Genome-wide association studies – Treatment induced ototoxicity in patients and survivors of childhood cancer

Princess Máxima Center - ototoxicity project
Van den Heuvel-Eibrink Group

Request for collaboration and proposed analysis plans for SJLIFE and CCSS

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In collaboration with and the DCCSS LATER group
In collaboration with and the PanCareLife group

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Introduction

1. Background

Cisplatin is a major chemotherapeutic drug used to treat several solid tumors and central nervous system (CNS) tumors. Ototoxicity is an irreversible and prevalent adverse event that occurs in an average of 50% of cisplatin-treated pediatric patients [1-3]. Cisplatin accumulates in the cochlea and damages cochlear hair cells through the formation of cross-links with DNA and the production of excessive amounts of reactive oxygen species, which lead to damage to outer hair cells in the cochlea, resulting in hearing loss, tinnitus, and vertigo [4]. CIHL can greatly impact children's speech-language development social behaviors, education and overall quality of life[5]. Cisplatin-induced ototoxicity occurs among children receiving treatment in similar clinical settings suggesting that cisplatin related ototoxicity might be partially influenced by genetic susceptibility [6, 7].

2. Aim

Our aim is to identify and validate nuclear and mitochondrial variants associated with cisplatin induced hearing loss and tinnitus. Currently, we have three active projects on cisplatin induced hearing loss (CIHL) and tinnitus in childhood cancer patients and survivors, that we would propose to collaborate on.

- **Project A:** Identification of nuclear variants associated with CIHL in children with cancer in the PCL cohort. (joint discovery)
- **Project B:** Identification of mitochondrial variants associated with CIHL in children with cancer in the PCL cohort. (joint discovery)
- **Project C:** Identification of nuclear variants associated with tinnitus in childhood cancer survivors in the DCCSS LATER cohort. (joint discovery)
- **Project D:** Identification of mitochondrial variants associated with tinnitus in childhood cancer survivors in the DCCSS LATER cohort. (joint discovery)

Additional projects are anticipated, for instance, the prospectively collected childhood cancer patient's cohort of the Princess Maxima Center, the SOUND study.

3. Study cohorts

3.1. PanCareLife cohort

This cohort is a multinational European consortium of pediatric cancer patients recruited between 2015 and 2018. Study participants were recruited through the collaboration of 14 institutions from 7 countries:

Austria, Czech Republic, Denmark, Germany, Italy, Netherlands, and Switzerland. This cohort pursued a study which included 1124 cancer patients treated with cisplatin, carboplatin, or cranial radiotherapy for childhood cancer. The PCL consortium (work package 5) is one of the largest European pediatric cancer cohorts focused on genetic susceptibility to gonadal impairment and ototoxicity as late effects to date. The ototoxicity sub-cohort (work package 4b) of PCL with 390 childhood cancer patients with a mean age of 9.8 (0-19.3), is focused on hearing loss after cisplatin treatment not confounded by radiotherapy. Moreover, there is a cisplatin treated replication sub cohort (work package 3) of 1100 childhood cancer patients in the PCL consortium [8].

Patients included in the PCL cohort were:

- Diagnosed with cancer before the age of 19 years
- Initially treated with cisplatin, as a single platinum drug during childhood cancer treatment, or switched from cisplatin to carboplatin during treatment
- Did not receive cranial or inner ear radiation
- Have bilateral hearing disfunction or no hearing loss as a result of platinum treatment
- Completed their chemotherapy treatment
- Have normal hearing at baseline and at least one pure tone audiometric evaluation available within two years after completion of chemotherapy
- Have either array or sequencing mtDNA data or have their biomaterial (blood or saliva) available for DNA extraction.
- Complete information of the following covariates is present:
  Age, sex, age at diagnosis, total cumulative cisplatin dosage, genetic principal components.

In this cohort two phenotype definitions will be tested. A binary outcome as hearing loss yes/no and a linear outcome to increase power based on Muenster/SIOP classification.

3.2. DCCSS LATER cohort

This cohort is a retrospective cohort of Dutch pediatric cancer survivors with a median age at diagnosis of 5.3(0-18) treated between 1963 to 2002. We collected the data for this project from the self-reported health questionnaire of the majority of childhood cancer survivors recruited for the Dutch Childhood Oncology Group - Long-Term Effects after Childhood Cancer (DCOG-LATER) cohort. 2088 of the survivors filled out the health questionnaire which included one item on tinnitus. The question was: “Have you had ringing in the ears, or do you currently have this condition?” (yes/no). Patient information on cancer type, gender, age at diagnosis, chemotherapy/radiotherapy, and surgery is also available in our DCCSS LATER database[9].

Patients included in the LATER-2 cohort were:

- Diagnosed with cancer before the age of 18.
- A participant of the DCCS LATER study.
- Answered to the tinnitus question in the questionnaire.
- Informed consent LATER 2 study signed

3.3. SOUND cohort

The SOUND cohort is a prospective study in the national childhood cancer center in the Netherlands. The initial aim of this cohort is to investigate the prevalence of hearing loss after cancer treatment in pediatric CNS and solid tumor patients. The prevalence of tinnitus and vertigo is also investigated. This investigation is pursued in the Princess Maxima center. Audiological testing and examinations are done in collaboration with audiological department of Wilhelmina Children's Hospital in the Netherlands on children of all ages according to recent guidelines[10]. Patients included in this study were diagnosed with CNS or solid tumor before the age of 19 between January 2021 and January 2023 and further divided in two strata. Patients treated with platinum agents (cisplatin, carboplatin, oxaliplatin), cranial radiotherapy, or Neuro/ENT surgery were part of stratum I. Patients treated with therapies not described in stratum I were included, with informed consent, in stratum II. The SOUND study will be available in the near future.

4. Materials and Methods

4.1. Endpoint and phenotype definition

4.1.1. Hearing loss definition:

Hearing loss has been categorized by Muenster and SIOP grading system. Since CIHL mostly presents with bilateral and asymmetrical hearing loss[11], the ear with the worst hearing outcome is used in the phenotype definition. Hearing deficiency was defined as a Muenster grade equal to or above 2b. It is noteworthy that phenotype definition based on SIOP grade is possible as well.

Here, we suspect that we are underpowered for such analysis. In order to test our suspicion, we aim to define a linear and two binary phenotypes which results in a quantitative and case-control association testing respectively.

- Binary definition 1

The phenotype is defined as a binary (categorical) variable, which results in a case-control study. Therefore, cases are patients with Muenster grade equal or above 2b and controls are patients with Muenster grade less than 2b or no hearing loss.

Muenster grade								
Controls			**Cases**					
0	**1a**	**2a**	**2b**	**2c**	**3a**	**3b**	**3c**	**4**

- Binary definition 2

Similar to the previous definition, this phenotype is defined as a binary (categorical) variable, which results in a case-control study. In order to have a clearer phenotype definition and a more distinct segregation between the cases and controls, patients with borderline hearing loss were excluded from the controls in

this definition. Therefore, cases are patients with Muenster grade equal or above 2b and controls are patients without hearing deficiency (Muenster grade = 0).

Muenster grade								
Controls	Excluded		Cases					
0	1a	2a	2b	2c	3a	3b	3c	4

- Linear definition

In order to add more power to the study, a linear phenotype was presumed here. Muenster grades are assigned to a score {0 to 8} and these scores are used instead of Muenster grades to mimic a discrete continuous variable. In this analysis genetic variances associated with differences in the linear interpretation of Muenster grade are identified.

Muenster grade									
Muenster grade	0	1a	2a	2b	2c	3a	3b	3c	4
Phenotypes	0	1	2	3	4	5	6	7	8

4.2. Tinnitus definition

Recruited patients of the retrospective LATER-2 cohort were asked a yes/no question on whether they have ringing in the ear (tinnitus) or not.

- Binary definition 3

Questionnaire (yes/no)	
Controls	Cases
No	Yes

4.3. Genotyping, quality control and imputation

Illumina Global Screening Array-MD v.1 (GSA-MDv1) has been used for genotyping in the PCL cohort and Illumina Global Screening Array-MD v.3 (GSA-MDv3) in the LATER cohort GSA arrays were chosen based on criteria explained elsewhere [9, 12]. Nuclear DNA variants are based on genome built 37 (GRCh37) and mitochondrial DNA variants are according to the revised Cambridge reference sequence (rCRS) for human mitochondrial genome. Both nuclear and mitochondrial DNA variants were quality controlled based on worldwide standards[8]. Nuclear DNA was imputed on to the Haplotype reference consortium (HRC r1.1) using the Michigan imputation server[7].

4.3.1. GWAS Analysis

A hypothesis free genome-wide association testing (GWAS) will be performed on nuclear DNA and mitochondrial DNA separately. Genetic tastings are explained in detail in analysis plans attached as supplements. We aim to perform a replication and/or meta-analysis with our collaborators to ensure a true association.

5. Summary of the request

- We propose a collaboration on ototoxicity GWAS projects mentioned above in childhood cancer patients and survivors of the PCL cohort and DCCSS LATER cohort. Each project is described in detail in sub-project descriptions A, B, C, and D.
- To date, GWAS data have been made available from the DCCS LATER cohort (n=2072), and the St. Jude Lifetime cohort study (SJLIFE) (n=3748). In addition, contact has been established with Prof. E. Dolan to request GWAS data from the AYA testicular tumor cohort within the Platinum study (n=1680), and with Prof. M. Ansari to request GWAS data from the Swiss Childhood Cancer Survivor Study (SCCSS).
- We would like to request collaboration with the Childhood Cancer Survivor Study (CCSS). With this collaboration, we aim to replicate previous identified hits associated with hearing loss and to identify novel (disease- or therapy related) SNPs that are associated with hearing loss and tinnitus in childhood cancer patients and survivors.

Project A: Identification of nuclear variants associated with CIHL in children with cancer in the PCL cohort. (Joint discovery)

1. Background

We previously performed a GWAS on the PCL WP4b with hearing loss classified by the Muenster [1] grade as its end point. In this analysis, hearing loss was treated as a binary variable with hearing levels above 2b denoted as hearing loss. TCERG1L allelic variation was discovered and validated in two independent cohorts and further validated in an in-vitro setting to be a risk factor in CIHL[7].

2. Aim

The aim of this project is to replicate the previous GWAS on identification of nuclear genetic variations associated with cisplatin induced hearing loss in childhood cancer patients and discover new variants through joint discovery.

3. Materials and methods

3.1. Quality control, Genotyping and imputation

We would like to suggest standard quality control (e.g., call rate, HWE, sample swap detection) as performed and explained in detail elsewhere[13]. For cohorts with genotyping data, we kindly ask imputation to the Haplotype reference consortium(HRC r1.1)[14] using the Michigan Imputation Server with default settings. For consistency between participating cohorts, we would like to ask you to liftover your genetic data to genome build.38 if it is not already the case using UCSC LiftOver application.

3.2. Phenotype Definition

In this study, the binary phenotype definition 1 and the linear phenotype definition of hearing loss explained in chapter 1 (page 6) will be used.

3.3. Genetic analysis

For Binary phenotypes logistic regression and for linear phenotypes linear regression can be performed. We suggest that Age, Age^2, Sex, TCCD and first 4 PCs be used as covariates.

3.3.1. Binary Model

We suggest that, for both binary outcomes logistic regression ("glm" function in rvtest) be used:

$$Phenotype(0,1) \sim SNP[i] + Sex + Age + TCCD + PCs$$

3.3.2. Linear Model

We request that you first check if the residuals of the linear regression of covariates is normal using the model below:

$$Phenotype[0,8] \sim Sex + Age + TCCD + PCs$$

If the residuals do not demonstrate normality, we suggest using rank based inverse transformation using R "qnorm" function:

- noraml_ranked_residuals <- qnorm((rank(Covar_residuals,na.last="keep")-0.5)/sum(!is.na(Covar_residuals)))
  - Covar_residuals: residuals calculated from the covariates model

After determining the transformations, the genetic analysis can be done as:

$$Normal\_ranked\_residuals \sim SNP(i)$$

The summary statistics of each model can then be shared to perform joint discovery. We suggest using the "metal" function in R for meta-analysis. The details of softwares and functions used for the final meta-analysis can be determined at a later meeting.

3.3.3. Significance threshold

Due to limited number of samples, we fear that a significance threshold of $P < 5 \times 10^{-8}$ is too stringent. Thus, we suggest variants with a suggestive level of significant ($P < 1 \times 10^{-5}$) be pursued[7].

4. Meta-analysis

Prior to meta-analysis: EasyQC will be performed to clean and harmonize the data. Variants with minor allele frequency (MAF) <0.05 will be excluded. P-values of less than 5x10-6 will be considered suggestive and p-values less than 5x10-8 will be considered statistically significant at the genome-wide level.

The results of the discovery and replication analyses will be pooled using the R package rmeta, with a fixed effects model or, in case of significant heterogeneity (p<0.05), a random effects model.

The summary statistics of each model can then be shared through a secured link using "SURFfilesender" to perform joint discovery. We suggest using "Metal" or "rmeta" package in R for the meta-analysis. We would also like to have a look at the TCERG1L allelic variant (rs893507) that was found in the previous GWAS[7].

Project B: Identification of mitochondrial variants associated with CIHL in children with cancer in the PCL cohort (Joint discovery)

1. Background

Mitochondria are important compartments of cells as the principal generators of cellular energy. Cochlear cells have high oxygen demands. Therefore, mitochondrial function is very important in these types of cells [15]. Mitochondrial DNA (mtDNA) variants have shown to be associated with genetic susceptibility to non-syndromic hearing loss in the general population [15-17]. Recently, mtDNA variants have been reported to be associated with a higher risk of pediatric cancer treatment-related toxicities, including sarcopenia, and cardiotoxicity [18, 19]. Moreover, it is observed that patients with CIHL cluster more with the rare European Haplogroup J [20, 21]. Although, there is evidence of differential responses of J haplogroup cytoplasmic hybrid (cybrid) cells to cisplatin cytotoxicity[22], the association of this haplogroup with CIHL has not been validated.

The overall aim of the current study is to identify and validate common mitochondrial genetic markers in the PCL WP4b, which may play a role in the risk of cisplatin-induced hearing loss in pediatric cancer patients, through joint discovery. Moreover, we are looking into the most appropriate way to determine haplogroups using array data and will include haplogroup analysis at a later date.

2. Aim

In this project we aim to identify homoplasmic mitochondrial DNA variants associated with cisplatin induced hearing loss in pediatric cancer patients

3. Materials and methods

3.1. mtDNA variant QC directly genotyped variants.

Besides the standard QC (e.g., call rate, HWE, sample swap detection), we request you perform some additional QC. As genotyping arrays and regular sequencing technology do not contain enough depth of coverage to assess low to mid-grade heteroplasmy (i.e., not all mtDNA molecules are the same), we opt to currently only investigate homoplasmy (i.e., all mtDNA molecules are identical).

The following additional QC should be performed:

- Heterozygote genotypes set to missing (i.e., only AA and BB genotypes remaining)
- Variants with MAF < 1% were excluded from analysis.

For ease of comparison, we are currently looking for cohorts with either genotype data using the Infinium© Global Screening Array (Illumina, San Diego, CA, USA) [12] or sequencing data with a good coverage of the mtDNA. We aim to include imputation methodologies of the mtDNA to allow participation of cohorts with other genotyping array data. SNP positions are based on the Revised Cambridge Reference Sequence (rCRS) of human mitochondrial DNA[23]. In the case of other references, we are happy to help with conversion to this reference sequence (supplementary file.1).

3.2. Phenotype Definition

In this study, the binary phenotype definition 1, binary phenotype definition 2, and the linear phenotype definition of hearing loss explained in chapter 1 (page 6 & 7) will be used.

3.3. Genetic testing

For Binary phenotypes logistic regression and for linear phenotypes linear regression can be performed. We suggest that Age, Age^2, Sex, TCCD and first 4 PCs be used as covariates.

3.3.1. Binary Regression Model

We suggest that, for both binary outcomes logistic regression ("glm" function in R) be used:

$$Phenotype(0,1) \sim SNP[i] + Sex + Age + Age2 + TCCD + PCs$$

3.3.2. Linear Regression Model

We request that you first check if the residuals of the linear regression of covariates is normal using the model below:

$$Phenotype[0,8] \sim Sex + Age + Age2 + TCCD + PCs$$

If the residuals do not demonstrate normality, we suggest using rank based inverse transformation using R "qnorm" function:

- noraml_ranked_residuals <- qnorm((rank(Covar_residuals,na.last="keep")-0.5)/sum(!is.na(Covar_residuals)))
  - Covar_residuals: residuals calculated from the covariates model

After determining the transformations, the genetic analysis can be done as:

$$Normal\_ranked\_residuals \sim SNP(i)$$

3.3.3. Significance threshold

As there are no conventional mitochondrial multiple hypothesis threshold, we would like to suggest that we use a Bonferroni correction for all the existing mtDNA haplotypes in the cohorts[24]. We are currently working on a method to accurately infer mtDNA haplotypes from our genotyping data.

4. Meta-analysis

The summary statistics of each model can then be shared through a secured link using “SURFfilesender” to perform joint discovery. We suggest using “Metal” software or “rmeta” package in R for the meta-analysis[7].

Project C: Identification of nuclear variants associated with tinnitus in childhood cancer survivors in the DCCSS LATER cohort (Joint discovery)

1. Background

Tinnitus, as a late effect in childhood cancer survivors, is a topic less investigated. Cisplatin treatment can cause hearing loss along with tinnitus. Tinnitus patients present with ringing, buzzing, or hissing sounds in their ear that cannot be recognized by others. This can cause patients a lot of stress, coping issues, and problems in keeping focus[9]. Multiple studies have investigated the association of tinnitus with genetic variations[25]. Several GWAS studies have investigated and identified genetic variants associated with tinnitus in the general population[26-29]. Tinnitus has also been found to have a shared genetic link with neuropsychiatric disorders[30, 31]. To our knowledge, the association of tinnitus with genetic variants has not been studied in a genome-wide fashion as a late effect of cancer treatment in childhood cancer survivors. We would like to ask you to join us for a joint discovery. The details of genome wide analysis are yet to be determined.

2. Aim

In this project we aim to identify nuclear DNA variants associated with tinnitus in childhood cancer survivors.

3. Materials and methods

3.1. Quality control, Genotyping and imputation

We would like to suggest standard quality control (e.g., call rate, HWE, sample swap detection) as performed and explained in detail elsewhere[13]. For cohorts with genotyping data, we kindly ask imputation to the Haplotype reference consortium(HRC r1.1)[14] using the Michigan Imputation Server with default settings. For consistency between participating cohorts, we would like to ask you to liftover your genetic data to genome build.38 if it is not already the case using UCSC LiftOver application.

3.2. Phenotype Definition

In this study, the binary phenotype definition 3 of tinnitus explained in chapter 1 (page 7) will be used.

3.3. Genetic analysis

For Binary phenotypes logistic regression and for linear phenotypes linear regression can be performed. We suggest that age at diagnosis, Sex(yes/no), total cumulative cisplatin dosage (TCCD), total radiotherapy dosage (TRD), Neuro/ENT surgery(yes/no) and first 4 PCs be used as covariates.

3.3.1. Binary Model

We suggest that, for binary outcomes logistic regression ("glm" function in rvtest) be used:

$$Phenotype(0,1) \sim SNP(i) + Sex + Age + TCCD + TRD + Surgery + PCs$$

3.3.2. Significance threshold

Due to limited number of samples, we fear that a significance threshold of $P < 5 \times 10^{-8}$ is too stringent. Thus, we suggest variants with a suggestive level of significant ($P < 1 \times 10^{-5}$) be pursued[7].

4. Meta-analysis

Prior to meta-analysis: EasyQC will be performed to clean and harmonize the data. Variants with minor allele frequency (MAF) <0.05 will be excluded. P-values of less than 5x10-6 will be considered suggestive and p-values less than 5x10-8 will be considered statistically significant at the genome-wide level.

The results of the discovery and replication analyses will be pooled using the R package rmeta, with a fixed effects model or, in case of significant heterogeneity (p<0.05), a random effects model.

The summary statistics of each model can then be shared through a secured link using "SURFfilesender" to perform joint discovery. We suggest using "Metal" or "rmeta" package in R for the meta-analysis.

Project D: Identification of mitochondrial variants associated with tinnitus childhood cancer survivors in the DCCSS LATER cohort (Joint discovery)

1. Background

Tinnitus, as a late effect in childhood cancer survivors, is a topic less investigated. Cisplatin treatment can cause hearing loss along with tinnitus. Tinnitus patients present with ringing, buzzing, or hissing sounds in their ear that cannot be recognized by others. This can cause patients a lot of stress, coping issues, and problems in keeping focus[9]. Multiple studies have investigated the association of tinnitus with genetic variations[25]. Several GWAS studies have investigated and identified genetic variants associated with tinnitus in the general population[26-29]. Tinnitus has also been found to have a shared genetic link with neuropsychiatric disorders[30, 31]. To our knowledge, the association of tinnitus with genetic variants has not been studied in a genome-wide fashion as a late effect of cancer treatment in childhood cancer survivors. We would like to ask you to join us for a joint discovery. The details of genome wide analysis are yet to be determined.

2. Aim

In this project we aim to identify homoplasmic mitochondrial DNA variants associated with tinnitus in childhood cancer survivors.

3. Materials and methods

3.1. mtDNA variant QC directly genotyped variants

Besides the standard QC (e.g., call rate, HWE, sample swap detection), we request you perform some additional QC. As genotyping arrays and regular sequencing technology do not contain enough depth of coverage to assess low to mid-grade heteroplasmy (i.e., not all mtDNA molecules are the same), we opt to currently only investigate homoplasmy (i.e., all mtDNA molecules are identical).

The following additional QC should be performed:

- Heterozygote genotypes set to missing (i.e., only AA and BB genotypes remaining)
- Variants with MAF < 1% were excluded from analysis.

For ease of comparison, we are currently looking for cohorts with either genotype data using the Infinium© Global Screening Array (Illumina, San Diego, CA, USA) [12] or sequencing data with a good coverage of the

mtDNA. We aim to include imputation methodologies of the mtDNA to allow participation of cohorts with other genotyping array data. SNP positions are based on the Revised Cambridge Reference Sequence (rCRS) of human mitochondrial DNA[23]. In the case of other references, we are happy to help with conversion to this reference sequence (supplementary file.1).

3.2. Phenotype Definition

In this study, the binary phenotype definition 3 of tinnitus explained in chapter 1 (page 7) will be used.

3.3. Genetic analysis

For Binary phenotypes logistic regression and for linear phenotypes linear regression can be performed. We suggest that age at diagnosis, Sex(yes/no), total cumulative cisplatin dosage (TCCD), total radiotherapy dosage (TRD), Neuro/ENT surgery(yes/no) and first 4 PCs be used as covariates.

3.3.1. Binary Model

We suggest that, for binary outcomes logistic regression ("glm" function in rvtest) be used:

$$Phenotype(0,1) \sim SNP(i) + Sex + Age + TCCD + TRD + Surgery + PCs$$

3.3.2. Significance threshold

As there are no conventional mitochondrial multiple hypothesis threshold, we would like to suggest that we use a Bonferroni correction for all the existing mtDNA haplotypes in the cohorts[24]. We are currently working on a method to accurately infer mtDNA haplotypes from our genotyping data.

4. Meta-analysis

The summary statistics of each model can then be shared through a secured link using "SURFfilesender" to perform joint discovery. We suggest using "Metal" software or "rmeta" package in R for the meta-analysis[7].

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