1.a. Full Title: Vitamin D, parathyroid hormone (PTH) and fibroblast growing factor (FGF) 23 in relation to colorectal cancer risk and mortality in the Atherosclerosis Risk in Communities Study

b. Abbreviated Title (Length 26 characters): Vitamin D and CRC

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I, the first author, confirm that all the coauthors have given their approval for this manuscript proposal. _AP_ [please confirm with your initials electronically or in writing]

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3. Timeline:
Visit 2 serum samples are currently being processed and anticipated to be finished by spring 2013. Analyses will begin after the ARIC Committee approves the proposal and all vitamin D measures are ready in spring-summer 2013.
4 Rationale:
Vitamin D is a fat-soluble hormone precursor obtained through cutaneous synthesis from sun exposure (vitamin D$_3$ (cholecalciferol)) and through oral intake from food and supplement sources (vitamin D$_2$ (ergocalciferol)) [1, 2] (for a more detail description of Vitamin D measures and their functions, please, see Appendix 1 and Figure 1 in the end of the proposal).

High levels of circulating vitamin D have been shown to reduce colorectal cancer risk [1]. It may promote differentiation and apoptosis and suppress cell proliferation and angiogenesis, alter the expression of genes that regulate inflammation, cell death and cell proliferation, and interfere with the growth-promoting actions of IGF-1 and other growth factors [3-5].

The first epidemiological evidence that circulating vitamin D is inversely associated with colorectal cancer (CRC) was reported in 1980 using ecologic data [6, 7]. Since that time, many observational studies have investigated an association of CRC risk or adenoma with various measures of Vitamin D status – circulating 25(OH) vitamin D levels [1, 6, 8-10], dietary intake [11, 12], and sun exposure estimates [13], or two latter measures combined. Most of these observational studies showed an inverse association between better vitamin D status and the CRC risk or CRC adenoma [1, 2, 6, 13-16]. The meta-analyses concluded that serum 25-(OH)D levels >80 nmol/l, compared with <30nmol/l were associated with 50% lower CRC risk [17]. However, two interventional studies reported inconsistent results: a large trial of 36,282 patients didn’t find any association (200 IU vitamin D$_3$ each day for 7 years) [18], whereas a small trial of 1179 post-menopausal women showed a protective role of 1100 IU vitamin D$_3$ intake in CRC prevention [19]. The unadjusted relative risks (RR) of incident CRC in the Ca + D and Ca-only groups were 0.402 (P = 0.01) and 0.532 (P = 0.06), respectively. When analysis was confined to cancers diagnosed after one year, RR for the Ca + D group fell to 0.232 (95%CI: 0.09-0.60) but did not change significantly for the Ca-only group. However, there is a concern that these findings could be spurious due to a small sample size and a failure of randomization [20].

Several studies also reported that vitamin D may be protective for CRC progression and mortality but this has not been clearly established [21-23].

Our understanding of the role of vitamin D in health and disease is developing. Recently it has been shown that in adult populations the epimer of vitamin D$_3$ (epi-D$_3$ isomer) constitutes 4-27% of the total 25(OH)D$_3$ [24-26] (discussed in the Appendix in more detail). Accounting for the epi-D$_3$ isomer will help to assess a 25(OH)D-CRC association more accurately.

The ARIC study presents a unique opportunity to further study an association between vitamin D and CRC risk and mortality since many biomarkers related to vitamin D status – 25(OH)D$_3$, 25(OH)D$_2$, epi-D3 isomer, PTH, and FGF23 (highlighted in the Figure 1 and discussed in Appendix 1) are being measured in stored serum from Visit 2 (1990-1992) in the whole ARIC cohort (n = 13,753).
The epidemiological data about PTH and FGF23 in relation to CRC are very limited. To our knowledge only one epidemiological study, nested case-control study of 1214 cases and 1214 controls within the EPIC cohort, has been conducted [27]. It reported that high levels of serum PTH (≥65 ng/L) compared with medium PTH levels of 30–65 ng/L were associated with increased CRC risk: RR = 1.41, (95% CI: 1.03–1.93) at all levels of vitamin D [27]. Of note, an inverse association of vitamin D and CRC risk was observed only when PTH was <65 ng/L.

Furthermore, the first epidemiological study examining circulating FGF-23 and the risk of metachronous colorectal adenoma has been conducted in 2011 [28]. This case-control study of 50 patients and 50 controls showed that compared to the lowest tertile of FGF-23, the adjusted odds ratios (95% CIs) for the second and third tertiles were 2.80 (0.94–8.31) and 3.41 (1.09–10.67), respectively (P-trend=0.03) [28]. In a linear regression model, there was also a statistically significant inverse relationship between FGF-23 and 1,25(OH)2D (β-coefficient=−1.2; P=0.001) but no statistically significant trend between FGF-23 and 25(OH)D concentrations (β-coefficient=0.55; P=0.10). Hence, FGF-23 activity may be mediated via 1,25(OH)2D or may act independent from the vitamin D pathway for instance through body size [28].

To the best of our knowledge, no study to date accounted for the epi-D3 isomer in relation to CRC risk or examined an association between FGF23 and CRC risk or potential confounding effect of FGF23 on the Vitamin D–CRC risk association.

**The goal of the current study** is to confirm an inverse association between vitamin D and CRC risk in the multiethnic ARIC cohort and refine this association by separately examining associations of CRC with each biomarker individually and their combination after careful adjustment for confounders. The greatest strength of the ARIC study is the availability of simultaneous measurements of different vitamin D measures, PTH, and FGF23. In addition, we will examine all the associations in relation to CRC-specific mortality in the same population.

**5. Main Hypothesis/Study Questions:**

- Examine association of 25(OH)D and its components with CRC risk and CRC-specific mortality:
  a) Examine separately associations of CRC risk and mortality with 25(OH)D2, 25(OH)D3, and 25(OH)D and as their sum;
  b) Examine association of CRC risk and mortality with epi-D3 isomer and 25(OH)D levels after subtraction of epi-D3 isomer levels;
  c) Examine separately associations of CRC risk with the sum of 25(OH)D2, 25(OH)D3, and epi-D3 to compare with previous studies.

- Assess associations of PTH and FGF23 with CRC risk and mortality:
  a) Explore whether PTH–CRC association is independent of 25(OH)D and the epi-D3 isomer;
  b) Evaluate whether relations of Vitamin D and FGF23 with CRC are mediated by PTH;
Currently, incident cancer cases have been ascertained until 2006 in the ARIC cohort. An U01 has been already funded to ascertain cancer cases beyond 2006. In a future study we are planning to examine SNPs in genes associated with vitamin D biosynthesis (CYP2R1, CYP27A1, CYP27B1, CYP24A1), transport (vitamin D–binding protein (DBP)), activity (vitamin D receptor (VDR), calcium-sensing receptor (CASR)) and catabolism (CYP24A1) in relation to CRC risk in the expanded cohort. Also, when the cancer registry re-linkage is complete, we will examine survival of colorectal cancer patients in relation to vitamin D levels.

6. Design and analysis (study design, inclusion/exclusion, outcome and other variables of interest with specific reference to the time of their collection, summary of data analysis, and any anticipated methodologic limitations or challenges if present).

**Study design:** Prospective cohort of all ARIC participants without prevalent cancer at Visit 2.

**Independent variables:** Serum 25(OH)D$_2$, 25(OH)D$_3$, epi-D$_3$ isomer, PTH and FGF23 from Visit 2, calcium from diet at Visit 1.

**Dependent Variable:** Colorectal cancer incidence (~300 cases) and CRC-specific mortality (~100 cases) through 2006.

**Other variables of interest:** age, race, sex, study site, season of blood draw, education, BMI, WHR, smoking status and number of pack-years of smoking, alcohol consumption, physical activity, diabetes, use of post-menopausal hormones (current/former/never use) for women, aspirin use.

**Analysis plan:** We will use a proportional hazard model to estimate the multivariate adjusted risk of CRC risk and mortality (separately) in relation to all markers at Visit 2. Participants began contributing time at risk at the second visit (1990-1992) through 12/31/2006. The proportional hazards assumption will be tested by graphing the log(-log(survival)) versus log(time). All serum biomarkers will be modeled as quartiles and as continuous variables. In addition, 25(OH)D will be categorized according to existing 25(OH)D cut-points (i.e. <10, 10-29, and ≥30 ng/mL). Cubic splines will be used to select the most appropriate representation of other biomarkers. Mediation will be evaluated through both mutual adjustment and, bi-directionally, through the residual method. Interaction with BMI and WHR, race, sex, dietary calcium and hormone replacement therapy (for women) will be assessed by including an interaction term into the model. In addition, we will examine associations of all the biomarkers with colon cancer risk. We will not have power to separately examine rectal cancer.

The following models will be used:
Model 1: adjusted for age, gender, race, ARIC study site center (proxy for latitude) and season of blood draw.
Model 2: Model 1 additionally adjusted for education, BMI, WHR, smoking status, number of pack-years of smoking, alcohol consumption, physical activity, diabetes, dietary calcium, use of post-menopausal hormones (current/former/never use) for women, and diet/calorie intake.

Of note, since serum calcium levels are tightly regulated in a narrow range [29], they are unlikely to be associated with CRC risk or modify vitamin D–CRC risk association. However, one case-control study reported decreased levels of serum calcium corrected for albumin in CRC patients compared to healthy controls [30]. Thus, in an exploratory analysis we will investigate the potential influence of serum calcium measured at Visit 2 and adjusted for serum albumin.

Inclusion/Exclusion: inclusion: all ARIC Visit 2 participants free of cancer; exclusion: those who did not give consent to participate in cancer studies, participants with missing data for serum biomarker in a corresponding analysis.

7.a. Will the data be used for non-CVD analysis in this manuscript?
___x__ Yes    ____ No

b. If Yes, is the author aware that the file ICTDER03 must be used to exclude persons with a value RES_OTH = “CVD Research” for non-DNA analysis, and for DNA analysis RES_DNA = “CVD Research” would be used?
___x__ Yes _____ No
(This file ICTDER03 has been distributed to ARIC PIs, and contains the responses to consent updates related to stored sample use for research.)

8.a. Will the DNA data be used in this manuscript?
___ Yes   x___ No

8.b. If yes, is the author aware that either DNA data distributed by the Coordinating Center must be used, or the file ICTDER03 must be used to exclude those with value RES_DNA = “No use/storage DNA”?
___x__ Yes _____ No

8.c. If yes, is the author aware that the participants with RES_DNA = ‘not for profit’ restriction must be excluded if the data are used by a for profit group?
___x__ Yes _____ No

9. The lead author of this manuscript proposal has reviewed the list of existing ARIC Study manuscript proposals and has found no overlap between this proposal and previously approved manuscript proposals either published or still in active status. ARIC Investigators have access to the publications lists under the Study Members Area of the web site at: http://www.cscs.unc.edu/ARIC/search.php

___x__ Yes _______ No
10. What are the most related manuscript proposals in ARIC (authors are encouraged to contact lead authors of these proposals for comments on the new proposal or collaboration)?
Lutsey ARIC Ancillary Study number 2009.17

11. a. Is this manuscript proposal associated with any ARIC ancillary studies or use any ancillary study data? __x__ Yes _____ No

11.b. If yes, is the proposal
_x__ A. primarily the result of an ancillary study (list number* _1995.04, 2009.17)
____ B. primarily based on ARIC data with ancillary data playing a minor role (usually control variables; list number(s)* ___________ __________)

*ancillary studies are listed by number at http://www.cscc.unc.edu/aric/forms/

12. Manuscript preparation is expected to be completed in one to three years. If a manuscript is not submitted for ARIC review at the end of the 3-years from the date of the approval, the manuscript proposal will expire.

Literature cited.

APPENDIX 1.

The potential functions of biomarkers related to vitamin D measured in ARIC (highlighted in Figure 1)

1) **25(OH)D** (comes from supplement/fortification origin) and **25(OH)D** (cutaneous synthesis and supplements). Total 25(OH)D is the sum of 25(OH)D and 25(OH)D concentrations. In most populations much more vitamin D is made from sun exposure than diet. However, vitamin D intake is an important contributor to 25(OH)D levels, especially in winter months in regions at high latitudes [1, 2]. Vitamin D and D are converted to 25-hydroxyvitamin D (25(OH)D) after 25-hydroxylation in the liver. 25(OH)D is then converted into the active metabolite 1,25(OH)D after 1-hydroxylation by the kidney and at the local tissue level (Figure 1). 25(OH)D is inactive, it has a half-life of about 2–3 weeks and varies with season. It has been also shown to be associated with age, race (skin pigmentation), sex, obesity, and dietary/supplemental vitamin D intake[31, 32]. In contrast, serum 1,25(OH)D is tightly biochemically regulated, has a circulating half-time of 5–15 hr and exhibits little seasonal variability [1]. For these reasons, serum 25(OH)D (the sum of 25(OH) vitamin D and vitamin D) is considered to be the most reliable measure of vitamin D status [1, 2]. In the human body, the highest concentration of 25(OH)D is noted in the plasma (usually measured in the serum as 20–150 nmol/L or 8–60 ng/mL), but the largest pool of 25(OH)D is in adipose tissue and muscle [33]. Measuring separately the two components of 25(OH)D may help distinguish between two sources of vitamin D.

2) **Vitamin D epimer [3-epi-25(OH)D]** is an emerging area of research. It has a similar chemical structure as 25(OH)D but differ in stereochemical configuration in a single site of molecular asymmetry (C-3α- vs. C-3β-hydroxy) [34]. It does not raise serum calcium but does suppress circulating PTH concentration in rats [46]. Given that the epimer constitutes 4-27% of the total 25(OH)D, and it is not explicitly measured by mass-spectrometry, vitamin D, and consequently of 25(OH)D, will be overestimated, as epimer values will be counted as vitamin D given its similar structure and mass [24, 25].

3) Serum **PTH** (parathyroid hormone) produced by the parathyroid gland is the most important endocrine regulator of circulating calcium and phosphorus concentration. It is also interrelated with Vitamin D [35]. A functional deficit of Vitamin D impairs calcium absorptive efficiency, leading, other things being equal, to a rise in PTH production [36]. Thus, increased exposure to UV light, increased dietary intakes of calcium and vitamin D have one common effect – they all down-regulate PTH production. PTH’s primary role in dietary calcium absorption is to stimulate renal conversion of 25(OH)D to 1,25(OH)D. An increase in vitamin D availability leads temporarily to an increase in its metabolite 1,25(OH)D, which in turn results in increased calcium absorption and an increase in serum free calcium. This suppresses PTH production, such that the efficiency of conversion of 25(OH)D to 1,25(OH)D declines, restoring the former levels of 1,25(OH)D while PTH remains somewhat suppressed [35].

It has been observed that patients with primary hyperparathyroidism are more likely to be diagnosed with a colon tumor [27]. PTH may affect cancer risk directly via
inflammatory, mitogenic and antiapoptotic pathways or indirectly via a number of different mechanisms - increased hepatic production of insulin growth factor-1 or enhanced intestinal calcium absorption and, consequently, to a potentially reduced concentration of calcium in the colon lumen [35]. If this is true, this may partially explain an inverse association between UV light, calcium, and vitamin D and decreased CRC risk. However, the range in reported serum 25(OH)D levels at which PTH levels are suppressed varies widely: from 37.5 to 125.0 nmol/L [29, 31, 37, 38]. Examining both PTH and 25(OH)D in relation to CRC in the ARIC cohort could help clarify the complex relation between these biomarkers and assess whether they are independently associated with CRC risk and mortality.

4) **FGF23** (fibroblast growth factor 23) is produced primarily by bone, and in particular by osteoblasts and osteocytes [38]. It was first identified as the causative factor for the hereditary disorder – autosomal dominant hypophosphatemic rickets [39]. It controls vitamin D metabolism through its actions on 1-α-hydroxylase and 24-hydroxylase [40, 41] (Figure 1). Pathologically, high circulating levels of FGF23 result in hypophosphatemia, decreased production of 1,25(OH)₂D, and elevated PTH [38, 40, 41]. In turn, an administration of 1,25(OH)₂D and phosphate increase circulating FGF-23 levels, comprising a feedback loop to maintain phosphate and vitamin D homeostasis [28]. FGF-23 can not only modulate serum 1,25(OH)₂D levels to regulate phosphate homeostasis, but it could also impact local production of 1,25(OH)₂D in the colon via CYP27B1 enzyme using serum 25(OH)D₃ as the metabolic precursor. The colonocytes possess FGF-23 receptors and also expresses the CYP27B1 enzyme [38, 40, 41].

5) **Dietary calcium** has been shown to be inversely associated with CRC risk [42]. Vitamin D is necessary for calcium absorption. Dietary calcium and dietary vitamin D intake are often highly correlated because of vitamin D fortification of milk products in the US. It is difficult to separate the effects of vitamin D and calcium because of the biological interactions between these substances [12, 43, 44]. A randomized trial showed that calcium supplementation reduced the risk of CRC adenoma recurrence only in individuals with vitamin D blood levels >73 nmol/L, whereas at lower vitamin D levels, calcium supplementation was not associated with a reduced risk [18]. Also, dietary calcium may have independent effects on CRC risk due to its tendency to chelate other molecules (especially bile acids) in the intestinal lumen [45].

Epidemiologic data from the ARIC study will be useful in clarifying the mechanisms between Vitamin D measures and CRC risk and mortality. This study may help to explore whether there may be clinical utility in screening for these biomarkers simultaneously.
Figure 1. Vitamin D metabolism in cancer [adapted from Deeb, 2007]