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BRSK1 modifies the risk of alkylating chemotherapy-related reduced ovarian function

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ABSTRACT

Background: Female childhood cancer survivors (CCS) show large inter-individual variability in the impact of DNA-damaging alkylating chemotherapy, given as treatment of childhood cancer, on ovarian function at adult age. Genetic variants in DNA repair genes affecting ovarian function might explain this variability.

Methods: To evaluate ovarian function, Anti-Müllerian hormone (AMH) levels were assessed in a discovery cohort of female CCS from the Dutch DCOG LATER-VEVO (N=285), and results were validated in the pan-European PanCareLIFE (N=465), and the USA-based St. Jude Lifetime Cohort (N=391). Using additive genetic models in linear and logistic regression, five genetic variants involved in DNA damage response were analyzed in relation to cyclophosphamide equivalent dose (CED) score and their impact on ovarian function.

Results: Meta-analysis across the three independent cohorts showed a significant interaction effect ($p = 3.0 \times 10^{-4}$) between rs11668344 of *BRSK1* (allele frequency = 0.34) among CCS treated with high dose alkylating agents (CED score ≥8,000 mg/m^2), resulting in a 3-fold increased odds of a reduced ovarian function (lowest AMH tertile) for CCS carrying one G allele compared to CCS without this allele (OR genotype AA: 1.8 vs OR genotype AG: 5.3).

Conclusions: Female CCS carrying a common *BRSK1* gene variant appear to be at 3-fold increased odds of a reduced ovarian function after treatment with high doses of alkylating chemotherapy. Genetic testing may inform future individualized counseling regarding treatment-related risks and fertility preservation services in girls with cancer, as well as of young adult survivors of childhood cancer.

INTRODUCTION

Advances in childhood cancer treatment has increased cancer survival rates, leading to a growing population of childhood cancer survivors (CCS)[1]. Abdominal-pelvic radiotherapy and alkylating agents may compromise ovarian function[2-4] and reduce their reproductive window. This may manifest as sub- or infertility[5,6] and a higher risk of premature menopause[7], which in turn may impair quality of life[8-13]. Substantial inter-individual variability in the impact of treatment on ovarian function in similarly treated CCS suggests a role for genetic factors in modifying the association between treatment and the risk of ovarian impairment.

Large-scale genome wide association studies (GWAS) in the general population have identified single nucleotide polymorphisms (SNPs) associated with age at natural menopause or premature ovarian insufficiency (POI)[14-19]. These SNPs include variants associated with the DNA damage response, and account for approximately 30% of the variance in early menopause[19]. Alkylating agents, common chemotherapeutic agents used in childhood cancer treatment, induce apoptosis of cancer cells by damaging DNA and inhibiting cellular metabolisms, DNA replication and transcription[20-23]. We hypothesized that girls and young women with less efficient DNA damage response systems are more vulnerable to the adverse effects of alkylating agents on ovarian function compared to women with a fully efficient DNA damage repair system, leading to ovarian dysfunction later in life.

Serum levels of anti-Müllerian hormone (AMH), produced by the granulosa cells of small growing follicles in the ovaries, are related to age at onset of menopause in healthy women[24] and can detect ovarian dysfunction prior to both detectible changes in FSH/LH or estrogen and clinical manifestations of menopause[25-28]. In addition, AMH, which is stable throughout the menstrual cycle, has been demonstrated as a useful and early surrogate marker of reduced ovarian function in cancer survivors[29-34]. This is convenient since many CCS cohort members are relatively young and have not yet reached menopausal age.

Identifying genetic risk factors for treatment-related reduced ovarian function may have clinical implications for risk assessment and medical decision-making regarding fertility preservation in newly diagnosed girls with cancer[35]. Moreover, this information may inform targeted counseling and surveillance strategies of compromised ovarian function and associated comorbidities in at-risk adult female survivors. The aim of the current study was, therefore, to evaluate whether SNPs in the DNA damage response pathway modify the adverse effect of alkylating agents on ovarian function in CCS.

METHODS

Study participants - Discovery cohort

CCS for the discovery cohort were identified from the Dutch Childhood Oncology Group (DCOG) LATER VEVO-study, a multi-center retrospective cohort study evaluating fertility, ovarian reserve and risk of premature menopause among adult female 5-year survivors of childhood cancer[36]. Data on prior cancer diagnosis and treatments were collected from medical files and information on use of hormones (contraceptives or hormonal replacement therapy (HRT)) and menopausal status at time of study was obtained from the DCOG LATER VEVO-study questionnaire[36]. The timing of serum sampling (menstrual cycle day 2-5, day 7 of hormone-free week, or anytime in case of no menstrual cycle or hormone releasing intrauterine device) was documented. The study was approved by the Medical Ethics Review Committee (IRB protocol number 2006/249, VUmc) and written informed consent was obtained from all participants.

Inclusion and exclusion criteria

Female 5-year CCS, diagnosed with cancer and treated with chemotherapy before the age of 25 years, and aged 18 years or older at time of study were enrolled in the current study. Eligible participants provided a blood sample to quantify AMH levels and extract DNA. To maximize the potential to detect a role of genetic variation, we excluded survivors who received treatments associated with extensive gonadal toxicity including allogeneic stem cell transplantation (SCT), Total Body Irradiation (TBI), bilateral ovary-exposing radiotherapy, cranial and/or craniospinal radiotherapy, or bilateral oophorectomy.

Study participants – Replication cohorts

PanCareLIFE cohort

PanCareLIFE (PCL) is a pan-European research project including 28 institutions from 13 countries addressing ototoxicity, fertility, and quality of life[37]. The first replication cohort included all adult 5-year female survivors from the PanCareLIFE cohort who were treated for cancer before the age of 25 years and fulfilled all inclusion criteria of this study[38]. Approval was obtained from all relevant local review boards and written informed consent from all participants.

St. Jude Lifetime Cohort

The St. Jude Lifetime Cohort Study (SJLIFE) is a cohort study among 10-year CCS in North America coordinated by the St. Jude Children's Research Hospital (Memphis, Tennessee, USA) combining treatment data, patient-reported outcomes and clinical assessment[39]. Participants in SJLIFE who fulfilled the inclusion criteria and had blood samples available for AMH and DNA analysis comprised the second replication cohort. Sex hormone use at time of study was documented.

Outcome and outcome definition

The outcome of this study was ovarian function, primarily determined by serum levels of AMH. AMH levels of all three cohorts were determined in the endocrine laboratory of VU University Medical Center Amsterdam by an ultra-sensitive Elecsys AMH assay (Roche Diagnostics GmbH, Mannheim, Germany) with an intra-assay coefficient of variation (CV) of 0.5% – 1.8%, a limit of detection (LoD) of 0.01 µg/L, and a limit of quantitation (LoQ) of 0.03 µg/L[40].

To account for age-dependency of AMH, participating women in each cohort were divided into four age categories: ≥18-25; ≥25-32; ≥32-40; ≥40 years. In each cohort and for each age category, AMH was divided into tertiles with exception of the last age category in which AMH levels varied too little to adequately define tertiles. CCS with an AMH level in the lowest tertile for their age category were defined as having a reduced ovarian function (case), while those with an AMH-value in the highest tertile for their age category were assumed not to have a reduced ovarian function (control). Women over 40 years of age were not considered a 'case' based on having an AMH-value in the lowest tertile, but on whether or not they had reported a premature menopause (absence of menses for > 12 months before the age of 40) at time of study. No 'control' subjects were defined in this age group due to the inability to identify with sufficient certainty those without a reduced ovarian function.

Candidate gene variant selection

SNPs were selected based on a literature search of recently published GWAS that identified loci associated with age at natural menopause[16,18,19,41]. Five GWAS hits in DNA damage response pathways, specifically in the inter-strand cross-link repair pathway, were selected based on the lowest *p*-value in the largest available GWAS meta-analysis, with the hypothesis that polymorphisms in these regions may increase the gonadotoxic effect of alkylating agents. The selected polymorphisms were in *UIMC1* (rs365132), *FANCI* (rs1054875), *RAD51* (rs9796), *BRSK1* (rs11668344) and *MCM8* (rs16991615). Details concerning the genotype data and quality control protocol are provided in the Supplementary Appendix.

Alkylating agents

For each survivor, the administered cumulative dose of alkylating agents was quantified using the validated Cyclophosphamide Equivalent Dose (CED)-score[42]. To evaluate the effects of no, low, medium and high dose alkylating agent exposure, the CED score was divided into four categories (0; >0 – 4,000 mg/m^2; ≥4,000 – 8,000 mg/m^2; ≥8,000 mg/m^2)[42]. Details on the administered chemotherapeutics, CED score in categories and a fractional polynomial selection procedure for CED score are further discussed in the Supplementary Appendix Tables S1-4.

Statistical analyses

Additive genetic associations, with AMH levels based on imputed allelic dosage, were evaluated by logistic and linear regression analyses based on two models: (1) *a main effect* model; and (2) *an interaction* model. Both models evaluated the association between reduced ovarian function and selected SNPs, adjusted for: ancestry and cohort effects using principle components, CED score (four categories using CED of zero as the reference category)[42], use of sex hormones (replacement or contraception) at time of study (yes/no), age at time of study (linear regression analysis only), and imputed numbers (0-2) of the alternative allele of the investigated variant (additive effects). The *interaction model* additionally included an interaction term (SNP*CED category) for genetic variant and CED score categories to evaluate the modifying effect of the variant on the impact of CED score on low AMH levels. Results of linear and logistic regression analyses are presented as regression coefficients (beta) with standard errors (se) and odds ratios (OR) with a 95% confidence interval (95% CI). For linear regression, AMH-levels were log-transformed to adjust for the skewed residuals distribution. Sensitivity analyses performed to assess the robustness of our findings, choices of the model and linkage disequilibrium (LD) are shown in Supplementary Appendix S5A-B.

SNPs that showed an association with log-transformed AMH levels or reduced ovarian function in either model, or an interaction effect with CED (p-values <0.05) were selected for replication. These analyses were conducted using SPSS (Statistical Package for Social Sciences (SPSS) version 24.0.0.1).

Replication and meta-analysis

Findings from the discovery cohort were assessed in both replication cohorts using identical models, except for sex hormone use at time of study, which was only available in SJLIFE. Data of the discovery and replication cohorts were combined and examined using meta-analytic approaches, in R version 3.5.1, package “rmeta”[43]. Details on the heterogeneity in the meta-analysis are described in the Supplementary Appendix, Tables S11-12. In the meta-analysis, p-values <0.01 (0.05/5 gene variants, correcting for multiple testing) were considered statistically significant.

RESULTS

Discovery cohort

In total, 285 CCS from the DCOG LATER-VEVO cohort participated in the current study (Table 1). Allele frequencies of the investigated SNPs are depicted in Table 2. All SNPs were in Hardy-Weinberg equilibrium (significance level $<1*10^{-7}$). Results from logistic regression analyses showed an association between *BRSK1* (rs11668344) and reduced ovarian func-

Table 1. Characteristics of participating CCS in the discovery and two replication cohorts

	Discovery DCOG LATER-VEVO (N=285)	Replication PanCareLIFE (N=465)	Replication St. Jude Lifetime (N=391)
Age at time of study (years)			
Median (range)	26.1 (18.3 – 52.4)	25.7 (18.0 – 45.0)	31.3 (19.1 – 59.5)
Age at diagnosis (years)			
Median (range)	5.8 (0.3 – 17.8)	10.4 (0.0 – 25.0)	6.9 (0.0 – 22.7)
18-25 years	0 (0)	21 (4.5)	16 (4.1)
Time since diagnosis (years)			
Median (range)	19.7 (6.7 – 41.4)	17.0 (5.0 – 39.1)	23.7 (11.0 – 46.2)
Diagnosis			
Leukaemia	112 (39.3)	109 (23.4)	121 (30.9)
Lymphoma	49 (17.2)	154 (33.1)	70 (17.9)
Renal tumors	37 (13.0)	35 (7.5)	27 (6.9)
CNS tumors	3 (1.1)	12 (2.6)	28 (7.2)
Soft tissue sarcoma	23 (8.1)	31 (6.7)	28 (7.2)
Bone tumors	26 (9.1)	45 (9.7)	34 (8.7)
Neuroblastoma	11 (3.9)	35 (7.4)	36 (9.2)
Other	24 (8.4)	44 (9.6)	47 (12.0)
Radiotherapy			
No	251(88.1)	297 (63.9)	268 (68.5)
Yes[a]	34 (11.9)	170 (36.1)	123 (31.5)
Thorax	22 (7.7)	88 (18.9)	71 (18.2)
Abdomen (above pelvic crest)	3 (1.1)	12 (2.6)	30 (7.7)
Unilateral ovarian[b]	0 (0)	9 (1.9)	3 (0.8)
Other	20 (7.0)	61 (13.1)	51 (13.0)
CED score			
0	106 (37.2)	161 (34.6)	198 (50.6)
> 0 – 4,000 mg/m^2	80 (28.1)	103 (22.2)	21 (5.4)
≥ 4,000 – 8,000 mg/m^2	52 (18.2)	68 (14.9)	78 (19.9)
≥ 8,000 mg/m^2	47 (16.5)	133 (28.6)	94 (24.0)
Hormone use at serum sampling			
No	199 (69.9)	232 (49.9)	263 (67.3)
Yes	86 (30.1)	116 (24.9)	128 (32.7)
Oral contraceptive-free day 7	70 (24.6)	3 (0.6)	n.a.
Anytime during oral contraceptive	n.a.	94 (20.2)	n.a.
HRT stop 7	2 (0.7)	20 (4.3)	n.a.
Anytime, with intrauterine device	14 (4.9)	n.a.	n.a.
Unknown	0 (0)	117 (25.2)	0 (0)

Table 1. Characteristics of participating CCS in the discovery and two replication cohorts (continued)

	Discovery DCOG LATER-VEVO (N=285)	Replication PanCareLIFE (N=465)	Replication St. Jude Lifetime (N=391)
Unilateral ovarian oophorectomy			
No	284 (99.6)	463 (99.6)	391 (100.0)
Yes	1 (0.4)	2 (0.4)	0 (0)
AMH level			
Median (range)	2.5 (<0.01 – 13.1)	2.1 (<0.01 – 18.5)	1.8 (<0.01 – 11.9)
Premature menopause (before age 40) and aged ≥40 years at study,	2 (0.7)	NA	4 (1.0)

Values represent the number (%) of women, unless indicated otherwise. [a]Not mutually exclusive; [b]Likely in radiotherapy field. CNS, central nervous system; CED, Cyclophosphamide Equivalent Dose; HRT, hormonal replacement therapy; n.a., not available

tion (OR 0.56, 95% CI 0.35 – 0.90; p-value = 0.016) in the main effect-model. In addition, a non-significantly modifying effect of *BRSK1* (rs11668344, minor allele frequency 0.34) on the effect of CED ≥8,000 mg/m^2 on reduced ovarian function (OR 5.02, 95% CI 0.76 – 33.08; p-value = 0.09) (Table 2) was observed in the interaction model. A significant modifying effect of a polymorphism in *FANCI* (rs1054875) on the effect of CED in the category >0 – 4000 mg/m^2 (OR 9.93, 95% CI 2.35 – 41.98; p-value = 0.002) was also observed (Table 2). Sensitivity analyses did not change these results (Table S5A-B of the Supplementary Appendix). Linear regression analysis showed a significant main effect of the *BRSK1* gene variant, but not of the other variants (Table S7 in the Supplementary Appendix). The two SNPs within the *BRSK1* and *FANCI* genes were assessed for replication in the two replication cohorts.

Replication and meta-analysis

The PanCareLIFE and SJLIFE replication cohorts included 465 and 391 female CCS, respectively (Table 1). Table 3 shows the combined analysis of both replication cohorts and the final meta-analysis including all three cohorts. Separate findings of the replication cohorts can be found in Table S9-10 in the Supplementary Appendix, full details of the meta-analysis in Tables S11-12. All three single-cohort analyses suggest a consistent modifying effect for the G allele of rs11668344 (*BRSK1*) on the effect of CED ≥8,000 mg/m^2 on reduced ovarian function. The meta-analysis showed an interaction effect of carrying the G allele of rs11668344 in *BRSK1* and an exposure to alkylating agents equivalent to a CED score ≥8,000 mg/m^2 of 3.81 (95% CI 1.85 – 7.86, $p = 3.0 \times 10^{-4}$). Table 4 shows the cumulative ORs for any genotype per CED category. Female CCS who received alkylating agents equivalent to a CED score ≥8,000 mg/m^2 had a 3-fold higher odds of having an AMH serum level in the lowest tertile for each additional G allele of rs11668344 in *BRSK1* (OR genotype AA 1.82 vs AG 5.27 vs GG 15.26).

Table 2. Association of single nucleotide polymorphisms with reduced ovarian function and CED-score in DCOG LATER-VEVO discovery cohort

Gene	Variant	Chrom	Ref.	Alt.	MAF	Model	Variant, interaction term	OR (95% CI)	P-value
BRSK1	**rs11668344**	19	A	G	0.34	1	rs11668344	0.56 (0.35 – 0.90)	0.016
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.43 (0.65 – 3.11)	0.374
							– ≥ 4,000 – 8,000	4.74 (1.92 – 11.71)	0.001
							– ≥ 8,000	5.04 (1.66 – 15.30)	0.004
							Hormones	2.02 (1.00 – 4.07)	0.049
						2	rs11668344	0.57 (0.25 – 1.31)	0.186
							CED: 0	1 (ref)	0.133
							– > 0 – 4,000	1.94 (0.62 – 6.07)	0.253
							– ≥ 4,000 – 8,000	5.46 (1.32 – 22.66)	0.019
							– ≥ 8,000	1.91 (0.44 – 8.29)	0.386
							SNP*CED: 0	1 (ref)	0.218
							– > 0 – 4,000	0.66 (0.21 – 2.13)	0.489
							– ≥ 4,000 – 8,000	0.85 (0.23 – 3.18)	0.807
							– ≥ 8,000	5.02 (0.76 – 33.08)	0.094
							Hormones	2.01 (0.98 – 4.14)	0.058
FANCI	**rs1054875**	15	A	T	0.36	1	rs1054875	1.01 (0.61 – 1.67)	0.975
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.37 (0.63 – 2.95)	0.425
							– ≥ 4,000 – 8,000	4.17 (1.73 – 10.05)	0.001
							– ≥ 8,000	4.98 (1.66 – 14.91)	0.004
							Hormones	1.79 (0.91 – 3.54)	0.094
						2	rs1054875	0.31 (0.11 – 0.90)	0.032
							CED: 0	1 (ref)	0.009
							– > 0 – 4,000	0.32 (0.10 – 1.06)	0.063
							– ≥ 4,000 – 8,000	2.19 (0.60 – 7.95)	0.235
							– ≥ 8,000	3.71 (0.84 – 16.38)	0.084
							SNP*CED: 0	1 (ref)	0.016
							– > 0 – 4,000	9.93 (2.35 – 41.98)	0.002
							– ≥ 4,000 – 8,000	3.49 (0.78 – 15.57)	0.102
							– ≥ 8,000	2.00 (0.38 – 10.44)	0.413
							Hormones	1.83 (0.90 – 3.73)	0.095
MCM8	**rs16991615**	20	G	A	0.08	1	rs16991615	0.90 (0.38 – 2.15)	0.817
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.37 (0.64 – 2.94)	0.420
							– ≥ 4,000 – 8,000	4.16 (1.74 – 9.97)	0.001
							– ≥ 8,000	4.96 (1.65 – 14.87)	0.004

Table 2. Association of single nucleotide polymorphisms with reduced ovarian function and CED-score in DCOG LATER-VEVO discovery cohort (continued)

Gene	Variant	Chrom	Ref.	Alt.	MAF	Model	Variant, interaction term	OR (95% CI)	P-value
							Hormones	1.80 (0.91 – 3.56)	0.089
						2	rs16991615	0.85 (0.21 - 3.39)	0.820
							CED: 0	1 (ref)	0.005
							– > 0 – 4,000	1.36 (0.59 – 3.14)	0.473
							– ≥ 4,000 – 8,000	4.48 (1.73 – 11.58)	0.002
							– ≥ 8,000	3.82 (1.22 – 11.95)	0.021
							SNP*CED: 0	1 (ref)	0.973
							– > 0 – 4,000	1.07 (0.14 – 8.06)	0.950
							– ≥ 4,000 – 8,000	0.61 (0.05 – 6.74)	0.683
							– ≥ 8,000	NA	NA
							Hormones	1.89 (0.95 – 3.75)	0.069
UIMC1	**rs365132**	5	G	T	0.5	1	rs365132	1.09 (0.70 – 1.69)	0.720
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.35 (0.63 – 2.91)	0.443
							– ≥ 4,000 – 8,000	4.18 (1.75 – 10.00)	0.001
							– ≥ 8,000	5.03 (1.68 – 15.11)	0.004
							Hormones	1.80 (0.91 – 3.54)	0.090
						2	rs365132	0.79 (0.39 - 1.61)	0.518
							CED: 0	1 (ref)	0.017
							– > 0 – 4,000	0.44 (0.11 – 1.82)	0.257
							– ≥ 4,000 – 8,000	4.05 (1.01 – 16.19)	0.048
							– ≥ 8,000	4.83 (0.78 – 29.90)	0.091
							SNP*CED: 0	1 (ref)	0.265
							– > 0 – 4,000	2.89 (0.93 – 8.98)	0.067
							– ≥ 4,000 – 8,000	1.04 (0.32 – 3.39)	0.948
							– ≥ 8,000	1.01 (0.17 – 5.98)	0.988
							Hormones	1.78 (0.89 – 3.57)	0.104
RAD51	**rs9796**	15	A	T	0.42	1	rs9796	0.94 (0.62 - 1.44)	0.787
							CED: 0	1 (ref)	0.001
							– > 0 – 4,000	1.37 (0.64 – 2.94)	0.419
							– ≥ 4,000 – 8,000	4.17 (1.74 – 9.99)	0.001
							– ≥ 8,000	4.98 (1.66 – 14.92)	0.004
							Hormones	1.79 (0.91 – 3.53)	0.092
						2	rs9796	0.92 (0.43 – 1.97)	0.838
							CED: 0	1 (ref)	0.167
							– > 0 – 4,000	1.66 (0.52 – 5.33)	0.397
							– ≥ 4,000 – 8,000	4.33 (1.18 – 15.91)	0.027
							– ≥ 8,000	2.34 (0.48 – 11.42)	0.291

Table 2. Association of single nucleotide polymorphisms with reduced ovarian function and CED-score in DCOG LATER-VEVO discovery cohort (continued)

Gene	Variant	Chrom	Ref.	Alt.	MAF	Model	Variant, interaction term	OR (95% CI)	P-value
							SNP*CED: 0	1 (ref)	0.546
							– > 0 – 4,000	0.81 (0.28 - 2.33)	0.692
							– ≥ 4,000 – 8,000	0.94 (0.29 - 3.16)	0.938
							– ≥ 8,000	2.82 (0.52 – 15.37)	0.230
							Hormones	1.70 (0.85 – 3.39)	0.135

Chrom., chromosome; MAF, minor allele frequency; CCS, childhood cancer survivors; CED, Cyclophosphamide Equivalent Dose; Ref, Reference allele; Alt, alternative allele. Position based on position build 37 on https://www.ncbi.nlm.nih.gov/snp/. Alt is reported as 0/1/2 (recalculated for presentation only, based on allelic dosage) for CCS with and without reduced ovarian function (see Methods section for details). Model 1: adjusted for principal components, use of hormone use and CED-categories. Model 2: additional to Model 1 interaction term of variant*CED category.

The modifying effect of >0 – 4,000 CED in *FANCI* (rs1054875) was non-significant in both replication cohorts. The three-cohort meta-analysis showed no significantly modifying effect on the association between >0 – 4,000 CED and reduced ovarian function (OR 2.76, 95% CI 1.17 – 6.53, p = 0.02) after correction for multiple testing.

DISCUSSION

This is the first study to assess the influence of genetic factors on alkylating chemotherapy-induced reduced ovarian function, using AMH as a biomarker, and incorporating two independent and identically phenotyped replication cohorts and a meta-analysis. We report a strong modifying effect of a common SNP (minor allele frequency 0.34) in the *BRSK1* gene on the toxicity of high dose alkylating agents, resulting in a 3-fold increased odds of a reduced ovarian function for CCS carrying one G allele compared to CCS without this allele (OR genotype AA: 1.8 vs OR genotype AG: 5.3) and a further 3-fold increased odds for CCS carrying two G alleles (OR genotype GG: 15.3).

One previous single center study evaluated the association between ovarian function in CCS with SNPs associated with age at menopause in the general population reporting that the T allele of rs1172822 of the *BRSK1* gene was inversely associated with serum AMH levels[41]. However, this study did not assess interaction between treatment and AMH levels or include validation using replication cohorts. Recently, a SJLIFE GWAS study identified a haplotype associated with an increased risk of premature menopause, especially in the subgroup of CCS who had received pelvic radiotherapy[44]. However, the haplotype is beyond the scope of this study as our population excluded survivors treated with bilateral ovarian radiotherapy due to low inter-individual variation of POI and the haplotype is not associated with DNA damage response genes.

Table 3. Association of single nucleotide polymorphisms with reduced ovarian function and chemotherapy in the meta-analyses

					Replication (PCL+SJLIFE) meta-analysis			Discovery + Replication (VEVO + PCL + SJLIFE) meta-analysis		
Gene	**Variant**	**Ref>Alt**	**Model**	**variant, interaction**	**OR (95% CI)**	**Direction**	**p-value**	**OR (95% CI)**	**Direction**	**p-value**
BRSK1	rs11668344	A>G	2	rs11668344	0.82 (0.54 – 1.24)	-+	0.349	0.76 (0.53 - 1.11)	--+	0.152
				CED: 0	1 (ref)			1 (ref)		
				– > 0 – 4,000	0.58 (0.21 – 1.58)	--	0.284	0.98 (0.46 – 2.09)	+--	0.964
				– ≥ 4,000 – 8,000	3.42 (1.52 – 7.67)	++	2.8×10^{-4}	3.83 (1.90 – 7.74)	+++	1.8×10^{-4}
				– ≥ 8,000	1.77 (0.18 – 17.60)	+-	0.627	1.82 (0.40 – 8.34)	++-	0.442
				SNP*CED: 0	1 (ref)			1 (ref)		
				– > 0 – 4,000	3.27 (1.11 – 9.66)	+-	0.032	1.37 (0.29 – 6.51)	-+-	0.690
				– ≥ 4,000 – 8,000	1.04 (0.44 - 2.48)	+-	0.922	0.98 (0.48 – 2.02)	-+-	0.960
				– ≥ 8,000	3.63 (1.66 - 7.95)	++	1.3×10^{-3}	3.81 (1.85 - 7.86)	+++	3.0×10^{-4}
FANCI	rs1054875	A>T	2	rs1054875	1.01 (0.65 - 1.56)	+-	0.977	0.85 (0.57 - 1.28)	-+-	0.432
				CED: 0	1 (ref)			1 (ref)		
				– > 0 – 4,000	0.88 (0.28 – 2.80)	+-	0.828	0.54 (0.23 – 1.24)	-+-	0.148
				– ≥ 4,000 – 8,000	5.29 (2.08 – 13.50)	++	4.7×10^{-4}	3.91 (1.83 – 8.33)	+++	4.1×10^{-4}
				– ≥ 8,000	3.69 (0.37 – 36.8)	++	0.266	3.70 (0.83 – 16.6)	+++	0.088
				SNP*CED: 0	1 (ref)			1 (ref)		
				– > 0 – 4,000	1.35 (0.46 - 3.96)	++	0.583	2.76 (1.17 – 6.53)	+++	0.021
				– ≥ 4,000 – 8,000	0.64 (0.29 - 1.40)	--	0.264	0.92 (0.46 - 1.86)	+--	0.823
				– ≥ 8,000	1.03 (0.53 - 2.03)	++	0.925	1.14 (0.61 - 2.12)	+++	0.691

PCL, PanCareLIFE cohort; SJLIFE, St. Jude Lifetime Cohort; VEVO, DCOG-LATER VEVO cohort; CED, Cyclophosphamide Equivalent Dose. Model 2: adjusted for principal components, hormone use (only for VEVO, SJLIFE) and CED-categories and the interaction term of variant*CED category. + = positive association of the SNP with reduced ovarian function in PCL and SJLIFE respectively. - = negative association of the SNP with reduced ovarian function in VEVO, PCL and SJLIFE respectively.

Table 4. Estimated cumulative OR per genotype of rs11668344 and CED score on reduced ovarian function, based on meta-analysis point estimates

	genotype AA		genotype AG		genotype GG	
CED in mg/m²	**N (%)**	**Estimated cumulative OR**	**N (%)**	**Estimated cumulative OR**	**N (%)**	**Estimated cumulative OR**
0	51 (40.8)	1 (ref)	36 (40.0)	0.76	14 (31.8)	0.58
> 0 – 4,000	19 (37.3)	0.98	19 (38.8)	1.02	5 (29.4)	1.06
≥ 4,000 – 8,000	36 (69.2)	3.83	36 (66.7)	2.85	7 (43.8)	2.12
≥ 8,000	43 (58.1)	1.82	62 (77.5)	5.27	18 (81.8)	15.26

N (%) represents the number of cases with reduced ovarian function (% of total) within each genotype group. OR, Odds ratio. Estimated ORs calculated by multiplying the corresponding ORs from the full model, for example for the estimate of genotype AG in CED category **≥ 8,000:** 1.82 * 0.76 * 3.81 = 5.27.

Our study revealed a strong *modifying* effect of a G allele of a genetic variant in *BRSK1* (rs11668344 A>G) on alkylating agent related reduced ovarian function. The meta-analysis on reduced ovarian function for the main effect of *BRSK1*, which is associated with an earlier age at menopause in the general population[16,18,19], did not find a significant association as the previous single center study reported[41]. Representing continuous variables such as CED-score in categories could lead to increased type I error for the detection of interaction effects[45]. Supplementary analyses using fractional polynomials (Supplementary Appendix, Tables S4) show that using the available data, estimating more flexible models to potentially avoid these spurious findings offers inconclusive results due to lack of power, while not contradicting the results found using the pre-defined categories.

Rs11668344 is an intronic variant in *THEM150B* and an expression quantitative trait locus that alters *BRSK1* RNA gene expression in whole blood (p-value = 2.4×10^{-19})[46] and has regulatory histone marks, suggesting a regulatory function. Several mechanisms for the modifying effect of *BRSK1* on reduced ovarian function in CCS can be considered. Alkylating agents are known to induce apoptosis of cancer cells by damaging DNA and inhibiting cellular metabolism, DNA replication and DNA transcription[20-23]. We hypothesize that due to a less efficient DNA damage response system, cancer patients carrying the G allele of rs11668344 in *BRSK1* are at an increased risk of the DNA-damaging impact of alkylating agents in healthy tissues most relevant to our outcome studied here, the ovary (Figure 1). It is plausible that the efficiency of the DNA damage response system becomes crucial upon treatment with alkylating agents amounting to high CED scores.

Future research will need to evaluate the relevant expression, which we would expect in granulosa cells or the primordial follicle pool – as opposed to the recruited and selected oocytes that have successfully progressed towards maturation. Several hypothetically relevant mechanisms of action require further research to elucidate causally biological pathways and target tissues involved in the modifying effect of *BRSK1* on alkylating agents-related low AMH levels (Supplementary Appendix).

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The identification of this genetic risk factor for alkylating agents-related low AMH levels, if confirmed for other measures of reduced ovarian function, may improve future risk prediction models including more adequate identification of groups with higher or lower risk of chemotherapy-induced ovarian impairment. Upfront fertility preservation programs, including ovarian tissue cryopreservation, will benefit from optimized prediction models as they can be directed to pediatric cancer patients at highest risk for gonadotoxicity for whom the balance of benefits/drawbacks -including ethical considerations- is most beneficial[47]. Moreover, female cancer survivors may also benefit from incorporating genetic testing to risk stratification in current targeted surveillance strategies of ovarian function and family planning counseling[48].

A major strength of this study is the inclusion of two replication cohorts. Yet, there were some differences in age at diagnosis and treatment exposures between the discovery and the replication cohorts. Survivors from the discovery cohort were younger at diagnosis, and were less often treated with alkylating agents amounting to CED score ≥8,000 mg/m^2. We therefore performed multiple sensitivity analyses to assess the choices of the model and cohort, but findings did not change our results. Another strength of this study is the measurement of AMH levels, as a marker for reduced ovarian function, with the same assay at one singular laboratory, eliminating between-assay differences. Previous studies demonstrated that alkylating agents are strongly associated with risk of reduced ovarian function as measured by decreased AMH levels in female CCS[4,28,49,50]. By using AMH levels as a marker of ovarian function, this study included a fairly substantial number of cases likely at increased risk of reduced fertility or a shorter reproductive window. However, while low AMH levels can also identify poor responders in assisted reproductive technology[51,52], it needs to be emphasized that AMH remains a *surrogate* marker of ovarian function. Validation using data collected long-term and using more definite and direct endpoints such as age at menopause, POI, or fecundity is needed to facilitate translation into clinical practice. In addition, larger cohorts would benefit the power of statistical tests.

In conclusion, this study shows that high dose alkylating chemotherapy-induced reduced ovarian function in female CCS is strongly modified by a common DNA variant (rs11668344) of the *BRSK1* gene. This is the first time a genetic risk factor has been described to modify the effect of chemotherapy on long-term ovarian function in three independent cohorts. This finding may serve as a starting point for individualized counseling regarding treatment-related risks and fertility preservation services in children with cancer as well as young adult survivors.

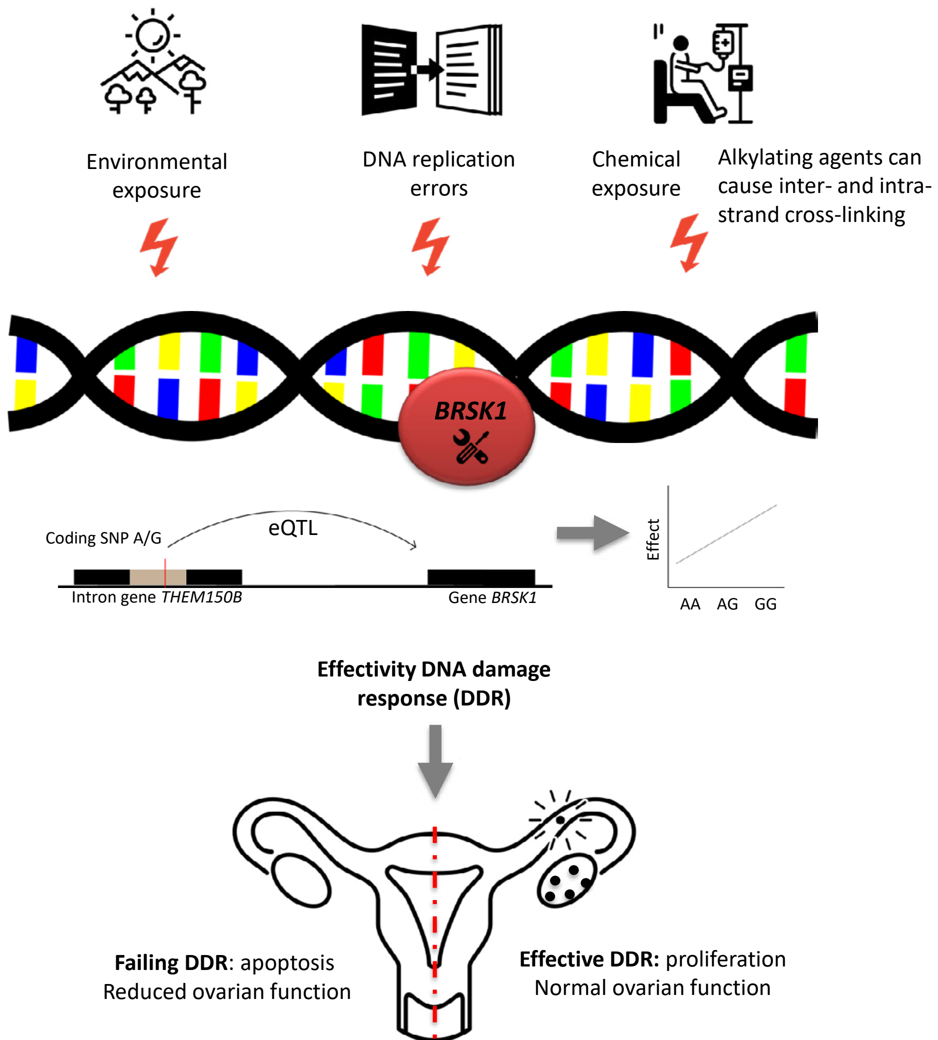

Figure 1. Simplified representation of the hypothesized biological plausibility
Simplified representation of the hypothesized biological plausibility of the effect of BRSK1 on reduced ovarian function. DNA damage can be the result of environmental exposure, DNA replication errors but also of chemical exposure. Alkylating agents are known to induce apoptosis of cancer cells by damaging DNA and inhibiting cellular metabolisms and DNA replication and transcription[20-23]. DNA damage response genes (BRSK1 is known to act as a DNA damage checkpoint) have previously been associated with age at natural menopause. Owing to a less efficient DNA damage response system, childhood cancer survivors carrying the G allele of rs11668344 (BRSK1) may be at an increased risk of the DNA-damaging impact of alkylating agents.

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PART III

Obstetric risks