Published in final edited form as:

Fertil Steril. 2012 July ; 98(1): 228–234. doi:10.1016/j.fertnstert.2012.03.029.

Circulating Vitamin D Correlates with Serum Anti-Mullerian Hormone Levels in Late Reproductive-Aged Women: Women's Interagency HIV Study

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Abstract

Objective—To study the correlation between circulating 25 hydroxy-vitamin D (25OH-D) levels and serum AMH in women enrolled in the Women's Interagency HIV Study (WIHS).

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Conflict of interest:

ZM: Nothing to disclose

D.B.S: Receives royalties related to the use of mullerian inhibiting substance for predicting ovarian response in women with infertility.

JW: Nothing to disclose

OA: Funding to her institution from NIH and from Merck

SH: Funding to her and to her institution from NIH

KA: Funding to her institution from NIH

EG: Funding to her institution from NIH

MY: Funding to her institution from NIH

RK: Nothing to disclose

RG: Funding to her and to her institution from NIH

HM: Funding to his institution from NIH

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Design—A cross-sectional study.

Setting—WIHS, a multicenter prospective study.

Patient(s)—All premenopausal women (n=388) with regular menstrual cycles were included and subdivided into three groups: group 1 with age <35 (N=128), group 2 with age 35 to 39 (N=119), and group 3 with age ≥ 40 (N=141).

Intervention(s)—Serum for 25OH-D, AMH, fasting glucose and insulin, and creatinine levels.

Main Outcome Measure(s)—Correlation between 25OH-D and AMH before and after adjusting for HIV status, BMI, race, smoking, illicit drug use, glucose and insulin levels, estimated glomerular filtration rate and geographic site of participation.

Result(s)—After adjusting for all covariates, the regression slope in all participants for total 25OH-D predicting $\log_{10}$ AMH for 25-year-olds (youngest participant) was -0.001 (SE=0.008, $p=0.847$); and for 45-year-olds (oldest participant), the corresponding slope was $+0.011$ (SE=0.005, $p=0.021$). Fasting insulin level was negatively correlated with serum AMH ($p=0.016$). The regression slope for the correlation between 25OH-D and AMH in group 1 was $+0.002$ (SE=0.006, $p=0.764$); in group 2 was $+0.006$ (SE=0.005, $p=0.269$); and in group 3 was $+0.011$ (SE=0.005, $p=0.022$). There was no association between HIV and AMH.

Conclusion(s)—A novel relationship is reported between circulating 25OH-D and AMH in women aged = 40 suggesting that 25OH-D deficiency might be associated with lower ovarian reserve in late reproductive-aged women.

Key terms

Vitamin D; anti-mullerian hormonem mullerian inhibiting substance; HIV; ovarian reserve; insulin resistance; obesity

INTRODUCTION

The classical actions of vitamin D include the regulation of calcium and phosphate metabolism; actions that determine the quality of bone mineralization, which are important for the prevention of rickets in children and osteomalacia or osteoporosis in adults (1). Along with the appreciation of a global epidemic of vitamin D insufficiency/deficiency (2), there has been a recognition that vitamin D has a much wider range of actions that include pro-differentiation, anti-proliferation, proapoptosis, immunosuppression, and anti-inflammation (3, 4). These actions have led to consideration of vitamin D as a potential treatment for diseases such as cancer, immunological diseases, diabetes, infection, and psoriasis among others (5–7).

Among the many physiologic processes influenced by vitamin D, a critical role in reproductive physiology has been suggested (8–10). Currently available data identify vitamin D as a key component in processes involved in reproductive success (9), and thereby suggest pathophysiological mechanisms for reproductive compromise in the setting of vitamin D deficiency. Recently, vitamin D has been shown to be a target for anti-mullerian hormone (AMH) gene expression in vitro (11). Anti-Mullerian hormone (AMH), also known as Mullerian-inhibiting substance (MIS), is a member of the transforming growth factor-beta superfamily (12). AMH is secreted by granulosa cells of follicles undergoing gonadotropin-independent development. Its production begins when follicles are recruited from the primordial pool to become primary follicles, and it ends when they reach the final size and differentiation state available for selection by pituitary follicle stimulating hormone (FSH) (12). AMH regulates the development of early preantral and small antral follicles and acts as an inhibitor of FSH sensitivity of growing antral follicles, which serves

to negatively modulate the FSH-dependent selection of dominant follicles. AMH is one of the most reliable markers for ovarian reserve and it is commonly used in assisted reproduction as a predictor of ovarian response in controlled ovarian hyperstimulation (13, 14). The highest values of AMH in women are attained after puberty and subsequently decrease with age, likely reflecting the age-related decline in ovarian reserve (15).

In human prostate cancer cells, the AMH gene is up-regulated by vitamin D via functional vitamin D response (VDR) elements that bind the vitamin D receptor (11). Additionally, co-expression of steroidogenic factor 1, a key regulator of AMH, increased basal AMH promoter activity that was further stimulated by vitamin D (11). Reports on vitamin D in in vitro fertilization (IVF) outcomes are conflicting. It was recently shown that serum and follicular fluid (FF) levels of 25 hydroxy-vitamin D (25OH-D) were highly correlated and that patients who achieved clinical pregnancies following IVF exhibited significantly higher FF levels of 25OH-D (9). However, another study revealed that FF 25OH-D was negatively correlated with embryo quality and clinical pregnancy rates following IVF (10).

We have recently reported that there is high prevalence of vitamin D deficiency (63%) in the Women's Interagency HIV Study (WIHS) (16). However, the role of vitamin D in ovarian reserve has been understudied. In this study, we assessed women enrolled in the WIHS to test the hypothesis that serum 25OH-D levels are correlated with serum AMH levels.

MATERIALS AND METHODS

Study Population

This study was nested within the WIHS, the largest ongoing multicenter prospective cohort study of HIV infection and related health conditions among HIV-infected women and high-risk seronegative women in the United States (17, 18). WIHS enrolled women in 1994–1995 (n= 2,623) and 2001–2002 (n= 1,143) at six sites in five cities in the United States: New York City (Brooklyn and Bronx/Manhattan, NY; San Francisco, CA; Los Angeles, CA; Washington DC, and Chicago, IL). The WIHS protocols and procedures have been described previously (18). Briefly, HIV-infected and -uninfected women were recruited from similar sources and frequency matched on age, race/ethnicity, and risk for HIV acquisition. Demographic data have been collected at study entry and include birth date and self-reported race/ethnicity. Information on medications, smoking status, illicit drug use and supplement intake has been collected at the same visit through interview. Semi-annual visits included interviewer-administered questionnaires, physical (including height and weight) and gynecological examinations, and collection of biologic specimens. This study was conducted under institutional review board approval of the study sites and the WIHS Data Management and Analysis Center. Written informed consent was obtained from all participants. HIV serostatus was determined using the Food and Drug Administration (FDA) - approved enzyme-linked immunosorbent assay testing and, if reactive, confirmed with the FDA approved Western blot HIV-1 confirmatory assay. AIDS was defined as reporting an AIDS-defining condition (19) and excluded the immunologic criterion of CD4 cell count less than 200 cells per microliter.

Inclusion criteria for this study included: 1) all premenopausal women with 2) regular menstrual cycles (excluding women with no period or 3-day irregularity in the prior 6 months), 3) a uterus and both ovaries intact, 4) available serum AMH data, 5) complete body mass index [BMI; calculated by weight (in kilograms) divided by the square of height (in meters)] and 6) available serum 25OH-D data. Samples were withdrawn from the repository accordingly, to investigate the relationship between serum 25OH-D and serum AMH. Participants who 1) had evidence of hepatic disease (such as hepatitis B and/or C), 2) any cancer (they might affect hormones production and/or clearance), 3) had prior

hysterectomy or oophorectomy, or 4) were pregnant were excluded from the study. We examined for a possible correlation between serum AMH and 25OH-D levels. AMH levels were measured on 1,121 stored sera from 946 subjects. Although AMH was measured at multiple time points for some women, serum 25OH-D measurements all came from a single 6-month period (October 2007 to April 2008) corresponding to study visit 27. AMH levels, current drug use and other time-dependent covariates used in analyses were all measured within 6 months of this window (i.e., at visit 26, 27, 28). Patients were not on any form of vitamin D replacement therapy. Kidney function was quantified by the estimated glomerular filtration rate (EGFR) calculated by the simplified Modification of Diet in Renal Disease equation, which takes into account a person's age, gender, race, and creatinine level (20). Fasting glucose, fasting insulin, and creatinine levels were determined for each participant (those with abnormal results were sent to be evaluated by a medical doctor).

Assays for AMH and vitamin D

Serum levels of AMH were measured with use of an ELISA kit (DSL-10-14400; Diagnostic Systems Laboratories, Webster, TX) according to the manufacturer's recommendations. Samples were run in duplicate by a single operator in the same laboratory without knowledge of group assignment. The lower limit of sensitivity was 0.1 ng/mL, and interassay coefficients of variation were 6%, 7%, and 10% at doses of 1.5, 2.2, and 8.1 ng/mL, respectively. Serum 25OH-D testing was performed by Quest Diagnostics using the liquid chromatography/mass spectroscopy/tandem spectroscopy (LC/MS/TS) method. The LC/MS/TS method is sensitive and equally specific for both forms of 25OH-D. The average inter-assay coefficient of variation across the analytical range of the assay was 7%.

Statistical Analysis

Data were expressed as medians [quartiles] because they were not normally distributed. In a multiple linear regression analysis, $\log_{10}$ AMH was correlated with the total 25OH-D, age and their interaction. In a further analysis, multiple linear regression was performed that adjusted for the following covariates: HIV status, BMI, race/ethnicity, smoking history, current illicit drug use, fasting glucose and insulin levels, EGFR and geographic site of participation (representative of sun exposure). Tests of interactions of HIV status with age and 25OH-D scores were conducted. Nonparametric Loess plots of continuous predictors against the dependent variable were constructed to assess the plausibility of linear relationship; quadratic or cubic terms in these predictors were introduced as necessary to cope with nonlinear relationships. Variance inflation factors were computed to assess the presence of severe multicollinearity. Residual plots were generated and inspected for signs of skew, outliers, and heteroskedasticity. Only 20 (5%) AMH measurements were below threshold, so for convenience the potential censoring problem was ignored, and sub-threshold results treated as if they had threshold values. Kruskal-Wallis test was used to assess if there was a difference in median ages among the six sites.

Regression models were repeated with age treated this time as categorical instead of continuous, by dividing subjects into three age groups: group 1 included women aged <35 (N=128), group 2 included women aged between 35 and 40 (N=119), and group 3 included women aged ≥ 40 (N=141). Pearson correlation between AMH and 25OH-D was calculated separately for each age group.

RESULTS

Of the 1,121 available AMH results, 690 were excluded based upon our inclusion and exclusion criteria. Of the 431 remaining samples, 388 had available 25OH-D. The demographics and clinical profiles of participants with both AMH and 25OH-D levels are

summarized in table 1. The youngest participant was 25 and the oldest was 45. Blacks and Hispanics represented the majority of the participants. The majority was also overweight, did not report any recent use of illicit drugs and had normal kidney function (represented by $\text{EGFR} = 90\text{mL/min/1.73m}^2$). As noted in table 1, the majority of the participants were vitamin D deficient (defined as 25OH-D levels $< 30\text{ ng/ml}$).

Analysis without covariates

In the multiple regression without covariates, there was a significant interaction of 25OH-D by age ($p=0.027$). According to this model, the regression slope for 25OH-D and $\log_{10}\text{AMH}$ for 25-year-olds was -0.01 ($\text{SE}=0.006$, $p=0.083$); for 45-year-olds, the corresponding slope was $+0.009$ ($\text{SE}=0.004$, $p=0.028$). In other words, for the youngest women in the study AMH may be negatively correlated with 25OH-D , whereas for the oldest women in the study the relationship was reversed. The mean age at which this relationship was reversed (*i.e.*, where the regression slope is zero) was estimated from this model to be 35.7 years (Figure 1).

Analysis with covariates

Increased age was the strongest predictor of low serum AMH (Table 2). Fasting insulin level was negatively correlated with serum AMH after adjusting for HIV status, BMI, race/ethnicity, smoking history, current illicit drug use, fasting glucose, EGFR and geographic site of collaboration (Table 2). Although AMH was significantly different at baseline among different study sites (sites 2 and 3 had significantly higher serum AMH levels than sites 5 and 6; Table 2), there was no notable difference by site in predicting serum AMH. Median ages for sites 1–6 respectively were 39.6, 37.2, 36.4, 37.4, 37.9 and 37.9 ($p=0.177$). There was no difference in median ages when comparing sites 2 and 3 with sites 5 and 6 ($p=0.347$).

According to this model, the regression slope for the correlation between 25OH-D and $\log_{10}\text{AMH}$ for 25-year-olds was -0.001 ($\text{SE}=0.008$, $p=0.847$); for 45-year-olds, the corresponding slope was $+0.011$ ($\text{SE}=0.005$, $p=0.021$) (Table 2, *p*Figure 1). However, the α -value for age-by 25OH-D interaction was now above 0.05. A test of the null hypothesis of no association between 25OH-D and $\log_{10}\text{AMH}$ (*i.e.*, that neither the 25OH-D coefficient nor the age-by- 25OH-D coefficient differed significantly from zero) yielded $p=0.049$. A test of the null hypothesis of no association between BMI and $\log_{10}\text{AMH}$ (*i.e.*, that neither the linear nor the quadratic coefficient in BMI differed significantly from zero) yielded $p=0.108$. A three-way interaction between HIV/AIDS group, age and 25OH-D , suggesting greater influence of age on the effect of 25OH-D on AMH among HIV+AIDS- than among either other group, fell short of statistical significance ($p=0.055$).

Analysis for three age groups

With age and 25OH-D as the only predictors, there was again a significant age by 25OH-D interaction ($p=0.019$). The regression slope for the correlation between 25OH-D and AMH was estimated for group 1 as -0.0086 ($\text{SE}=0.004$, $p=0.054$); for group 2 as $+0.002$ ($\text{SE}=0.004$, $p=0.6$); and for group 3 as $+0.008$ ($\text{SE}=0.004$, $p=0.041$). AMH by 25OH-D correlation for group 1 was -0.21 ($p=0.019$); for group 2 was $+0.05$ ($p=0.662$); and for group 3 was $+0.16$ ($p=0.063$).

With other covariates in the model, there was no significant age by 25OH-D interaction ($p=0.427$). The regression slope for the correlation between 25OH-D and AMH was estimated for group 1 as $+0.002$ ($\text{SE}=0.006$, $p=0.764$); for group 2 as $+0.006$ ($\text{SE}=0.005$, $p=0.269$); and for group 3 as $+0.011$ ($\text{SE}=0.005$, $p=0.028$). A partial F -test of the null hypothesis of no main effect of 25OH-D and no 25OH-D by age interaction yielded

$p=0.111$. A partial F -test of the null hypothesis of no linear or quadratic effect of BMI yielded $p=0.167$. The effect of HIV/AIDS fell short of statistical significance ($p=0.078$), and there was no interaction of HIV status with 25OH-D ($p=0.378$).

Figure 1 represents a model for the regression slope for serum 25OH-D and serum AMH. In women aged 45, serum 25OH-D was significantly and positively correlated with serum AMH after adjusting for HIV status, BMI, race/ethnicity, smoking history, current illicit drug use, fasting glucose, fasting insulin, EGFR and geographic site of collaboration. At age 25, the slope was negative (although it did not reach significance in multivariate analysis). However the slope was positive at age 45 (and remained statistically significant after adjusting for the covariates noted above).

DISCUSSION

We demonstrated that circulating 25OH-D was positively correlated with serum AMH levels in late reproductive-aged (age more than 40) women independent of HIV serostatus and adjusting for BMI, race/ethnicity, smoking history, current illicit drug use, glucose, insulin, EGFR and geographic site of participation. As expected and established, age was the strongest predictor of serum AMH. Our results also indicated that fasting insulin level was negatively correlated with serum AMH. The difference in AMH noted at baseline between sites (2 and 3 versus 5 and 6) should be investigated in further studies, and cannot be explained by age discrepancy as median ages did not differ among sites. Although it is beyond the scope of the current study, other factors like race/ethnicity, BMI and illicit drug use might be different among different sites of participation in the WIHS and might explain the difference in AMH between sites 2 and 3 versus 5 and 6.

AMH and Vitamin D

This is the first report that assessed the relationship between circulating vitamin D level and ovarian reserve, as reflected in serum AMH levels- the most reliable marker of ovarian reserve to date. The effect of vitamin D on AMH gene expression was evaluated in prostate cancer cell line. In that *in vitro* study, vitamin D was found to be a target for AMH gene expression (11). The AMH gene was up-regulated by vitamin D via functional vitamin D response elements that bind the vitamin D receptor in human prostate cancer cells (11). Additionally, co-expression of steroidogenic factor 1, a key regulator of AMH, increased basal AMH promoter activity that was further stimulated by vitamin D (11). Although we did not look at AMH gene expression in granulosa cells of our subjects, our results in serum might be in harmony with this *in vitro* study in males. Of note, the only source of circulating AMH are granulosa cells, making serum AMH a measure of AMH release function by granulosa cells. Additionally, the relationship between circulating (serum and FF) vitamin D and reproductive potential in women has been studied and has shown conflicting results (9, 10). In one study (9) of 84 women undergoing IVF, serum and FF levels of 25OH-D were highly correlated and there was a significantly higher clinical pregnancy and implantation rates with increase in FF 25OH-D. Patients achieving clinical pregnancy following IVF ($n=26$) exhibited significantly higher FF levels of 25OH-D. Multivariable logistic regression analysis adjusting for age, BMI, ethnicity, and number of embryos transferred confirmed FF 25OH-D levels as an independent predictor to success of an IVF cycle (9). That study implied that there might be a potential benefit of vitamin D supplementation on treatment success in infertile patients undergoing IVF. On the other hand, Anifandis et al (10) studied 101 women who underwent IVF and correlated FF 25OH-D with IVF cycle outcome. Their results indicated that FF 25OH-D levels were negatively correlated to the quality of embryos and that the higher values of 25OH-D were associated with lower possibility to achieve pregnancy. Altogether, to date, there is insufficient data to accurately evaluate the effects of vitamin D in women undergoing IVF. A possible reason for the discrepancy between those

two studies was their outcomes (pregnancy and embryo quality) which heavily rely on other factors like sperm quality/quantity and endometrial receptivity.

There is some evidence suggesting that vitamin D deficiency might be involved in the pathogenesis of insulin resistance and the metabolic syndrome in polycystic ovarian syndrome (PCOS). In PCOS women, low 25OH-D levels are associated with obesity, metabolic and endocrine disturbances (21–23), and vitamin D supplementation might improve menstrual frequency and metabolic disturbances in those women (24, 25). Moreover, vitamin D might influence steroidogenesis (26) and ovarian follicular development (27). Although AMH is most known for its activity in initiating regression of Mullerian structures during male fetal development (28), it has several postnatal actions. Similar to vitamin D, AMH exhibits important actions on steroidogenesis (29), ovarian follicular development (30) and PCOS (31). Because vitamin D induces the AMH gene (11) and because we found here that vitamin D and AMH are positively correlated, it is possible that vitamin D contributes to AMH actions by increasing AMH production by granulosa cells. These speculations warrant further investigation.

AMH and Insulin Resistance

In our study, we found a negative correlation between fasting insulin and serum AMH. These results are in agreement with other data (32, 33). For instance, Park et al (32) studied 120 healthy, reproductive-aged women without PCOS and found that insulin resistance and hyperinsulinemia were associated with lower levels of AMH suggestive of the effect of abnormal insulin action on AMH secretion by granulosa cells.

The mechanisms by which abnormal insulin levels, and hence its action, exerts an effect on AMH have not been investigated. In women, AMH is produced by granulosa cells of preantral and small antral follicles. Insulin resistance has been reported to have a negative effect on ovarian granulosa cell function (34). Additionally, factors that may disturb granulosa cell function may also affect the production or secretion of AMH. Therefore, we hypothesize that hyperinsulinemia negatively impacted granulosa cell AMH secretion in our cohort. The decreased AMH level may be caused by insulin-induced oxidative injuries and suboptimal follicular vascularization. A large body of evidence suggests that insulin resistance is associated with compromised vascularization, characterized by endothelial cell dysfunction and oxidative stress (35, 36). Additionally, reproductive function is tightly coupled with energy hemostasis (37). Because the ovary is an energy-competent organ that contains all the components of insulin signaling required for glucose metabolism, it is not surprising that abnormal insulin action alters AMH secretion or production by granulosa cells. The clinical significance of lower AMH levels in normo-ovulatory women, like our subjects, is not known. Whether the present relationship means poor ovarian reserve or ovarian senescence is still uncertain.

Conclusion

A novel relationship is reported herein between circulating vitamin D and serum AMH suggesting that circulating vitamin D is associated with ovarian reserve in late reproductive-aged women. With the global epidemic of vitamin D insufficiency among HIV-infected and -uninfected women and given our findings, assessment of vitamin D status might be considered as a part of routine workup in premenopausal women in their late reproductive years since appropriate supplementation of those deemed depleted of vitamin D might translate to improved fertility outcome. Future studies should consider replacing vitamin D in women with vitamin D deficiency, and assessing consequent AMH blood levels.

Acknowledgments

Funding:

Data in this manuscript were collected by the Women's Interagency HIV Study (WIHS) Collaborative Study Group with centers (Principal Investigators) at New York City/Bronx Consortium (Kathryn Anastos); Brooklyn, NY (Howard Minkoff); Washington DC, Metropolitan Consortium (Mary Young); The Connie Wofsy Study Consortium of Northern California (Ruth Greenblatt); Los Angeles County/Southern California Consortium (Alexandra Levine); Chicago Consortium (Mardge Cohen); Data Coordinating Center (Stephen Gange).

The WIHS is funded by the National Institute of Allergy and Infectious Diseases (U01-AI-35004, U01-AI-31834, U01-AI-34994, U01-AI-34989, U01-AI-34993, and U01-AI-42590) and by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (U01-HD-32632). The study is co-funded by the National Cancer Institute, the National Institute on Drug Abuse, and the National Institute on Deafness and Other Communication Disorders. Funding is also provided by the National Center for Research Resources (UCSF-CTSI Grant Number UL1 RR024131). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

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Figure 1.

A model for regression slope pattern between serum 25OH-D and serum AMH in the WIHS participants after adjusting for HIV status, BMI, race/ethnicity, smoking history, current illicit drug use, fasting glucose, fasting insulin, EGFR and geographic site of collaboration. Slope is zero at age 35.7. * $P < 0.05$ for women aged 40 and above.

Table 1

Demographics and clinical profile of participants with available serum 25 Hydroxy- vitamin D and available serum AMH (n= 388)

	Median [25 th – 75 th quartiles] or N (%)	Minimum	Maximum
Age (years)	37.82 [33.11–41.4]	25	45
BMI^a (kg/m²)	28.50 [24.60–34.60]	15	74
Race/Ethnicity			
African American	226 (58%)		
Caucasian	30 (8%)		
Hispanic	118 (30%)		
Other	14 (4%)		
Tobacco use			
Current	142 (37%)		
Former	97 (25%)		
Never	148 (38%)		
Illicit drug use			
Current	71 (18%)		
Not current	317 (82%)		
Site of Collaboration			
Bronx	82 (21%)		
Brooklyn	90 (23%)		
Washington D.C.	53 (14%)		
Los Angeles	72 (19%)		
San Francisco	41 (11%)		
Chicago	50 (13%)		
HIV/AIDS^b status			
HIV –	141 (36%)		
HIV + AIDS –	194 (50%)		
AIDS +	53 (14%)		
Fasting Glucose (mg/dl)	87 [81–91]	63	277
Fasting Insulin (μIU/ml)	9 [5–14]	2	96
EGFR^c (mL/min/1.73m²)	100 [85–115]	6	185
AMH^d (ng/ml)	1.12 [0.4–2.4]	0.07	12.9
25 Hydroxy-vitamin D (ng/ml)	14 [9–21]	4	50

^aBMI, body mass index

^b AIDS defining illness is a prior report of conditions consistent with the 1993 CDC surveillance definition, excluding the criterion of CD4<200 cells/mL

^c EGFR, estimated glomerular filtration rate

^d Anti-mullerian hormone

Table 2

Regression coefficients between all covariates and serum AMH

	Estimate $\pm$ SE ^a	t-value	p-value
Intercept	1.226 $\pm$ 0.500	2.45	0.015
Age (years)	-0.061 $\pm$ 0.009	-6.57	<.0001
Race/Ethnicity			0.571
Caucasian vs other	-0.120 $\pm$ 0.172	-0.70	
African American vs other	0.016 $\pm$ 0.143	0.12	
Hispanic vs other	-0.054 $\pm$ 0.150	-0.36	
Illicit drug use	0.062 $\pm$ 0.071	0.87	0.382
Smoking status			0.185
Current smoker vs never smoker	-0.086 $\pm$ 0.064	-1.33	
Former smoker vs never smoker	0.034 $\pm$ 0.068	0.50	
HIV/AIDS status			0.117
HIV- vs AIDS+	0.164 $\pm$ 0.088	1.85	
HIV+AIDS- vs AIDS+	0.070 $\pm$ 0.084	0.83	
Fasting glucose (mg/dl)	0.001 $\pm$ 0.001	0.81	0.419
Fasting insulin (μIU/ml)	-0.007 $\pm$ 0.002	-2.43	0.016
EGFR (mL/min/1.73m²)	0.002 $\pm$ 0.001	1.59	0.112
Site of participation^b			0.004
Site 1 vs site 6	0.119 $\pm$ 0.092	1.29	
Site 2 vs site 6	0.241 $\pm$ 0.091	2.65	
Site 3 vs site 6	0.208 $\pm$ 0.102	2.04	
Site 4 vs site 6	0.182 $\pm$ 0.099	1.82	
Site 5 vs site 6	-0.054 $\pm$ 0.126	-0.43	
BMI (kg/m²)	0.030 $\pm$ 0.014	2.12	0.035
BMI * BMI	-0.0003 $\pm$ 0.0002	-2.04	0.042
25 Hydroxy-vitamin D (ng/ml)	-0.017 $\pm$ 0.020	-0.86	0.390
Age(ng/ml) * 25Hydroxy-vitamin D(ng/ml)	0.0006 $\pm$ 0.0005	1.21	0.226

^aStandard error.^bSite 1= Bronx, Site 2= Brooklyn, Site 3= Washington D.C., Site 4= Los Angeles, Site 5= San Francisco, Site 6= Chicago. Overall R²=0.41