Statistical Plan

Sample Size and Data Analysis: The study will use a single site randomized, double-mask design. Our primary analysis to examine the effect of betaine supplementation will be by change from baseline to 3-month values compared to placebo in completer analysis for all endpoints of Aims 1-3. Safety will be assessed by intention to treat. Prior to data analysis, the data will be examined for outliers. Continuous measures whose distributions are not symmetric will be transformed. In secondary analysis for all outcomes we will determine if baseline betaine determines response to supplementation.

Insulin Sensitivity-Basis for power calculations: In an overweight prediabetes population of 14 persons similar to that proposed in this grant and using a similar clamp protocol, we demonstrated insulin sensitivity of 5.9±2.1 mg/kg/min, a value significantly lower than 8.3±1.8 in our normoglycemic control group of 27 patients (*P*=0.0005). This demonstrates likelihood that the cohort proposed will be insulin resistant. We also recently assessed insulin sensitivity by clamp before and after *placebo* in a cohort of 29 persons with impaired glucose tolerance in another 3-month randomized study. Baseline values were 4.0±1.5 mg/kg/min and 3 months later 4.1±1.7, P=0.3. The placebo change over time was 0.16±0.81, or variance <4%. This demonstrates the high reproducibility of clamp measures. A total of 30 patients entering this two-treatment parallel-design study, provides 80% probability of detecting a treatment difference at a paired two-sided comparison with type 1 error 0.05 significance, if the standard deviation is 0.81 and the true difference between treatments is 0.85 mg/kg/min (a between-group difference of 20% change from baseline). Given the short duration of the study, we anticipate ≥80% retention rate, but even 24 completers would detect difference between treatment groups of 0.96 mg/kg/min (25% change from baseline between groups).

Glycemia is a secondary end-point of strong interest. In a recent study of patients with prediabetes mean/SD fasting glucose was 103±9.5 mg/dl, and did not change over 3-months (0.28±10.3 mg/dl, P=0.9). With 30 patients in a two-treatment parallel-design study, and standard deviation of 10, the probability is 80% that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference between treatments is 7 mg/dl. This would be clinically significant, and 1/4-1/3 the magnitude of the placebo-corrected reduction in fasting glucose of 25% seen in HFD-fed mice with betaine supplementation.

Change in liver fat. Previous studies suggest high reproducibility of liver fat assessed by MR over 28 weeks. The mean hepatic fat content, determined by magnetic resonance spectroscopy, was higher in subjects with NASH than healthy controls (19.5±3.1% vs. 3.0±0.7%, respectively, P<0.001). Overweight, dysglycemic persons are anticipated to fall between this range. However, assuming variability between images equals the variability seen in patients with NASH (i.e. an assumption that the SD of the response variable change in hepatic fat over the treatment interval is 3.1, a likely overestimate), and with a total of 30 patients entering this two-treatment parallel-design study, there is 80% probability that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference in change from baseline between treatments is 3.3% of liver fat.

Endothelial function. We will determine if betaine supplementation compared to placebo changes endothelial function. In a secondary analysis for this aim we will determine if either baseline betaine concentration determines endothelial responsiveness. The latter will test the hypotheisis that folate status may modulate the effect of betaine on homocysteine response to methionine load.

To calculate sample size, we estimated mean and standard deviations of flow-mediated vasodilation from our previously reported findings for a placebo-treated group composed of patients with T2D. The reported change from baseline following placebo was -0.9 ± 1.2% (from a baseline value of 5.8 ± 1.1% to 4.9 ± 0.9% at six month follow up). It is anticipated that betaine will increase flow-mediated, endothelium-dependent vasodilation by 25% compared with placebo. Therefore, we expect the mean change in the betaine group to be 1.45% (from a baseline value of 5.8% to a three month value of 7.25%). Although we will analyze the data using a mixed model, to estimate required sample sizes we used the methods of Cochran and Cox for a two-sample t-test with an alpha of 0.05 for a two-tailed test of significance and 80% power..

Secondary Outcomes. Secondary outcomes are considered exploratory. We recognize that this study will allow for detection of only relatively large effects.