

Serum Alanine Aminotransferase Levels Decrease Further with Carbohydrate than Fat  
Restriction in Insulin Resistant Adults

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Running Title: Effect of a Carbohydrate Restricted Diet on ALT

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## Abstract

**Objectives:** While weight loss interventions have been shown to reduce steatosis in nonalcoholic fatty liver disease (NAFLD), the impact of dietary macronutrient composition is unknown. We assessed the effect on serum ALT concentrations of two hypocaloric diets, varying in amounts of carbohydrate (CHO) and fat; in obese, insulin-resistant individuals: a population at high risk for NAFLD.

**Research Design and Methods:** Post-hoc analysis was performed of ALT concentrations in 52 obese subjects with normal baseline values, and insulin resistance as quantified by the steady-state plasma glucose test (SSPG), who were randomized to hypocaloric diets containing either 60%CHO-25%fat or 40%CHO-45%fat (15% protein), for 16 weeks. Primary endpoint was change in ALT, which was evaluated according to diet, weight loss, SSPG, and daylong insulin concentrations.

**Results:** Although both diets resulted in significant decreases in weight, SSPG, daylong insulin, and serum ALT concentrations, the 40% CHO diet resulted in greater decreases in SSPG ( $p<0.04$ ), circulating insulin ( $p<0.01$ ), and ALT ( $9.5\pm 9.4$  vs.  $4.2\pm 8.3$  U/L,  $p<0.04$ ) concentrations. ALT changes correlated with improvement in insulin sensitivity ( $p=0.04$ ) and daylong insulin ( $p<0.01$ ). Individuals with ALT concentrations above proposed upper limits experienced significant declines in ALT, unlike those with lower ALT levels.

**Conclusions:** In a population at high risk for NAFLD, a hypocaloric diet moderately lower in CHO decreased serum ALT concentrations to a greater degree than a higher CHO-low fat diet, despite equal weight loss. This may result from a relatively greater decline in daylong insulin concentrations. Further research with histological endpoints is needed to further explore this finding.

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathologic syndrome that encompasses a spectrum of conditions ranging from simple hepatic fat (steatosis) to steatohepatitis, fibrosis and end stage liver disease. (1-3). NAFLD affects up to 74% of obese individuals (4, 5), and is almost always associated with both hepatic and peripheral insulin resistance (6-9).

There has been recent interest in the optimal diet to reduce the cardiometabolic complications of obesity-associated insulin resistance. As the same population is also at risk of NAFLD, it is of interest to identify the optimal dietary approach to prevent steatosis, the initial lesion, in this group. Furthermore, the development of steatosis per se, even in the absence of fibrosis, may stimulate inflammatory pathways that adversely affect metabolism (10-12), possibly contributing to systemic insulin resistance and risk for cardiovascular disease and diabetes.

Current therapeutic guidelines for NAFLD advocate weight loss as a first line treatment (13). While a number of studies have demonstrated a reduction in steatosis with weight loss (14-17), there are no published studies comparing the relative benefits for NAFLD of a hypocaloric diet that is restricted in fat versus carbohydrate (CHO). In this regard, it seems intuitive that a low-fat diet would be preferable to a high-fat diet in preventing or reversing established steatosis. Indeed, the American Heart Association, American Diabetes Association (ADA), and National Heart Lung & Blood Institute all recommend for weight loss a diet with fewer than 30% of total calories derived from fat. However, we and others have previously shown that

diets enriched in CHO lead to increased circulating insulin concentrations (18,19), which contribute to elevated fasting and daylong triacylglycerol (TG) concentrations and lower high-density lipoprotein cholesterol (HDL-C) concentrations under isocaloric conditions. Even under weight loss conditions, diets that are lower in CHO and higher in fat have relatively greater benefits on insulin, TG, and HDL-C concentrations than similarly hypocaloric, low-fat diets (20 - 22). We hypothesized that a lower-CHO diet, via greater reductions in daylong insulin concentrations, may also be relatively more effective in reducing intrahepatic fat in the insulin-resistant obese population. This hypothesis is based on the observation that de novo lipogenesis in the liver is increased 4-fold in insulin-resistant individuals (23), possibly as a result of insulin-stimulation of the transcription factor SREBP-1c, which regulates expression of lipogenic genes in the liver (24). Thus we sought to determine, in an obese, insulin-resistant population without established liver disease, whether restriction in dietary CHO or fat would yield relatively greater reduction in serum ALT concentrations during dietary weight loss.

As it has been suggested that the upper limits of normal for serum ALT concentrations set in the 1980's may have been falsely elevated by undiagnosed NAFLD in the reference population (25), a secondary goal of this study was to determine if ALT values within the current normal limits decrease with weight loss, presumably to truly "normal" concentrations. Insulin-sensitizing thiazolidenedione treatment has been demonstrated to cause a decrease in ALT

concentrations in the normal range (26), and we hypothesized that a similar decrease would be seen with weight loss. Thus, we compared change in ALT in those above and below the newly proposed upper limit for the normal range (30 U/L men; 19 U/L women) (25).

#### Research Design and Methods

The study population consisted of 52 apparently healthy, obese, and insulin-resistant individuals, selected on the basis of baseline ALT concentrations in the normal range at our institution (<60U/L), from a larger group of subjects (n=57) who participated in a weight loss study (20). All participants gave written informed consent, and the protocol was approved by the Stanford University Human Subjects Committee. To determine eligibility, blood frozen at -80° C was sent to the clinical laboratory at Stanford Medical Center for measurement of baseline serum ALT.

Inclusion criteria included body mass index (BMI) 30.0-36.0 kg/m<sup>2</sup>, fasting plasma glucose < 126 mg/dL, stable body weight, and no pre-existing heart disease, anemia, or kidney disease. Subjects with positive viral serology, previously abnormal serum hepatic transaminases, or alcohol use of more than 2 standard alcoholic drinks per day were excluded.

Insulin-mediated glucose uptake (IMGU) was quantified by a modification (27) of the insulin suppression test as originally described and validated (28, 29). This approach to the quantification of IMGU has been used for over 30 years, and the results are highly correlated with the euglycemic hyperinsulinemic clamp technique (29). Briefly, after a 12 hour overnight fast, an intravenous catheter is placed into each of the patient's arms. A 180-minute infusion of somatostatin (0.27

µg/m<sup>2</sup>/min), insulin (32 mU/m<sup>2</sup>/min), and glucose (267 mg/m<sup>2</sup>/min) is administered into one arm. Blood is drawn from the other arm at 30-minute intervals, increasing to 10-minute intervals for the last 30 minutes of the study, to determine steady-state plasma glucose (SSPG) and insulin (SSPI) concentrations. As SSPI concentrations are similar for all subjects, the SSPG directly measures the subject's ability to dispose of the glucose load. All subjects entering the dietary intervention phase were required to be insulin resistant on the basis of a SSPG concentration ≥ 180 mg/dL. This level corresponds to the most insulin-resistant third of 490 apparently-healthy individuals (30); a cut point that predicts the development of a variety of adverse clinical outcomes (31, 32).

Daylong plasma glucose and insulin concentrations were determined as described previously (33), at hourly intervals before and after two standardized meals given at 8 AM and noon. Both meals contained (as percentages of total calories) 15% protein, 43% carbohydrate, and 42% fat (<10% saturated fat), with breakfast comprising 20% and lunch comprising 40% of estimated daily caloric requirements. Insulin was measured in a stepwise sandwich enzyme-linked immunosorbent assay procedure on an ES 300 (Boehringer Mannheim Diagnostics). Glucose was measured using the hexokinase method on the Hitachi 747 (Boehringer Mannheim Diagnostics).

Following baseline testing, subjects were randomized to follow a hypocaloric diet containing either 60% CHO/25% fat or 40% CHO/45% fat. Both diets contained 15% protein and 7% saturated fat. ADA modified diabetes exchange lists were used. Choices included complex

carbohydrates with high fiber content, lean protein, and low saturated fat. All subjects were encouraged to attempt to consume 25 grams of fiber. No glycemic index targets were set. Following 2 hours of individualized dietary instruction, meals were prepared at home according to weekly diet plans from the study dietitians. Calorie deficit for both diets was -750 kcal/day, as estimated by the Harris Benedict equation (34) and an activity factor for each subject. Subjects were required to keep a daily food diary, which was reviewed weekly with study dietitians to monitor weight and enhance compliance. Actual macronutrient intake was estimated by FOOD PROCESSOR software (version 8.0, ESHA, Salem, OR) analysis of food diaries. Adjustments in intake were recommended as needed to enhance compliance with assigned diets. Sixteen weeks of a hypocaloric diet was followed by 2 weeks of weight maintenance with calorie requirements for each subject recalculated according to their current weight. Following the weight maintenance phase, baseline measurements were repeated.

In order to test our theory that the current normal range of serum ALT included undiagnosed NAFLD, a secondary analysis was performed. Subjects were divided into 2 groups regardless of diet, based on whether their baseline ALT was above (“abnormal”) or below (“normal”) the cut-points suggested by Prati et al. The decline in ALT in the two groups was then compared, with the hypothesis that those with the abnormal ALT at baseline, suggestive of NAFLD, would have a greater decline in serum ALT with the hypocaloric diet.

Results are expressed as mean  $\pm$  SD unless otherwise stated. Between-group

differences in experimental variables were assessed with Student’s unpaired t-tests, while within-group changes resulting from the diets were assessed with Student's paired t-tests. Differences in categorical variables were tested with chi-squared analysis. SSPG, glucose and insulin area-under-the curve (AUC) values were log-transformed for normality: all other variables were normally distributed. For clarity of presentation, actual values of variables are presented: p-values were done with log-transformed values as per above. Due to the relatively great number of potential confounders for our sample size, stepwise multiple linear regression models were employed for the magnitude of change in each dependent variable (ALT concentration, weight, insulin, glucose, and SSPG concentrations) to assess the independent effect of dietary group (between-group difference). Potential confounders entered into each model included age, gender, baseline concentration of the dependent variable, and amount of weight lost. In all cases, p values  $<0.05$  was considered statistically significant. Analyses were performed using SAS 9.2 and SPSS (version 12, Point Richmond, CA, USA).

## Results

Twenty-six subjects from each dietary group were eligible for the current analysis. As shown in Table 1, demographic characteristics did not differ significantly between the 2 groups. Among women, postmenopausal status was not significantly different and only 2 women in each group used hormone replacement therapy. There were also no significant differences between the baseline metabolic variables of weight, BMI, SSPG, and plasma insulin, glucose and ALT concentrations for the two diet

groups. Table 1 also details the estimated actual macronutrient composition of meals consumed: amounts of CHO and total/mono/polyunsaturated fats differ significantly between groups.

Both groups experienced a significant decrease in weight ( $7.0 \pm 3.8$  kg,  $p < 0.001$  vs  $5.7 \pm 4.1$  kg,  $p < 0.001$ ) as a result of the dietary intervention (Table 2), but the amount of weight loss did not differ significantly between the two groups. Serum ALT concentrations decreased twice as much in the group assigned to the 40% CHO as compared with the 60% CHO diet group ( $-9.5 \pm 9.4$  vs  $-4.2 \pm 8.3$ ,  $p < 0.01$ ). This difference remained statistically significant ( $p < 0.02$ ) after adjusting for baseline ALT concentration, gender, age, and weight loss.

Insulin resistance, as quantified by SSPG, declined significantly in both groups (Table 2). The magnitude of decline was greater in the 40% CHO group but after adjustment for age, gender, baseline SSPG and weight loss, this difference was not statistically significant. Daylong insulin concentrations declined significantly only in the 40% CHO diet group, which was significantly greater than the decline in the 60% CHO group even after adjustment for baseline insulin concentration, age, gender, and weight loss. Daylong glucose concentrations did not change significantly in either group.

Based upon the proposal that the upper limits of normal for ALT concentrations should be adjusted to be 30 U/L for men and 19 U/L for women (25), we divided our study subjects into those who had baseline ALT concentrations above (“abnormal”) or below (“normal”) these proposed alternative cut points. There

were no significant differences between the abnormal versus normal ALT groups in terms of age ( $51 \pm 11$  vs  $52 \pm 10$ ,  $p = 0.83$ ), number of women (64% vs 42%,  $p = 0.14$ ), postmenopausal status (27% vs 37%,  $p = 0.56$ ), BMI ( $32.5 \pm 2.6$  vs  $32.5 \pm 1.9$ ,  $p = 0.97$ ), SSPG ( $237 \pm 38$  vs  $234 \pm 41$ ,  $p = 0.83$ ), glucose AUC ( $859 \pm 90$  vs  $864 \pm 98$ ,  $p = 0.85$ ), or insulin AUC ( $391 \pm 164$  vs  $534 \pm 539$ ,  $p = 0.18$ ), and the mean baseline ALT concentrations for two groups were significantly different ( $19.5 \pm 5.5$  vs  $32.9 \pm 8.6$  U/L,  $p < 0.01$ ). Comparison of the ALT responses of those with “abnormal” or “normal” baseline ALT concentrations to the 40% and 60% CHO diets is shown in Figure 1. Irrespective of dietary macronutrient content, the decline (mean + SD) in ALT concentrations with weight loss in the 33 subjects with baseline “abnormal” ALT concentrations was 10-fold greater than in the 19 with “normal” baseline values ( $-10.2 \pm 9.3$  U/L vs.  $-1.0 \pm 5.4$  U/L,  $p < 0.01$ ). In addition, in the “abnormal” baseline ALT group, concentrations fell to a greater degree in the those assigned to the 40% CHO diet ( $n = 18$ ) as compared to those on the 60% CHO diet ( $n = 15$ ), ( $-12.8 \pm 8.8$  U/L vs.  $-7.2 \pm 9.1$  U/L,  $p < 0.01$  after adjustment for age, gender, baseline ALT and weight loss). Among those with “normal” baseline ALT concentrations ( $n = 19$ ), ALT decreases were  $-2.0 \pm 1.4$  U/L vs  $-0.8 \pm 0.6$  U/L,  $p = 0.97$ ), on the 40% and 60% CHO diets, respectively.

## Conclusions

The main finding of this study is that among obese, otherwise healthy, insulin-resistant individuals, a hypocaloric diet moderately-restricted in CHO (40%) and enriched in fat (45%), as compared with a traditional low-fat hypocaloric diet (25% fat and 60% CHO), lowers serum ALT

concentrations to a significantly greater degree, even after adjustment for amount of weight loss and other potential confounders, including baseline concentration of ALT. Of note, the subjects in our study were selected to have no prior diagnosis of liver disease and baseline ALT concentrations that did not exceed the current upper limit of the normal range. Thus, it is important to consider that even in apparently-healthy obese, insulin-resistant individuals; weight loss can reduce ALT concentrations, suggesting that steatosis was present. Furthermore, our study adds to the accumulating data suggesting that among this population at particularly high risk for obesity-related morbidities, hypocaloric diets that differ in macronutrient composition yield different weight-loss-related benefits. For example, we and others have previously shown that hypocaloric diets lower in CHO are more effective in reducing triglyceride and increasing HDL-cholesterol concentrations (20-22,36), which may be related to relatively greater reductions in insulin concentrations (18,20,21,36) as a result of lower dietary CHO intake. Indeed, it is plausible that relatively greater reductions in ambient insulin concentrations in the current study also contributed to the lowering of serum ALT, given that significantly greater reductions were seen on the 40% as compared with the 60% CHO diet.

There are several lines of evidence supporting a biological link between insulin concentrations and hepatic steatosis. Firstly, strong correlations have been observed between the degree of hyperinsulinemia and the extent of hepatic steatosis (9, 37). Also, in NAFLD, the area of the lobule containing the highest number of steatotic hepatocytes is Zone 3;

the area closest to portal drainage with the highest concentration of insulin (38). The more insulin resistant an individual, and the higher the daylong insulin and free fatty acid (FFA) concentrations, the greater is hepatic very low-density lipoprotein triglyceride (VLDL-TG) synthesis and secretion (39-42). Furthermore, ex vivo hepatic perfusion studies have shown that the higher the ambient in vivo insulin concentration, the greater the stimulatory effect of a given increment in perfusate FFA concentration on hepatic triglyceride synthesis and secretion (43). In regard to ALT, links between glucose-mediated insulin sensitivity and serum ALT have been demonstrated previously (37,44,45). On a molecular level, sterol regulatory binding protein 1 (SREBP1), the peroxisome proliferator activated receptor (PPAR) system, and Fas genes are key transcriptional factors regulated by insulin. In situations of insulin and FFA abundance, such as obesity, insulin binding increases the expression of SREBP-1c, leading to increased expression of lipogenic genes and a consequent increase in de novo lipogenesis (46). All of these observations support the biologic plausibility of our observation that those assigned to the 40% CHO diet experienced a greater decline in ALT concentrations as a result of greater reductions in ambient insulin concentrations.

Alternatively, there is a growing body of evidence that intrahepatic fat accumulation potentiates insulin resistance via stimulation of inflammatory pathways regulated by NF-KB (10-12). In this regard, prevention/reduction of early intrahepatic fat accumulation may not only prevent the development of NASH and subsequent cirrhosis in a minority of

affected patients, but may potentiate improvement in insulin sensitivity and other metabolic parameters.

Baseline ALT concentrations for our subjects did not exceed the upper limit of the normal range (60 U/L), but 63% of subjects had a baseline ALT above that of new, more conservative limits (< 19 U/L for females and < 30 U/L for males) (25). Our results further support these limits, as individuals with ALT levels above these limits decreased with dietary weight loss, while those below the new upper limits did not. Furthermore, even in this smaller subgroup, the impact of dietary macronutrient composition was statistically significant, with greater reductions in ALT observed in the 40% CHO diet group. While ALT is not a precise marker of NAFLD, these results suggest that the majority of our subjects did indeed have some degree of steatosis.

Our findings are limited to the obese, insulin-resistant population, who: 1) have compensatory hyperinsulinemia and may be more sensitive to variations in dietary macronutrient composition with regard to insulin concentrations; and 2) have a high likelihood of steatosis (5,6,9). Furthermore, our subjects were engaged in dietary weight loss, and thus, we cannot extrapolate the results of our study to eucaloric diets. We also did not quantify variability in micronutrients consumed, including relative proportions of refined and unrefined carbohydrates, types of carbohydrate, or n-6/n-3 polyunsaturated fats, and thus cannot rule out the possibility that these factors and/or glycemic index, or differences in fiber intake in our two study diets had an impact on our results. Finally, we did not quantify hepatic steatosis radiologically or

histologically, but given the current findings, it would be reasonable to employ more costly and/or invasive studies in this subject pool as well as in those with established steatohepatitis to evaluate the potential benefits of moderate reductions in dietary CHO.

In summary, it appears that a hypocaloric diet moderately restricted in CHO may be beneficial in reducing hepatic steatosis in obese, insulin-resistant adults. Given the ongoing debate as to which macronutrient composition is optimal for reducing health risks associated with obesity, our results demonstrate yet another benefit of a CHO-restricted diet moderately enriched in fat as compared with the currently recommended higher CHO diets typically prescribed for weight loss in this population (47-49). Further research is needed to identify the mechanism by which lower CHO can more effectively lower ALT concentrations in patients with steatosis, and the relative benefit in those with more advanced NAFLD.

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Table 1: Baseline characteristics and reported macronutrient composition consumed by subjects assigned to 60% versus 40% carbohydrate diet. (mean  $\pm$  SD).

	60% Carbohydrate Diet (n = 26)	40% Carbohydrate Diet (n= 26)	p
Age (yrs)	54 $\pm$ 10	49 $\pm$ 11	0.08
Gender (M:F)	14 : 12	12 : 14	0.48
Postmenopausal women (N, %)	9 (35%)	7 (27%)	0.56
Ethnicity (% Caucasian)	92%	93%	0.89
BMI (kg/m <sup>2</sup> )	33.0 $\pm$ 2.4	32.3 $\pm$ 1.8	0.20
Weight (kg)	95.1 $\pm$ 13.0	94.2 $\pm$ 12.3	0.80
*SSPG (mg/dL)	227 $\pm$ 26	247 $\pm$ 47	0.77
*AUC Glucose (mg/dL•8 hrs)	869 $\pm$ 88	852 $\pm$ 98	0.53
*AUC Insulin (uU/mL•8 hrs)	435 $\pm$ 439	459 $\pm$ 256	0.82
Serum ALT (U/L)	27.0 $\pm$ 10.4	29.0 $\pm$ 9.5	0.49
Carbohydrate (%)	58 $\pm$ 4	42 $\pm$ 5	< 0.01
Protein (%)	18 $\pm$ 2	18 $\pm$ 2	0.69
Total Fat (%)	23 $\pm$ 4	39 $\pm$ 5	<0.01
Saturated Fat (%)	8 $\pm$ 2	9 $\pm$ 2	0.14
Monounsaturated Fat (%)	8 $\pm$ 2	13 $\pm$ 2	<0.01

Polyunsaturated Fat (%)	4 ± 1	7 ± 2	0.01
Fiber (g)	26 ± 6	23 ± 8	0.27
Kcal/day	1699 ± 334	1595 ± 262	0.24

p-value calculated using Student's unpaired t-test

\*p-values for variables that are not normally distributed refer to analyses performed with logarithmically transformed data.

2 women in each dietary group were taking hormone replacement therapy

Table 2: Change in metabolic variables (mean  $\pm$  SD) as a result of hypocaloric dietary intervention in individuals assigned to the 40% versus 60% CHO diet.

	60% Carbohydrate Diet (n = 26)				40% Carbohydrate Diet (n= 26)					
	Pre	Post	Delta	p	Pre	Post	Delta	p	P	P#
Weight (kg)	95.1 $\pm$ 13.0	89.4 $\pm$ 13.0	-5.7 $\pm$ 4.1	<0.01	94.2 $\pm$ 12.3	87.1 $\pm$ 11.3	-7.0 $\pm$ 3.8	0.20	0.20	0.23
ALT (U/L)	27.0 $\pm$ 10.4	22.9 $\pm$ 7.7	-4.2 $\pm$ 8.3	0.02	29.0 $\pm$ 9.5	19.5 $\pm$ 4.7	-9.5 $\pm$ 9.4	<0.01	<0.01	0.02
SSPG (mg/dL)	226.5 $\pm$ 26.4	194.1 $\pm$ 42.9	-32.4 $\pm$ 40.7	<0.01	246.6 $\pm$ 47.2	185.4 $\pm$ 67.9	-61.3 $\pm$ 52.3	<0.02	<0.02	0.06
AUC Insulin ( $\mu$ U/mL • 8 hrs)	435 $\pm$ 439	400 $\pm$ 383	-35 $\pm$ 115	0.14	459 $\pm$ 256	295 $\pm$ 180	-164 $\pm$ 151	0.03	0.03	0.04
AUC Glucose (mg/dL • 8 hrs)	869 $\pm$ 88	873 $\pm$ 87	-4 $\pm$ 68	0.75	852 $\pm$ 98	826 $\pm$ 92	26 $\pm$ 76	0.12	0.16	0.74

p: within-group comparison for each diet (paired Student's t-test).

P: unadjusted between-group comparison (unpaired Student's t-test) of the change in dependent variable.

P# : adjusted for age, gender, baseline concentration of variable, and weight loss (stepwise multiple linear regression analysis).

## Figure Legend

Figure 1: The change in ALT is shown in the two diet groups divided into those with an “abnormal” and a “normal” baseline ALT based on proposed upper limits of 19 U/L for women and 30 U/L for men (24).  $P < 0.01$  for the decrease in ALT in those with “abnormal” versus “normal” ALT at baseline (both dietary groups combined,  $n = 52$ ). In the “abnormal ALT” group ( $n=33$ ),  $P < 0.01$  for the between-diet comparison of the decrease in ALT after adjustment for age, gender, baseline ALT and weight loss by stepwise multiple linear regression.  $P = 0.97$  for the between-diet comparison of the decrease in ALT in the “normal” baseline group ( $n = 19$ ).

