Title: A Genome-Wide Association Study of Stroke and Myocardial Infarction among Long-term Survivors of Childhood Cancer

Working Groups: Genetics
Chronic Disease

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Background & Rationale:

With contemporary therapy, five-year survival rates for many childhood malignancies exceed 80%.[1] As the number of childhood cancer survivors continues to grow,[2] reducing the adverse consequences of treatment is increasingly important in this vulnerable population. Unfortunately, curative chemotherapy and radiation therapy is associated with significant cerebrovascular and cardiovascular morbidity. These treatment-related complications threaten long-term quality of life and contribute to premature death among survivors of childhood cancer.[3, 4] A recent report from the CCSS examined clinical risk factors and created a risk prediction model for stroke and ischemic heart disease among childhood cancer survivors.[5] The model identified radiation therapy exposure to the chest and male sex as being associated with ischemic heart disease and cranial radiation exposure, radiation dose of ≥ 35 Gy to the chest, and alkylating agent chemotherapy being associated with stroke. Mechanisms underlying individual genetic susceptibility to cerebrovascular and cardiovascular complications remains incompletely understood, which severely limits precision prevention efforts. Therefore, the objective of this study is to identify inherited genetic variation associated with the incidence of cerebrovascular and cardiovascular complications among childhood cancer survivors enrolled in the Childhood Cancer Survivor Study (CCSS).

Cerebrovascular Late Effects: Stroke is a devastating late effect as these events often lead to long-term adverse physical and emotional sequelae. Several studies, including both large, multicenter cohort studies and smaller institutional case series, have demonstrated that childhood cancer survivors have increased rates of late-occurring transient ischemic attacks and strokes compared to unaffected populations.[6-14] In these reports, the incidence rates of first onset, non-perioperative stroke among childhood cancer survivors ranges between 57.9 – 628 strokes per 100,000 person years of follow-up.[6, 7, 13, 15] In a large cohort study involving long-term survivors of teenage and young adult cancer (the Teenage and Young Adult Cancer Survivor Study),[16] cancer survivors experienced a 40% increased risk of developing any cerebrovascular event compared with that expected from the general population (standardized hospitalization ratio [SHR] 1.4, 95% CI 1.3-1.4). Among brain tumor and leukemia survivors, exposure to cranial radiation therapy (CRT),[6, 7, 13, 17] dose of CRT,[15] and radiation exposure to the circle of Willis have been linked to stroke.[7, 13] Similarly, radiation exposure to the neck is associated with increased risks of stroke among Hodgkin's disease survivors.[14, 18] In addition to exposure to radiation, identified risk factors for stroke among childhood cancer survivors include hypertension,[15] severe headaches,[19] and neurofibromatosis

type-1.[8] Interestingly, established risk factors for stroke in the general adult population, including obesity, dyslipidemia, splenectomy, smoking, and use of oral contraception, were not associated with stroke risk among survivors of childhood cancer within the first two decades following treatment.[6, 12, 14] The pathophysiology of stroke following CRT is believed to be a result of thickening and fibrosis of the intima media of large cerebral arteries, leading to stenosis and occlusion of these vessels and subsequently to stroke.[9, 18, 20] Given the unique adverse effects of childhood cancer therapies, the biology underlying stroke in this population likely differs from the general population.

Cardiovascular Late Effects: Multiple studies have established that cardiotoxicity occurs as a result of exposure to radiotherapy and certain chemotherapeutic agents, particularly anthracyclines.[21-23] Cardiovascular co-morbidities (i.e., myocardial infarction) also arise as an unintended consequence of childhood cancer treatment. In the St. Jude Lifetime Cohort Study, the prevalence of major electrocardiographic abnormalities, including myocardial infarction, among survivors of childhood cancer was more than double that of unaffected controls.[24] Among survivors, the presence of major abnormalities was associated with anthracycline doses ≥300 mg/m^2 and cardiac radiation doses as low as 1-1,999 cGy. In the CCSS,[25] survivors of childhood cancer were significantly more likely than siblings to report myocardial infarction (hazard ratio [HR] 5.0, 95% CI 2.3-10.4). The same study showed that radiation exposure (doses ≥3500 cGy) led to an increased risk of myocardial infarction (P for trend <0.001), whereas anthracycline treatment and dose was not significantly associated with increased risk of myocardial infarction. Additional evidence suggests that survivors spared cardiotoxic therapy (i.e., anthracycline, cardiac-directed radiation) still experience an excessive risk of cardiovascular complications.[26, 27]

Genetics of Cerebrovascular and Cardiovascular Events: Stroke and myocardial infarction are leading causes of long-term disability and death among adult populations in the United States, and there are several established and potentially modifiable risk factors for these events, including but not limited to: hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking.[28] Studies in these populations have also indicated that inherited genetic variation is an important predictor of stroke and myocardial infarction risk;[29-34] however, the impact of genetic variation on the occurrence of these outcomes among childhood cancer survivors has not been fully explored.

We hypothesize that there are unique genetic loci that influence susceptibility to stroke and myocardial infarction secondary to childhood cancer therapy. The following specific aims outline a coordinated and comprehensive assessment of genetic susceptibility to cerebrovascular and cardiovascular complications among childhood cancer survivors. We will use a three-prong approach to evaluate our hypotheses: (1) a genome-wide association study (GWAS) to identify common variants associated with stroke or myocardial infarction; (2) a gene-based approach to evaluate the role of rare variants on the risk of stroke or myocardial infarction; and (3) a biologically driven approach to identify genetic variants that may influence these phenotypes through the alteration of gene expression.

Specific Aims:

1. Identify common genetic variants associated with occurrence of cerebrovascular and cardiovascular complications among long-term survivors of childhood cancer.

1.a. Discover common genetic variants (minor allele frequency [MAF ≥5%]) associated with stroke among 5,324 childhood cancer survivors of European ancestry enrolled in the CCSS.

1.b. Discover common genetic variants (MAF ≥5%) associated with myocardial infarction among 5,324 childhood cancer survivors of European ancestry enrolled in the CCSS.

2. Evaluate the contribution of low- and intermediate-frequency genetic variants to cerebrovascular and cardiovascular complications among long-term survivors of childhood cancer.

2.a. Utilize a gene-based aggregation test of rare to low frequency genetic variants (MAF <5%) to identify genes associated with stroke among 5,324 childhood cancer survivors of European ancestry enrolled in the CCSS.

2.b. Utilize a gene-based aggregation test of rare to low frequency genetic variants (MAF <5%) to identify genes associated with myocardial infarction among 5,324 childhood cancer survivors of European ancestry enrolled in the CCSS.

Analysis Framework:

This analysis will leverage existing data within the CCSS to address each specific aim. The proposed study population, variables of interest, and analytic plan for each aim are outlined below. Final decisions on the methods will be reached with input from CCSS statisticians and collaborators:

Outcomes of Interest: The two primary outcomes of interest for this study are self- or proxy-reported: stroke (CTCAE grades 3 – 5) and myocardial infarction (CTCAE grades 3 – 5). Each outcome will be dichotomous (yes/no) documentation of first-stroke or first-myocardial infarction reported on an Original Cohort CCSS questionnaire (Baseline or Follow-up 1 - Follow-up 5).

Study Population: The study population will consist of the 5,324 childhood cancer survivors of European ancestry enrolled in the Original CCSS Cohort (diagnosed 1970-1986) with available genotype data. In this eligible population, 163 survivors reported stroke and 121 reported myocardial infarction as of the June 1, 2017 data release. We will conduct secondary analyses for each outcome restricted to high-risk sub-populations survivors:

- Stroke Subset 1: Radiation (>1 cGy) to the head or neck within 5 years of primary cancer diagnosis
- MI Subset 1: Radiation (>1 cGy) to the heart within 5 years of primary cancer diagnosis
- MI Subset 2: Anthracycline (>1 mg/m^2) treatment within 5 years of primary cancer diagnosis
- MI Subset 3: Radiation (>1 cGy) to the heart and anthracycline (>1 mg/m^2) treatment within 5 years of primary cancer diagnosis

Exploratory Variables: As outlined in the analytic approach for each specific aim, the primary exploratory variables (predictor variables) will include:

- Imputed genotypes available through the Request for Proposals for Genome-Wide Investigation of Late-Effects using the CCSS Cohort. Genotypes are imputed based on the 1000 Genomes Project.
- Gene expression levels imputed from the available genotype data

Additional Covariates considered in the analysis will include:

- Cancer Diagnosis
- Year of Diagnosis
- Age at Diagnosis
- Age at Last Follow-up
- Age at SMN (censor follow-up)
- Age at Event (for stroke and myocardial infarction)
- Vital Status at Last Follow-up
- Sex
- Genetically determined ancestry (e.g., calculated ancestry-specific principal components)

- Reported Co-Morbidities (e.g., Hypertension, Obesity, Diabetes Mellitus)
- Behavioral Risk Factors (e.g., Smoking Status, Physical Activity)
- Cumulative Chemotherapy Dose (i.e., anthracycline dose, alkylating agent score)
- Radiation Dose: Estimated from the maximum treatment dose to the: 1) cranium, 2) pituitary gland (surrogate for the circle of Willis), 3) neck (surrogate for carotid arteries, and 4) chest/heart. Segments will be considered to fall within the radiation field if greater than half of the segment was included in the primary radiation field. Radiation Dose will be evaluated according to region of interest:
  - Cranial/neck radiation dose: (1) None; (2) < 20 Gy; (3) 20 – 29 Gy; (4) 30 – 49 Gy; (5) ≥50 Gy.
  - Chest/heart radiation dose: (1) None; (2) < 5 Gy; (3) 5 – 14 Gy; (4) 15 – 34 Gy; (5) ≥35 Gy.

Analytic Approach: We will work with the CCSS statistical team to finalize the appropriate analysis for the proposed study. Descriptive statistics will be generated and compared between survivors with and without the outcomes of interest (stroke or myocardial infarction). For each aim, we will conduct regression diagnostics to evaluate the assumptions and overall goodness of fit for the most significant findings. Appropriate steps will be taken to address multiple comparisons (i.e., Bonferroni-corrected p-values), influential observations, and violations of the regression model assumptions. Study characteristics will be displayed in tables such as example Table 1 at the end of the proposal.

Analysis for Aim 1: Aim 1 will test the association of common genetic variants (MAF ≥5%) with occurrence of stroke or myocardial infarction. We will construct logistic regression models to calculate effect estimates (odds ratio [OR], 95% confidence intervals [CI] and p-values for the association between each imputed genetic variant and stroke/myocardial infarction. The proposed analyses will be performed using available software packages (e.g., SNPTEST v2.5.4 software, GenABEL or the R GENESIS software[35]), assuming additive allelic effects. When the outcome of interest has low prevalence, it is possible that for some combination of the predictors (i.e., variant alleles) of all observations have the same event status. Should this happen, we would apply exact methods. Quality control of the imputed data set will remove with a MAF <5% or imputation quality score (R2) <0.30. We will adjust for covariates, including primary cancer diagnosis, age at cancer diagnosis, sex, radiation site and dose. We will construct Cox proportional hazards models to take advantage of time to event data and calculate hazard ratios (HR) for the association between each SNP and time from diagnosis to stroke/myocardial infarction. To the extent possible, we will conduct secondary analyses for SNPs associated with the phenotype of interest (e.g., p-value $<5x10^{-6}$) including covariates for behavioral factors and co-morbidities (e.g., participant-reported obesity, hypertension, smoking at entry into the cohort) to evaluate the potential impact of these establish risk factors on the observed associations. Therapeutic subgroup analyses will be restricted to the high-risk survivor populations previously identified (see Study Population). Statistical significance will be defined at a genome-wide p-value $<5x10^{-8}$.[36] Results will be displayed in tables (see example Table 2) as well as figures (see example Figure 1 and 2).

Statistical Power: Power to detect genome-wide significant association signals (p-value $<5x10^{-8}$) was calculated with Quanto software, conservatively assuming 163 cases of stroke were compared to 1,600 unaffected controls. The proposed study has >80% power to detect genetic variants with effect sizes (OR) >2.65 at a MAF=10% and >2.10 at a MAF=40%. Similarly, assuming 121 cases of myocardial infarction are compared to 1,200 similarly treated controls, the proposed study is powered to discover SNPs with effect sizes >3.00 at a MAF=10% and >2.35 at a MAF=40%.

Table 2. Results from common variant analyses

Chr.	BP	SNP ID	Gene	Functional annotation	A1	A2	N	OR	95% CI	p-value
1	2345	Rs6789	*ABC*	Exonic	A	C	5324	2.5	1.6-3.4	$1x10^{-4}$

Chr.=chromosome; BP=base pair; SNP ID=single nucleotide polymorphism identifier; A1=allele 1; A2=allele 2

Figure 1. Manhattan Plot

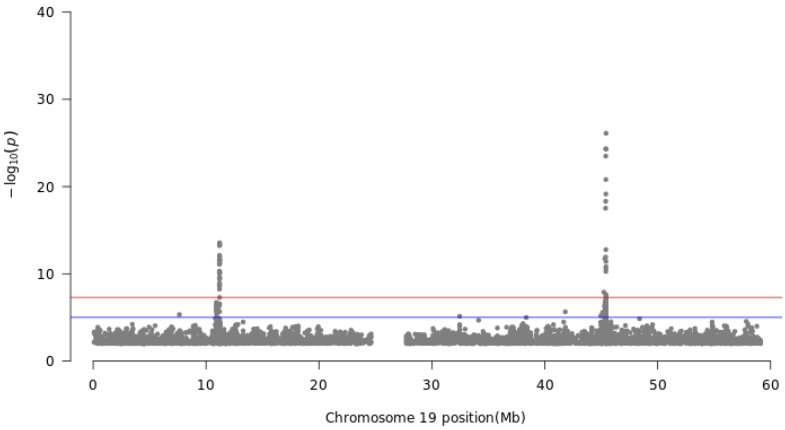
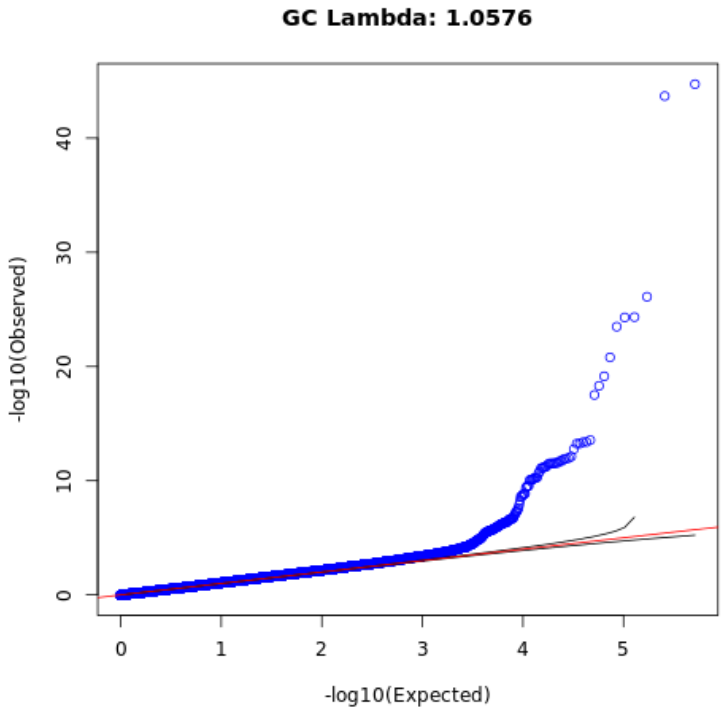


Figure 2. QQ Plot



Analysis for Aim 2: The goal of this aim is to assess the contribution of low- and intermediate-frequency genetic variation (MAF <5%) to the occurrence of stroke or myocardial infarction by applying tests that aggregate variants within an annotated gene, with secondary analyses focusing on a combined outcome of stroke or myocardial infarction. We will utilize the WGS Annotator (WGSA) pipeline that is driven by tools and databases that reflect decades of work with clinical/Mendelian genomes and large epidemiologic projects.[37, 38] Variant-centric annotation includes functional and deleteriousness prediction incorporating regulatory and epigenetic annotation from ENCODE,[39] Roadmap,[40] and FANTOM5.[41] Within each gene, a burden test (T5) and the optimized Sequence Kernel Association Test (SKAT-o) will be used while adjusting for age at cancer diagnosis, age at last follow-up or time of event, sex, and treatment exposures. SKAT-O maximizes power by adaptively using the data to optimally combine the burden test and the nonburden sequence kernel association test (SKAT). Burden tests are more powerful when most variants in a region are causal and the effects are in the same direction, whereas SKAT is more powerful when a large fraction of the

variants in a region are noncausal or the effects of causal variants are in different directions. SKAT-O is computationally efficient and has been applied to multiple genome-wide studies using SNP array data.[42-44] Variants included in the tests are annotated as loss-of-function, splicing, or nonsynonymous. The T5 test collapses variants with MAF <5% into a single genetic score and assumes all variants in gene will have effects in the same direction, while SKAT-o allows for omnidirectional effects of the variants. To incorporate Cox regression into the aggregate tests are statistically challenging, no such software currently exists. Therefore, we propose to conduct logistic regressions in the current application and will apply Cox model if appropriate method is developed. The current approaches employ a generalized linear mixed regression framework, and all analyses will be carried out using the R GENESIS package.[45] In association tests involving variants with low minor allele frequency or count, it is recommended to use a logistic regression model implementing Firth's bias reduction method,[46] which introduces a more effective score function by adding an term that counteracts the first-order term from the asymptotic expansion of the bias of the maximum likelihood estimation. Gene-based analyses will include all gene regions with ≥2 genetic variants present. Therapeutic subgroup analyses will be restricted to the high-risk survivor populations previously identified (see Study Population). Statistical significance will be defined at a p-value $<2.5x10^{-6}$ (Bonferroni correction assuming 20,000 gene tests). Results will be displayed in tables (see example Table 3) as well as figures (see example Figure 1 and 2).

Table 3. Results from gene-based SKAT-o analyses

Chr.	Gene	Start (BP) and Stop (BP) position	N	cMAF	# variants in the test	p-value
1	*ABC*	2345 to 10987	5324	0.092	20	$1x10^{-4}$

Chr.=chromosome; BP=base pair; cMAF=cumulative minor allele frequency

Special Consideration:

Plans for Replication: We have identified several potential avenues for replicating the top candidate loci identified in the CCSS Original Cohort. Childhood cancer survivors included in the replication phase will meet the same eligibility criteria as survivors in the discovery population. We have formal agreements in place (see letters of support) to replicate our findings in the following populations: 1) Children's Oncology Group study ALTE03N1: Key Adverse Events After Childhood Cancer: Survivors of childhood cancer (aged ≤21 years at diagnosis) with an incident stroke are matched 2:1 with similarly treated unaffected survivor controls (~70 cases/140 matched controls), and 2) St. Jude LIFE cohort: survivors of childhood cancer (not co-currently enrolled in CCSS) with an incidence of stroke (~79 cases) or myocardial infarction (~108 cases) will be matched to similarly treated unaffected survivors from a population of more than 3,600 participants. If necessary, we may also seek permission to replicate our findings in the CCSS Expansion Cohort, comprised of survivors of childhood cancer (diagnosed 1980-1999). If available, we will leverage existing genotype data from the replication population.

Biological generalizability: As a future direction, in order to provide biological context for the findings from Aims 1-3 of this proposal, we will perform "look-ups" in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium genome-wide association studies for several cardiovascular outcomes. CHARGE is integrated into the largest GWAS for coronary artery disease and myocardial infarction (CARDIoGRAMplusC4D[47]) and also for stroke (MEGASTROKE). Genetic loci identified in Aims 1-3 may be interrogated for evidence of an effect on cardiovascular outcomes in the general population. The studies used to assess generalizability include the resources shown in Table 4. These consortia also primarily include individuals of European ancestry.

Table 4. Characteristics of the available CHARGE GWAS

Phenotype	Consortium	Sample size*	Status	Availability
Coronary artery disease	CARDIoGRAMplusC4D	60,801/123,504	Published[47]	Public
Myocardial infarction	CARDIoGRAMplusC4D	43,676/128,198	Published[47]	Public
Stroke	MEGASTROKE	67,162/454,450	Submitted	Request

*Cases/control

Functional validation: Additionally as a future direction, for any candidate genes identified as a part of Aims 1-3, the team has the ability to elucidate the biological basis for of the observed association through phenotypic profiling of iPSC-derived cardiomyocyte and endovascular cell lines exposed to chemotherapeutic agents and radiation. We can define the molecular changes that occur in response to treatment through analysis of RNAseq, metabolomic, mitochondrial function, cardiac contractility, and cell viability data. We have used iPSC-cardiomyocytes to determine if candidate cardiotoxicity susceptibility genes are anthracycline-responsive in the heart. We have also completed pilot studies of other relevant functional assays to model doxorubicin-induced cardiotoxicity. This framework can easily be applied to other chemotherapeutic agents or radiation exposure. iPSC-endovascular cells can also be profiled in a similar manner to explore changes in the vascular cells.

Table 5: Characteristics of Childhood Cancer Survivors who Did and Did Not Develop Stroke or Myocardial Infarction				
Characteristic	**Stroke (n = ____)**	**No Stroke (n = ____)**	**Myocardial Infarction (n = ____)**	**No Myocardial Infarction (n = ____)**
Median age at diagnosis of primary cancer, years (range)				
Age at original diagnosis, years, *n (%)*				
< 5				
5 – 10				
11 – 15				
>15				
Median age at last follow-up, years (range)				
Current age, years, *n (%)*				
< 20				
20 – 29				
30 – 39				
> 40				
Median duration of follow-up, years (range)				
Sex, *n (%)*				
Males				
Females				
Race/ethnicity, *n (%)*				
White, non-Hispanic				
Black, non-Hispanic				
Hispanic, non-Hispanic				
Other				
Primary cancer diagnosis, *n (%)*				
Leukemia				
CNS tumors				
Hodgkin's disease				
Non-Hodgkin's lymphoma				
Kidney Tumors				
Neuroblastoma				
Soft tissue sarcoma				
Bone tumors				
Dose of radiation to cranium / neck (*Stroke) n (%)*				
None				
< 20 Gy				
20 – 29 Gy				
30 – 48 Gy				
≥ 50Gy				
Dose of radiation to heart / chest *(Myocardial Infarction)* n (%)				
None				
< 5 Gy				
5 - 14 Gy				
15 - 34 Gy				
≥ 35 Gy				
Anthracycline Dose, *n (%):*				
None				
1 – 99				
100 – 249				
>250				
Unknown				
Alkylating agent score, *n (%):*				
0				
1 – 2				
3 – 4				
≥ 5				
Unknown				
Co-Morbidities:				
Hypertension				
Dyslipidemia				
Obesity				
Diabetes Mellitus				
Reported ever smoking				
Physical Activity				
Vital status, number alive, *(%)*				

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