25-hydroxyvitamin D Levels and Juvenile Idiopathic Arthritis: is there an Association with Disease Activity?

A thesis

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Abstract:

Background: The inverse association between disease activity and serum levels of 25-hydroxyvitamin D [25(OH)D] demonstrated in adults with rheumatoid arthritis has not been explored in juvenile idiopathic arthritis (JIA). The aims of this study were: to examine the association between serum levels of 25(OH)D and disease activity in JIA, to determine the prevalence of vitamin D deficiency [25(OH)D≤19 ng/ml] and insufficiency [25(OH)D 20-29 ng/ml], and to determine factors associated with lower serum levels of 25(OH)D in this population. **Methods:** In this cross-sectional study, conducted between Oct 2009 and Sep 2010, disease activity was measured using JADAS-27 (Juvenile Arthritis Disease Activity Score 27), as well as its individual components (physician global assessment of disease activity, parent global assessment of child's well-being, count of joints with active disease, and erythrocyte sedimentation rate). Linear regression models were developed to analyze the association between serum 25(OH)D levels and JADAS-27, and to determine variables associated with serum 25(OH)D levels. **Results:** A total of 154 patients (61% females, 88% non-Hispanic whites) were included. Mean age was 10.6 years. Mean serum 25(OH)D level was 29.2 ng/ml. Vitamin D deficiency was detected in 13% of patients and insufficiency in 42%. JADAS-27 had a median value of 5.2 (range 0-30.7). In univariate and multivariate analyses, 25(OH)D levels were not associated with JADAS-27, nor with its individual components. However, in a subset analysis including all new onset (time since disease onset ≤ 3 months) JIA patients (n=27) there was a non-significant negative correlation between serum 25(OH)D levels and JADAS-27 (r=-0.29, p=0.14). In the univariate and multivariate analyses, age, ethnicity, BMI, and season were significantly associated with serum 25(OH)D levels, but not total vitamin D intake. **Conclusions**: More than one half of JIA patients had serum 25(OH)D levels below 29 ng/ml, however there was no association between serum 25(OH)D levels and disease activity in this sample of children and adolescents with JIA. Age, ethnicity, BMI, and season were associated

with serum 25(OH)D levels. Future larger, long-term studies evaluating patients with new-onset JIA are needed to further explore and elucidate the association between serum 25(OH)D levels and disease activity.

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25-hydroxyvitamin D levels and Juvenile Idiopathic Arthritis: is there an Association with Disease Activity?

Background

Vitamin D plays an important role in the immune system and has multiple immunosuppressant properties [1-9]. Serum vitamin D levels have been negatively associated with disease activity in autoimmune disorders in adults across multiple observational studies [10-20]. For example, serum 25-hydroxyvitamin D [25(OH)D] levels have been found to correlate inversely with disease activity in adults with rheumatoid arthritis (RA) and in those with newly diagnosed inflammatory polyarthritis [21, 22]. Similarly, serum 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] concentration has been found to be negatively correlated with disease activity in RA and ankylosing spondylitis [23, 24]. (Appendix 1)

Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic disease of childhood and is an important cause of short- and long-term disability in children [25]. In a previous study we showed that children with autoimmune disorders, including JIA, were more likely to be vitamin D deficient than controls [submitted paper, Appendix 2]. Similarly, others have showed decreased serum levels of 25(OH)D in children with JIA, compared to healthy controls [26]. However, the association between disease activity and serum levels of 25(OH)D demonstrated in adults with RA has not to our knowledge been reported in children with JIA. If this association exists in JIA, it may represent a promising therapeutic target.

The primary aim of this study was to examine the association between serum levels of 25(OH)D and disease activity in children and adolescents with JIA. The secondary aims were to determine the prevalence of vitamin D deficiency and insufficiency according to the conventionally used (but non-validated) cutoffs of serum

25(OH)D (≤19 ng/ml and 20-29 ng/ml) in children and adolescents with JIA, and to determine factors associated with lower serum levels of 25(OH)D in this population.

Methods

This was a cross-sectional study, conducted between October 2009 and September 2010, at the Pediatric Rheumatology clinic of the Floating Hospital for Children at Tufts Medical Center. Children and adolescents with JIA (subtypes: oligoarthritis, rheumatoid factor-positive polyarthritis, rheumatoid factor-negative polyarthritis, systemic-onset arthritis, enthesitis-related arthritis, and psoriatic arthritis), between the ages of 2 and 19 years, who had blood drawn for routine clinical monitoring and agreed to participate in the study were enrolled.

Subjects who had any conditions, or were using any medications, that would affect vitamin D metabolism were excluded. These conditions included: use of prednisone (any dose, on the previous 3 months), concurrent medical problems (diabetes type 1, inflammatory bowel disease, celiac disease, immunodeficiency), and pregnancy. Subjects with history of a recent infection (within 2 weeks) were also excluded, since this could increase the inflammatory markers used to measure JIA disease activity. This project was approved by the Institutional Review Board at Tufts Medical Center. Children 7 years or older signed assent forms and parents of minors signed consent forms. Participants who were 18 years or older signed consent forms themselves. From all subjects who were invited to participate in the study, only one declined.

Disease activity measurement was compiled using a previously validated score, JADAS- 27 (Juvenile Arthritis Disease Activity Score 27) [27]. This score includes four measures: physician global assessment of disease activity using a visual analog scale (VAS), parent global assessment of child's well-being determined by a VAS, count of joints with active disease, and erythrocyte sedimentation rate (ESR). The physician assessment of disease activity is based on a 10 cm VAS, where 0 corresponds to no activity and 10 corresponds to maximum activity. The parent's assessment of child's well-being is also based on a

10 cm VAS, where 0 corresponds to very well and 10 to very poor. The count of active joints in JADAS-27 evaluates 27 joints: cervical spine, elbows, wrists, metacarpophalangeal joints (from first to third), proximal interphalangeal joints, hips, knees, and ankles. Each active joint scores 1 point, and the total goes from 0 to 27. ESR is normalized to a score ranging from 0 to 10, by the formula (ESR-20)/10. JADAS-27 is then calculated as the simple linear sum of the scores of its 4 components, which yields a total score of 0 to 57, with higher scores representing worse disease activity. To illustrate clinically, a JADAS-27 of 5 would correspond to mild disease activity, for example, a child with 2 active joints, normal ESR, and 1.5 (out of 10) in the physician and parent VAS.

Serum 25(OH)D was measured by LC/MS/MS (Waters Acquity UPLC with TQD triple quadrupole mass spectrometer). This method separates and quantifies circulating $25(OH)D_2$ and $25(OH)D_3$. The lab participates in the College of American Pathologists proficiency testing and the Vitamin D External Quality Assessment Scheme (deqas.org), an international consortium of laboratories set up to ensure the analytical reliability of 25(OH)D. The lab also validated the assay using the Standard Reference Material (SRM 972), which was developed by the National Institute of Standards and Technology (NIST). Inter-assay CVs (coefficient of variation) are 6.5-11% for $25(OH)D_3$ over the range of 5-80 ng/mL and 9-13% for $25(OH)D_2$ over the range of 2.5-60 ng/mL.

A structured interview was conducted with parents. Participants who were 18 years or older completed this interview themselves. The structured interview was designed to collect important demographic and health information, including, date and season of evaluation, child's age, gender, ethnicity, JIA subtype, time interval since disease onset, body mass index (BMI), current medications, use of supplements containing vitamin D, use of oral contraceptives, history of recent travel or tanning, and diet history (food frequency questionnaire). Season in which

the study visit was conducted was registered, since serum 25(OH)D levels vary considerably by season. BMI was calculated dividing the weight (in kg) by the square of the height (in m). Children were classified as underweight if their BMI was under the 5th percentile, obese if their BMI was greater than or equal to the 95th percentile, and adequate if BMI was between the 5th and 94th percentiles [28]. Medications used for JIA treatment were documented (non-steroidal anti-inflammatory drugs, methotrexate, and biologic drugs including etanercept, adalimumab, canakinumab, and abatacept), as well as the duration of use of each medication. We only considered the use of methotrexate or a biologic drug if the duration of use was at least 2 months. The use of oral contraceptives was registered, as estrogens may influence serum 25(OH)D levels [29]. We also documented trips to areas of increased sun exposure, in relation to Boston, as well as tanning, since these 2 factors could influence serum 25(OH)D levels [30-32]. Vitamin D ingested from diet was calculated based on a food frequency questionnaire [33]. We estimated the total daily vitamin D intake, in IU, by adding the amount ingested from diet to any amount ingested from supplements.

Statistical analyses

Approximately 180 patients with JIA are seen at the Pediatric Rheumatology clinic in the Floating Hospital for Children each year. *A priori* sample size calculations showed that recruitment of 150 subjects would yield 80% power to detect a correlation between serum 25(OH)D levels and JADAS-27 of 0.23. Sample size calculation was constrained by feasibility, rather than a clinically meaningful correlation of 0.23.

Scatter plots of serum 25(OH)D levels and JADAS-27 results were constructed to examine linearity and to check for potential outliers. A linear regression model was developed to analyze the association between serum

25(OH)D levels and the outcome JADAS-27. The model was adjusted for potential confounders, identified *a priori*, including age, gender, JIA subtype, ethnicity (non-Hispanic whites versus others), medications (none, non-steroidal anti-inflammatory drugs, or immunosuppressants - methotrexate and/or biologics), season (summer versus others), and time since disease onset. We checked for interactions between serum 25(OH)D and medications, since we thought there could be a differential effect of serum 25(OH)D on disease activity at different levels of this variable. The variables ethnicity and season were collapsed into binary variables for the model to avoid overfitting.

The prevalence of vitamin D deficiency and insufficiency as defined by conventionally used cutoffs for serum 25(OH)D (≤19 ng/ml and 20-29 ng/ml) was determined. Despite being widely used in the literature, the cutoffs mentioned above have not been validated in children and therefore were not employed in our primary analyses [34, 35].

Two approaches were used to determine factors associated with low serum 25(OH)D levels. We used a logistic regression model to identify variables associated with low vitamin D levels, defined as the lowest quartile of our sample [serum 25(OH)D <23 ng/ml], vs. higher levels of serum 25(OH)D (≥23 ng/ml). The variables of interest were: age (up to 10 years old vs. older than 10), ethnicity (non-Hispanic whites vs. other ethnicities), BMI (obese vs. non-obese), and season (summer vs. non-summer).

A linear regression model was also developed to analyze the variables associated with serum 25(OH)D levels, including age, ethnicity (non-Hispanic whites vs. other ethnicities), BMI (obese vs. non-obese), season (summer vs. non-summer), and total daily vitamin D intake.

JMP version 8 (SAS Institute Inc., Cary, NC) was used for data analyses. $P<0.05$ was considered significant for the linear regression models. For the logistic

regression model, which was exploratory only, $P<0.1$ was considered significant. We checked model diagnostics and goodness of fit for all 3 models (Appendix 3).

Results

A total of 154 patients were enrolled and included in the analysis. Patient characteristics are shown in Table 1. The average age of participants was 10.6 years, 61% were female, and 88% were non-Hispanic white. The number of patients enrolled during each season was not significantly different. Only a small number of patients were noted to have documented trips to areas of increased sun exposure or tanning (N=7) or to have used contraceptives (N=5). Among the 57 patients (37%) taking supplements containing vitamin D, the dose ranged from 400 to 2,400 IU of vitamin D_3. Total daily vitamin D intake ranged from 0.03 to 2,764 IU.

The mean serum 25(OH)D level was 29.2 ng/ml. The lowest quartile of serum 25(OH)D consisted in levels <23.0 ng/ml; the second quartile included levels from 23.0 to <28.5 ng/ml; the third quartile was between 28.5 and 34.0 ng/ml and the highest quartile consisted of serum 25(OH)D above 34.0 ng/ml. The values of serum 25(OH)D_3 and 25(OH)D_2 are shown in Table 2. Serum 25(OH)D levels of 19 ng/ml or less were detected in 13% of patients and serum 25(OH)D levels from 20 to 29 ng/ml in 42% (Table 2).

The score of JIA disease activity, JADAS-27, had a median value of 5.2 (range 0 to 30.7). Results of the four components of JADAS-27 are detailed in Table 3.

In the univariate linear regression analysis serum 25(OH)D levels were not associated with JADAS-27 (p=0.97), nor with any of the four separate components of JADAS-27 (p-value range 0.32-0.89).

A similar lack of association was found in multivariate linear regression analysis adjusting for age, gender, JIA subtype, ethnicity, JIA medications, season, and time since disease onset (p=0.67) (Table 4). However, this multivariate analysis revealed nominally significant associations between JADAS-27 and JIA subtype (p=0.003), and ethnicity (p=0.006). Patients with rheumatoid factor-positive

polyarticular JIA had estimated JADAS-27 scores that were 4.9 points higher on average than patients with oligoarticular JIA. Non-Hispanic white patients had JADAS-27 scores that were 1.9 points lower, on average, than patients of all other ethnicities (Table 4).

In a subset analysis, we examined the correlation of serum 25(OH)D levels with JADAS-27 separately for all new onset (time since disease onset ≤ 3 months) JIA patients (N=27) as opposed to patients with long-term disease. This subset analysis was not planned *a priori*; we performed to explore whether measured and unmeasured confounders associated with long-term disease (e.g., medications, vitamin D supplementation, lifestyle) could be responsible for a falsely negative result. In this subset analysis, there was a non-significant negative correlation between serum 25(OH)D levels and JADAS-27 in new-onset patients (r=-0.29, p=0.14), while there was no correlation in patients with long-term JIA (r=0.06, p=0.52).

Regarding variables possibly associated with the lowest quartile of serum 25(OH)D levels, in the multivariate logistic regression analysis, ethnicities other than non-Hispanic white (OR=3.1; 95%CI=1.1, 8.7; p=0.04), age older than 10 (OR=2.5; 95%CI=1.1, 5.5; p=0.03), and obesity (OR=2.4; 95%CI=0.9, 6.1; p=0.07) were marginally associated with higher odds of this endpoint.

In the multivariate linear regression analysis using serum 25(OH)D levels as the outcome, for each increase in age by 1 year, serum 25(OH)D decreased an estimated 0.3 ng/ml (p=0.04); non-Hispanic white patients had estimated serum 25(OH)D levels that were on average 2.6 ng/ml higher than other ethnicities (p=0.01); non-obese patients had estimated serum 25(OH)D levels that were 2.5 ng/ml higher, on average, than obese patients (p=0.006); and estimated serum 25(OH)D levels were on average 2.3 ng/ml higher in summer than in other times of

year (p=0.01) (Table 5). Self-reported total vitamin D intake was not associated with serum 25(OH)D levels (p=0.86).

Discussion

There was no association between serum 25(OH)D levels and JIA disease activity, measured by JADAS-27, in either univariate or multivariate analyses, nor between serum 25(OH)D levels and individual JADAS-27 components.

Although we did not find an association between serum 25(OH)D levels and JADAS-27, there was a significant association between JIA subtypes with this disease activity score. This suggests that the overall null result of our primary analysis was unlikely to be due to substantial measurement error of disease activity or lack of meaningful variation in this outcome, since we did find the association that has been established before and was expected [25].

There are several reasons why we may have failed to detect an association between serum 25(OH)D levels and disease activity in patients with JIA. First, JIA is a different disease than RA [25], and the association between serum 25(OH)D levels and disease activity described in adult patients with inflammatory arthritis may not exist in childhood arthritis.

A second possibility is that we analyzed data from patients with established ongoing disease. These patients could have several measured and unmeasured confounders, which could induce the apparent lack of an existing association between serum 25(OH)D levels and disease activity. Many of these patients were taking methotrexate or a biologic drug, for example, which modifies the disease course. Considering this possibility, we conducted a subset analysis of new patients, in which we found an intriguing though statistically non-significant negative association between serum levels of 25(OH)D and JADAS-27. The lack of statistical significance of this finding could have been due to inadequate power, as there was a very small sample of new-onset JIA. Hence, future studies of the association between serum 25(OH)D levels and disease activity, enrolling only newly diagnosed JIA patients, may be useful.

A third aspect to be considered is that chronic exposures, such as serum 25(OH)D levels in our study, may have different effects on short- or long-term outcomes [36]. The lack of an association between serum 25(OH)D levels and JIA disease activity measured at the same point in time does not preclude an association with disease activity in the long-term.

Another aspect to be considered is that the apparent effect of risk factors on exacerbations might be attenuated when there is congruence between the risk factors that cause the disease to develop in the first place and risk factors that cause an acute exacerbation [36]. It is not known if serum 25(OH)D levels are a risk factor for the development of JIA, but if so, the effects of serum 25(OH)D levels on JIA exacerbations could have been attenuated. We did not analyze patients enrolled in our study separately by disease flare or remission.

A fifth possibility for the lack of an association between serum 25(OH)D levels and JIA activity in our study is that there was confounding by vitamin D supplementation that was not captured in our model. While we collected information on current vitamin D supplementation, we did not capture information on duration of vitamin D supplementation. It is known that at least 3 months of vitamin D supplementation are necessary to reach a steady state and physiologic effects of vitamin D deficiency or insufficiency may persist for even longer [37]. These effects could obscure any relationship between cross-sectionally measured serum 25(OH)D levels and JIA disease activity.

Another possibility is that the association between serum 25(OH)D levels and disease activity in JIA might be weaker than our study would be able to detect, due to inadequate statistical power.

An interesting aspect of this study is that disease activity was worse in African American, Hispanic, and Asian patients. Although the association of ethnicity and JIA disease activity has not been published before, others have reported a higher

incidence of rheumatoid factor-positive polyarticular JIA in non-white populations [38, 39]. Several factors could be responsible for the worse disease activity in these minorities in our sample, including different socioeconomic status, cultural aspects, genetics, or even adherence to treatment. Future studies are needed to further explore JIA disease activity in different ethnicities.

The variables associated with serum 25(OH)D levels in this sample of children with JIA were the same as those described for healthy children and adults in other studies, including age, ethnicity, BMI, and season [9, 30-32, 40-44]. Unexpectedly, total vitamin D intake was not associated with serum 25(OH)D levels. The lack of association may have been due to recently initiated treatment of vitamin D deficient patients with high doses of vitamin D supplements who did not achieve steady state of serum 25(OH)D levels yet.

This study had some limitations, including the cross-sectional design, which impacted the probability of detecting an effect if serum 25(OH)D levels influenced disease activity over time. Also JADAS-27 as a measurement of disease activity has yet to be validated for enthesitis-related arthritis and psoriatic arthritis. However, we conducted separate analyses with the individual components of JADAS-27, which are used in clinical practice to determine disease activity. There is the possibility of selection bias, as only patients who were having blood drawn for clinical reasons were invited to be enrolled in the study. Nevertheless, the study enrolled patients during a period of one year, and virtually every patient with JIA would have blood drawn at least once a year.

The major strength of this study is that it was, to the best of our knowledge, the first study to examine the association between serum 25(OH)D levels and disease activity in children and adolescents with JIA. Another strength was the study duration of one year, which was important to register the seasonal variation in serum 25(OH)D levels, and also to maximize the number of patients enrolled. This

study adds evidence to the growing knowledge regarding vitamin D and autoimmunity.

In conclusion, more than one half of JIA patients had serum 25(OH)D levels below 29 ng/ml, however there was no association between serum 25(OH)D levels and disease activity in this sample of children and adolescents with JIA. Age, ethnicity, BMI, and season were associated with serum 25(OH)D levels in patients with JIA. Future larger, long-term studies evaluating patients with new-onset JIA are needed to further explore and elucidate the association between serum 25(OH)D levels and disease activity.

Table 1: Demographic characteristics of enrolled patients

Age (mean ± SD, range)	10.6 ± 4.5, 2 – 19
Females (N, %)	94, 61%
Ethnicity (N, %)	
Non-Hispanic white	135, 88%
African American	4, 2.5%
Hispanic	11, 7%
Asian	4, 2.5%
JIA subtype (N, %)	
Oligoarthritis	71, 46%
Rheumatoid factor-negative polyarthritis	33, 21.5%
Rheumatoid factor-positive polyarthritis	4, 2.5%
Systemic-onset arthritis	4, 2.5%
Enthesitis-related arthritis	29, 19%
Psoriatic arthritis	13, 8.5%
Time since JIA onset in months (median, interquartile range, range)	28, 6 – 66, 2 – 197
Medications (N, %)	
Non-steroidal anti-inflammatory drugs	77, 50%
Methotrexate	28, 18%
Biologics	25, 16%
Intra-articular steroids	6, 4%
None	50, 32.5%
Time on medications in months (median, interquartile range)	
Non-steroidal anti-inflammatory drugs	8, 2 – 22
Methotrexate	15, 7 – 47
Biologics	19, 6 – 33
Season (N, %)	
Fall	49, 32%
Winter	39, 25%
Spring	36, 23.5%
Summer	30, 19.5%
Body mass index (BMI) category (N, %)	
Adequate	122, 79%
Obese	28, 18%
Underweight	4, 3%
BMI absolute value (median, interquartile range, range)	18.6, 16.2 – 21.9, 13.4 - 36.7
BMI percentile (median, interquartile range)	67, 41 – 88
Vitamin D ingestion	
Dose of vitamin D_3 on supplements in IU (median, interquartile range)	400, 400 - 1,000
Vitamin D ingested from diet in IU (median, interquartile range, range)	239, 136 – 368, 0.03 - 1,210
Total daily vitamin D intake in IU (median, interquartile range)	368, 172 - 687

Table 2: Serum 25(OH)D levels

Total serum 25(OH)D in ng/ml (mean ± SD, range)	29.2 ± 9.2, 6 – 58
Serum 25(OH)D_3 in ng/ml (mean ± SD, range)	27.9 ± 9.3, 6 – 58
Serum 25(OH)D_2 in ng/ml (mean ± SD, range)	1.3 ± 3.1, 0 – 29
Serum 25(OH)D ≤19 ng/ml (N, %)	20, 13%
Serum 25(OH)D 20-29 ng/ml (N, %)	64, 42%

Table 3: JADAS-27 and its individual components

	Median	**Interquartile range**	**Range**
Parent VAS	1.4	0.6 – 2.8	0 – 8.9
Physician VAS	1.2	0.2 – 2.5	0 – 8
ESR normalized	0	0 - 0	0 - 8.2
Joint count	2	0 - 4	0 – 18
JADAS-27	5.2	2 – 10.1	0 – 30.7

Table 4: Multivariate linear regression analysis of the effect of serum 25(OH)D levels on JADAS-27

Term	Estimate	Standard Error	95% Confidence interval	p-value
Serum 25(OH)D (ng/ml)	0.02	0.05	-0.08, 0.12	0.67
Age (years)	-0.01	0.13	-0.26, 0.24	0.92
Gender (male)	0.007	0.48	-0.93, 0.95	0.98
JIA subtype				0.003
Oligoarthritis	Reference group			
Psoriatic arthritis	-1.78	1.49	-4.70, 1.14	0.23
Rheumatoid factor-negative polyarthritis	1.75	1.10	-0.41, 3.91	0.11
Rheumatoid factor-positive polyarthritis	4.94	2.47	0.10, 9.78	0.05
Systemic-onset arthritis	-0.16	2.39	-4.84, 4.52	0.94
Enthesitis-related arthritis	-1.68	1.22	-4.07, 0.71	0.17
Ethnicity (non-Hispanic white)	-1.94	0.70	-3.31, -0.57	0.006
Medications				0.13
Methotrexate and/or biologics	Reference group			
Non-steroidal anti-inflammatory drugs only	0.77	0.66	-0.52, 2.06	
None	-1.36	0.68	-2.69, -0.03	
Season (non-Summer)	0.09	0.61	-1.11, 1.29	0.87
Time since disease onset (months)	-0.0005	0.01	-0.02, 0.02	0.96

Table 5: Multivariate linear regression analysis of factors associated with serum 25(OH)D levels

Term	Estimate	Standard Error	95% Confidence interval	p-value
Age (years)	-0.32	0.15	-0.61, -0.03	0.04
Ethnicity (non-Hispanic white)	2.64	1.06	0.56, 4.72	0.01
Body mass index (non-obese)	2.51	0.91	0.73, 4.29	0.006
Season (non-Summer)	-2.28	0.90	-4.04, -0.52	0.01
Total daily vitamin D intake (IU)	0.0002	0.001	-0.002, 0.002	0.86

Appendix 1: Extended Background

1,25-dihydroxyvitamin D [1,25$(OH)_2$D] has many roles in the immune system (Table A1.1). The overall effect of vitamin D is immunosuppressive [2].

Because of its immunosuppressive activity, the effect of vitamin D has been investigated in various experimental models of autoimmunity. For example, in the murine model of collagen-induced arthritis (the animal model for rheumatoid arthritis), vitamin D receptor agonists prevented disease expression and also suppressed established disease [45, 46].

There are many difficulties in establishing an association between vitamin D deficiency and autoimmune rheumatologic disorders in humans. Different cut-offs for vitamin D deficiency and insufficiency are used in different studies; some studies are based on the reported vitamin D intake rather than on serum 25(OH)D levels; the rarity of the diseases make it difficult to obtain large samples of subjects; there are many confounding factors associated with those diseases, including prednisone intake, photosensitivity and recommended use of sunscreen; and the outdated limit for unsupervised intake impairs dose-appropriate interventional studies.

The hypothesis that vitamin D relates to autoimmune disorders emerged from the observation that people living near the equator were at a decreased risk of developing common autoimmune diseases [41]. Furthermore, several surveys of rheumatology patients found reduced levels of serum 25(OH)D. For example, in a study involving 1029 patients with different rheumatologic autoimmune disorders, including RA, patients had lower serum 25(OH)D levels than controls [47].

There are different types of evidence linking RA with vitamin D deficiency, like the latitude-related prevalence of RA, which coincides with areas where vitamin D deficiency is more prevalent [10, 18, 46, 48]. Although intake report is not the ideal way of evaluating vitamin D status, greater intake of vitamin D inversely related to

the risk of developing RA in a prospective cohort study of 29,368 women [19]. Vitamin D has also been related to disease activity in RA; serum 25(OH)D levels negatively correlated with RA clinical status [18, 48], and low $1,25(OH)_2D_3$ levels were associated with higher RA disease activity [1]. On the other hand, not all studies confirm this association. In a small sample of patients with RA (n=29), serum 25(OH)D levels did not differ from controls [13]. Besides, in a prospective analysis involving over 180,000 women followed for up to 22 years, vitamin D intake did not relate to the risk of developing RA [20].

Table A1.1: 1,25 dihydroxyvitamin D actions in the immune system

Decreases the antigen-presenting activity of macrophages to lymphocytes [42, 49]
Increases apoptosis induced by dendritic cells and T lymphocytes – tolerance [7, 8, 12]
Inhibits the maturation of monocytes into dendritic cells [1, 47]
Induces activation of T reg and natural killer T cells [2, 48]
Inhibits the Th1 profile [1, 2, 4, 47] • Decreases IL-2 and IFN-γ synthesis [1, 6, 42, 47, 49]
Stimulates the Th2 dominance [1, 2, 4, 47] • Increases IL-4, IL-5 and IL-10 synthesis [1, 7, 49]
Inhibits the synthesis of IL-12, IL-1, IL-6 and TNFα [6, 40, 42, 50]
Inhibits B cell proliferation, plasma cell differentiation, and antibodies production [1, 3, 5, 47, 49]

Appendix 2: (paper submitted to Journal of Rheumatology on 01/25/2011)

25-hydroxyvitamin D levels and Vitamin D deficiency in Children with Rheumatologic Disorders

Background

Vitamin D has multiple immunosuppressant properties [1]. In vitro, 1,25-dihydroxy-vitamin D [1,25$(OH)_2$D] inhibits the activation of monocytes to dendritic cells, decreasing IL-12 production; it inhibits B cell proliferation, decreasing production of antibodies; and it also inhibits T cell proliferation and activation. 1,25$(OH)_2$D inhibits the Th1 profile, decreasing IL-2, IFN-γ, IL-1, IL-6 and TNF-α synthesis and promotes Th2 dominance, increasing IL-4, IL-5 and IL-10 synthesis [2-8, 32, 41].

Studies in animal models of lupus, rheumatoid arthritis, and inflammatory bowel disease have used 1,25$(OH)_2D_3$ analogs not only to prevent, but also to treat ongoing disease [5, 7, 8, 45, 46, 51-53].

Vitamin D deficiency has been linked to several autoimmune diseases in humans, including inflammatory bowel disease, type 1 diabetes, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (SLE) [8, 11, 12, 14, 17, 31, 40, 42, 46, 47, 49, 51]. Most studies in humans have documented lower serum levels of vitamin D in patients with autoimmune diseases. Serum vitamin D levels in adults with SLE are significantly lower than in controls [8, 13, 15, 16, 47, 48, 51].

Considerably less information is known about serum levels of 25-hydroxyvitamin D [25(OH)D] in children with rheumatologic disorders. The chief aim of this observational study was to determine the prevalence of vitamin D deficiency [defined as serum 25(OH)D <20 ng/ml] and insufficiency [defined as serum 25(OH)D 20-29 ng/ml] [32], as well as factors associated with serum 25(OH)D levels, in

children and adolescents attending a pediatric rheumatology clinic. A second aim was to ascertain if there was a difference in serum levels of 25(OH)D and vitamin D deficiency rates among children with autoimmune disorders as compared to those with non-autoimmune conditions.

Methods

We performed a cross-sectional analysis of serum 25(OH)D levels of patients attending the pediatric rheumatology clinic in the Floating Hospital for Children at Tufts Medical Center between November 2008 and October 2009. All patients who had routine phlebotomy performed were included. This study was approved by the Institutional Review Board of Tufts Medical Center.

Serum 25(OH)D was measured by LC/MS/MS (Waters Acquity UPLC with TQD triple quadrupole mass spectrometer). This method separates and quantifies circulating $25(OH)D_2$ and $25(OH)D_3$. The lab participates in the College of American Pathologists proficiency testing and the Vitamin D External Quality Assessment Scheme (deqas.org), an international consortium of laboratories set up to ensure the analytical reliability of 25(OH)D. The lab also validated the assay using the Standard Reference Material (SRM 972), which was developed by the National Institute of Standards and Technology (NIST). Inter-assay CVs (coefficients of variation) are 6.5-11% for $25(OH)D_3$ over the range of 5-80 ng/mL and 9-13% for $25(OH)D_2$ over the range of 2.5-60 ng/mL.

Patients were classified into 3 mutually exclusive categories, according to their vitamin D status, as follows: adequate [serum 25(OH)D level ≥30 ng/ml], insufficient [serum 25(OH)D 20-29 ng/ml], or deficient [serum 25(OH)D < 20 ng/ml] [32].

Information concerning patients' demographics (sex, age, and ethnicity), body mass index (BMI), diagnosis, disease activity, use of medications and supplements, and season of serum 25(OH)D measurement were identified based on the review of medical records.

Patients were classified into two distinct groups based on their clinical diagnoses. The first group included patients with definite autoimmune disorders, including juvenile idiopathic arthritis (JIA), juvenile SLE, juvenile dermatomyositis,

systemic sclerosis, mixed connective tissue disease (MCTD), and vasculitis, while the second included patients who had a non-autoimmune condition (e.g., orthopedic or infectious diseases, pain amplification syndrome, hypermobility, and growing pains).

Disease activity was evaluated only for patients with autoimmune disorders. This was defined as active or inactive based on the attending physician's assessment at the time of the index clinic visit.

BMI was divided into 3 categories: normal, obese, and underweight. Children were classified as underweight if their BMI was under the 5^{th} percentile, obese if their BMI was greater than or equal to the 95^{th} percentile, and normal if BMI was between the 5^{th} and 94^{th} percentiles [28].

Statistical analyses

Descriptive statistics were used to analyze the data. We determined the rates of vitamin D deficiency and insufficiency in the study sample. Linear regression was used to evaluate the association between age and serum 25(OH)D levels. Student's T-test was used to analyze the influence of gender, disease activity (active or inactive), BMI (obese or non-obese), and group (autoimmune or non-autoimmune) on serum 25(OH)D levels. One-way ANOVA was used to examine the association of ethnicity, diagnosis, medications, season, and use of supplements (no use, use of ≤ 400 IU of vitamin D_3, or use of >400 IU of vitamin D_3) with serum 25(OH)D levels. Chi-square test was used to analyze if there was an association between vitamin D status (adequate, insufficient, or deficient) and disease group (autoimmune or non-autoimmune). A multivariate linear regression was used to analyze the association between autoimmune and non-autoimmune diseases with 25(OH)D levels, adjusting for potential confounding variables (age, supplement use, BMI, ethnicity, and season). A multivariate logistic regression was used to determine the odds ratio of

vitamin D deficiency in patients with autoimmune disorders, after adjusting for confounders (supplement use, BMI, ethnicity, and season). All tests were two-sided and statistical significance was established with an alpha of 0.05.

Results

Sample

A total of 254 patients had serum 25(OH)D measurements performed during the study period; of these, 169 patients (67%) had autoimmune disorders and 85 had non-autoimmune conditions. The average age of all children was 12.3 (±4.5) years, 67% were female, and 80% were Caucasian. There were no differences in various demographic characteristics according to disease group (Table A2.1).

Vitamin D deficiency and insufficiency

Mean serum level of 25(OH)D in the complete sample was 28.6 (±11) ng/ml. The range was 2 to 59 ng/ml. Average serum levels of 25(OH)D in patients with autoimmune disorders were 28.1 (±11.4) ng/ml, compared to 29.7 (±10.3) ng/ml in patients with non-autoimmune disorders (p=0.25).

In patients with autoimmune disorders, 44% had adequate levels of vitamin D, 33% had vitamin D insufficiency, and 23% had vitamin D deficiency. In patients with non-autoimmune conditions, 48% had adequate levels of vitamin D, 38% were vitamin D insufficient and 14% were vitamin D deficient (p=0.24).

The odds ratio of patients with autoimmune disorders being vitamin D deficient, after adjusting for supplement use, BMI, ethnicity, and season, was 2.28, in relation to patients with non-autoimmune disorders (p=0.041) (Table A2.2).

Variables associated with serum 25(OH)D Levels

Age, ethnicity, BMI, season, and use of supplements containing vitamin D were significantly associated with serum 25(OH)D levels, as expected (Table A2.3).

Serum 25(OH)D and Medications

Among patients with autoimmune disorders, 60 (36%) were not taking any medications. Of the remainder, medication use included non-steroidal anti-inflammatory drugs (39, 23%), prednisone (24, 14%), biologic drugs (24, 14%), disease modifying anti-rheumatic drugs (methotrexate, cyclosporine, cyclophosphamide, azathioprine, and hydroxycloroquine) (20, 12%), and intravenous immunoglobulin infusions (2, 1%). Mean serum 25(OH)D levels did not differ in relation to the use of particular medications (p=0.12) (Table A2.4). No patients with non-autoimmune conditions were taking long-term medications, other than vitamins.

Serum 25(OH)D and Rheumatologic Disorders

Serum 25(OH)D levels did not vary by diagnosis in patients with autoimmune disorders (Table A2.4), although there was a trend towards lower levels in patients with SLE and MCTD (p=0.054).

One hundred seventeen patients (69%) with autoimmune disorders had active disease. Mean serum 25(OH)D levels did not differ in patients with active (27.9 ± 11.7 ng/ml) or inactive (28.5 ± 10.8 ng/ml) disease (p=0.78).

In patients with non-autoimmune conditions, mean serum 25(OH)D levels did not differ between different diagnoses (p=0.96).

In the multivariate linear regression, after adjusting for age, supplement use, BMI, ethnicity, and season, there was a trend towards lower serum 25(OH)D levels in patients with autoimmune disorders, in comparison to non-autoimmune disorders (p=0.06). Patients with autoimmune disorders had estimated serum 25(OH)D concentrations on average 1.1 ng/ml lower than patients with non-autoimmune conditions.

Discussion

In this sample of children and adolescents with autoimmune and non-autoimmune conditions, we found high rates of vitamin D deficiency and insufficiency. Serum levels of 25(OH)D were associated with age, ethnicity, BMI, season, and use of supplements. There was a trend towards lower serum 25(OH)D levels in patients with SLE and MCTD, in comparison to patients with other autoimmune disorders. After adjusting for confounding variables, there was a trend towards lower serum 25(OH)D levels in patients with autoimmune compared to non-autoimmune disorders. Patients with autoimmune disorders were 2.28 times more likely to be vitamin D deficient than patients with non-autoimmune conditions.

Our findings are consistent with a large number of cross-sectional studies on vitamin D status and autoimmune rheumatologic diseases in adults [14, 17, 21, 22, 45, 51-54]. Interventional studies also show an interesting effect of vitamin D in autoimmunity. For example, high-dose oral α-calcidiol therapy reduced disease activity in 89% of 19 patients with rheumatoid arthritis [11]. In another uncontrolled study of 11 adults with systemic sclerosis, $1,25(OH)_2D_3$ administered for a period of 6 months to 3 years, was associated with significant improvement in clinical parameters [55].

There are no previously published studies in children comparing serum levels of 25(OH)D in patients with autoimmune disorders and non-autoimmune disorders, or healthy controls. One of the few studies assessing serum 25(OH)D levels in children with rheumatologic disorders did not show decreased levels either in patients with active or inactive disease [56]. In a case series, subnormal vitamin D levels were found in 7 out of 12 adolescents with SLE [57]. However, in this study vitamin D status was tested using measures of serum $1,25(OH)_2D$, instead of 25(OH)D, which is a better marker of vitamin D status [8, 30-32, 40, 42, 44, 46].

There was a trend for lower serum 25(OH)D levels in patients with MCTD and SLE, in comparison to other autoimmune disorders. Lower serum levels of 25(OH)D in SLE patients have been previously reported [8, 45, 47, 48, 51-53]. These findings could relate to the photosensitivity associated with these conditions and the special recommendation to use sunscreen and avoid sun exposure. Other possibilities include associated renal disease or medications [10, 49, 53]. Hence, patients with SLE and MCTD may be at increased risk for vitamin D deficiency and insufficiency. Regular monitoring of serum 25(OH)D levels in this population may therefore be recommended [40, 53].

Unlike other studies, we did not find an association between disease activity and serum levels of 25(OH)D in patients with autoimmune disorders. Others have found lower levels of serum 25(OH)D in adult patients with active SLE, in comparison to patients with inactive disease [14, 17, 48, 53], and negative correlations between serum 25(OH)D levels and disease activity in adults with rheumatoid arthritis [21, 22, 48]. However, other studies failed to show any correlation between disease activity and serum 25(OH)D levels in SLE patients [45-47]. The lack of association between disease activity and serum 25(OH)D levels in our study might be explained by the tool used to measure disease activity, which was based on the attending physician's assessment only.

Although prednisone use is known to cause reduced serum levels of 25(OH)D [40],we did not find a significant association between this use and serum 25(OH)D levels. Similar findings were reported by others in adults with SLE [51], or Behcet's disease [58].

Our study had several limitations, including being retrospective, lack of information available about diet, sun exposure, and use of sunscreen [all of which influence serum 25(OH)D levels]c. One possible explanation why we did not find a significant difference in serum 25(OH)D levels between patients with autoimmune

and non-autoimmune disorders was our relatively small sample size. Another possibility is that serum 25(OH)D levels actually differ by autoimmune disorder, and this difference was missed when different diagnoses were combined. However, this is the first report comparing serum 25(OH)D levels between children with autoimmune rheumatologic disorders and non-autoimmune conditions.

In conclusion, more than one half of children and adolescents attending a pediatric rheumatology clinic were vitamin D deficient or insufficient. Serum 25(OH)D levels were associated with age, ethnicity, BMI, season, and use of supplements. There was a trend towards lower serum 25(OH)D levels in patients with autoimmune compared to non-autoimmune disorders; patients with autoimmune disorders were 2.28 times more likely to be vitamin D deficient than patients with non-autoimmune conditions. Future studies evaluating serum 25(OH)D levels and disease activity in childhood rheumatologic disorders are needed.

Table A2.1: Demographic variables by disease group

Demographic data	**Autoimmune disorders** (N=169)		**Non-autoimmune disorders** (N=85)		**p-value**
Age (mean ± SD)	12.3 ± 4.7		12.4 ± 4.3		NS
Female sex (N,%)	115	68%	56	66%	NS
Ethnicity (N, %)					
Caucasian	136	80%	68	80%	NS
African American	13	8%	6	7%	
Hispanic	13	8%	6	7%	
Asian	7	4%	5	6%	
BMI (N, %)					
Non-obese	145	86%	70	82%	NS
Obese	24	14%	15	18%	
Season (N, %)					
Fall	40	24%	14	16%	NS
Winter	51	30%	27	32%	
Spring	40	24%	24	28%	
Summer	38	22%	20	24%	
Vitamin D supplementation (N, %)					
None	139	82%	70	82%	NS
≤ 400 IU of VD_3 daily	24	14%	13	15%	
>400 IU of VD_3 daily	6	4%	2	2%	
Diagnosis (N, %)	• Juvenile idiopathic arthritis - 124, 73% • Systemic lupus erythematosus - 18, 11% • Vasculitis (Behcet's disease, Wegener's granulomatosis, Takayasu's arteritis, Henoch-Schönlein purpura) - 9, 5% • Dermatomyosistis - 8, 5% • Mixed connective tissue disease - 3, 2% • Scleroderma - 3, 2 % • Sjogren's - 2, 1% • Rheumatic Fever - 1, 0.5% • Neonatal lupus - 1, 0.5%		• Orthopedic disorders (patello-femoral syndrome, osteochondritis dissecans, meniscus strain, fractures, Osgood-Schlatter) - 28, 33% • Other (asthma, fatigue, arthralgias) - 11, 13% • Infectious diseases (Lyme, viral infections, bacterial infections) - 11, 13% • Abnormal labs - 10, 12% • Pain amplification syndrome - 10, 12% • Hypermobility and growing pain - 9, 10% • Acrocyanosis - 6, 7%		N/A

Table A2.2: Multivariate analysis of variables associated with vitamin D deficiency [serum 25(OH)D < 20ng/ml]

Variable	Estimate	Std Error	P value	Odds ratios
Season (fall, winter, and spring)	1.140	0.386	0.0032	9.78
Ethnicity (Caucasian)	-1.043	0.195	<.0001	0.12
Body mass index (non-obese)	-0.503	0.234	0.0317	0.36
Disease (autoimmune disorders)	0.412	0.209	0.0489	2.28
Supplementation (none)	0.792	0.330	0.0166	4.87

Table A2.3: Multivariate analysis of variables associated with serum 25(OH)D levels

Variable	Estimate	Std Error	P value
Age	-0.304	0.128	0.019
Vitamin D supplementation (none)	-5.701	1.177	<0.0001
Vitamin D supplementation (≤ 400 IU of VD_3 daily)	-2.221	1.423	0.12
Autoimmune disorder	-1.112	0.604	0.06
Body mass index (non-obese)	2.5462	0.798	0.001
Ethnicity (Caucasian)	3.9210	0.741	<0.0001
Season (fall)	-0.265	1.060	0.8
Season (winter)	-3.643	0.925	0.0001
Season (spring)	-2.487	0.993	0.013

Table A2.4: Mean serum 25(OH)D level by autoimmune disorder and medication use

	Mean serum 25(OH)D level in ng/ml (±SD)
Autoimmune disorder	
Juvenile idiopathic arthritis	28.9 (±10.6)
Systemic lupus erythematosus	21.3 (±12)
Vasculitis	29.3 (±12.5)
Dermatomyosistis	32.1 (±16)
Mixed connective tissue disease	21.3 (±14.6)
Scleroderma	26 (±1.7)
Sjogren's	32 (±1.4)
Medication used	
None	29.7 (±11.4)
Non-steroidal anti-inflammatory drugs	28.7 (±11.3)
Prednisone	22 (±10.7)
Biologics	27.9 (±12)
Disease modifying anti-rheumatic drugs	29.3 (±10.8)
Intravenous immunoglobulin	32.5 (±6.4)

Appendix 3: Model Diagnostics

The best linear form for continuous variables was analyzed and the assumption of linearity was met, except for age in model 2.

In the first linear regression model (Table A3.1), we looked at the association of serum 25(OH)D levels with JADAS-27, and we adjusted for potential confounders, identified a priori, including age, gender, JIA subtype, ethnicity, (non-Hispanic whites versus others), medications (none, non-steroidal anti-inflammatory drugs, or immunosuppressants - methotrexate and/or biologics), season (summer versus others), and time since disease onset. Only JIA subtype (rheumatoid factor-positive polyarticular JIA) and ethnicity were significantly associated with JADAS-27.

We looked at the model diagnostics. The residuals were normally distributed (Figure 1). There was a slight fan-shaped pattern on the distribution of the residuals, which was consistent with a violation of the assumption of homoscedasticity (Figure 2). We did a log transformation of the outcome (JADAS-27) and re-ran the model. Regarding our variable of interest, serum 25(OH)D levels, the p-value remained quite similar (Table A3.2). The residuals of this new model were not normally distributed (Figure 3), although the assumption of homoscedasticity was met (Figure 4). Hence, since the model did not change significantly, and the model residuals fit better in the original model, we decided to keep the original outcome JADAS-27, instead of the log transformation.

In the original model, there were some outliers and influential points in the Cook's D influence and leverage plots (Figure 5). So we excluded the 8 influential points (116, 61, 103, 51, 144, 26, 121, and 88) and ran a new model (Table A3.3). There was not a significant change regarding betas and p-values in this model without the influential points, except for other subtypes of JIA becoming significant. However, our predictor of interest, serum 25(OH)D had an even weaker beta. As those other variables were just being included in the model as possible confounders,

and our variable of interest did not have an important change, we kept the original model, with all 154 subjects in it. Besides, there was no reason to think that there was any error in measurement or recording of these points.

The second model was a logistic regression model that analyzed variables associated with low vitamin D levels, defined as the lowest quartile of our sample [serum 25(OH)D <23 ng/ml], vs. higher levels of serum 25(OH)D (≥23 ng/ml). The variables of interest were: age (up to 10 years old vs. older than 10), ethnicity (non-Hispanic whites vs. other ethnicities), body mass index (obese vs. non-obese), and season (summer vs. non-summer) (Table A3.4). In this model age was dichotomized, instead of being used as a continuous variable, due to non-linearity. The median age was used to determine the cut-off point for the dichotomization. As this model was exploratory, we considered a $p<0.1$ as significant. The residuals and DFBETAS showed some outliers and influential points (Figure 6). We excluded the 6 influential points (79, 148, 3, 92, 41 and 48) and re-ran the model (Table A3.5). The model did not have dramatic changes after excluding the influential points, and there was no reason to think that there was any error in measurement or recording of the data, so we kept the original model. Hosmer and Lemeshow test was performed to asses goodness of fit and showed a good fit of the model ($p=0.75$) (Table A3.6). The area under the curve for the ROC curve was 0.69 (Figure 8).

The third model was a linear regression model developed to analyze variables possibly associated with serum 25(OH)D levels, including age, ethnicity (non-Hispanic whites vs. other ethnicities), BMI (obese vs. non-obese), season (summer vs. non-summer), and total daily vitamin D intake (Table A3.7). In the model diagnostics, the assumption of normality and homoscedasticity were met (Figures 9 and 10). However, there were some outliers and influential points on Cook's D influence and leverage plots (Figure 11). We excluded 7 influential points

(151, 28, 150, 144, 125, 61, and 9) and re-ran the model (Table A3.8). The changes in betas and p-values were not dramatic, and we looked at those individual points, and there was no reason to think that there was any error in measurement or recording of the data. So we kept the original model, with all the 154 points.

Table A3.1: Multivariate linear regression model to determine the association between serum 25(OH)D levels and JADAS-27

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	9.04509	2.40574	3.76	0.0002*
age	-0.012995	0.133048	-0.10	0.9223
gender[0]	0.0075413	0.484759	0.02	0.9876
JIA 2[0]	-1.780859	1.488302	-1.20	0.2335
JIA 2[1]	1.7550581	1.104493	1.59	0.1143
JIA 2[2]	4.9439069	2.466851	2.00	0.0470*
JIA 2[3]	-0.161761	2.393614	-0.07	0.9462
JIA 2[4]	-1.678578	1.21847	-1.38	0.1705
eth col[0]	-1.94077	0.697421	-2.78	0.0061*
meds[0]	-1.363686	0.678153	-2.01	0.0463*
meds[1]	0.7689796	0.665516	1.16	0.2499
season col[0]	0.0927715	0.612767	0.15	0.8799
timedis	-0.000597	0.01274	-0.05	0.9627
25OHD	0.022224	0.05215	0.43	0.6706

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
age	1	1	0.28167	0.0095	0.9223
gender	1	1	0.00715	0.0002	0.9876
JIA 2	5	5	572.29776	3.8765	0.0025*
eth col	1	1	228.64970	7.7439	0.0061*
meds	2	2	123.13366	2.0851	0.1281
season col	1	1	0.67679	0.0229	0.8799
timedis	1	1	0.06478	0.0022	0.9627
25OHD	1	1	5.36235	0.1816	0.6706

Table A3.2: Multivariate linear regression model after log-transformation of the outcome (JADAS-27)

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.8589614	0.499887	3.72	0.0003*
age	-0.009195	0.028548	-0.32	0.7479
gender[0]	0.0204226	0.101399	0.20	0.8407
JIA 2[0]	-0.282297	0.312731	-0.90	0.3683
JIA 2[1]	0.2626787	0.231743	1.13	0.2590
JIA 2[2]	0.1623665	0.512171	0.32	0.7517
JIA 2[3]	0.652993	0.567168	1.15	0.2516
JIA 2[4]	-0.353168	0.261104	-1.35	0.1785
eth col[0]	-0.297619	0.145946	-2.04	0.0434*
meds[0]	-0.181645	0.144272	-1.26	0.2102
meds[1]	0.1641235	0.140249	1.17	0.2440
season col[0]	0.0161518	0.129044	0.13	0.9006
timedis	-0.000727	0.002678	-0.27	0.7865
25OHD	0.0059588	0.010948	0.54	0.5872

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
age	1	1	0.129519	0.1037	0.7479
gender	1	1	0.050647	0.0406	0.8407
JIA 2	5	5	12.695487	2.0337	0.0778
eth col	1	1	5.192030	4.1585	0.0434*
meds	2	2	2.572617	1.0303	0.3597
season col	1	1	0.019560	0.0157	0.9006
timedis	1	1	0.091984	0.0737	0.7865
25OHD	1	1	0.369854	0.2962	0.5872

Table A3.3: Multivariate linear regression model to determine the association between serum 25(OH)D levels and JADAS-27 after exclusion of 8 influential points

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	10.475961	1.950491	5.37	<.0001*
age	-0.092205	0.108193	-0.85	0.3956
gender[0]	0.1780321	0.384104	0.46	0.6438
JIA 2[0]	-2.772902	1.209916	-2.29	0.0235*
JIA 2[1]	1.2675523	0.881588	1.44	0.1529
JIA 2[2]	8.8349555	2.165688	4.08	<.0001*
JIA 2[3]	-0.544476	1.866902	-0.29	0.7710
JIA 2[4]	-3.285978	0.992187	-3.31	0.0012*
eth col[0]	-1.686222	0.55003	-3.07	0.0026*
meds[0]	-0.76733	0.541562	-1.42	0.1589
meds[1]	-0.03516	0.527538	-0.07	0.9470
season col[0]	0.3991912	0.48825	0.82	0.4151
timedis	-0.009041	0.010506	-0.86	0.3911
25OHD	0.0030022	0.041979	0.07	0.9431

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
age	1	1	0.53802	0.0179	0.8939
gender	1	1	4.44285	0.1475	0.7015
JIA 2	5	5	503.48008	3.3432	0.0071*
eth col	1	1	277.72454	9.2208	0.0029*
meds	2	2	146.88754	2.4384	0.0912
season col	1	1	0.44756	0.0149	0.9032
timedis	1	1	0.08967	0.0030	0.9566
25OHD	1	1	5.09564	0.1692	0.6815

Table A3.4: Multivariate logistic regression model to determine variables associated with the lowest quartile of serum 25(OH)D

Term	Estimate	Std Error	ChiSquare	Prob>ChiSq	Lower 95%	Upper 95%
Intercept	-0.775865	0.3836122	4.09	0.0431*	-1.574759	-0.0477073
age c2[0]	-0.4505969	0.205574	4.80	0.0284*	-0.8678577	-0.0566519
eth col[0]	-0.5592947	0.2656478	4.43	0.0353*	-1.0817909	-0.0277099
BMI col[0]	-0.4381783	0.2381491	3.39	0.0658	-0.9043713	0.03758732
season col[0]	0.32083007	0.2979793	1.16	0.2816	-0.222067	0.97443148

Odds Ratios

For VDS 2 odds of 0 versus 1

Odds Ratios for age c2

Level1	/Level2	Odds Ratio	Reciprocal
1	0	2.4625413	0.4060846

Odds Ratios for eth col

Level1	/Level2	Odds Ratio	Reciprocal
1	0	3.0605342	0.3267403

Odds Ratios for BMI col

Level1	/Level2	Odds Ratio	Reciprocal
1	0	2.402132	0.4162969

Odds Ratios for season col

Level1	/Level2	Odds Ratio	Reciprocal
1	0	0.5264178	1.8996319

Table A3.5: Multivariate logistic regression model to determine variables associated with the lowest quartile of serum 25(OH)D after exclusion of 6 influential points

Term	Estimate	Std Error	ChiSquare	Prob>ChiSq	Lower 95%	Upper 95%
Intercept	-0.7800421	0.3949622	3.90	0.0483*	-1.5997976	-0.028299
age c2[0]	-0.5404951	0.2160797	6.26	0.0124*	-0.9831758	-0.1295111
eth col[0]	-0.6151366	0.272002	5.11	0.0237*	-1.1533702	-0.0735874
BMI col[0]	-0.3972643	0.2574625	2.38	0.1228	-0.89892	0.12204173
season col[0]	0.30415169	0.3020302	1.01	0.3139	-0.2474327	0.9646246

For log odds of 0/1

Odds Ratios

For VDS 2 odds of 0 versus 1

Odds Ratios for age c2

Level1	/Level2	Odds Ratio	Reciprocal
1	0	2.9475968	0.3392594

Odds Ratios for eth col

Level1	/Level2	Odds Ratio	Reciprocal
1	0	3.4221645	0.2922127

Odds Ratios for BMI col

Level1	/Level2	Odds Ratio	Reciprocal
1	0	2.2133973	0.4517942

Odds Ratios for season col

Level1	/Level2	Odds Ratio	Reciprocal
1	0	0.5442735	1.8373115

Table A3.6: Hosmer and Lemeshow test (model 2)

Partition for the Hosmer and Lemeshow Test

Group	Total	VDS 2 = 0		VDS 2 = 1	
		Observed	Expected	Observed	Expected
1	16	1	1.16	15	14.84
2	37	4	4.80	33	32.20
3	11	1	1.78	10	9.22
4	12	4	3.16	8	8.84
5	47	14	12.62	33	34.38
6	11	5	3.57	6	7.43
7	20	8	9.90	12	10.10

Hosmer and Lemeshow Goodness-of-Fit Test

Chi-Square	DF	Pr > ChiSq
2.6648	5	0.7515

Table A3.7: Multivariate linear regression model to determine variables associated with serum 25(OH)D levels

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	30.239463	2.093896	14.44	<.0001*
age	-0.31659	0.155293	-2.04	0.0433*
eth col[0]	2.6402208	1.06205	2.49	0.0140*
BMI col[0]	2.5116424	0.91177	2.75	0.0066*
season col[0]	-2.276567	0.904532	-2.52	0.0129*
total VD	0.0002046	0.001186	0.17	0.8632

Table A3.8: Multivariate linear regression model to determine variables associated with serum 25(OH)D levels after exclusion of 7 influential points

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	29.452164	1.899151	15.51	<.0001*
age	-0.249836	0.140731	-1.78	0.0780
eth col[0]	1.9085422	0.96855	1.97	0.0507
BMI col[0]	2.2030836	0.842798	2.61	0.0099*
season col[0]	-2.378762	0.823857	-2.89	0.0045*
total VD	0.0013687	0.001141	1.20	0.2324

Figure 1: Residuals' distribution and normal probability plot (model 1)

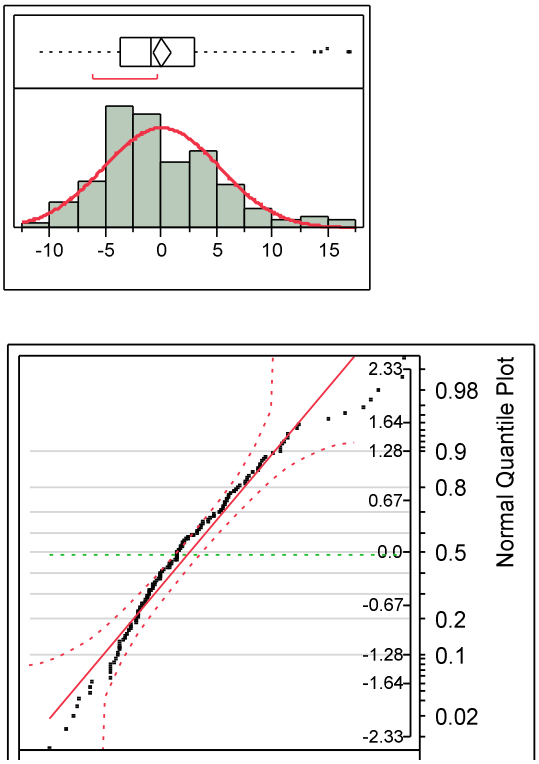
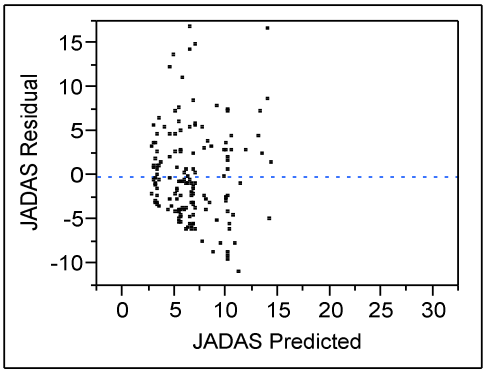


Figure 2: Residuals by predicted plots (model 1)



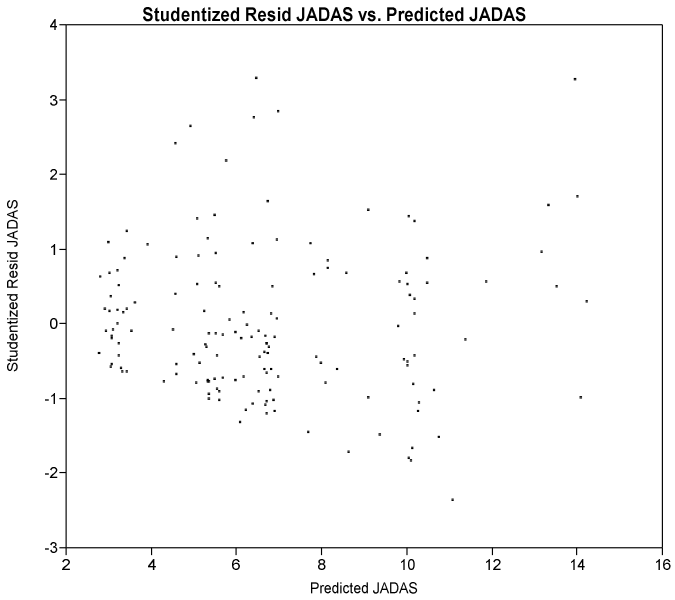
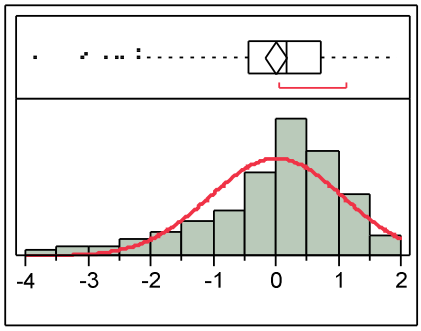


Figure 3: Residuals' distribution and normal probability plot (model 1 after log transformation of JADAS-27)



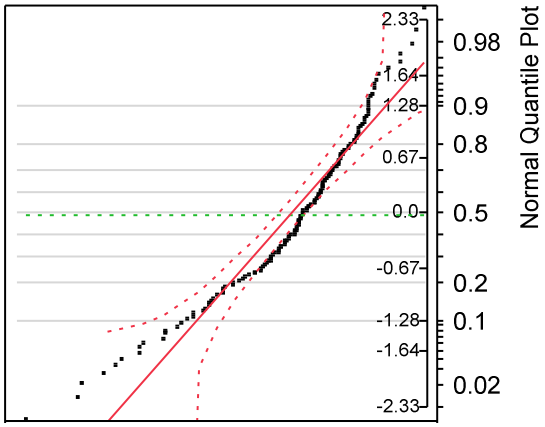
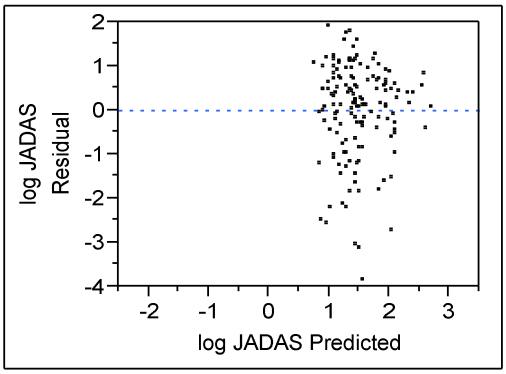


Figure 4: Residuals by predicted plots (model 1 after log transformation of JADAS-27)



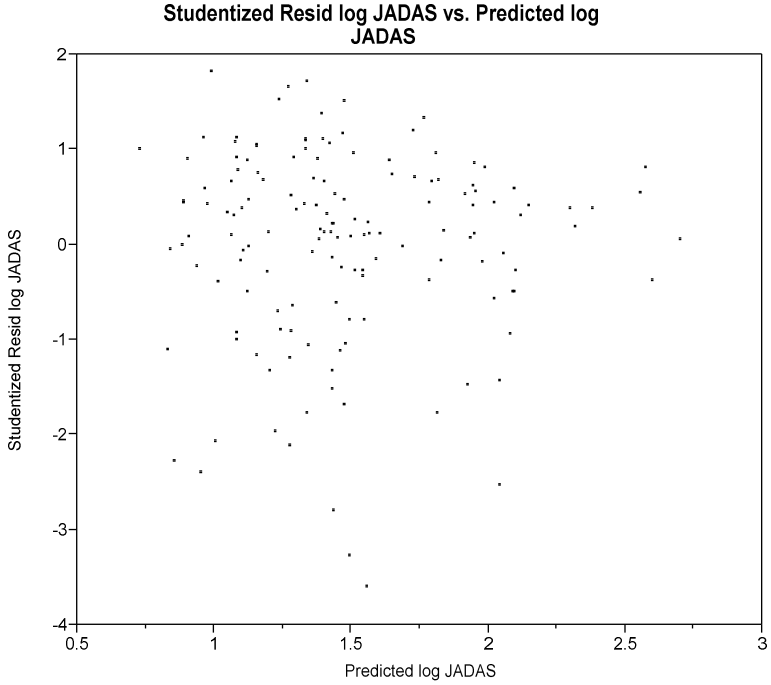
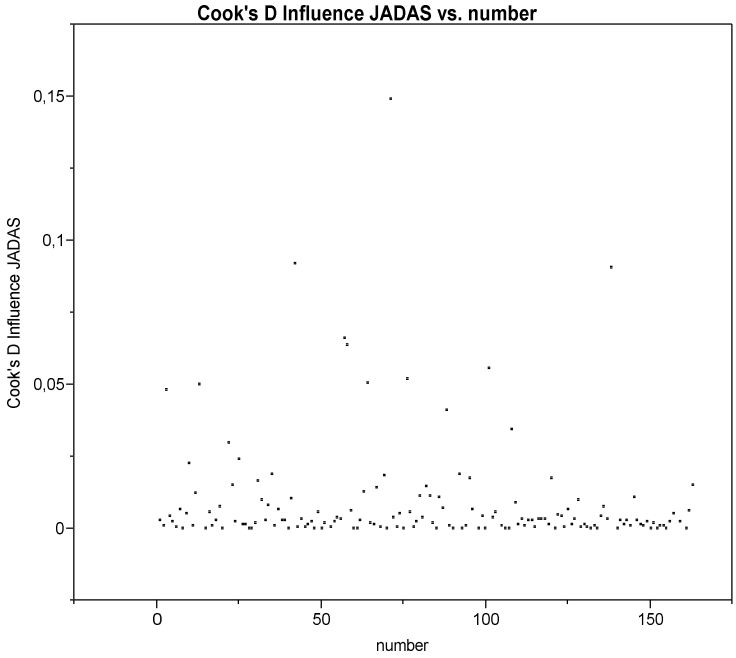
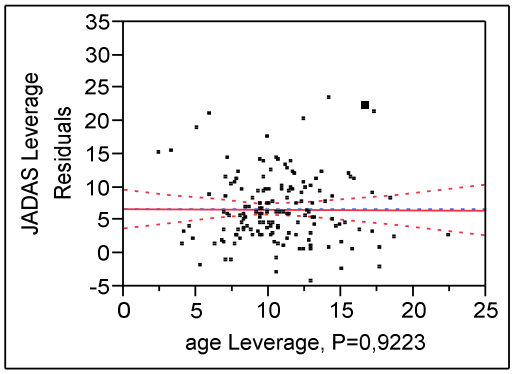
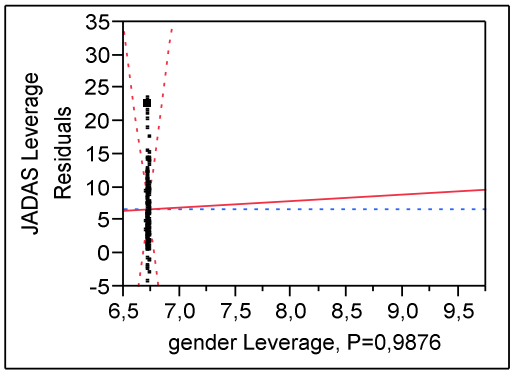
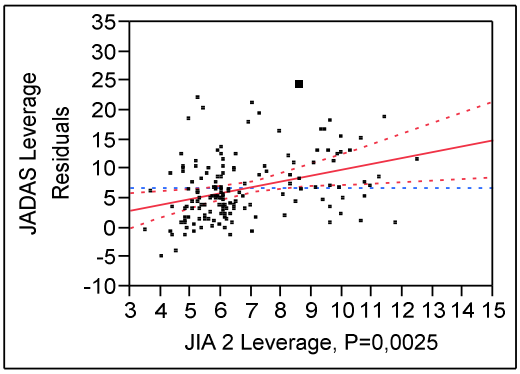


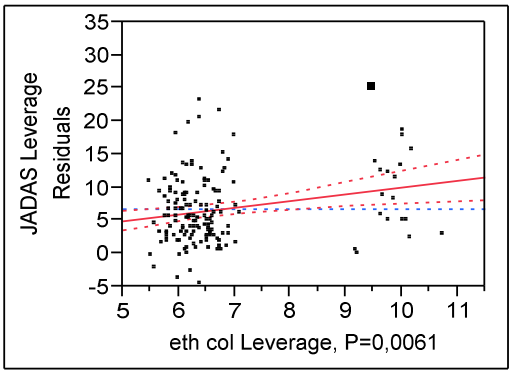
Figure 5: Cook's D influence and leverage plots (model 1)








JADAS Leverage Residuals
35
30
25
20
15
10
5
0
-5
-10
3 4 5 6 7 8 9 10 11 12 13 14 15
JIA 2 Leverage, P=0,0025


JADAS Leverage Residuals
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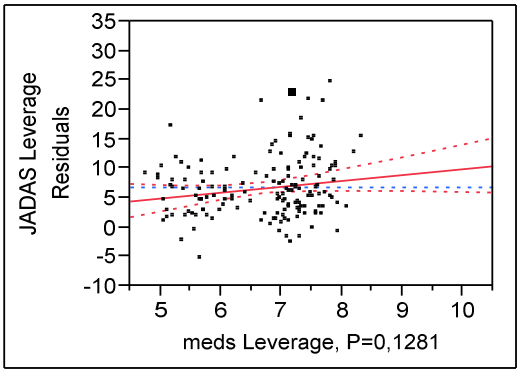
30
25
20
15
10
5
0
-5
5 6 7 8 9 10 11
eth col Leverage, P=0,0061


JADAS Leverage Residuals
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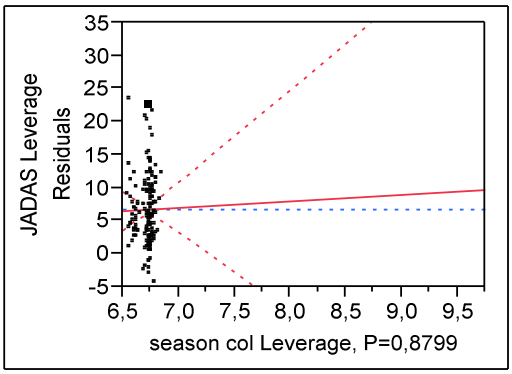
30
25
20
15
10
5
0
-5
-10
5 6 7 8 9 10
meds Leverage, P=0,1281


JADAS Leverage Residuals
35
30
25
20
15
10
5
0
-5
6,5 7,0 7,5 8,0 8,5 9,0 9,5
season col Leverage, P=0,8799

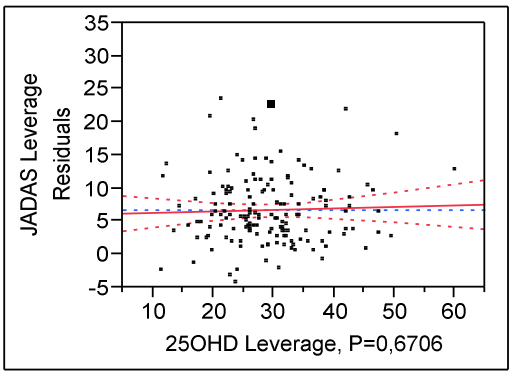
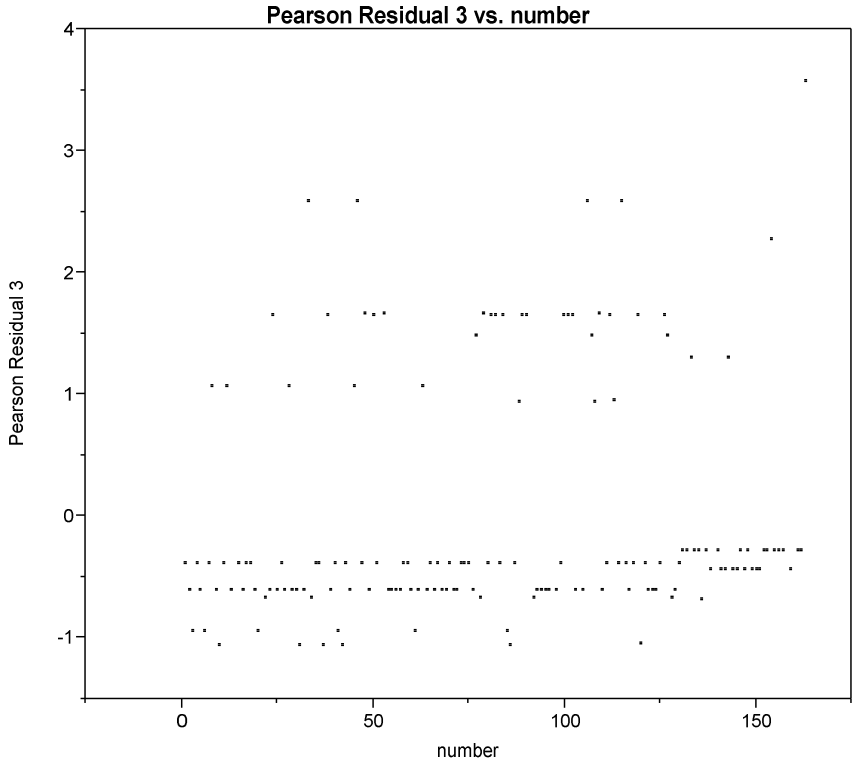
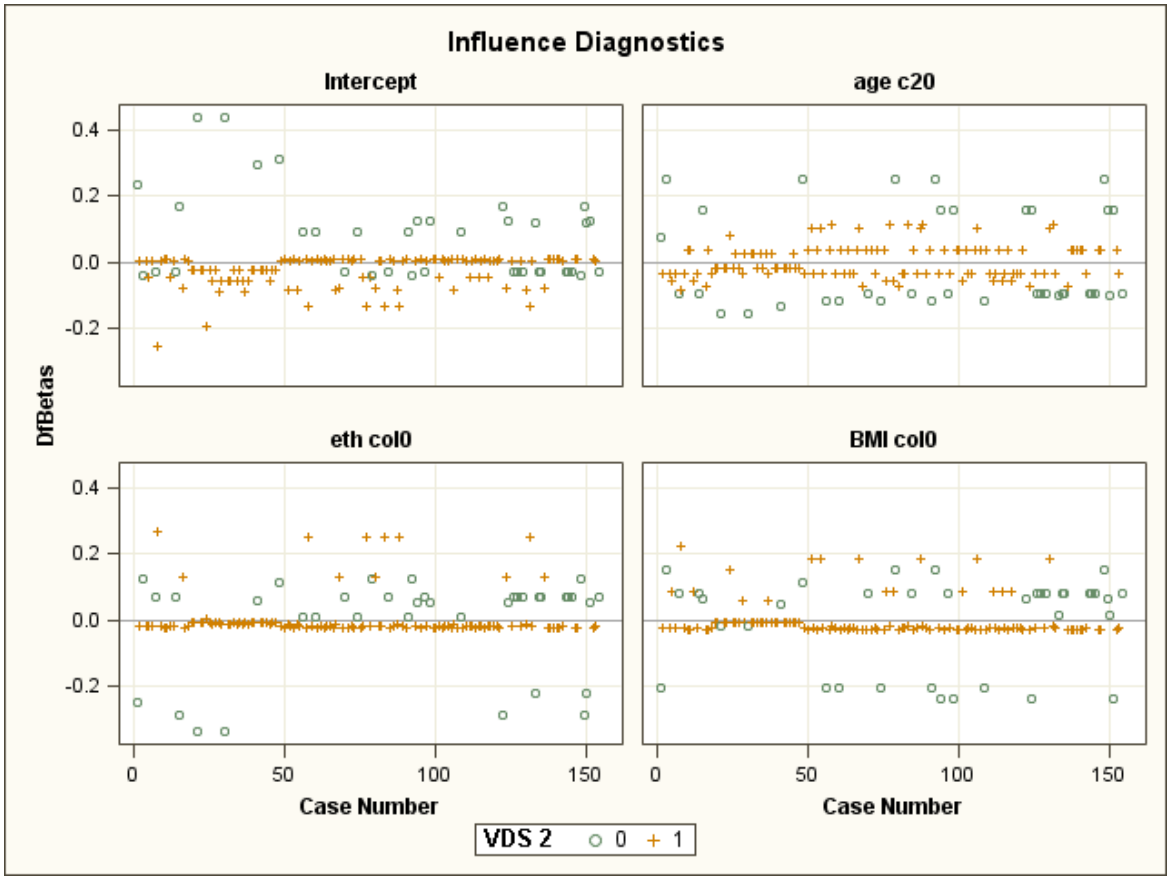

JADAS Leverage Residuals
35
30
25
20
15
10
5
0
-5
10
20
30
40
50
60
25OHD Leverage, P=0,6706

Figure 6: Residuals and DFBETAS plots (model 2)





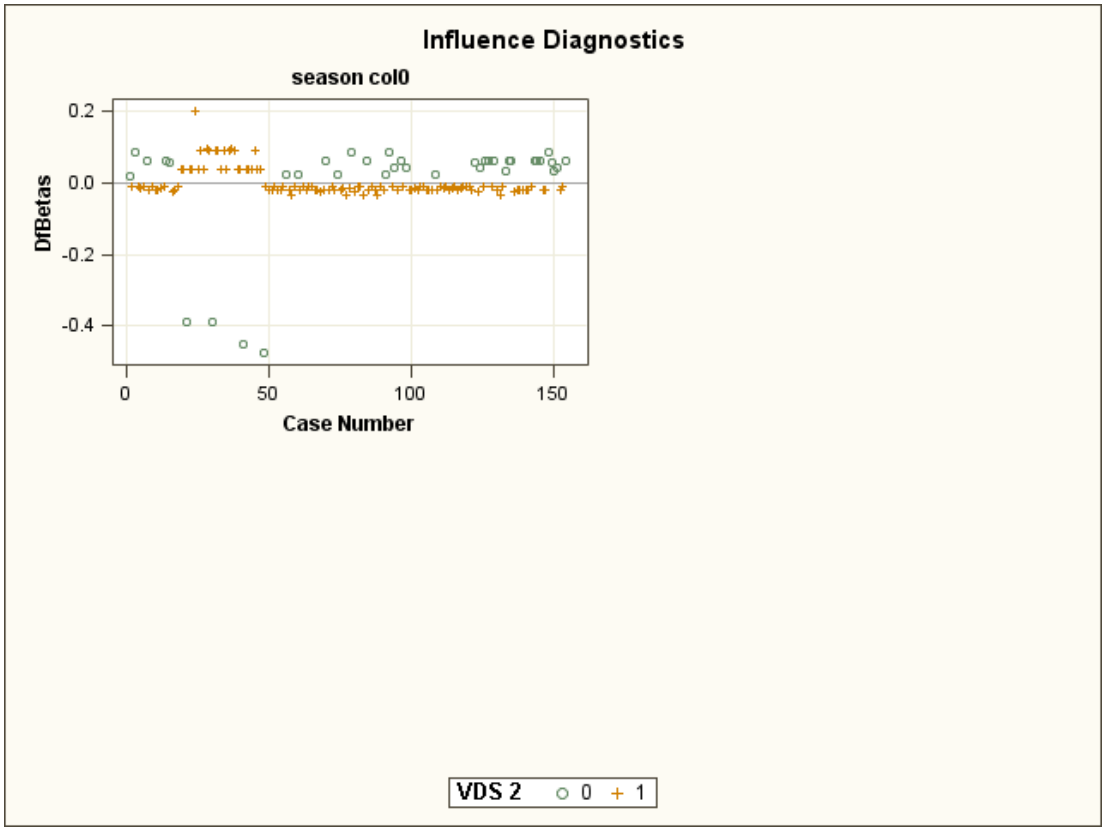
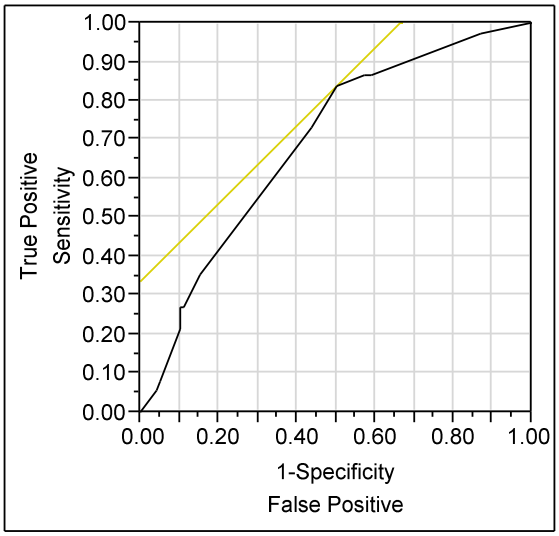
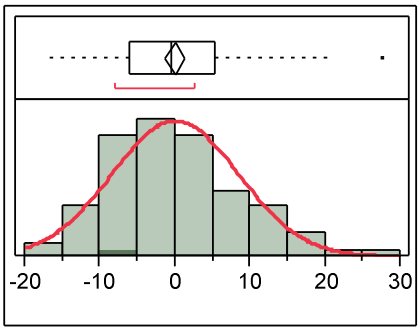

Influence Diagnostics
season col0
DfBetas
0.2
0.0
-0.2
-0.4
0
50
100
150
Case Number
VDS 2
0
1

Figure 7: ROC curve (model 2)



Area Under Curve = 0.68965

Figure 8: Residuals' distribution and normal probability plot (model 3)



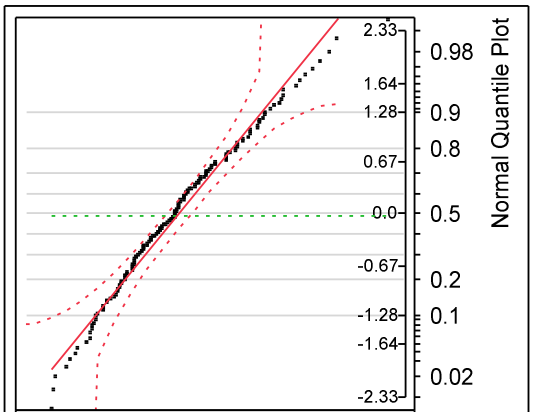
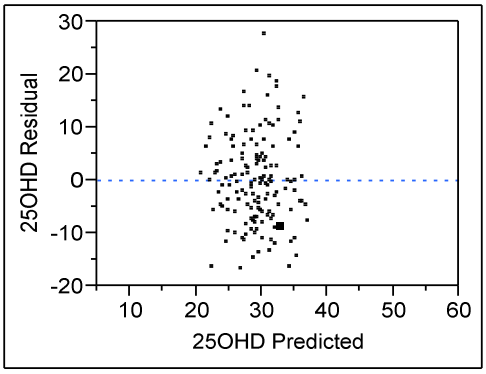


Figure 9: Residuals by predicted plots (model 3)



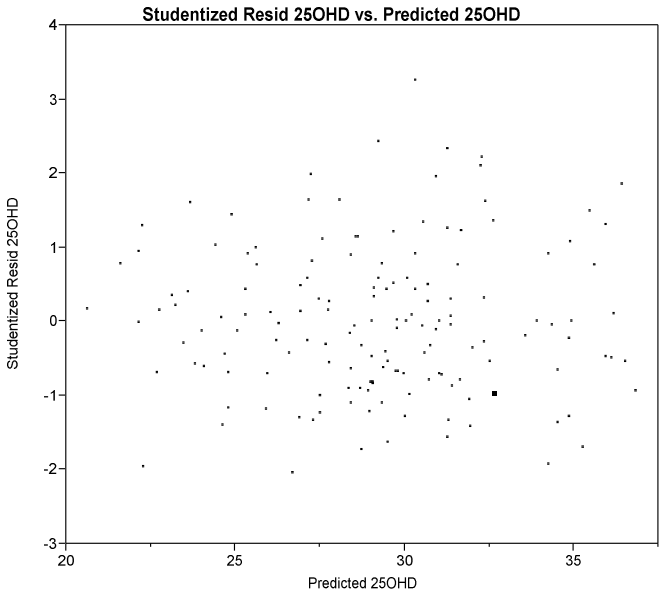
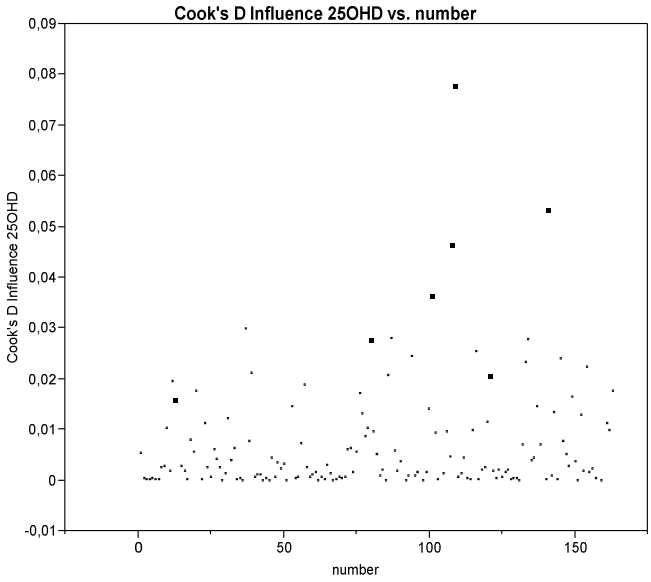
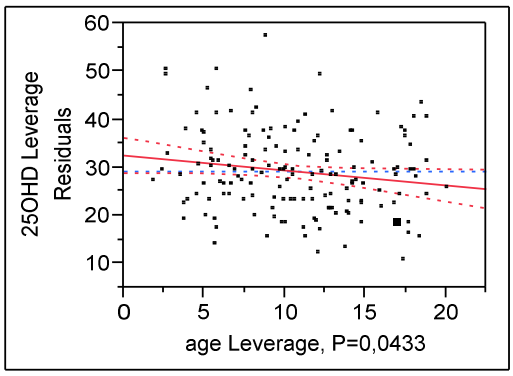
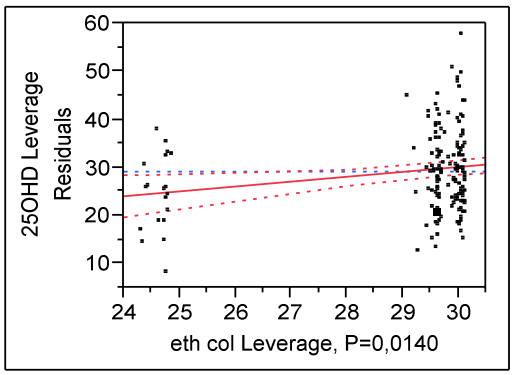
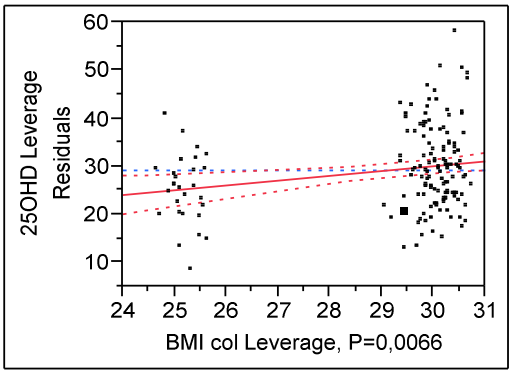


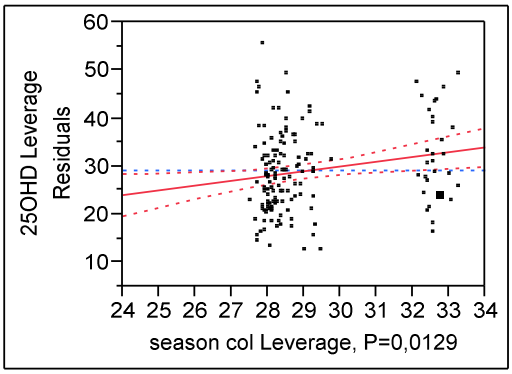
Figure 10: Cook's D influence and leverage plots (model 3)

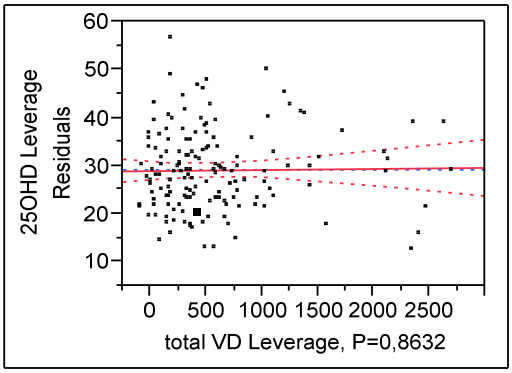







25OHD Leverage Residuals
60
50
40
30
20
10
24 25 26 27 28 29 30 31
BMI col Leverage, P=0,0066


25OHD Leverage Residuals
60
50
40
30
20
10
24 25 26 27 28 29 30 31 32 33 34
season col Leverage, P=0,0129


25OHD Leverage Residuals
60
50
40
30
20
10
0 500 1000 1500 2000 2500
total VD Leverage, P=0,8632

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