Relationship of total and free 25-hydroxyvitamin D to biomarkers and metabolic indices in healthy children

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Precis: We compared total 25-OHD and various free 25-OHD measures as indicators of vitamin D status and conclude that free 25-OHD does not improve upon total 25-OHD as a clinical measure in healthy children.

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ABSTRACT

Context: Vitamin D status is usually assessed by serum total 25-hydroxyvitamin D. Whether free 25-hydroxyvitamin D measures better correlate with various clinical outcomes is unclear.

Objective: To identify correlations between total 25-hydroxyvitamin D (t25-OHD), calculated and direct measures of free 25-OHD, and to identify associations of these measures with other outcomes in children, across the 6 common *GC* haplotypes.

Design: Healthy urban-dwelling children underwent measurement of relevant variables.

Setting: Academic medical center

Participants: 203 healthy, urban-dwelling children, 6 months to 10 yrs old, predominantly of Hispanic background and representative of all common *GC* haplotypes.

Intervention: None

Main Outcome Measures: Total and free 25-OHD and $1,25(OH)_2D$, calcium, phosphate, PTH, glucose, insulin, aldosterone, and renin.

Results: Mean t25-OHD [26.3±6.7ng/ml; 65.8±16.8nmol/L] were lowest in the *GC*2 genotype. Mean $t1,25(OH)_2D$ [57.6±16.5pg/ml; 143.9±41.3pmol/L], were lowest in *GC*1f/1f, *GC*1f/2, and *GC*2/2 groups. T25-OHD correlated strongly with calculated free 25-OHD (cf25-OHD) (r=0.89) and moderately with directly measured free 25-OHD (dmf25-OHD) (r=0.69). Cf25-OHD correlated with dmf25-OHD (r=0.69) ($p<0.001$ for all). t25-OHD inversely correlated with BMI (r=-0.191; p=0.006), skin reflectometry, and systolic blood pressure. T25-OHD correlated with fasting insulin and HOMA-IR, however significance for these correlations were not evident after adjustment for BMI. PTH inversely correlated with all measures of 25-OHD, but most strongly with t25-OHD.

Conclusions: Measure of circulating total and free 25-OHD are comparable measures of vitamin D status in heathy children. Correlations are similar with other outcome variables, however t25-OHD remains the strongest correlate of circulating PTH and other variables. These data argue against routine refinement of the t25-OHD measure using currently available assessments of free 25-OHD.

INTRODUCTION

Assessment of vitamin D status has been widely employed in clinical settings to guide nutritional supplementation for optimal bone health. More recently, broader health concerns have been reported to be associated with vitamin D status, including risk for colon cancer (1), progression of multiple sclerosis (2), obesity, and metabolic syndrome (3), although vitamin D supplementation has not had marked effects on the outcomes of these conditions (4). In clinical practice vitamin D stores have most frequently been assessed by measurement of the most-abundant circulating vitamin D metabolite, 25-hydroxyvitamin D (25-OHD), in serum or plasma. Two major isoforms, 25-OHD_2 and 25-OHD_3 comprise the total 25-OHD, and a new generation of LC/MS/MS methodologies allow for specific quantification of the concentration of each of these two isoforms. The most frequently employed vitamin D assays have assessed the "total" circulating 25-OHD, comprised of the fraction bound to the plasma proteins albumin and vitamin D binding protein (DBP), also known as "group-specific complement" or GC as well as the unbound or "free" fraction (5). Refinement of total 25-OHD assays for more precise clinical utility has been considered a potentially valuable addition to the clinical chemistry armamentarium. Methodologic development and standardization of available assays are ongoing practices. Moreover, there has been a long-standing dialog regarding the consideration that the concentration of free, unbound 25-hydroxyvitamin D fraction may better represent functional availability of vitamin D for further metabolism or action (6). Thus, assessment of free 25-OHD has been proposed as a means to improve the clinical significance of the estimation of an individual's vitamin D status. Calculated estimates of free 25-OHD concentrations have generally relied upon formulas incorporating the circulating concentrations of total 25-OHD, albumin and DBP, and the affinity constants of 25-OHD for DBP and albumin, respectively (7). This approach was employed for years in related fields, as witnessed by the use of calculated free thyroxine measures in the clinical evaluation of thyroid disorders, however direct measures are now frequently employed. Similarly, various estimates of circulating testosterone are often utilized, including assays for free, total, and bioavailable testosterone (8), and the adoption of free prostate-specific antigen

(PSA) measures have been added recently to the urological clinical testing battery (9). Attention to the "free-hormone hypothesis" (10, 11) in the vitamin D field has generated studies that suggest that calculated free 25-OHD levels (based on the circulating vitamin D binding protein and albumin levels) are better correlates of such clinical outcomes as bone mineral density (7).

Further complexity of this system is evidenced by natural genetic variation in *GC*. Six major species of *GC* are found in humans, due to haplotype differences resulting in common amino acid variants at residues 243 and 246 in the DBP protein (5). We and others have shown that circulating concentrations of DBP vary across the 6 different haplotypes, as well as circulating concentrations of (total) 25-OHD (7, 12). As each of the six major species differ structurally, it is possible that different 25-OHD binding affinities may exist for each. However, this molecular variance occurs at residues distant from the vitamin D binding site of the molecule thus reducing the likelihood of this consideration (5). Nevertheless, it has been proposed that calculation of free 25-OHD, when using a composite affinity constant averaged across all GC species, may not accurately represent the affinity of a given individual's DBP for 25-OHD, and that calculated free 25-OHD using an affinity constant specific for the subject's haplotype would provide a more accurate estimate of the true free 25-OHD concentration (13). Finally, newer assays providing direct measurement of free 25-OHD offers yet another means of determining this measure (14). Therefore we have employed both direct and calculated approaches to determination of free 25-OHD in this study.

With regards to assessment of vitamin D status in the context of bone and mineral homeostasis, numerous reports have demonstrated correlations of circulating 25-OHD levels with indices of a variety of disorders and metabolic parameters (For review see 15). Most of these studies have investigated adult cohorts, and a paucity of related data is available in children. We previously

identified demographic, dietary, and biochemical correlates of total 25-OHD levels in a large cohort of inner city children, predominantly of Hispanic ethnicity, and found strong relationships of vitamin D status with *GC* haplotype (12). Here we extend this work to explore the clinical utility of total and free circulating 25-OHD measures as correlates of markers of bone and mineral status and additional metabolic parameters including circulating levels of glucose, insulin, aldosterone, and renin. Directly measured free 25-OHD was performed, as well as calculated free 25-OHD using both a single DBP affinity constant for 25-OHD for all DBP haplotypes, and haplotype-specific affinity constants. Finally, we examined whether any associations identified between various measures of 25-OHD and the potentially related variables in the overall population similarly occurred within individuals in each of the six common *GC* haplotypes.

METHODS

Subjects and Study design

Healthy, urban-dwelling children, aged 6 months to 10 years, were recruited to participate in a vitamin D supplementation study. Families were from neighborhoods in New Haven, Connecticut reflecting a largely Hispanic community. Participating families from prior observational studies were contacted, and postings on www.clinicaltrials.gov (NCT01050387), in local medical offices and other community sites served as additional means of recruitment. Children were excluded from participation if there was any history of bone and mineral disorders or any use of medications known to affect vitamin D metabolism (e.g., systemic glucocorticoids, pharmacologic vitamin D metabolites, or vitamin D supplements in excess of 400 IU/d). The study was approved by the Yale University Human Investigation Committee and written informed consent was obtained from the appropriate parent or guardian.

Data Collection

A visit to our out-patient research center was arranged where height, weight, and blood pressure were measured. Erythematous and melanin-based components of skin tone were assessed at forehead and axillary sites using a Cortex DSM II reflectometer (16). A fasting blood sample was obtained for measurement of vitamin D metabolites, DBP, and biochemical variables as detailed below. DNA for determination of *GC* haplotype was obtained. Ultrasound determination of Speed of Sound (SOS) was determined at the radius using the Sunlight Pediatric Ultrasound device, as described previously (17). Baseline clinical and biochemical data were obtained prior to beginning the supplementation phase of the study and provided the data for the current report.

Analytical Methods

Biochemical assays

Serum calcium was performed by the Clinical Chemistry Laboratory at Yale-New Haven Hospital using a Roche Diagnostics DPP modular autoanalyzer. Serum phosphorus, albumin and alkaline phosphatase activity were performed using standard colorimetric methods with an Alfa Wasserman autoanalyzer, by the Yale Center for Clinical Investigation Core Laboratory. Serum glucose was measured using a Nova Strip Glucose meter (Nova Biomedical, Waltham, MA). Total serum 25-OHD and 1,25$(OH)_2$D were measured by radioimmunoassay kit methodology (DiaSorin, Stillwater, MN). Results of samples analyzed in our 25-OHD assay are consistently found to agree with the mid-range of outcomes of those using this assay in the international DEQAS standardization system (18). The inter- and intra-assay coefficients of variation for the 25-OHD assay in our hands are 9.6% and 6.6 %, respectively. Serum DBP was determined by previously described radial immunodiffusion methodology in the laboratory of one of the authors (19). Serum PTH was measured with antisera to the mid-region of human PTH using the ^{125}I-labeled 43-68 residue fragment of human PTH as

radioactive trace, as described (20); inter- and intra-assay coefficients of variation are 13.1% and 15.0 %, respectively.

Serum osteocalcin was measured by an in-house ELISA method which recognizes intact osteocalcin and the 1-43 major fragment with equimolar reactivity (21). The bone formation marker, serum N-terminal propeptide of type 1 collagen (P1NP), was measured using an ELISA-based kit (Immunodiagnostic Systems; Gaithersburg, MD), as was the bone resorption marker, serum C-terminal telopeptide of type I collagen (CTX). Serum intact FGF23 was measured using an ELISA-based kit (Kainos Laboratories; Tokyo, Japan). The vitamin D associated cytokines CCL13 and cathelicidin (LL37) were measured in serum using ELISA kits from R&D Systems (Minneapolis, MN) and Hycult Biotech (Plymouth Meeting, PA), respectively. Plasma aldosterone and renin were measured by RIA (Diagnostic Systems Laboratories; Webster, TX). Plasma insulin was measured by RIA using a kit from EMD Millipore (Billerica, MA). Hemoglobin $A1_C$ was assessed using a DCA Vantage analyzer (Siemens, USA).

Calculated free 25-OHD (cf25-OHD) and calculated free $1,25(OH)_2D$ [cf$1,25(OH)_2D$] were calculated using serum DBP and albumin concentrations, and their reported dissociation constants for these vitamin D metabolites respectively (7). Genotype-specific free 25-OHD (gsf25-OHD) was calculated using 25OHD/DBP dissociation constants specific for each individual's haplotype (13). Direct measurement of free 25-OHD (dmf25-OHD) was performed using an ELISA-based kit (DIAsource ImmunoAssays; Louvain-la-Neuve, Belgium). HOMA-IR was calculated according to the formula: [Glucose (nmol/L) x Insulin (uU/mL) / 22.5] (22).

Genotype analysis

The p.D432E (rs7041) and p.T436K (rs4588) SNPs of *GC*/DBP were genotyped with phase assignment based on allele-specific amplification of the p.T436K site followed by restriction endonuclease digestion of the p.D432E site, as previously described (12). As there were no recombinants between the two polymorphic loci, assignment of a diplotype for each subject, based on the three haplotype alleles – wild type (electrophoretic variant 1f), mutant 432E (electrophoretic variant 1s), and mutant 436K (electrophoretic variant 2) – was unambiguous.

Statistical analysis

Descriptive statistics were used to summarize the data. Circulating 25-OHD measures and metabolic indices (glucose, insulin, hemoglobin A1c, HOMA-IR) expected to be affected by BMI were analyzed with and without adjustment for BMI, which has been repetitively shown to be significantly associated with total 25-OHD (t25-OHD) (23). Procedural means, frequencies for continuous and categorical variables, and univariate regression were computed (SAS software version 9.2). Parametric or non-parametric (ANOVA or Kruskal-Wallis) testing with multiple comparison testing was performed using GraphPad, PRISM® Software v8.00. Correlation analysis was performed and as the distributions of nearly all measures were not normal, the non-parametric Spearman correlation coefficients were calculated.

RESULTS

After obtaining appropriate consent, we enrolled 225 healthy children, aged 7 months to 10 years from a largely Hispanic/Latino background (74% of subjects). Sample collection was successful in 203 of the children. Other characteristics of the study subjects are shown in Table 1.

Measures of vitamin D metabolites

The mean total circulating 25-OHD values for the entire cohort was 26.3 ± 6.7 ng/ml [65.8 ± 16.8 nmol/L] (mean +/- SD). Values for the entire group and for each *GC* haplotype group are shown in Figure 1A. Kruskal-Wallis analysis showed that differences were apparent across the haplotypes ($p < 0.002$), and multiple comparison testing showed that values for the *GC* 1s/1s haplotype were significantly greater than those for the *GC* 1f/1s and *GC* 2/2 haplotypes ($p < 0.005$ and $p = 0.012$ respectively). As shown previously (11) groups containing the "K" allele (*GC* 2 groups) were lower than others. Mean serum DBP for the entire group was 291 ± 31 ug/ml (Figure 1B) and as with total 25-OHD, significant differences were observed across the haplotype groups ($P < 0.002$). As shown previously, serum DBP levels were lower in those groups containing a *GC 2* ("K") allele, and greatest for those homozygous for the *GC* 1s allele.

Values for cf25-OHD, using constant affinity across haplotypes, and the values using the haplotype specific constants (gsf25-OHD) are shown in Figures 2A and 2B, respectively. Mean values for the entire cohort are comparable between the two methods (6.8 ± 2.7 pg/ml utilizing the single affinity constant vs. 6.9 ± 2.7 pg/ml, utilizing the haplotype-specific constants). Values across haplotypes were comparable for cf25-OHD ($p > 0.50$). Values for dmf25-OHD determined by direct measurement are shown in Figure 2C for each *GC* haplotype group, and had comparable relative values across haplotype groups ($p = 0.093$), as was shown for the cf25-OHD and t25-OHD levels. When the haplotype-specific affinity constants are used (Figure 2B), a different pattern emerges with significant differences across the haplotype groups ($p < 0.001$); relatively low values were observed in the *GC1f/1f* group, and relatively high values observed in the *GC2/2* group.

Values for total $1,25(OH)_2D$ [$t1,25(OH)_2D$] and $cf1,25(OH)_2D$ levels are shown in Figure 3A and 3B respectively. Mean $t1,25(OH)_2D$ for the entire cohort is 57.6 ± 16.5 pg/ml [143.9 ± 41.3 pmol/L] (Figure 3A). For $t1,25(OH)_2D$, minor, but significant differences existed across haplotype groups ($P < 0.001$) with the lower values observed in the *GC1f/1f, GC1f/2*, and *GC2/2* groups. Specifically the *GC 1f/1s* group was greater than the 3 groups noted above with the lowest values ($P < 0.02$). Mean $cf1,25(OH)_2D$ for the entire cohort was 0.24 ± 0.07 pg/ml [0.60 ± 0.18 pmol/L] (Figure 3B), and minor differences were apparent across haplotype groups ($P = 0.026$). The only significant pairwise difference was observed between *GC1f/1s* and *GC1s/1s* groups, suggesting slightly lower values for values in the *GC1f/1f* compared to the other groups, consistent with findings for $t1,25(OH)_2D$. As no DBP haplotype specific binding constants are available for $1,25(OH)_2D$, only a composite affinity constant was employed for these calculations.

Correlation analysis

Correlation among 25-OHD measures

Circulating t25-OHD correlated strongly with cf25-OHD ($r=0.89$, $p<0.001$), and moderately with dmf25-OHD ($r=0.69$, $p<0.001$) (24), but somewhat less so with gsf25-OHD ($r=0.47$, $p<0.001$). Cf25-OHD also was moderately correlated with dmf25-OHD ($r=0.69$, $p<0.001$) (24), and again, somewhat less so with gsf25-OHD ($r=0.58$, $p<0.001$). Dmf25-OHD also correlated with gsf25-OHD ($r=0.41$, $p<0.001$).

Other clinical and biochemical correlates of 25-OHD

Associations were determined between each of the four measures of circulating 25-OHD (total, calculated free, directly measured free, and haplotype-specific calculated free) and the clinical,

metabolic, and bone-related variables are shown in Table 2. For clinical variables, t25-OHD was inversely correlated with BMI (r = -0.191; p= 0.006), melanin-dependent and erythema-dependent measures of skin reflectometry, as expected, and also with systolic blood pressure. This pattern was generally true for associations with cf25-OHD and gsf25-OHD, however correlation of gsf25-OHD with BMI was weaker, and not of statistical significance, and an association with systolic blood pressure was not evident (r = -0.05). Overall, the gsf25-OHD had poorer correlation with most of the end-points in this study than either dmf 25-OHD or cf25-OHD.

For the metabolic variables, serum insulin and HOMA-IR were significantly correlated in inverse manner with all four 25-OHD measures. We further analyzed this relationship in a regression analysis as to ascertain whether these findings primarily represented an effect of BMI on insulin and HOMA-IR; the significance of these correlations was eliminated when adjusting for BMI (P = 0.147 for insulin; P = 0.069 for HOMA-IR). There were no significant correlations between hemoglobin A1c and any of the 25-OHD measures. Circulating aldosterone levels correlated positively with all measures of 25-OHD, but did not achieve statistical significance for cf25-OHD. Similar findings were seen for plasma renin activity, although associations were weaker for this measurement. In contrast to insulin and HOMA-IR, adjusting for BMI effects did not alter the positive correlations of aldosterone and renin with 25-OHD measures.

Correlation analyses of all 25-OH vitamin D measures and bone-related variables revealed that all measures of 25-OHD correlated inversely with serum PTH, as expected, and the strongest relationship was with t25-OHD. Dmf25-OHD correlated with circulating cathelicidin and CCL13 levels, and very weak negative correlations of CTX and P1NP with the gsf25-OHD level, however the significance of these weak correlations is not clear. There were no other significant correlations of

25-OHD with cytokines or other bone and mineral biomarkers, and no relationship to ultrasound determination of SOS at the radius.

Correlates within each GC haplotype subgroup

We extended this analysis to determine if patterns of any significant correlations identified for the overall group were evident in each of the haplotype groups, which would represent structural differences in DBP. Associations of t25-OHD with variables that significantly correlated with t25-OHD in the overall group were similar within most haplotype groups although the sample size within each group was too small for these to achieve statistical significance (24). Inverse associations persisted between t25-OHD and insulin, HOMA-IR, PTH, BMI, height Z-score, and systolic blood pressure within these groups with the minor exceptions of BMI and height Z-score in the *GC*1s/1s subgroup. Positive associations persisted between t25-OHD and aldosterone and plasma renin activity within these groups with the minor exception for aldosterone in the *GC*1s/2 subgroup.

Correlates of $1,25(OH)_2D$

Circulating $t1,25(OH)_2D$ correlated strongly with $cf1,25(OH)_2D$ ($r=0.94$, $p<0.001$). Neither $t1,25(OH)_2D$ nor $cf1,25(OH)_2D$ levels correlated with any of the clinical or metabolic variables examined (Table 3). In contrast,associations between both measures of $1,25(OH)_2D$ and several bone-related variables were evident. Both $t1,25(OH)_2D$ and $cf1,25(OH)_2D$ levels were directly correlated with serum phosphorus, and inversely correlated with the cytokines CCL13 and cathelicidin (LL37). There were modest inverse correlations of $t1,25(OH)_2D$ and the bone turnover markers osteocalcin and CTX. Overall, total and $cf1,25(OH)_2D$ did not show substantively different relationships with the variables analyzed.

DISCUSSION

Results of this study suggest that both total and calculated free 25-OHD levels are associated with several metabolic variables in childhood, however calculated methods for free 25-OHD only modestly differ from total circulating 25-OHD as a marker for these measures. Nor did assessment of directly measured free 25-OHD substantially improve the nature of these correlations. Furthermore, we found comparable associations between vitamin D status and those associated variables within all of the six *GC* haplotype groups. Thus, our data do not support the notion that circulating free 25-OHD measures are more robust markers of disease than standard total 25-OHD concentrations. Our study participants were healthy children, largely of Hispanic descent, living in northern US latitudes, and perhaps at relatively higher risk for vitamin D deficiency than in other populations.

Improving assessment and interpretation of clinical vitamin D status has been a substantial clinical concern over the past few decades. The considerable variability in assay results has led to international standardization efforts (18) and more recently, to the adoption of NIST standards for 25-OHD_2 and 25-OHD_3 (25). The free hormone hypothesis, that free 25-OHD is the moiety accessible to cellular uptake and physiologic function has informed the prevalent view (10), in which the protein bound compartment represents a secondary reservoir of total stores, but not a biologically active complex. Alternative hypotheses have included the possibility that cellular uptake of vitamin D metabolites may be facilitated by DBP (6), thus indicative of a vitamin D-related functional role for DBP beyond that of a carrier protein.

Recent studies have shown differing DBP circulating concentrations across the common genetic haplotypes of *GC* in both adults (7) and children (12). Moreover, although one study has shown that

DBP binding affinity for 25-OHD may differ among the common DBP variants (13), most other studies have not found major differences in affinity (6). Finally, the recent report of a woman with biallelic deletion of *GC* has shed light on this issue with respect to the extremely low circulating t25-OHD concentrations occurring in the absence of any typical features of vitamin D deficiency (26). These recent findings support the notion that relatively (very) low concentrations of free 25-OHD are adequate to maintain normal vitamin D status under most conditions. This observation, in fact, mirrors the description in an earlier report of the of *Gc*-null murine model of DBP deficiency, where biochemical manifestations of vitamin D deficiency were not apparent in the setting of very low circulating levels of t25-OHD (27).

Our data provide an instructive comparison of total, calculated free, and directly measured free 25-OHD levels in a large childhood cohort representing the six common *GC* haplotypes. These measures are inherently correlated with one another, with the strongest correlation between t25-OHD and cf25-OHD levels. In confirmation of an earlier study of DBP effects in a larger childhood cohort, we observed that DBP and t25-OHD levels were slightly lower in haplotype groups incorporating the *GC2* ("K") allele (12). Moreover, total 25-OHD is a robust inverse correlate of several clinical measures, including dermal reflectometry measures, BMI, and systolic (but not diastolic) blood pressure. It is of interest that this well-described inverse correlation between BMI and 25-OHD may be explained, in part, by the down regulation of the *Cyp2r1* enzyme encoding the major vitamin 25-hydroxylase enzyme in obese mice (28). In our cohort, the inverse associations of 25-OHD measures with insulin and HOMA-IR were explained by the association with BMI. Weak positive associations of 25-OHD measures with circulating aldosterone levels and plasma renin activity were not explained by BMI and suggest that there may be inherent vitamin D relationships to these metabolic actors in early life. These findings differ somewhat from those identified in an adult hypertension/heart-failure cohort, in which vitamin D supplementation did not affect circulating renin or aldosterone

levels, however in a subset of patients with low baseline 25-OHD there was a mild decrease in renin (29). Furthermore, Cyp27b1 (vitamin D 1α hydroxylase) null mice have been shown to have elevated levels of circulating aldosterone and renin, which correct with administration of $1,25(OH)_2D$ (30, 31). The only bone-related parameter that consistently associated with all measures of 25-OHD was, as might be expected, serum PTH levels. Refinements of the t25-OHD measure by calculating or directly measuring the free fraction of this metabolite did not substantially improve the overall clinical utility of the measure. Moreover the use of separate affinity constants for each DBP haplotype did not improve correlations and indeed for the most established correlate, PTH, the relationship was weaker for gscf25-OHD than the other measures of free 25-OHD (cf25-OHD and dmf25-OHD) as well as t25-OHD, as has been previously suggested (6).

In contrast, measures of $1,25(OH)_2D$ were most strongly correlated with serum phosphate, perhaps reflecting strong effects on intestinal absorption of this mineral in childhood. Indeed, in some clinical contexts (e.g., phosphate deprivation) an inverse correlation between serum phosphate and $1,25(OH)_2D$ would be expected in view of the phosphate/FGF23 effects on the vitamin D hydroxylases. Inverse correlations with the cathelecidin and CCL13, cytokines related to vitamin D status were found, but are not typical of the relationships described in adulthood, when $1,25(OH)_2D$ has been reported as a stimulus for cathelicidin production (32). As with 25-OHD, calculation of the free fraction of $1,25(OH)_2D$ did not substantially alter relevant biologic correlates of the total $1,25(OH)_2D$ measure. Finally, the direction of change for variables that correlated with 25-OHD or $1,25(OH)_2D$ was generally comparable in this small study, when examining separately each of the haplotype groups. However, a limitation of this analysis is the potentially confounding effect introduced by the relatedness of subjects. There were 40 families with sibling participation in which 2 children shared the same haplotype.

In conclusion, this study compares various measures of vitamin D in a childhood population. The data argue that the total circulating 25-OHD level is an adequately robust clinical tool, at least as applied to healthy children, and various assessments of the free fraction do not substantially enhance clinical utility of the measure. In addition, assessment of calculated free $1,25(OH)_2D$ offers no significant advantage over total $1,25(OH)_2D$ measures. Moreover, the relationship of vitamin D measures to various biological variables are generally comparable across the common *GC* haplotypes with modest differences. All of these findings would support the contention that total 25-OHD serves as a reasonable routine measure of vitamin D status, at least in healthy children, and more refined measures of the free fraction should be reserved for special situations, such as case of abnormally low or high DBP concentrations and certainly in the rare case of suspected deficiency of DBP.

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FIGURE LEGENDS

FIGURE 1: Box-and-whisker plots for the entire cohort (shaded boxes on left) and of each of the 6 common *Gc* haplotype groups for total circulating 25-OHD (panel A) and vitamin D binding protein (panel B). Upper and lower extremes of the whiskers represent maximum and minimum values; upper and lower borders of the boxes represent 75th and 25th centile values, and the middle bars represent the median value. Significantly different groups by multiple comparison testing are indicated by brackets; * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

FIGURE 2: Box-and-whisker plots for the entire cohort (shaded boxes on left) and of each of the 6 common *Gc* haplotype groups for calculated free (cf) 25-OHD (panel A), genotype specific calculated free (gscf) 25-OHD (panel B), and directly measured free (dm) 25-OHD (Panel C). Upper and lower extremes of the whiskers represent maximum and minimum values; upper and lower borders of the boxes represent 75th and 25th centile values, and the middle bars represent the median value. Differences across haplotypes were not evident for cf 25-OHD and dm 25-OHD. Significant differences by multiple comparison testing across haplotype groups for gscf 25-OHD are indicated by brackets; * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

FIGURE 3: Box-and-whisker plots for the entire cohort (shaded boxes on left) and of each of the 6 common *Gc* haplotype groups for total circulating $1,25(OH)_2D$ (t1,25D) (panel A) and calculated free $1,25(OH)_2D$ (panel B). Upper and lower extremes of the whiskers represent maximum and minimum values; upper and lower borders of the boxes represent 75th and 25th centile values, and the middle bars represent the median value. Significantly different groups by multiple comparison testing are indicated by brackets; * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

TABLE 1. CHARACTERISTICS OF STUDY SUBJECTS

Age N=203	5.6±2.3 yrs
Race/Ethnicity N=203	Hispanic/Latino= 156 (76.8%) African American= 29 (14.3%) Caucasian= 1 (0.5%) Other*= 16 (7.9%) Unreported**= 1 (0.5%)
Sex N=203	Male= 102 Female= 101
Height N=202	112.7±16.3 cm
Height z-Score N=196	0.86±1.25
Weight N=203	24.3±11.8 Kg
BMI N=202	18.1±4.2 Kg/m^2

Values are mean ± standard deviation.

TABLE 2. CORRELATES OF MEASURES OF 25-HYDROXYVITAMIN D

Clinical Variables							
	BMI	Axill-E	Axill-M	Forehd-E	Forehd-M	Systolic BP	Diastolic BP
T25D n	202	200	200	201	201	193	189
r	-0.19**	-0.19**	-0.22**	-0.17*	-0.19**	-0.16*	-0.5
cf 25D n	180	178	179	179	179	172	168
r	-0.21**	-0.16*	-0.20**	-0.15*	-0.20**	-0.22**	-0.14
gscf 25D n	180	178	179	179	179	172	168
r	-0.09	-0.30***	-0.34***	-0.19**	-0.27**	-0.05	0.02
dmf 25D n	197	195	196	196	196	188	184
r	-0.30***	-0.16*	-0.14	-0.18**	-0.15*	-0.23**	-0.1

Metabolic Variables							
		Glucose	Insulin	HOMA-IR	HgbA1c	Aldosterone	Renin
T25D	n	182	189	170	196	197	187
	r	-0.11	-0.27**	-0.23**	-0.01	0.16*	0.15*
cf 25D	n	163	171	152	177	180	169
	r	-0.19*	-0.38***	-0.36***	-0.04	0.13	0.09

gscf 25D	n	163	171	152	177	180	169
	r	-0.08	-0.22**	-0.23**	-0.01	0.20**	0.16*
dmf 25D	n	177	185	166	191	192	185
	r	-0.18*	-0.33***	-0.32***	0.02	0.23**	0.12

Bone Related and Immunologic Variables										
		Ca	Phos	AlkPhos	PTH	FGF23	OC	CTx	P1NP	Cathd	CCL13
T25D	n	200	193	195	201	197	197	198	198	190	190
	r	0.09	0.07	-0.07	-0.28***	0.07	-0.14	-0.02	-0.01	0.04	0.64
cf 25D	n	180	176	178	180	179	179	180	180	172	170
	r	0.01	0.03	-0.05	-0.20**	0.04	-0.08	0.03	0.03	0.05	0.06
gscf 25D	n	180	176	178	180	179	179	180	180	172	170
	r	-0.05	0.10	-0.11	-0.15*	-0.01	-0.13	0.15*	-0.15*	0.07	0.07
dmf 25D	n	194	188	190	196	191	192	193	193	185	184
	r	-0.02	0.01	-0.04	-0.24***	0.02	-0.11	0.04	-0.04	0.24**	0.20**

Sample size (n) and Pearson correlation coefficient, r, are shown in rows for each pair of variables listed. P value for the significance of the correlation are denoted as follows: * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Abbreviations: *T25D*, total 25-hydroxyvitamin D; *cf 25D*, calculated free 25-hydroxyvitamin D; *gscf 25D*, genotype-specific calculated free 25-hydroxyvitamin D; *dmf 25D*, directly measured free 25-hydroxyvitamin D; *BMI*, body mass index; *Axill-E*, erythema-dependent component of skin tone, axillary site; *Axill-M*, melanin-dependent component of skin tone, axillary site ; *Forehd-E*, erythema-dependent component of skin tone, forehead site; *Forehd-M*, melanin-dependent component of skin tone, axillary site; *BP*, blood pressure; *HOMA-IR*, homeostatic model assessment for insulin resistance; *Hgb A1c*, hemoglobin A1c; *Ca*, calcium; *Phos*, phosphate; *alk phos*, alkaline phosphatase; *PTH*, parathyroid hormone; *FGF23*,

fibroblast growth factor 23; *OC*, osteocalcin; *CTx*, c-terminal telopeptide type I collagen; *P1NP*, procollagen type I propeptide; *Cathd*, cathelicidin; *CCL13*, chemokine ligand 13.

TABLE 3. CORRELATES OF MEASURES OF 1,25-DIHYDROXYVITAMIN D

Metabolic Variables							
		Glucose	Insulin	HOMA-IR	HgbA1c	Aldosterone	Renin
T 1,25D	n	172	187	169	186	187	185
	r	-0.04	-0.08	-0.08	0.07	0.04	0.11
cf 1,25D	n	155	170	152	168	171	167
	r	-0.09	-0.13	-0.12	-0.09	0.02	0.07

Bone Related and Immunologic Variables											
		Ca	Phos	AlkPhos	PTH	FGF23	Ostcln	CTx	P1NP	Cathd	CCL13
T 1,25D	n	189	183	185	188	187	187	188	188	188	183
	r	0.04	0.24**	0.02	-0.07	0.02	-0.15*	-0.15*	0.03	-0.21**	-0.27***
cf 1,25D	n	171	167	169	171	170	170	171	171	170	165
	r	-0.01	0.22**	0.05	0.01	0.02	-0.14	-0.13	0.04	-0.23**	-0.28**

Sample size (n) and Pearson correlation coefficient, R, are shown in rows for each pair of variables listed. P value for the significance of the correlation are denoted as follows: * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Abbreviations: *T 25D*, total 1,25-dihydroxyvitamin D; *cf 1,25D*, calculated free 1,25-dihydroxyvitamin D; *BMI*, body mass index ; *Axill-E*, erythema-dependent component of skin tone, axillary site; *Axill-M*, melanin-dependent component of skin tone, axillary site ; *Forehd-E*, erythema-dependent component of skin tone, forehead site; *Forehd-M*, melanin-dependent component of skin tone, axillary site; *BP*, blood pressure; *HOMA-IR*, homeostatic model assessment for insulin resistance; *Hgb A1c*, hemoglobin A1c; *Ca*, calcium; *Phos*, phosphate; *alk phos*, alkaline phosphatase; *PTH*, parathyroid hormone; *FGF23*, fibroblast growth factor 23; *OC*, osteocalcin; *CTx*, c-terminal telopeptide type I collagen; *P1NP*, procollagen type I propeptide; *Cathd*, cathelicidin; *CCL13*, chemokine ligand 13.

Fig 1.

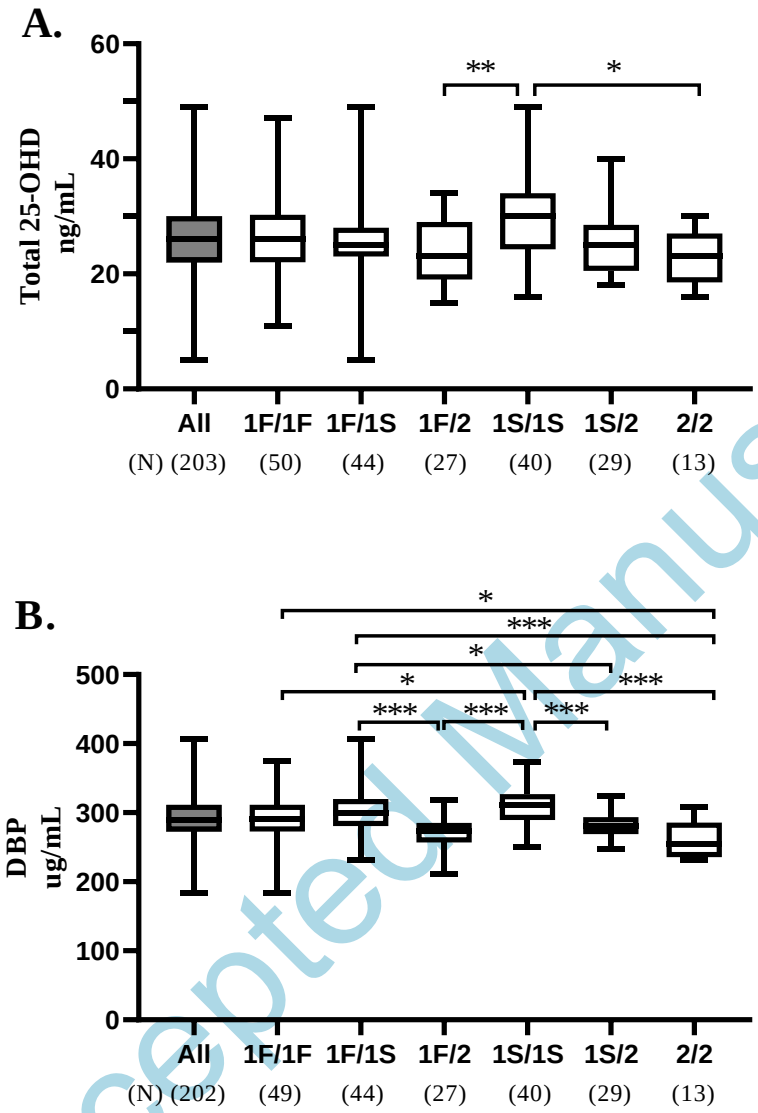

FIGURE 1: Box-and-whisker plots for the entire cohort (shaded boxes on left) and of each of the 6 common *Gc* haplotype groups for total circulating 25-OHD (panel A) and vitamin D binding protein (panel B). Upper and lower extremes of the whiskers represent maximum and minimum values; upper and lower borders of the boxes represent the 75th and 25th centile values, and the middle bars represent the median value. Significantly different groups by multiple comparison testing are indicated by brackets; * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Fig 2.

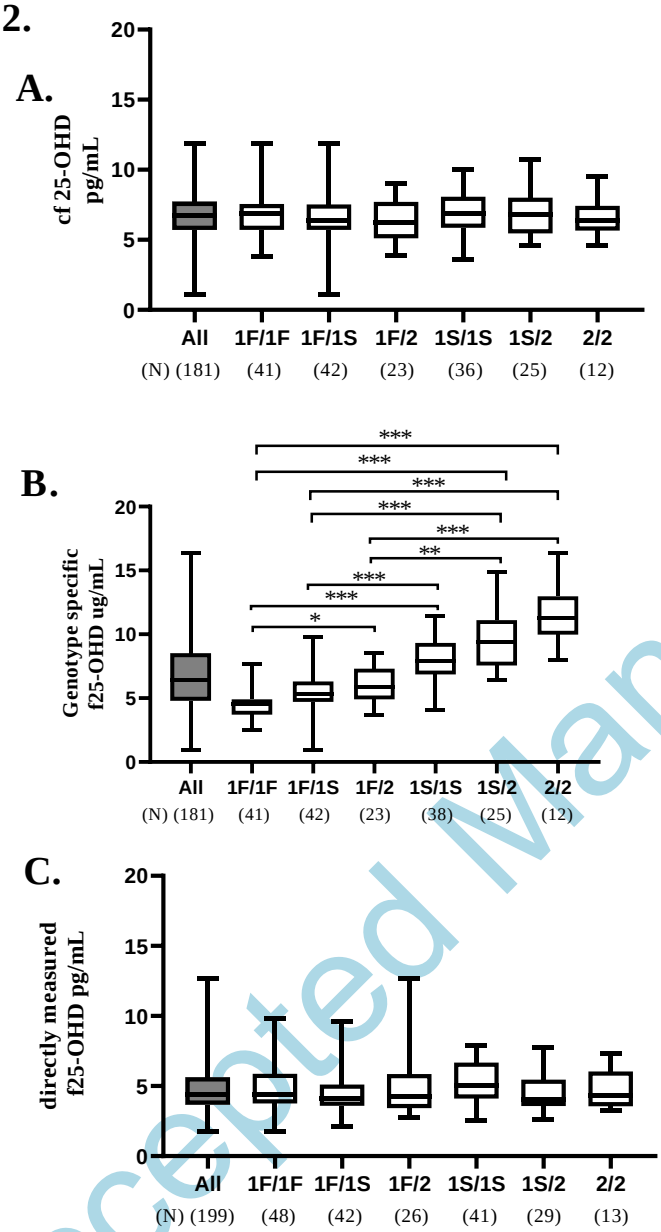

FIGURE 2: Box-and-whisker plots for the entire cohort (shaded boxes on left) and of each of the 6 common *Gc* haplotype groups for calculated free (cf) 25-OHD (panel A), genotype specific calculated free (gscf) 25-OHD (panel B), and directly measured free (dm) 25-OHD (Panel C). Upper and lower extremes of the whiskers represent maximum and minimum values; upper and lower borders of the boxes represent 75th and 25th centile values, and the middle bars represent the median value. Differences across haplotypes were not evident for cf 25-OHD and dm 25-OHD. Significant differences by multiple comparison testing across haplotype groups for gscf 25-OHD are indicated by brackets; * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Fig. 3

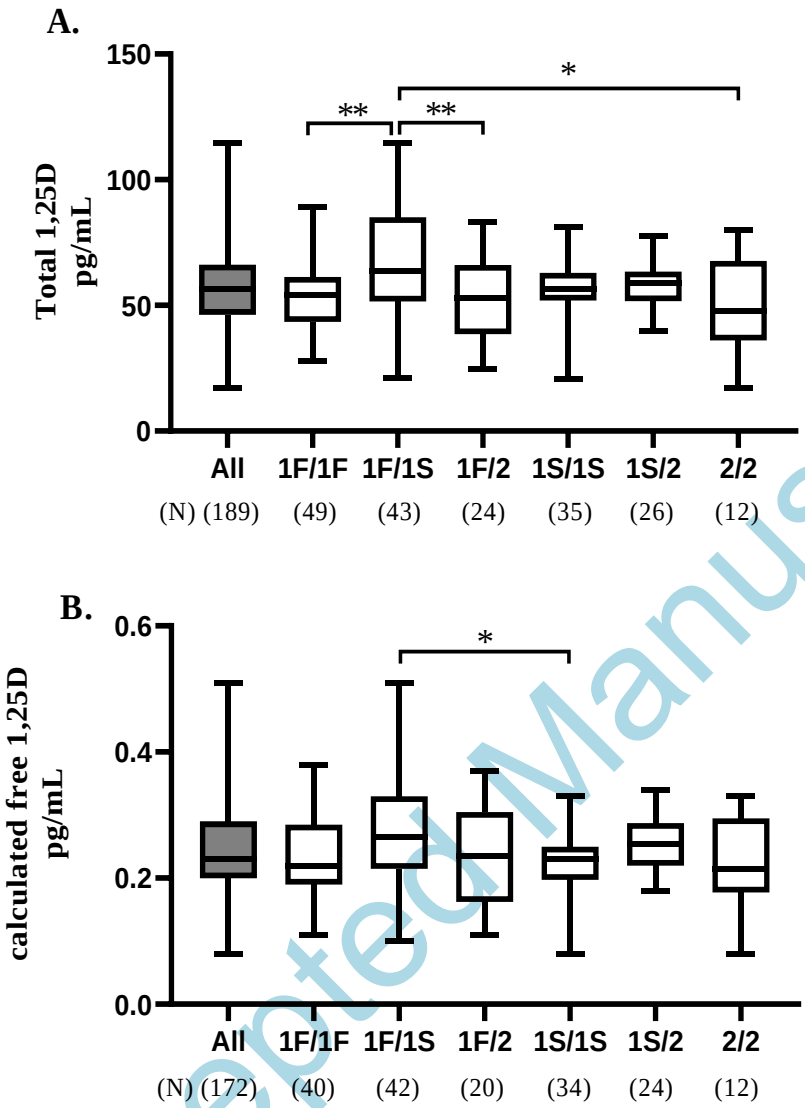

FIGURE 3: Box-and-whisker plots for the entire cohort (shaded boxes on left) and of each of the 6 common *Gc* haplotype groups for total circulating $1,25(OH)_2D$ (t1,25D) (panel A) and calculated free $1,25(OH)_2D$ (panel B). Upper and lower extremes of the whiskers represent maximum and minimum values; upper and lower borders of the boxes represent 75^{th} and 25^{th} centile values, and the middle bars represent the median value. Significantly different groups by multiple comparison testing are indicated by brackets; * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.