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Association of Vitamin D Concentration and Leukocyte Telomere Length

A. Study Purpose and Rationale

Study Purpose:
To determine the association between vitamin D and leukocyte telomere length in a large population-based cohort.

Background and Rationale:
Telomeres are specialized regions at the end of chromosomes that play a critical role in maintaining genomic stability during repeated cycles of cell replication. Telomeres consist of noncoding repetitive sequences (TTAGGG) that extend for several thousand base pairs. The telomeric complex also includes the enzyme telomerase and associated structural proteins. Telomerase is a ribonucleoprotein reverse transcriptase that functions to elongate the telomere by 5' to 3' polymerization along an RNA template. The telomere is a dynamic structure, and telomere length is regulated by a variety of factors. Cells with critically short telomeres enter replicative senescence, losing their ability to proliferate, and often undergo apoptosis. Telomere length depends upon the rate of cell proliferation as well as the balance of factors that affect telomerase activity and stability of the telomeric complex.[1]

Leukocyte telomere length, in particular, has emerged as a marker of biological aging in epidemiologic studies. Previously it was thought that telomerase existed only in germ line cells and not in somatic cells; however, it has since been shown that telomerase can be expressed in somatic cells, including lymphocytes.[2] The current understanding is that leukocyte telomere length reflects the cumulative burden of inflammation and oxidative stress. At a fundamental level, telomere length is determined by the rate of hematopoietic stem cell proliferation and the rate of telomere attrition with each leukocyte replication. The latter is influenced by genetic, epigenetic, and environmental factors that affect telomere stability and telomerase activity, many of which remain to be elucidated.[3]

Vitamin D deficiency has been shown to be associated with a variety of human ailments, including inflammatory conditions like rheumatoid arthritis, inflammatory bowel disease, lupus, and multiple sclerosis,[4,5] and age-related diseases like cardiovascular disease and cancer.[6] Certain diseases, such as multiple sclerosis and inflammatory bowel disease, have long been noted to be more prevalent in high latitude regions that receive less sunlight. Furthermore, vitamin D supplementation in these conditions has therapeutic effects, such as decreasing relapse rate in inflammatory bowel disease and multiple sclerosis.[4] In accordance with these observations, Vitamin D has been shown to exert anti-inflammatory and immunomodulatory effects in cell culture models.[7,8] The vitamin D receptor is expressed by many immune cells including circulating monocytes, macrophages, dendritic cells, and activated T cells.[4] Quiescent CD4+ T cells express VDR at low levels, which increases fivefold after activation.[9] 1,25 dihydroxyvitamin D

inhibits Th1 response and promotes Th2 dominance by decreasing expression of IFN-γ and IL-2 and increasing expression of IL-5 and IL-10.[9] Given the direct actions of vitamin D on leukocytes and the association of vitamin D with conditions also associated with telomere shortening, there is a potential link between vitamin D and leukocyte telomere dynamics.

Although observational studies have established links between leukocyte telomere length and cardiovascular disease,[10] obesity,[11] insulin resistance,[12] dementia,[13] and other disease states, there have been few studies exploring a possible relationship between leukocyte telomere shortening and vitamin D deficiency. In a large cross-sectional observational study of female twin pairs (n=2160), Richards and colleagues showed a positive association between vitamin D concentration and leukocyte telomere length.[14] Pearson's correlation coefficient was 0.07 ($p=0.0010$), and the association was strengthened with age-adjusted leukocyte telomere length ($r=0.09$, $p<0.0001$). Their findings remained statistically significant when adjusted for additional covariates including age, season of vitamin D measurement, menopausal status, use of hormone replacement therapy, and physical activity (covariates found to be potential confounders by the Bayesian Information Criterion). The adjusted difference in telomere length between the highest and lowest tertile of vitamin D concentrations was 107.1 base pairs ($p=0.0009$), which is equivalent to approximately 5 years of telomeric aging. However, there are significant limitations inherent to their cohort population. First, female gender is independently associated with higher telomerase activity and longer telomere length due to the effects of estrogen. Second, outcomes in twin pairs are not independent and twin pairs may not be fully representative of the general population. Although the investigators made corrections in the statistical model to control for non-independence, it remains a significant concern whether these results can be applied to a general population.

In addition to epidemiologic evidence, Zhu et al conducted a small intervention study of vitamin D_3 supplementation in overweight African Americans. They demonstrated a 19.2% increase in peripheral mononuclear cell (PBMC) telomerase activity (1.56 ± 0.29 absorbance reading unit (AU) to 1.86 ± 0.42 AU, $p<0.0001$) over 16 weeks.[15] Similarly, a retrospective case-control study of hemodialysis patients showed longer PBMC telomere length in patients treated with active vitamin D_3 compared to untreated matched controls (8.8 ± 1.5 kbp vs. 8.4 ± 0.2 kbp; $p = 0.003$).[16]

Given the overlap between disease phenotypes associated with low vitamin D concentrations and short leukocyte telomere length, we hypothesize that there may be an association between serum vitamin D concentration on leukocyte telomere length. We propose two hypotheses regarding the mechanism of the association, although this determination lies outside the scope of the present study. Hypothesis 1: Given the immunomodulatory and anti-inflammatory effects of vitamin D, vitamin D may directly act via vitamin D receptors on immune cells to have a protective effect on telomere length (for example, by increasing telomerase activity or promoting structural stability). Hypothesis 2: Alternatively, there may be residual confounding due to an unknown factor (for example, genetic polymorphisms with pleotropic effects that influence both vitamin D levels and telomere length).

B. Study Design and Statistical Analysis

Study Population

The 1995 Nova Scotia Health survey (NSHS95) is a population-based survey implemented by Heart Health Nova Scotia and the Nova Scotia Department of Health. Potential participants were randomly identified based on a probability sample by Statistics Canada, the national statistical agency and census bureau, and were selected to be representative of the Nova Scotia population by age, sex, and geographic location. Participants consisted of non-institutionalized, non-pregnant Nova Scotia residents, ages 18 years or older, who were listed in the registry of Medical Services Insurance, the government-sponsored universal health insurance plan. Of 4,500 targeted participants, 3227 (72%) provided informed consent and were enrolled. Propensity score analyses revealed no meaningful response biases between those enrolling versus those not enrolling in the study. Participants provided written informed consent to participate in this study, which allowed for linkage to their health care use data and for the storage and future use of blood assays.[17]

Study Design

Cross-sectional observational study

Statistical Analysis

We will use SPSS to analyze the association of serum vitamin D concentration and leukocyte telomere length. We will conduct multiple linear regression analysis to determine the unadjusted association between vitamin D concentration and leukocyte telomere length. Vitamin D concentration will be the independent variable and leukocyte telomere length will be the independent variable. The primary analysis will consider both leukocyte telomere length and vitamin D as continuous variables. We will optimize transformation of these variables to approximate a normal distribution. We will adjust this model for demographic and clinical covariates, including age, sex, body mass index, smoking status, physical activity level, menopausal status, use of hormone replacement therapy, estimated sunlight hours, and biomarkers of inflammation (C-reactive protein, Interleukin-6, sICAM-1). Nonlinearity will be explored by LOESS models. In addition, we plan to conduct secondary analyses dividing the study population into tertiles of vitamin D in which the lowest tertile will be considered the reference group. We will conduct the analysis with predefined categories of vitamin D deficiency as follows: deficient (<20ng/ml); insufficient (20-30ng/ml) and optimal (>30ng/ml). Furthermore, we will approximate the seasonal variation of vitamin D levels using a fitted sinusoidal model in order to overcome the limitation of having a single vitamin D measurement.[18] Additional analyses will done to evaluate the effect of vitamin D supplementation on leukocyte telomere length if this data is available.

Power analysis

Of the 3,227 participants enrolled, there were 2,242 subjects with data on leukocyte telomere length, 1,864 subjects with 25-hydroxyvitamin D measurements, and 1,828 subjects with both LTL and 25-hydroxyvitamin D measurements who will be included in the present analysis. Given a fixed sample size of 1,828, we conducted a post hoc power analysis. To estimate the effect size, we looked at previous results from Richard and

colleagues that showed a Pearson's correlation coefficient (r) = 0.07 for the unadjusted association between leukocyte telomere length and serum vitamin D concentration.[14] Assuming a sample size of 1828, a null hypothesis of no association (r=0), and a two-tailed test, the present study has 85% power to detect a correlation of this magnitude. A limitation of our study population is that if the effect size in our cohort is smaller than previously noted, there may not be sufficient power to detect a true correlation. However, given that the expected correlation is small, a further diminished effect size would render a finding clinically insignificant.

C. Study Procedures

A group of 29 public health nurses attended a five-day training session to learn the techniques for contacting participants and collecting data. From March through November 1995, nurses contacted survey participants and interviewed those who agreed to participate. Participants were asked for consent to allow linkage of their NSHS95 study data to centralized, computerized, health care utilization data. Participants also attended one clinic visit to obtain anthropometric measures (such as body mass index), and have blood drawn and stored for those who consented. A full medical history was assessed and medication use was recorded at a home visit.[17]

Measurement of serum 25-hydroxyvitamin D was performed using the Diasorin radioimmunoassay method (Stillwater, MN). This assay measures two forms of 25(OH)D: cutaneously-derived vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) derived from supplements or fortified foods. Normal levels of 25(OH)D are >30ng/mL. Inter-assay precision is 15% at 17ng/mL and 15% at 57ng/mL; assay sensitivity is 1.5ng/mL.

Measurement of leukocyte telomere length was conducted as previously described in Shaffer et al.[17] Measurements were performed on coded samples by laboratory personnel blinded to participant characteristics. DNA was extracted from frozen buffy coat samples. Average leukocyte telomere length was determined using a polymerase chain reaction (PCR) method modified from that of Cawthon and colleagues.[19] Real-time quantitative PCR was performed using a CFX384 thermocycler (Biorad, Richmond, CA). The assay method was optimized for use of both telomere (T) and single copy gene (S) amplifications on the same 384-well plate, with reference standard DNA samples on each plate. Assay coefficient of variance was 5% to 8%. Given that the T/S ratio depends on the particular DNA standards used, T/S ratios were converted to telomere base pairs using a formula (base pairs = (1,585*T/S ratio)+3,582) derived from co-analysis of selected DNA samples with both PCR and terminal restriction fragment methods (non-radioactive TeloTAGGG Telomere Length, Roche Diagnostics, Mannheim, Germany).

D. Study Drugs*

None

E. Medical Device.*

None

F. Study Questionnaires

Study questionnaires were previously completed by participants as detailed above. No new study questionnaires will be competed for the purpose of the present analysis.

G. Study Subjects

Inclusion criteria were non-institutionalized, non-pregnant adults age 18 years or older, who were listed in the registry of Medical Services Insurance, the government-sponsored universal health insurance plan.

Subjects were excluded from analysis for missing assessments of 25-hydroxyvitamin D concentration or leukocyte telomere length.

H. Recruitment of Subjects

Subjects were previously recruited for the NSHS95 study as detailed above. No new subjects will be recruited for the present analysis.

I. Confidentiality of Study Data

All data has previously been coded and de-identified. Data is stored on a secure password-protected computer accessible only to the study investigators.

J. Potential Conflict of Interest

None of the investigators have any financial interest in the components of the study.

K. Location of the Study

The study was previously conducted in Nova Scotia. All data analysis for the present study will be completed at CUMC.

L. Potential Risks

There no significant risks anticipated as the result of additional data analysis. To anticipate the potential risk of compromising patient privacy, precautions have been taken as described above.

M. Potential Benefits

No individual benefit to subjects is anticipated as a result of participation in the study.

N. Alternative Therapies

Not applicable.

O. Compensation to Subjects

No additional compensation will be provided to subjects for these additional analyses. Informed consent was obtained at the time of enrollment allowing for use of their de-identified data by additional investigators for research purposes.

P. Costs to Subjects

None

Q. Minors as Research Subjects

No minors were enrolled in the course of the study.

R. Radiation or Radioactive Substances

Not applicable.

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