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## Uric acid suppresses 1 alpha hydroxylase in vitro and in vivo

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### Abstract

**Objective**—Patients with gout have lower calcitriol levels that improve when uric acid is lowered. The mechanism of these observations is unknown. We hypothesized that uric acid inhibits 1- $\alpha$ hydroxylase.

**Materials and methods**—*In vivo*, Sprague Dawley rats were randomized to control (n=5), allantoxanamide (n=8), febuxostat (n=5), or allantoxanamide+febuxostat (n=7). Vitamin D, PTH, and 1- $\alpha$ hydroxylase protein were evaluated. In order to directly evaluate the effect of uric acid on 1- $\alpha$ hydroxylase, we conducted a series of dose response and time course experiments *in vitro*. Nuclear factor  $\kappa$ -B (NF $\kappa$ B) was inhibited pharmacologically. Finally, to evaluate the potential implications of these findings in humans, the association between uric acid and PTH in humans was evaluated in a cross-sectional analysis of data from the NHANES (2003-2006); n= 9773.

**Results**—1,25(OH)<sub>2</sub>D and 1- $\alpha$ hydroxylase protein were reduced in hyperuricemic rats and improved with febuxostat treatment. Uric acid suppressed 1- $\alpha$ hydroxylase protein and mRNA expression in proximal tubular cells. This was prevented by NF $\kappa$ B inhibition. In humans, for every 1 mg/dL increase in uric acid, the adjusted odds ratio for an elevated PTH (>65 pg/mL) was 1.21 (95% C.I. 1.14, 1.28; P< 0.0001), 1.15 (95% C.I. 1.08, 1.22; P<0.0001), and 1.16 (95% C.I. 1.03, 1.31; P=0.02) for all subjects, subjects with estimated GFR  $\geq$  60, and subjects with estimated GFR <60 mL/min/1.73 m<sup>2</sup> respectively.

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**Conclusion**—Hyperuricemia suppresses 1- $\alpha$ hydroxylase leading to lower 1,25(OH)<sub>2</sub>D and higher PTH in rats. Our results suggest this is mediated by NF $\kappa$ B. The association between uric acid and PTH in NHANES suggests potential implications for human disease.

### Keywords

uric acid; parathyroid hormone; mineral and bone disorders

## Introduction

Chronic kidney disease (CKD) is associated with significant morbidity and mortality [1]. In addition to well-known risk factors such as hypertension and diabetes, several “nontraditional” risk factors may contribute to the higher risk of death in patients with CKD compared to the general population [2-6]. Inflammation and CKD mineral and bone disorder (MBD) are two risk factors that have taken center stage in CKD patients [7-8]. In particular, the progressive decline in 1,25 dihydroxy-vitamin D (1,25(OH)<sub>2</sub>D) levels that occurs as glomerular filtration rate (GFR) falls has been associated with increased activity of the renin angiotensin system [9], insulin resistance [10], and progressive inflammation [11-12]. In turn, inflammation itself may suppress vitamin D activation thus contributing to the osteopenia seen in inflammatory diseases such as ankylosing spondylitis [13].

Vitamin D, provided in the diet or generated in the skin by ultraviolet light, is normally converted to 25 hydroxyvitamin D (25(OH)D) in the liver. 25(OH)D is then further hydroxylated by a rate-limiting step to 1,25(OH)<sub>2</sub>D by the enzyme 1- $\alpha$  hydroxylase (CYP27B1); present primarily in proximal tubular cells in the kidney [14]. A decrease in 1,25(OH)<sub>2</sub>D secondary to reduced nephron mass and/or 1- $\alpha$  hydroxylase enzyme activity contributes to the development of secondary hyperparathyroidism in patients with CKD [15].

The potential for a direct relationship between hyperuricemia and vitamin D metabolism was initially raised more than 20 years ago [16-17], and was subsequently explored by Takahashi et al [18], where patients with gout were found to have lower 1,25(OH)<sub>2</sub>D levels than normal controls. The same group showed that treatment of hyperuricemia increases 1,25(OH)<sub>2</sub>D levels with no change in 25(OH)D [19] suggesting that hyperuricemia may have a suppressive effect on 1- $\alpha$  hydroxylase activity. The mechanism underlying such observations remains unknown. We hypothesized that high uric acid levels reduces 1,25(OH)<sub>2</sub>D levels by suppressing 1- $\alpha$  hydroxylase and, since low 1,25(OH)<sub>2</sub>D levels can stimulate parathyroid hormone (PTH), that high uric acid levels should be associated with elevated PTH levels. To test this, we also conducted studies *in vivo* where we utilized an animal model of acute hyperuricemia and *in vitro* using human proximal tubular cells. Furthermore, we evaluated the relation between high uric and elevated PTH levels utilizing data from the National Health and Nutrition Examination Survey (NHANES 2003-2006), a large cross sectional sample of the US population.

## Methods

### Animal Study

Male Sprague-Dawley rats ( $n = 25$ ) weighing 150 g (Charles Rivers Laboratories, Wilmington, MA) were randomly assigned to one of 4 groups: control ( $n=5$ ), allantoxanamide at a dose of 150 mg/kg intraperitoneally ( $n=8$ ), febuxostat at a dose of 30mg/kg by gavage ( $n=5$ ), or allantoxanamide and febuxostat ( $n=7$ ). Allantoxanamide is a uricase inhibitor that has been shown to acutely raise serum uric acid when injected intraperitoneally in rats [20] at a dose of 100-200 mg/kg [20-21]. Febuxostat is a non-purine

selective inhibitor of xanthine oxidase. Contrary to allopurinol, febuxostat does not inhibit other enzymes in purine and pyrimidine metabolism pathways [22], yet has a more potent uric acid lowering effect than allopurinol *in vitro* and *in vivo* [23]. Considering the potent effect of allantoxanamide, febuxostat was administered 1 hour prior to allantoxanamide and at a high dose to ensure the uric acid lowering effect. After 24 hours, blood samples were obtained and all animals were sacrificed under light anesthesia. Kidneys were immediately fixed in Methyl-Carnoy's solution. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado Denver.

### Biochemical data

Serum chemistries including uric acid, creatinine, calcium, and phosphorus were measured by autoanalyzer (VetAce machine; Alpha Wasserman, West Caldwell, NJ). As previously published by other groups, the ratio of 1,25(OH)<sub>2</sub>D to 25(OH)D was used as an indirect assessment of 1- $\alpha$  hydroxylase activity [24]. 1,25(OH)<sub>2</sub>D and 25(OH)D levels were measured by Enzyme-linked ImmunoAssay (ELISA) kits (Immunodiagnostic systems). The reported intra-assay CV% for 25(OH)D ranges between 5.3-6.7% and the interassay CV% ranges from 4.6-8.7%. For 1,25(OH)<sub>2</sub>D, the intra-assay CV% ranges between 9.3-10.7% and the interassay CV% ranges from 17.1-19.7%. Similarly, intact PTH was measured by ELISA (Immunotopics, Inc.).

### Renal histology and Immunofluorescence

Kidneys were fixed in paraffin, sectioned (2  $\mu$ m thickness), and stained by Periodic Acid-Schiff (PAS) for histological analysis. For immunofluorescence, heat-induced epitope retrieval was achieved in antigen retrieval citrate solution (BioGenex, San Ramon, CA) for 1- $\alpha$  hydroxylase. After rinsing the sections in PBS, they were blocked in 1% normal goat serum for 1 hour at room temperature. A rabbit polyclonal anti-rat 1- $\alpha$  hydroxylase antibody (Santa Cruz, CA) was used as primary antibody, and Alexa fluor 568 conjugated goat polyclonal anti-rabbit antibody (Invitrogen, Carlsbad, CA) as secondary antibody. Images were analyzed using Axio Vision image analyzer (Carl Zeiss, Thornwood, NY) at 20X and 40X. The same settings of the microscope were applied to all the images being compared. Nonspecific staining with secondary antibody was negligible.

### Immunoblotting

Whole kidney or stimulated HK2 cells were lysed in lysis buffer (20 mM Tris-HCl [pH 8.0], 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% Glycerol, and 0.5 mM PMSF). Nuclear and cytosolic proteins were extracted with Biovision extraction kit (Mountain View, CA). Equal amounts of protein were resuspended in SDS sample buffer, boiled for 5 min, and analyzed on 4 to 20% SDS-PAGE gels. The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-ECL; Amersham, Piscataway, NJ) and probed with the following antibodies: 1- $\alpha$  hydroxylase and 24 hydroxylase (Santa Cruz, Santa Cruz, CA), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The antigen-antibody complexes were detected by the ECL protocol using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG as secondary antibody. The immunoblots shown are representative of the animal groups.

### Cell culture and reagents

In order to evaluate the effect of uric acid on 1- $\alpha$  hydroxylase more directly, we conducted a series of *in vitro* experiments. Human proximal tubular cells (HK2 cells) were cultured in Keratinocyte-SFM basal media (Invitrogen), supplemented with Bovine Pituitary Extract (20-30  $\mu$ g/mL) and recombinant Epidermal Growth Factor (0.1-0.2 ng/mL), 5% Fetal Bovine Serum, 100 U/ml penicillin, and 10 g/ml streptomycin (Invitrogen). Cells were

cultured at 37°C in 95% air-5% CO<sub>2</sub> until they were 90% confluent, and then allowed to differentiate for 5-7 days. After serum starvation for 24 hours, cells were stimulated with uric acid. For the dose response experiments, uric acid was given at 2.5, 5.0, 7.5, and 10 mg/dL and the cells were collected after 24 hours of treatment. Uric acid was used at 10 mg/dL for the time course experiments and the cells were collected at: 1, 2, 4, 8, 16, and 24 hours. To prevent uric acid crystal formation in the media, uric acid was dissolved in prewarmed media, and kept at 37°C for a minimum of 30 minutes prior to application in cell culture. For cell viability assessment, the cells were treated with 2.5, 5.0, 7.5, and 10 mg/dL uric acid and collected after 24 hours, then incubated for 3 min at room temperature with an equal volume of 0.4% (w/v) trypan blue solution, and counted using a dual-chamber hemocytometer and a light microscopy. Viable and nonviable cells were recorded separately. The means of three independent cell counts were pooled for analysis. Considering prior evidence that nuclear factor  $\kappa$ -B (NF $\kappa$ B) may inhibit 1- $\alpha$  hydroxylase transcription [25], and that uric acid has been shown to activate NF $\kappa$ B[26-27], we examined the role of NF $\kappa$ B in mediating uric acid effects. BAY 11-7082, an NF $\kappa$ B inhibitor (Sigma) was used. A dose of 5 $\mu$ M/mL of BAY 11-7082 was determined by a series of viability experiments, and was applied 1 hour prior to uric acid treatment (10mg/dL).

The cell culture experiments were repeated twice to ensure reproducibility. Quantitative data are presented as mean  $\pm$  SEM and were analyzed either by paired t-test or ANOVA as appropriate. Significance was assigned at  $P < 0.05$ . All statistical analyses were performed by using InStat Version 3.06 (GraphPad Software, San Diego, CA).

### **Methods for the correlation between uric acid and PTH in humans**

**Study population and sample:** Data were obtained from the NHANES (2003-2006), a cross sectional survey conducted by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and prevention between 2003 and 2006. A stratified, multistage sampling design was used, with over sampling of non-Hispanic Blacks, Mexican-Americans, and persons over the age of 60 years. Subjects underwent a standardized questionnaire, administered during a home interview followed by a detailed physical examination and blood specimens' collection at a mobile examination center or at the participants' homes [28]. The analysis was restricted to adult participants 18 years of age or older who had complete serum PTH and uric acid data (n= 9773 individuals).

**Primary predictor and outcome:** The independent variable used in this analysis was serum uric acid modeled as a continuous variable. Serum uric acid was measured by oxidization with the specific enzyme uricase (Hitachi model 737 Multichannel Analyzer; Boehringer Mannheim Diagnostics, Indianapolis, IN) and is reported in mg per dL. Details about quality-control procedures have been published elsewhere [28].

The dependent variable of interest was serum PTH levels (modeled as a continuous and categorical variable). PTH was tested for normality and found to be non-normally distributed, hence it was log transformed. Categorically, elevated PTH was defined as  $> 65$  pg/mL on the basis of previous studies [15]. Intact PTH levels were measured by Elecsys 1010 analyzer using ECL/Origen electrochemiluminescent process (reference range: 10–65 pg/mL) [28].

**Other measurements:** Potential confounding factors were chosen based on prior studies or on their biological plausibility. The following covariates were included in the analysis: age, sex, race/ethnicity, diabetes, hypertension, BMI, serum levels of 25-hydroxyvitamin D (25(OH)D), calcium, phosphorus, and serum bicarbonate levels, in addition to the

Modification of Diet in Renal Disease (MDRD)- estimated glomerular filtration rate (eGFR), and use of diuretics.

**Statistical analysis:** Univariate modeling was first performed to determine if a directional trend existed between the selected variables and elevated PTH. Categorical variables were examined with a Chi-square test and continuous variables examined with two-tailed T test. The independent relationship between serum uric acid and elevated PTH levels was investigated using logistic regression models adjusting simultaneously for several potential confounders. The analysis was then stratified by serum phosphorus levels  $>4.5$  and  $4.5$  mg/dL based on the normal adult reference range. In addition, we stratified by serum calcium levels  $>9.5$  and  $9.5$  mg/dL. The number of participants with serum calcium levels  $>10$  mg/dL was small (3%), and the cut-off of  $9.5$  mg/dL was chosen based on prior evidence that serum calcium  $>9.5$  mg/dL is associated with an increased risk of death in patients with CKD [29]. Due to known confounding effect of kidney function on uric acid and PTH levels, we examined the association between serum uric acid and PTH in groups stratified by MDRD-estimated GFR. The absence of significant CKD was defined as MDRD-estimated GFR  $\geq 60$  mL/min/1.73 m<sup>2</sup> and significant CKD was defined as MDRD-estimated GFR  $<60$  mL/min/1.73 m<sup>2</sup>. Considering the evidence that PTH levels begin to increase once the GFR is  $<60$  mL/min/1.73 m<sup>2</sup> [15] and the variability of the MDRD equation in individuals with GFR  $\geq 60$  mL/min/1.73 m<sup>2</sup> we avoided further definition of GFR  $\geq 60$  mL/min/1.73 m<sup>2</sup> into 60-89 and  $\geq 90$  mL/min/1.73 m<sup>2</sup> [30]. To characterize the relationship between uric acid and PTH in CKD, we stratified CKD status as follows: MDRD-eGFR 45-59, 30-44, and 15-29 mL/min/1.73 m<sup>2</sup>. Linear regression models were also applied. Only a few of NHANES participants had eGFR  $<15$  mL/min/1.73 m<sup>2</sup> and were not included in the analysis. Analyses were conducted using SAS software, version 9.1.3 (Research Triangle Institute, Research Triangle Park, NC). P values  $<0.05$  were considered statistically significant.

## Results

### Acute hyperuricemia significantly reduces 1- $\alpha$ hydroxylase activity and protein expression in vivo

Mean serum uric acid for the different treatment groups are shown in Figure 1A. Animals with hyperuricemia had a significantly lower ratio of 1,25(OH)<sub>2</sub>D/25(OH)D when compared to the control group, as shown in Figure 1B. 1,25(OH)<sub>2</sub>D levels for the control, allantoxanamide, febuxostat, allantoxanamide + febuxostat groups were  $836 \pm 56$ ,  $708 \pm 73$ ,  $756 \pm 55$ , and  $795 \pm 56$  pmol/L, respectively (Figure 1C). Mean 25(OH)D levels were lower in the hyperuricemic rats but this did not achieve statistical significance (Figure 1D). Immunofluorescence indicated reduced expression of 1- $\alpha$  hydroxylase protein in the proximal tubular nephron of the hyperuricemic rats (Figure 2A). Consistently, immunoblotting showed reduced expression of 1- $\alpha$  hydroxylase in whole kidney lysates of hyperuricemic rats and restoration of protein expression in the rats treated with both allantoxanamide and febuxostat, thus excluding direct toxicity of allantoxanamide as causative for reduced 1- $\alpha$  hydroxylase expression (Figure 2B and 2C). 24-hydroxylase expression was unchanged in response to hyperuricemia. Median (IQR) PTH levels were: 499 (451, 571), 984 (459, 1789), 596 (452, 836), and 776 (730, 1131) pg/mL for the control, allantoxanamide, febuxostat, and allantoxanamide + febuxostat groups respectively (P=0.14). Calcium and phosphorus levels are shown in Table 1. While serum phosphorus levels increased in the hyperuricemic group compared to the control, this was not statistically significant. Of note, acute hyperuricemia was associated with increased serum creatinine and tubular dilatation as shown in Figure 3. The serum creatinine for the allantoxanamide group was significantly higher than the control group ( $1.9 \pm 1.2$  versus

0.4±0.1 mg/dL). The acute kidney injury was only partially reversed by the xanthine oxidase inhibitor, febuxostat (serum creatinine 0.7±0.2 mg/dL).

### **Acute hyperuricemia significantly reduces 1- $\alpha$ hydroxylase mRNA and protein expression in human HK2 cells and inhibition of NF $\kappa$ B restores 1- $\alpha$ hydroxylase mRNA and protein expression**

Figure 4A and 4B illustrate decreasing expression of 1- $\alpha$  hydroxylase mRNA and protein in HK2 tubular cells in response to increasing uric acid concentrations. The reduction in 1- $\alpha$  hydroxylase protein was notably significant at 10 mg/dL of uric acid. Time course experiments revealed that 1- $\alpha$  hydroxylase mRNA was significantly suppressed at 6 hours (Figure 4D), whereas protein expression was significantly inhibited after 16 hours of treatment with uric acid (Figure 4C). Pretreatment of the HK2 cells with Bay 11-7082, an inhibitor of NF $\kappa$ B prevented uric acid - mediated suppression of 1- $\alpha$  hydroxylase mRNA and protein (Figures 4E and 4F). These findings suggest that uric acid suppresses 1- $\alpha$  hydroxylase mRNA and protein expression by activating NF $\kappa$ B. Protein expression of 24 hydroxylase was unaffected by uric acid in this cell model (data not shown). In addition, the percent of viable cells was 95, 94, 94, 95, and 95% for uric acid treatment groups: control, 2.5, 5, 7.5, and 10 mg/dL respectively. No significant differences were observed for all group comparisons.

### **The relation between uric acid and PTH in humans**

**Characteristics of the participants according to PTH:** Participants with high PTH >65 pg/mL (n=1338) were older and more likely to be of non- Hispanic Black ethnicity. Compared to the individuals with normal PTH, the participants with increased PTH had higher body mass index (BMI), and greater prevalence of hypertension and diabetes. High PTH was also associated with lower 25(OH)D levels and a lower MDRD- estimated GFR (Table 2). Additionally, participants with elevated PTH had higher serum uric acid levels (P < 0.0001).

**Cross sectional association between serum uric acid and elevated PTH in all participating subjects (n=9773):** Mean serum uric acid was 5.3 ± 1.4 mg/dL. In the unadjusted analysis, the odds ratio for an elevated PTH >65 pg/mL was 1.32 (95% C.I. 1.26, 1.37; P<0.0001) for every 1 mg/dL increase in uric acid. After adjusting for age, sex, race/ ethnicity, diabetes, hypertension, BMI, serum levels of 25(OH)D, calcium, phosphorus, and serum bicarbonate, MDRD- estimated GFR, and diuretic use, the odds of an elevated PTH level was 1.21 (95% C.I. 1.14, 1.28; P<0.0001) for every 1mg/dL increase in serum uric acid. These findings remained unchanged when subjects on diuretics or allopurinol were excluded. The association between uric acid and PTH >65 pg/mL remained significant in the following subgroups: calcium >9.5 and 9.5 mg/dL, and phosphorus >4.5 and 4.5 mg/dL (data not shown).

Linear regression analysis yielded a  $\beta$  estimate for log transformed PTH on uric acid of 0.031 (95% C.I. 0.028, 0.034; P<0.0001) in unadjusted analysis, and 0.030 (95% C.I. 0.027, 0.033; P<0.0001) in the multivariate model.

**Cross sectional association between serum uric acid and PTH by chronic kidney disease status:** 768 of the 9773 participants (8%) were found to have CKD (defined as MDRD-estimated GFR < 60 mL/min/1.73 m<sup>2</sup>). In individuals without CKD (MDRD-estimated GFR  $\geq$  60 mL/min/1.73 m<sup>2</sup>), the odds ratios for an elevated PTH level of >65 pg/mL for every 1 mg/dL increase in uric acid were: 1.24 (95% C.I. 1.19, 1.30; P< 0.0001) in unadjusted analysis, and 1.15 (95% C.I. 1.08, 1.22; P< 0.0001) in the adjusted analysis. In individuals with CKD, the unadjusted analysis revealed an odds ratio of 1.36 (95% C.I. 1.23,

1.50;  $P < 0.0001$ ), and the adjusted analysis revealed an odds ratio of 1.16 (95% C.I. 1.03, 1.31;  $P = 0.02$ ).

On further examination of the relationship between uric acid and log transformed PTH across kidney function levels, serum uric acid was significantly correlated with log transformed PTH for the eGFR groups:  $\geq 60$ , 45-59 and 30-44 mL/min/1.73 m<sup>2</sup>, with  $\beta$  estimates of 0.025 (95% C.I. 0.022,0.025;  $P < 0.0001$ ), 0.025 (95% C.I. 0.012,0.032;  $P < 0.0001$ ), 0.037 (95% C.I. 0.008,0.066;  $P = 0.014$ ) respectively. However, after adjusting for age, sex, race, diabetes, hypertension, 25(OH)D levels, and MDRD estimated GFR, serum uric acid and log PTH were significantly correlated only in eGFR groups  $\geq 60$  and 45-59 mL/min/1.73 m<sup>2</sup>, with estimates of 0.020 (95% C.I. 0.016,0.023;  $P < 0.0001$ ) and 0.020 (95% C.I. 0.006,0.034;  $P < 0.0001$ ) respectively. We found no correlation between uric acid and log PTH in eGFR groups 30-44 and 15-29 mL/min/1.73 m<sup>2</sup>. These findings are shown in Table 4. Characteristics of the subjects by GFR category are shown in Table 3.

## Discussion

We report that experimental hyperuricemia suppresses 1- $\alpha$  hydroxylase activity in rats leading to lower 1,25(OH)<sub>2</sub>D levels. In addition, we found that increased serum uric acid is independently associated with increased odds of elevated PTH, defined as an iPTH  $> 65$  pg/mL, in a large number of NHANES participants. Further analysis revealed serum uric acid correlates positively with log PTH in individuals with MDRD- estimated GFR  $< 60$  mL/min/1.73 m<sup>2</sup>. While these finding could potentially be attributed to reduced kidney function and reduced functioning nephron mass, our *in vitro* experiments suggest that uric acid itself suppresses 1- $\alpha$  hydroxylase mRNA and protein expression. This inhibitory effect appears to be mediated by NF $\kappa$ B.

Our findings are consistent with previous studies reporting uric acid to activate NF $\kappa$ B in a variety of cell culture models including proximal tubular cells [26-27], and the identification of NF $\kappa$ B binding element in the 1- $\alpha$  hydroxylase promoter [25]. The potential for a suppressive effect of hyperuricemia on vitamin D metabolism is further supported by reports of lower 1,25(OH)<sub>2</sub>D in patients with gout [18]. In one study of 114 males with gout, treatment of hyperuricemia with either a xanthine oxidase inhibitor (allopurinol) or with a uricosuric agent (benzbromarone) increased 1,25(OH)<sub>2</sub>D with no change in 25(OH)D levels [19]. The observation that lowering serum uric acid via a uricosuric agent increases 1,25(OH)<sub>2</sub>D levels in humans with gout suggests that uric acid inhibits 1- $\alpha$  hydroxylase activity.

In the general population, and in the absence of significant kidney disease, hyperparathyroidism is most commonly due to low 25- hydroxyvitamin D [31]. We, however, show a correlation between uric acid and elevated PTH that is independent of serum 25(OH)D, calcium, and phosphorus suggesting that other mechanisms may underlie this association. Although the exact mechanism underlying the association between high uric acid and high PTH cannot be elucidated from such a cross sectional analysis, our experimental findings suggest that uric acid inhibits 1- $\alpha$  hydroxylase activity by inhibiting 1- $\alpha$  hydroxylase protein expression. Inhibition of 1- $\alpha$  hydroxylase results in lower 1,25(OH)<sub>2</sub>D levels. As such the elevated PTH levels in our study population would be a surrogate marker for lower calcitriol levels.

Of interest is the association between uric acid and PTH in individuals with MDRD- estimated GFR  $< 60$  mL/min/1.73 m<sup>2</sup>. This association is likely driven by the correlation between uric acid and PTH levels in the group with MDRD- eGFR 45-59 mL/min/1.73 m<sup>2</sup>. We found this correlation was independent of GFR, suggesting that hyperuricemia may

affect vitamin D metabolism independently of renal dysfunction. The possibility of a link between uric acid and CKD-MBD was explored by Hsu et al. who showed that sodium urate suppressed the synthesis of 1,25(OH)<sub>2</sub>D when infused into normal rats [16]. Subsequently 9 patients with stable CKD were treated with allopurinol [17]. Daily treatment with allopurinol for 1 week lowered serum uric acid and increased 1,25(OH)<sub>2</sub>D, but had no effect on 25(OH)D levels. Collectively, such data suggest that lowering uric acid could modulate MBD in patients with CKD despite reduced functioning nephron mass.

Our study has several limitations. To induce hyperuricemia, we used the uricase inhibitor, allantoxanamide, and this approach resulted in some tubular injury and in abnormalities in calcium and phosphorus levels. This raises the possibility that the effects seen on vitamin D metabolites are due to the tubular damage rather than the hyperuricemia. However, treatment with febuxostat only partially reversed the biochemical and histological abnormalities in renal function, and yet completely restored 1- $\alpha$  hydroxylase protein expression. This complete restoration of 1- $\alpha$  hydroxylase protein expression coincided with the complete resolution of hyperuricemia in this model despite residual kidney injury. Changes in serum calcium levels were not statistically significant (possibly due to the small number of animals used), but it is plausible that hypercalcemia contributed to lower 1,25(OH)<sub>2</sub>D as higher calcium levels coincided with lower 1,25(OH)<sub>2</sub>D in the febuxostat group in the animal study. Although our *in vitro* data showed that uric acid suppressed 1- $\alpha$  hydroxylase mRNA and protein expression in dose dependent and time course experiments. These data suggest that uric acid itself may have a suppressive effect on vitamin D metabolism. Of note, there are also limitations with the epidemiological cross sectional analysis. Despite our data that uric acid suppresses 1- $\alpha$  hydroxylase experimentally, we cannot exclude other potential mechanisms may be involved in humans. For example, primary hyperparathyroidism has long been associated with hyperuricemia [32-33], and PTH has been shown to stimulate uric acid uptake by the renal cortical tubules of rabbits [34]. In addition, CKD-MBD involves complex interrelationships between calcium, phosphorus, PTH and other cofactors such as fibroblast growth factor -23 that were not characterized in our study. Other limitations include the small number of participants in the more advanced stages of CKD and we cannot be certain that there is no correlation between uric acid and PTH in the more advanced stages of CKD.

While our study and others [17] would suggest that treatment of hyperuricemia may improve vitamin D metabolism in CKD, some studies do not [35], and it is important to examine this question with properly designed studies in CKD. In addition, our study only examines the effect of uric acid on 1- $\alpha$  hydroxylase in the kidney and should not be generalized to extrarenal tissues. The regulation of 1- $\alpha$  hydroxylase protein expression and activity varies depending on the organ and the cell type [14] and some inflammatory pathways have been shown to upregulate 1- $\alpha$  hydroxylase in the endothelium and in monocytes [36-37]. If and how uric acid may modulate vitamin D in such tissues needs to be explored.

Notwithstanding these limitations, our study has several strengths including a combination of cell culture and animal studies supported by an epidemiological study of a large number of participants, where minorities (namely African Americans and Hispanics) are well represented.

In conclusion, acute hyperuricemia suppresses 1- $\alpha$  hydroxylase activity *in vivo* due to reduced 1- $\alpha$  hydroxylase mRNA and protein expression. Our *in vitro* studies suggest that this is mediated by NF $\kappa$ B. Consistent with these findings, increased uric acid is independently associated with an increased odds of abnormally elevated PTH level and correlates significantly with PTH in the general population and in patients with mild to



moderate CKD (eGFR = 45 mL/min/1.73 m<sup>2</sup>). Our results should prompt further investigation into the potential link between uric acid and CKD- MBD.

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## List of Abbreviations

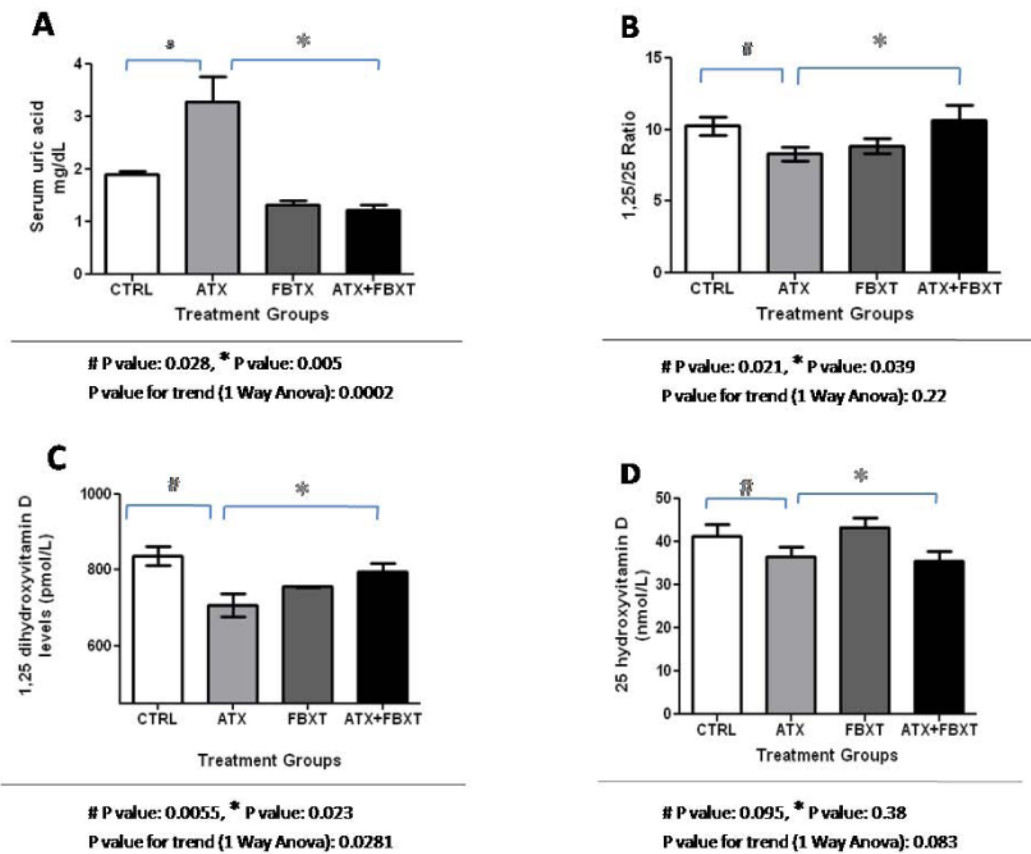
<b>CKD</b>	chronic kidney disease
<b>MBD</b>	Mineral and bone disorder
<b>1,25(OH)<sub>2</sub>D</b>	1,25 dihydroxy-vitamin D
<b>25(OH)D</b>	25 hydroxyvitamin D
<b>PTH</b>	parathyroid hormone
<b>IQR</b>	inter-quartile range
<b>NHANES</b>	National Health and Nutrition Examination Survey
<b>MDRD</b>	Modification of Diet in Renal Disease
<b>GFR</b>	glomerular filtration rate
<b>BMI</b>	body mass index
<b>ATX</b>	allantoxanamide
<b>FBXT</b>	febuxostat
<b>NFκB</b>	nuclear factor κ- B

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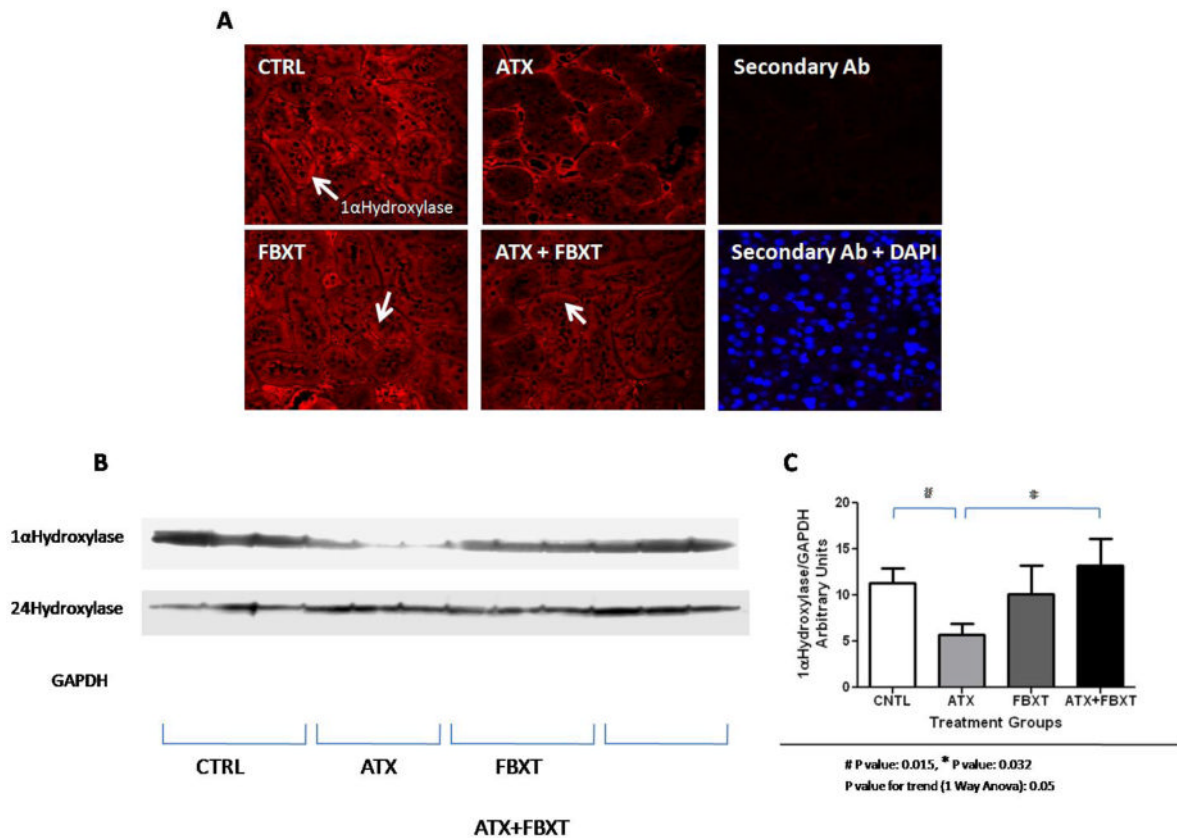
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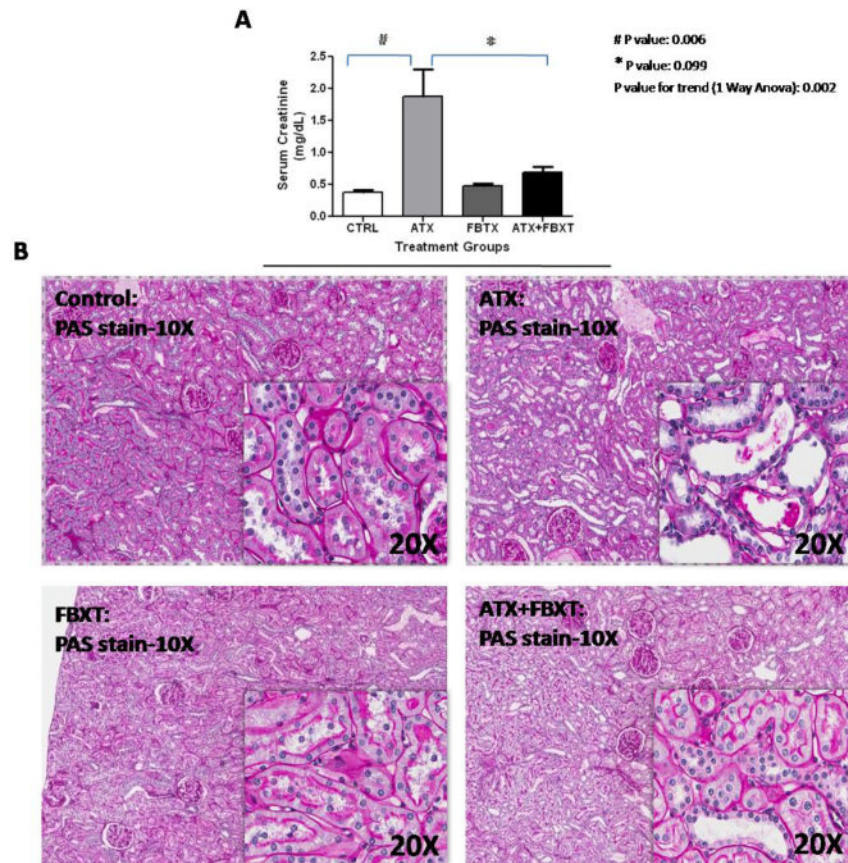
**Figure 1.**

Allantoxanamide successfully induces acute hyperuricemia and significantly inhibits 1- $\alpha$  hydroxylase activity in rats. **Figure 1A:** After 24 hours, mean serum uric acid was  $1.9 \pm 0.1$ ,  $3.3 \pm 1.4$ ,  $1.3 \pm 0.2$ , and  $1.2 \pm 0.3$  mg/dL for the control, allantoxanamide, febuxostat, and allantoxanamide + febuxostat groups respectively. **Figure 1B:** Acute hyperuricemia leads to reduced 1- $\alpha$  hydroxylase activity estimated by the ratio of 1,25(OH)<sub>2</sub>D to 25(OH)D. **Figure 1C:** Mean  $\pm$  SD of 1,25(OH)<sub>2</sub>D levels (pmol/L) in different treatment groups. **Figure 1D:** Mean  $\pm$  SD of 25(OH)D levels (nmol/L) according to treatment group. CTRL: control, ATX: allantoxanamide, FBXT: febuxostat, and ATX + FBXT: allantoxanamide + febuxostat.



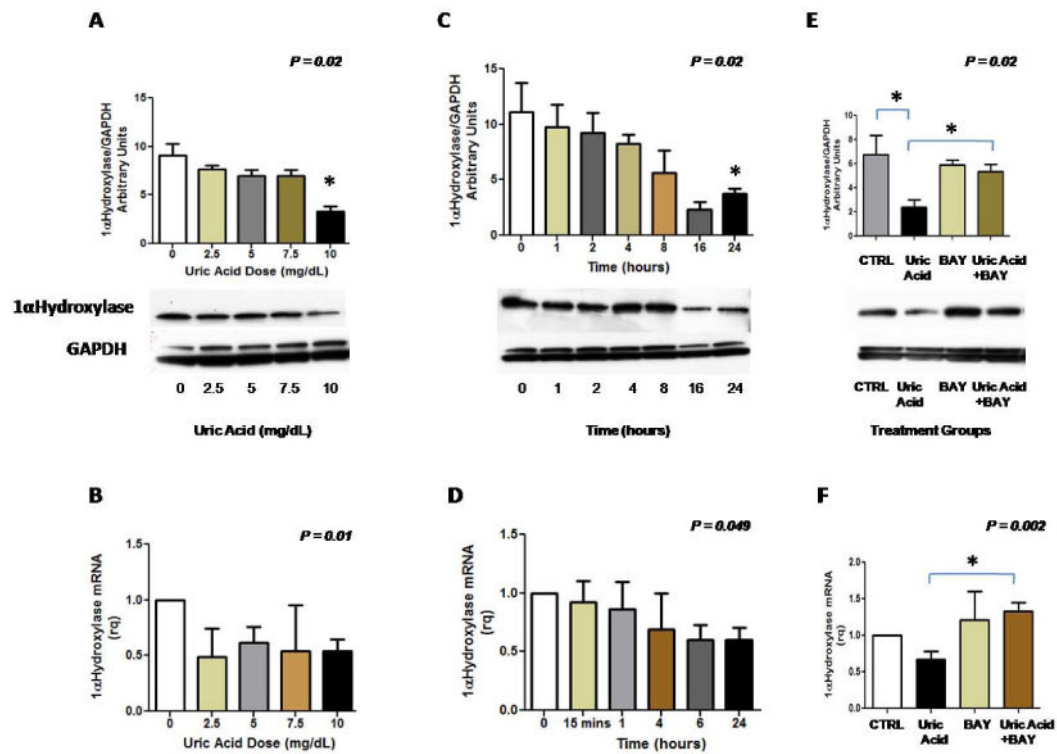
**Figure 2.**

1- $\alpha$  hydroxylase protein expression is significantly reduced in hyperuricemic rats. Acute hyperuricemia had no significant effect on 24-hydroxylase protein expression. **Figure 2A:** Immunofluorescence for 1- $\alpha$  hydroxylase protein according to treatment group. **Figure 2B:** Western blotting for whole kidney lysates showing the expression of 1- $\alpha$  hydroxylase, 24-hydroxylase, and GAPDH proteins. The results shown are representative of all the animals included in the study. **Figure 2C:** Densitometry results comparing 1- $\alpha$  hydroxylase protein expression after normalization to GAPDH protein expression between the different treatment groups. The densitometry summarizes normalized 1- $\alpha$  hydroxylase protein expression for all the animals included in the study. CTRL: control, ATX: allantoxanamide, FBXT: febuxostat, and ATX + FBXT: allantoxanamide. + febuxostat.



**Figure 3.**

Allantoxanamide treatment was associated with increased serum creatinine and tubular dilatation. Serum creatinine for the hyperuricemic group was significantly higher than the control group ( $1.9 \pm 1.2$  versus  $0.4 \pm 0.1$  mg/dL). The acute kidney injury was partially reversed by the xanthine oxidase inhibitor, febuxostat (creatinine  $0.7 \pm 0.2$  mg/dL). **Figure 3A:** Mean  $\pm$  SD of serum creatinine (mg/dL) are shown in the different treatment groups, **Figure 3B:** Allantoxanamide was associated with acute tubular toxicity. CTRL: control, ATX: allantoxanamide, FBXT: febuxostat, and ATX + FBXT: allantoxanamide. + febuxostat.



**Figure 4.**

Uric acid suppresses 1- $\alpha$  hydroxylase mRNA and protein expression in human proximal tubular and inhibition of NF $\kappa$ B ameliorates the suppressive effect of uric acid on 1- $\alpha$  hydroxylase mRNA and protein. **Figure 4A:** 1- $\alpha$  hydroxylase protein expression is increasingly suppressed with increasing treatment dose of uric acid. **Figure 4B:** 1- $\alpha$  hydroxylase mRNA expression is suppressed with increasing treatment dose of uric acid. **Figure 4C:** 1- $\alpha$  hydroxylase protein expression is evident after 16 hours of treatment with uric acid (10mg/dL). **Figure 4D:** 1- $\alpha$  hydroxylase mRNA expression is significantly reduced after 6 hours of uric acid treatment (10mg/dL). **Figure 4E:** Inhibition of NF $\kappa$ B prevents the suppressive effect of uric acid on 1- $\alpha$  hydroxylase protein; Bay 11-7082 dose= 5  $\mu$ M/mL, uric acid dose=10 mg/dL. **Figure 4F:** Inhibition of NF $\kappa$ B prevents the suppressive effect of uric acid on 1- $\alpha$  hydroxylase transcription. Results for each figure represent the findings of 3 independent experiments.

**Table 1**

Serum calcium and phosphorus levels for the *in vivo* model.

	Treatment group			P for trend	
	Control	Allantoxanamide	Febuxostat		Allantoxanamide + Febuxostat
Calcium	8.4 ± 0.9	9.2 ± 1.2	10.0 ± 1.4	9.5 ± 0.9	0.19
Phosphorus	5.9 ± 1.5	8.5 ± 2.6	6.3 ± 1.7	5.8 ± 0.3	0.14

Values are presented as mean ± standard deviation



**Table 2**  
**Clinical Characteristics of the Participants According to Parathyroid Hormone Levels**

Characteristic	Parathyroid Hormone Level		p-value
	65 pg/mL (n=8435)	> 65pg/mL (n=1338)	
Age (years)	45 ± 20	55 ± 21	< 0.0001
Sex (%)			0.28
Male	49	47	
Female	51	53	
Race/Ethnicity (%) *			< 0.0001
Non-Hispanic white	50	42	
Non-Hispanic black	21	31	
Mexican American	25	23	
Other	4	4	
BMI (kg/m <sup>2</sup> )	28 ± 6	31 ± 8	< 0.0001
Diabetes (%)	8.5	12	< 0.0001
Hypertension (%)	28	46	< 0.0001
25-hydroxyvitamin D (ng/mL)	22 ± 10	17 ± 8	< 0.0001
Calcium (mg/dL)	9.5 ± 0.4	9.4 ± 0.4	< 0.0001
Phosphorus (mg/dL)	3.8 ± 0.5	3.7 ± 0.6	< 0.0001
Serum bicarbonate (mmol/L)	25 ± 2	25 ± 3	0.86
Serum uric acid (mg/dL)	5.2 ± 1.4	5.9 ± 1.6	< 0.0001
Estimated GFR (mL/min/1.73m <sup>2</sup> )	98 ± 27	82 ± 31	< 0.0001
Diuretic use (%)	10%	25%	<0.0001
Loop diuretics	2%	12%	
Thiazide diuretics	7%	12%	

Values are expressed as means ± standard deviation or %=percent of patients; BMI=body mass index; GFR=glomerular filtration rate

\* The definition of race/ethnicity can be found at: <http://grants.nih.gov/grants/guide/notice-files/NOT-OD-01-053.html>

Conversion units: uric acid: mg/dL × 59.48 = μmol/L; calcium: mg/dL × 0.25 = mmol/L; vitamin D: pg/mL × 2.5 = pmol/L; phosphorus: mg/dL × 0.32 = mmol/L.

**Table 3**  
**Clinical characteristics of the participants according to stages of chronic kidney disease**

Characteristic	Chronic Kidney Disease Stages MDRD- estimated GFR (mL/min/1.73m <sup>2</sup> )				p-value
	60 N=9001	45-59 N=537	30-44 N=166	15-29 N=48	
Age (years)	43 ± 17	70 ± 11	73 ± 9	70 ± 10	<0.0001
Sex (%)					<0.0001
Male	48	35	36	43	
Female	52	65	64	57	
Race/Ethnicity (%)					<0.0001
Non-Hispanic white	48	73	69	50	
Non-Hispanic black	23	10	20	23	
Mexican American	25	13	6	23	
Other	4	4	5	4	
BMI (kg/m <sup>2</sup> )	29 ± 7	29 ± 5	30 ± 6	31 ± 6	0.003
Diabetes (%)	7	21	36	51	<0.0001
Hypertension (%)	25	67	72	8	<0.0001
25-hydroxyvitamin D (ng/mL)	22 ± 10	22 ± 8	20 ± 7	18 ± 7	0.004
Estimated GFR (mL/min/1.73m <sup>2</sup> )	99 ± 24	53 ± 4	38 ± 3	24 ± 3	<0.0001
Serum uric acid (mg/dL)	5.2 ± 1.4	6.2 ± 1.3	7.2 ± 1.2	7.9 ± 2.3	<0.0001
% with PTH >65 pg/mL	11	31	54	88	<0.0001

Values are expressed as means ± standard deviation or %—percent of patients; BMI=body mass index; GFR=glomerular filtration rate

**Table 4**  
**Linear regression analysis showing  $\beta$  estimates (95% CI) for the association between serum uric acid and log transformed PTH levels across stages of chronic kidney disease**

	Chronic Kidney Disease Stages MDRD- estimated GFR (mL/min/1.73m <sup>2</sup> )			
	60 N=9001	45- 59 N=537	30-44 N=166	15-29 N=48
Unadjusted (95% CI)	0.025 (0.022, 0.025) P<0.0001	0.025 (0.012, 0.032) P<0.0001	0.037 (0.008, 0.066) P=0.014	0.022 (-0.011, 0.055) P=0.19
Adjusted * (95% CI)	0.020 (0.016, 0.023) P<0.0001	0.02 (0.006, 0.034) P=0.003	0.02 (-0.009, 0.049) P=0.17	0.026 (-0.01, 0.065) P=0.22

\* Parsimonious model: age, sex, race/ethnicity, diabetes, hypertension, body mass index, 25(OH)D, and MDRD- estimated GFR.