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Effects of Vitamin D and Calcium Supplementation on Leukocyte Telomere Length in Colorectal Adenoma Patients: A Randomized Clinical Trial

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Effects of Vitamin D and Calcium Supplementation on Leukocyte Telomere Length in Colorectal Adenoma Patients: A Randomized Clinical Trial

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Abstract

Effects of Vitamin D and Calcium Supplementation on Leukocyte Telomere Length in Colorectal Adenoma Patients: A Randomized Clinical Trial

By Evelyn Wang

Background:

Epidemiologic studies have shown that calcium and vitamin D are associated with decreased risk for colorectal cancer. However, data on this association in the context of leukocyte telomere length is limited. This study investigates the efficacy of a calcium and vitamin D intervention in persons at increased risk for colorectal cancer – leukocyte telomere length is used as a proxy for chemoprevention success. Baseline associations between leukocyte telomere length and patient lifestyle characteristics are also investigated.

Methods:

Blood specimens were collected before and after supplementation with calcium and vitamin D, alone and in combination, in a 6-month, pilot, double-blinded, placebo controlled, randomized clinical trial in 92 patients previously diagnosed with sporadic colorectal adenoma. Real-time qPCR was performed to measure relative leukocyte telomere length, which was then translated into base-pair length. Fisher's exact test for categorical variables and ANOVA for continuous variables were used to test for differences among treatment groups. Linear mixed effects models were used to estimate the treatment effects for leukocyte telomere length and plasma 25-OH-vitamin D, the best indicator of vitamin D exposure.

Results:

After 6 months of treatment, among 24 participants that had both baseline and follow-up leukocyte telomere length data, 25-OH-vitamin D increased 43%, 75%, and 3% in the calcium, vitamin D_3, and calcium plus vitamin D_3 groups relative to the placebo group, respectively. Relative to the placebo group, mean telomere length decreased by 16%, 18%, and 10% in the calcium, vitamin D, and calcium plus vitamin D groups, respectively. Analysis of 46-baseline participants and 37 follow-up participants that had at least one telomere length measurement revealed numbers consistent with the 24 participants (except for the calcium plus vitamin D_3 group, in which telomere length increased). Several lifestyle and dietary factors for colorectal cancer were associated with baseline leukocyte telomere length in the hypothesized direction.

Conclusion:

Our preliminary results suggest that calcium and vitamin D, individually or together, may not have an effect on leukocyte telomere length after 6 months of treatment.

Key Words: Leukocyte Telomere Length, Vitamin D, Calcium, Colorectal Neoplasms, Randomized Clinical Trial

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Table of Contents

Background

Telomeres are protective, noncoding nucleotide sequences characterized by "TTAGGG" repeats at the 3' ends of eukaryotic DNA. These nucleoprotein complexes prevent chromosome deterioration and loss during cellular mitosis. Telomere length is regulated by telomerase, a ribonucleoprotien enzyme. Telomerase functions by adding additional "TTAGGG" repeat sequences to existing telomere strands, essentially extending telomere length (1). Without telomerase, the Hayflick limit for cells will eventually be reached and apoptosis occurs. Telomere shortening places cells at increased risk of abnormal proliferation, senescence, and cell death, and has been linked to accelerated aging and early mortality in humans (2,3). Thus, longer telomere length is generally regarded as a biomarker of cellular longevity and overall health (4).

Short telomeres might be associated with colorectal cancer (CRC) risk, progression, and mortality after cancer diagnosis (5). In histopathology findings, cancerous colon tissue cells had noticeably shorter telomeres compared to adjacent, healthy specimens (6). A Chinese case-control study of 628 CRC cases and 1,256 age and sex frequency matched cancer-free controls found that having shorter leukocyte telomeres increased the risk of CRC by 13% (OR = 1.13; 95%CI: 1.00-1.28) (7). In another case-control study (n = 598 CRC cases), those with microsatellite stable CRCs exhibited shorter blood leukocyte telomere lengths compared to matched controls. However, this association varied with age of cancer onset, signifying that telomere dynamics may be both age and disease dependent (8). A long-term prospective study of 47,102 Danish participants found very short telomere length was associated with reduced survival after CRC diagnosis, the hazard ratio for premature death 1.31 (95% CI: 1.14-

1.52). However, short telomere length was not associated with CRC risk, a multivariable adjusted hazard ratio of 0.98 was observed (95% CI: 0.88 to 1.08) (9).

A meta-analysis of 27 retrospective case-control and prospective studies found a strong association between short LTL and cancer risk, an OR of 1.96 (95% CI: 1.37-2.81, P = 0.0001) (10). Notably, summary ORs for CRC risk can differ by study type, where stronger associations are found in retrospective "traditional" case-control studies, compared to prospective nested case-control studies. Pooley et al. conducted both study types and confirmed this trend. Their prospective case-control study of 185 CRC cases and 370 matched cancer free controls found that the odds ratio for shortest verses the longest quartile of telomere length was 1.13 (95% CI: 0.54-2.36). However, their retrospective case-control study of 2,249 CRC cases and 2,161 cancer free controls yielded an OR of 2.14 (95% CI: 1.77-2.59) (11). Similarly, in a prospective cohort of 134 incident cases of colorectal carcinoma and 357 matched controls, no association between mean leukocyte telomere length and CRC risk was observed (OR=0.94; 95% CI: 0.65-1.38) (12). Another prospective cohort study of 191 incident CRC cases and 306 matched controls (matched by age, smoking status, and follow-up length) also found no association between mean telomere length and CRC risk (OR= 1.25; 95% CI: 0.86-1.80) (13). These findings suggest that reverse causation may have contributed to some of the associations observed in retrospective studies.

It is hypothesized that extreme telomere shortness prompts carcinogenesis since this condition suggests that normal Rb and p53 signal pathways have been disrupted, and timely apoptosis will not occur (14). These cells, unchecked by neither growth suppressors nor subject to division checkpoints, eventually via uncontrolled proliferation

and accumulation of genetic mutations, may lead to the development of cancerous lesions.

More recent findings suggest that having abnormally long telomeres is also deleterious to human health. That is, both overly long and short telomeres increase the risk of developing CRC. This association has also been observed in cancers of the breast, lung, kidney, and lymphatic system (5). A case-control study nested within the Shanghai Women's health study found a U-shaped association between peripheral blood cell telomere length and CRC risk. Subjects with atypically short telomeres had a 56% higher risk of CRC than the control group (OR=1.56; 95% CI: 0.92-2.64); those with abnormally long telomeres had a 61% higher risk of developing CRC (OR=1.61; 95% CI: 0.94-2.75). Researchers further concluded that the lowest cancer risk group had average telomere lengths in the third quintile (40^{th}-60^{th} percentile), not too long and not too short (15). Relatedly, a case control study also found the risk of CRC to be correlated with extremes in telomere length. Subjects younger than 50 with longer telomeres (80-99^{th} percentiles) had 6 times the risk of developing CRC than matched controls, while subjects older than 50 with shorter telomeres (1-10^{th} percentiles) had 12 times the risk of developing CRC (8). It is hypothesized that longer telomeres increase cancer risk due to delayed apoptosis. Increased cellular lifespan allows for extended exposure to harmful environmental toxins, genetic mutations, or accumulation of both (16).

The rate of telomere loss is dependent upon cell division frequency, environmental factors, and telomerase activity; it is therefore plausible that telomere length can be modulated by environmental and lifestyle factors (17-19). At least three randomized clinical trials have found that telomere length could be modified through

stress reduction (20), dietary intervention (21), and weight loss (22). In addition, several cross-sectional studies found that several lifestyle factors (e.g., physical inactivity, obesity, smoking), diet, and personality traits (e.g., pessimism) are correlated with shorter telomere length (23-25). However, the effects of dietary and lifestyle factors on the LTL are largely unknown.

Vitamin D is a fat-soluble prohoromone vital to maintaining a healthy immune system as well as normal growth and formation of the skeletal system. Serum/plasma 25-hydroxy vitamin D [25-OHD] is a reliable indicator of vitamin D status (26). It is recommended that children and adults maintain a vitamin D level of at least 50 ng/ml. A low level of vitamin D (below 50 ng/ml) may increase the risk of certain cancers and susceptibility to illnesses such as multiple sclerosis, rickets and osteoarthritis (27). Deficient patients are normally advised by clinicians to take vitamin D supplements, sometimes in conjunction with calcium, to treat and prevent calcium deficiencies. The current adult recommended daily allowance (RDA) for vitamin D is 600 IU and 1,200 mg for calcium. Overconsumption of calcium is rarely life threating but hypervitaminosis D is, and can result in hypercalcemia, nausea, vomiting, asthenia, polyuria, and renal failure (28).

Vitamin D aids in the absorption of calcium by forming the hormone calcitrol, which increases the amount of calcium in the blood stream for subsequent gastrointestinal absorption. Calcium, like Vitamin D, has important biochemical functions that, aside from strengthening bone, include binding to bile and fatty acids, resulting in compounds that are less likely to damage DNA in colonocytes. Several epidemiologic studies have shown that calcium, taken alone or in conjunction with vitamin D, can lower the risk of

CRC (29-31). Calcium by itself may protect against colorectal neoplasms. A randomized clinical trial of 832 subjects (calcium group=409; placebo group=423) found that calcium supplementation decreases the risk of adenoma occurrence (RR = 0.81, 95% CI: 0.67 to 0.99; P=0.04) (32). A secondary re-analysis of the data from this trial found a potential synergism between calcium supplementation and serum vitamin D levels in lowering the risk of colorectal adenoma occurrence. It was found that adenoma risk for the calcium group was contingent on serum 25-(OH)-vitamin D levels. For those with baseline serum vitamin D levels below the median (29.1 ng/mL), calcium supplementation was not associated with adenoma occurrence. However, for those with serum vitamin D levels above the median, calcium supplementation was associated with a reduced risk (RR = 0.71; 95 % CI: 0.57-0.89) (33).

Vitamin D's anti-carcinogenic effects include regulation of the cell cycle, modulation of growth factor signaling, DNA repair, and immune support (34). An ecologic study found US colon cancer mortality rates are highest in regions with the lowest levels of ultraviolet radiation, suggesting vitamin D has chemopreventive properties (35,36). A 20-year longitudinal study of the National Health and Nutrition Examination Survey (NHANES III) participants found that a serum vitamin D level of 81.3 nmol/l or higher was associated with a 72% reduced risk of CRC mortality in blacks (37). Numerous prospective studies have found associations between higher circulating vitamin D with reduced risk of CRC (38). A meta-analysis of 28 observational studies found a lower colorectal adenoma risk for those in the highest quintile of vitamin D intake (OR=0.89; 95% CI: 0.78-1.02) compared to those in the lowest quintile of vitamin D intake (39). A meta-analysis of 8 longitudinal studies found that a 50 nmol/l increase in

serum vitamin D was associated with a 60% lower CRC risk (OR=0.41; 95% CI: 0.11-1.49) (40).

Positive associations between blood vitamin D level and leukocyte telomere length have been observed (see below). It is possible that this association is a result of vitamin D's anti-inflammatory and anti-proliferative systemic effects that may influence white blood cell turnover, thereby maintaining longer leukocyte telomere length (41). A United Kingdom study of 2,160 women (18-79yo) found a positive correlation between serum vitamin D level and leukocyte telomere length; a 107.1 base pair difference (equal to 5 years of cellular aging) was observed between the highest and lowest tertiles of serum vitamin D (42). In addition, a Nurses Health study found that higher serum 25-(OH)D level was associated with longer telomere length, an OR of 1.59 observed between the highest and lowest quartiles of telomere length (43). While these cross-sectional analyses have supported use of vitamin D to obtain longer leukocyte telomere length, no randomized clinical trial to date has investigated this claim. We have therefore utilized previously collected biosamples and lifestyle data from an already completed randomized clinical trial to accomplish this goal.

We decided to study the vitamin D-telomere length relationship in the context of colon adenoma disease because the condition is a precursor to colon cancer, and is thus a pivotal time point to examine the effectiveness of a chemopreventive intervention. Our research hypothesis is that 2 g of supplemental calcium and 800 IU of vitamin D_3 administered daily, alone or in combination, will prevent leukocyte telomere length shortening in patients with colorectal adenomas compared to the placebo group in 6 months time.

Methods

Study Design

The study design of the parent study is a pilot, double-blinded, placebo controlled, randomized clinical trial to test the effects of calcium and vitamin D_3, alone and in combination, on markers of CRC risk in normal colorectal mucosa. Our study is a biomarker based adjunct study to the parent study utilizing additional telomere length data measured by qPCR. The outcome of interest is telomere length, measured in base pairs.

Our study makes use of already collected biosample and questionnaire data of the parent study with no additional contact with study participants. All analyses were conducted on the de-identified data and is therefore considered "non-human subject research", exempt from IRB submission (**Appendix 1**). The parent study was approved by the Emory University IRB, all study participants having submitted written informed consent.

Sample size

Study participants were recruited from a patient population of the Emory University Digestive Diseases Clinic. Out of 533 patients who have had at least one pathology-confirmed adenomatous colonic or rectal polyp within the past 36 months, 244 were invited to participate in the study. Of them, 105 entered the placebo run-in period, after which 92 were randomized to study treatment (44) (**Appendix 2**).

The exclusion criteria for recruitment included adherence to the placebo run-in trial (took at least 80% of their pills), medication use, medical conditions, and willingness to provide blood and rectal biopsy samples. The 92 subjects were divided randomly among 4 treatment groups: the placebo group (n=23), the 2.0 g elemental calcium group (n=23), the 800 IU vitamin D_3 group (n=23), and the 2.0g elemental calcium and 800 IU vitamin D_3 group (n=23). Tishcon Corporation manufactured all study tablets (placebo and non-placebo) to be identical in shape, color, size, and appearance. Elemental calcium tablets were comprised of calcium carbonate and vitamin D tablets were comprised of Vitamin D_3. These nutrient forms were chosen due to their relative inability to cause toxicity and their widespread, successful use in past clinical trials.

At baseline and final follow-up visits 6 months apart, questionnaires were administered and blood draws were performed to measure vitamin D and calcium levels, as well as other biomarkers and clinical indicators of interest. We were able to obtain sufficient buffy coat DNA from 62 participants at baseline and 75 participants at follow-up, and leukocyte telomere length data for 46 participants at baseline and 37 participants at follow-up. Only 24 subjects had both baseline and follow-up DNA extracted [Placebo group (n=6), Calcium group (n=4), Vitamin D_3 group (n=5) and Calcium + Vitamin D_3 group (n=9)]. Due to small sample size, the results of this study are considered preliminary and will be used to obtain initial evidence on the effects of vitamin D and calcium supplementation on leukocyte telomere length.

Laboratory test

Plasma 25-OH-vitamin D quantification

Radioimmunoassay method was employed (45,46) by Dr. Bruce Hollis at the Medical University of South Carolina. Blinded laboratory testing was carried out on all baseline and follow-up plasma samples, as well as quality control samples. The average intra-assay coefficient of variation for 25-OH-vitamin D was 2.3%.

DNA Extraction:

DNA extraction from frozen buffy coats was performed using the *Qiagen FelxiGene DNA Kit* according to manufacturer instructions. Isolated DNA was eluted in 200-350 µl of sterile DNA-grade water to achieve consistent DNA concentration. Samples were then divided into separate batches for gel electrophoresis, telomere analysis, and storage. All samples were stored at -80°C.

Gel Electrophoresis:

Up to 12ul of eluded DNA was used for gel electrophoresis. DNA concentration was maintained at 20 ng/ul. Electrophoresis was performed to visualize DNA and gauge quality and size. 95% of all samples showed visible DNA in fluorescence, and gel images depicted clear bands (**Appendix 3**). Numerical DNA concentrations were derived by microblotting 2 ul of eluded DNA onto a Take3 plate (BioTek Epock machine). DNA concentrations varied dramatically, ranging from 2 - 1744 ng/ml.

Preparation of Standards for qPCR

Three cancer cell lines were included in each PCR run as reference. This was done to quantify targeted templates in experimental samples. Relative length was then determined by the standard curve method. H1299 was used as the long telomere standard, Hela used as the short telomere standard, and UMUC3 as the transfection agent. Each PCR run included these three standards, diluted with TE buffer to 26, 8.75, 2.9,0.97,0.324 and 0.108 ng/ml concentrations (21).

Quantitative PCR reactions

PCR reactions were made to a final volume of 25 µl using Invitrogen Platinum qPCR master mix according to manufacturer's recommendations. Approximately 10 µl of DNA sample was used per well, and contained at least 20 ng of DNA (47). All qPCR reactions were processed in a CFX96 real-time system thermal cycler (Bio-Rad Machine) with the following cycling parameters: 1. Experimental samples – initial denaturation at 96°C for 1 s, anneal/extend at 54°C for 60s; followed by 39 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 min, fluorescence data collection, 30 cycles 2.) Single copy genes – initial denaturation at 95°C for 15 s, anneal at 58°C for 1s, extend at 72°C for 20s, 8 cycles; followed by denaturation at 96°C for 1 s, anneal at 58°C for 1s, extend at 72°C for 20s, hold at 83°C for 5s with data collection, 35 cycles (21).

The primer used for telomere PCR was *tel1b* [5'-CGGTTT$(GTTTGG)_5$GTT-3'] at a final concentration of 100 nM and tel2b [5'-GGCTTG$(CCTTAC)_5$CCT-3'] at a final concentration of 900 nM. The primers for single-copy gene PCR were *hbg1* [5'-

GCTTCTGACACAATGTGTTCACTAGC -3’] at a final concentration of 300nM and hbg2 [5’-CACCAACTTCATCCACGTTCACC-3’] at a final concentration of 700 nM.

Statistical Analysis

The primary outcome is telomere length, which was calculated based on the sample and control Cq values from rt-PCR. Leukocyte telomere length was measured at baseline and at 6 months follow-up for each study participant. Fisher's exact test for categorical variables and ANOVA for continuous variables were used to test for differences among treatment groups.

The means and standard deviations for serum 25-OHD level and telomere length were summarized by treatment group at both baseline and follow-up. Absolute differences were calculated by comparing the measures between the two time points. The significances of the differences were tested by Wilcoxon signed rank tests.

To visually examine the change in the leukocyte telomere length by the treatment group, spaghetti plots were used to show the changes in telomere length over time by treatment groups at both individual and group levels. A mixed linear regression model was used to fit the fixed effect and random effect of telomere length outcome with only the treatment group and time, and their interaction, as predictors.

Results

Study participants

Treatment groups did not differ significantly in characteristics measured at baseline (**Table 1**). The mean age of participants was 58 years old, 58.3% were men, 66.7% were Caucasian, 79.2% were college graduates and 25% had a family history of CRC in a first-degree relative. Most participants were nonsmokers, overweight, and were non-users of multivitamins and NSAID medications. What is worth mentioning is that the proportion of those with a family history of CRC and the proportion of those who regularly use aspirin differ quite substantially among the four treatment groups. While the study population that has both LTL measurements is small (n=24), they are not much different compared to all study participants (n=92) at baseline. The average age of participants for both groups is 58 years old. Total dietary intake calories is 1595.91 kcal/day for the study population (n=24), similar to the 1595.81 kcal/day for all study participants (n=92). In addition, calcium and vitamin D intake levels between the n=24 and n=92 groups were 642.74 mg/d and 618.57 mg/d, and 285.37 IU/d and 277 IU/d, respectively (**Table 1 and Appendix 4**).

Treatment groups did not differ significantly in terms of dietary intake (**Table 1**). The mean total energy intake across groups was 1595.99 kcal/d (SD: 493.04) a nd the mean total calcium level across groups was 642.74 mg/d (SD: 374.51). The mean t otal vitamin D intake was 285.37 IU/d (SD: 200.36), with the highest intake in the Calciu

m + Vitamin D_3 group, at 374.98 IU/d (SD: 208.91) and the lowest intake in the Calcium group at 216.43 IU/d (SD: 145.8). However, the difference in daily vitamin D intake acro ss treatment groups was still not statistically significant (p=0.14). In addition, the mean to tal fat intake was 65.84 gm/d (SD: 23.67) while the mean dietary fiber and mean alcohol i ntake were 14.76 gm/d (SD: 7.35) and 10.58 gm/d (SD: 19.13), respectively.

Plasma vitamin D

At baseline, there were no significant differences between the four study groups in plasma 25-OHD (p=0.22). The Calcium + Vitamin D_3 group had the highest 25-OHD concentration at 26.87 ng/ml (SD:16.83), followed by the Calcium group and Vitamin D_3 group at 23.77 ng/ml (SD: 7.51) and 19.19 ng/ml (SD: 9.51), respectively. The lowest mean measure of 25-OHD was in the placebo group, at 17.28 ng/ml (SD: 6.4). The absolute differences in 25-OHD measures between 6-mo follow-up and baseline show increased levels in all groups except the Placebo group where a decrease of 1.1 ng/mL (SD: 5.0) was observed. The largest increase in serum 25-OHD was observed in the Vitamin D_3 group at 11.4 ng/ml (SD: 10.2) (p=0.25). The Calcium group had an increase of 7 ng/ml (SD: 14.0) in serum 25-OHD (p=0.63) and the Calcium + Vitamin D_3 group had a moderate increase of 5.3 ng/ml (SD: 6.7) in serum 25-(OH)D (p=0.16). However, Wilcoxon signed rank tests did not detect any statistical significances for these 25-OHD increases (**Table 2 and Figure 1** bottom). Relative to the placebo group, 25-(OH)D increased by 43%, 75%, and 3% in the calcium, vitamin D, and calcium plus vitamin D groups, respectively.

Telomere Length

At baseline, there were also no significant differences between the four study groups in telomere length (p=0.54). The Vitamin D_3 group had the highest mean in telomere length at 5374.5 bp (SD: 3621.6), followed by the Calcium + Vitamin D_3 group and Placebo group at 5374.5 bp (SD: 3621.6) and 3983.6 bp (SD: 841.4), respectively. The lowest mean measure of telomere length appeared in the placebo group, at 3620.7 bp (SD: 429.8). The absolute differences in telomere length between 6-month follow-up and baseline show increased but varied magnitude in telomere length. Surprisingly, the placebo group had the greatest increase in telomere length at 1529.8 bp (SD: 3078.1). The Vitamin D_3 group and Calcium + Vitamin D_3 group gained moderate increases in telomere lengths at 724 bp (SD: 5283.5) and 1126 bp (SD: 3254.1), respectively. The calcium group had the least increase in telomere length at 91.1 bp (SD: 904.7). However, Wilcoxon signed rank tests did not detect any statistical significances for these telomere length increases (**Table 2 and Figure 1** top). Relative to the placebo group, mean telomere length decreased by 16%, 18%, and 10% in the calcium, vitamin D, and calcium plus vitamin D groups, respectively.

Visual inspection of treatment effects of Ca and VitD on LTL

Next, we visually inspected which treatment intervention confers the greatest telomere length increase. In Figure 2, similar to the results in Table 2, the Placebo group had the largest positive slope in the summary line whereas the Calcium group only had a marginal positive slope in the summary line. These trend lines demonstrate that all

treatment methods led to longer telomere lengths, but the Placebo group experienced the largest gain in telomere length over 6 months (**Figure 2**).

Telomere length at baseline and follow-up of all participants with at least one telomere length measurement

Due to small sample size, we decided to also include participants who have had at least one telomere length measurement either at baseline or at follow-up visit. Assuming that randomization was successful, we would expect that the differences in LTL are due to treatment at the end of follow-up. However, we should interpret the results with caution since the measurements were done on different subjects at baseline and at follow-up. The number of baseline participants is 46 and the number of follow-up participants is 37. At baseline, we observe that the vitamin D_3 group had, on average, the longest telomere length of 5017.02 bp (SE: 508.81). The calcium group had, on average, at the second longest telomere length of 4384.08 bp (SE: 482.70), followed by the placebo group of 3820.02 (SE: 440.64) and the calcium + vitamin D_3 group of 3713.08 (SE: 394.12). At follow-up, the vitamin D_3 group still had, on average, the longest telomere length at 5770.44 (SE: 937.34). The calcium + vitamin D_3 group had the second longest telomere length of 5446.8 (SE: 847.85), followed by the placebo group of 4778.25 (SE: 937.34) and finally the calcium group of 4114.51 bp (SE: 994.19). We can infer from these results that the vitamin D_3 treatment was the most effective, followed by the Calcium + Vitamin D_3 group, placebo group, and the calcium group **(Table 3)**. Overall, these numbers are consistent with the numbers in Table 2 (except for the Calcium +

Vitamin D_3 group), such that the placebo group experienced the highest increase in LTL. Relative to the change in the placebo group, LTL did not change in the calcium group, decreased in the vitamin D_3 group, and increased in the Calcium + Vitamin D_3 group.

Associations of Lifestyle and CRC risk factors with LTL

We then looked for associations of baseline LTL with potential risk factors for CRC. We found that younger participants < 55 years old had longer telomere length (4405.75 bp) compared to older participants ≥55 years old (4001.28 bp). Females had, on average, slightly longer telomere length than men (4191.55 bp compared to 4112.90 bp). We observed that participants who have a family history of CRC had, on average, longer telomere length (4296.09 bp) than those who did not (3587.1 bp). Using BMI WHO-defined categories (normal: 18.50-24.99 kg/m^2; overweight: 25.00-29.99 kg/m^2; obese: ≥30 kg/m^2) participants of normal weight had, on average, longer telomere length (4507.01bp) than those who were overweight/obese (4087.21). Those who have never smoked had longer telomeres (4173.85 bp) than those who had smoked or are currently smoking (3440.55 bp). Those who partook in higher levels of physical activity (more than 5 hours / week), had, on average, longer telomere lengths (4597.08 bp) than those who engaged in lower levels of physical activity (4025.41 bp) **(Table 4)**.

In regards to food /nutrient /drug consumption, longer telomere length was associated with a higher fiber intake, lower intake of calcium, intake of NSAIDS, higher red and processed meat consumption, lower fruits and vegetable intake, and lower alcohol intake. It is worthy to note that longer telomere length with lower calcium consumption was the only statistically significant association observed.

Notable, large proportional differences in telomere length were found in the following risk factor categories: family history of CRC (19.7%), smoking (21.3%), fiber intake (18.4%), calcium intake (19.6%), and fruit and vegetable intake (17.8%). This suggests that these risk factors may have greater effects on telomere length than others (**Table 4**).

Discussion

Initial analysis of 24 participants (that had both baseline and follow-up telomere length data) revealed that all treatment groups experienced increased telomere length at 6 months follow-up, with the placebo group experiencing the greatest increase. This indicated that relative to the placebo, Vitamin D_3 supplementation was not effective in preventing leukocyte telomere shortening. Overall, our preliminary data did not indicate that Vitamin D_3 supplementation will prevent telomere shortening. However, anti-neoplastic effects of vitamin D_3 may be tissue specific, and changes not evident in blood. Including additional participant data (that had at least one telomere length data at either baseline or follow-up) revealed that the Calcium + Vitamin D_3 treatment, relative to the placebo group, could have resulted in longer LTL, but the interpretation of these results is very limited because LTL was not measured on the same subjects before and after supplementation with Calcium + Vitamin D_3. Overall our findings suggest that the calcium and vitamin D intervention, alone and in combination, do not have an effect on telomere length over the 6-month study period.

Our study found that risk factors associated with shorter telomere length include being older than 55 years old, being male, and having a family history of CRC. Modifiable risk factors such as BMI, smoking, NSAID intake, physical activity, fiber

intake, and alcohol consumption coincides with current medical recommendations for better health. Our findings coincide with previously published studies that found pro-agers such as smoking and obesity (24), as well as alcohol consumption (25) are associated with shorter telomere length. However, some discrepancies have been observed such as longer telomere length for those who consume red and processed meats and lower fruits and vegetables. This however, may be explained by over or underestimations when completing questionnaires, and perhaps the fact that all participants were at increased risk for CRC.

Telomere length (as a biomarker) may be the future of health assessment, disease diagnosis and cancer therapy. However, further data are needed to promote its use as a clinical indicator. The results of this pilot study has determined that while a vitamin D and calcium intervention does raise serum 25-(OH)D levels, it does not appear to have an effect on telomere length. However, due to small sample size, this finding may not be conclusive.

Our study has several limitations. First, it is still unclear if qPCR is the most accurate, standardized assay for measuring telomere length. In a blinded study comparing LTL measurement techniques, measurement error (defined by inter-assay CV) from qPCR method was 6.45%, compared to 1.74% from southern blot. A 6% error equates to about a 360 bp change, nearly a 13-year difference (48). Also, pre-analytical conditions such as blood collection method, day-to-day intra-individual variability, and residual PCR inhibitors may account for tremendous variability in qPCR (49). Lastly, insufficient buffy coat (80% of original samples were < 1 ml in volume) resulted in difficulties in acquiring sufficient leukocyte DNA. 19% of our buffy coat samples yielded DNA

concentrations below 14 ng/ml, which could explain why telomere length data was unavailable for many samples. Missing data at either baseline or follow-up resulted in severe reductions in overall sample size, greatly impacting analysis.

Despite these limitations, our study embraces several strengths. To our knowledge, it is one of the first randomized clinical trials to evaluate the effect of vitamin D and calcium supplementation on telomere length. The novel idea to study this relationship in colorectal adenoma patients allows for a more comprehensive understanding of nutrient chemoprevention. The randomization of treatment groups guaranteed equal distribution of confounding variables and eliminated bias introduced from unknown confounders. Lastly, our establishment of a working clinical trial design and laboratory methods can pave the way for future, larger studies.

Future directions

Clearly, more studies on the effect of increased serum 25-(OH)D on telomere length are needed before any nutritional guidelines can be established. The next step would be to create a more standardized biosample collection protocol for future RCTs to ensure sufficient buffy coat collection and precise telomere assessment. It would be interesting to recruit study participants from other cancer risk groups to further establish Vitamin D as a chemopreventive agent; telomere length data used as an ancillary biomarker to determine therapy constructiveness.

References

1. Blackburn, E.H., *Telomere states and cell fates.* Nature, 2000. **408**(6808): p. 53-6.
2. Cassidy, A., et al., *Associations between diet, lifestyle factors, and telomere length in women.* Am J Clin Nutr, 2010. **91**(5): p. 1273-80.
3. Epel, E.S., et al., *The rate of leukocyte telomere shortening predicts mortality from cardiovascular disease in elderly men.* Aging (Albany NY), 2009. **1**(1): p. 81-8.
4. Terry, D.F., et al., *Association of longer telomeres with better health in centenarians.* J Gerontol A Biol Sci Med Sci, 2008. **63**(8): p. 809-12.
5. Ma, H., et al., *Shortened telomere length is associated with increased risk of cancer: a meta-analysis.* PLoS One, 2011. **6**(6): p. e20466.
6. Engelhardt, M., et al., *Telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer.* Clin Cancer Res, 1997. **3**(11): p. 1931-41.
7. Qin, Q., et al., *Telomere length in peripheral blood leukocytes is associated with risk of colorectal cancer in Chinese population.* PLoS One, 2014. **9**(2): p. e88135.
8. Boardman, L.A., et al., *The association of telomere length with colorectal cancer differs by the age of cancer onset.* Clin Transl Gastroenterol, 2014. **5**: p. e52.
9. Weischer, M., et al., *Short telomere length, cancer survival, and cancer risk in 47102 individuals.* J Natl Cancer Inst, 2013. **105**(7): p. 459-68.
10. Wentzensen, I.M., et al., *The association of telomere length and cancer: a meta-analysis.* Cancer Epidemiol Biomarkers Prev, 2011. **20**(6): p. 1238-50.
11. Pooley, K.A., et al., *Telomere length in prospective and retrospective cancer case-control studies.* Cancer Res, 2010. **70**(8): p. 3170-6.
12. Lee, I.M., et al., *Mean leukocyte telomere length and risk of incident colorectal*

carcinoma in women: a prospective, nested case-control study. Clin Chem Lab Med, 2010. **48**(2): p. 259-62.

13. Zee, R.Y., et al., *Mean telomere length and risk of incident colorectal carcinoma: a prospective, nested case-control approach.* Cancer Epidemiol Biomarkers Prev, 2009. **18**(8): p. 2280-2.
14. Gilley, D., et al., *Factors impacting human telomere homeostasis and age-related disease.* Mech Ageing Dev, 2008. **129**(1-2): p. 27-34.
15. Cui, Y., et al., *Association of leukocyte telomere length with colorectal cancer risk: nested case-control findings from the Shanghai Women's Health Study.* Cancer Epidemiol Biomarkers Prev, 2012. **21**(10): p. 1807-13.
16. Cheng, A.J., et al., *Possible role of telomerase activation in the cancer predisposition of patients with hereditary nonpolyposis colorectal cancers.* J Natl Cancer Inst, 1998. **90**(4): p. 316-21.
17. Monaghan, P. and M.F. Haussmann, *Do telomere dynamics link lifestyle and lifespan?* Trends Ecol Evol, 2006. **21**(1): p. 47-53.
18. Ornish, D., et al., *Effect of comprehensive lifestyle changes on telomerase activity and telomere length in men with biopsy-proven low-risk prostate cancer: 5-year follow-up of a descriptive pilot study.* Lancet Oncol, 2013. **14**(11): p. 1112-20.
19. Babizhayev, M.A. and Y.E. Yegorov, *Smoking and health: association between telomere length and factors impacting on human disease, quality of life and life span in a large population-based cohort under the effect of smoking duration.* Fundam Clin Pharmacol, 2011. **25**(4): p. 425-42.
20. Biegler, K.A., et al., *Longitudinal change in telomere length and the chronic stress response in a randomized pilot biobehavioral clinical study: implications for cancer prevention.* Cancer Prev Res (Phila), 2012. **5**(10): p. 1173-82.
21. Kiecolt-Glaser, J.K., et al., *Omega-3 fatty acids, oxidative stress, and leukocyte telomere*

length: A randomized controlled trial. Brain Behav Immun, 2013. **28**: p. 16-24.

22. Garcia-Calzon, S., et al., *Longitudinal association of telomere length and obesity indices in an intervention study with a Mediterranean diet: the PREDIMED-NAVARRA trial.* Int J Obes (Lond), 2014. **38**(2): p. 177-82.
23. O'Donovan, A., et al., *Pessimism correlates with leukocyte telomere shortness and elevated interleukin-6 in post-menopausal women.* Brain Behav Immun, 2009. **23**(4): p. 446-9.
24. Valdes, A.M., et al., *Obesity, cigarette smoking, and telomere length in women.* Lancet, 2005. **366**(9486): p. 662-4.
25. Strandberg, T.E., et al., *Association between alcohol consumption in healthy midlife and telomere length in older men. The Helsinki Businessmen Study.* Eur J Epidemiol, 2012. **27**(10): p. 815-22.
26. Bischoff-Ferrari, H.A., *Optimal serum 25-hydroxyvitamin D levels for multiple health outcomes.* Adv Exp Med Biol, 2008. **624**: p. 55-71.
27. Forrest, K.Y. and W.L. Stuhldreher, *Prevalence and correlates of vitamin D deficiency in US adults.* Nutr Res, 2011. **31**(1): p. 48-54.
28. Atkinson, S.A., *[The new dietary reference intakes from the Institute of Medicine for calcium and vitamin D].* Perspect Infirm, 2011. **8**(5): p. 5.
29. Newmark, H.L. and R.P. Heaney, *Calcium, vitamin D, and risk reduction of colorectal cancer.* Nutr Cancer, 2006. **56**(1): p. 1-2.
30. Sun, Z., et al., *Calcium and vitamin D and risk of colorectal cancer: results from a large population-based case-control study in Newfoundland and Labrador and Ontario.* Can J Public Health, 2011. **102**(5): p. 382-9.
31. Zhang, X. and E. Giovannucci, *Calcium, vitamin D and colorectal cancer chemoprevention.* Best Pract Res Clin Gastroenterol, 2011. **25**(4-5): p. 485-94.
32. Baron, J.A., et al., *Calcium supplements for the prevention of colorectal adenomas.*

Calcium Polyp Prevention Study Group. N Engl J Med, 1999. **340**(2): p. 101-7.

33. Grau, M.V., et al., *Vitamin D, calcium supplementation, and colorectal adenomas: results of a randomized trial.* J Natl Cancer Inst, 2003. **95**(23): p. 1765-71.
34. Lamprecht, S.A. and M. Lipkin, *Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms.* Nat Rev Cancer, 2003. **3**(8): p. 601-14.
35. Garland, C.F. and F.C. Garland, *Do sunlight and vitamin D reduce the likelihood of colon cancer?* Int J Epidemiol, 2006. **35**(2): p. 217-20.
36. Grant, W.B., *Ecologic studies of solar UV-B radiation and cancer mortality rates.* Recent Results Cancer Res, 2003. **164**: p. 371-7.
37. Fiscella, K., et al., *Racial disparity in death from colorectal cancer: does vitamin D deficiency contribute?* Cancer, 2011. **117**(5): p. 1061-9.
38. Touvier, M., et al., *Meta-analyses of vitamin D intake, 25-hydroxyvitamin D status, vitamin D receptor polymorphisms, and colorectal cancer risk.* Cancer Epidemiol Biomarkers Prev, 2011. **20**(5): p. 1003-16.
39. Wei, M.Y., et al., *Vitamin D and prevention of colorectal adenoma: a meta-analysis.* Cancer Epidemiol Biomarkers Prev, 2008. **17**(11): p. 2958-69.
40. Yin, L., et al., *Meta-analysis: longitudinal studies of serum vitamin D and colorectal cancer risk.* Aliment Pharmacol Ther, 2009. **30**(2): p. 113-25.
41. Welsh, J., *Vitamin D and cancer: integration of cellular biology, molecular mechanisms and animal models.* Scand J Clin Lab Invest Suppl, 2012. **243**: p. 103-11.
42. Richards, J.B., et al., *Higher serum vitamin D concentrations are associated with longer leukocyte telomere length in women.* Am J Clin Nutr, 2007. **86**(5): p. 1420-5.
43. Liu, J.J., et al., *Plasma vitamin D biomarkers and leukocyte telomere length.* Am J Epidemiol, 2013. **177**(12): p. 1411-7.
44. Fedirko, V., et al., *Effects of vitamin D and calcium supplementation on markers of apoptosis in normal colon mucosa: a randomized, double-blind, placebo-controlled*

clinical trial. Cancer Prev Res (Phila), 2009. **2**(3): p. 213-23.

45. Hollis BW. Quantitation of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D by radioimmunoassay using radioiodinated tracers. Methods Enzymol. 1997;282:174–86.
46. Hollis BW, Kamerud JQ, Kurkowski A, Beaulieu J, Napoli JL. Quantification of circulating 1,25-dihydroxyvitamin D by radioimmunoassay with 125I-labeled tracer. Clin Chem. 1996;42(4):586–92.
47. Cawthon, R.M., *Telomere length measurement by a novel monochrome multiplex quantitative PCR method.* Nucleic Acids Res, 2009. **37**(3): p. e21.
48. Aviv, A., et al., *Impartial comparative analysis of measurement of leukocyte telomere length/DNA content by Southern blots and qPCR.* Nucleic Acids Res, 2011. **39**(20): p. e134.
49. Zanet, D.L., et al., *Blood and dried blood spot telomere length measurement by qPCR: assay considerations.* PLoS One, 2013. **8**(2): p. e57787.

Tables and Figures

Table 1. Baseline Demographics and Clinical Measurements of Study Population

Characteristics	Treatment Group					
	All Groups Mean (SD) | N (%)	Placebo Mean (SD) | N (%)	Calcium Mean (SD) | N (%)	Vitamin D_3 Mean (SD) | N (%)	Calcium + Vit. D_3 Mean (SD) | N (%)	P-value [b]
	(n=24)	(n = 6)	(n = 4)	(n = 5)	(n = 9)	
Demographics, medical history, habits, anthropometrics						
Age, years	58.17 (7.61)	55.5 (7.12)	58 (9.38)	59.2 (5.76)	59.44 (8.8)	0.80
Men (%)	14 (0.58)	2 (0.33)	2 (0.5)	3 (0.6)	7 (0.78)	0.40
White (%)	16 (0.67)	4 (0.67)	4 (1)	3 (0.6)	5 (0.56)	0.50
College graduate (%)	19 (0.79)	5 (0.83)	4 (1)	3 (0.6)	7 (0.78)	0.71
Family history of colorectal cancer (%)	6 (0.25)	2 (0.33)	3 (0.75)	1 (0.2)	0 (0)	0.02
Take NSAID [c] regularly [d] (%)	4 (0.17)	3 (0.5)	0 (0)	0 (0)	1 (0.11)	0.12
Take aspirin regularly (%)	9 (0.38)	0 (0)	2 (0.5)	1 (0.2)	6 (0.67)	0.05
Current smoker (%)	1 (0.04)	0 (0)	1 (0.25)	0 (0)	0 (0)	0.17
Take multivitamin (%)	8 (0.33)	1 (0.17)	1 (0.25)	1 (0.2)	5 (0.56)	0.45
BMI, kg/m^2 [e]	32.04 (6.84)	32.57 (7.81)	26.2 (3.07)	33.68 (6.1)	33.38 (7.3)	0.48
Mean dietary intakes [f]						
Total energy intake, kcal/d	1595.99 (493.04)	1468.13 (491.36)	1754.27 (594.69)	1469.2 (666.34)	1681.31 (387.73)	0.58
Total [g] calcium, mg/d	642.74 (374.51)	605.1 (464.66)	770.36 (250.82)	740.45 (597.17)	556.84 (207.19)	0.72
Total [g] vitamin D, IU/d	285.37 (200.36)	227.31 (163.81)	216.43 (145.8)	248.88 (253.98)	374.98 (208.91)	0.14
Total fat, g/d	65.84 (23.67)	61.68 (23.73)	75.24 (33.11)	49.81 (22.39)	73.35 (17.78)	0.58
Dietary fiber, g/d	14.76 (7.35)	14.77 (8.84)	14.69 (5.23)	18.29 (11.86)	12.83 (3.72)	0.70
Alcohol intake, g/d	10.58 (19.13)	3.86 (4.94)	16.05 (19.29)	9.19 (14.99)	13.41 (26.94)	0.47
Total serum vitamin D						
25-OH-vitamin D, ng/mL	22.73 (12.4)	17.28 (6.4)	23.77 (7.51)	19.19 (9.51)	26.87 (16.83)	0.22

[a] Data are given as means (SD) unless otherwise specified.
[b] By Fisher's exact test for categorical variables, and ANOVA for continuous variables.
[c] Nonsteroidal anti-inflammatory drug.
[d] At least once a week.
[e] Body mass index
[f] All nutrients energy adjusted using residual method
[g] Diet plus supplements.

Table 2. Plasma 25-OH-vitamin D and Telomere Length at Baseline and 6-mo Follow-up by Treatment Groups

	Baseline				6-mo follow-up				Absolute difference				Abs. Treatment Effect [§]	Rel. Effect [¶]
	n	Mean	SD	P-value [*]	n	Mean	SD	P-value *	n	Mean	SD	P-value [#]		
25-OH-vitamin D (ng/mL)														
Placebo	6	17.3	6.4		6	15.7	6.4		6	-1.1	5.0	0.81	-	-
Calcium	4	23.8	7.5	0.22	4	30.8	21.4	0.11	4	7.0	14.0	0.63	8.1	1.43
Vitamin D3	5	19.2	9.5		5	30.6	5.1		5	11.4	10.2	0.25	12.5	1.75
Calcium + Vit. D3	9	26.9	16.8		9	25.1	6.2		9	5.3	6.7	0.16	6.4	1.03
Telomere Length (bp)														
Placebo	6	3983.6	841.4		6	5513.4	2727.2		6	1529.8	3078.1	0.44	-	-
Calcium	4	3620.7	429.8	0.54	4	3711.8	681.5	0.66	4	91.1	904.7	0.63	-1438.7	0.74
Vitamin D3	5	5374.5	3621.6		5	6098.5	3598.4		5	724.0	5283.5	0.63	-805.8	0.82
Calcium + Vit. D3	9	3933.5	754.9		9	5059.5	3118.9		9	1126.0	3254.1	0.43	-403.8	0.90

[*] p-values were calculated by ANOVA F-tests

[#] Wilcoxon Signed rank test p-values are calculated

[§] Absolute treatment effect was calculated by the formula: ΔLTL in treatment group - ΔLTL in placebo group

[¶] Relative effect was calculated by the formula: [(Treatment LTL at follow-up) / (Treatment LTL at Baseline)] / [(Placebo LTL at follow-up) / (Placebo LTL at Baseline)]

Table 3. Telomere Length at Baseline and Follow-up by Treatment Groups for Participants who had at least one Telomere Length measurement

Characteristic	Baseline (N=46)			6-Month Follow-up (N=37)		
	n	Telomere Length (SE)	P*	n	Telomere Length (SE)	P
Treatment						
Placebo	12	3820.02 (440.64)	0.19	9	4778.25 (937.34)	0.62
Calcium	10	4384.08 (482.70)		8	4114.51 (994.19)	
Vitamin D3	9	5017.02 (508.81)		9	5770.44 (937.34)	
Calcium + Vitamin D3	15	3713.08 (394.12)		11	5446.8 (847.85)	
% Difference: Calcium vs Placebo[§]		15%			-14%	
% Difference: Vitamin D3 vs Placebo		31%			21%	
% Difference: Calcium + Vitamin D3 vs Placebo		-3%			14%	

[*] Based on the F-test for significance of generalized linear model

[§] % Difference is calculated by the formula (measure B - measure A) / measure A × 100%

Table 4. Association of telomere length with potential risk factors of colorectal cancer at baseline

Characteristic	Baseline (N=46)		
	n	Telomere Length (SE)	P*
Age			
Younger Age (<55 YO)	16	4405.75 (391.01)	0.41
Older Age (≥55 YO)	30	4001.28 (285.55)	
% Difference		-9%	
Sex			
Male	29	4112.90 (292.64)	0.87
Female	17	4191.55 (382.21)	
% Difference		2%	
Family history of colorectal cancer			
Yes	10	3587.10 (489.44)	0.21
No	36	4296.09 (257.96)	
% Difference		20%	
BMI¶			
Normal	6	4507.01 (640.84)	0.54
Overweight/Obese	40	4087.21 (248.19)	
% Difference		-9%	
Smoking			
Past or current	2	3440.55 (1109.41)	0.52
Never	44	4173.85 (236.52)	
% Difference		21%	
NSAID intake£			
Yes	7	4284.20 (595.36)	0.80
No	39	4116.43 (252.23)	
% Difference		-4%	
Physical activity #			
Lower	16	4025.41 (432.11)	0.34
Higher	19	4597.08 (396.53)	
% Difference		14%	
Fiber intake			
Lower	24	4553.77 (312.29)	0.07
Higher	21	3712.54 (333.85)	
% Difference		-18%	
Calcium intake			
Lower	24	4581.51 (310.48)	0.05
Higher	21	3680.83 (331.92)	
% Difference		-20%	
Red and processed meat intake			
Lower	22	3996.34 (336.94)	0.50
Higher	23	4318.88 (329.54)	

% Difference		8%	
Fruits and vegetable intake			
Lower	24	4539.6 (313.16)	0.08
Higher	21	3728.73 (334.78)	
% Difference		-18%	
Alcohol intake			
Lower	22	4365.31 (335.97)	0.40
Higher	23	3965.95 (328.58)	
% Difference		-9%	

[*] Based on the F-test for significance of generalized linear model

[§] % Difference is calculated by the formula (measure B - measure A) / measure A × 100%

[¶] No patient with underweight status in the study cohort, so only two BMI categories are listed

[£] Not including Aspirin

[#] Lower and Higher levels of variables Physical activity, fiber intake, calcium intake, red and processed meat intake, fruits and vegetable intake and alcohol intake are classified by the median

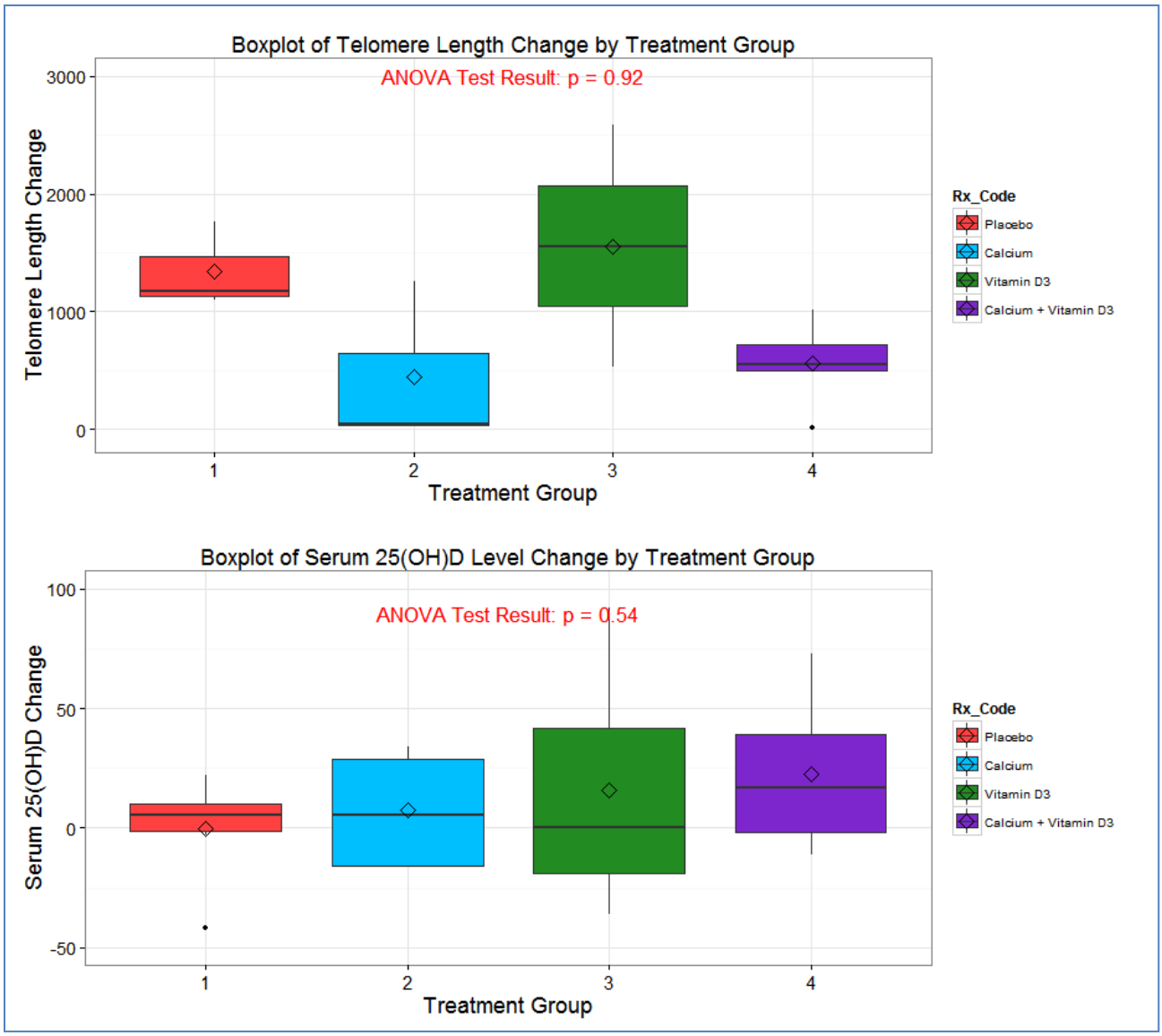


Figure 1. Boxplot of outcome measures by treatment groups. In our defined population, the telomere length change and serum 25(OH)D change are calculated by the measures at baseline and 6 month follow-up**.** The distributions of the outcome changes are represented by boxplots color-coded for treatment groups. ANOVA analyses are carried out to compare the statistical differences in Telomere length change (upper) and serum 25(OH)D change (bottom).

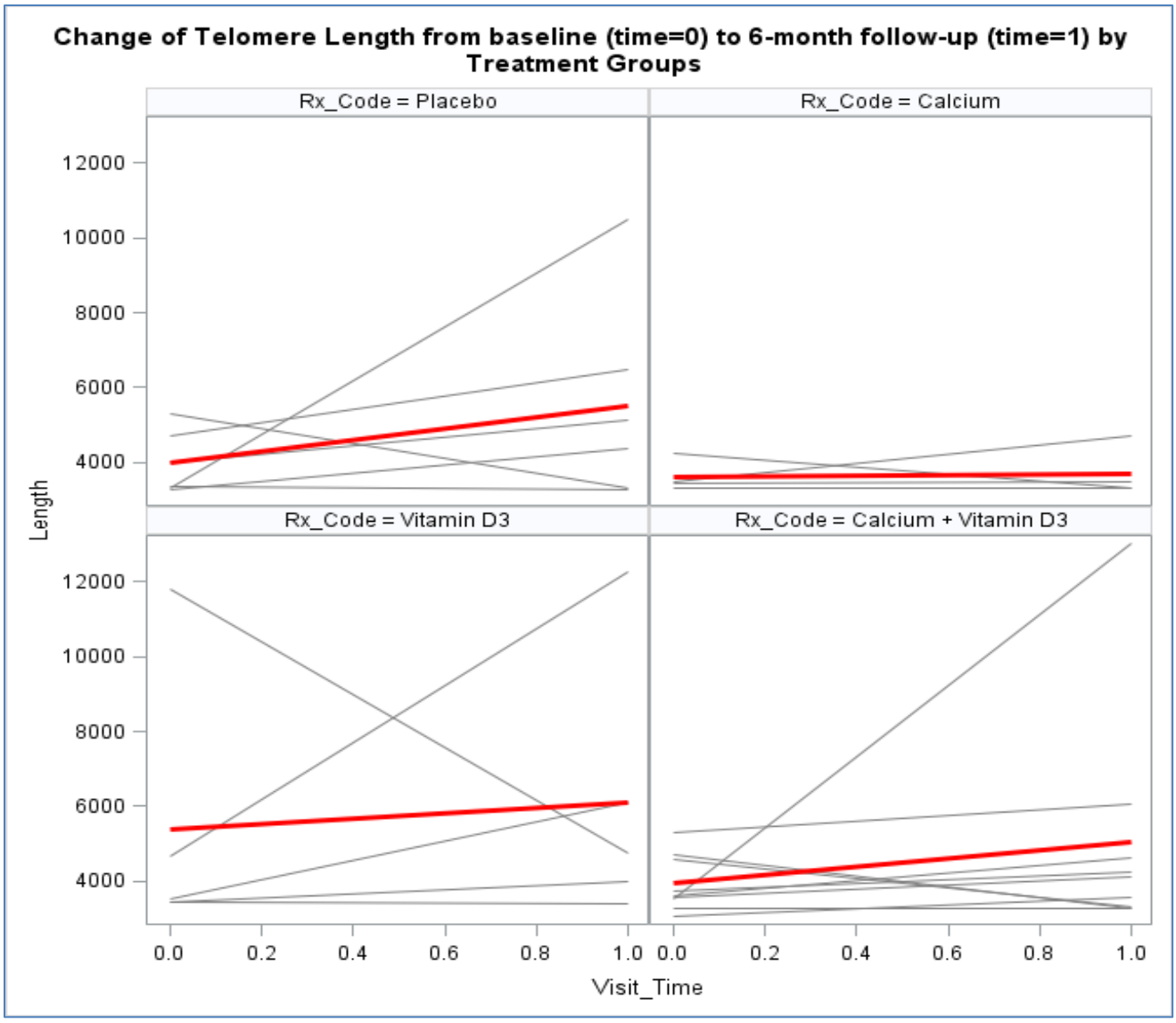


Figure 2. Panel Plot of Telomere length change over 6-month follow-up by Treatment Groups. Patients were divided into four treatment groups. Telomere length was measured at baseline and 6 month follow-up. Each patient's telomere length change is represented by a grey line and the trend of in-group telomere length change is summarized by a red line.

Appendices

DATE: April 16, 2014

RE: **Determination:** No IRB Review Required
Project Topic: *Secondary Analysis of Baseline Associations between Telomere Length and Vitamin D Levels*
Investigator: Evelyn Wang

Dear Ms. Wang:

Thank you for requesting a determination from our office about the above-referenced project. Based on our review of the information you provided, we have confirmed that your project did not require IRB review because it did not meet the definition of involving "human subjects" as set forth in Emory Policies and Procedures or federal regulations (45 CFR 46.102). In particular, this project aimed to use a dataset from a previous 2009 study to perform a secondary analysis of baseline associations between telomere length and vitamin D levels. In order to investigate this aim, you used solely a deidentified data set through which you had no access or links to identifiable information. You had no interaction or intervention with individuals, and no identifiable information was viewed or retained.

Please note that this determination does not affect the ability to publish the results. If you have questions about this issue, please contact the IRB.

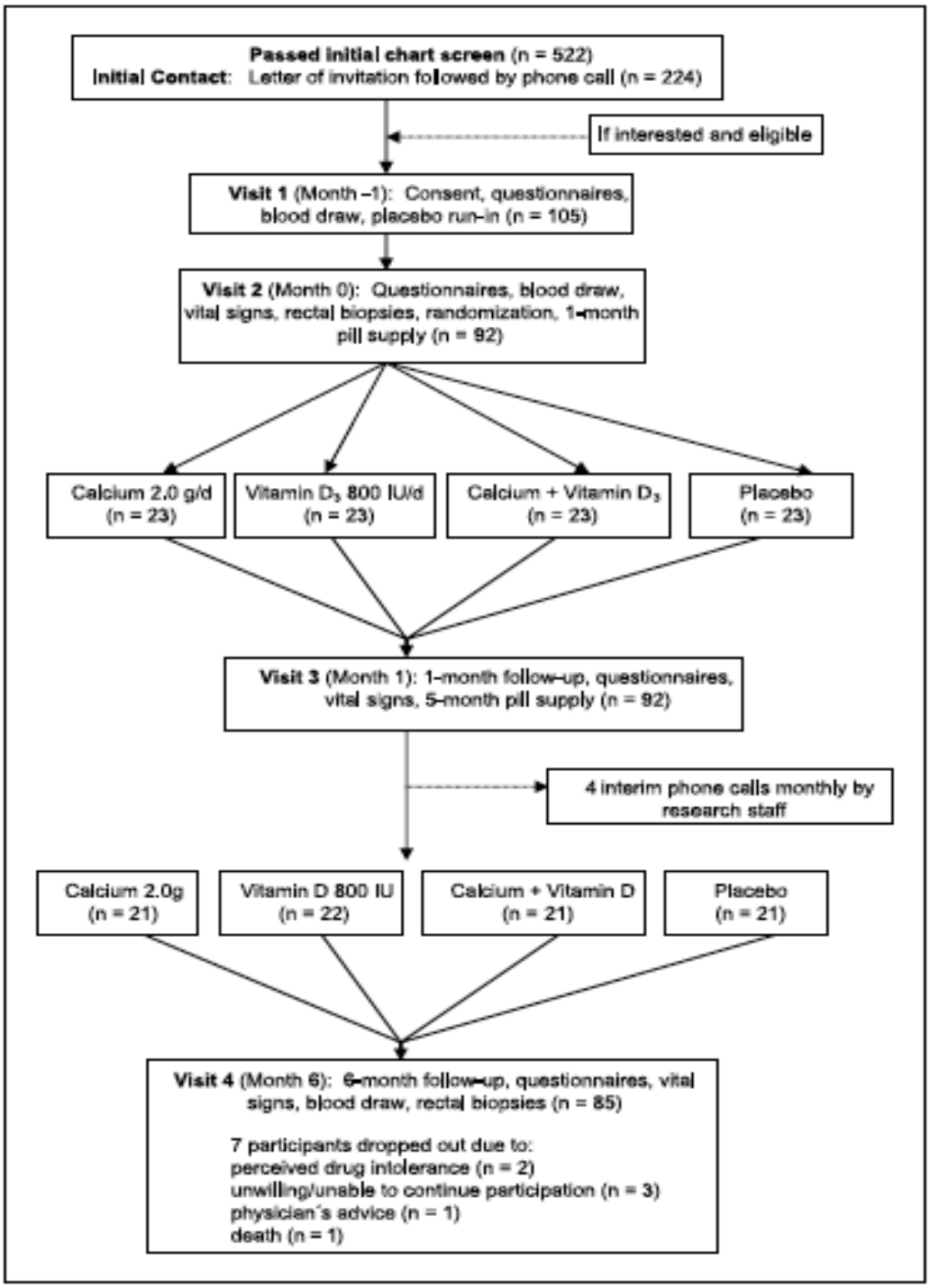
This determination could be affected by substantive changes in the study design, subject populations, or identifiability of data. If the project changes in any substantive way, please contact our office for clarification.

Thank you for consulting the IRB.

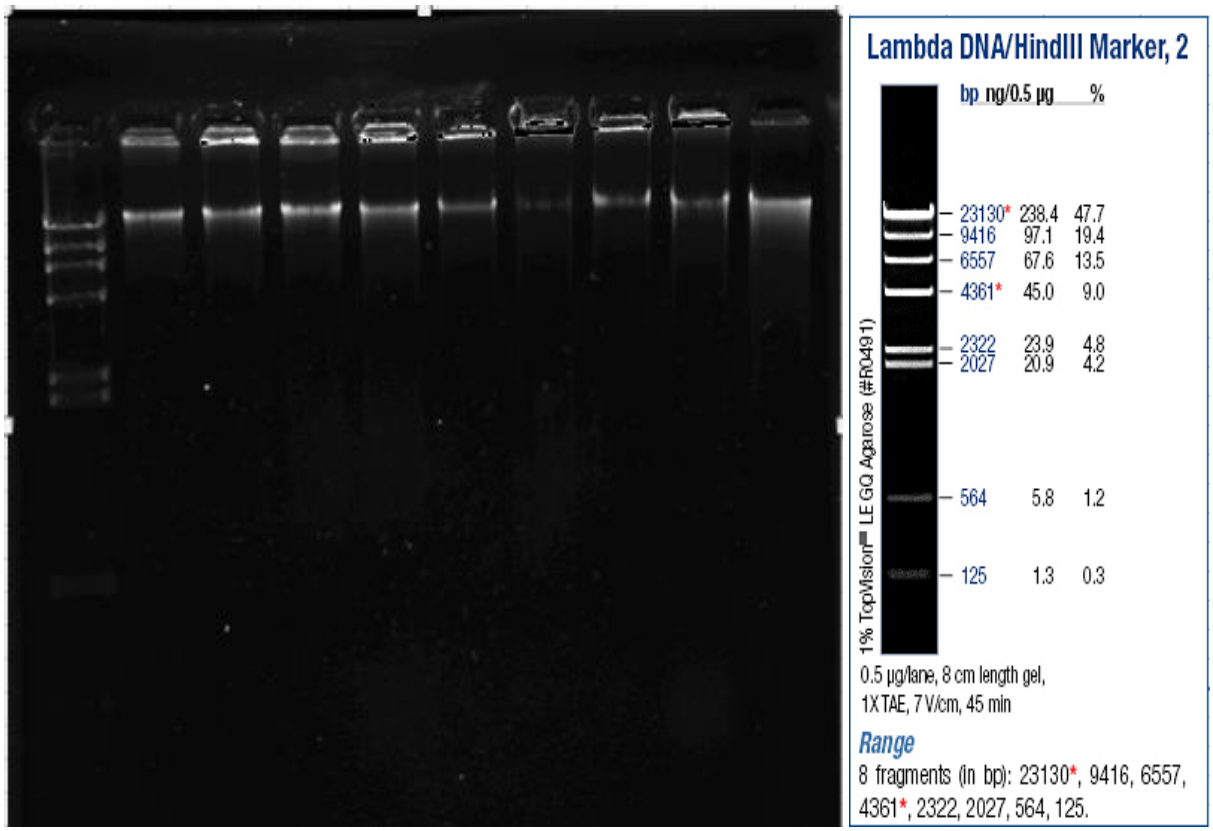
Sincerely,

Kevin Wack, MA, MTS, CIP
Education and Quality Assurance
Emory University Institutional Review Board
1599 Clifton Rd, Atlanta, GA 30322

Appendix 1. IRB waiver letter indicating that no review was required for the thesis study titled: Effects of Vitamin D and Calcium Supplementation on Leukocyte Telomere Length in Colorectal Adenoma Patients: A Randomized Clinical Trial



Appendix 2. Flow Diagram of a Trial of Supplemental Calcium and Vitamin D_3, Alone and in Combination vs. Placebo over Six Months on Markers of Apoptosis in the Normal Colorectal Mucosa.



Appendix 3. Argarose gel electrophoresis visualized with *GelRed* nucleic acid stain under fluorescence. Hindll digest of lambda DNA used as a molecular weight standard to approximate DNA mass.

Appendix 4. Baseline characteristics of all study participants (n=92) who participated in the parent study

Characteristics	Treatment Group				
	Placebo (n = 23)	**Calcium** (n = 23)	**Vitamin D_3** (n = 23)	**Calcium + Vit. D_3** (n = 23)	**P-value** [b]
Demographics, medical history, habits, anthropometrics					
Age, years	58.52 (8.16)	61.91 (8.15)	60.22 (8.07)	62.09 (7.5)	0.39
Men (%)	70	70	70	70	1
White (%)	74	83	65	61	0.39
College graduate (%)	65	61	57	44	0.53
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.6
Take NSAID [c] regularly [d] (%)	22	13	9	22	0.6
Take aspirin regularly (%)	22	52	30	56	0.05
If woman (n = 28), taking estrogens (%)	4	9	4	4	1
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Body mass index (BMI), kg/m^2	30.61 (7.22)	29.35 (5.50)	28.86 (5.56)	31.55 (5.97)	0.44
Mean dietary intakes [e]					
Total energy intake, kcal/d	1595.81 (527.69)	1787.92 (691.02)	1848.11 (820.74)	1844.71 (751.86)	0.59
Total [f] calcium, mg/d	618.57 (307.86)	745.78 (334.88)	843.12 (525.79)	823.57 (713.85)	0.41
Total [f] vitamin D, IU/d	277.44 (229.93)	335.79 (202.15)	360.47 (317.08)	414.92 (315.50)	0.4
Total fat, gm/d	66.80 (32.22)	72.31 (34.88)	69.83 (31.91)	73.76 (27.68)	0.59
Dietary fiber, gm/d	14.77 (7.24)	17.37 (8.82)	17.53 (9.11)	17.15 (10.61)	0.97
Alcohol intake, gm/d	8.57 (14.34)	10.94 (15.11)	13.83 (18.36)	10.16 (19.61)	0.84
Total serum vitamin D					
1,25-(OH)2vitamin D (pg/mL)	39.23 (7.63)	45.41 (35.34)	44.51 (21.66)	37.91 (12.58)	0.6
25-OH-vitamin D, ng/mL	20.42 (7.61)	25.7 (7.67)	20.99 (8.32)	20.94 (9.67)	0.12

[a] Data are given as means (SD) unless otherwise specified.

[b] By Fisher's exact test for categorical variables, and ANOVA for continuous variables.

[c] Nonsteroidal anti-inflammatory drug.

[d] At least once a week.

[e] All nutrients energy adjusted using residual method

[f] Diet plus supplements.