correlation with rs1172822 (β: −0.15, 95% CI: −5.94 to 0.42, $P = 0.088$) (Table V). When performing analyses without adjustment for confounders, the rs1172822 was borderline significantly associated with predicted age at menopause in the Dutch population model (β: −0.64, 95% CI: −1.29 to 0.01, $P = 0.054$) and was still not associated with age in the Iranian population model (β: −2.99, 95% CI −6.12 to 0.15, $P = 0.062$).

Discussion

We evaluated the importance of SNPs, which had been shown previously to be associated with age at natural menopause in healthy women, to the ovarian reserve in female CCSs. We found that the CT genotype of rs1172822 of the *BRSK1* gene was negatively

Table IV Distribution of allelic frequencies associated with age at menopause in 176 Caucasian CCSs.

SNP (minor allele)	β (log AMH)	95% CI	P-value
rs7333181 (A)	0.06	−0.22 to 0.51	0.431
Age AMH	−0.24	−0.08 to −0.02	0.001
AAD score	−0.22	−0.36 to −0.08	0.002
RT abdomen (Gy)	−0.34	−0.11 to −0.05	<0.0001
rs1172822 (T)	−0.16	−0.62 to −0.06	0.017
Age AMH	−0.23	−0.08 to −0.02	0.001
AAD score	−0.19	−0.32 to −0.06	0.006
RT abdomen (Gy)	−0.34	−0.11 to −0.05	<0.0001
rs236114 (T)	0.07	−0.15 to 0.49	0.291
Age AMH	−0.24	−0.08 to −0.02	0.001
AAD score	−0.22	−0.35 to −0.08	0.002
RT abdomen (Gy)	−0.36	−0.11 to −0.05	<0.0001
rs271924 (T)	0.05	−0.17 to 0.39	0.445
Age AMH	−0.23	−0.08 to −0.02	0.001
AAD score	−0.21	−0.35 to −0.08	0.002
RT abdomen (Gy)	−0.34	−0.11 to −0.05	<0.0001
rs9457827 (T)	−0.05	−0.65 to 0.31	0.483
Age AMH	−0.23	−0.08 to −0.02	0.001
AAD score	−0.20	−0.34 to −0.07	0.004
RT abdomen (Gy)	−0.34	−0.11 to −0.05	<0.0001
rs909253 (G)	−0.01	−0.30 to 0.25	0.862
Age AMH	−0.21	−0.07 to −0.02	0.004
AAD score	−0.17	−0.32 to −0.04	0.014
RT abdomen (Gy)	−0.35	−0.11 to −0.05	<0.0001

CCSs, childhood cancer survivors; AAD score, alkylating agent dose score; RT, radiotherapy; CI, confidence interval.

Table V Predicted age at menopause and BRSK1 polymorphism in 176 Caucasian CCSs.

	OR	95% CI	P-value
Method of Broer et al. (2011) (Dutch population)			
rs1172822 (T)	−0.17	−1.18 to −0.02	**0.044**
AAD score	−0.22	−0.56 to −0.08	0.010
Abdominal RT (Gy)	−0.27	−0.18 to −0.05	0.001
Method of Tehrani et al. (2011) (Iranian population)			
rs1172822 (T)	−0.15	−5.94 to 0.42	0.088
AAD score	−0.16	−2.56 to 0.04	0.058
Abdominal RT (Gy)	−0.17	−0.74 to 0.01	0.055

Bold values significant at $P < 0.05$.
CCSs, childhood cancer survivors; AAD score, alkylating agent dose score; RT, radiotherapy; OR, odds ratio; CI, confidence interval.

associated with serum AMH levels after correction for multiple testing. Moreover, the T-allele of rs1172822 was associated with the predicted age at menopause of the Dutch population model.

Serum AMH levels were used as a proxy for the ovarian reserve. As most of our patients were in the premenopausal age range, we needed a marker that does not fluctuate during the menstrual cycle and is representative of the ovarian reserve. AMH is strongly correlated with the antral follicle count and is relatively constant during and between menstrual cycles, in contrast to FSH (Hehenkamp et al., 2006; La Marca et al., 2006; Tsepelidis et al., 2007; Streuli et al., 2008). Although some studies did report variations during the cycle (Wunder et al., 2008; Streuli et al., 2009; Sowers et al., 2010; Robertson et al., 2011), the differences were small in amplitude and similar to the reported minor variability between cycles, which suggests that AMH production is independent of gonadotrophins. Therefore, AMH is a useful serum marker for the ovarian reserve. Moreover, two studies showed that serum AMH levels can be used to predict the age at menopause (Broer et al., 2011; Tehrani et al., 2011), and therefore serum AMH levels were used in our study. An AMH level of 1 μg/l has been used as a cut-off value to identify a decreased ovarian reserve. Several studies have tried to identify a cut-off value for AMH to classify decreased fertility, poor response after IVF and non-pregnancy, and these studies have been reviewed (Broer et al., 2009). The mean of these AMH cut-off values is ∼1 μg/l and we used this value as it is the best available estimate. However, we also performed our quantitative analyses with AMH levels and found a borderline significant association with the minor allele (T) of rs1172822. This association may not be as significant as the analysis of the subgroups owing to the small number of patients ($n = 21$) homozygous for the minor allele (T). Therefore, a linear regression of AMH levels and the three genotypes (CC, CT, TT) has been performed. A highly significant negative association between AMH and the heterozygote CT genotype of rs1172822 has been observed (β: −0.63, 95% CI −1.02 to −0.24, $P = 0.002$), which supports the negative association between rs1172822 and AMH levels.

A low serum AMH level in adult female CCSs was associated with the CT genotype of rs1172822, which is consistent with previous findings in healthy women in which this polymorphism was associated with a decrease in the age at menopause of 4 months per T-allele (Stolk et al., 2009; He et al., 2010). Rs1172822 is a polymorphism in the BR serine/threonine kinase 1 gene (*BRSK1*), which is located on chromosome 19q13.4, intron 17 and encodes an AMP-activated protein kinase (AMPK)-related kinase. It is highly expressed in the human forebrain (Kishi et al., 2005) and moderately expressed in mammalian ovaries (GeneAtlas). Interestingly, the downstream targets of *BRSK1* are several members of the family of AMPK-related kinases phosphorylate tau, a microtubule-associated protein that regulates the stability of the microtubule network (Kishi et al., 2005). This includes the maternal embryonic leucine zipper kinase (MELK2; chromosome 9) that is highly expressed in spermatogonia and oocytes (Lizcano et al., 2004; Thelie et al., 2007). *BRSK1* is specifically activated by phosphorylation, together with 12 other AMPKs, including MELK, through the serine/threonine protein kinase 11, named STK11 or LKB1 (Bright et al., 2008). LKB1 (19p13.3) is considered a master regulator of cell polarity (by regulating cytoskeletal dynamics) and, being the only protein identified with this activity, it is expressed in mouse oocytes (Szczepanska and Maleszewski, 2005). Mutation of *LKB1* affects epithelial, neuronal and oocyte polarity, thereby influencing cell growth. More recently, *BRSK1* was found to be essential for

centriole duplication and therefore plays an important role in cell–cycle progression (Alvarado-Kristensson *et al*., 2009). *BRSK1* is therefore important for cells to function normally. Recent hypotheses concerning an association between genome-stability genes and ageing have been proposed: recent studies link specific deficiencies in genome maintenance to symptoms of premature ageing, which are likely related to a tissue-specific spectrum of DNA lesions caused by the unique metabolic profile of each particular organ or tissue (Lans and Hoeijmakers, 2012). This may explain the involvement of *BRSK1* in the ovarian ageing process, possibly resulting in reduced AMH levels.

In the present study, only one of the six previously identified polymorphisms in the healthy population was associated with AMH levels in adult female CCSs. These findings suggest that the effects of gonadotoxic treatment exceed the influence of these genetic polymorphisms. However, most of these SNPs have a low MAF (Table I). The lower the MAF, the higher the number of survivors needed to achieve sufficient power to observe an influence on AMH levels. Therefore, final conclusions regarding these associations cannot be drawn, given the relatively small effect size of those polymorphisms and the large effect of alkylating agents and pelvic radiotherapy on the ovarian reserve in a small sample of CCSs. Our observations should be investigated further in a larger sample.

Rs1172822 is significantly associated with the predicted age at menopause of the Dutch population model by Broer *et al*. (2011). This is in line with a previous report that the rs1172822 polymorphism is associated with the age at menopause in the healthy population (Stolk *et al*., 2009). Still, the effect of treatment with alkylating agents or pelvic radiotherapy seemed to be stronger than the effect of the rs1172822 polymorphism on the predicted age at menopause. We found no association between rs1172822 and the predicted age at menopause using the Iranian population model: the different genetic background of the women in the Iranian population model is most likely the cause. Indeed, the rs1172822 MAF in Asian subjects is 10-fold lower than in the European population (dbSNP).

We realize that our pilot study only shows preliminary data. To confirm our findings and enlarge the study, a nationwide GWA project on the ovarian reserve in female CCSs should be performed, including a replication cohort.

In conclusion, we showed that the CT genotype of rs1172822 of the *BRSK1* gene in female adult CCSs is associated with a decreased serum AMH level and a lower predicted age at menopause, based on a Dutch population model. These findings support the idea that previously identified polymorphisms which are associated with age at menopause in healthy women may also have an effect on the onset of menopause in female CCSs. This pilot study appears to show a new aspect of the influence of genetic variants on the ovarian reserve following treatment of childhood cancer and should be investigated further in a nationwide GWA study. Eventually, this information can help us to improve counselling on fertility preservation prior to cancer treatment, based on genetic factors in individual patients.

Supplementary data

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

Authors' roles

W.D. acquired, analysed and interpreted data, drafted the manuscript and designed the study. M.M.H.E. designed the study, drafted the manuscript and critically revised the manuscript for intellectual content and data interpretation. L.S. analysed data and critically revised the manuscript for intellectual content. R.P. critically revised the manuscript for intellectual content. A.G.U. designed the study and critically revised the manuscript for intellectual content. J.A.V. critically revised the manuscript for intellectual content and data interpretation. J.S.E.L. designed the study, drafted the manuscript, interpreted data and critically revised the manuscript for intellectual content.

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Conflict of interest

J.S.E.L. has received fees and grant support from the following companies (in alphabetic order): Ferring, Genovum, Merck-Serono, Organon, Schering Plough and Serono. All other authors have nothing to disclose.

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