Title
Nutrient Modulators of Inflammation in Obese and Lean Adolescents

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Nutrient Modulators of Inflammation in Obese and Lean Adolescents

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Clinical Research

by

Emily Clark King

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ABSTRACT OF THE THESIS

Nutrient Modulators of Inflammation in Obese and Lean Adolescents

by

Emily Clark King

Master of Science in Clinical Research

University of California, Los Angeles, 2017

Professor Robert M. Elashoff, Chair

Background: Obese individuals are at risk for iron deficiency due to both insufficient dietary iron intake and trapping of iron through an interleukin-6 (IL-6) dependent pathway. Vitamin D deficiency, which is also common in obese individuals, contributes to the pro-inflammatory state of obesity. Obese individuals are also at increased risk for insulin resistance, which may be worsened by iron and vitamin D deficiency.

Objective: To evaluate the associations of, and interaction between, iron and vitamin D with inflammation and insulin resistance.

Design/Methods: 19 obese (BMI Z-score ≥ 2.0) and 28 lean (BMI Z-score -2.0 to 1.0) subjects were recruited (mean age 14.5 years, SD 2.1 years) in a cross-sectional study. Fasting serum was analyzed for iron status [soluble transferrin receptor (sTfR)], IL-6, and 25-hydroxy vitamin D (25[OH]D). Insulin sensitivity (Si) was determined by frequently sampled intravenous glucose tolerance test (FSIGT.) Iron deficiency was defined as sTfR level>8.3 mg/L. Vitamin D deficiency was defined as 25(OH)D level<20 ng/mL. Linear regression was used to measure the associations of, and interaction between, 25(OH)D and sTfR with IL-6 and insulin sensitivity.

Results: The prevalence of vitamin D deficiency was 37% in obese vs. 10% in lean subjects. STfR was negatively associated with Si ($p<0.01$) and positively associated with IL-6 ($p<0.0001$). 25(OH)D trended toward a positive association with Si ($p=0.08$) and was negatively associated with IL-6 ($p<0.01$). There was no interaction between sTfR and 25(OH)D in the associations with either Si or IL-6.

Conclusions: Higher vitamin D levels and better iron status are associated with increased insulin sensitivity and decreased inflammation in our small cohort. Micronutrient modulation may have a future role in dietary interventions for the prevention of insulin resistance and complications of chronic inflammation.

The thesis of Emily Clark King is approved.

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University of California, Los Angeles

2017

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Acknowledgments

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Introduction

There is a growing consensus that obesity is an inflammatory state and that this inflammation contributes to the increased risk for insulin resistance and cardiovascular disease in obese individuals. In addition, multiple nutrient deficiencies are common in obese individuals and may contribute to the inflammatory state. Our study focuses on iron and vitamin D deficiencies.

Iron deficiency is common among obese children and adolescents (1–5). In the United States, iron deficiency in obese children and adolescents is hypothesized to result from both 1) regular consumption of excess calories but insufficient micronutrients including iron, and 2) obesity-related inflammation (3,4,6).

Inflammation impacts iron trafficking in the following fashion. Hepcidin is a hepatic protein that decreases iron efflux out of macrophages and enterocytes through degradation of the membrane-bound protein ferroportin. Hepcidin, and other markers of iron status such as ferritin, are upregulated in response to the pro-inflammatory cytokine interleukin-6 (IL-6). In contrast, soluble transferrin receptor (sTfR), a marker of availability of iron to the bone marrow, does not fluctuate based on concurrent inflammation. Similar to IL-6, vitamin D deficiency also increases the expression of hepcidin (7). Consequently, in the face of acute or chronic inflammation, or in the pro-inflammatory state associated with vitamin D deficiency, as a result of increased hepcidin, iron that would be recycled from senescent red blood cells instead becomes trapped in macrophages, and dietary iron that would be absorbed becomes trapped in enterocytes. These processes result in a functional iron deficiency. Whether dietary or functional in origin, iron deficiency restricts the availability of body iron for key enzymatic processes, which may then increase inflammation and also possibly insulin resistance.

Although most previous studies have suggested that iron excess is associated with the development of insulin resistance (8), these studies relied on surrogate measures of insulin resistance (e.g. HOMA-IR). In addition, they used measures of iron status that are confounded by inflammation (e.g. ferritin). Thus, the association between iron status and insulin resistance has not been adequately assessed. In contrast to these studies, there is reason to believe that iron *deficiency* could contribute to insulin resistance. For example, stearoyl-CoA desaturase enzyme 1 (SCD1) is an iron-dependent enzyme that produces monounsaturated fatty acids from saturated fatty acids, thereby decreasing inflammation. In animal studies, low iron availability decreases SCD1 activity. Therefore, with low iron availability, in humans, there may be less SCD1 activity and therefore increased inflammation which may then promote insulin resistance (9,10)

Vitamin D deficiency is common in obese individuals, including obese children and adolescents (11,12) and is associated with insulin resistance and a higher risk for cardiovascular disease (13). Vitamin D deficiency may contribute to the inflammatory environment in obese individuals. For example, the active form of vitamin D, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], has been shown to directly suppress inflammation through a toll-like receptor-2 dependent pathway and activation of regulatory T cells (14–16). Thus, vitamin D deficiency is associated with increased systemic inflammation and decreased insulin sensitivity in obese children (17–20). Vitamin D deficiency is associated with elevated inflammatory markers independent of obesity; for example, IL-6 concentrations and CRP levels are higher in overweight/obese children with 25(OH)D < 20ng/mL than in the overweight/obese children with 25(OH)D levels > 20ng/mL (21–23). Furthermore, clinical trials of vitamin D have shown improvement in insulin sensitivity, decreased hypercholesterolemia, decreased inflammatory processes, and decreased microvascular complications in pediatric diabetic patients (13,24,25).

The objective of this exploratory study was to examine the complex interplay in obese and lean individuals among iron, 25(OH)D, inflammation, and insulin resistance, where low vitamin D may directly and indirectly decrease iron availability, and iron deficiency may then be involved in a feed-forward loop between iron deficiency and inflammation. Combined, this could result in increased long-term risk for insulin resistance and cardiovascular disease. We hypothesized that iron deficiency and vitamin D deficiency would be identified more in obese compared to lean adolescents, and that lower levels of iron availability and vitamin D concentrations may have a synergistic effect on worsening inflammation and insulin resistance.

Research Design and Methods

Study Subjects

Stored samples from 19 obese (BMI z-score > 2.0) and 28 lean (BMI z-score -2.0 – 1.0) but otherwise healthy adolescents with an age range 9 – 17 years (median 13 years) were used for this study. Plasma, serum, and urine samples were collected previously during a study of the associations between bone and energy metabolism (26). These subjects were recruited from the community around the University of Minnesota, Minneapolis, MN. Exclusion criteria included diagnosis of diabetes mellitus, polycystic ovarian syndrome, treatment with a medication that alters insulin sensitivity, secretion, or beta-cell mass, participation in a concurrent intervention trial, and pregnancy. Informed consent was obtained from the parents or guardians of all participants that included the storage of samples and use of samples in future research. Assent was obtained from all participants. Approval for the inclusion of human subjects was obtained from the Institutional Review Board at the University of Minnesota.

Clinical Measures

A physical examination was performed on all subjects by a physician for pubertal staging by the Tanner method. Anthropometric measurements included height measured by wall-mounted stadiometer (without shoes) to the nearest 0.1 cm (average of 3 measurements taken) and weight by electronic scale to the nearest 0.1 kg. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Bone age was determined by plain radiograph of the left hand and wrist using the reference standards published by the Greulich and Pyle method (27).

Lab Measures

All participants underwent an insulin modified frequently sampled IV glucose tolerance test (FSIGT) following a 10-hour fast (28,29). Insulin sensitivity (SI) was determined by the minimal model (MINMOD) method for interpretation of FSIGT. SI measures the capacity of insulin to promote the clearance of glucose and inhibit endogenous glucose production; higher values indicate increased insulin sensitivity. Total 25(OH)D was measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). Vitamin D deficiency was defined as 25(OH)D concentration of < 20 ng/mL. IL-6 was measured by ELISA (Fairview Advanced Research and Diagnostic Lab, Minnesota). Iron status was assessed by sTfR using ELISA (Ramco Lab, Minnesota) because it is a measure of iron availability that is not influenced by inflammation. Iron deficiency was defined as sTfR concentration of > 8.3 mg/L.

Statistical Methods

The t-test was used to compare continuous variables, and the chi-squared test and one-sided Fisher's exact test were used to compare categorical variables in obese vs. lean groups. Skewed variables were log transformed. P-values <0.05 were considered statistically significant. Linear regression was used on the combined data set to measure the associations of 25(OH)D and Log(sTfR) with Log(IL-6) and Log(Si). Interactions between 25(OH)D and Log(sTfR), 25(OH)D and obese vs. lean, and Log(sTfR) and obese vs. lean, in the associations with Log(IL-6) and Log(Si) were tested. If a significant interaction was found with obese vs. lean status, within group analyses were also performed. All analyses were made with a type one error of 0.05. STATA IC 14 was the statistical software used.

Results

Subject Characteristics

19 obese participants and 28 lean participants were matched for age (Table 1) (mean 14.5 +/- 2.1 years) and skeletal age (mean 15 +/- 2.1 years). Of note, there was a greater proportion of females in the lean group. The racial composition differed in the two groups ($p<0.01$), with a greater proportion of white participants in the lean group (75% vs. 42%) and a greater proportion of black participants in the obese group (21% vs. 3.6%). In obese participants, 5% were identified as iron deficient, while 0% of the lean group were iron deficient; 37% of the obese group were vitamin D deficient, compared to 10% of the lean group ($p<0.05$).

Table1. Population characteristics.		
	Obese[1]	Lean[2]
Variable	**Mean (SD) or N (%)** (N=19)	**Mean (SD) or N (%)** (N=28)
Age, years	14.6 (2.1)	14.5 (2.1)
Skeletal age, years	15.2 (2.1)	14.9 (2.2)
Gender, number of females (%)	8 (42%)	16 (57%)
Race**		
White	8 (42%)	21 (75%)
Black	4 (21%)	1 (3.6%)
American Indian	0 (0%)	1 (3.6%)
Other/mixed	6 (32%)	1 (3.6%)
Unknown	1 (5.3%)	4 (14.3%)
BMI Z-score	2.42 (0.22)	0.17 (0.71)
IL-6, pg/mL***	2.8 (1.5)	1.0 (0.67)
Insulin sensitivity, $(mU/L)^{-1}\ min^{-1}$***	1.4 (0.6)[2]	4.9 (2.6)[1]
sTfR (mg/L)***	5.5 (1.7)[1]	3.85 (1.28)
Iron deficient	1 (5.6%)[1]	0
25(OH)D, ng/mL**	21.4 (8.3)	37.4 (7.8)
Vitamin D deficient*	7 (37%)	3 (10%)
BMI=body mass index; 25(OH)D=25-hydroxyvitamin D; IL-6=Interleukin 6; sTfR=soluble transferrin receptor. [1]Obese = BMI z-score >2.0 [2]Lean = BMI z-score -2.0 – 0.99 *= p<.05, **=p<.01, ***=p<.001 Superscript indicates # missing data		

Table 2. Regression Analyses of Vitamin D and Iron status on Inflammation and Insulin Sensitivity.							
		Log (IL-6)			Log (Si)		
Model	Predictors	Regression coefficient	95% CI	P-value	Regression coefficient	95% CI	p-value
1	Log (sTfR)	0.98	0.48, 1.49	<0.001	-0.68	-1.17, -0.19	0.008
2	25(OH)D	-0.04	-0.07, -0.01	0.005	0.024	-0.003, 0.05	0.08
3	Log (sTfR)*25(OH)D	-0.018	-0.09, 0.05	0.61	-0.032	-0.11, 0.05	0.44
	Log (sTfR)	1.44	-0.43, 3.3	0.13	0.11	-2.04, 2.27	0.92
	25(OH)D	-0.011	-.12, .10	0.84	0.072	-0.05, 0.19	0.24

Table 3. Regression Analyses of Iron Status and obese category (lean vs obese) on Inflammation and Insulin Sensitivity.

Model	Predictors	Log (IL-6)			Log (Si)		
		Regression coefficient	95% CI	P-value	Regression coefficient	95% CI	p-value
1	obese	1.07	0.69, 1.45	<0.001	-1.20	-1.48, -0.91	<0.001
2	Log (sTfR)	0.98	0.48, 1.49	<0.001	-0.68	-1.17, -0.19	0.008
3	Obese*Log (sTfR)	-0.19	-1.24, 0.85	0.72	-0.30	-1.08, 0.47	0.44
	obese	1.15	-0.47, 2.77	0.16	-0.76	-1.97, 0.44	0.21
	Log (sTfR)	0.57	-0.22, 1.36	0.15	0.20	-0.38, 0.79	0.49

Table 4. Regression Analyses of Vitamin D status and obese category (lean vs obese) on Inflammation and Insulin Sensitivity.

Model	Predictors	Log (IL-6)			Log (Si)		
		Regression coefficient	95% CI	P-value	Regression coefficient	95% CI	p-value
1	obese	1.07	0.69, 1.45	<0.001	-1.20	-1.48, -0.91	<0.001
2	25(OH)D	-0.04	-0.07, -0.01	0.005	0.024	-0.003, 0.05	0.08
3	Obese*25(OH)D	-0.0014	-0.05, 0.05	0.95	-0.035	-0.07, 0.0005	0.053
	obese	0.99	-0.24, 2.22	0.11	-0.34	-1.26, 0.58	0.46
	25(OH)D	-0.019	-0.05, 0.01	0.24	0.015	-0.008, 0.04	0.2

Associations between Iron and Vitamin D with Insulin Sensitivity and Inflammation

We found no association between 25(OH)D and sTfR levels. In addition, there was no significant interaction between sTfR and 25(OH)D on the association with either IL6 or insulin

sensitivity (Table 2). Therefore, associations of sTfR and 25(OH)D with IL6 and insulin sensitivity are reported separately.

Greater sTfR, reflective of poorer iron status, was positively associated with IL6 ($p<0.001$) and negatively associated with insulin sensitivity (Figure 1). There was no interaction between obese status and sTfR on the association of sTfR with either IL6 or insulin sensitivity (Table 3).

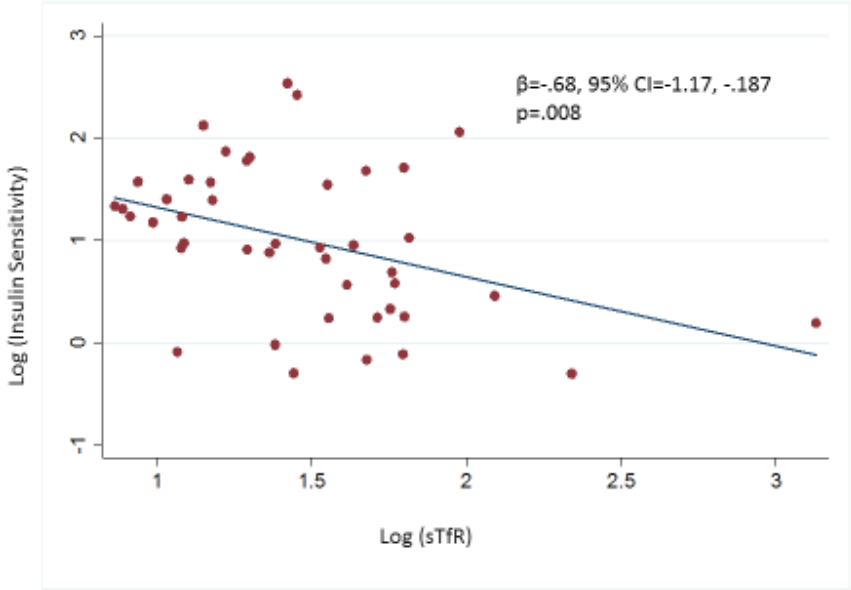

Figure 1. Associations of sTfR with Insulin Sensitivity

25(OH)D was negatively associated with IL6 ($p<0.01$) and trended towards a positive association with insulin sensitivity (Figure 2). There was no interaction between obese status and 25(OH)D on the association with IL6; however, there was a trend towards an interaction between obese status and 25(OH)D on the association with insulin sensitivity ($p=0.053$) (Table 4). Therefore, we tested the association of 25(OH)D with insulin sensitivity within each group. In

these sub-analyses, we found no association in the obese group (p=0.11) or the lean group (p=0.23).

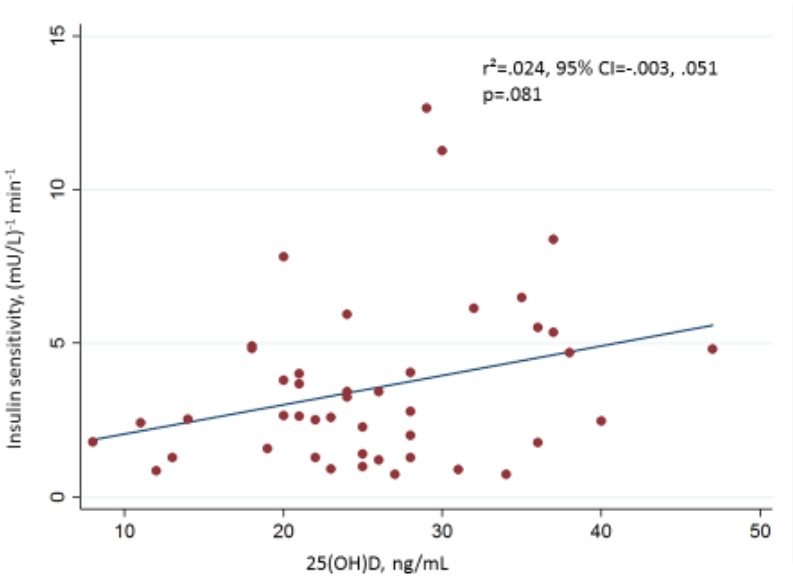

Figure 2. Associations of 25(OH)D with Insulin Sensitivity

Discussion

Obese individuals are known to have more inflammation and insulin resistance than lean individuals. Moreover, obese individuals are more likely to be vitamin D deficient. We sought to determine whether obese individuals are also more likely to be iron deficient compared to lean individuals and to determine if there is a synergistic interaction between iron deficiency and vitamin D deficiency that would thereby exacerbate the inflammation and insulin resistance in obese individuals.

In the present study, we found that sTfR was positively associated with IL6 and negatively associated with insulin sensitivity. Thus, poorer iron status is associated with increased inflammation and insulin resistance. The association of higher sTfR with inflammation is not surprising, but the association with insulin resistance is novel. In addition, we found an

interaction between obese status and vitamin D on insulin sensitivity; in other words, being obese amplified the relationship between lower vitamin D levels and worse insulin resistance. Finally, our data did not support our hypothesis of a synergistic interaction between iron deficiency and vitamin D deficiency on inflammation or insulin resistance.

The prevalence of iron deficiency was not statistically significantly higher in our obese subgroup compared to our lean subgroup. This is likely due to a relatively small sample size because it is inconsistent with other studies that have found a higher prevalence of iron deficiency in obese compared to lean children (1,3–5). Regardless of BMI status, we found that greater sTfR, reflective of poorer iron status, is associated with increased insulin resistance. This is contrary to most previous studies, which have shown that iron excess is associated with insulin resistance (8). However, these studies used surrogate measures of insulin resistance (e.g. HOMA-IR) and measures of iron that may be confounded by inflammation (e.g. ferritin). In contrast, our study had improved scientific rigor by using the gold standard for measures of insulin sensitivity (FSIGT) and a measure of iron status that is more resistant to inflammation (sTfR).

Not surprisingly, the prevalence of vitamin D deficiency was higher in our obese subgroup compared to our lean subgroup, which is consistent with previous studies of children (21). Similarly, decreased levels of vitamin D were associated with increased levels of IL6 in our study group. Vitamin D deficiency was found to be associated with insulin resistance in our group, just as other studies have shown decreased levels of vitamin D to be associated with insulin resistance, especially in overweight and obese pediatric populations (20,22).

The primary limitation of this study was the small sample size. This limits the application of our findings to a larger scale and our ability to detect differences based on a p-value of 0.05. In addition, our study may have been under-powered to detect an interaction between vitamin D deficiency and iron deficiency on either inflammation or insulin resistance, even though such an interaction may in fact exist. Likewise, our obese subgroup may be too small to find a significantly higher prevalence of iron deficiency, compared to the lean group, which has been identified in other studies. As well, the racial differences between our obese and control groups could serve as confounding variables. Finally, given our study subjects were recruited in Minnesota, the population is not representative of children from different parts of the U.S. or from different latitudes, which potentially could affect their vitamin D levels and/or dietary intake of nutrients. In spite of these limitations, however, we were still able to demonstrate associations between iron and vitamin D levels with inflammatory markers and insulin sensitivity.

In conclusion, iron and vitamin D deficiency are common in the obese pediatric population and may place such children at risk for future insulin resistance and cardiovascular disease. Poorer iron status as measured by sTfR was associated with increased insulin resistance and this relationship did not differ by BMI or vitamin D status. To the best of our knowledge, finding a relationship between higher sTfR and lower insulin sensitivity is a novel finding. Therefore, iron may play an independent role in prevention of diabetes mellitus and the ideal iron intake for individuals at risk for diabetes mellitus deserves further study. Finally, the different relationship between 25(OH)D and insulin resistance that our data revealed is important for planning and interpreting clinical trials of the impact of vitamin D on insulin resistance.

Statistical Appendix

We used linear regression to test for the associations between 25(OH)D and sTfR on IL6 and insulin sensitivity. Iron status, measured by sTfR, was negatively associated with insulin sensitivity ($p<0.01$).

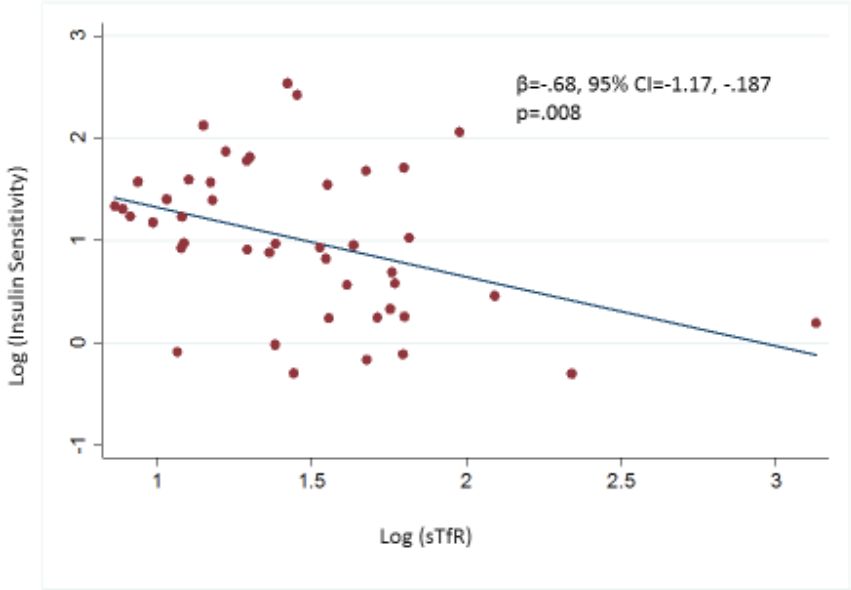

Figure 1. Associations of sTfR with Insulin Sensitivity

In order to examine the model assumptions for linear regression, we plotted the residuals of sTfR. The observed values are symmetrically distributed around the predicted line, and they tend to cluster toward the middle of the plot. In addition, there is not a clear pattern to the residuals. Thus, this model assumption for using linear regression is adequate.

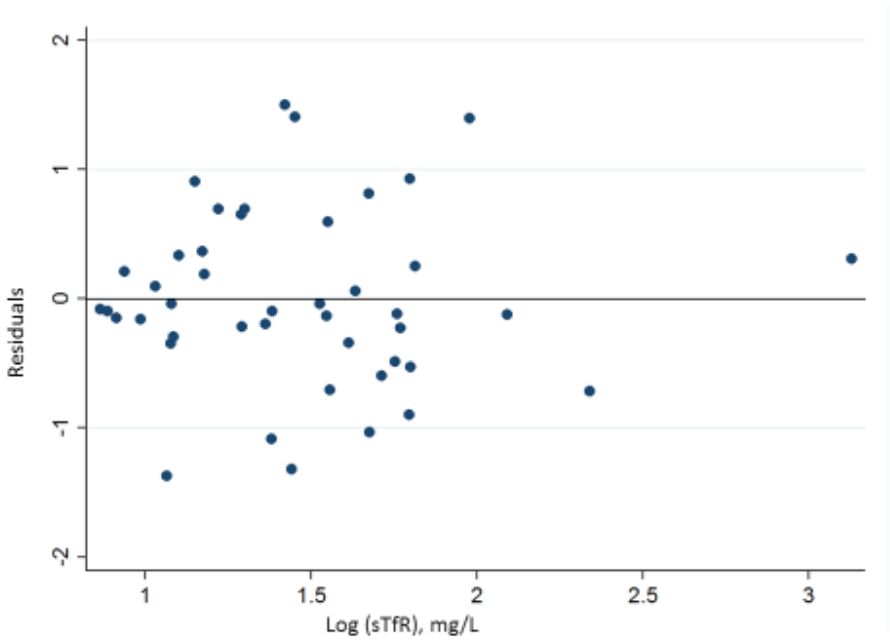

Figure 1A. Residuals of sTfR on Insulin Sensitivity

25(OH)D trended towards a positive associated with insulin sensitivity (p=0.08). There was a trend towards an interaction between obese status and 25(OH)D on the association with insulin sensitivity (p=0.053). Therefore, we tested the association of 25(OH)D with insulin sensitivity within each group. In these sub-analyses, we found a trend towards a negative association between 25(OH)D and insulin sensitivity in the obese group (p=0.11) and a trend towards a positive association in the lean group (p=0.23). When we regress 25(OH)D and BMI z-score on insulin sensitivity in the obese subgroup, the slope between 25(OH)D and insulin sensitivity is positive (β=0.26, p=0.09). This may be because with increase in BMI z-score there is less of an effect of 25(OH)D on insulin sensitivity, and with increase in 25(OH)D, there is less of an effect of BMI z-score on insulin sensitivity.

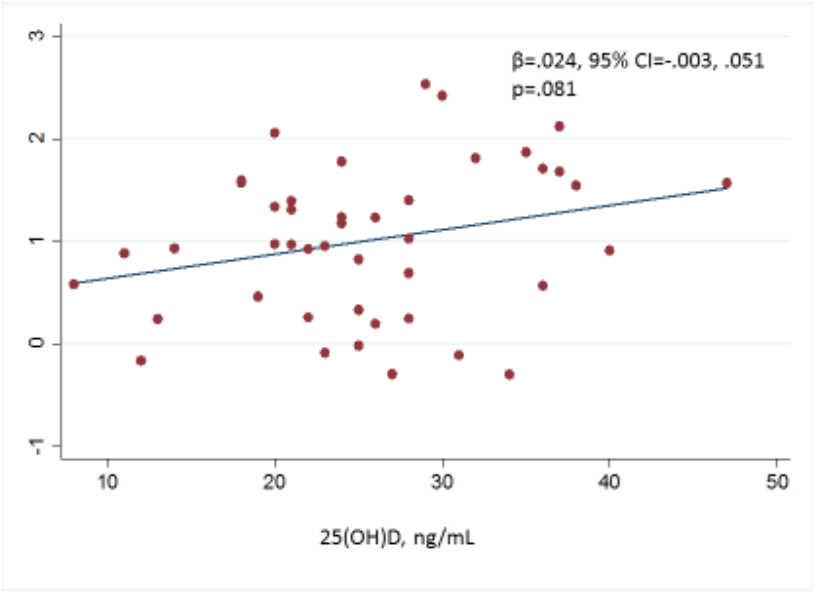

Figure 2. Associations of 25(OH)D with Insulin Sensitivity

When we examine the residuals of 25(OH)D on insulin sensitivity, the observed values are randomly clustered around the predictive line, and they are symmetric with no clear patterns. Therefore, this model assumption is met.

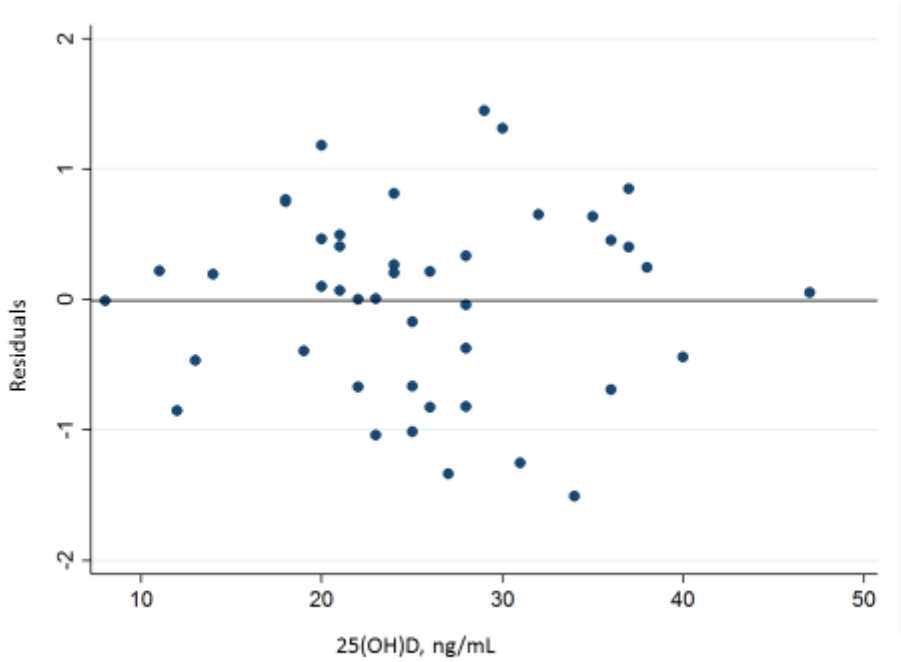

Figure 2A. Residuals of 25(OH)D on Insulin Sensitivity

Iron status (sTfR) was positively associated with IL6 ($p<0.001$).

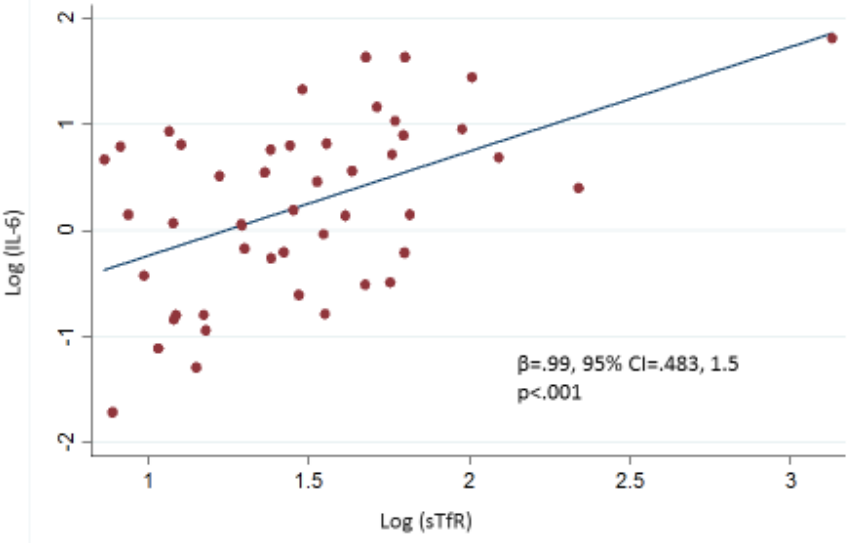

Figure 3. Associations of sTfR with IL-6

Again, when we examine the residuals in this model it is apparent that they are symmetrically distributed about the predicted line; however, they are not clustered around the small integers, and there is quite a range of residuals. Therefore, this assumption is partially met.

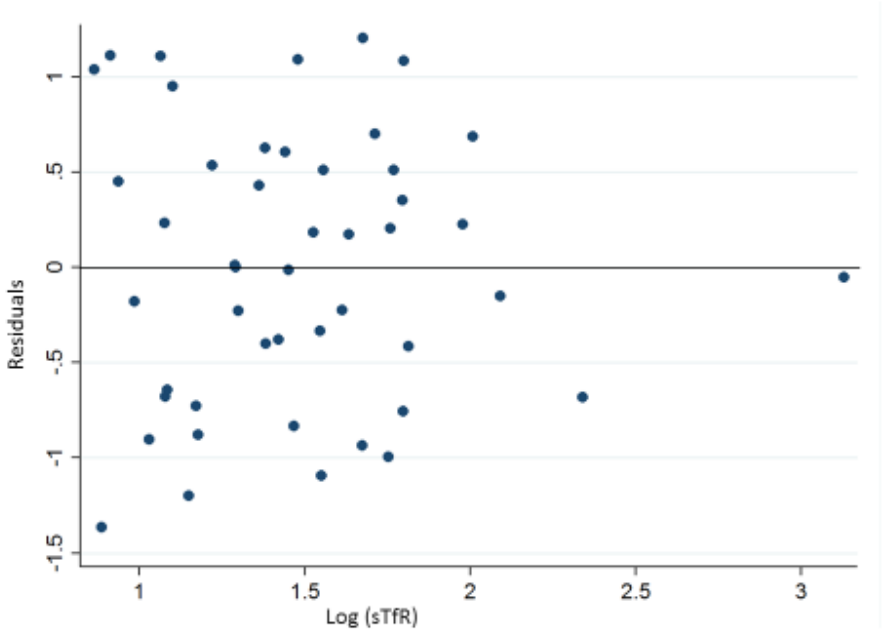

Figure 3A. Residuals of Log (sTfR) on Log (IL-6)

25(OH)D was negatively associated with IL6 ($p<0.01$). There was no interaction between obese status and 25(OH)D on the association with IL6 ($p=0.95$).

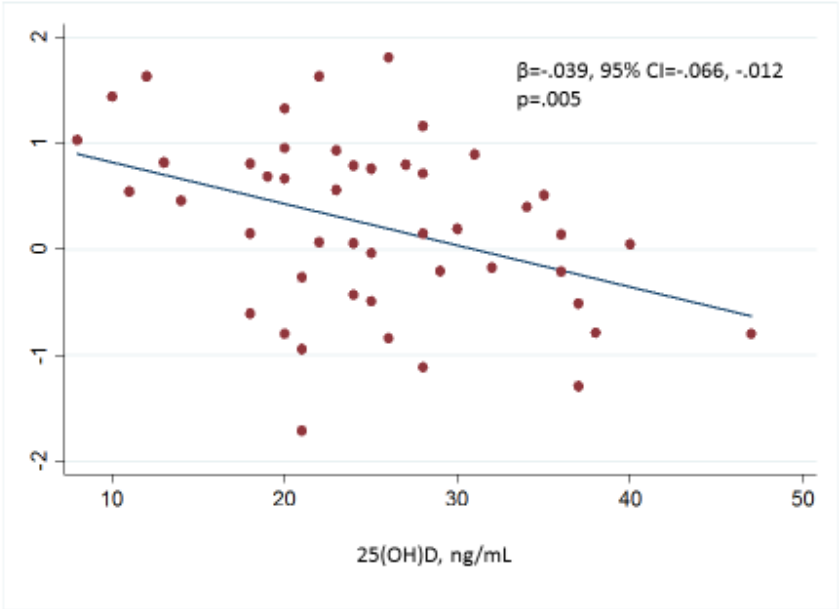

Figure 4. Associations of 25(OH)D with IL-6

In examining the residuals for this analysis, they appear to be symmetrically distributed around the predicted line, with most observed values clustered within small integer values, therefore this assumption is met.

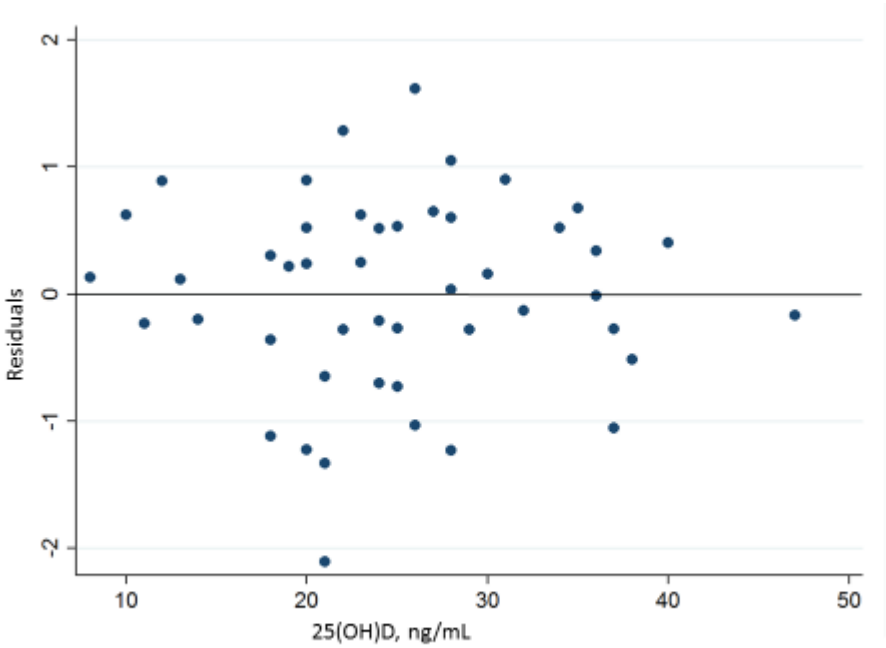

Figure 4A. Residuals of 25(OH)D on IL-6

We found no association between 25(OH)D and sTfR levels. In addition, there was no significant interaction between sTfR and 25(OH)D on the association with either IL6 (p=0.61) or insulin sensitivity (p=0.44).

There was no interaction between obese status and sTfR on the association of sTfR with either IL6 (p=0.72) or insulin sensitivity (p=0.44).

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