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The Association of Mitochondrial Copy Number with Sarcopenia in Adult Survivors of Childhood Cancer

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The Association of Mitochondrial Copy Number with Sarcopenia in Adult Survivors of Childhood Cancer

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An abstract of
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Abstract

The Association of Mitochondrial Copy Number with Sarcopenia in Adult Survivors of Childhood Cancer

By
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Background: Adult childhood cancer survivors (CCS) are at increased risk of frailty, with loss of muscle mass and function (sarcopenia) frequently observed. While various cancers and treatment-related regimens are known to provoke sarcopenia, the mechanism behind these insults is elusive. Although loss of functioning mitochondria has been implicated in the pathobiology of aging in the general population, their role as agents of pathological change in CCS has yet to be explored. Using germline (GL) peripheral blood (PBL) mitochondrial copy number (mtCN) as a proxy for functional mitochondria, this cross-sectional study investigates the association of mtCN with sarcopenia among CCS.

Methods: Participants were enrolled in a retrospective St Jude Lifetime Cohort clinical study designed to prospectively assess long-term health outcomes among adult CCS. mtCN estimates were derived from whole-genome sequencing (WGS) data collected from 1,762 PBL GL samples, and validated by qRT-PCR. Sarcopenia was the primary outcome, with haplogroup, demographic, clinical and treatment-related factors included as covariates.

Results: The prevalence of sarcopenia among CCS was 27%, with females significantly more afflicted (31.5% vs 22.9%; P <.0001). Age of diagnosis was significant (P=.04), with 51.7% of CCS with sarcopenia diagnosed between 4 and 13 years of age. Additionally, 39.0% of CCS with sarcopenia had CNS malignancies. CCS who received radiation had twice the odds (OR, 1.8; 95% CI 1.4 to 2.2) of having sarcopenia, while glucocorticoids were protective (OR, 0.7; 95% CI 0.5 to 0.8). WGS mtCN estimates were positively correlated with qRT-PCR ($r = 0.589$; $p < .0001$). Multivariable logistic regression indicated that mtCN decreased with age ($\beta = -0.77$; P =.001), and was higher in females ($\beta = 10.38$; P =.01) and non-Hispanics ($\beta = 40.66$; P =.01). Logistic modeling revealed that the odds of sarcopenia increased 21% (aOR, 0.8; 95% CI 0.7 to 0.9) for every standard deviation decrease in mtCN.

Conclusion: The early appearance of a geriatric syndrome among young survivors suggests a pathobiology irrespective of normal aging. While a growing body of evidence supports PBL mtCN as a biomarker for adverse health outcomes, this study is the first to find an association between mtCN and sarcopenia among CCS.

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All of these beautiful people in my life, I am simply in awe.

~Kelly

TABLE OF CONTENTS

INTRODUCTION 1
METHODS 2
Study Population 2
Outcome 3
Mitochondria copy number (mtCN) 4
Covariates 5
Statistical Analysis 5
RESULTS 6
Population Characteristics 6
Treatment 7
Factors associated with sarcopenia 7
Mitochondria copy number (mtCN) 8
Sarcopenia and mitochondria copy number (mtCN) 9
DISCUSSION 9
REFERENCES 18
TABLE 1: Characteristics of 1,762 Childhood Cancer Survivors With and Without Sarcopenia 23
TABLE 2: Associations between survivor characteristics and sarcopenia 26
FIGURE 1: qPCR validation of WGS mtCN estimates. 29
FIGURE 2: mtCN and age among males and females, by ethnic group. 30
FIGURE 3: Final Model Odds Ratios 31

INTRODUCTION

As five-year survival rates for pediatric cancers have approached 85% [1], by 2020 an estimated 500,000 U.S. childhood cancer survivors will be at increased risk for accelerated aging [2-4], with perturbed body mass composition and reduced strength conferring disproportionate risk for chronic disease and mortality [2, 3, 5, 6]. Accordingly, reductions in lean muscle mass and strength deficits are frequently observed among survivors [2, 5, 7] and recapitulate the age-related loss of skeletal muscle mass and functional capacity that are characteristic of sarcopenia, canonically a geriatric determinant of frailty [5, 6]. Although primary cancer diagnosis and cancer therapy-related modalities have been implicated as risk factors for muscle weakness in cancer survivors [2, 4, 8-10], the pathobiology through which these insults operate has yet to be elucidated [8].

Regardless of etiology, mitochondria play an integral role towards delineating the sarcopenia phenotype [11-13]. Skeletal muscles are highly metabolic tissues that are densely packed with mitochondria [14-16], a double-membraned organelle that is exclusively tasked with energy (ATP) production in cells via oxidative phosphorylation (OXPHOS). Within each mitochondria, multiple copies of a circular and double-stranded, 16.5 kb mitochondrial genome encode the 13 polypeptides required for cellular respiration [17, 18]. When mitochondrial DNA (mtDNA) integrity is compromised, either through mutation or replication error, OXPHOS capacity is impeded [19, 20], and cellular function is impaired. These perturbations eventually culminate into tissue dysfunction and pathological states ensue. Genetically heterogeneous mitochondrial diseases are exemplary in this respect, as persistence of mtDNA mutations frequently

perturb neuromuscular function and herald myopathies [21, 22]. Compelling evidence also comes from mtDNA-mutator mouse models of accelerated aging, where unfettered adulteration of mtDNA disrupts mitochondrial respiratory complexes and precedes loss of skeletal muscle mass [23-25].

Age-related declines in mitochondrial function have also been explored in numerous studies, with mitochondrial DNA copy number (mtCN) used as a proxy for mitochondrial and OXPHOS capacity [16, 26]. Inverse relationships between copy number and age have been observed in both skeletal muscle and peripheral blood samples from healthy individuals [27-30], although some studies have indicated that copy number losses are not apparent until middle age [31, 32], and a few studies have suggested that changes are not even detected [33, 34]. Associations between peripheral blood copy number and frailty or its components have also been investigated in the general population [27, 35], although with conflicting results. While the prognostic value of mtCN as a harbinger of pathological change has been addressed in the general population, little is known about the relationship between mitochondrial function and sarcopenia in adult survivors of childhood cancer. To this end, this study seeks to understand the association between mtCN and sarcopenia among survivors.

METHODS

Study Population

Participants were enrolled into the institutional review board–approved, retrospective St Jude Lifetime Cohort (SJLIFE) as part of a clinical study designed to prospectively assess long-term health outcomes among adult survivors of childhood cancer [36, 37]. Patients ≥ 18 years of age at follow-up who were diagnosed with

childhood malignancy ≥ 10 years ago and who were treated at St. Jude Children's Research Hospital (SJCRH) were eligible for enrollment. Informed written consent was obtained from each participant prior to clinical evaluation and collection of both physical performance metrics and demographic information. Consent was also obtained for collection and banking of biological specimens dedicated for use in genomic analyses. Of the 1,848 survivor germline samples that were sequenced, the analysis was restricted to the 1,762 survivors (95.3%) that had low muscle mass or grip strength measures available, and who did not receive allogeneic stem-cell transplantation.

Outcome

Sarcopenia, as defined by low lean muscle mass or weak grip strength, was the primary outcome. Relative appendicular lean muscle mass was determined from dual x-ray absorptiometry and height, wherein the sum of lean mass in arms and legs was divided by height in meter squared. Isometric hand-grip strength was measured in kilograms, using a Jamar hand-held dynamometer, with each participant seated with the shoulder at 0° to 10°, the elbow at 90° of flexion, and the forearm in a neutral position [38, 39]. Individuals were classified as having low lean muscle mass when their relative lean mass was < -1.5 standard deviations below age-, sex-, and race-specific values derived from the National Health and Nutrition Examination Study (NHANES) [40]. Muscle weakness was determined from body mass index (BMI) –specific cut-points for strength [41], with individuals <-1.5 standard deviations classified as weak.

Mitochondria copy number (mtCN)

Whole genome sequencing (WGS) estimation. During the initial clinical evaluation, peripheral blood samples were obtained from 1,848 SJLIFE participants and subsequently cryopreserved. Germline DNA was extracted using the DNA Blood Mini kit (Qiagen, Valencia, CA), and 1ug of fluorometrically quantified material was subjected to whole genome pair-end sequencing on a HiSeq X Ten System (Illumina Inc., San Diego, CA) at the HudsonAlpha Institute for Biotechnology Genomic Services Laboratory (Huntsville, AL). Using Burrows-Wheeler Aligner (BWA) v.0.7.12 [42], sequencing reads were aligned to both the human reference genome build 37 (GRCh37) and the revised human mitochondrial reference (NC_012920) [42]. Average coverage for both genomic and mitochondrial DNA was called using the depth command in SAMtools v.1.2 (http://samtools.sourceforge.net/) [43], with mitochondrial copy number (mtCN) estimated as previously described [35].

qPCR validation. WGS estimates of mtCN were validated for a subset (n=95) of survivors by real-time quantitative PCR (qPCR) using SsoAdvanced™ Universal SYBR® Green Supermix reagent (Bio-Rad, Hercules, CA) and 10ng of germline DNA from survivors. DNA from a 143B osteosarcoma cell line that lacks mitochondrial DNA (ρ0) was also included as a negative control. Primers against the mitochondrial-encoded NADH dehydrogenase 2 (MT-ND2) gene were designed using NCBI Primer Blast (forward: 5'-ACCAAACCCAGCTACGCAAA-3'; reverse: 5'-AGTAGTAGGGTCGTGGTGCT-3') and primers used against the nuclear-encoded β2-microglobulin (β2M) gene (forward: 5'-TGCTGTCTCCATGTTTGATGTATCT-3'; reverse: 5'-TCTCTGCTCCCCACCTCTAAGT-3') were published previously [44]. The

qPCR reaction and thermocycling conditions were followed according to the manufacturer's protocol and the assay was performed on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative mtCN of each individual was determined by taking the difference in C_T values between the β2M and MT-ND2 genes and accounting for diploid nuclear DNA ($2 \times 2^{\Delta CT}$) [44].

Covariates

Factors that were potentially associated with sarcopenia included sex, race, ethnicity, cancer group diagnosis (blood, brain, solid), and smoking status. Age at diagnosis, age of assessment, and survival time were also considered as categorical variables (rounded quartiles). Treatment variables such as type of chemotherapeutic agent used and radiation site exposure were also included and were considered as binary variables. Data regarding demographic characteristics and treatment exposures were extracted from medical records, whereas smoking status, ethnicity, and race were self-reported on questionnaires administered during the initial SJLIFE evaluation. For mitochondrial DNA haplogroup assignment, sequencing data was submitted through HaploFind [45] and haplogroups were further categorized as "Ancestral", "Asian", "European", or "Reference" based on human mtDNA migratory patterns (https://www.mitomap.org/).

Statistical Analysis

All analyses were conducted with SAS software version 9.4 (SAS Institute, Inc., Cary, NC). Bivariate analysis was used to characterize demographic and treatment differences between survivors with and without sarcopenia. A two-sided p-value ≤ 0.05

was considered significant with $\chi 2$ and Fisher's exact tests used for categorical variables, while Wilcoxon rank-sum test was used for continuous variables. Crude associations (odds ratios [OR] and 95% CIs) between these same variables and sarcopenia were also explored with logistic regression models. In order to identify factors that impacted mtCN, a stepwise multivariable linear regression was utilized, with significant factors subsequently modeled in concert with independent predictors of sarcopenia during multivariable logistic regression modeling. During this latter stage of logistic modeling, mtCN was no longer defined by a single copy of mtDNA but was instead measured in terms of standard deviation changes in copy number. Interaction terms were evaluated in the full model, with the final model exploring the association between sarcopenia and mtCN while controlling for sex, corticosteroid treatment, and radiation.

RESULTS

Population Characteristics

Among 1,762 adult survivors of childhood cancer (Table 1), the prevalence of sarcopenia was 27%, with females significantly more afflicted than males (31.5% vs 22.9%; P <.0001). Differences in haplogroup, race, and ethnicity were insignificant, although the majority of the cohort was white (84.1%) and non-Hispanic (98.2%). The majority of the cohort had survived 8 to 19 years (47.6%), with no differences in median (IQR) age observed between those with (28.9 yrs (14.7)) and without sarcopenia (29.6 yrs (13.1)), which was expected since the outcome was adjusted for age. Significant differences were observed, however, based on age of diagnosis (P=.04), with a higher proportion of survivors with sarcopenia diagnosed between the ages of 4 and 13 (51.7%) as compared to those without sarcopenia (44.9%). Type of cancer diagnosis also

impacted groups differently, as 39.0% of survivors that had central nervous system (CNS) malignancies also suffered from sarcopenia versus those survivors that had solid tumor (29.9%) and blood malignancies (22.3%).

Treatment

In terms of treatment modality (Table 1), any form of radiation exposure was significant (<.0001), with 31.1% of survivors with sarcopenia having been exposed. At least one-third of any site-specific (brain, abdominal, or heart) radiation exposures were associated with sarcopenia, although survivors that were exposed to abdominal radiation had the highest prevalence of sarcopenia (36.0%). Glucocorticoids and anthracyclines were the most significant chemotherapeutics (P<.0001 for both) and exerted a protective effect; over 77% of survivors exposed to either of these agents did not have sarcopenia. However, the apparent benefit of anthracycline treatment was dependent upon radiation dose, as less anthracyclines were administered to those survivors who had received considerable doses of radiation. Indeed, in the subset of survivors (n = 756) who never received radiation, anthracyclines were not significant (P = 0.39). Alkylating agents were not significant (P =0.62) between groups either nor were there any significant differences observed among the 1.5% of individuals that received non-allogeneic stem-cell transplants (P= .66).

Factors associated with sarcopenia

For every standard deviation decrease in mtCN, survivors were 20% more likely to have sarcopenia, with females 1.6 times more likely to develop the condition than males (95% CI, 1.3 to 1.9) (Table 2). Mantel-Haenszel tests for trend did not identify

changes in associations between sarcopenia and increasing age of diagnosis, increasing age of assessment, or increasing survival time; however, survivors diagnosed between the ages of 4 and 8 (OR, 1.5; 95% CI, 1.1 to 2.1) and between the ages of 9 and 13 (OR, 1.4; 95% CI 1.1 to 2.0) were significantly more likely to have sarcopenia as compared to those diagnosed between the ages of 14 and 24 (the reference group). Treatment exposures impacted the likelihood of developing sarcopenia, as individuals who received any radiation were almost twice as likely to have sarcopenia, with those that received abdominal radiation having the highest odds (OR, 1.8; 95% CI 1.4 to 2.2). Conversely, survivors that received glucocorticoids during cancer treatment were 30% less likely to develop sarcopenia (OR, 0.7; 95% CI 0.5 to 0.8). No associations between smoking behavior, race, ethnicity, type of cancer diagnosis, or exposure to non-allogeneic stem-cell transplants were observed.

Mitochondria copy number (mtCN)

For the subset of whole-genome sequenced samples that were validated by qPCR, mtCN values were positively correlated and a significant linear relationship was observed (Pearson correlation, $r = 0.589$; $p < .0001$) (Fig 1). In terms of factors that influenced mtCN, stepwise multivariable linear regression analysis identified age, sex, and ethnicity as significant predictors (Fig 2). Here, higher mtCN was associated with female sex ($\beta = 10.38$; $P = .01$) and non-Hispanic race/ethnicity ($\beta = 40.66$; $P = .01$), whereas a depreciation in mtCN was seen with increasing age ($\beta = -0.77$; $P = .001$). Significant differences in mtCN also existed based on sarcopenia status, with median copy numbers of 301 and 310 observed among survivors with and without sarcopenia, respectively ($P < .01$).

Sarcopenia and mitochondria copy number (mtCN)

The most valid measure of the association indicated that for every standard deviation decrease in mtCN, the odds of having sarcopenia increased by 21% (aOR, 0.8; 95% CI 0.7 to 0.9). This estimate was obtained in a full logistic model that adjusted for all independent predictors of sarcopenia status and where no significant interactions were identified. In subsequent modeling, although sequential removal of independent predictors did not alter the OR greater than 10%, the final model (Fig 3) was ultimately adjusted for sex, exposure to any radiation, and corticosteroids due to the significance of these factors in the current analysis as well as in the literature. Indeed, the odds of sarcopenia were more pronounced in females, who were 63% more likely to have the condition (OR, 1.6; 95% CI 1.3 to 2.0), and in those survivors with a history of radiation exposure, who were 70% more likely (OR, 1.7; 95% CI 1.4 to 2.1). Corticosteroids exerted a protective effect, as survivors that received these agents were 55% less likely to develop sarcopenia (OR, 0.7; 95% CI 0.5 to 0.8).

DISCUSSION

Adult CCS are at increased risk for physiological frailty, with concomitant reduction of muscle mass or function frequently observed [3]. Accordingly, our study found that the prevalence of sarcopenia among SJLIFE survivors was 27%, and that women were disproportionately affected (31.5% vs 22.9%; P <.0001). By comparison, and using a similar definition, an evidence-based evaluation of nine studies comprising 10,063 participants reported that 19.5% of men and 23.9% of women aged 65 years and older were sarcopenic [46]. Given that the median age of our SJLIFE cohort was only

29.4 years, the early appearance of a geriatric syndrome among young survivors suggests a pathobiology irrespective of normal aging.

We hypothesized that mitochondrial adulterations could account for the sarcopenia phenotype observed in our population, and that these changes would manifest in a reduction of mtCN. Indeed, we found that the odds of having sarcopenia were increased 21% with each standard deviation decrease in mtCN. This association is supported by previous work involving progeroid mouse models that express a proofreading-deficient version of the nuclear-encoded mtDNA polymerase gamma (PolG). In these studies, accelerated accumulation of mtDNA mutations hastened the onset of skeletal muscle loss and subsequent loss of mtCN was observed [24, 47]. Further support for loss of mitochondrial capacity has also been demonstrated in aging humans, where mtDNA oxidative damage and impaired ATP production were associated with decreased mtDNA content and reduced skeletal muscle aerobic performance and function [29, 48].

However, fundamental differences exist in the mitochondrial underpinnings between normal aging and sarcopenia. For example, divergent mitochondrial quality control processes are observed between sarcopenic mice and those that are chronologically aged. In skeletal muscles of POLG mice, increases in mitochondrial fission protein (Fis1) and recruitment of the autophagy machinery (Atg5, p62) have been correlated with decreases in mtCN. This was in striking contrast to normally aged mice, who exhibited marked increases in fusion-related proteins (Mfn1, Mfn2), reductions in autophagy-related proteins (Beclin-1, p62), and mild increases in mtDNA copy number. Collectively, this suggests opposing modes of managing mitochondrial dysfunction, with

normally aged mice diluting out negative contributions, whereas sarcopenic mice upregulate mechanisms that target irreversibly damaged mitochondria for removal [47, 49].

As a consequence of cancer therapy, many survivors were exposed to various cytotoxic modalities that are capable of damaging mitochondria. Anthracyclines such as doxorubicin, for example, were predominantly used in the treatment of acute lymphoblastic leukemia (ALL), until long-term doses were proven to induce cardiac and skeletal muscle dysfunction through ROS-mediated impairment or deletion of mtDNA [50-54]. Our study failed to find an association between sarcopenia and administration of anthracyclines, due to the combination of lower doses of doxorubicin and the possible co-administration of the iron-chelator, dexrazoxane. These modifications may have impeded oxidative stress and exerted skeletomuscular protective effects [55, 56]. Alkylating agents were also found to be inconsequential to sarcopenia among our survivors, despite evidence that the mitochondrial electron transport system may be disrupted by acrolein, an end-product of cyclophosphamide metabolism [57, 58]. In this case, co-administration of antioxidants such as probucol may have protected patients from muscle damage.

However, one-third of CCS with sarcopenia received ionizing radiation, an agent capable of generating unrepairable double strand breaks (DSBs) in DNA [59]. This is exquisitely detrimental for the mitochondrial genome, which cannot avail itself of the protective histones and repair mechanisms afforded to nuclear DNA, and as a result is vulnerable to a specific 4977-bp common deletion as a result of exposure [60, 61]. Additional evidence in mice have indicated that mitochondrial deletions may persist in tissues for months after radiation exposure [62, 63]. More importantly, mouse models of

inducible DSBs that exclusively target mitochondria have offered compelling evidence that transient mitochondrial damage may be sufficient to provoke sarcopenia. This phenotype was observed months after DSB induction and was characterized by mtDNA depletion, more pronounced loss of muscle mass in females, and a reduction of myosatellite cell populations [64].

The prospect of delayed skeletal muscle dysfunction as a result of short-term mitochondrial damage to muscle satellite cells (MSCs) is intriguing. During postnatal development, MSCs actively proliferate and give rise to a burgeoning population of myonuclei that eventually support muscle fiber growth [65]. Although MSCs support hypertrophic expansion of muscle fibers throughout early adulthood, activity stops during puberty, where the total number of fibers is maintained until middle age [10, 66]. After puberty, MSCs are relegated to quiescent niches until muscle injury elicits their re-entry into the cell cycle to either repair muscle fibers or self-renew and return to the niche [67]. In our cohort, 51.7% of CCS with sarcopenia were diagnosed between the ages of 4 and 13 years, a time when MSCs should either be actively proliferating or contributing to skeletal muscle mass. Given the heightened sensitivity of MSCs to chemotherapy-induced oxidative stress or mitochondrial DSBs [68], it is possible that the finite supply of MSCs becomes either prematurely exhausted or that they are impaired and acquiesce into early senescence, thereby perpetuating an accelerated sarcopenic phenotype.

This study provides evidence for the role of mitochondrial dysfunction in the early appearance of sarcopenia among CCS. Considering the racial structure of the SJLIFE cohort, our results are analogous to a 2015 general population study that found that prevalent frailty was significantly associated with reduced mtCN in 13,133 older,

white participants [27]. Although this same study, plus another 2015 study that examined mtCN associations among 2,077 Sardinians [35], failed to find an association between mtCN and the weakness component of frailty, we find that the odds of sarcopenia were increased by 27% (aOR, 0.7; 95% CI 0.6 to 0.9) when looking at grip strength alone. Our finding is substantiated by similar mtCN and grip strength results from a large population-based Danish study that found that increases in mtCN were associated with improved health in the elderly [32]. Fundamental differences in the pathobiology between aging in the general population and the accelerated phenotype that occurs in CCS may account for the discrepancies among studies.

In terms of factors that affect mtDNA abundance, sex, ethnicity, and age were initially found to be significant independent predictors. That females had higher PBL mtCN than males is consistent with other studies [27, 30, 35], although one study has suggested that these differences were not apparent [32], and two studies have reported conflicting results using the same population, but differing only by sample size [69, 70]. This suggests that sample size may confound true differences between males and females, and that effect sizes should be reported instead. Indeed, in our study, the effect size was small (d = 0.11) and indicated that mtCN differences between males and females were actually negligible. With respect to self-reported ethnicity, non-Hispanics had 40 more copies of mtDNA than Hispanics; however, it is worth noting that Hispanics were under-represented in the cohort and that mitochondrial haplogroup assignment, which can account for more admixture in a population, had no bearing on mtCN, a finding that has also been substantiated elsewhere [30, 71].

Age was significantly associated with PBL mtCN, with a mean loss of 0.77 copies with every advancement in year. Compared to other investigations of the general population, mtCN depletion in CCS was more pronounced, as Mengel-From and colleagues reported a loss of 0.54 copies per year after the age of 50, Ding and colleagues reported a loss 0.15 copies per year, and Zhang et al reported 0.4 copy losses per year in a cohort of British women [27, 32, 35]. Using a different metric, Asher et al reported 0.14 mtCN standard deviations were lost over 10 years in older individuals, whereas we found a loss of 0.09 mtCN standard deviations over the same time period. While our results slightly differ from Ashar's frailty study, it is possible that the inclusion of younger participants in our cohort diminished the degree of mtCN loss. Nevertheless, our results and the aforementioned studies indicate that PBL mtCN depreciates with advancing age.

The ability of WGS to estimate mtCN per cell has been the subject of multiple investigations of varying health outcomes [30, 35, 71-73], with the most common algorithm for inferring mtCN derived from taking the ratio of average mitochondrial sequence coverage over that of average nuclear sequence coverage, and then adjusting the estimate for the diploid nature of autosomal DNA [35]. Additionally, qRT-PCR is often utilized as a means of either validating next-generation sequencing platforms or as the primary method to estimate mtCN [32, 35, 70]. To demonstrate the efficacy of both platforms, DNA from a 143B osteosarcoma cell line that lacks mitochondrial DNA (ρ0) was included as a negative control in both of our assays, and the results demonstrated that WGS estimates (0.17 mtCN) were superior to estimates derived by qRT-PCR (2.0 mtCN). Further research into the qRT-PCR primers used for MT-ND2 detection indicated that this region shares similarity with known NUMTS (http://genome.ucsc.edu/

, reference human genome assembly GRCh37) [74], or nuclear sequences that harbor insertions of mtDNA into the nuclear genome [75], and that this possibly inflated qRT-PCR estimates. Since our WGS estimate was well within the range of what would reasonably be expected for the ρ0 cell line, we consider the mtCN estimates for the CCS cohort to be accurate. Furthermore, in a robust study that compared three different methods (shotgun sequencing, capture-enriched sequencing, droplet digital PCR) of mtCN estimation across multiple tissues, WGS was also found to be the most precise [30].

Despite the widespread use of PBL mtCN as a biomarker in health outcome studies, the appropriateness of using PBL mtCN as a surrogate for muscle mtDNA content merits discussion. Dramatic increases in mtCN have been observed in post-mitotic tissues with higher energy requirements, with skeletal muscle typically harboring thousands of copies of mtDNA as compared to leukocytes, which contain hundreds. That mtCN is regulated in a tissue-specific manner is apparent [30, 33, 34, 76]; however, there is discordance as to whether mtCN are correlated among tissues within the same individual. In a 2016 study that estimated mtCN across various autopsied tissues from 152 individuals, Wachsmuth and colleagues found that intra-individual mtCN was mostly uncorrelated across tissues (e.g. blood vs. skeletal muscle), with only similar tissues (e.g. small vs. large intestine) exhibiting positive correlations. This finding is supported by another study that examined mtCN in the context of m.3243A>G mitochondrial disease burden in adults; here, decline in skeletal muscle mtCN was associated with disease burden, but no correlation was found between blood and skeletal muscle mtCN [77]. Conversely, Frahm et al. found intra-individual correlations between brain tissues and

skeletal and heart muscle, and further suggested that systemic stress coordinated tissue mtCN responses [33]. In the absence of a consensus, more studies that directly measure the correlation between PBL and skeletal muscle mtCN are needed.

The scope of this study was limited to mtCN and did not investigate the potential contributions of PBL genetic or mitochondrial variants to the sarcopenia phenotype. Considering that roughly 900 gene products are required to sustain mitochondria function, variants that impact the proteins that are encoded by the nucleus are certainly germane [78]. Precedence for a mitochondrial variant, though, has been attained in frailty research, where an inherited PBL SNP that predisposes healthy individuals to weakness has been attributed to a minor C allele SNP at the mt204 locus in the mitochondrial D-loop [79]. The location of this variant could negatively impact mtDNA replication and was the premise for the group's subsequent investigation of mtCN as a determinant of frailty within this same population [27, 80]. Thus, the potential presence of a D-loop variant that predisposes CCS to sarcopenia could not only explain the observed reduction in mtCN, but could also identify at-risk survivors for intervention-based measures.

This study finds that the odds of sarcopenia are increased 21% with every standard deviation decrease in mtCN, and that this association is significant even when considering a more stringent definition of sarcopenia. For example, when requiring both mass *and* function as the criteria, the prevalence of sarcopenia in our cohort dropped to 3.5%. However, the stringent model now emphasizes the debilitating odds of cranial radiation (aOR, 3.8; 95% CI 2.2 to 6.4), the increased odds for females (aOR, 2.7; 95% CI 1.5 to 4.6), while strengthening the protective influence of mtCN (aOR, 0.7; 95% CI

0.5 to 0.9). In either context, the evidence suggests a compelling role for mitochondria as agents of pathological change among survivors. Considering that skeletal muscles can occupy 40-50% of total body mass and are central to physiological reserve [81], it follows that depletion of muscle mass and function has systemic consequences and suggests that sarcopenia is not merely a component of, but rather a precursor to frailty.

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TABLE 1: Characteristics of 1,762 Childhood Cancer Survivors With and Without Sarcopenia

	Sarcopenia + **N=476 (27.0%)**	**Sarcopenia -** **N=1,286 (73.0%)**	**P-value**
Age, median (IQR)	28.9 (14.7)	29.6 (13.1)	0.45 †
Sex			<.0001
Female, n (%)	268 (31.5)	584 (68.5)	
Male, n (%)	208 (22.9)	702 (77.1)	
Race			0.57 ^
White, n (%)	394 (26.6)	1,088 (73.4)	
Black, n (%)	79 (29.2)	192 (70.9)	
Other, n (%)	3 (33.3)	6 (66.7)	
Ethnicity			0.80
Non-Hispanic, n (%)	468 (27.1)	1,262 (73.0)	
Hispanic, n (%)	8 (25.0)	24 (75.0)	
Age at diagnosis, yrs, n (%)			0.04
0-3	137 (26.2)	386 (73.8)	
4-8	136 (30.4)	312 (69.6)	
9-13	110 (29.3)	265 (70.7)	
14-24	93 (22.4)	323 (77.6)	
Age of assessment, yrs, n (%)			0.18
18-24	167 (29.3)	404 (70.8)	
25-30	107 (26.1)	303 (73.9)	
31-37	93 (23.3)	306 (76.7)	
38-64	109 (28.5)	273 (71.5)	
Time since diagnosis, yrs, n (%)			0.08
8-19	234 (27.9)	604 (72.1)	
20-29	130 (23.6)	420 (76.4)	

30-49	112 (30.0)	262 (70.1)	
Tumor Group, n (%)			<.0001^
Blood, n (%)	206 (22.3)	720 (77.8)	
Brain, n (%)	90 (39.0)	141 (61.0)	
Solid, n (%)	178 (29.9)	417 (70.1)	
Other, n (%)	2 (20.0)	8 (80.0)	
Any radiation, n (%)			<.0001
Yes	313 (31.1)	693 (68.9)	
No	163 (21.6)	593 (78.4)	
Abdominal radiation, n (%)			<.0001
Yes	145 (36.0)	258 (64.0)	
No	331 (24.4)	1,028 (75.6)	
Cranial radiation, n (%)			<.0001
Yes	178 (33.8)	349 (66.2)	
No	298 (24.1)	937 (75.9)	
Heart radiation, n (%)			<.001
Yes	183 (33.0)	371 (67.0)	
No	293 (24.3)	915 (75.8)	
Anthracycline, n (%)			<.0001
Yes	237 (22.6)	811 (77.4)	
No	239 (33.5)	475 (66.5)	
Alkylating agents, n (%)			0.62
Yes	285 (27.5)	753 (72.5)	
No	191 (26.4)	533 (73.6)	
Glucocorticoids, n (%)			<.0001
Yes	174 (22.4)	604 (77.6)	
No	302 (30.7)	682 (69.3)	
Bone marrow transplant, n (%)			0.66

Yes	8 (30.8)	18 (69.2)	
No	468 (27.0)	1,268 (73.0)	
Smoking, n (%)			0.44
Never	313 (28.6)	848 (73.0)	
Current	97 (28.6)	242 (71.4)	
Past	57 (23.9)	182 (76.2)	
mtCN, median (IQR)	301.0 (98.4)	309.8 (118.4)	<.01†
Haplogroup, n (%)			0.38
Ancestral	85 (28.8)	210 (71.2)	
Asian	10 (21.7)	36 (78.3)	
European	200 (28.5)	503 (71.6)	
Reference	180 (25.2)	534 (74.3)	

IQR= Interquartile Range

† = Wilcoxon rank-sum test

^ = Fisher's Exact

mtCN = mitochondrial DNA copy number

Missing: Haplogroup data for 1 survivor with sarcopenia and 3 survivors without sarcopenia, smoking data for 9 survivors with sarcopenia and 14 survivors without sarcopenia, and 86 survivors missing measurements for sarcopenia.

TABLE 2: Associations between survivor characteristics and sarcopenia

	Survivors with Sarcopenia N = 476			
Factors	**n** (%)	**OR**	**95 % CI**	**trend ¥ p-value**
mtCN		0.8	0.8 to 0.9	
Sex				
Female	268 (31.5)	1.6	1.3 to 1.9	
Male	208 (22.9)	Reference		
Race				
White	394 (26.6)	0.7	0.2 to 2.9	
Black	79 (29.2)	0.8	0.2 to 3.4	
Other	3 (33.3)	Reference		
Ethnicity				
Non-Hispanic	468 (27.1)	1.1	0.5 to 2.5	
Hispanic	8 (25.0)	Reference		
Age at diagnosis, yrs				
0-3	137 (26.2)	1.2	0.9 to 1.7	
4-8	136 (30.4)	1.5	1.1 to 2.1	
9-13	110 (29.3)	1.4	1.1 to 2.0	
14-24	93 (22.4)	Reference		0.23
Age of assessment, yrs				
18-24	167 (29.3)	Reference		0.45
25-30	107 (26.1)	0.9	0.6 to 1.1	
31-37	93 (23.3)	0.7	0.6 to 1.0	

38-64	109 (28.5)	1.0	0.7 to 1.3	
Time since diagnosis, yrs				
8-19	234 (27.9)	Reference		0.82
20-29	130 (23.6)	0.8	0.6 to 1.0	
30-49	112 (30.0)	1.1	0.8 to 1.4	
Tumor Group				
Blood	206 (22.3)	1.1	0.2 to 5.4	
Brain	90 (39.0)	2.6	0.5 to 12.3	
Solid	178 (29.9)	1.7	0.4 to 8.1	
Other	2 (20.0)	Reference		
Any radiation				
Yes	313 (31.1)	1.6	1.3 to 2.1	
No	163 (21.6)	Reference		
Abdominal radiation				
Yes	145 (36.0)	1.8	1.4 to 2.2	
No	331 (24.4)	Reference		
Cranial radiation				
Yes	178 (33.8)	1.6	1.3 to 2.0	
No	298 (24.1)	Reference		
Heart radiation				
Yes	183 (33.0)	1.5	1.2 to 1.9	
No	293 (24.3)	Reference		
Anthracycline				
Yes	237 (22.6)	0.6	0.5 to 0.7	
No	239 (33.5)	Reference		

Alkylating agents			
Yes	285 (27.5)	1.1	0.9 to 1.3
No	191 (26.4)	Reference	
Glucocorticoids			
Yes	174 (22.4)	0.7	0.5 to 0.8
No	302 (30.7)	Reference	
Bone marrow transplant			
Yes	8 (30.8)	1.2	0.5 to 2.8
No	468 (27.0)	Reference	
Smoking			
Never	313 (27.0)	Reference	
Current	97 (28.6)	1.1	0.8 to 1.4
Past	57 (23.9)	0.9	0.6 to 1.2
Haplogroup			
Ancestral	85 (28.8)	1.2	0.9 to 1.6
Asian	10 (21.7)	0.8	0.4 to 1.7
European	200 (28.5)	1.2	0.9 to 1.5
Reference	180 (25.2)	Reference	

mtCN = mitochondrial DNA copy number

¥ Mantel-Haenszel chi-square

Missing: Haplogroup data for 1 survivors with sarcopenia and 3 survivors without sarcopenia, smoking data for 9 survivors with sarcopenia and 14 survivors without sarcopenia, and 85 survivors missing measurements for sarcopenia.

FIGURE 1: qPCR validation of WGS mtCN estimates.

MtCN estimates from 95 randomly selected whole-genome sequenced samples are plotted along the x-axis versus mtCN estimates derived from qPCR of the same samples, plotted along the y-axis.

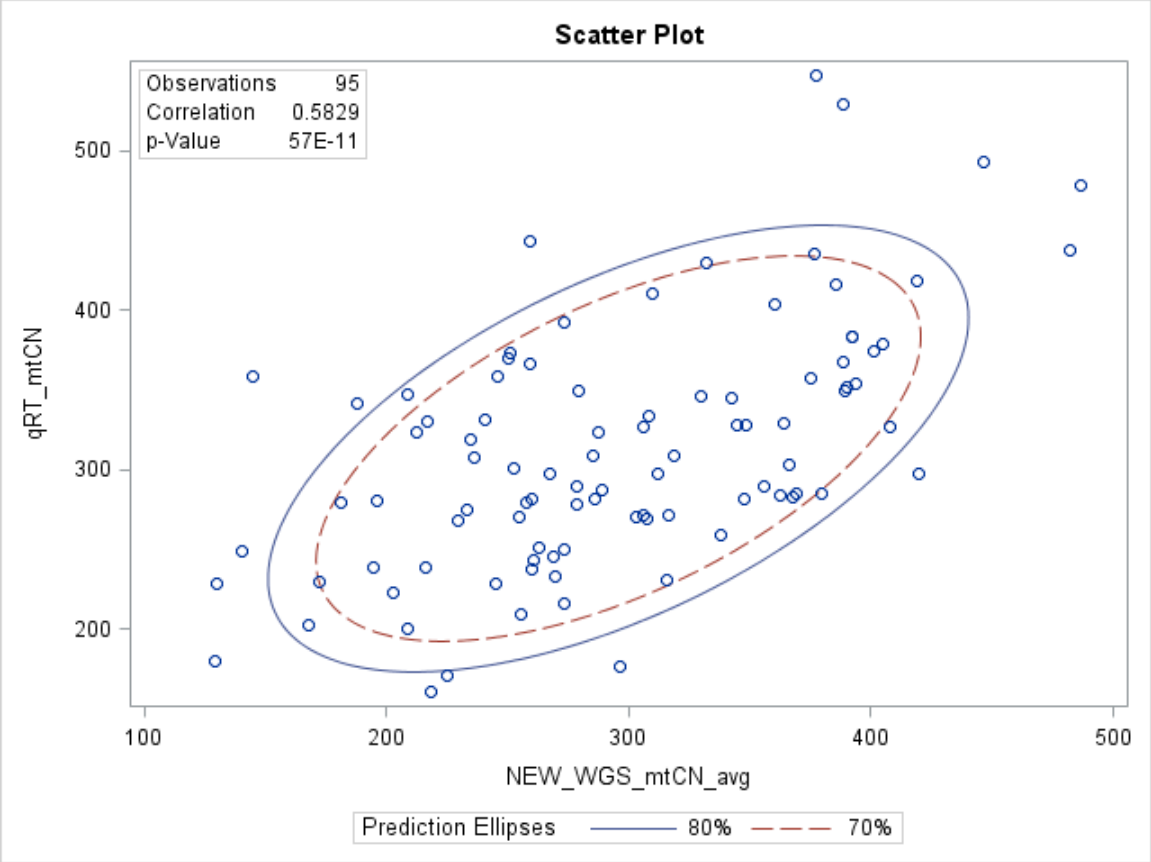

FIGURE 2: mtCN and age among males and females, by ethnic group.

Two pairs of regression lines are presented, with the upper pair reflecting higher mtCN observed among non-Hispanics versus the lower pair of regression lines that represent Hispanics. Females (blue and green lines) have higher mtCN than males (red and brown) in both ethnic groups. Relative depreciation of mtCN with increasing age is observed, regardless of sex or ethnicity.

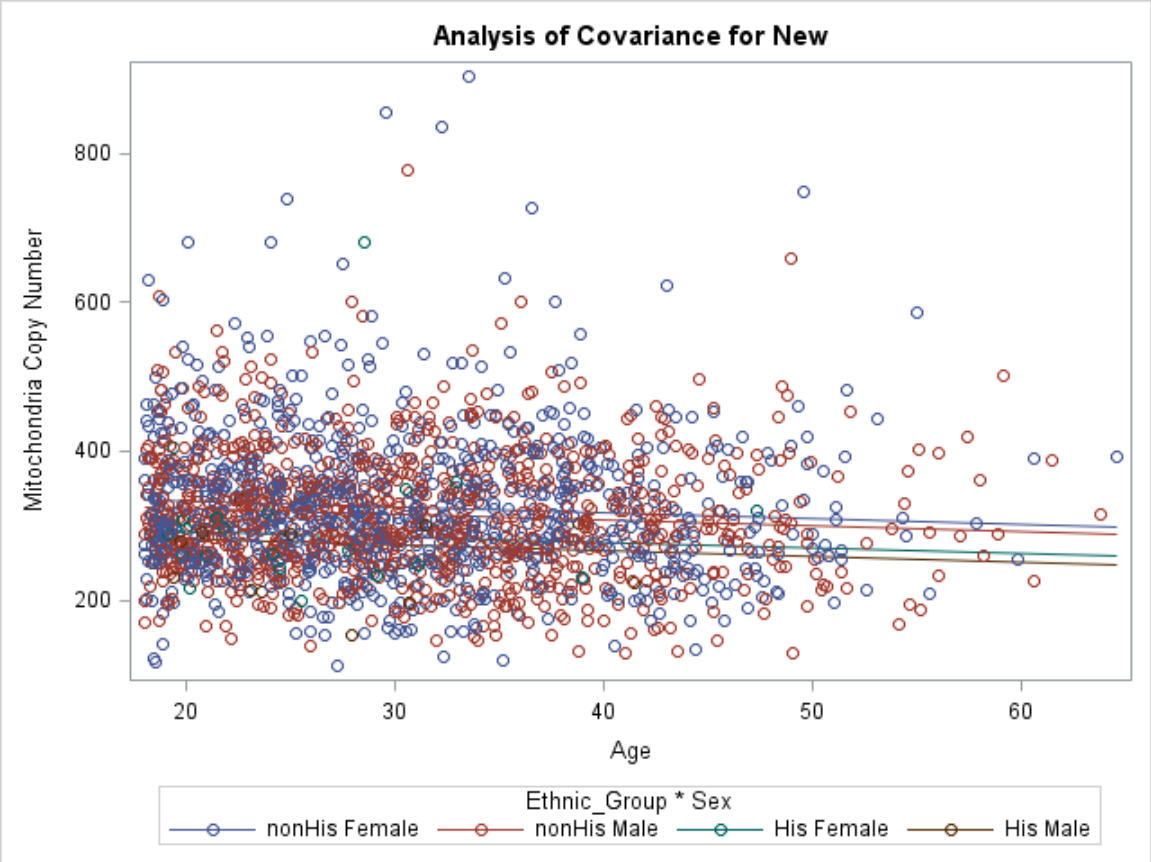

FIGURE 3: Final Model Odds Ratios

Final model demonstrating the association between standard deviation changes in mitochondrial copy number (mtCNSD) and odds of developing sarcopenia.