

1. **Study title:** Genetic Association Study of Cardiac Toxicity Following Chest Radiotherapy

2. **Working group and investigators:**

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3. **Background and rationale:**

Cardiac disease is among the most common causes of severe or disabling chronic conditions in childhood cancer survivors in long-term survivors of childhood cancer (1-3). A significant proportion of pediatric cancer patients receive radiation therapy (RT) as part of their cancer treatment. While RT is a necessary component of therapy for many pediatric cancers, it is also a known risk factor for late cardiac diseases including pericarditis, pericardial fibrosis, myocardial fibrosis and cardiomyopathy, coronary artery disease, valvular dysfunction, and arrhythmias (2-4). The highest risks of late cardiac disease are for children and young adults who received higher heart doses (4). The relative risk of late cardiac disease is up to 60-fold higher in childhood cancer survivors receiving higher heart dose from RT (>15 Gy and >30 Gy mean heart dose associated with highest risks) (5, 6). A recent study using the Childhood Cancer Survivor Study (CCSS) by Bates et al. JCO 2019 found that large volumes (>50%) of heart receiving low-moderate (5-20 Gy) doses or smaller volumes (1-30%) of heart receiving high (>20 Gy) doses had RR for cardiac disease of 1.6 - 2.4 (4). Despite compelling evidence for radiation causing a variety of cardiovascular complications, currently there is no way to predict who is at higher or lower risk of cardiac toxicity from radiation, and the critical factors that alter radiation-induced cardiotoxicity are not completely clear. This leads to a one-size-fits-all approach to limiting cardiac doses for radiation treatments, which is suboptimal.

While the pathophysiology of radiation-induced cardiac dysfunction is multifactorial, many of the clinical manifestations of cardiac radiation exposure stem from collagen and extracellular matrix deposition and subsequent fibrosis and/or vascular damage (1, 2). These events can occur in all areas of the heart, including the pericardium, the vasculature, the cardiac valves, and the myocardium. Valvular dysfunction and arrhythmias are late effects of cardiac radiation exposure (1, 2). Valvular dysfunction can be caused by damage to the cardiac valves directly and/or the surrounding myocardium. This can lead to fibrosis and thickening of the valves, calcification, and/or valvular retraction (3). Similarly, the conduction system of the heart can be damaged by radiation-induced pathways that result in changes in the extracellular matrix, vascular function, and fibrosis. Dysfunction along the entire conduction system have been described after cardiac radiation exposure, resulting in a number of types of arrhythmias (4, 5). While valvular

dysfunction and arrhythmias manifest with different clinical findings, they can stem from similar injury mechanisms: extracellular matrix changes and inflammation, leading to fibrosis and/or vascular damage. Because this study aims to find variants that cause changes in pathways leading to radiation-induced cardiac dysfunction, examining phenotypes that can manifest from similar radiation-induced fibrosis and/or vascular function. Although there are smaller number of events when arrhythmias and valvular dysfunction are examined separately, we also will examine the phenotypes separately for this project.

Radiosensitivity is a complex genetic trait, but little is known about the specific genetic variants associated with cardiac toxicity following radiotherapy. Rare, single-gene syndromes associated with hypersensitivity to radiation include Ataxia-Telangiectasia, in which mutations in the DNA strand break sensing and repair gene *ATM* result in extreme radiosensitivity and cancer predisposition (7), as well as related syndromes resulting from rare mutations in other DNA repair genes including *NBS1*, *MRE11* and *LIG4* (8, 9). In addition, common genetic variants, such as single nucleotide polymorphisms (SNPs), also impact the response of normal tissues to radiation, predisposing individuals to the development of toxicities. In fact, heritability is estimated to range from 58% to 82% (10-16). More data are needed regarding potential factors, including genetic variation, which can influence the sensitivity of patients to cardiac radiation as part of their cancer care. The CCSS provides an ideal tool to begin to examine whether genetic variants influence the development of long-term cardiac toxicities after radiation in childhood cancer survivors. A genetic risk score for development of late cardiac toxicity could be used to determine screening and intervention strategies and, thus, have a major impact on survivorship care.

We hypothesize that germline genetic variants (SNPs) increase the risk for development of cardiac toxicity with increasing radiation dose to the heart received during the course of cancer treatment in children. Our specific aims involve SNP discovery, as well as targeted assessment of SNPs with a previously reported role in cardiac disease in the general population. We will use discovery genome-wide association approaches to identify novel risk SNPs for which the frequency differs significantly comparing childhood cancer survivors who developed cardiac toxicity post-radiotherapy to those survivors who did not develop such outcomes, controlling for important covariates such as length of follow-up and chemotherapy and radiation exposure. To assess the role of known cardiac disease predisposition SNPs, we will compute a polygenic risk score (PGRS) combining allelic information from a panel of published coronary heart disease SNPs (17), and this PGRS will be tested for association with cardiac toxicity in childhood cancer survivors, again controlling for important covariates. The rationale for selecting this PGRS is that there are likely common molecular pathways that, when disrupted, predispose to both radiotherapy induced heart damage and coronary heart disease. As noted above, arrhythmias and valvular dysfunction stem from radiation-induced cell death and aberrant signaling that result in extracellular matrix changes and inflammation, leading to fibrosis and/or vascular damage. The genes and molecular pathways involved in this damage likely overlap, at least in part, with the genes and molecular pathways involved in coronary heart disease more generally. For example, a locus associated with coronary heart disease lies within *NOS3*, encoding nitric oxide synthase 3, which is involved in production of nitric oxide, a potent vascular smooth muscle relaxant (18). It is plausible that aberrant function of vascular smooth muscle cells would predispose to radiation-induced vascular damage that could result in increased risk for development of arrhythmia and/or valvular dysfunction. Another coronary heart disease-associated SNP is intronic to *SMAD3* (18), a downstream mediator of TGF- β , both of which are well-characterized signaling molecules centrally involved in radiation-induced tissue fibrosis (19). Other SNPs comprising the PGRS for coronary heart disease also likely tag genes involved in pathways related to radiation-induced damage.

In both approaches - GWAS and PGRS - we will investigate potential interactions between genetic variants and radiation exposure to assess effect modification. Finally, we will explore potential biologic mechanisms using genetically-predicted gene expression patterns from our

genome-wide association results. Our analysis will focus specifically on post-radiotherapy cardiac valvular disease and arrhythmias (combined as a single outcome), as these have not, to our knowledge, been studied previously in the context of genetic predisposition in childhood cancer survivors. These outcomes are relatively rare, and inclusion of all CCSS participants for whom genetic and toxicity data is available in a single discovery cohort will maximize statistical power. If we find that there are a sufficient number of participants from CCSS for whom genetic and toxicity data is available, we may divide the cohort into a discovery and validation set.

4. **Specific aims/objectives/research hypotheses:**

Aim 1. Identify SNPs associated with the development of cardiac valvular disease and/or arrhythmias in childhood cancer survivors and evaluate the effect of chest RT.

1a. Conduct a genome-wide association study (GWAS) of cardiac valvular disease and/or arrhythmia among survivors of childhood cancer, adjusting for clinical covariates found to be important.

1b. Perform separate GWAS in subgroups stratified by receipt of RT to determine whether genetic factors modify the effect of radiation on risk of developing valvular disease and/or arrhythmias.

Aim 2. Determine whether a PGRS for coronary artery disease (20) is associated with increased risk of cardiac valvular disease and/or arrhythmia following RT.

2a. Test association of the PGRS (in which each SNP is weighted based on the published coronary artery disease association (20)) with cardiac valvular disease and/or arrhythmia, adjusting for clinical covariates found to be important.

2b. Determine if dose to the heart modifies the effect of the weighted PGRS on risk of cardiac valvular disease and/or arrhythmia following radiotherapy in childhood cancer survivors.

Aim 3. Explore mechanisms of radiotherapy-related cardiac valvular disease and/or arrhythmia through *in silico* bioinformatic analysis.

3a. Overlap top-ranking GWAS-identified SNPs with tissue-specific bioinformatics reference data on genomic regulatory markers and chromatin state available through the ENCODE database (21).

3b. Test top-ranking GWAS-identified SNPs for association with gene expression using the GTEx tissue database (22).

3c. Use PrediXcan (23) to explore the predicted gene expression patterns and molecular mechanisms through which top-ranking GWAS-identified SNPs affect radiotherapy-related cardiac toxicity.

5. **Analysis framework:**

Outcome(s) of interest:

Cardiac arrhythmia and cardiac valvular disease will be defined using similar methods to the recent CCSS paper by Bates et al. (4). Specifically, using the National Cancer Institute Common Terminology Criteria for Adverse Events (version 5), conditions will be categorized as mild (grade 1), moderate (grade 2), severe or disabling (grade 3), life threatening (grade 4), or fatal (grade 5). When insufficient information is available to distinguish between grades, the lower-severity grade will be assigned. Our analysis will be restricted to grade 3 to 5 arrhythmias and valvular disease. Valvular disease and arrhythmia will be analyzed as a single, combined outcome (primary analysis) as well as separate outcomes (secondary analysis).

Subject population to be included:

This study will include all childhood cancer survivors who have data available on one or both cardiac outcomes, data on receipt of chest radiotherapy (minimum yes/no), as well as genome-wide SNP data. Individuals must be free from the given outcome (arrhythmia or valvular disease) at the time of CCSS cohort entry in order to be included in analysis for that particular outcome. All individuals with data available will be included (i.e. toxicity cases and controls will not be pre-selected). From Mulrooney et al. (6), we estimate having 104 cases in total (68 with grade 3+ arrhythmia and 36 with grade 3+ valvular disease), though we will need to confirm all have genomic data available. Subjects may be included from both the original CCSS cohort as well as the expanded cohort, pending data availability.

Exploratory variables:

Age at diagnosis

Year (decade) of diagnosis

Ethnicity

Sex

Cardiotoxic chemotherapy use (type of drug, dose), noting those who received anthracyclines and cumulative dose of anthracycline

Heart dose and dose volume metrics (using methods by Bates et al (4), including mean heart dose, volume of heart receiving ≥ 5 Gy, maximum heart dose < 20 Gy, and volume of the heart receiving ≥ 20 Gy

History of smoking (time dependent)

Diabetes (time dependent, if sufficient data on age at diagnosis available)

Hypertension (time dependent, if sufficient data on age at diagnosis available)

Dyslipidemia (time dependent, if sufficient data on age at diagnosis available)

Coronary artery disease (time dependent)

Analytic Approach:

Aim 1 proposes to use standard GWAS approaches with the QC criteria previously used by the CCSS GWAS studies to identify novel risk SNPs ($MAF \geq 0.05$) for cardiac valvular disease and/or arrhythmia, defined as a single binary outcome (primary analysis). Analysis in Aim 1a will include all participants, regardless of RT exposure. Analysis in Aim 1b will be stratified by heart dose and dose-volume metrics, comparing those with either $>50\%$ of heart receiving low-moderate (5-20 Gy) radiation or 1-30% of heart receiving high (>20 Gy) radiation to those receiving lower (or no) dose/volumes. If we find that the number of participants in the high dose/volume group is very few, we would consider instead stratifying by mean heart dose ≥ 10 Gy and comparing to those receiving a mean heart dose < 10 Gy. For all analyses, we will use Cox regression assuming an additive genetic model to test each SNP, adjusting for clinical covariates. Important clinical covariates will first be selected univariately, allowing a liberal threshold for inclusion as adjustment factors (p -value < 0.2). Statistical significance for SNP-toxicity associations will be defined as $p < 5 \times 10^{-8}$, which is the typical threshold applied in GWAS and represents a Bonferroni correction for 1 million independent SNP tests. In the event that no SNPs meet this stringent threshold, we will apply a more relaxed threshold, for example $p < 5 \times 10^{-6}$, for subsequent analysis of effect modification and exploratory bioinformatic analysis. We will also explore gene- and pathway-based analyses using Pascal (24) to explore results further, given that the limited sample size means we will likely miss many true positive single-SNP associations with small effect sizes. We will also compute the Bayes false discovery probability (BFDP) (25) for each significant SNP as an additional measure of the likelihood that our statistically significant associations represent false positives.

Example Table 1. Association results for SNPs significantly associated with arrhythmia and valvular disease at the genome-wide significance threshold of $p < 5 \times 10^{-8}$ (alternatively – top SNPs if none reach genome-wide significance).

SNP	Location (chr, BP)	Minor Allele	MAF	OR (95% CI)	P	BFDP
rs11111	chrX:NNN	A/T/C/G	%			%

Effect modification will be evaluated by adding an interaction terms to Cox model(s) evaluating the main effects of the SNP(s) and radiation exposure, where the SNPs are top SNPs of the stratified analyses stratified by chest RT exposure. In the event that we identify a significant interaction ($p < 0.05/2k$ for the interaction term for k top hits from the two stratified analyses), a table will be included reporting the full model results (i.e. SNP, all covariates, and interaction).

Aim 2 proposes evaluating a PGRS for coronary artery disease in the general population to determine whether this score is also a risk factor for valvular disease and/or arrhythmia in childhood cancer survivors exposed to chest radiotherapy. As in the discovery GWAS, we will use Cox regression to test the PGRS (as a continuous variable) for association with toxicity, adjusting for clinical covariates. Because the PGRS is tested as a single variable representing the cumulative effect of multiple SNPs, there is no need to correct for multiple comparisons, and statistical significance will be defined as $p < 0.05$ for the PGRS. To calculate the weighted PGRS, the number of alleles for each SNP is first multiplied by the regression coefficient representing the effect size on coronary artery disease in the general population (reported in (20)) prior to summing the alleles across all SNPs included in the score. We will report histograms of each PGRS in addition to association test results (see Example Table 2). Effect modification will be evaluated by testing an interaction term in the multivariable Cox regression model, as for the single-SNP analysis described above.

Example Table 2. Association between polygenic risk score and each of arrhythmia and valvular disease, adjusted for clinical covariates.

	Odds Ratio (95% CI)	P
PGRS		
Clinical covariate 1		
Clinical covariate 2		

In Aim 3, we will use PrediXcan to explore potential biologic mechanisms underlying SNP-toxicity associations identified (23). PrediXcan can directly test the molecular mechanisms through which SNPs affect a given phenotype, such as radiotherapy-related cardiac toxicity. This analytic tool estimates the component of gene expression determined by an individual's genetic (i.e. SNP) profile and correlates this estimated gene expression with the phenotype/disease to identify genes involved in the etiology. A major advantage of PrediXcan is that it can use reference transcriptome data sets that are publically available, requiring only genome-wide SNP data from the study population under investigation. Results from a PrediXcan analysis can point to novel pathways and aid in the design of follow-up experiments, as well as point to potential targets for intervention. An important limitation, however, is that these tissues are not radiation exposed, and thus we may not detect gene expression effects of SNPs that exert their effect only in the presence of radiation exposure. In addition, there are limited tissue types available, and the target tissue(s) or cell(s) of importance for development of radiation induced valvular disease or arrhythmias may not be available. However, these *in silico* analyses are relatively easy and low-cost to perform, and they could provide clues as to how to design subsequent functional studies using the appropriate tissue and/or cell types.

Power: Statistical power for the proposed project is limited by Aim 1 due to stringent correction applied for multiple testing. As our primary aim is to identify SNPs associated with the combined phenotype of arrhythmia and valvular disease, statistical power is determined by the combined number of cases, the minor allele frequency (MAF) of risk SNP(s), and the effect size of such

SNP(s). We estimate having 104 cases (68 with grade 3+ arrhythmia and 36 with grade 3+ valvular disease, from Mulrooney et al. (6)) and sufficient controls with no documented arrhythmia or valvular disease. The number of available samples will be higher if data is available from both the original CCSS cohort and the expanded cohort. Based on these numbers, we have >80% statistical power for detection of SNPs with MAF \geq 15% and with hazard ratios \geq 3.0, and approximately 50% power for less common SNPs. Power for stratified analyses will depend on the numbers of patients within each dose group. As noted above, gene- and pathway-based analyses will be used to maximize knowledge gained from the study given the limited statistical power for rare outcomes like cardiac radiotoxicity.

6. Special considerations:

Because ancestry is a potential confounder in genetic association studies, SNP analyses will be performed first among only individuals of Caucasian-ancestry (determined using genetically derived principal components from analysis with the 1000 Genomes reference population). A secondary analysis will be performed among all study subjects, and ancestry-derived principal components will be included as covariates.

Plans for replication: We plan to replicate our findings using data from the St. Jude Lifetime Cohort, which could serve as a validation set for any significant or suggestive associations identified in the proposed CCSS analysis. Specifically, we will test any SNP(s) reaching genome-wide significance in the CCSS cohort for association with the appropriate phenotype in St. Jude Lifetime Cohort (either the combined phenotype or individual phenotype). Additionally, we will perform an individual patient data GWAS meta-analysis, combining the beta coefficients and standard errors from Cox regression models, for the two cohorts. This approach will maximize our statistical power to detect SNPs that may have been missed in the CCSS analysis due to limited power, as prior work has shown that meta-analysis is more powerful than staged analysis for SNP discovery (26). We will use a fixed-effects meta-analysis using inverse-variance weighting. Cochran's Q statistic and associated p-values will test for across-study heterogeneity.

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