STUDY PROTOCOL

Choline Nutritional Status: Development of a Biomarker Panel

NCT number NCT03726671
Document Date 12/18/2018

INTERVENTIONAL STUDY

Complete Title: Choline nutritional status: development of a biomarker panel

Short Title: Choline Status

Drug or Device Name(s): N/A

FDA IND/IDE (if applicable): N/A

Sponsor: NIH & Balchem

Protocol Date: 8/4/2017

Amendment 1 Date: 4/12/2018

Amendment 2 Date: 12/18/2018

Amendment 3 Date:

Amendment 4 Date:

Lead Investigator:

Steven Zeisel, M.D., Ph.D.

University of North Carolina at Chapel Hill

Protocol Version: 3

Version Date: December 18, 2018

I confirm that I have read this protocol and understand it.

Principal Investigator Name: _____

Principal Investigator Signature: _____

Date: _____

TABLE OF CONTENTS

ABBREVIATIONS AND DEFINITIONS OF TERMS

Abbreviation	Definition
AE	adverse event
AI	**adequate intake**
ANOVA	**analysis of variance**
ASA24	**Automated Self-Administered 24-hour Dietary Assessment Tool**
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BMI	body mass index
BUN	blood urea nitrogen
CAP	controlled attenuation parameter
Cho	choline
Cho-d9	choline chloride (trimethyl-D9)
CK	creatine phosphokinase
CRF	case report form
FDR	false discovery rate
GC-TOF-MS	gas chromatography-time of flight-mass spectrometry
GWA	genome wide association
IPA®	Ingenuity Pathway Analysis (Qiagen)
UPLC-MS/MS	ultra-performance liquid chromatography mass spectrometry mass spectrometry
mcmol/L	micromole per liter
MRS	mass resonance spectroscopy
MRI	mass resonance imaging
m/z	**mass to charge ratio**
NAFLD	**Non-alcoholic fatty liver disease**
NCI	**National Cancer Institute**
NIH C-F ERCMRC	**NIH Common Fund Eastern Regional Comprehensive Metabolomics Resource Core**
OPLS-DA	**Orthogonal Partial Least Squares Discriminant Analysis**
OPLS-EP	**Orthogonal Partial Least Squares Effect Projections**
OPLS-R	**Orthogonal Partial Least Squares Regression**
PtdCho	**phosphatidylcholine**
PEMT	**phosphatidylethanolamine-*N*-methyltransferase**
QC	**quality control**
RDI	**recommended daily intake**
SAE	**serious adverse event**
SNP	single nucleotide polymorphisms
TPN	**total parenteral nutrition**
UL	**upper limit**
UP	**unexpected problem**
VIP	**variable importance to projection**

PROTOCOL SYNOPSIS

Study Title	Choline Nutritional Status: Development of a Biomarker Panel
Funder	NIH, Balchem
Clinical Phase	N/A
Study Rationale	Cho is an important nutrient, for which Adequate Intake and Reference Daily Intake values have been established, however Cho consumption is lower than recommended in most people, which can lead to liver and/or muscle dysfunction. Plasma Cho concentrations are a poor biomarker for choline status because it is homeostatically regulated. We have previously identified biomarkers that changed in people who became sick due to Cho depletion. We propose that a score for Cho status based on measurements of these markers can be used by clinicians and scientists to identify people who may be at risk of developing organ dysfunction due to Cho deficiency unless their Cho intake is increased.
Study Objective(s)	**Aim 1**: Determine whether a Cho status score based on a panel of biomarkers correlates well with measures of Cho pool size as assessed using an isotope dilution method. **SubAim 1a**: To test assumptions used in the choline isotope dilution method for estimating choline pool size, subjects will undergo MRS of Cho compound peaks in liver. Fatty liver will be assessed by MRI to compare to and validate the Fibroscan methodology. **SubAim 1b**: Determine whether there are additional biomarkers from untargeted metabolomic profiling that, when added to the panel developed in Aim 1, are better at predicting Cho status in people. **SubAim 1c**: Determine whether people with genetic polymorphisms (SNPs) known to increase risk for developing Cho deficiency-induced organ dysfunction are also more likely to have a worse Cho status score and a reduction in choline pool size assessed using isotope dilution.
Test Article(s) *(If Applicable)*	The experimental diets, delivering 100%, 50% (to be determined by a pilot study) and 25% of the recommended intake of Cho (in all forms) (550 mg Cho/70 kg body weight/d), are identical except for the bread that we offer (Cho in the bread will be varied to deliver the desired Cho above that delivered by the base 25% Cho diet as described previously (1)). On day 12 of a diet period, subjects will consume 250mg Cho in the form of Cho chloride (trimethyl-d9). We will confirm total Cho content of diets by LC-MS/MS analysis of samples of duplicate food portions as previously described (1).

Study Design	Healthy volunteers (n=50 males, 50 premenopausal females and 50 postmenopausal females; 30 additional subjects for dropout replacement), will be randomly assigned to an order of 2 week intervals of diets where they consume meals that we provide containing 100% of the recommended intake of Cho (550 mg Cho/day), 50% of the recommended intake of Cho (275 mg Cho), and 25% of the recommended intake of Cho (137.5 mg Cho) for 2 weeks each. Each dietary interval is followed by a minimum 2 week washout period. On day 12 of each diet period, subjects will consume a bolus of 250mg of Cho in the form of Cho chloride-(trimethyl-d9). Before this bolus, a plasma sample will be collected to measure baseline enrichment of Cho isotopes. At the end of each diet period, plasma and urine samples will be collected for biomarker assays and isotopic dilution estimation of Cho pool size, and we will perform transient elastography (Fibroscan) of liver to assess liver fat. (see Fig. 1, appendix).
Subject Population key criteria for Inclusion and Exclusion:	Inclusion Criteria 1. Subjects age 17 – 70 years 2. Male & female; representative of the Kannapolis-Charlotte metro area **Exclusion Criteria** 1. Implantable devices, BMI > 32, smokers, pregnant or planning to become, allergies to food to be consumed, chronic systemic diseases, consuming Cho-containing supplements, consuming drugs or medications known to cause liver or muscle damage or to alter Cho metabolism (e.g. methotrexate). For MRI/MRS we will exclude claustrophobia, pacemaker, artificial heart valve, metal plate, pin, or other metal implant, intrauterine devices such as Copper-7 IUD, insulin or other drug pump, aneurysm clips, previous gunshot wound, cochlear implant, or other hearing device, employment history as a metal worker, permanent (tattoo) eye-liner.
Number Of Subjects	180
Study Duration	Each subject's participation will last 6 weeks and a few days. This does not include the washout period between the dietary arms (minimum of 2 weeks each). The entire study is expected to last 4 years.
Study Phases Screening Study Treatment Follow-Up	(1) Screening: screening will take place in years 1-3 with the goal of consenting 50 subjects per year to complete the study. (2) Intervention: the study will take place in years 1-3. Subjects will be placed on diets as described in the study design (figure 1, app).

	(3) Follow-up: year 4 of the study will involve data analysis and interpretation, and writing of a manuscript for publication.
Efficacy Evaluations	Expected Results and interpretation: Our aim is to develop a panel of biomarkers and an algorithm for calculating a Cho status score that correlates well with measures of Cho pool size by isotope dilution. We will define the minimum set of biomarkers that provides >90% accuracy in predicting Cho pool size measured by isotopic dilution. We expect to see incremental declines in Cho status, as reflected in the panel of biomarkers. Results on the 100% diet should be similar to what we previously published. In the earlier studies, subjects were fed the 10% diet for a period of 2 to 7 weeks; in the proposed study, we feed 25%, 50%, and 100% (adequate intake) diets for two weeks (order randomly assigned) with 2 week washout periods in between. We expect that our results on the 25% & 50% diets will be less than those previously described; i.e. there will be smaller difference in the biomarkers than previously described. We expect that the 25% & 50% diets will show changes in Cho pool size and in biomarkers that fall between 100% and 10% values. Genotype will change the rate at which a diet treatment alters Cho pool size but will not affect whether our biomarker panel correlates with the pool size achieved. Thus, we do not have to power the study for genotype differences.
Pharmacokinetic Evaluations	*N/A*
Safety Evaluations	After each diet period a clinical chemistry panel will be run to assess potential liver and muscle damage. If any damage is noted after any of the dietary periods (liver damage = >1.5-fold increase in aspartate aminotransferase (AST) alanine transaminase (ALT)or muscle damage = >5-fold increase in creatine phosphokinase (CK)), affected subjects will follow the abnormal lab value protocol chart (See Appendix Figure 6). This will be reported to the IRB and the subject will be monitored and re-tested according to protocol and until AST/ALT and CK values return to normal.
Statistical And Analytic Plan	(Aim 1, SubAim 1a,1b). Unsupervised principal component analysis will first be used to examine the clustering of samples from the diet periods, and to ensure that the QC pool replicates center in the samples from which they were derived. Supervised Orthogonal Partial Least Squares Regression (OPLS-R) will then be used to identify a panel of biomarkers, assign weight to each biomarker, and calculate a composite score to predict choline pool size. We will analyze all of the samples using OPLS-R to identify a panel of biomarkers that predicts choline pool size universally for all three diet periods (healthy men, pre- and post-menopausal women). We will also consider performing the analysis for each diet period separately in case the universal panel does not provide high accuracy in prediction. Variable Importance to Projection (VIP) score will be calculated based on the OPLS weights and the variability explained in OPLS-R. Biomarkers with VIP>1.5 will be considered the most important biomarkers responsible for prediction.

	Pathway analysis will be performed using GeneGo metacore (Thomson Reuters, https://portal.genego.com) and QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).
DATA AND SAFETY MONITORING PLAN	Steven Zeisel (PI), Martin Kohlmeier MD, and study physician, Mark Vincent, MD will monitor subject data for safety continuously throughout the study and as new subjects are recruited.

1 BACKGROUND AND RATIONALE

1.1 Introduction

Scientific Premise

Choline (Cho) is an essential nutrient and most Americans' diets do not achieve the recommended intake (2). Diets low in Cho are associated with liver and muscle disease (3-5) and with suboptimal fetal development (6, 7), while diets too high in choline may be associated with increased risk for heart disease (8, 9). There is no validated biomarker for choline status (the availability of the various forms of Cho needed to sustain optimal cellular function); measurement of plasma Cho concentrations is not adequate because plasma choline is homeostatically regulated. Based on extensive preliminary and published data from our lab (10), we identified a panel of potential biomarkers that could be used to assess Cho status, and we propose studies to validate this biomarker panel against measures of Cho pool size using isotope dilution.

Impact

A biomarker panel for assessing Cho status would be used by clinicians and scientists to identify people with depleted choline stores and who may be at risk of developing organ dysfunction due to choline deficiency if their choline intake is not increased. This would enhance clinical care for pregnant women, people with obesity and metabolic syndrome, fatty liver, muscle disease and for people on parenteral nutrition (where choline deficiency is a problem) (11). It would also enhance clinical and epidemiological studies attempting to link choline status to disease risk. Finally, it may permit refinement of recommendations for dietary choline intake.

Innovation

There is no previous work attempting to establish a biomarker panel to assess Cho status. We propose to use cutting edge techniques to develop such a panel. The innovative biomarker panel that we propose for choline status is based on previous work by our research group (10) and has never been considered as markers for choline status prior to the proposed studies. The use of isotope dilution to measure Cho pool size is innovative; though it has been tried for Vitamin A status assessment (12), it has not been attempted for choline status. The investigators have been among the leaders in the area of choline nutrient requirements.

Background:

People need to have a source of Cho*:* Cho has several important functions. It is (i) a source of methyl-groups needed to make the primary methyl donor, *S*-adenosylmethionine; (ii) a part of the neurotransmitter acetylCho; and (iii) a component of the major phospholipids in membranes (phosphatidylCho (PtdCho) and sphingomyelin)

(13). The Cho derivative, PtdCho is a main constituent of very low density lipoproteins (VLDL) and is required for VLDL secretion and the export of fat from liver (14). Cho also is important for normal fetal development (15, 16).

Cho is a required nutrient and in 1998, an Adequate Intake (AI) and a Tolerable Upper Limit (UL) for Cho was established (17). In 2016, the US Food and Drug Administration (FDA) set a Recommended Daily Intake (RDI) for Cho based on the AIs as part of the new Nutrition Facts label for packaged foods (published in the Federal Register on May 27, 2016; FDA-2012-N-1210-0875, Federal Register Number:2016-11867). The AI/RDI varies by age and gender, but is 550 mg/d in adult men and 425 mg/d in adult women (more in pregnant and lactating women).

***Dietary Cho intake varies over a 3-fold range**:* There is a wide variation in Cho intake in the diet; in several human cohorts, Cho intake varies by approximately 3-fold – the US National Health and Nutrition Examination Survey (NHANES 2009-2012) data show that only 11% of adult Americans achieve the Adequate Intake level of Cho, with mean intake being 300 mg/day (10%ile being 200 mg/d; 90%ile being 500 mg/d) (2). Similar ranges of intake were reported in the Framingham Offspring Study (18), the Atherosclerosis Risk In Communities study (19, 20) and the Nurse's Health Study (21). Intake of Cho is even lower in low-income countries such as Jamaica and The Gambia (22, 23).

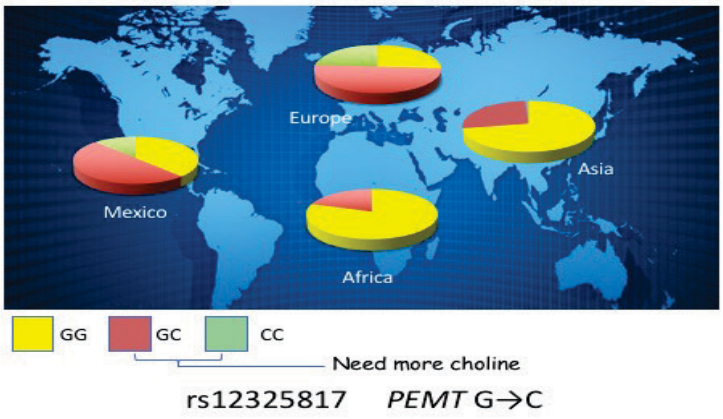
***Clinical studies show that people fed diets low in Cho develop liver and muscle damage**:* Diets low in Cho result in fatty liver, liver damage and muscle damage in people (3-5). The dietary requirement is highest for men and postmenopausal women while premenopausal women may have a reduced requirement for Cho, as discussed later (4, 11, 17, 24-26). Nonalcoholic fatty liver (NAFLD), that occurs as humans become obese, can be predicted by a genetic signature (pattern of SNPs) in Cho-related genes (27), and higher dietary intake of Cho reduces the risk of developing fatty liver (28). At present, Cho is not routinely added to commercial parenteral solutions for infants and adults (29, 30). However, adults and infants receiving total parenteral nutrition (TPN) over the long term have low plasma Cho concentrations and often develop hepatic abnormalities, including NAFLD (31-33). In men, dietary intakes in excess of the Cho RDI-AI are needed to optimize homocysteine disposal after a methionine load as well as for the removal of fat from liver (34). A Cho intake exceeding current dietary recommendations was also shown to preserve markers of cellular methylation and attenuate DNA damage in a genetic subgroup of folate-compromised men (35).

***Population studies also suggest that there is a range for healthful Cho intake**:* Women eating diets low in Cho during pregnancy (about 200 mg/d) are more likely to give birth to a child with birth defects than are women eating 500 mg/d (6, 36, 37). However, other studies have found no relationship between plasma or serum Cho concentrations during pregnancy and neural tube defects in offspring (38, 39) (likely because plasma Cho is not an adequate marker of Cho status). In addition, low dietary intake of Cho (about 150mg/d) was associated with decreased cognitive function in the Framingham Offspring Cohort (40). Higher Cho intake in pregnant women in Massachusetts was associated with modestly (but significantly) better child visual memory at age 7 years (41). In one observational study in 2,195 adults aged 70–74 years in Norway, participants with plasma Cho concentrations lower than 8.4 mcmol/L (20^{th} percentile of concentrations in the study population) had poorer sensorimotor speed, perceptual speed, executive function, and global cognition than those with Cho concentrations higher than 8.4 mcmol/L (42). Lower dietary intake of Cho was associated with an increased risk of breast cancer (43, 44) and colorectal cancer (45). Diets low in Cho increased the overall relative risk for developing cancer in a quantitative meta-analysis of 11 papers published on this topic (46), with the largest reported effects found for lung (30% increase; (47)), nasopharyngeal (58% increase; (47)) and breast cancer

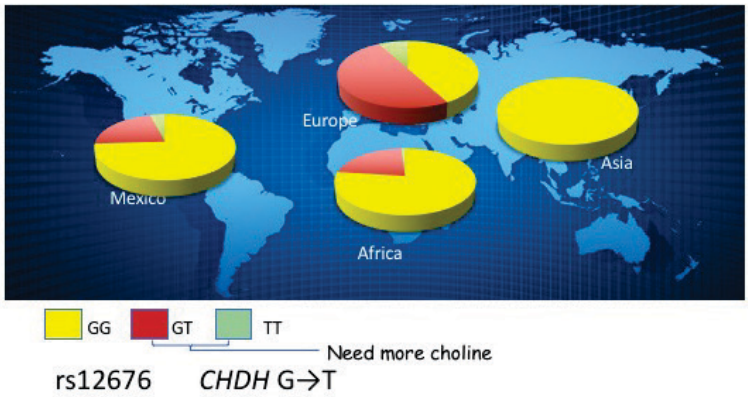
(60% increase; (48)). An increment of 100 mg/day of Cho and betaine (a metabolite derived from Cho) intake helped reduce cancer incidence by 11% (46).

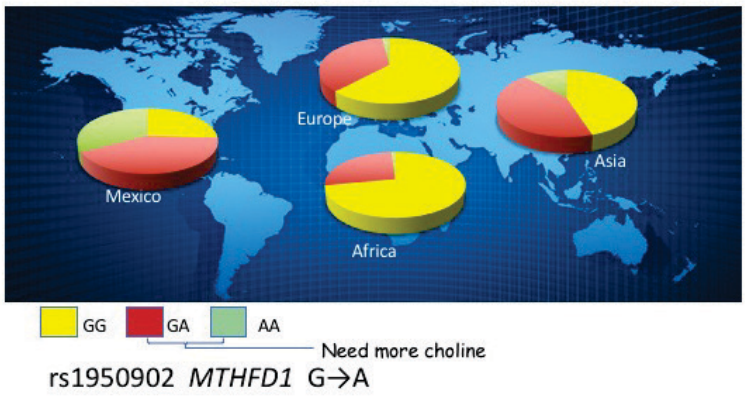
At the same time, diets high in Cho may be associated with an increased risk for prostate cancer progression (49), for colorectal adenomas (21) and for heart disease (8, 9). Analysis of data on 80,978 women from the Nurses' Health Study and 39,434 men from the Health Professionals Follow-Up Study found an increased risk of mortality in those consuming higher levels of Cho (50). Thus, it appears that diets at the lower end of normal intake for Cho have adverse health consequences, while diets at the higher end of normal intake also have adverse consequences. This U-shaped risk-benefit curve defines a relatively narrow range for optimal Cho intake, making the development of better biomarkers of Cho status very important.

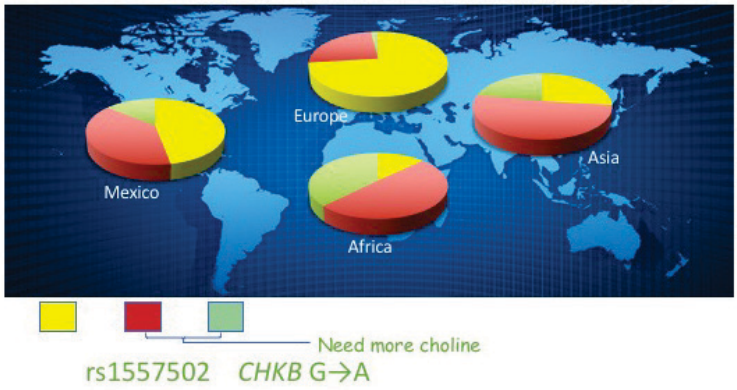
***Genetic variation influences risk for becoming Cho depleted**:* Single nucleotide polymorphisms (SNPs) in several genes related to Cho and folate metabolism alter the risk for developing organ dysfunction when people become Cho depleted (25, 35, 51, 52). PEMT (phosphatidylethanolamine-*N*-methyltransferase) catalyzes the formation of Cho moiety as part of PtdCho (53) and this pathway can reduce the amount of Cho that must be obtained from the diet (3). This gene is induced by estrogen; with maximum induction at the concentrations achieved in women during pregnancy (54). Women with the C allele of *PEMT* rs12325817 (74% of Caucasian women) are more susceptible to liver damage when consuming a low Cho diet (51, 55) because this allele abrogates estrogen-induced expression of the gene (56). Other *PEMT* SNPs also increase Cho requirement (rs4646343-A and rs3760188-A) (52). A number of other common SNPs in genes related to choline metabolism alter the requirement for Cho (25, 35, 51, 52, 57-59). Thus, similar dietary intakes may lead to very different Cho status, depending on genotype.



Given the narrow range for healthy intake of Cho, given the establishment of a RDI/AI for Cho to maintain health, given the 3-fold variation in dietary intake in the US, given the effects of common SNPs on the dietary requirement for Cho, and because plasma Cho concentrations do not adequately reflect status (see below), it is important that a good biomarker panel for assessing Cho status be established.







Potential biomarkers for Cho status: Self-reported dietary intake is very hard to measure accurately, and for Cho we have demonstrated that self-report can significantly underestimate actual intake (60). Thus, we need a set of biomarkers with which to assess Cho status in people. The largest stores of Cho are located in the liver, and mass resonance spectroscopy of liver has been used in the past to assess Cho status in humans (3, 25, 51). This method is not practical for use as a biomarker in clinical or public health practice as it is expensive and the availability of the instrumentation is limited. Liver biopsy is risky and not practical, making measurement of hepatic Cho and Cho metabolite concentrations a poor choice for assessing Cho status. Measurement of Cho concentrations in plasma alone is not an adequate marker for Cho status because Cho concentrations drop quickly to 70% of normal when a person is fed a low Cho diet, but do not decrease further with more severe depletion (3, 4) even in people starved for 7 days (61). The concentrations of PtdCho and sphingomyelin also decrease in people fed a low Cho diet (3, 4), but these phospholipids alone are not adequate biomarkers for Cho status because they are carried as part of plasma lipoproteins, and vary in concentration with changes in fat and cholesterol transport by these particles. Thus, such measurements are useful but not sufficient as biomarkers for Cho status.

Perhaps there is a panel of biomarkers that together will more accurately and reliably reflect Cho status. We admitted people (n=169; 60 premenopausal women, 52 postmenopausal women and 57 men) to our CTSA and fed them a diet containing 100% of recommended intake (550 mg/d) for one week, and then fed them a low Cho diet (10% normal Cho) for up to 7 weeks, or until they developed liver or muscle damage, and then fed them a Cho replete diet until such damage was completely resolved (24, 25, 52, 55). In these carefully controlled studies, using n=44 of the subjects, a comparison of metabolomic profiles between the two diet periods identified a number of potentially useful biomarkers of Cho status (10) (**Figs 2 & 3**).

The concentrations of plasma Cho, betaine, sarcosine, dimethylglycine, methionine, and PtdCho were significantly decreased, and homocysteine was significantly increased during Cho depletion in people (10). Cho

depletion was associated with abnormal renal function in animal models (62); a number of metabolites that accumulate in people with diminished renal function, including 3-carboxy-4-methyl-5-propyl-2-furanpropano-ate (CMPF), hippurate and 3-indolepropionate also were significantly increased by Cho depletion in our study subjects (10). Cho is important for mitochondrial function (63, 64), and in our study, plasma concentrations of several acylcarnitines were increased, reflecting perturbed mitochondrial function (65). In addition, people with SNPs that increase susceptibility to Cho deficiency had > 50% increased concentrations of propionylcarnitine (10). Plasma concentrations of a number of amino acids and their metabolites increased in Cho depletion (glutamine, pyroglutamate, glycine, valine, glutamyl-valine, and leucine) (10). Finally, plasma concentrations of a number of metabolites released from dying cells (urate and pseudouridine) were elevated in Cho depleted subjects (10). The alterations associated with Cho depletion in most metabolites were at least partially corrected by Cho repletion (10).

When people were depleted of Cho they developed abnormal aspartate transaminase (AST; marker of liver damage) and creatine phosphokinase (CK; marker of muscle damage) activities in blood (24, 52). This occurs because Cho deficiency induces apoptosis in tissues (26, 66, 67). Development of fatty liver was also a marker for Cho depletion in people fed low Cho diets (24, 25, 52, 55). Previously, we used mass resonance imaging to quantitate the fatty liver that occurred with Cho depletion (24) but this method is expensive and we believe that other non-invasive methods can be used to assess fatty liver. Controlled Attenuation Parameter (CAP) is a new ultrasound-based technique to measure steatosis simultaneously with assessment of liver stiffness using transient elastography (TE); it has been used in research studies (68-72) and is being used in hepatology clinics (73, 74). The measures of organ dysfunction discussed above, could improve a panel of other biomarkers by providing information assessing likely organ effects associated with Cho depletion. Though we expect elevations in these markers, we do not anticipate clinically significant damage based on this study design (see Safety).

Each of the above biomarker measures alone could be changed by multiple physiological inputs, however, based our data from the above study (10), we hypothesize that the combined panel of biomarkers provides a pattern of changes unique to Cho status, with more severe Cho deficiency having larger changes in the biomarkers. We suggest that a score based on some or all of the panel of biomarkers described above will provide a much more accurate and specific assessment of Cho status than measurement of plasma concentrations of Cho or PtdCho alone (**Fig. 4**).

Our preliminary data only represent fully replete and very depleted people, with no information about the people with in-between Cho status. In this grant proposal, we propose to generate data on people with more moderate depletion of Cho and develop a panel of biomarkers and use these measures to develop a Cho status score. By making measurements in people fed incrementally reduced amounts of Cho, and comparing the biomarker measures to body total Cho pool size assessed using isotope dilution (a proxy for the availability of the various forms of Cho), we will be able to identify the combination of biomarkers and algorithm for calculating a Cho status score that best predicts total Cho pool size, and therefore predicts choline nutritional status (the availability of the various forms of Cho needed to sustain cellular function).

1.2 Name and Description of Investigational Product or Intervention

Diets: The experimental diets, delivering 100%, 50% and 25% of the recommended intake of Cho (in all forms) (550 mg Cho/70 kg body weight/d), are identical except for the bread that we offer (Cho in the bread will be varied to deliver the desired Cho above that delivered by the base 25% Cho diet as described previously (1)). The base 10% Cho diet is composed of 0.8 g high biologic value protein/kg [current Recommended Dietary

Allowance (RDA)], with 30% kcal coming from fat and the remaining kcal from carbohydrate; diets are purchased commercial meals that have been measured for total nutritional values to acquire specific choline amounts for controlled choline intake. These diets were designed to bracket the range of dietary intake observed in several large published human population studies (18-23, 75). We have experience preparing and using diets with similar choline controls for more than 60 days per subject in our previously published studies (10, 24, 25, 51, 55).

Total food intake will be adjusted to be isocaloric (using snacks that contain minimal choline in any form) and to provide adequate intakes of macro- and micronutrients. All diets meet or exceed the Estimated Average Requirement for methionine plus cysteine, and the RDI for vitamins B_{12}, B_6, and folate. A multivitamin supplement (Kirkland Signature Daily Multi Vitamins and Minerals; Costco, Seattle, WA) provides vitamins and minerals at or above the RDI or Adequate Intake. Subjects will also be given a dietary supplement containing the RDI of magnesium and 1.5 times the Adequate Intake of calcium (Calcium Magnesium Complex; Vitamin Shoppe, North Bergen, NJ). We will use a 3-day diet menu of equivalent diets to allow for meal diversity. On the last 5 days of a diet period, subjects will consume 22% of their total Cho in the form of Cho chloride-(trimethyl-d9; 98%; Cambridge Isotope Laboratories, Tewksbury, MA, USA) replacing non-labeled Cho mole for mole in the bread. We will confirm total Cho content of diets by LC-MS/MS analysis of samples of duplicate food portions as previously described (1). Cho content and stability during storage of Cho in the bread will be separately determined.

Compliance with the diet plan will be assessed by having subjects bring back empty containers, by verbal check every week when new food is picked up, and by checking biomarker values and determining whether they deviate from gender-matched group mean data by more than 2 standard deviations. In addition, we will assess plasma and urine samples for presence of elevated riboflavin levels as a compliance biomarker, as riboflavin will be included in the dinner rolls used to supplement Cho intake.

1.3 Non-Clinical and Clinical Study Findings

There are no expected benefits to the subjects in the study.

Safety: Participants will remain on the above-noted experimental diets for the indicated time periods, or until they develop organ dysfunction associated with Cho deficiency (liver damage = >1.5-fold increase in aspartate aminotransferase (AST) or alanine aminotransferase (ALT), or muscle damage = >5-fold increase in creatine kinase (CK)). A clinical chemistry panel will be run at the beginning of the study (Screening) and at the beginning and end of each dietary period of the study. If they develop organ dysfunction at the end of any of the experimental diet periods, subjects will be monitored according to the protocol established for abnormal lab values (See Appendix Figure 6). We have extensive experience with these diet formulations (24, 52, 55) and have not observed clinically significant liver or muscle damage in individuals fed the 10% Cho diet for 2 weeks or less. After longer periods on a 10% Cho diet, clinically liver and muscle damage was noted, and reversed with 2 weeks of feeding 100% Cho diet. No other toxicity was observed after as long as 7 weeks on the 10% Cho diet (24, 52, 55). A urine pregnancy test will be performed on premenopausal women at the beginning and end of each dietary arm.

1.4 Relevant Literature and Data

Please see references throughout the rationale & background material.

2 STUDY OBJECTIVES

The goal of this study is to identify a biomarker panel that correlates well with measured Cho pool size across a range of different degrees of Cho depletion. We hypothesize that, as body stores of Cho diminish, cells and organs will reach the point when metabolism/function in the cell is altered, and that this will result in a progression of changes in biomarkers that reflect Cho status.

2.1 Primary Objective

Determine whether, in people, a choline status score based on measurements of a panel of biomarkers correlates well with measures of choline pool size as assessed using an isotope dilution method.

2.2 SubAims

SubAim 1a: Test assumptions used in the choline isotope dilution method for estimating choline pool size.

a) collect urine after first day of diet administration of deuterated choline and develop an estimate of % loss of administered label from body pool; b) measure choline pool size by isotope dilution and compare results to changes seen using mass resonance spectroscopy (MRS) of choline compound peaks in liver (the major storage pool for choline); c) assess fatty liver by mass resonance imaging (MRI) and compare results to those obtained using Fibroscan methodology.

These experiments will help us to refine the assumptions that we make to model choline pool size, and will validate the use of Fibroscan data as a measure of fatty liver.

SubAim 1b: Determine whether there are additional biomarkers from untargeted metabolomic profiling that, when added to the panel developed in Aim 1, are better at predicting choline status in people (using choline pool size determined by isotope dilution as a proxy for choline status).

In the experiment described in Aim 1 we will add consideration of data from untargeted metabolomic profiles at the end of each diet period. This data will be used to identify metabolites that, when added to the biomarker panel developed in Aim 1, improve the correlation with choline pool size assessed using isotope dilution.

SubAim 1c: Determine whether people with genetic polymorphisms known to increase risk for developing choline deficiency-induced organ dysfunction are also more likely to have a worse choline status score and a reduction in choline pool size assessed using isotope dilution.

A number of SNPs in genes of choline and folate metabolism have been associated with increased risk for developing organ dysfunction on a low choline diet. We hypothesize that people with these SNPs have a lower choline pool size/worse choline status score for any given choline dietary intake than do people without these SNPs.

3 INVESTIGATIONAL PLAN (brief overview)

3.1 Study Design

This is a crossover study wherein each subject will serve as their own control. Comparisons will be made between the low choline diet periods to the 100% choline diet at the beginning and end of the 6 weeks on the three dietary intervals (see Fig. 1, Appendix).

Screening: Before entry to the study the participants will be seen at the University of North Carolina Nutrition Research Institute Outpatient Clinical facility where a physical exam by a physician and a Clinical Chemistry panel will be performed to ascertain that they are healthy. Subjects will have mass resonance spectroscopy to measure Cho compounds in liver, and mass resonance imaging to assess fatty liver to validate the isotope dilution methodology and Fibroscan technology, respectively.

Intervention: Recruited subjects will be asked to fill out the Automated Self-Administered 24-hour Dietary Assessment Tool (ASA24) found on the NCI web site (https://epi.grants.cancer.gov/asa24/) for 3 days the week prior to the first diet intervention. This will estimate the choline intake level for subjects beginning the study. The week after screening, subjects will be placed on one of the three dietary choline levels (100%, 50%, and 25% - order randomly assigned) with a minimum 2 week washout between. On day 12 of each diet period, subjects will receive a 250mg bolus dose of the Cho delivered as Cho-d9 in order to estimate Cho pool sizes by isotope dilution (see Fig. 1, Appendix).

Follow-up: If the clinical laboratory data remain within normal values, this will conclude the subject's participation. If not, see section 4.3 (below).

Unscheduled Visits: Subjects may have to make unscheduled visits to pick up food if their freezer storage space is limiting.

3.2 Allocation to Treatment Groups and Blinding (if applicable):

All subjects will be randomly assigned to the order of the diet interventions. Investigators making metabolite, MRI, MRS, or Cho pool analyses will be blinded to diet assignments.

3.3 Study Duration, Enrollment and Number of Subjects:

The study lasts 10 weeks and a few days for each subject. We plan to enroll 150 subjects: 50 males, 50 pre-menopausal females, and 50 post-menopausal females. We will recruit an additional 30 subjects to account for drop out replacement, so the total number of subjects is 180.

3.4 Study Population

Inclusion criteria: Participants, ages 17 years - 70 years, will be recruited from the greater Kannapolis-Charlotte metro area (58% Caucasian, 33% African American; 3.5% Asian); we will make special efforts to assure that they reflect the race and ethnicity demographics of the region. Healthy men, pre- and post-menopausal women (defined as having had their last spontaneous menstrual bleeding >12 mo previously and as having a follicle stimulating hormone concentration >30 IU/L) and children (ages 17-18 years) will be asked to participate in a protocol approved by the Institutional Review Board at the University of North Carolina at Chapel Hill (n=60/group (we estimate that n=50/group will complete study) x 3 groups). Written informed consent will be obtained from all participants before initiating any study activities. Inclusion will be contingent on being in a good state of health as determined by physical examination and standard clinical laboratory tests with BMI <32.

Exclusion criteria: Subjects will be excluded if they are using drugs or medications known to be damaging to liver or muscle at typically prescribed doses or that have the potential to alter Cho metabolism (e.g., methotrexate); or if the volunteer has a history of hepatic, renal, or other chronic systemic disease. Individuals who are current smokers, who consume >2 alcoholic beverages/d or >14/wk, who are substance abusers or drug addicted, who are eating unusual diets that would interfere with the study, who have an allergy to soy (soy protein is used in experimental diet formulation), or people who are using Cho-containing dietary supplements are likewise ineligible.

Before entry to the study the participants will be seen at the University of North Carolina Nutrition Research Institute Outpatient Clinical facility where a physical exam by a physician and a Clinical Chemistry panel will be performed to ascertain that they are healthy.

For MRS and MRI testing, we will exclude subjects with: claustrophobia; cardiac pacemaker or artificial heart valve, metal plate, pin, or other metallic implant, intrauterine device, such as Copper-7 IUD, insulin or other drug pump, aneurysm clips, previous gunshot wound, cochlear implant or other hearing device, employment history as a metalworker (had metal in eye), permanent tattoos.

4 STUDY PROCEDURES (what will be done)

4.1 Screening/Baseline Visit procedures:

Subjects will be screened by telephone interview to make sure they meet the inclusion/exclusion criteria. Upon arrival to the Nutrition Research Institute volunteers will be asked to read and sign the consent forms for participation in the study. They will complete a medical history questionnaire to include a complete list of medications they are currently taking. They will be asked to update the information if anything were to change during the study period. A physical exam will be performed by a licensed physician and a blood specimen will be collected via venipuncture. If the subject is a pre-menopausal female, a urine pregnancy test will be performed. Subjects will be asked about food allergies, and must confirm they can eat the following foods that will be in the commercially prepared frozen foods they will consume:

The following have been tested for total Cho content and have been deemed appropriate for this study

'Evol Egg Whites, Kale, Roasted Tomato & Goat Cheese', 'Amy's Vegetable Pie Pocket Sandwich', 'Evol Egg Whites & Spinach Burrito', 'Evol Butternut Squash Lasagna', 'Lean Cuisine Four Cheese Pizza', 'Lean Cuisine Five Cheese Rigatoni', 'Lean Cuisine Four Cheese Pizza', 'Banquet Chicken Pot Pie', 'Jimmy Dean Delights English Muffin Applewood Smoke Chicken Sausage, Egg Whites Cheese', 'Jimmy Dean Delights English Muffin, Turkey Sausage, Egg Whites, Cheese', 'El Monterey Beef & Bean Burrito', 'Jimmy Dean Delights Croissant Turkey Sausage, Egg Whites & Cheese', 'Amy's Broccoli & Cheddar Bake Bowl', 'Amy's Macaroni and Cheese', 'Amy's Pesto Tortellini Bowl', 'Amy's Spinach Béchamel Lasagna', 'Amy's Casserole Chile Relleno Bowl', 'Lean Cuisine BBQ Recipe Chicken Pizza', 'Healthy Choice Three Cheese Tortellini', 'Amy's Vegetable Lasagna', 'Evol Portabella & Goat Cheese Ravioli', 'Amy's Vegetable Pot Pie', 'Banquet Chicken Nuggets and Fries', 'Amy's Country Cheddar Bowl', 'Jimmy Dean Delights, Turkey Sausage, Egg Whites, Potatoes, Cheese', 'Stouffer's Lasagna with Meat Sauce', 'Stouffer's Turkey Tetrazzini', 'Banquet Spaghetti and Meatballs', 'Amy's Broccoli & Cheddar Bake Bowl', 'Healthy Choice Chicken Parmigiana', 'Evol Ziti & Meat Sauce', 'Amy's Indian Mattar Paneer', 'Amy's Enchilada Verde, Spinach & Cheese Enchilada', 'Healthy Choice Sweet Sesame Chicken', 'Devour White Cheddar Mac &

Cheese', 'Evol Chicken Enchiladas', 'Evol Siracha Chicken', 'Healthy Choice Power Bowls', 'Cuban-Inspired Pork Bowl', 'Luvo Chicken Enchiladas', 'Amy's Chinese Noodles & Veggies w/ Cashew Cream Sauce', 'Healthy Choice Power Bowls', 'Adobo Chicken Bowl', 'Lean Cuisine Chicken Fried Rice', 'Healthy Choice Chicken Fried Rice', 'Evol Fire Grilled Steak', 'Jimmy Dean Ranchero Steak & Eggs Bowl', 'Jimmy Dean Burrito Breakfast Bowl'. We also provide fried rice and plain steamed rice to make up for calories as may be required (these items are low in Cho). Other items given as low Cho snacks are: Coca Cola, Dr. Pepper, Strawberry Jell-O, Sprite, Ocean Spray Cran-Apple Juice, Pure Leaf Sweet Tea, Dole Diced Pears, Celestial Seasonings Chamomile Tea, and Folgers Classic Roast Instant Coffee.

If the subject meets the inclusion/exclusion criteria, they will be given a study id, user name, and password in order to complete an online 3-day Food Frequency Questionnaire (FFQ). They must complete the FFQ prior to the beginning of the dietary intervention.

4.2 Intervention/Treatment procedures (by visits):

Visit 1: Upon arrival to the Nutrition Research Institute, the subjects will read and sign the consent forms for participation in the study. If a pre-menopausal female, a pregnancy urine test will be performed on the first visit and and last visit of each dietary interval. Female subjects must be using birth control during the study. Estrogen birth control is acceptable, but because estrogen can affect gene expression, estrogen use and time of menstrual cycle will be added to our data collection. During this visit a baseline blood sample will be collected and a saliva sample for DNA analysis. They will deliver a baseline 24 hour urine collection (kit given at screening). A body composition and heart rate measure will be obtained. A Fibroscan will be performed to assess the fat content of the liver. Food will be provided for 3 days of the 100% Cho diet. They will also be given multivitamins and a dietary supplement containing the RDI of magnesium and 1.5 times the adequate intake level of calcium. These will be supplied for each of the 2-week dietary interventions. Subjects will take home food for days 1-4 of the study. They will be given a 24 hour urine collection kit that will be used to test for dietary compliance on day 5.

Visit 2: On day 5 of the study subjects will drop off the urine sample and pick up food for days 4-9.

Visit 3: On day 9 of the study subjects will pick up food for days 9-11. Subjects will be instructed to fast for day 12 serum sample.

Visit 4: On day 12 subjects will return to the NRI to pick up meals to be consumed on days 12-14. They will provide a fasted serum sample via venipuncture for baseline metabolomics and –d9 content of Cho metabolites and 0 hour and 6 hours (30 minute, 1 hour and 2 hour samples may also be collected). A bolus dose of Cho d9 will be given in a beverage immediately following the 0 hour blood draw. Subjects will also complete a simple muscle test (Biodex) consisting of bicep curls. Subjects will be instructed to fast for day 13 serum sample. Participants will collect urine for 24 hours after bolus dose.

Visit 5: On day 13 subjects will provide fasted blood sample again and return 24 hour urine collection. They will pick up kit for subsequent 24 hour urine collection. Subjects will be instructed to fast for day 14 serum sample.

Visit 6: Subjects will provide fasted blood sample again and return 24 hour urine collection. They will pick up kit for subsequent 24 hour urine collection. Subjects will be instructed to fast for day 15 serum sample. UNC NRI will transport participants to Wake Translational Imaging Program in Winston Salem to complete MRS/MRI scans.

Visit 7: Subjects will arrive the morning of Day 15 for serum sample via venipuncture for metabolomics and Cho-d9 measurements. Subjects will return their 24 hour urine sample. A fibroscan test will be performed and, if female, a urine pregnancy test will be done. A heart rhythm strip and body composition measure will be obtained. This will complete the first arm of the study and will be followed by a two week washout period.

Visits 8-14: for the second arm of the study are the same as visits above, with the exception of the saliva for DNA.

Visits 15-21: for the third arm of the study are the same as visits above, with the exception of the saliva for DNA.

This concludes study participation.

4.3 Follow-up procedures (by visits):

If the clinical laboratory data remains within normal values, subjects will continue on to the next diet period. If any of the laboratory data is not within normal values at the end of any study period, subjects will monitored as directed by abnormal lab values protocol (See Appendix Figure 6).

4.4 Unscheduled Visits:

There may be subjects that do not have the freezer capacity to store food and will have to make more frequent visits to pick up food.

4.5 Concomitant Medication documentation:

All concomitant prescription medications taken during study participation will be recorded on the case report forms (CRFs). For this protocol, a prescription medication is defined as a medication that can be prescribed only by a properly authorized/licensed clinician. Medications to be reported in the CRF are concomitant prescription medications, over-the-counter medications and non-prescription medications.

4.6 Rescue medication administration: N/A.

Subject Completion/Withdrawal procedures: Subjects can withdraw from the study at any time without penalty. The investigators have the right to stop their participation any time due to any clinical adverse event (AE), laboratory abnormality, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant, failure to follow instructions (noncompliance with administered diet), or because the entire study has been stopped. Participants who withdraw or are terminated from the study will have normal Comprehensive metabolic panel results at the time of discharge. If they do not, they will follow the established abnormal lab values protocol (See Appendix Figure 6), and the Comprehensive metabolic panel will be repeated weekly until all values are within normal range. Subjects will be compensated for the days spent in the study.

4.7 Screen Failure procedures:

Subjects failing the screening visit (Visit 1) will receive $25 cash and will be informed that they are not eligible.

5 STUDY EVALUATIONS AND MEASUREMENTS (how measurements will be made)

5.1 Efficacy Evaluation (if applicable):

Biomarker panel:

Based on the discussion above, we selected the following biomarker panel for evaluation: Direct measures of Cho-derived pools: Cho, betaine, dimethylglycine, homocysteine, methionine, PtdCho, sphingomyelin, sarcosine, trimethylamine and trimethylamine oxide.

Measures of Cho-sensitive renal function: Plasma 3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF), hippurate and 3-indolepropionate.

Measures of Cho-sensitive mitochondrial function: Plasma acylcarnitines.

Measures of Cho-sensitive amino acid metabolism: Plasma glutamine, glutamyl-valine, glycine, leucine, pyroglutamate and valine.

Measures of Cho-sensitive liver & muscle damage: Plasma AST and CK; Controlled Attenuation Parameter (CAP).

This panel of biomarkers will be measured by targeted metabolomic profiling using LC-MS and GC-MS platforms, by targeted standard clinical assays of AST and CK activities in plasma, and by vibration-controlled transient elastography (Controlled Attenuation Parameter) assessment of fatty liver using a Fibroscan® M Probe.

Targeted metabolomic profiling of Cho-related metabolites**:** Plasma Cho, betaine, sphingomyelin and PtdCho (76, 77), dimethylglycine (78) and homocysteine (79) will be measured using liquid chromatography multiple reaction monitoring mass spectrometry coupled to stable isotope dilution. These methods are running in our laboratory and we have published extensively using the methods (e.g. (52, 55, 77, 80-83)). We use some deuterated internal standards in this assay, and plan to use 13C and 15N internal standards to replace these because of the use of deuterium in the isotope dilution studies. We will perform appropriate QC analysis to assure these substitutions do not alter the accuracy of our assays. Our method easily distinguishes deuterated Cho-containing compounds from C_{12} forms.

Acylcarnitines: Plasma acylcarnitines will be measured using liquid chromatography multiple reaction monitoring mass spectrometry coupled to stable isotope dilution (84, 85). Acylcarnitines are byproducts of lipid oxidation and even-chain species arise from incomplete β-oxidation of fatty acids, whereas odd-chain species such as C3 and C5 are produced during amino acid catabolism (86-88).

Clinical chemistry panel (comprehensive metaboloic panel {CMP})**:** At beginning and the end of each diet period (6 times total) all subjects will have urine and a blood sample obtained. Blood or urine will be tested for a Comprehensive Metabolic Chemistry Panel (Chem12) that includes: glucose, calcium, albumin, total protein, sodium, potassium, CO_2, Chloride, BUN, creatinine, CK, ALP, ALT, AST, and bilirubin. This test will be performed Carolinas Medical Center (CMC), which is both Clinical Laboratory Improvement Act and College of American Pathologists accredited. Creatine kinase (CK) will also be obtained.

Liver fat will be assessed using vibration-controlled transient elastography (Controlled Attenuation Parameter; CAP) using a Fibroscan® M Probe (Echosens, Paris, France) which is validated for fatty liver analysis in people of

BMI up to 32 (68-72). CAP is calculated from liver ultrasonic attenuation rate, reflecting the degree of hepatic steatosis (69). We have this instrumentation in place at the UNC NRI and have validated it against ultrasound analysis of liver fat.

During the first year, in a validation study (**SubAim 1a**) on the first 10 subjects (see isotope dilution section later) we will obtain both the Fibroscan assessment for fatty liver and quantitation of liver fat using mass resonance imaging using a method we previous published (24). Liver fat content is estimated by whole-body 3.0-T Siemens Sykra MR system using a modified "In and Out of Phase" procedure (18, 28). This approach involves the use of the differences in transverse magnetization intensity after an ultrabrief time interval (FLASH; echo time = 2.2 and 4.5 ms, flip angle = 80 °, and repetition time = 140 ms). Processing of successive FLASH MRI images with software from Siemens Medical Solutions (Malvern, PA) is used to estimate fat content. Quantification of organ fat content is based on measurements across 5 images (liver slices) per subject and standardized by relating the results to the fat content of similarly measured images of spleen. (It is assumed that spleen fat remained constant during the study and could be used to normalize values over time.). We will use this comparison to further validate the use of Fibroscan data to assess liver fat.

Isotope dilution estimation of body Cho pool: Using isotope dilution can provide an estimate of the size of the body pool of Cho. Our proposed method is conceptually similar to the method for measuring total body water from a bolus dose of labeled water (90). Similar methodology was used recently in studies of metabolic flux of Cho in pregnant women (89). Isotope dilution is a well-established method used to estimate pool size for other nutrients, such as vitamin A (12). Similar to vitamin A, the major storage pools for Cho are in the liver, and ingested Cho is rapidly absorbed and accumulated by liver (13, 89).
On the last 5 days of a diet period, subjects will consume 22% of their total dietary Cho in the form of Cho chloride-(trimethyl-d9; 98%; Cambridge Isotope Laboratories, Tewksbury, MA, USA). This proportion was shown by others to result in adequate enrichment of isotopic Cho in adults (89). This Cho will be diluted in the plasma pool of Cho and then in the liver and total body tissue pool of Cho, and metabolites will be formed (**Figure 5, appendix**). We will collect a plasma sample before (baseline) and after (post dosing) each 5-day feeding of deuterated Cho.

Cho is stored in the body primarily as Cho, phosphoCho, glycerophosphoCho, PtdCho and sphingomyelin. Acetylcholine and CDP-Cho constitute a very small portion of the Cho pool and will not be measured. In plasma, phosphoCho is hydrolyzed to Cho by alkaline phosphatase; thus, measuring enrichment of Cho includes a measure of phosphoCho. New Cho enters the pool from the diet and from *de novo* synthesis catalyzed by PEMT. The sum of the metabolite enrichments of Cho-d9, glycerophosphoCho-d9, sphingomyelin-d9 and PtdCho-d9 will be calculated relative to the sum of Cho compounds with no d9. The presence of Cho d3 compounds will be a measure of recycling of a methyl group into Cho via the PEMT pathway, and will be considered to be part of the unenriched Cho pool. Betaine d3 will not be considered part of the Cho pool, but its formation will allow us to adjust our assumptions as to how much of administered label is lost from the Cho pool compartment. We expect that betaine formation will be significant.

Isotope enrichments in each of the Cho-containing compounds will be measured using LC-MS/MSas described above (89). We will calculate enrichment for each Cho-containing molecule as well as for the combination of the Cho molecules (total); we expect that total Cho enrichment will be what is used in our analyses, but examination of the enrichment of the individual Cho-containing species may allow us to refine our mathematical

compartment models for Cho distribution at 5 days.

Estimated total body Cho pool size is calculated using the following mass balance equations:

1. $(F_a \times a) + (F_b \times b) = (F_c \times c)$

Where $\mathbf{F_a}$ is the isotope abundance of deuterium in the isotopic dose; **a** is the amount of the absorbed isotopic dose (absorption assumed to be 100%); $\mathbf{F_b}$ is the isotope abundance of deuterium in the endogenous Cho pool (measured at baseline before 5-day deuterium treatment); **b** is the amount of the endogenous Cho pool at baseline; $\mathbf{F_c}$ is the isotope abundance of deuterium in the total Cho pool ($F = {}^2H/[{}^1H + {}^2H]$) after 5-day dosing; **c** is the amount of the total Cho pool after dosing.

F_a, F_b and F_c are measured by isotope ratio mass spectrometry.
a is calculated from administered dose

2. $c = a + b$
3. b = body pool at baseline (unknown) = $a \left(\frac{F_a - F_c}{F_c - F_b}\right)$

Though we will call the estimate derived from the above a measure of total body pool size, it is really just an estimate of the size of the pool that dietary Cho equilibrates with by 5 days (likely smaller than the total body pool). We must make a number of assumptions, discussed below, to arrive at such an estimate. Each assumption certainly affects our estimations of pool size, and as we collect data we will have to model outcomes if we alter these assumptions.

The use of isotope dilution to estimate Cho pools size has not been used before, but as noted above is an approach that has been proved successful for total body water (90) and Vitamin A (12). For both of these methods, a single bolus of isotopic label can be used. For both of these it is important that the isotopic label equilibrates rapidly with the body pool. We know that Cho equilibrates within minutes with the vascular and liver pools of Cho, but that equilibration with the PtdCho-bound Cho takes hours to days. For this reason, we propose to administer deuterated Cho for 5 days. We realize that our diet depletion protocol will result in a decline in body pool size during these 5 days, but suggest that the decline during the previous 11 days will be larger and make the resulting error in pool size estimation acceptable. Caudill used 3 weeks as her equilibration period (89), but such a long period would provide an estimate of average Cho pool size during the diet intervention rather than at the end of the intervention. Based on our best estimates of Cho turnover in the free and esterified pools, we believe that a 5-day isotope equilibration will work best for the purposes of the study. The equation for calculating Vitamin A total body pool also includes a correction factor for efficiency of absorption of Vitamin A (12). For Cho, we are assuming that efficiency of absorption is 100% (studies described below in SubAIm 1a will help us to test/revise this assumption). This likely is an overestimate, but will result in an overestimation of pool size that will be a constant error for all diet periods, thereby the calculation of the change in pool size caused by a diet period should not be appreciably altered. Similarly, the assumption that little Cho is lost to renal excretion is not true, as the metabolites betaine and TMA are excreted in the urine. Such losses of label in urine will result in an underestimate of Cho pool sizes, but this underestimation of pool size will be a constant error for all diet periods, and changes in pool size should not be affected. We also assume

that *de novo* formation of PtdCho by phosphatidylethanolamine N-methyltransferase (PEMT) during the 5 days of isotope administration is small compared to body pool size; in women with estrogen this dilution of isotopic label would be greater than in men and postmenopausal women. We believe that the contribution of PEMT will not appreciably change the estimates of pool size that we obtain. To reiterate, as long as the assumptions are uniform in the equation used across diet periods, treatment effects should not be affected.

We propose to use deuterium labeling rather than ^{13}C. The natural abundance of deuterium is ~0.015% compared to hydrogen at >99.9%, whereas those of ^{13}C and ^{12}C are 1.1% and 98.9%, respectively. This makes it easier to attain satisfactory enrichment with lower doses of deuterium. However, deuterium can shift within or between molecules through proton exchange, but this usually occurs under harsh conditions (12). The Caudill data using deuterated Cho suggests that proton exchange is not a significant problem in human studies (89). Using d9-Cho relative to 1,1,2,2 –d4-Cho allows us to better differentiate by mass (9 vs 4 mass units). Deuterium labeled Cho is also 6x less expensive than ^{13}C-Cho.

We will conduct studies during year 1 to better validate the isotope dilution methodology. Through the MRI/MRS studies we will test some of these assumptions (**SubAim 1a**). We will collect a 24 h urine sample after the isotope administration periods, and measure the amount of label lost in the urine (likely in the form of deuterated betaine and TMAO) during 24 hr periods. We will also use mass resonance spectroscopy of liver to estimate Cho pool size in liver. This measure should be related to choline pool size as measured by isotope dilution because liver is the major storage pool for choline. At the end of each diet period we will perform proton magnetic resonance spectroscopy (^{1}H-MRS) with a clinical whole-body 3.0-T Siemens Sykra MR system with an 8-element phased array surface coil (see letter of Support from Wake Medical Center) to quantify liver Cho *in vivo* using unsuppressed water as an internal reference (91, 92). This method has been used in people to measure Cho compound concentrations in liver tumors and these studies in 90 subjects successfully measured Cho compounds in surrounding normal liver tissue (92). In another component of the validation study, we will use Cho-1,2-$^{13}C_2$ chloride (Sigma Aldrich, St. Louis) as well as d9-Cho in 5 of the subjects to determine if there is an isotope-related difference in our estimate of Cho pool size by isotope dilution (see discussion above). These studies during the first year should permit us to test many of the assumptions that we need to make to measure pool size using isotopic labeling.

Liver mass resonance imaging and spectroscopy: As discussed earlier, we will perform mass resonance imaging and spectroscopy to assess Cho compounds in liver and fatty liver. Scanning will be performed with the 3.0-T Siemens Sykra MR system (see above). Axial, sagittal and coronal T2-weighted single-short fast spin echo sequences are performed in each subject for 1 H MRS localization. A 2 x 2 x 2 cm^3 voxel is placed in the right lobe of liver, avoiding large vessels, bile ducts and adjacent structures. PRESS sequences are used to allow for spatial localization of the 1 H MRS voxel. Spectroscopy will use respiratory gating and a scanning time of 138 s and total measurement time of 5 to 8 min including both acquisition and resting time. Liver fat (normalized to spleen fat) swill be quantitated using a modified “In and Out of Phase” procedure as previously described (24). This approach uses the differences in transverse magnetization intensity after an ultrabrief time interval (FLASH; echo time = 2.2 and 4.5 ms, flip angle = 80 °, and repetition time = 140 ms). Processing of successive FLASH MRI images with software from Siemens Medical Solutions (Malvern, PA) is used to estimate fat content. We have experience using these methods in human studies where we fed people low Cho diets and observed proportional decreases in the size of Cho-compound peaks in liver MRS spectra (24, 55).

Metabolomic profiling: In **SubAim 1b**, we will explore whether there are metabolites detected using untargeted metabolic profiling that enhance the Cho status score so that it better predicts Cho pool size as measured using isotope dilution. Broad-spectrum methods will be conducted by the NIH Common Fund Eastern Regional Comprehensive Metabolomics Resource Core (NIH C-F ERCMRC), under the direction of Dr. Susan Sumner, by UPLC-TOF-MS and GC-TOF-MS (see letter of Support). The UPLC-TOF-MS method was adapted from the Human Serum Metabolome Consortium (HUSERMET) (93, 94). Briefly, plasma will be extracted, lyophilized, and reconstituted for UPLC-MS/MS analysis, and data will be acquired on an Acquity UPLC system coupled to a SYNAPT G2 Time of Flight (TOF) mass spectrometer (Waters Corporation, Milford, MA). Chromatographic separation of metabolites will be performed using reversed-phase chromatograph, as previously described (95). Broad-spectrum MS data will be processed using Progenesis QI for spectral alignment, deconvolution, peak picking, and matching to the ERCMRC in-house RT-MS library of approximately 900 metabolites and additional public databases, such as METLIN and HMDB. A list of ion intensities for each feature detected will be generated using retention time (RT) and m/z data pairs as the identifiers. Ion intensities for each detected peak will be normalized to the sum of the peak intensities (excluding standards) within that sample. The resulting normalized peak intensities contain a three-dimensional (3D) data matrix with RT, m/z, and ion intensities for each feature. Ion peaks are filtered based on the quality and reproducibility of the data in the pooled QC aliquots (see below). The ERCMRC uses a GC-TOF-MS broad spectrum metabolomics method developed by the Fiehn lab (96, 97). Briefly, plasma will be extracted, derivatized using a two-step procedure, and spiked with fatty acid methyl esters (FAMEs, as RI markers). Broad-spectrum GC-TOF-MS metabolomics data will be acquired on Pegasus GC x GC TOFMS (Leco Inc., St. Joseph, MI). Data files will be processed for deconvolution using ChromaTOF® software and transferred to BinBase (96, 98) for peak retention index calculations, metabolite identification, and generation of a table of peak identifications and intensities. Each peak intensity is normalized to the sum intensities of the metabolites and used for statistical investigation. In addition to BinBase, NIST library matches will be conducted. The ERCMRC has used these methods for analysis of plasma, serum, urine, feces, ovary, brain, saliva, sweat, breast tissue, muscle, kidney, and cells. System suitability checks will be performed before analysis of samples, to evaluate sensitivity, retention time, and mass calibration for tolerance within a pre-established range. In every analysis batch, at least 10% of the samples are QC standards, which are dispersed throughout the analytical run to evaluate any variability across or between plates. QC measures include reviewing the quality of each run, stability of internal standards signal, and drifts in retention time. All samples will be randomized prior to analysis to prevent potential bias towards diet periodss. In addition to analysis of each individual study sample, small aliquots of each of the dietary period study samples will be combined to create a pool, and then prepared in replicate. These quality control pools will be randomized with the individual study samples prior to data acquisition. For untargeted metabolomics analysis, an unsupervised multivariate analysis will be used to ensure that the QC pool samples cluster tightly and are in the center of the study samples from which they were derived (93, 95). If the QC pools do not cluster, the signals which introduce the variability will be removed from further analysis. After statistical and multivariate analysis are conducted which demonstrate which signals differentiate the study diet periods (described below), these QC pools (created for the diet periods) will also be used to obtain fragmentation patterns to identify the signals deemed important for distinguishing the diet periods.

Genotyping: In **SubAim 1c**, we will test the validity of our Cho score and isotope dilution measures by asking whether people with genetic polymorphisms known to increase risk for developing Cho deficiency-induced organ dysfunction have values for Cho score and isotope dilution that show that they have smaller pool size for any given diet treatment (and perhaps have altered choline biomarkers). For example, as discussed earlier about half of premenopausal women with a common PEMT SNP do not induce the gene and make less Cho and

therefore must eat Cho. Based on our previous studies, we expect that 56% of premenopausal women will attenuate the rate at which they deplete of Cho because they can induce PEMT activity with estrogen (44% cannot due to *PEMT* SNPs), and we expect them to show will show intermediate changes in Cho pool size and thus, in the biomarkers. For the *PEMT* rs12325817-C SNP we genotype using RT-PCR (56) (it is in a region of Alu repeats that makes the genotyping array fail for this SNP). *PEMT* rs4646343-A and rs3760188-A are obtained with the array below.

In an exploratory study, we will ask whether other SNPs that create inefficiencies in choline metabolism also are associated with decreased choline pools size and alter choline biomarkers. We will use a custom Illumina Expanded Multi-Ethnic genotyping array (Mega-EX). This chip contains >2 million markers plus 899 added SNPs in genes of Cho and 1-carbon metabolism that were selected based on our published identification of SNPs (24, 25, 52, 55, 56) that altered Cho status. We will assess genotype cluster calls for accuracy, using Illumina's included sample-dependent and independent controls for assay performance, and replica samples will be included with every set of ~95 samples to assess allele calling consistencies. Poorly-performing DNA samples, markers that deviate from HWE (P <0.0001) or have >5% assay failure, will be removed. Genotypic data will be stored as comma delimited text files and integrated into the database at UNC-NRI, where subsets are retrieved for analyses using established scripts. Haplotypes and frequency estimates for them will be obtained with a maximum likelihood method that accounts for pedigree structure using Sequential Oligogenic Linkage Analysis Routines (SOLAR) software (99). CNVs will be assessed as previously described (100).

5.2 Pharmacokinetic Evaluation: N/A.

5.3 Safety Evaluations:

Participants will remain on the experimental diets for the indicated time periods, or until they develop organ dysfunction associated with Cho deficiency (liver damage = >1.5-fold increase in aspartate aminotransferase (AST) or muscle damage = >5-fold increase in creatine kinase (CK)). A clinical chemistry panel will be run at the beginning of the study (Screening) and at the beginning and end of each dietary period of the study (see Figure 1, appendix). If they develop organ dysfunction at the end of any of the experimental diet periods, subjects will follow the abnormal lab values protocol (Appendix Figure 6). We have extensive experience with these diet formulations (24, 52, 55) and have not observed clinically significant liver or muscle damage in individuals fed the 10% Cho diet for 2 weeks or less. After longer periods, clinical liver and muscle damage was noted, and reversed with 2 weeks of feeding 100% Cho diet. No other toxicity was observed after as long as 7 weeks on the 10% Cho diet (24, 52, 55). A urine pregnancy test will be performed on pre-menopausal women at the beginning and end of each dietary arm.

6 STATISTICAL CONSIDERATION

6.1 Primary Endpoint

SubAim 1a: Test assumptions used in the choline isotope dilution method for estimating choline pool size. In the subjects studied: a) collect urine after administration of deuterated choline and develop an estimate of % loss of administered label from body pool; b) measure choline pool size by isotope dilution and compare results

to changes seen using mass resonance spectroscopy (MRS) of choline compound peaks in liver (the major storage pool for choline); c) assess fatty liver by mass resonance imaging (MRI) and compare results to those obtained using Fibroscan methodology. These experiments will help us to refine the assumptions that we make to model choline pool size, and will validate the use of Fibroscan data as a measure of fatty liver.

SubAim 1b: Determine whether there are additional biomarkers from untargeted metabolomic profiling that, when added to the panel developed in Aim 1, are better at predicting choline status in people (using choline pool size determined by isotope dilution as a proxy for choline status). In the experiment described in Aim 1 we will add consideration of data from untargeted metabolomic profiles at the end of each diet period. This data will be used to identify metabolites that, when added to the biomarker panel developed in Aim 1, improve the correlation with choline pool size assessed using isotope dilution.

6.2 Secondary Endpoint

SubAim 1c: Determine whether people with genetic polymorphisms known to increase risk for developing choline deficiency-induced organ dysfunction are also more likely to have a worse choline status score and a reduction in choline pool size assessed using isotope dilution. A number of SNPs in genes of choline and folate metabolism have been associated with increased risk for developing organ dysfunction on a low choline diet. We hypothesize that people with these SNPs have a lower choline pool size/worse choline status score for any given choline dietary intake than do people without these SNPs.

6.3 Statistical Methods

Statistical methods (Aim 1, SubAim 1a,1b**).** Unsupervised principal component analysis will first be used to examine the clustering of samples from the diet periods, and to ensure that the QC pool replicates center in the samples from which they were derived. This will enable the evaluation of the stability of the platform across the plate and between days for the signals which have not yet been assigned, to remove any signals that are too random and prevent the desired clustering, and to identify subjects who outlie from their perspective diet periods. Supervised Orthogonal Partial Least Squares Regression (OPLS-R) will then be used to identify a panel of biomarkers, assign weight to each biomarker, and calculate a composite score to predict choline pool size. When we applied Orthogonal Partial Least Squares Effect Projections (OPLS-EP) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) to our previous data, we identified a panel of biomarkers and calculated a composite score that successfully predicted choline status (baseline or depletion) for both men and women (**Figure 1**). In this proposed study, we will use OPLS-R instead of OPLS-DA, because choline pool size is a continuous variable, while choline status in our previous study was a categorical variable. We will analyze all of the samples using OPLS-R to identify a panel of biomarkers that predicts choline pool size universally for all three diet periods (healthy men, pre- and postmenopausal women). We will also consider performing the analysis for each diet period separately in case the universal panel does not provide high accuracy in prediction. For the purpose of validation, we will not split samples into training and testing sets, as it reduces the number of samples used for model building. Instead, prediction error will be estimated using the Bootstrap method, which was found by recent studies as providing “nearly unbiased estimates of future model performance without holding back data when making the final estimates of model parameters” (101). Variable Importance to Projection (VIP)

score will be calculated based on the OPLS weights and the variability explained in OPLS-R. Biomarkers with VIP>1.5 will be considered the most important biomarkers responsible for prediction. Pathway analysis will be performed using GeneGo metacore (Thomson Reuters, https://portal.genego.com) and QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Statistical methods (SubAim 1c**).** Linear model with repeated measures will be used to identify SNPs that increase (or decrease) the risk for developing choline deficiency-induced organ dysfunction. This analysis will be performed for each diet period (healthy men, pre- and postmenopausal women) separately. In each linear model, the choline status score defined by Aim 1 is the response variable. Diet effect (fixed), genotype effect (fixed), subject effect (random) and diet×genotype interaction effect are the independent variables. In the repeated measures design, each subject serves as his/her own control. Therefore, confounding effect from genotype is not our concern. However, it is possible that genotype serves as a moderator, which interacts with the diet effect, and affects the direction or strength of the diet effect. For example, the change in choline status score in responding to dietary choline may be observed in subjects with one genotype (responders), but not in subjects with other genotypes (non-responders). The genotypes that modify the diet effect will be identified by the diet×genotype interaction term. This analysis will be performed in the MIXED procedure in SAS (SAS Institute, Inc., Cary, NC). Benjamini-Hochberg method for False Discovery Rate (FDR) correction will be used for multiple testing correction. To improve the normality of the data, data transformation such as log transformation will be applied to metabolomics data, and outliers with studentized residue > 3 or <-3 will be excluded. Post hoc comparisons will be performed to identify metabolites associated with different levels of dietary choline and different genotypes. The analysis described above will also be applied to choline pool size data to identify significant diet×genotype interaction effect for choline pool size.

6.4 Sample Size and Power

Sample size estimation (Aim 1, SubAim 1a,1b**).** Similar to OPLS-DA, OPLS-R is a multivariate method for prediction. Different from hypothesis testing methods such as t-test or ANOVA, where sample size is determined by the power of rejecting null hypotheses when the alternative hypothesis is true, the sample size of OPLS-R is determined by its accuracy of prediction. In this study, we propose to recruit three groups of subjects (healthy men, pre- and postmenopausal women) with n=60/group (estimated that n=50/group will complete study). We plan to analyze all three groups together (n=50*3=150 subjects) to identify a formula for composite score that can predict choline pool size for all three groups. In case the universal score does not perform well, we will analyze each group (n=50 subjects) separately. Using our previous data with n=44 subjects, we successfully identified a panel of metabolites that can be used to predict whether a sample is taken at baseline or after choline deficient diet. As shown by the ROC plot, the area under curve was 98.77%, which indicates very high sensitivity and specificity (Figure 2). Our previous study indicates that there is a strong association between the panel of biomarkers and choline status even when the model was developed with a relatively small number of samples.

Sample size estimation (SubAim 1c**).** This analysis will first be applied to the *PEMT* SNPs (rs12325817-C; rs4646343-A and rs3760188-A) that are known to interact with the effect of dietary choline in women but not in men (24). This analysis serves to confirm the consistency between the performance of the choline status score and existing knowledge. Assuming the effect size of the diet×genotype interaction is 0.6 (moderate effect), we

will have 89.61% of power to detect it at P<0.05 cutoff. The same analysis will then be performed for all other SNPs (>2 million) measured in this study. We will be under-powered for the global analysis after multiple testing adjustment for 2 million tests, so this analysis will be an exploratory analysis. To increase statistical power, we will use SNP based pathway enrichment analysis method (102). This analysis was developed for GWA studies, and addresses the lack of power issue in GWA studies. It will identify SNPs with jointly significant genetic effect, i.e. sharing the same biological pathways, even when the individual P value for each SNP may not be significant. The pathways identified from this analysis may provide insight into the regulation of choline metabolism.

7 STUDY INTERVENTION (drug, device or other intervention details)

Diets: The experimental diets, delivering 100%, 50% and 25% of the recommended intake of Cho (in all forms) (550 mg Cho/70 kg body weight/d), are identical except for the bread that we offer (Cho in the bread will be varied to deliver the desired Cho above that delivered by the base Cho diet as described previously (1)). The base Cho diet is composed of 0.8 g high biologic value protein/kg [current Recommended Dietary Allowance (RDA)], with 30% kcal coming from fat and the remaining kcal from carbohydrate; diets are prepared in-house to protocol specifications and are similar to those described in detail in a publication in JADA (1). These diets were designed to bracket the range of dietary intake observed in several large published human population studies (18-23, 75). We have experience using these limited Choline level diets for more than 60 days per subject in our previously published studies (10, 24, 25, 51, 55).

Total food intake will be adjusted to be isocaloric (using snacks that contain minimal choline in any form) and to provide adequate intakes of macro- and micronutrients. All diets meet or exceed the Estimated Average Requirement for methionine plus cysteine, and the RDI for vitamins B_{12}, B_6, and folate. A multivitamin supplement (Kirkland Signature Daily Multi Vitamins and Minerals; Costco, Seattle, WA) provides vitamins and minerals at or above the RDI or Adequate Intake. Subjects will also be given a dietary supplement containing the RDI of magnesium and 1.5 times the Adequate Intake of calcium (Calcium Magnesium Complex; Vitamin Shoppe, North Bergen, NJ). We will use a 3-day diet menu of equivalent diets to allow for meal diversity. On the day 12 of a diet period, subjects will consume 250mg Cho in the form of Cho chloride-(trimethyl-d9; 98%; Cambridge Isotope Laboratories, Tewksbury, MA, USA) replacing non-labeled Cho mole for mole in the bread. We will confirm total Cho content of diets by LC-MS/MS analysis of samples of duplicate food portions as previously described (1). Cho content and stability during storage of Cho in the bread will be separately determined.

8 STUDY INTERVENTION ADMINISTRATION

This is a crossover study wherein each subject will serve as their own control. Comparisons will be made between the low choline diet periods to the 100% choline diet at the beginning and end of the eight week period (see Fig. 1, Appendix). No randomization will take place as the study design requires all subjects start with a 100% Cho diet in order to compare biomarker changes to the graded decrease in Cho levels over subsequent weeks. All subjects will spend the last two weeks of the study on the 100% Cho replete diet.

Investigators making metabolite, MRI, MRS, metabolomics analyses, or Cho pool analyses, will be initially blinded to diet assignments. Only personnel performing work necessary for data interpretation leading to a publishable manuscript will be un-blinded.

9 SAFETY MANAGEMENT

We plan to apply for NC TraCS DSMB support if the IRB approves of the study. We will submit ongoing reports to the committee according to the frequency of reports that is stipulated if the study is accepted by the DSMB. We anticipate that they will request an interim review after 10 subjects have completed the study.

Serious adverse event (SAE) or serious suspected adverse reaction. An adverse event (AE) or suspected adverse reaction is considered "serious" if, in the view of either the investigator, it results in any of the following outcomes: death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

*Unanticipated problems involving risks to participants or others to include, in general, any incident, experience, or outcome that meets **all** of the following criteria:*

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied;
- Related or possibly related to participation in the research ("possibly related" means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

For AEs not included in the protocol defined grading system, the following guidelines will be used to describe severity.

- ***Mild*** *– Events require minimal or no treatment and do not interfere with the participant's daily activities.*
- **Moderate** – Events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- **Severe** – Events interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually potentially life-threatening or incapacitating.

For all collected AEs, the clinician who examines and evaluates the participant will determine the AE's causality based on temporal relationship and his/her clinical judgment. The degree of certainty about causality will be graded using the categories below.

- **Definitely Related** – There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out. The clinical event, including an abnormal laboratory test result, occurs in a plausible time relationship to diet administration and cannot be explained by concurrent disease or other drugs or chemicals. The event must be pharmacologically or phenomenologically definitive, with use of a satisfactory rechallenge procedure if necessary.
- **Probably Related** – There is evidence to suggest a causal relationship, and the influence of other factors is unlikely. The clinical event, including an abnormal laboratory test result, occurs within a reasonable time after administration of the diet, is unlikely to be attributed to concurrent

  disease or other drugs or chemicals, and follows a clinically reasonable response on withdrawal (dechallenge). Rechallenge information is not required to fulfill this definition.

- **Possibly Related** – There is some evidence to suggest a causal relationship (e.g., the event occurred within a reasonable time after administration of the trial diet). However, other factors may have contributed to the event (e.g., the participant's clinical condition, other concomitant events). Although an AE may rate only as "possibly related" soon after discovery, it can be flagged as requiring more information and later be upgraded to "probably related" or "definitely related," as appropriate.
- **Unlikely to be related** – A clinical event, including an abnormal laboratory test result, whose temporal relationship to diet administration makes a causal relationship improbable (e.g., the event did not occur within a reasonable time after administration of the trial medication) and in which other drugs or chemicals or underlying disease provides plausible explanations (e.g., the participant's clinical condition, other concomitant treatments).
- **Not Related** – The AE is completely independent of study diet administration, and/or evidence exists that the event is definitely related to another etiology. There must be an alternative, definitive etiology documented by the clinician].

The study physician, Dr. Torres, will be responsible for determining whether an AE is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study agent.

The occurrence of an AE or SAE may come to the attention of study personnel during study visits and interviews of a study participant presenting for medical care, or upon review by a study monitor.

All AEs including local and systemic reactions not meeting the criteria for SAEs will be captured on the appropriate CRF. Information to be collected includes event description, time of onset, clinician's assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis), and time of resolution/stabilization of the event. All AEs occurring while on study must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution.

Any medical condition that is present at the time that the participant is screened will be considered as baseline and not reported as an AE. However, if the study participant's condition deteriorates at any

time during the study, it will be recorded as an AE. UPs will be recorded in the data collection system throughout the study.

Changes in the severity of an AE will be documented to allow an assessment of the duration of the event at each level of severity to be performed. AEs characterized as intermittent require documentation of onset and duration of each episode.

The PI will record all reportable events with start dates occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. At each study visit, the investigator will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization.

AEs will be reported to the IRB within 24h after the study physician becomes aware of them.
The study physician shall report Serious Adverse Events to the IRB as soon as possible, but in no event later than 24 h after the investigator first learns of the event.
To satisfy the requirement for prompt reporting, Unexpected problems (Ups) will be reported using the following timeline:

- UPs that are SAEs will be reported to the IRB within 24h of the investigator becoming aware of the event.
- Any other UP will be reported to the IRB within 10 days of the investigator becoming aware of the problem.

Data Safety Monitoring Plan: Participant confidentiality is strictly held in trust by the participating investigators and their staff. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party.
The study participant's contact information will be securely stored for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by local IRB and Institutional regulations.

This study is a genome-wide association study and will comply with the NIH Policy for Sharing of Data Obtained in NIH Supported or Conducted GWAS, which calls for investigators funded by the NIH for GWAS to 1) share de-identified genetic (genotypic and phenotypic) data through a centralized NIH data repository; and 2) submit documentation that describes how the institutions have considered the interests of the research participants, such as privacy and confidentiality. Submission of data to the NIH GWAS repository will be consistent with the permissions and limitations delineated on the study consent signed by study participants.

Intended use of stored samples, specimen or data. We will store DNA from each subject so that we can measure gene polymorphisms and other variants that are discovered by others to be relevant to choline metabolism after the completion of this study.

We will store plasma from each subject so that we can measure metabolites and other biomarkers are discovered by others to be relevant to choline metabolism after the completion of this study.

With the participant's approval de-identified biological samples will be stored at -80 degrees C in freezers at the UNC Nutrition Research Institute.

Study participants who request destruction of samples will be notified of compliance with such request and all supporting details will be maintained for tracking.

Access to stored samples will be limited to study investigators. Samples and data will be stored using codes assigned by the investigators. Data will be kept in password- protected computers. Only investigators will have access to the samples and data.

10 DATA COLLECTION AND MANAGEMENT

Steven Zeisel (PI) and the medical director Jomari Torres, MD will monitor subject data for safety continuously throughout the study and as new subjects are recruited. Data will be transmitted electronically via secured email communications among study personnel, including Dr. Steven Zeisel, Dr. Martin Kohlmeier, Stephen Orena, Julie Stegall, and Dr. Isis Trujillo. All data transmission between team members will use the study ID to reference individual participation. De-identified data will be transmitted in SAS via encrypted hard drives or via secure UNC email accessed through a secure network. Sharing of data by email will use the secure protocol required of University personnel.

Once the study has ended, data and human biological specimens will be labeled only with coded study IDs for duration of the study and analysis and should not be identifiable in any way. Personally identifiable data collected for the purpose of payment to study subjects for participation (SSN, name, address, etc.) will be located in locked offices. Any electronically stored information will be kept in password-protected files. Specimens will be stored indefinitely and not anonymized. The specimens will not be shared with other researchers.

Genetic samples will be banked and genotyping will be conducted at a later date when we are certain that a follow-on study will occur. Genetic samples mean DNA for genotyping. Identifiable data and biological materials from study subjects who do not sign the optional storage consent will be destroyed by deleting electronic files and shredding hard copy.

11 RECRUITMENT STRATEGY

All study subjects will volunteer for participation in the study by responding by telephone or in person to recruitment materials. Subjects who respond to recruitment materials will be asked questions from the screening questionnaire to insure they are eligible for participation in the study. Study subjects will be recruited from the Charlotte metro region. Flyers will be posted on college campuses and other public buildings. Recruitment ads will be placed on the website and listservs of the UNC Nutrition Research Institute and the North Carolina Research Campus. Interviews will be scheduled in private rooms. Telephone numbers and email addresses will be stored in locked cabinets.

12 CONSENT PROCESS

The investigator will ensure that this study is conducted in full conformity with Regulations for the Protection of Human Subjects of Research codified in 45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 56, and/or the ICH E6.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the IRB for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. All changes to the consent form will be IRB approved; a determination will be made regarding whether previously consented participants need to be re-consented.

Consent forms describing in detail the study agent, study procedures, and risks are given to the participant and written documentation of informed consent is required prior to starting intervention/administering study product. The following consent materials are submitted with this protocol:

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continues throughout the individual's study participation. Extensive discussion of risks and possible benefits of participation will be provided to the participants and their families. Consent forms will be IRB-approved and the participant will be asked to read and review the document. The investigator will explain the research study to the participant and answer any questions that may arise. All participants will receive a verbal explanation in terms suited to their comprehension of the purposes, procedures, and potential risks of the study and of their rights as research participants. Participants will have the opportunity to carefully review the written consent form and ask questions prior to signing.

The participants should have the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. The participant will sign the informed consent document prior to any procedures being done specifically for the study. The participants may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the participants for their records. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

13 PLANS FOR PUBLICATION

Year 5 of the study will entail data processing and interpretation and writing of a paper for publication.

14 REFERENCES

1. Busby, M. G., Fischer, L., da Costa, K. A., Thompson, D., Mar, M. H., and Zeisel, S. H. (2004) Choline- and betaine-defined diets for use in clinical research and for the management of trimethylaminuria. *Journal of the American Dietetic Association* **104**, 1836-1845
2. Wallace, T. C., and Fulgoni, V. L., 3rd. (2016) Assessment of Total Choline Intakes in the United States. *Journal of the American College of Nutrition* **35**, 108-112
3. Fischer, L. M., daCosta, K. A., Kwock, L., Stewart, P. W., Lu, T. S., Stabler, S. P., Allen, R. H., and Zeisel, S. H. (2007) Sex and menopausal status influence human dietary requirements for the nutrient choline. *The American journal of clinical nutrition* **85**, 1275-1285
4. Zeisel, S. H., daCosta, K.-A., Franklin, P. D., Alexander, E. A., Lamont, J. T., Sheard, N. F., and Beiser, A. (1991) Choline, an essential nutrient for humans. *FASEB J.* **5**, 2093-2098

5. Buchman, A., Dubin, M., Moukarzel, A., Jenden, D., Roch, M., Rice, K., Gornbein, J., and Ament, M. (1995) Choline deficiency: a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. *Hepatology* **22**, 1399-1403
6. Shaw, G. M., Carmichael, S. L., Yang, W., Selvin, S., and Schaffer, D. M. (2004) Periconceptional dietary intake of choline and betaine and neural tube defects in offspring. *Am J Epidemiol* **160**, 102-109
7. Shaw, G. M., Finnell, R. H., Blom, H. J., Carmichael, S. L., Vollset, S. E., Yang, W., and Ueland, P. M. (2009) Choline and risk of neural tube defects in a folate-fortified population. *Epidemiology* **20**, 714-719
8. Wang, Z., Klipfell, E., Bennett, B. J., Koeth, R., Levison, B. S., Dugar, B., Feldstein, A. E., Britt, E. B., Fu, X., Chung, Y. M., Wu, Y., Schauer, P., Smith, J. D., Allayee, H., Tang, W. H., DiDonato, J. A., Lusis, A. J., and Hazen, S. L. (2011) Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* **472**, 57-63
9. Tang, W. H., Wang, Z., Shrestha, K., Borowski, A. G., Wu, Y., Troughton, R. W., Klein, A. L., and Hazen, S. L. (2015) Intestinal microbiota-dependent phosphatidylcholine metabolites, diastolic dysfunction, and adverse clinical outcomes in chronic systolic heart failure. *J Card Fail* **21**, 91-96
10. Sha, W., da Costa, K. A., Fischer, L. M., Milburn, M. V., Lawton, K. A., Berger, A., Jia, W., and Zeisel, S. H. (2010) Metabolomic profiling can predict which humans will develop liver dysfunction when deprived of dietary choline. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **24**, 2962-2975
11. Buchman, A. L., Ament, M. E., Sohel, M., Dubin, M., Jenden, D. J., Roch, M., Pownall, H., Farley, W., Awal, M., and Ahn, C. (2001) Choline deficiency causes reversible hepatic abnormalities in patients receiving parenteral nutrition: proof of a human choline requirement: a placebo-controlled trial. *J Parenter Enteral Nutr* **25**, 260-268
12. Gannon, B. M., and Tanumihardjo, S. A. (2015) Comparisons among Equations Used for Retinol Isotope Dilution in the Assessment of Total Body Stores and Total Liver Reserves. *J Nutr* **145**, 847-854
13. Zeisel, S. H. (2006) Choline: critical role during fetal development and dietary requirements in adults. *Annu Rev Nutr* **26**, 229-250
14. Yao, Z. M., and Vance, D. E. (1988) The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J. Biol. Chem.* **263**, 2998-3004
15. Zeisel, S. H. (2009) Importance of methyl donors during reproduction. *Am J Clin Nutr* **89**, 673S-677S
16. Jiang, X., Yan, J., West, A. A., Perry, C. A., Malysheva, O. V., Devapatla, S., Pressman, E., Vermeylen, F., and Caudill, M. A. (2012) Maternal choline intake alters the epigenetic state of fetal cortisol-regulating genes in humans. *FASEB J* **26**, 3563-3574
17. Institute of Medicine, and National Academy of Sciences USA. (1998) Choline. In *Dietary reference intakes for folate, thiamin, riboflavin, niacin, vitamin B12, panthothenic acid, biotin, and choline* Vol. 1 pp. 390-422, National Academy Press, Washington D.C.
18. Cho, E., Zeisel, S. H., Jacques, P., Selhub, J., Dougherty, L., Colditz, G. A., and Willett, W. C. (2006) Dietary choline and betaine assessed by food-frequency questionnaire in relation to plasma total homocysteine concentration in the Framingham Offspring Study. *Am J Clin Nutr* **83**, 905-911
19. Bidulescu, A., Chambless, L. E., Siega-Riz, A. M., Zeisel, S. H., and Heiss, G. (2007) Usual choline and betaine dietary intake and incident coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) study. *BMC Cardiovasc Disord* **7**, 20
20. Bidulescu, A., Chambless, L. E., Siega-Riz, A. M., Zeisel, S. H., and Heiss, G. (2009) Repeatability and measurement error in the assessment of choline and betaine dietary intake: the Atherosclerosis Risk in Communities (ARIC) study. *Nutr J* **8**, 14
21. Cho, E., Willett, W. C., Colditz, G. A., Fuchs, C. S., Wu, K., Chan, A. T., Zeisel, S. H., and Giovannucci, E. L. (2007) Dietary choline and betaine and the risk of distal colorectal adenoma in women. *J Natl Cancer Inst* **99**, 1224-1231
22. Dominguez-Salas, P., Moore, S. E., Cole, D., da Costa, K. A., Cox, S. E., Dyer, R. A., Fulford, A. J., Innis, S. M., Waterland, R. A., Zeisel, S. H., Prentice, A. M., and Hennig, B. J. (2013) DNA methylation potential: dietary intake and blood concentrations of one-carbon metabolites and cofactors in rural African women. *The American journal of clinical nutrition* **97**, 1217-1227

23. Gossell-Williams, M., Fletcher, H., McFarlane-Anderson, N., Jacob, A., Patel, J., and Zeisel, S. (2005) Dietary intake of choline and plasma choline concentrations in pregnant women in Jamaica. *The West Indian medical journal* **54**, 355-359
24. Fischer, L. M., daCosta, K., Kwock, L., Stewart, P., Lu, T.-S., Stabler, S., Allen, R., and Zeisel, S. (2007) Sex and menopausal status influence human dietary requirements for the nutrient choline. *Am. J. Clin. Nutr.* **85**, 1275-1285
25. Kohlmeier, M., da Costa, K. A., Fischer, L. M., and Zeisel, S. H. (2005) Genetic variation of folate-mediated one-carbon transfer pathway predicts susceptibility to choline deficiency in humans. *Proc Natl Acad Sci U S A* **102**, 16025-16030
26. da Costa, K. A., Badea, M., Fischer, L. M., and Zeisel, S. H. (2004) Elevated serum creatine phosphokinase in choline-deficient humans: mechanistic studies in C2C12 mouse myoblasts. *The American journal of clinical nutrition* **80**, 163-170
27. Corbin, K. D., Abdelmalek, M. F., Spencer, M. D., da Costa, K. A., Galanko, J. A., Sha, W., Suzuki, A., Guy, C. D., Cardona, D. M., Torquati, A., Diehl, A. M., and Zeisel, S. H. (2013) Genetic signatures in choline and 1-carbon metabolism are associated with the severity of hepatic steatosis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **27**, 1674-1689
28. Yu, D., Shu, X. O., Xiang, Y. B., Li, H., Yang, G., Gao, Y. T., Zheng, W., and Zhang, X. (2014) Higher dietary choline intake is associated with lower risk of nonalcoholic fatty liver in normal-weight Chinese women. *The Journal of nutrition* **144**, 2034-2040
29. Vanek, V. W., Borum, P., Buchman, A., Fessler, T. A., Howard, L., Jeejeebhoy, K., Kochevar, M., Shenkin, A., and Valentine, C. J. (2012) A.S.P.E.N. position paper: recommendations for changes in commercially available parenteral multivitamin and multi-trace element products. *Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition* **27**, 440-491
30. Buchman, A. L. (2009) The addition of choline to parenteral nutrition. *Gastroenterology* **137**, S119-128
31. Buchman, A. L., Sohel, M., Brown, M., Jenden, D. J., Ahn, C., Roch, M., and Brawley, T. L. (2001) Verbal and visual memory improve after choline supplementation in long-term total parenteral nutrition: a pilot study. *JPEN. Journal of parenteral and enteral nutrition* **25**, 30-35
32. Buchman, A. L., Moukarzel, A., Jenden, D. J., Roch, M., Rice, K., and Ament, M. E. (1993) Low plasma free choline is prevalent in patients receiving long term parenteral nutrition and is associated with hepatic aminotransferase abnormalities. *Clinical nutrition (Edinburgh, Scotland)* **12**, 33-37
33. Sentongo, T. A., Kumar, P., Karza, K., Keys, L., Iyer, K., and Buchman, A. L. (2010) Whole-blood-free choline and choline metabolites in infants who require chronic parenteral nutrition therapy. *Journal of pediatric gastroenterology and nutrition* **50**, 194-199
34. Veenema, K., Solis, C., Li, R., Wang, W., Maletz, C. V., Abratte, C. M., and Caudill, M. A. (2008) Adequate Intake levels of choline are sufficient for preventing elevations in serum markers of liver dysfunction in Mexican American men but are not optimal for minimizing plasma total homocysteine increases after a methionine load. *Am J Clin Nutr* **88**, 685-692
35. Shin, W., Yan, J., Abratte, C. M., Vermeylen, F., and Caudill, M. A. (2010) Choline intake exceeding current dietary recommendations preserves markers of cellular methylation in a genetic subgroup of folate-compromised men. *J Nutr* **140**, 975-980
36. Shaw, G. M., Finnell, R. H., Blom, H. J., Carmichael, S. L., Vollset, S. E., Yang, W., and Ueland, P. M. (2009) Choline and Risk of Neural Tube Defects in a Folate-Fortified Population. *Epidemiology* **epub**, 10.1097/EDE.1090b1013e3181ac1099fe1097
37. Shaw, G. M., Carmichael, S. L., Laurent, C., and Rasmussen, S. A. (2006) Maternal nutrient intakes and risk of orofacial clefts. *Epidemiology* **17**, 285-291
38. Signore, C., Ueland, P. M., Troendle, J., and Mills, J. L. (2008) Choline concentrations in human maternal and cord blood and intelligence at 5 y of age. *Am J Clin Nutr* **87**, 896-902

39. Mills, J. L., Fan, R., Brody, L. C., Liu, A., Ueland, P. M., Wang, Y., Kirke, P. N., Shane, B., and Molloy, A. M. (2014) Maternal choline concentrations during pregnancy and choline-related genetic variants as risk factors for neural tube defects. *The American journal of clinical nutrition* **100**, 1069-1074
40. Poly, C., Massaro, J. M., Seshadri, S., Wolf, P. A., Cho, E., Krall, E., Jacques, P. F., and Au, R. (2011) The relation of dietary choline to cognitive performance and white-matter hyperintensity in the Framingham Offspring Cohort. *Am J Clin Nutr* **94**, 1584-1591
41. Boeke, C. E., Gillman, M. W., Hughes, M. D., Rifas-Shiman, S. L., Villamor, E., and Oken, E. (2013) Choline intake during pregnancy and child cognition at age 7 years. *Am J Epidemiol* **177**, 1338-1347
42. Nurk, E., Refsum, H., Bjelland, I., Drevon, C. A., Tell, G. S., Ueland, P. M., Vollset, S. E., Engedal, K., Nygaard, H. A., and Smith, D. A. (2013) Plasma free choline, betaine and cognitive performance: the Hordaland Health Study. *The British journal of nutrition* **109**, 511-519
43. Xu, X., Gammon, M. D., Zeisel, S. H., Bradshaw, P. T., Wetmur, J. G., Teitelbaum, S. L., Neugut, A. I., Santella, R. M., and Chen, J. (2009) High intakes of choline and betaine reduce breast cancer mortality in a population-based study. *FASEB J* **23**, 4022-4028
44. Du, Y. F., Luo, W. P., Lin, F. Y., Lian, Z. Q., Mo, X. F., Yan, B., Xu, M., Huang, W. Q., Huang, J., and Zhang, C. X. (2016) Dietary choline and betaine intake, choline-metabolising genetic polymorphisms and breast cancer risk: a case-control study in China. *Br J Nutr* **116**, 961-968
45. Lu, M. S., Fang, Y. J., Pan, Z. Z., Zhong, X., Zheng, M. C., Chen, Y. M., and Zhang, C. X. (2015) Choline and betaine intake and colorectal cancer risk in Chinese population: a case-control study. *PLoS One* **10**, e0118661
46. Sun, S., Li, X., Ren, A., Du, M., Du, H., Shu, Y., Zhu, L., and Wang, W. (2016) Choline and betaine consumption lowers cancer risk: a meta-analysis of epidemiologic studies. *Sci Rep* **6**, 35547
47. Ying, J., Rahbar, M. H., Hallman, D. M., Hernandez, L. M., Spitz, M. R., Forman, M. R., and Gorlova, O. Y. (2013) Associations between dietary intake of choline and betaine and lung cancer risk. *PLoS One* **8**, e54561
48. Zhang, C. X., Pan, M. X., Li, B., Wang, L., Mo, X. F., Chen, Y. M., Lin, F. Y., and Ho, S. C. (2013) Choline and betaine intake is inversely associated with breast cancer risk: a two-stage case-control study in China. *Cancer Sci* **104**, 250-258
49. Richman, E. L., Stampfer, M. J., Paciorek, A., Broering, J. M., Carroll, P. R., and Chan, J. M. (2010) Intakes of meat, fish, poultry, and eggs and risk of prostate cancer progression. *Am J Clin Nutr* **91**, 712-721
50. Zheng, Y., Li, Y., Rimm, E. B., Hu, F. B., Albert, C. M., Rexrode, K. M., Manson, J. E., and Qi, L. (2016) Dietary phosphatidylcholine and risk of all-cause and cardiovascular-specific mortality among US women and men. *Am J Clin Nutr* **104**, 173-180
51. da Costa, K. A., Kozyreva, O. G., Song, J., Galanko, J. A., Fischer, L. M., and Zeisel, S. H. (2006) Common genetic polymorphisms affect the human requirement for the nutrient choline. *Faseb J* **20**, 1336-1344
52. da Costa, K. A., Corbin, K. D., Niculescu, M. D., Galanko, J. A., and Zeisel, S. H. (2014) Identification of new genetic polymorphisms that alter the dietary requirement for choline and vary in their distribution across ethnic and racial groups. *FASEB J*
53. DeLong, C. J., Shen, Y. J., Thomas, M. J., and Cui, Z. (1999) Molecular distinction of phosphatidylcholine synthesis between the CDP- choline pathway and phosphatidylethanolamine methylation pathway. *J Biol Chem* **274**, 29683-29688
54. Resseguie, M., Song, J., Niculescu, M. D., da Costa, K. A., Randall, T. A., and Zeisel, S. H. (2007) Phosphatidylethanolamine N-methyltransferase (PEMT) gene expression is induced by estrogen in human and mouse primary hepatocytes. *Faseb J* **21**, 2622-2632
55. Fischer, L. M., da Costa, K. A., Kwock, L., Galanko, J., and Zeisel, S. H. (2010) Dietary choline requirements of women: effects of estrogen and genetic variation. *Am J Clin Nutr* **92**, 1113-1119
56. Resseguie, M. E., da Costa, K. A., Galanko, J. A., Patel, M., Davis, I. J., and Zeisel, S. H. (2011) Aberrant estrogen regulation of PEMT results in choline deficiency-associated liver dysfunction. *J Biol Chem* **286**, 1649-1658
57. West, A. A., and Caudill, M. A. (2010) Genetic variation: impact on folate (and choline) bioefficacy. *Int J Vitam Nutr Res* **80**, 319-329

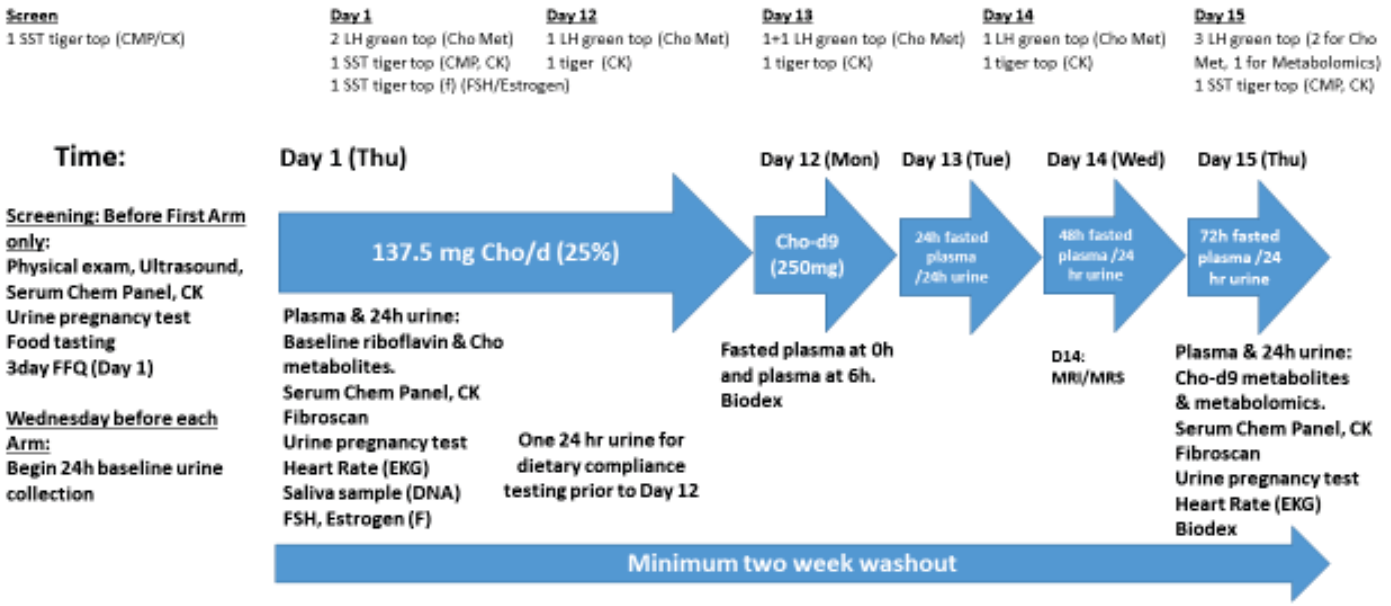
58. Michel, V., and Bakovic, M. (2009) The solute carrier 44A1 is a mitochondrial protein and mediates choline transport. *Faseb J*
59. Michel, V., and Bakovic, M. (2012) The ubiquitous choline transporter SLC44A1. *Central nervous system agents in medicinal chemistry* **12**, 70-81
60. Fischer, L. M., Scearce, J. A., Mar, M. H., Patel, J. R., Blanchard, R. T., Macintosh, B. A., Busby, M. G., and Zeisel, S. H. (2005) Ad libitum choline intake in healthy individuals meets or exceeds the proposed adequate intake level. *J Nutr* **135**, 826-829
61. Savendahl, L., Mar, M.-H., Underwood, L., and Zeisel, S. (1997) Prolonged fasting results in diminished plasma choline concentration but does not cause liver dysfunction. *Am. J. Clin. Nutr.* **66**, 622-625
62. Anonymous. (1974) Nutrition classics from The Journal of Biological Chemistry, 131:567-577, 1937. Choline metabolism. I. The occurrence and prevention of hemorrhagic degeneration in young rats on a low choline diet. By Wendell H. Griffith and Nelson J. Wade. *Nutrition Review* **32**, 112-114
63. Johnson, A., Lao, S., Wang, T., Galanko, J., and SH, Z. (2012) Choline dehydrogenase polymorphism rs12676 Is a functional variation and Is associated with changes in human sperm cell function. *PLoS ONE* **7**, e36047 doi:36010.31371/journal.pone.0036047
64. Johnson, A. R., Craciunescu, C. N., Guo, Z., Teng, Y. W., Thresher, R. J., Blusztajn, J. K., and Zeisel, S. H. (2010) Deletion of murine choline dehydrogenase results in diminished sperm motility. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **24**, 2752-2761
65. Adams, S. H., Hoppel, C. L., Lok, K. H., Zhao, L., Wong, S. W., Minkler, P. E., Hwang, D. H., Newman, J. W., and Garvey, W. T. (2009) Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women. *J Nutr* **139**, 1073-1081
66. da Costa, K. A., Niculescu, M. D., Craciunescu, C. N., Fischer, L. M., and Zeisel, S. H. (2006) Choline deficiency increases lymphocyte apoptosis and DNA damage in humans. *Am J Clin Nutr* **84**, 88-94
67. Shin, O. H., Mar, M. H., Albright, C. D., Citarella, M. T., daCosta, K. A., and Zeisel, S. H. (1997) Methyl-group donors cannot prevent apoptotic death of rat hepatocytes induced by choline-deficiency. *J. Cell. Biochem.* **64**, 196-208
68. Myers, R. P., Pollett, A., Kirsch, R., Pomier-Layrargues, G., Beaton, M., Levstik, M., Duarte-Rojo, A., Wong, D., Crotty, P., and Elkashab, M. (2012) Controlled Attenuation Parameter (CAP): a noninvasive method for the detection of hepatic steatosis based on transient elastography. *Liver Int* **32**, 902-910
69. Fujimori, N., Tanaka, N., Shibata, S., Sano, K., Yamazaki, T., Sekiguchi, T., Kitabatake, H., Ichikawa, Y., Kimura, T., Komatsu, M., Umemura, T., Matsumoto, A., and Tanaka, E. (2016) Controlled attenuation parameter is correlated with actual hepatic fat content in patients with non-alcoholic fatty liver disease with none-to-mild obesity and liver fibrosis. *Hepatol Res*
70. de Ledinghen, V., Vergniol, J., Foucher, J., Merrouche, W., and le Bail, B. (2012) Non-invasive diagnosis of liver steatosis using controlled attenuation parameter (CAP) and transient elastography. *Liver Int* **32**, 911-918
71. Chon, Y. E., Jung, K. S., Kim, S. U., Park, J. Y., Park, Y. N., Kim do, Y., Ahn, S. H., Chon, C. Y., Lee, H. W., Park, Y., and Han, K. H. (2014) Controlled attenuation parameter (CAP) for detection of hepatic steatosis in patients with chronic liver diseases: a prospective study of a native Korean population. *Liver Int* **34**, 102-109
72. Masaki, K., Takaki, S., Hyogo, H., Kobayashi, T., Fukuhara, T., Naeshiro, N., Honda, Y., Nakahara, T., Ohno, A., Miyaki, D., Murakami, E., Nagaoki, Y., Kawaoka, T., Tsuge, M., Hiraga, N., Hiramatsu, A., Imamura, M., Kawakami, Y., Aikata, H., Ochi, H., Takahashi, S., Arihiro, K., and Chayama, K. (2013) Utility of controlled attenuation parameter measurement for assessing liver steatosis in Japanese patients with chronic liver diseases. *Hepatol Res* **43**, 1182-1189
73. Leung, J. C., Loong, T. C., Wei, J. L., Wong, G. L., Chan, A. W., Choi, P. C., Shu, S. S., Chim, A. M., Chan, H. L., and Wong, V. W. (2016) Histological severity and clinical outcomes of nonalcoholic fatty liver disease in nonobese patients. *Hepatology* **10.1002/hep.28697**
74. Dyson, J. K., Anstee, Q. M., and McPherson, S. (2014) Non-alcoholic fatty liver disease: a practical approach to diagnosis and staging. *Frontline Gastroenterol* **5**, 211-218

75. Wallace, T. C., McBurney, M., and Fulgoni, V. L., 3rd. (2014) Multivitamin/mineral supplement contribution to micronutrient intakes in the United States, 2007-2010. *J Am Coll Nutr* **33**, 94-102
76. Koc, H., Mar, M. H., Ranasinghe, A., Swenberg, J. A., and Zeisel, S. H. (2002) Quantitation of choline and its metabolites in tissues and foods by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry. *Anal Chem* **74**, 4734-4740
77. Zhao, X., Zeisel, S. H., and Zhang, S. (2015) Rapid LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine N-oxide, and creatinine in human plasma and urine. *Electrophoresis* **doi 10.1002/elps.201500055**
78. Allen, R. H., Stabler, S. P., and Lindenbaum, J. (1993) Serum betaine, N,N-dimethylglycine and N-methylglycine levels in patients with cobalamin and folate deficiency and related inborn errors of metabolism. *Metabolism* **42**, 1448-1460
79. Ubbink, J. B., Hayward Vermaak, W. J., and Bissbort, S. (1991) Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. *Journal of chromatography* **565**, 441-446
80. Adams, K. M., Lindell, K. C., Kohlmeier, M., and Zeisel, S. H. (2006) Status of nutrition education in medical schools. *Am J Clin Nutr* **83**, 941S-944S
81. Mar, M. H., Ridky, T. W., Garner, S. C., and Zeisel, S. H. (1995) A method for the determination of betaine in tissues using high performance liquid chromatography. *J. Nutr. Biochem.* **6**, 392-398
82. Pomfret, E. A., daCosta, K., Schurman, L. L., and Zeisel, S. H. (1989) Measurement of choline and choline metabolite concentrations using high-pressure liquid chromatography and gas chromatography-mass spectrometry. *Anal. Biochem.* **180**, 85-90
83. Zeisel, S. H., Mar, M. H., Howe, J. C., and Holden, J. M. (2003) Concentrations of choline-containing compounds and betaine in common foods. *J Nutr* **133**, 1302-1307
84. Haqq, A. M., Lien, L. F., Boan, J., Arlotto, M., Slentz, C. A., Muehlbauer, M. J., Rochon, J., Gallup, D., McMahon, R. L., Bain, J. R., Stevens, R., Millington, D., Butler, M. D., Newgard, C. B., and Svetkey, L. P. (2005) The Study of the Effects of Diet on Metabolism and Nutrition (STEDMAN) weight loss project: Rationale and design. *Contemp Clin Trials* **26**, 616-625
85. Millington, D. S., Kodo, N., Norwood, D. L., and Roe, C. R. (1990) Tandem mass spectrometry: a new method for acylcarnitine profiling with potential for neonatal screening for inborn errors of metabolism. *J Inherit Metab Dis* **13**, 321-324
86. Sampey, B. P., Freemerman, A. J., Zhang, J., Kuan, P. F., Galanko, J. A., O'Connell, T. M., Ilkayeva, O. R., Muehlbauer, M. J., Stevens, R. D., Newgard, C. B., Brauer, H. A., Troester, M. A., and Makowski, L. (2012) Metabolomic profiling reveals mitochondrial-derived lipid biomarkers that drive obesity-associated inflammation. *PLoS One* **7**, e38812
87. Boyle, K. E., Canham, J. P., Consitt, L. A., Zheng, D., Koves, T. R., Gavin, T. P., Holbert, D., Neufer, P. D., Ilkayeva, O., Muoio, D. M., and Houmard, J. A. (2011) A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. *The Journal of clinical endocrinology and metabolism* **96**, 775-781
88. Makowski, L., Noland, R. C., Koves, T. R., Xing, W., Ilkayeva, O. R., Muehlbauer, M. J., Stevens, R. D., and Muoio, D. M. (2009) Metabolic profiling of PPARalpha-/- mice reveals defects in carnitine and amino acid homeostasis that are partially reversed by oral carnitine supplementation. *FASEB J* **23**, 586-604
89. Ganz, A. B., Shields, K., Fomin, V. G., Lopez, Y. S., Mohan, S., Lovesky, J., Chuang, J. C., Ganti, A., Carrier, B., Yan, J., Taeswuan, S., Cohen, V. V., Swersky, C. C., Stover, J. A., Vitiello, G. A., Malysheva, O. V., Mudrak, E., and Caudill, M. A. (2016) Genetic impairments in folate enzymes increase dependence on dietary choline for phosphatidylcholine production at the expense of betaine synthesis. *FASEB J*
90. van Marken Lichtenbelt, W. D., Westerterp, K. R., and Wouters, L. (1994) Deuterium dilution as a method for determining total body water: effect of test protocol and sampling time. *Br J Nutr* **72**, 491-497
91. Shen, Z. W., Cao, Z., You, K. Z., Yang, Z. X., Xiao, Y. Y., Cheng, X. F., Chen, Y. W., and Wu, R. H. (2012) Quantification of choline concentration following liver cell apoptosis using 1H magnetic resonance spectroscopy. *World J Gastroenterol* **18**, 1130-1136

92. Zhang, L., Zhao, X., Ouyang, H., Wang, S., and Zhou, C. (2016) Diagnostic value of 3.0T (1)H MRS with choline-containing compounds ratio (CCC) in primary malignant hepatic tumors. *Cancer Imaging* **16**, 25
93. Dunn, W. B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., Brown, M., Knowles, J. D., Halsall, A., Haselden, J. N., Nicholls, A. W., Wilson, I. D., Kell, D. B., Goodacre, R., and Human Serum Metabolome, C. (2011) Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat Protoc* **6**, 1060-1083
94. Milner, J. J., Rebeles, J., Dhungana, S., Stewart, D. A., Sumner, S. C., Meyers, M. H., Mancuso, P., and Beck, M. A. (2015) Obesity Increases Mortality and Modulates the Lung Metabolome during Pandemic H1N1 Influenza Virus Infection in Mice. *J Immunol* **194**, 4846-4859
95. Dhungana, S., Carlson, J. E., Pathmasiri, W., McRitchie, S., Davis, M., Sumner, S., and Appt, S. E. (2016) Impact of a western diet on the ovarian and serum metabolome. *Maturitas* **92**, 134-142
96. Kind, T., Wohlgemuth, G., Lee, D. Y., Lu, Y., Palazoglu, M., Shahbaz, S., and Fiehn, O. (2009) FiehnLib: mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry. *Anal Chem* **81**, 10038-10048
97. Fiehn, O., Garvey, W. T., Newman, J. W., Lok, K. H., Hoppel, C. L., and Adams, S. H. (2010) Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. *PLoS One* **5**, e15234
98. Want, E. J., Wilson, I. D., Gika, H., Theodoridis, G., Plumb, R. S., Shockcor, J., Holmes, E., and Nicholson, J. K. (2010) Global metabolic profiling procedures for urine using UPLC-MS. *Nat Protoc* **5**, 1005-1018
99. Almasy, L., and Blangero, J. (1998) Multipoint quantitative-trait linkage analysis in general pedigrees. *Am J Hum Genet* **62**, 1198-1211
100. Bejjani, B. A., and Shaffer, L. G. (2006) Application of array-based comparative genomic hybridization to clinical diagnostics. *The Journal of molecular diagnostics : JMD* **8**, 528-533
101. Harrell, F. J. (2015) *Regression Modeling Strategies*, Springer International, Switzerland
102. Weng, L., Macciardi, F., Subramanian, A., Guffanti, G., Potkin, S. G., Yu, Z., and Xie, X. (2011) SNP-based pathway enrichment analysis for genome-wide association studies. *BMC Bioinformatics* **12**, 99

15 APPENDIX

Figure 1



Repeat the above for 50% Cho (275 mg/d) and 100% Cho (550 mg/d). Arms performed in random order with minimum 2 week washout in between.

Figure 1 outlines what each 2 week intervention period is comprised of. This will be completed 3 times per subject.

Figure 2

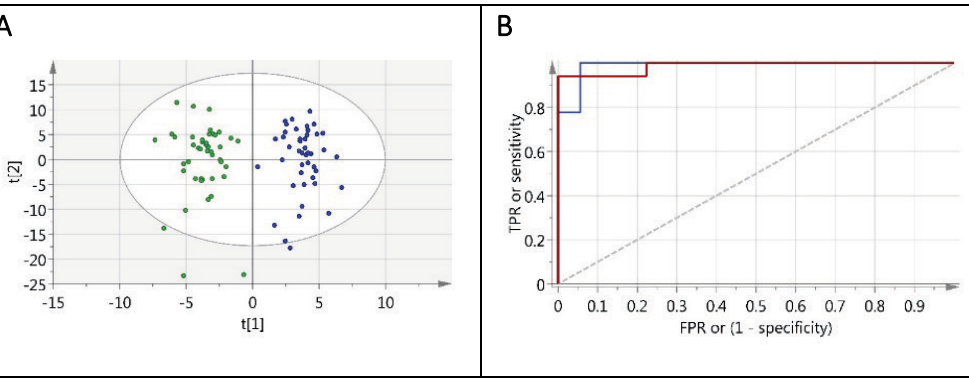


Figure 2. OPLS-DA successfully separates and predicts samples taken at baseline or after choline deficient diet. a) The scatter plot of OPLS-DA shows the complete separation between baseline samples (green) and samples taken after choline deficient diet (blue). R2Y=0.891, Q2Y=0.768 which indicates very good model fit and prediction ability. b) ROC plot with area under curve = 98.77% for both baseline (blue) and depletion (red), which indicates very high sensitivity and specificity of the model in predicting choline status. Modified from (10).

Figure 3

Pathway	Metabolite	Platform[2]	Fatty liver		Muscle dysfunction		No dysfunction	
			D/B[1]	R/D[1]	D/B[1]	R/D[1]	D/B[1]	R/D[1]
Choline metabolism	Choline	T-LC/MS	0.67	1.50	0.66	1.25	0.71	NA
	Sarcosine	T-GC/MS	0.47	2.00	0.60	1.08	0.56	NA
	Dimethylglycine	T-GC/MS	0.51	1.81	0.55	1.41	0.59	NA
	Betaine	T-LC/MS	0.45	1.66	0.53	1.07	0.47	NA
	Methionine	T-GC/MS	0.86	1.10	0.93	1.01	0.96	NA
	Phosphatidylcholine (total)	T-LC/MS	0.89	1.08	0.90	1.06	0.97	NA
	Homocysteine	T-GC/MS	1.16	0.97	1.19	0.99	1.27	NA
Carnitine metabolism	Carnitine	N-LC/MS+	1.41	0.84	1.23	1.02	1.42	NA
	Butyrylcarnitine	N-LC/MS+	1.23	0.89	1.03	1.13	1.30	NA
	Isobutyrylcarnitine	N-LC/MS+	1.09	1.12	0.88	1.44	1.41	NA
	Isovaleryl-L-carnitine	N-LC/MS+	0.75	1.36	1.01	0.88	0.87	NA
	Propionylcarnitine	N-LC/MS+	1.02	0.99	1.38	1.38	1.78	NA
Renal transporter	Hippurate	N-LC/MS+	0.53	1.88	0.93	0.72	0.75	NA
	3-indolepropionate	N-LC/MS+	0.74	1.03	1.08	0.62	0.50	NA
	CMPF	N-LC/MS-	0.46	0.86	0.57	0.74	0.32	NA
	Creatinine	N-LC/MS+	1.00	0.95	1.00	1.00	1.12	NA
	Myo-inositol	N-GC/MS	0.70	1.22	0.85	1.13	0.76	NA
Keto acid metabolism	Pyridoxate	N-LC/MS-	0.71	1.18	0.93	1.09	0.82	NA
	Erythronate	N-GC/MS	1.11	0.90	1.21	0.97	1.24	NA
Cell death	Urate	T-Clin Lab	1.19	0.90	1.39	0.92	1.22	NA
	Pseudouridine	N-GC/MS	1.17	0.92	1.28	0.98	1.20	NA
Amino acid metabolism	Glutamine	N-LC/MS+	1.07	0.87	1.06	0.98	1.12	NA
	Pyroglutamate	N-LC/MS+	1.29	1.06	1.08	1.03	1.36	NA
	Valine	N-LC/MS+	1.03	1.01	0.97	1.06	1.13	NA
	Glutamylvaline	N-LC/MS+	1.19	0.91	1.37	1.20	1.47	NA
	Glycine	N-GC/MS	1.12	0.89	1.09	0.98	1.09	NA
	Leucine	N-LC/MS+	1.01	1.03	0.87	1.45	1.25	NA
	Trans-4-hydroxyproline	N-GC/MS	1.01	1.35	0.70	0.89	1.18	NA
Diet	Stachydrine	N-LC/MS+	0.06	5.39	0.10	0.86	0.03	NA
Fatty liver	Ratio of liver fat to spleen fat	MRS	1.55	0.72	1.18	0.82	1.12	NA
Unknown	X - 02249	N-LC/MS-	0.63	1.60	0.57	0.98	0.67	NA
	X - 09789	N-LC/MS-	0.30	2.98	0.23	1.71	0.32	NA
	X - 11315	N-LC/MS+	0.82	0.95	1.03	0.92	0.84	NA
	X - 11787	N-LC/MS+	1.46	0.84	1.39	1.19	1.74	NA
	X - 11849	N-LC/MS-	1.50	1.28	5.76	1.37	6.00	NA
	X - 11876	N-LC/MS-	1.88	0.69	1.45	1.25	1.74	NA

Fig. 3. Metabolites that significantly changed in intensity after Cho depletion. Human subjects were fed diets with Cho, then deprived of Cho, and then repleted with Cho. Some of the subjects developed organ dysfunction (liver or muscle) when deprived of Cho, others did not (No dysfunction). Plasma samples were collected at the end of each diet period and analyzed by targeted and nontargeted biochemical assays. Fold change between depletion and baseline (D/B) and between repletion and depletion (R/D) are shown, with significant changes (FDR adjusted $P<0.05$) underscored. Fold change that indicates an increase by Cho depletion or repletion is shaded red; change that indicates a decrease is shaded green. NA, not performed. From (10).

Figure 4

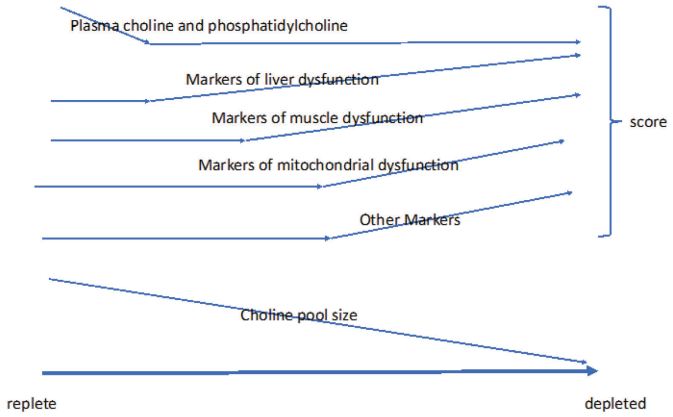


Figure 4: Cho status score correlates with Cho pool size. We propose that as Cho pools become depleted, a decrease in concentrations of plasma Cho and PtdCho occurs early. As Cho pools decrease further, other markers of altered 1-carbon metabolism change and cellular dysfunction due to lack of Cho starts, and various metabolite, enzyme and functional markers reflecting this dysfunction begin to rise. Though there may be many causes of dysfunction for a particular organ, the pattern of organ dysfunction combined with specific Cho metabolites provides a distinct signature for Cho depletion. A Cho status score based on this signature should correlate well with Cho pool size as measured by isotopic dilution.

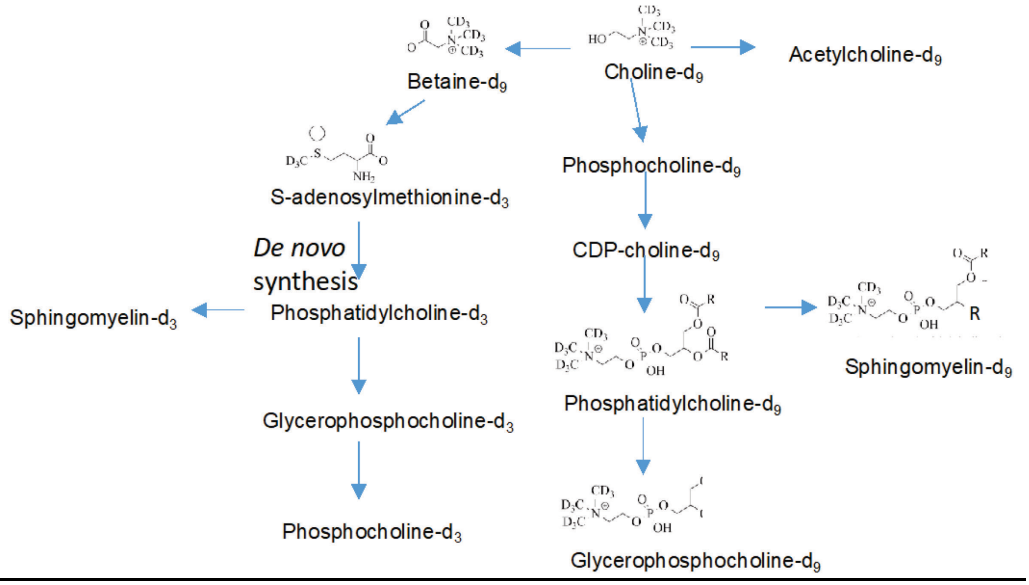


Figure 5: (modified from (89)). Distribution of deuterium from Cho chloride-(trimethyl-d9) in metabolites that appear in plasma.

Figure 6: Abnormal lab results – Action Protocol

CK level ***Normal range*** Males – 24-204 U/L Females – 24-173 U/L	***Real change*** is considered ≥ 5X upper range of normal – level required to be considered a real change for statistical outcome calculations	***Monitoring level*** - ≥50X upper range of normal. This level indicates that additional monitoring should be performed to assess clinical relevance.	***Stopping level*** (STOP) – ≥125X upper range normal. This level requires stopping subject's continued participation in the study.
	No need for IRB – AE reporting	Report to IRB with the description of plan of action.	Report to IRB as AE with the description of plan of action.
	Plan of action: 1.Repeat labs to include (CK/CMP) in 1 week – if still high and increasing then repeat in 24hrs (Discussed with MD plan of action) -if still high but decreasing then repeat in 2 weeks until normalization	Plan of action: 1. Repeat labs (CK/CMP) 2. Add myoglobin urine test 4. Assess eGFR (from CMP), determine if eGFR has decreased from baseline values to <60. Send ALL labs STAT. *If Myoglobin is <5mg/L and if eGFR has not declined from baseline values, then consider keeping subject in the study as long as the CK normalizes before next ARM of the study. * Recheck CK in 1 week again until normalization. * If Myoglobin in urine is >5mg/L or if eGFR has declined to <60 –stop subject's continued participation in the study and feed a diet containing 100% choline requirement until value normalizes. 5. Refer subject to their own physician for followup.	Plan of action: 1. STOP subject's participation in the study. 2. Repeat labs (CK/CMP) 3. Add GGT blood test 4. Add myoglobin urine test 5. Assess eGFR (from CMP), determine if eGFR has decreased from baseline values to <60. Send ALL labs STAT. 6. Subject to be placed on 100% choline diet. 7. Follow up labs weekly until normalization. 8. Refer subject to their own physician for followup.

AE – adverse event, CK – creatine kinase, CMP – comprehensive metabolic panel, eGFR-estimated glomerular filtration rate, GGT-gamma-glutamyl transferase

Myoglobin in urine – Normal levels are usually 0 to less than 1 mg/L.
Results between 1 and 15 mg/L are associated with vigorous exercise, myocardial infarct, mild muscle injury, and other conditions.
Patients with urine myoglobin greater than 15 mg/L are at risk of acute renal failure.
Tea color urine (visible myoglobinuria) – usually occurs when myoglobin >250 micrograms/ml (normal is < 5nanograms/ml) – which corresponds to a destruction of > 100gm of muscle tissue.
Scalco RS, et al. BMJ Open Sport Exerc Med 2016;2:e000151. Doi:10.1136/bmjsem-2016-000151

Abnormal AST/ALT levels in the ABSENCE of increased CK – protocol for action

AST/ALT results ***AST Normal range*** 0-40 IU/L ***ALT Normal range*** Males – 0-44 IU/L Females – 0-32 IU/L	***Real change*** is considered ≥1.5X upper range of normal – level required to be considered a real change for statistical outcome calculations	***Monitoring level*** is ≥2.5X upper range of normal. This level indicates that additional monitoring should be performed to assess clinical relevance.	***Stopping level*** (STOP) is ≥5X upper range normal. This level requires stopping subject's continued participation in the study.
	No need for IRB –AE reporting	Report to IRB with the description of plan of action.	Report to IRB as AE with the description of plan of action.
	Plan of action: 1.Repeat labs (CMP only) in 1 week – if still high and increasing then repeat in 24hrs (Discussed with MD plan of action/possible etiology) --if still high but decreasing then repeat in 2 weeks until normalization	Plan of action: 1. Repeat labs (CMP) 2. Add GGT blood test (if ≥2.5x upper range of normal possible indication of alcohol consumption and noncompliance with study) May consider then moving to stop subject from the study. Send ALL labs STAT 3. GGT is <2.5x the upper range of normal then consider keeping the subject in the study as long as the AST/ALT and GTT normalize before next ARM. * Recheck AST/ALT and GTT in 1 week again until normalization.	Plan of action: 1. STOP subject participation in study. 2. Repeat labs (CMP) 3. Add GGT blood test. Send ALL labs STAT. 4. Subject to return to his general normal diet. 5. Follow up labs weekly until normalization. 6. Refer subject to their own physician for followup

ALT-alanine aminotransferase, AST-Aspartate aminotransferase