Protocol Title: Intermittent Calorie Restriction, Brain Insulin Resistance, and Biomarkers of Brain Function
Abbreviated Title: Calorie Restriction and Brain Insulin Resistance
Protocol Number: 15-AG-0123
NCT Number: NCT02460783
Date of This Submission/Version: 07/27/2016

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Total requested accrual *(separately specify planned accrual for each subject group)*
(0) Patients
(40) Healthy Volunteers (We request permission to screen up to 100 potential participants, in order to allow for 60 potential screen failures/dropouts; and 40 completers)

Project Uses Ionizing Radiation: No x Yes (*attach RSC/RDRC documentation)*
Medically-indicated only
x Research-related only
Both

IND/IDE x No oYes *(attach FDA documentation)*
Drug/Device/#_____
Sponsor: _____

Durable Power of Attorney x No o Yes

Multi-institutional Project x No o Yes

Institution#1_NIA/NIH_____ FWA #_____
Date of IRB approval _____*(attach IRB documentation)*

Institution#2_____ FWA #_____

Date of IRB approval _____*(attach IRB documentation)*

Data and Safety Monitoring Board x No o Yes

Technology Transfer Agreement x No o Yes
Agreement type and number _____ Expiration Date_____

Samples are being stored o No x Yes

Flesch-Kincaid reading level of consent form: 9.7

PRECIS

Being overweight or obese can cause insulin resistance (IR), which is defined as reduced responsiveness to insulin by the cells of various tissues or organs. IR at midlife increases the risk of developing Alzheimer's disease (AD). We recently discovered novel biomarkers of brain IR (altered Tyr and Ser phosphorylated forms of insulin receptor substrate 1; IRS-1) in plasma exosomes enriched for neuronal origin. Moreover, IR is associated with AD biomarkers including deficits in resting state brain activity and cognitive performance. Calorie restriction is defined as consuming fewer calories than what is considered normal without a lack of nutrients. A certain type of calorie restriction, in which one consumes 500-600 calories a day for two consecutive days, followed by non-restricted eating for 5 days (5-2 CR), has been shown to lower peripheral insulin resistance effectively. Effects of CR and lowering peripheral IR on brain IR and cognition are unknown. The goal of this exploratory pilot study is to provide proof-of-concept that 5-2 CR at midlife can reverse brain IR, lower peripheral IR, improve cognitive performance, and increase brain activation at rest and during tasks. Specifically, we will study the effects of 8 weeks of 5-2 CR versus a control dietary intervention on brain and peripheral IR, memory and executive function, resting state default mode network activity, brain metabolism, and AD biomarkers. Forty overweight to obese women and men (between 55 and 70 years of age) will be randomized 1:1 into 5-2 CR and control groups. In the 5-2 CR group, participants will be offered "healthy living" dietary counseling at baseline, which they will be instructed to implement for five days/week. For each of the other two consecutive days/week, they will consume two shakes (Boost **®,** CWI Medical), providing a total of 480 Kcal/day. In the control group, participants will be offered "healthy living" dietary counseling at baseline, which they will be asked to implement for every day of the week. Participants will undergo screening including a history and physical examination, calculation of Body Mass Index (BMI, which must be ≥ 27) and a blood draw for insulin and glucose to determine whether they have insulin resistance. If participants meet eligibility criteria, they will continue with a baseline visit involving anthropometric measures, questionnaires, tests of cognitive function, brain MRI, blood draws for plasma and peripheral blood mononuclear cells, and lumbar puncture for cerebrospinal fluid biomarkers of AD. After 8 weeks, we will collect the same measures. To assess and reinforce compliance with their respective diet, participants will come into the clinic every 2 weeks to discuss compliance, measure their body weight and perform blood draws for measurement of ketones to objectively confirm energy restriction for the 5-2 CR group. We will also contact participants every week to further ensure compliance. To assess the effects of the diets on physical activity, participants will be asked to wear an accelerometer for 96 hours before and after they are on the diet.

List of Abbreviations

Abbreviation	Definition
5-2 CR	5-2 Calorie Restriction
$A\beta_{1-42}$	Amyloid-beta (1-42) peptide
AD	Alzheimer's disease
AE	Adverse Event
AGES	Advanced Glycation End Products
AL	Ad libitum
ALT	Alanine Aminotransferase
AOPP	Advanced Oxidation Protein Products
AST	Aspartate transaminase
ATP10A	aminophospholipid translocase
BDNF	Brain Derived Neurotropic Factor
BMI	Body Mass Index
BRC	Biomedical Research Center
CA3	Cornus Ammoni 3
CBC	Complete blood count
CMP	Complete metabolic panel
CSF	Cerebral Spinal Fluid
CVLT	California Verbal Learning Test
DMN	Default Mode Network
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
fMRI	Functional Magnetic Resonance Imaging
FWA	Federal Wide Assurance
GABA	gamma-Aminobutyric acid
GIP	Gastric Inhibitory Peptide
GLP-1	Glucagon Like Peptide-1
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIPAA	Health Insurance Portability and Accountability
HEENT	Head, eyes, ears, nose, throat
HIV	Human immunodeficiency virus
HOMA-IR	Homeostasis Model Assessment-Insulin Resistance
IL-6, 8, 12, 23	Interleukin-6, 6, 12, 23
INR	International normalized ratio
IR	Insulin Resistance
IRB	Institutional Review Board
IV	Intravenous
Kg	Kilogram
LP	Lumbar Puncture
mL	Milliliter

MMSE	**Mini Mental State Exam**
MMT	**Mixed Meal Test**
mRNA	**Messenger RNA**
MRS	**Magnetic Resonance Spectroscopy**
NAA	**N-Acetyl Aspartate**
NIA	**National Institute on Aging**
OGTT	**Oral Glucose Tolerance Test**
PANAS	**Positive and Negative Affect Schedule**
PBMC	**Periperial Blood Mononuclear cells**
pcASL	**Pseudo-continuous arterial spin labeling**
PSA	**Public service announcement**
PT	**Prothrombin time**
PTT	**Partial thromboplastin time**
QA	**Quality Assurance**
SAT	**Subcutaneous adipose tissue**
S_i	**Insulin Sensitivity**
SOP	**Standard Operating Procedure**
TNF-α	**Tumor Necrosis Factor**
TSH	**Thyroid stimulating hormone**
UP	**Unanticipated Problem**
vmPFC	**Ventromedial prefrontal cortex**
WTI	**Wilms tumor protein**

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1. Introduction

Insulin signaling dysfunction and Alzheimer's disease

Type 2 diabetes increases the risk for developing sporadic Alzheimer's disease (AD).[1] Insulin Resistance (IR) is defined broadly as reduced cellular responsiveness to insulin.[2] Peripheral IR, characterized by progressive hyperinsulinemia needed to maintain normoglycemia, may promote AD pathogenesis in the absence of hyperglycemia. For example, in the Rotterdam study, a two-fold increase in baseline insulin or in an index of IR over a 3-year period corresponded to a 40% greater likelihood of conversion to AD among participants without a history or present clinical diagnosis of type 2 diabetes.[3]

In the brain, insulin normally facilitates microvascular blood flow, glucose uptake and metabolism,[4] reduces accumulation and oligomerization of Aβ, [5-7] and inhibits phosphorylation of tau fibrils.[8] Currently, there are no in vivo measures of brain IR, but it is assumed that it accompanies peripheral IR,[9] which can easily be measured in vivo and studied. Peripheral IR has been associated with impaired glucose uptake in precuneus and prefrontal cortex[10] and dysregulated brain insulin binding. Brain IR has recently been suggested to occur in AD even in the absence of peripheral IR,[11] while AD-related pathology (Aβ-containing plaques and tau-containing tangles) and atrophy first occur in brain areas with a high density of insulin receptors that are also affected by early AD. These areas include the hippocampus, prefrontal cortex, cingulate cortices, and posteriomedial areas in parietal lobe such as the precuneus.[12-14] Furthermore, peripheral IR, in rhesus macaques[15,16] and humans,[17-19] predicts neural atrophy in these regions. Finally, chronic insulin administration in rodents prevents atrophy in CA3 and neocortex in streptozotocin-induced diabetes.[20] Thus, IR affects several physiological processes that are involved in neurodegeneration and AD etiopathogenesis.

In terms of epidemiology, IR increases with age, lack of exercise, high calorie diets, and higher visceral adiposity.[12,21,22] IR is present in many middle-aged[23] and aged[24,25] adults with a BMI above 27.[26] Specifically, higher visceral adiposity due to being overweight or obese corresponds to higher IR in 25-40% of late middle-aged to geriatric Caucasian (Wilson and Kannel, 2002) and Asian[27] populations. Overweight to obese participants (BMI ≥ 27) aged 55-70 years have insulin sensitivity values of approximately 7.6 $min^{-1}/(\mu U\ mL^{-1}) \times 10^{-4}$.[24,25] By extension, older overweight adults are at risk for developing AD[12] and may show deficits in memory and executive function due to the effects of IR on the brain, as well as physiological changes associated with higher IR.

CR improves insulin signaling and slows neurodegeneration in animals

Most studies find that a restriction in caloric intake, without nutrient deficiency, extends lifespan and maintains optimal insulin signaling in mammalian species including rodents and non-human primates when the control animals are fed ad libitum and so develop age-related insulin resistance; CR eliminates aged non-human primates reduces or

eliminates the development of glucoregulatory impairment and cardiovascular disease.[28-30] An important exception is recent work by Mattison and colleagues,[31] who found that CR does not extend lifespan in the NIA CR rhesus macaque cohort, in which the monkeys in the control diet group were not overfed and not obese. Interestingly, monkeys who began CR in middle age (13-17 years) had significantly less glucoregulatory dysfunction versus controls, but not monkeys who began CR as juveniles. This NIA study underscores the importance of studying CR effects on middle age. Furthermore, long-term 30% CR relative to baseline intake increased insulin sensitivity in the Wisconsin National Primate Research Center monkey cohort, which began CR exclusively in middle age. Regarding the brain, this increased sensitivity was associated with improved cognition relative to controls, as well as more gray matter volume in hippocampus, prefrontal cortices, and other areas affected by early AD.[16]

Recent findings from animal studies suggest that alternate day fasting can have similar beneficial effects, even with relatively modest 10-25% reductions in overall calorie intake.[32-34] The brain effects of CR may be due to several biological mechanisms impacted by improved insulin signaling, such as: 1) increased brain-derived neurotrophic factor (BDNF), which is significantly lower after diet-induced IR in mice; 2) decreased pro-inflammatory cytokine levels; 3) decreased production of reactive oxygen species (ROS) that damage macromolecules; and 4) improved energy metabolism manifested by increased insulin sensitivity and changes in leptin, adiponectin, and other hormones.[32,33,35-38] We found that an alternate day CR regimen over 8 weeks, in overweight asthma patients, reduced peripheral IR and plasma inflammatory markers.[39]

A study comparing continuous restriction and 5-2 CR in overweight women, defined as 2 consecutive days of consuming 500-600 kilocalories followed by non-restricted eating for 5 days, indicated that both regimens produced comparable effects to alternate day restriction.[40] Both continuous CR and 5-2 CR were tailored to result in a net 20% energy restriction. Critically, 5-2 CR led to significantly higher insulin sensitivity relative to continuous restriction and was easier for participants to maintain. We previously showed that 8 weeks of alternate day CR induces multiple effects of interest,[39] such as decreased weight (~8% of baseline body weight), decreased TNF-α (Figure 1), and decreased levels of ROS-induced 8-isoprostane, protein carbonyl, and nitrotyrosine (Figure 2).

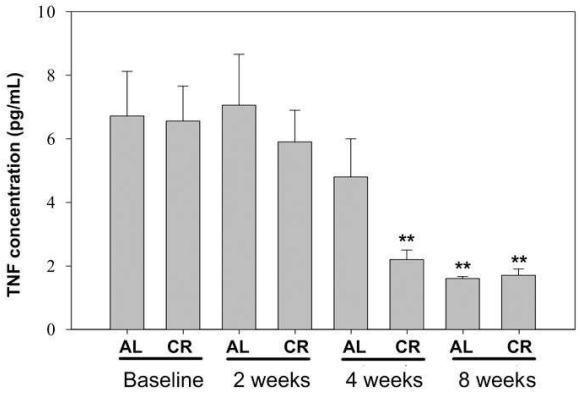

Figure 1. The effect of alternate day calorie restriction on serum TNF-α. By the 8-week time point, concentrations were greatly reduced regardless of assessment on ad libitum (AL) or restricted (CR) days.

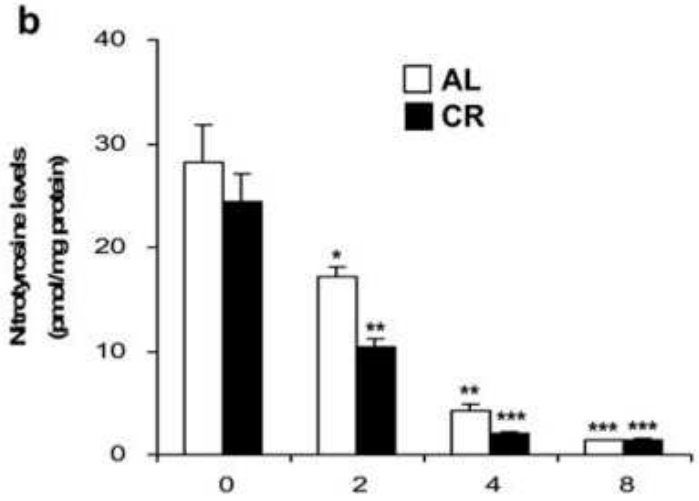

Figure 2. The effect of alternate day calorie restriction on serum levels of nitrotyrosine. By the 8-week time point levels decreased regardless of assessment on ad libitum (AL) or restricted (CR) days.

Study aims and overview

This study is a proof-of-concept exploratory pilot study, which will generate several kinds of preliminary data to guide further studies in the field. Using a randomized clinical trial design, we will compare the effects of 5-2 CR and a control diet. We will compare various outcome measures between groups, but also to baseline on a within-groups basis.

The following main outcomes will be assessed: 1) The ratio of Tyr and Ser phosphorylated forms (p-panY and pSer312) of insulin receptor substrate 1 (IRS-1) in plasma exosomes enriched for neuronal origin, which is a novel biomarker of brain IR (primary) (44); 2) peripheral insulin sensitivity derived from a mixed meal tolerance test;

3) brain fMRI activation during presentation of appetitive "junk" foods versus non-appetitive foods; 4) brain fMRI activation during rest; 5) brain metabolism measured by magnetic resonance spectroscopy (MRS); and 6) cognitive performance on memory and executive function tasks. Due to the exploratory nature of this study, we will also collect many secondary outcomes that inform on the relevant mechanisms for our primary outcome of interest, or are biomarkers of AD. Secondary measures include but are not limited to anthropometric measures of obesity (body weight, waist circumference, BMI, and sagittal diameter, which reflects visceral fat particularly well[41]), physical activity measured by monitoring devices, blood and CSF biomarkers relevant to AD, and other parameters detailed below.

Study population: We will study women and men aged 55-70 years with BMI ≥ 27 and confirmed peripheral IR at baseline. Peripheral IR is mostly present in middle-aged[23] and aged[24,25] adults with a BMI above 27, even in the absence of type 2 diabetes. Specifically, higher visceral adiposity due to being overweight or obese corresponds to higher IR in 25-40% of late middle-aged to geriatric Caucasian[42] and Asian[27] populations. Overweight to obese participants (BMI ≥ 27) aged 55-70 years have insulin sensitivity values of approximately 7.6 $min^{-1}/(\mu U\ mL^{-1}) \times 10^{-4}$.[24,25] We will study participants aged 55-70 years to examine brain IR and other AD-associated biomarkers and measures during late middle-age/early old age, which temporally correspond to the preclinical stage of AD. Late middle-life/early old age overweight adults are at higher risk for developing AD[12] and may show mild deficits in executive function due to the effects of IR on the brain, as well as physiological changes associated with higher IR.

We will exclude subjects with stroke and other neurological diseases of the central nervous system that may impact structural integrity, metabolism and function in affected brain regions and confound the results of MRI, MRS, as well as CSF and exosomal biomarkers. We will exclude subjects that abuse illicit drugs since these may impact brain metabolism and function and may confound the effects of the two diets on brain biomarkers. We will exclude subjects with clinically significant endocrine disorders such as hypothyroidism, Cushing's Syndrome, etc. can alter peripheral and brain metabolism, body weight, and appetite and may confound the effects of the two diets on relevant outcome measures. We will exclude subjects with history of anorexia nervosa or bulimia, since these disorders may complicate compliance with the two diets or raise concerns for the safety of implementing a new dietary pattern. In addition, subjects with Type 2 diabetes, taking anti-diabetic medications or with history of hypoglycemia may not be able to safely engage in 5-2 CR, due to the possibility of developing symptomatic hypoglycemia. We will exclude subjects with chronic HIV, HCV, or HBV infections, since these are associated with metabolic and endocrine abnormalities and may confound the effects of the two diets. We will exclude subjects with low hematocrit, since it might suggest an undiagnosed underlying condition and limit our ability to obtain blood samples at various times during the study. We will exclude subjects with elevated AST and/or ALT > 1.5 times the upper normal limit suggesting significant liver disease, which may have an impact on the effects of the two

diets. We will exclude subjects receiving systemic corticosteroids that can induce IR and alter multiple aspects of metabolism and alter the effects of the two diets. Given the profound effects of APOE genotype on AD biofluid and imaging biomarkers and AD risk in midlife,[43-46] it is customary for studies of AD biomarkers and cognition in preclinical cohorts, to control for APOE-associated variance as a covariate.[19,47] In addition, there is evidence for a complex interaction of APOE, sex and IR regarding their effects in AD.[48] Therefore, APOE genotyping will be performed for all participants.

Brain IR (primary outcome): We have recently developed a methodology for exosome isolation from stored plasma samples and enrichment for neuronal-origin based on immunoreactivity towards neuronal cell adhesion molecules (NCAM and/or L1)[49] expressed on the surface of a sub-population of these exosomes. First, we implemented this methodology in a cross-sectional study measuring AD pathogenic proteins ($A\beta_{42}$, total Tau, p-T181-Tau, p-S396-Tau) in L1/NCAM expressing exosomes isolated from blood samples of 54 AD patients and 54 cognitively normal age- and sex- matched controls. Exosomal $A\beta_{42}$, p-T181-Tau, and p-S396-Tau were significantly higher in AD patients compared to controls achieving near-perfect discrimination between patients and controls with an ROC AUC ~ 1.[50] Subsequently, we performed a study looking for biomarkers of brain IR in 26 patients with AD, 26 cognitively normal age- and sex-matched controls without diabetes and 20 age- and sex-matched controls with diabetes. We measured two phosphorylated forms of IRS-1 that differ in terms of downstream insulin signaling, one relatively active (p-panY-IRS-1) and one relatively inactive (p-S312-IRS-1); the ratio of the two forms in a tissue is a measure of insulin resistance. We found that AD patients had significantly lower p-panY-IRS-1 and higher p-S312-IRS-1 compared to both diabetic and non-diabetic controls, while diabetic controls had intermediate values between AD patients and non-diabetic controls.[51] These findings suggest the presence of brain selective IR in patients with AD and, to a lesser extent, in individuals with diabetes. In addition, in a longitudinal series of 24 patients, with samples 1 - 10 years prior to diagnosis of AD or amnestic MCI and at the time of diagnosis, and 24 age- and sex- matched controls, we found that p-panY-IRS-1, p-S312-IRS-1, and their ratio, are abnormal 1-10 years prior to AD/aMCI diagnosis (**Figure 3**). Therefore, brain IR is present at the preclinical stage of AD and these brain IR exosomal biomarkers may be used to predict AD at the preclinical stage as early as 10 years prior to diagnosis.[51]

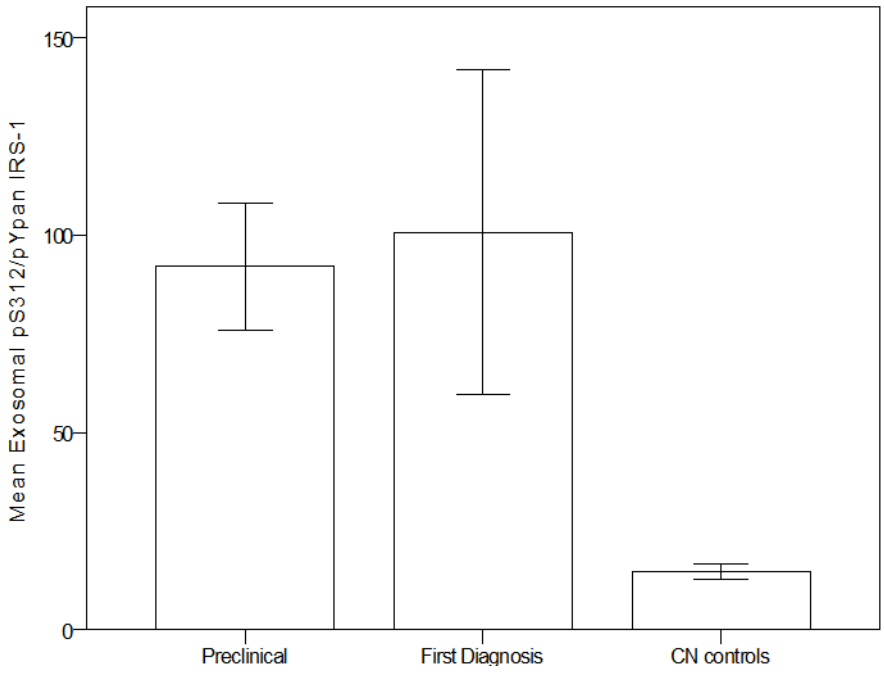

Figure 3. Mean exosomal pS312-IRS-1/p-panY-IRS-1 in neuronal enriched exosomes in 22 patients with AD at the time of first diagnosis and 1-10 years preclinically, as well as in cognitively normal age and sex-matched controls.[51]

In the present study, we hypothesize that brain IR biomarkers will respond to CR and that the 5-2 CR group will show increased p-panY-IRS-1, decreased p-S963-IRS-1, and increased ratio, compared to baseline and to controls.

Peripheral Insulin Sensitivity: HOMA-IR and peripheral insulin sensitivity (S_I) will be used to assess peripheral IR. HOMA-IR is calculated from fasting glucose and insulin. HOMA-IR strongly corresponds to insulin sensitivity determined from euglycemic or hyperinsulinemic clamp.[52] HOMA-IR will primarily be used to screen participants to see if they have peripheral insulin resistance (see below). S_I will be derived from a mixed meal tolerance test. The typical S_I measurement is derived from a 75 g oral glucose tolerance test (OGTT) and shows high correspondence with the "gold standard" euglycemic-hyperinsulinemic clamp method.[53] The mixed meal has the advantages of enabling the assessment of incretins such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP), and acting as a more natural stimulus of islet beta cells that secrete insulin.[54] Lower S_I corresponds to higher IR. For defining a cut-off inclusion criterion for this study, we consider an HOMA-IR value greater than 1.8 as evidence of IR in overweight, late middle-aged to aged participants without type 2 diabetes,[52] whereas median HOMA-IR was 2.89 among participants with type 2 diabetes in the Matthews et al. study. We hypothesize that the 5-2 CR group will show increased S_I compared to baseline and to controls.

In addition, we will assess the effects of the two diets on these related secondary

measures: anthropometric measures of obesity, such as body weight, BMI, waist circumference, sagittal diameter[41]; and clinical laboratory measures reflecting peripheral metabolism and glucoregulatory control, such as plasma lipids and HbA1c.

Neuroimaging - Brain function: We will use fMRI to probe brain activation during a food rating task concerning appetitive "junk" foods versus non-appetitive foods. Moderate CR in humans typically does not affect appetite.[39,55] Instead, restriction decreases food cravings for and consumption frequency of energy-dense foods during CR[55,56] and several months after resumption of regular diet.[29] The neural correlates of this change in behavioral responsiveness have not been investigated. We hypothesize that viewing images of energy-dense foods activates meso-cortico-limbic regions involved in reward, like striatum, prefrontal cortex, insula, and ventral tegmental area[57]; deactivation of these regions has been seen with voluntary cognitive reappraisal.[58] We hypothesize that participants on 8 weeks of 5-2 CR will show decreased liking ratings for energy-dense foods and corresponding decreased fMRI activation compared to controls. The fMRI paradigm we will use was developed by Antonio Rangel at the California Institute of Technology and has been used in multiple published studies (such as Harris et al., Temporally dissociable mechanisms of self-control: early attentional filtering versus late value modulation, J Neuroscience 2013; Hare et al., Focusing attention on the health aspects of foods changes value signals in vmPFC and improves dietary choice, J Neuroscience 2011; etc) to probe perceived value and salience of food stimuli that drive dietary choices, as well as mechanisms of self control.

We will also assess the effects of the two diets on intrinsic functional connectivity of the default mode network (DMN), a major and one of the earliest targets of AD pathology.[59] IR is related to decreased glucose uptake in the precuneus, the key node of the DMN, in cognitively normal older adults with pre-diabetes.[10] Conversely, acute intranasal insulin administration in participants with type 2 diabetes increases DMN activity, suggesting that insulin modulates DMN neural metabolism and firing. The levels of CSF AD biomarkers ($A\beta_{1\text{-}42}$, total tau, p_{181}Tau) in cognitively intact adults are also associated with changes in DMN connectivity.[60] We hypothesize that participants on 8 weeks of 5-2 CR will improve DMN connectivity compared to controls eating normally for 8 weeks.

Finally, as a secondary measure, we will assess the effects of the two diets on brain activity underlying executive function performance. This task is exploratory because no existing studies examine the effect of IR or IR-targeted therapies on Stroop-induced brain activity. To this end, we will perform fMRI during a Stroop Color-Word task, a standard test of executive function.[61] The Stroop effect[62] in this paradigm consists of reading a color word (e.g. the word "red") and making a selection of either the color word or the font color. The font color can be either congruent (the word "red" is colored red) or incongruent (e.g. the word "red" is colored yellow) to the color word. Inhibitory control is needed to disregard the incongruent color word and select the font color. This Stroop effect produces robust activation in prefrontal cortex,[61] a target area of interest, since its activity is negatively associated with IR.[19] We hypothesize that participants on 8

weeks of 5-2 CR will show improved performance on Stroop in association with decreased activation (suggesting lesser effort) compared to controls.

Neuroimaging - Brain metabolism: To assess the effects of the two diets on brain energy metabolism we will use magnetic resonance spectroscopy (MRS), a technique used to measure chemical concentrations in the brain. Specifically, within regions of interest, we will measure concentrations of N-Acetyl Aspartate (NAA), glucose, and lactate normalized to creatine to assess: 1) neural density (NAA), 2) glucose metabolism, reflected by brain glucose concentration reflecting the balance between glucose delivery through the blood brain barrier and consumption, and 3) metabolic reliance on glycolysis (lactate). These metabolites will be measured in the hippocampus and prefrontal cortex, areas preferentially vulnerable to AD pathology that show impaired glucose uptake as IR increases.[10] We will also derive concentrations of additional metabolites, such as neurotransmitters like glutamate and GABA,[63] which are decreased in AD as a result of impaired brain metabolism.[64] We hypothesize that, by improving brain metabolism, participants on 8 weeks of 5-2 CR will show increased concentrations of glutamate and GABA and decreased glucose (due to increased utilization) and lactate (due to decreased production) compared to controls.

Cognition and affect: Moderate CR has profoundly beneficial effects on memory, such as the California Verbal Learning Test (CVLT), in sedentary aged adults at risk for AD,[65] an effect that may be associated with insulin resistance. Consequently, we will assess verbal memory for unstructured information with the CVLT, as well as structured information with Logical Memory I and II; these tests are common neuropsychological measures. Memory recall generally begins to decline starting in the fifth decade of life,[66] while peripheral IR may be related to subclinical declines in memory performance within the range of normal variability, but also subclinical hippocampal microstructural changes.[67,68] In addition, higher IR in older adults predicts executive function deficits in Trails A and B [69], as well as the Symbol Digit Modalities Test and Stroop Color-Naming task.[70] We intend to measure executive function using the newly developed computerized assessment tool called "Examiner" (http://examiner.ucsf.edu/). This software reliably and validly assesses domains of executive function and is intended to become the "gold standard" for clinical trials. We hypothesize that participants on 8 weeks of 5-2 CR will show better performance on executive function and memory tasks compared to baseline and controls, within the normal range.

Regarding secondary measures specific to cognition, we screen out subjects with subclinical cognitive dysfunction by using the Mini Mental State Exam (MMSE) and an inclusive cut-off of 26 out of 30. Given the possibility of psychological stress associated with the two diets, we will gauge mood using the Positive and Negative Affect Schedule, or PANAS[71] and the general quality of life of participants using the RAND Health 36-Item Short Form Health Survey (RAND SF-36) as previously described.[40] These questionnaires use Likert scales that assess mood, level of energy, difficulty concentrating, headaches, feeling cold, constipation, specific negative emotions (depression, anxiety, and

irritability), hunger and preoccupation with food. We hypothesize that participants on 8 weeks of 5-2 CR will show improved positive over negative affect and general quality of life compared to controls.

AD biofluid biomarkers: To assess the effect of 5-2 CR on AD pathogenesis, we will assess additional plasma exosomal protein and mRNA exosomal biomarkers of AD.[50] In addition, we will assess traditional AD biomarkers in CSF: $A\beta_{1-42}$, total tau, p_{181}Tau, and soluble APP fragments. Extracellular Aβ oligomerization resulting in decreased CSF $A\beta_{1-42}$ is thought to begin during the preclinical phase of AD, perhaps decades before clinical disease onset. Even mild glucoregulatory dysfunction in older adults diagnosed with AD was associated with greater Aβ burden.[72] By extension, IR in late middle age may also be related to Aβ burden. Intracellular tau deposition (and increased CSF concentration) increases during preclinical and clinical disease, in concert with neurodegeneration and cognitive deficits.[73] For tau protein, insulin resistance dysregulates phosphorylation of the β subunit.[74] We hypothesize that participants on 8 weeks of 5-2 CR may show increased CSF $A\beta_{1-42}$, no change in total tau, but decreased p_{181}Tau compared to controls.

In addition, we also intend to assess a number of less well-established or novel AD biomarkers, which reflect various aspects of AD etiopathogenesis. These include chemicals in CSF, plasma, serum and whole blood, such as neurotrophic factors, cytokines and inflammatory factors and cells, various peptides, proteins, lipids, and RNAs. In terms of neurotrophic factors, BDNF is an important pleiotropic factor that promotes synaptic plasticity and protects against the cytotoxic effects of Aβ, where AD patients illustrate worse cognitive performance in association with lower CSF BDNF levels.[75] Pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and IL-12 are released by microglia and predict elevated Aβ levels in transgenic[76,77] and wild-type rodents,[78] as well as reduced synaptic plasticity[79] and other aspects of neurodegeneration. Increased production of reactive oxygen species mediates neural atrophy by damaging mitochondrial DNA and degrading macromolecules such as proteins and sugars,[80] leading to advanced gylcation end products (AGEs) and advanced oxidation protein products (AOPP) formation and further cellular damage. Importantly, a previous 8-week intermittent CR study[39] showed that markers of inflammation and oxidative stress in blood were significantly reduced (Figures 1 and 2).

Finally, we will measure proteins and RNAs implicated in AD pathogenesis and insulin signaling in neuronal-origin plasma exosomes. These include, but are not limited to $A\beta_{1-42}$, total tau, p_{181}Tau, p-S396-Tau, p-S312-IRS-1, p-panY-IRS-1, cathepsin-D, ubiquitin, LAMP2, HSP70, and CD81.[81]

Physical activity: To assess baseline activity-associated variance in other outcomes, as well as the effects of the two diets on physical activity, we will use accelerometry. We hypothesize that the 5-2 CR group will not show any differences in physical activity

compared to baseline and the control group. Nonetheless, this analysis is expected in many manuscripts on dietary interventions, in order to rule out effects attributed to differences in physical activity.

Gene expression: To understand the molecular mechanisms of the benefits of calorie restriction, several studies have utilized gene expression changes using microarray in mammals. In a recent meta-analysis of publically available CR microarray data from mouse, rat and pigs in gene expression changes were analyzed across various tissues including liver, kidney, heart, mammary glands, muscle, lung, brain, and adipose.[82] There were 174 genes that were significantly over- or under-expressed, some of which were tissue-specific but others were found in more than 3 tissue types. The functional categories that were enriched included genes involved in growth hormone signaling, lipid metabolism, immune response, retinol metabolism, copper ion detoxification, and circadian rhythms. These results highlighted the complex patterns of changes that occur with CR that span across many domains.

A handful of studies have examined the gene expression changes that occur with caloric restriction in humans, particularly in fat cells. In a study of 36 healthy young (mean age ~36.8 + 10 years old) overweight individuals, randomized to control, 25% CR and 12.5% caloric restriction with 12.5% increase energy expenditure (CREX) the changes in expression of mitochondrial biogenesis genes in skeletal muscle cells was tested.[83] Compared to control group, subjects in CR and CREX group showed increase expression of mitochondrial genes (*PPARGC1A*, *TFAM*, *eNOS*, *SIRT1*, and *PARL*). In particular, expression of *SIRT1* was increased by 113% in CR and 61% in CREX group. In a study of 3-month CR study in 22 obese women, genome-wide expression analysis in subcutaneous adipose tissue (SAT) showed a down-regulation of 474 adipocyte genes and up-regulation of 511 macrophage-related inflammation genes.[84] In a second study, 7 low- and high- weight loss responders to a 6-month hypo-caloric diet were assessed for genome-wide expression differences SAT.[85] While no differences in gene expression were observed before the intervention, 644 genes were differentially expressed after the dietary intervention. Pathway analysis of differentially expressed genes suggests an enrichment of genes involved in angiogenesis and cerebellum function. Interestingly, up-regulation of *eNOS* observed in muscle cells was also observed in SAT. It is unclear whether there were other overlaps between SAT and skeletal muscle in response to CR. In a slightly different study design, the final study looked at gene expression differences for ~8500 genes in SAT between women who were successful and unsuccessful at weight-loss following 6-month dietary intervention on one of 4 standard diets that varied in glycemic index and macronutrient composition following an 8-week period of CR.[86] The global expression pattern was able to discriminate between responders (N=22) and non-responders (N=22) to the weight loss protocols. These studies provide promising evidence for the feasibility to detect changes in gene expression under different dietary conditions in humans.

We expect to find a unique gene expression signature in whole blood after 5-2 CR. We

anticipate that several expression changes in domains similar to mammalian studies will be observed in humans including genes that encode proteins involved in growth hormone signaling, lipid metabolism, and immune response. We will take two approaches to analyze the expression data. First expression changes in genes previously implicated in CR will be tested. These include genes involved in mitochondrial function, insulin signaling, BDNF pathways, autophagy, mTOR pathways, DNA repair, antioxidant system and cell survival. Second, an unbiased approach will be taken by analyzing genome-wide expression changes.

Methylation in Leukocytes: There are two studies that have looked at genome-wide methylation in SAT and peripheral blood mononuclear cells (PBMCs) in humans.[85,87] In the study of DNA methylation in SAT following 6-month CR, differences at 35 loci were found between the high and low weight loss responders at baseline suggesting methylation signature can be predictive of those who will respond to CR intervention. Three of these regions showed differential methylation after CR.[85] Not surprisingly, differential methylation was observed in genomic regions that contain genes linked with weight control and insulin regulation including the cholecystokinin B receptor gene. In the second study, differential methylation in PBMC was studied in 12 subjects who were either low- or high- weight loss responders to 8-weeks of caloric restriction.[87] At baseline, several CpG located in the aminophospholipid translocase (*ATP10A*) and cell surface glycoprotein 44 (*CD44*) genes differed between the two groups suggesting that methylation patterns in this region are important for weight loss in response to CR. At the end of the study, methylation differences were observed at the promoter region of Wilms tumor protein (*WT1*). Interestingly, the methylation at *ATP10A* and *WT1* significant changed during the 8-week intervention. The biological relevance of these genes with caloric restriction is unknown. One of the limitations of the study reported by Milagro and colleagues is that their analyses did not account for white blood cell differential counts. This is particularly concerning when studying weight loss since changes in leukocytes have been documented and shown to explain 10% of the variance in weight loss over 2 years.[88] Therefore, in our study, we intend to measure white blood cell and lymphocyte differential counts at the times of PBMCs acquisition. We expect to find a unique methylation signature with 5-2 CR in PBMCs. As with the expression data, we will use a two-pronged approach for the analysis of methylation data where we take a targeted candidate pathway approach and an agnostic genome-wide methylation change approach. Furthermore, our study may replicate some of the PBMC DNA methylation changes observed in CR findings reported by Milagro.[87]

Future studies: Finally, CSF and plasma samples will be saved for future studies on the effects of the two diets on metabolism (e.g. hormones of metabolism, such as incretins, glucagon, etc.), proteomic and metabolomic studies.

2. Study Objectives

Hypotheses and Specific Aims

1. To assess the effects of the two diets on exosomal p-S312-IRS-1, p-panY-IRS-1, and their ratio (brain IR);
2. To assess the effects of the two diets on other exosomal protein and mRNA biomarkers of AD;
3. To assess the effects of the two diets on S_I and HOMA-IR (peripheral IR);
4. To assess the effects of the two diets on liking ratings for calorie-rich foods and associated fMRI activation versus non-appetitive foods;
5. To assess the effects of the two diets on Stroop-associated fMRI activation;
6. To assess the effects of the two diets on intrinsic functional connectivity of the (DMN) assessed by resting fMRI;
7. To assess the effects of the two diets on glutamate, GABA, glucose and lactate in prefrontal cortex and hippocampus;
8. To assess the effects of the two diets on memory and executive function;
9. To assess the effects of the two diets on mood, level of energy, difficulty concentrating, headaches, feeling cold, constipation, negative emotions (depression, anxiety, irritability), hunger and preoccupation with food, and quality of life; To assess the effects of the two diets on clinical Labs: lipid panel, uric acid;
10. To assess the effects of the two diets on plasma ketones, such as β-hydroxybutyrate and other metabolites;
11. To assess the effects of the two diets on plasma and CSF AD biomarkers, including $A\beta_{1\text{-}42}$and p_{181}Tau;
12. To assess the effects of the two diets on plasma and CSF neurotrophic factors, such as BDNF;
13. To assess the effects of the two diets on plasma and CSF reactive oxygen species, advanced glycation end-products and advanced oxidation protein products;
14. To assess the effects of the two diets on plasma and CSF pro-inflammatory cytokines, such as IL-6, IL-8, IL-12 and IL-23;
15. To assess the effects of the two diets on anthropometric measures (weight, waist circumference, sagittal diameter);
16. To assess the effects of the two diets on physical activity as measured by accelerometry;
17. To assess the effects of the two diets on expression pattern of genes in candidate pathways;
18. To assess the effects of the two diets on the global gene expression pattern in PBMCs;
19. To assess the effects of the two diets on methylation patterns in genes of candidate CR pathways;
20. To assess the effects of the two diets on global methylation patterns in PBMCs.

2.a. Primary objectives

Primary objective is to demonstrate that 8-weeks of 5-2 CR plus “healthy living”

counseling in a group of overweight, late middle-aged to aged participants will significantly increase the ratio of plasma exosomal p-panY-IRS-1 to p-S312-IRS-1, compared to baseline and to controls who only receive “healthy living” counseling.

2.b. Secondary objectives

Secondary objectives are noted under specific aims 5-21. Collectively, we aim to demonstrate that 8-weeks of 5-2 CR plus “healthy living” counseling in a group of overweight, late middle-aged to aged participants will significantly improve peripheral insulin sensitivity, decrease fMRI activation in meso-cortico-limbic areas involved in appetitive food salience and reward pathways, increase intrinsic functional connectivity of the DMN, improve brain metabolism, AD biomarker profile and cognitive performance compared to baseline and controls who only receive “healthy living” counseling.

3. Subjects

3.a. Description of study population

The study population will consist of overweight, older (55-70 years old), men and women with evidence of IR and central obesity. The requirement for IR was set because it defines a population at increased risk for AD,[89] and IR may be responsive to brief[39] or extended[90] CR in overweight human participants. The upper and lower age limits were set because we are interested in the effects of IR and of 5-2 CR on the preclinical phase of AD, at midlife, 15-20 years prior to the onset of clinical disease.

The accrual target is 100 participants to allow for 40 participants that will be successfully enrolled and complete the study and 60 screen failures and withdrawals. Withdrawals will be replaced.

3.b. Inclusion criteria

1. BMI ≥ 27; in addition, weight ≤ 350 lbs (weight limit for MRI scanner);
2. Age of 55-70 years;
3. HOMA-IR ≥ 1.8;
4. MMSE ≥ 26

3.c. Exclusion criteria

1. History of cardiovascular disease;
2. History of clinically significant stroke or other neurological disease of the central nervous system;
3. History of substance abuse in the past 6 months or positive urine drug screen;
4. History of clinically significant endocrine disorders (common mild endocrine

disorders, such as untreated subclinical hypothyroidism with TSH < 10 mU/l or successfully treated hypothyroidism may be allowed);
5. History of eating disorders, significant GI disorders or malabsorption disorders;
6. History of type 2 diabetes; and/or use of anti-diabetes medications or insulin; and/or type 2 diabetes diagnosed during the screening visit based on fasting glucose > 125 mg/dL;
7. History of hypoglycemia; and/or a fasting glucose < 70 mg/dL during the screening visit.
8. Current use of systemic corticosteroids;
9. Positive screening tests for HIV, HCV or HBV;
10. Hematocrit less than 35% or hemoglobin less than 11 mg/dL;
11. ALT or AST > 1.5 times the upper normal limit;
12. Contraindications for MRI (pacemakers, ferrous metal implants or shrapnel in or around the head, etc.).
13. Contraindications to LP, such as Coumadin, coagulopathy (international normalized ratio, or INR > 1.5; prothrombin time (PT), partial prothrombin time (PTT) > 1.5 x upper normal limit). Aspirin 81 mg qd is allowed. Aspirin up to 325 mg qd is allowed, if withheld for 7 days prior to the LP.
14. Pregnancy or nursing.
15. Refusal to consent to genetic testing for APOE.

4. Study Design and Methods

4.a. Study overview

This study is a randomized clinical trial, where participants will be assigned into two groups. In the 5-2 CR group, participants will be offered "healthy living" dietary counseling at baseline, consisting exclusively of published literature from the CDC and USDA, which they will be instructed to implement for five days/week. In addition, they will be instructed to consume only two shakes (Boost®, CWI Medical) providing a total of 480 Kcal/day for two consecutive days/week. In the control group, participants will be offered "healthy living" dietary counseling at baseline, which they will be instructed to implement for every day of the week.

Should a participant not be able to keep their scheduled study visits due to an unforeseen circumstance, and they are on 5-2 CR, she or he may be asked to implement CR on another day, or to extend the 5-2 CR diet for an additional week. Voluntary dropouts will be replaced until we have acquired complete data from 40 participants.

4.a.1. Experimental conditions: Both groups will receive "healthy living" brochures published by the CDC and USDA at baseline. In the 5-2 CR group, participants will be encouraged to implement "healthy living" diet for five days/week, and not to intentionally overeat. For 2 consecutive days per week, participants in the 5-2 CR group will consume 480 kilocalories in the form of two meal replacement shakes (Boost®, CWI

Medical). These shakes will be supplied at the end of the Baseline Visit, as well as during the Compliance Visits every 2 weeks. This dietary regimen achieves about 20% net restriction compared to their weekly caloric intake, assuming a baseline 2000 Kcal/day intake. Participants in the 5-2 CR group will have the opportunity to taste the meal replacement shake (Boost®, CWI Medical) at the end of the Baseline Visit and choose a flavor they prefer. In the control group, participants will be encouraged to follow the "healthy living" diet every day.

Rationale for control condition: We wish to implement a low intensity form of dietary intervention as an active placebo, by providing published material on "healthy living" diet to both groups. In general, counseling has very modest effects on body weight, if any. For example, Noda and colleagues[91] examined weight loss over 8 weeks among participants who initially received 30-60 minutes of dietary counseling (n=44) vs. no counseling (n=47). Whereas the dietary counseling group showed modest weight loss (-0.6 kg) compared to baseline and weight in the no counseling group did not change (0 kg), there was no overall significant effect of Intervention Group. Six months of intensive dietary counseling, defined as weekly consultation for the first month and biweekly thereafter, led to modest weight loss (-2.5 kg)[92] – in this study, we will implement a much less intensive form of dietary counseling. Therefore, dietary counseling alone is not an effective way for achieving weight loss. By contrast, Harvie et al.[40] found that 8 weeks of 5-2 CR led to a reduction of 6.5 kg. In addition to providing an active placebo, implementing the same dietary counseling to both groups, we intend to make the 5-2 CR group's diet during non-CR days less variable and similar to that of the "Healthy living" group. Finally, by offering an intervention to control subjects, we intend to increase their motivation for completing the study. Counseling is routinely used as a control in studies implementing behavioral interventions, such as diet and exercise.[93]

Although, we do not wish to implement detailed dietary counseling besides providing subjects with published material and reviewing it with them, the study Investigators have received advice by long-time collaborator at the US Department of Agriculture's Beltsville Human Nutrition Research Center Dr. David Baer. The Center is specialized in testing novel dietary interventions and implementing comprehensive behavioral interventions to ensure compliance. Study investigators and the Clinical Research Coordinator have become acquainted with their methods and will continue to consult with them throughout this study whenever applicable. The Clinical Research Coordinator or the PI will review the informational materials with participants and provide any necessary clarifications.

4.a.2. Compliance: We will assess and reinforce diet compliance with correspondence by telephone or electronic mail on week 1, 3, 5 and 7, as well as with Compliance Visits on weeks 2, 4, and 6. To assess compliance during Compliance Visits, we will be measuring plasma β-hydroxybutyrate. Values around 2-2.5 μM/mL during CR days suggests compliance during 5-2 CR. β-hydroxybutyrate values of 0.5-1.0 μM/mL suggest non-compliance with 5-2 CR, but would be expected for non restricted days and for the

control group.[39] In addition, we will be measuring body weight. Weight loss for 5-2 CR is expected to be about 2% per week based on our previous work.[39] Therefore, a progressive decrease seen after 2 weeks of 5-2 CR and persisting in the 5-2 CR group over 8 weeks suggest compliance to 5-2 CR,[39] while a smaller decrease in body weight is expected for the control group.

In addition, we will contact participants by telephone or electronic mail (using the Secure Email & File Transfer Service: https://secureemail.nih.gov/bds/Login.do) weekly to assess not only compliance with the diets, but also mood, level of energy, difficulty concentrating, headaches, feeling cold, constipation, negative emotions (sadness/depression, anxiety, irritability), hunger and preoccupation with food.[39,40,94] For e-mail communications, we will provide participants with the web-address of the Secure Email & File Transfer Service with instructions on how to acquire login credentials.

4.b. Recruitment

Participants may be recruited through direct advertisement. Brochures and flyers will be used. Recruitment letters may be mailed to health care providers and to those participants who have requested information. We may place ads and/or public service announcements (PSA) on websites, in radio broadcasts, television, as well as newspapers, newsletters, and magazines. Direct mailing through established marketing and advertising organizations might also be used. Pre-screening over the telephone will utilize the eligibility checklist. Participants that we believe are likely to be eligible will come to the NIA-Clinical Research Unit to undergo screening.

We anticipate an accrual rate of 2-6 participants per month.

4.c. Screening

Subjects will arrive on the NIA-Clinical Research Unit after a 12-hour fast from the previous night. Informed consent will be obtained from the subject prior to any procedures. The Screening Visit will include vital signs, completion of a symptoms review questionnaire, medical history, physical examination, anthropometric measurements (weight, height), screening clinical blood tests (HIV, HBV and HCV, complete metabolic panel, including glucose, fasting insulin to calculate HOMA-IR, liver function tests, complete blood counts, PT, PTT, and INR, fasting lipid panel, uric acid, HbA_{1c}, and TSH (to assess thyroid function), screening urine drug test, urine pregnancy test (only in women who are able to become pregnant), and administration of the MMSE. The visit will last approximately 3 hours. About 28 mL of blood will be taken during the screening visit. If inclusion criteria are met, participants will participate in a Baseline Visit.

At the end of the Screening visit, eligible participants will be given an accelerometer to

wear for 96 hours. They will be given instructions on how to wear the device and to mail it back to the unit via a pre-paid envelope. To ensure more accurate estimates of physical activity, participants will keep an activity log of when they sleep, exercise, or bathe.

4.d. Study procedures

4.d.1.Chronological ordering of procedures (see Table 1 and 2)

All procedures are conducted solely for research purposes. This protocol has no relationship to other protocols. Radiation will be used only for fluoroscopy-guided Lumbar Puncture (LP) (up to twice for the duration of the study). To facilitate scheduling (e.g., if fluoroscopic LP cannot be scheduled on the same day) or satisfy a given participant's request, we may split the various procedures of the Baseline Visit over two days within a week from each other.

The study is comprised of: an initial telephone pre-screening to describe the study and inclusion/exclusion criteria to potential participants; a "Screening Visit"; randomization into one of two groups; a "Baseline Visit"; the "Outcome Visit" after 8 weeks. During the 8-week period for the 5-2 CR or control groups, participants will return every two weeks for "Compliance Visits", to assess and reinforce dietary compliance in keeping with previous protocols.[39,40,94] The Outcome Visit will take place at the conclusion of the 8-week phase. Specifically for the 5-2 CR group, the Outcome Visit will be timed during the second of the two CR days. In total, there will be 6 outpatient visits to the NIA Clinical Unit: Screening Visit, Baseline Visit, Week 2 Compliance Visit, Week 4 Compliance Visit, Week 6 Compliance Visit, and the Week 8 Outcome Visit. There will be an additional follow-up phone call or electronic mail within 3 days of completing the Week 8 Visit to assess any problems related to their final visit. Accelerometry will be implemented on two occasions for 96 hours each time: once between the screening and baseline visits and again after the Week 8 Outcome Visit. Throughout the study, additional safety visits may take place for any clinically significant. Participants will be involved in the study for approximately 8 – 11 weeks (allowing for an up to 3 week gap between Screening and Baseline Visits). Study participation will end with completion of accelerometry after the Week 8 Outcome Visit.

It is anticipated that participants in this study will occasionally miss or fail to complete an assessment or procedure, such as a completion of a rating scale or a blood draw. Omissions such as these will be considered expected events and not protocol deviations provided they are infrequent and do not include data needed to assess safety or the primary study outcome. Cumulative proportions of these missed events in the study population will be presented to the IRB annually. In addition, the rate of omissions will be monitored by the Investigators. If an individual misses more than 15% of the required assessments/procedures or if more than 15% of the participants miss completion of the same assessment or procedure, it will be considered a deviation and a deviation report

will be sent to the IRB within two weeks.

Table 1: Procedures

	Visit 1	Visit 2		Visit 3, 4, 5	Visit 6	
		Week 0		Weeks 2, 4, 6	Week 8	
	1 Day 3-hours	Day 1 7-9 hours	Day 2 optional	1 Day 1-hour	Day 1 7-9 hours	Day 2 optional
Procedures	**Screening**	**Baseline**		**Compliance (Outpatient)**	**Outcome**	
Informed Consent	X					
History and Physical (MMSE Visit 1 only)	X				X	
Review Inclusion/Exclusion Criteria	X	X				
Symptoms Review Questionnaire	X	X			X	
PANAS, RAND SF-36		X		X	X	
Cognitive Testing		X			X	
MRI		X			X	
Accelerometers	X				X	
Vital Signs	X	X		X	X	
Anthropometric measurements	X	X		X	X	
Urine Drug Screen	X					
Urine Pregnancy test	X	X			X	
Fasting Blood Draw	X	X		X	X	
Mixed Meal Tolerance Test		X			X	
Lumbar Puncture		X			X	
Dispense Diet Shakes (5-2 CR Group)		X		X		
Dietary Counseling-ALL Groups		X		X		
Meals / Snacks		X		X	X	
Follow-up Phone Call or Email				**Weeks 1, 3, 5, 7**	**Follow-up**	
Payment	**$50.00**	**$350.00**		**$150.00***	**$500.00**	

Table 2: Laboratory

	Visit 1	Visit 2		Visit 3, 4, 5	Visit 6	
		Week 0		Weeks 2, 4, 6	Week 8	
	1 Day 3-hours	Day 1 7-9 hours	Day 2 optional	1 Day 1-hour	Day 1 7-9 hours	Day 2 optional
Laboratory	**Screening**	**Baseline within 4 weeks of Screening Visit**		**Compliance (Outpatient)**	**Outcome**	
Clinical Labs: CBC with differential, CMP, HbA1c, Phosphate, Magnesium, TSH, Liver Panel, Lipid Panel, Uric Acid, PT/INR, PTT, insulin, HIV, HBV, HCV	**X**					
Research Lab: Insulin	**X**					
Clinical Labs: CBC and PT/INR, PTT		**X**				
Research Labs: AD Biomarkers, Cytokines/Chemokines, Neurotrophic Factors, Exosomes, Oxidative Stress Biomarkers, gene expression and DNA methylation, WBC differential counts, β-hydroxybuterate		**X**				
CSF		**X**			**X**	
Research Labs: β-hydroxybuterate, gene expression and DNA methylation, WBC differential counts, plasma sample for AD Biomarkers, Cytokines/Chemokines, Neurotrophic Factors, Exosomes, Oxidative Stress Biomarkers				**X**		
Clinical Labs: CBC with differential, CMP, HbA1c, Phosphate, Magnesium, TSH, Liver Panel, Lipid Panel, Uric Acid, PT/INR, PTT, insulin					**X**	
Research Labs: AD Biomarkers, Cytokines/Chemokines, Neurotrophic Factors, Exosomes, Oxidative Stress Biomarkers, gene expression and DNA methylation, WBC differential counts, insulin, β-hydroxybuterate					**X**	

Amount of Blood	**28 ml**	**168 ml**	**62 ml/visit**	**174 ml**

4.d.2. Baseline visit

This visit will occur within four weeks from the Screening Visit. Participants will arrive on the NIA-Clinical Research Unit after a 12-hour fast. The following procedures will be done: vital signs, anthropometric measurements (weight, waist circumference, sagittal diameter), and a blood draw for clinical labs (CBC, PT, PTT, and INR to ensure safety of the LP), urine pregnancy test (only in women who are able to become pregnant), and research labs including DNA collection (for APOE genotyping and gene expression studies). A 4-hour mixed meal tolerance test will be done. They will also have cognitive testing and will be asked to complete the Symptoms Review Questionnaire, PANAS, and RAND SF-36. A lumbar puncture (LP) will take place; up to a total of 15 mL of CSF will be collected. Participants will have a brain MRI. About 168 mL of blood will be taken during the visit. To facilitate scheduling or satisfy a given participant's request, we may split the various procedures of the Baseline Visit over two days within a week from each other.

4.d.3. 5-2 CR or control groups

At the end of the Baseline Visit, all participants will receive informational materials and one-on-one dietary counseling about "healthy living", consisting of providing and reviewing published material (by the CDC and USDA) on meal portion control, calories in common beverages, meal substitutions for controlling calorie intake, and other dietary considerations. Participants will then be randomized by study investigators into one of two groups to either 1) implement 8 weeks of 5-2 CR (cases); or 2) implement "healthy living" diet (controls).

Participants in the 5-2 group will receive all shakes (Boost®, CWI Medical) from the NIA-Clinical Research Unit.

4.d.4. Compliance visits

To assess and reinforce compliance with their respective diet, we will contact participants by phone or email (via the Secure Email & File Transfer Service: https://secureemail.nih.gov/bds/Login.do) at weeks 1, 3, 5, 7 and four days after the Outcome Visit. In addition, we will conduct 2, 4, and 6 week Compliance Visits. The same procedure described below will occur during each one of these.

These visits will take place during the second of the two consecutive restriction days for the 5-2 CR group or any day for the "healthy living" diet group after an 8 hour fast. A blood draw to measure β-hydroxybutyrate, vital signs and anthropometric

measurements (weight, waist circumference, sagittal diameter) will be done. Participants will provide feedback regarding physical and psychological symptoms by completing a Symptoms Review Questionnaire and the RAND SF-36. Samples for β-hydroxybutyrate levels will be sent to Harbor Hospital Lab. In addition, a plasma sample will be sent to the NIA Core Lab for future studies. These visits will take about 1 hour.

In addition, blood will be collected for isolation of total RNA and genomic DNA for target pathways gene expression and methylation analyses, and PBMCs counts. 62 ml of blood will be collected during each one of these visits.

4.d.5.Outcome visit

The Outcome Visit will occur at the end of the 8-week period in the 5-2 CR or control group. This visit will take place during the first of the two consecutive constriction days for 5-2 CR participants or at a convenient time during the week for control participants. This visit will occur after a 12-hour fast, to ensure comparability of the mixed meal test with the Baseline Visit. The visit procedures will be similar to the Baseline Visit, including a lumbar puncture (LP) and a medical history and physical exam.

Participants will arrive on the NIA-Clinical Research Unit after a 12-hour fast. The following procedures will be done: vital signs, anthropometric measurements (weight, waist circumference, sagittal diameter), and a blood draw for clinical labs (CBC, PT, PTT, INR, CMP, lipid panel, uric acid, HgbA1c, insulin, β-hydroxybutyrate), urine pregnancy test (only in women who are able to become pregnant), and research labs including DNA collection (for gene expression studies). A 4-hour mixed meal tolerance test will be done. They will also have cognitive testing and will be asked to complete the Symptoms Review Questionnaire, PANAS, and RAND SF-36. A lumbar puncture (LP) will take place and up to a total of 15 mL of CSF will be collected. Participants will also have a brain MRI. About 174 mL of blood will be taken during each visit (including clinical and research labs). To facilitate scheduling or satisfy a given participant's request, we may split the various procedures of the Outcome Visit over two days within a week from each other.

At the end of the Outcome visit, participants will be given an accelerometer to wear for 96 hours. They will be given instructions on how to wear the device and to mail it back to the unit via a pre-paid envelope. To ensure more accurate estimates of physical activity, participants will keep an activity log of when they sleep, exercise, or bathe.

4.d.6. Procedures

Medical history: A NIA licensed practitioner (Physician or Nurse Practitioner) will obtain standardized medical history including past medical history, current review of systems, medications and supplements, as well as family history of Alzheimer's disease and dementia.

Physical examination: Vital signs including body temperature, blood pressure, and heart rate will be obtained. A NIA licensed practitioner (Physician or Nurse Practitioner) will conduct screening head, eyes, ears, nose, and throat (HEENT), cardiovascular, respiratory, abdominal, neurological exams.

Anthropometric measurements: Height, weight, waist circumference, and sagittal diameter will be obtained with the subject in a hospital gown without shoes.

PANAS: Participants will report on Likert scales how they feel emotionally. The PANAS is a common index of emotional state.[71]

Symptoms Review Questionnaire: Participants will report on Likert scales their mood, level of energy, difficulty concentrating, headaches, feeling cold, constipation, negative emotions (depression, anxiety, irritability), hunger and preoccupation with food during constriction and non-constriction days for the week prior to the visit.

RAND SF-36 scale: This questionnaire gauges physical and psychosocial effects of the dietary interventions. It has been used as a quality of life assessment in our previous 5-2 CR study.[40]

Screening blood tests: After a 12-hour fast, 28 mL of blood will be drawn for the following blood tests: complete metabolic panel, liver function tests, complete blood counts, PT, PTT, INR, fasting lipid panel, Hba1c, TSH, insulin, HIV, HBV, and HCV.

Screening urine toxicology screen: Urine will be collected during the screening visit for a standard toxicology screen.

Urine pregnancy test: A urine pregnancy test (only in women who are able to become pregnant) will be performed at screening and before MRI or fluoroscopic LP procedures at the Baseline and Outcome Visits.

4-hour mixed meal tolerance test: After fasting for at least 12 hours, participants will be asked to drink 5 mL/Kg of the dietary supplement shake Ensure Plus®. Ensure Plus® is a mixed liquid meal; one bottle (480 mL) contains 95 g carbohydrate as dextrose, 26 g protein, and 25 g fat, providing 710 kilocalories. In addition, to inducing a change in glucose and insulin, the mixed meal tolerance test is ideal for testing changes in GLP-1, GIP, ghrelin, glucagon, pancreatic polypeptide, C-peptide, and other hormones. A blood draw will take place before subjects consuming the liquid meal Plasma, serum and whole blood biomarkers will be measured from this blood draw. for biomarkers, hormones, and cytokines (approximately 35 mL). Then subjects will ingest the liquid meal, followed by blood draws every 20 minutes (5 mL per sample). Typically, participants will have an intravenous (IV) line placed in an arm vein for blood draws.

Lumbar puncture (LP): Participants will have LPs performed during the Baseline Visit and Outcome Visit at 8 weeks, to provide CSF for measurement of biomarkers. Up to a total of 15 mL of CSF will be collected on each of those two visits. CBC, PT, and PTT results should be available prior to each LP taking place. Participants will be encouraged to be well hydrated on the day of the LP.

In individuals in whom a landmark-guided LP at the bedside is deemed technically difficult by the medical staff (such as in the presence of degenerative lumbar spine disease, prior lumbar spine surgery, kyphosis/scoliosis or other conditions) or fails, a licensed Interventional Radiologist may perform the procedure under ultrasound or X-ray guidance (fluoroscopy-guided LP).

Cognitive testing: Logical Memory and CVLT will be administered using standard procedures. The MMSE will be administered only during the Screening Visit. In addition, we will administer EXAMINER, a software program (http://examiner.ucsf.edu/index.htm) implementing a battery of neuropsychological tests that reliably and validly assesses domains of executive function. This software was kindly given via a technology transfer request from Dr. Joel Kramer at the University of California – San Francisco. We will assess working memory, inhibition, set shifting, fluency, planning, insight, and social cognition and behavior. Neuropsychological testing will take approximately 1 hour to complete.

Brain MRI: In total, structural MRI, fMRI and MRS will last about 2 hours.

- Structural MRI -- Standard structural brain images will be collected for coregistration with fMRI and to allow for clinical over-read.

- Functional MRI (fMRI) --

    I. fMRI Study 1 will implement an event-related design, with two intermixed conditions (appetitive "junk" foods vs. non-appetitive foods), in two runs. Appetitive food trials will be pseudo-randomly intermixed with non-appetitive food trials. Each trial will last for 4 seconds and will be followed by a variable interstimulus interval during which a fixation cross will be displayed. In each trial, they will be presented with a picture of a food item. Participants will be asked "how much they would like to eat" a given food, as it appears on screen by selecting one of 4 responses: ''Really like it'', ''Like it,'' ''Do not like it,'' or ''Really do not like it.'' Each of the 60 appetitive or non-appetitive food items will be shown about twice during each run.
    II. fMRI Study 2 will be a block design of a color-word Stroop task adapted from Nestor and colleagues.[61] The study consists of congruent, incongruent, and rest blocks, presented over two counterbalanced runs. Each run consists of 8 congruent blocks, 8 incongruent blocks, and 15 rest blocks. Each block consists of 15 trials. The order of blocks will cycle through a congruent block

to a rest block to an incongruent block; the pattern will then repeat. The counterbalanced run will begin in the opposite order and similarly cycle. During congruent or incongruent trials, the participant will be asked to select what the color of the word is on the screen: red, green, blue, or yellow.

III. fMRI Study 3 will be a resting state fMRI study in a single run. Subjects will be asked to keep their eyes open and fixate on a cross. This study will be used to assess intrinsic functional connectivity between different brain regions.

- MRS -- We will assess the concentrations of several brain metabolites, including glutamate, GABA, NAA, creatine, lactate, and glucose, using J-PRESS MRS, followed by fitting of metabolites using ProFit.[63,95,96] Each of these metabolites has a characteristic pattern of spectral lines defined by the energy of transitions that occur when nuclei are excited into a higher energy state through radio frequency pulses. The spectral lines from all metabolites present, taken together, make up the observed spectrum. Concentrations of individual metabolites can be determined through evaluation of the relative sizes of the spectral lines in the observed spectra. The reliability of the measurement and fitting procedure for each metabolite can be assessed using Cramer-Rao lower bounds.[97] We will use standard localization techniques based on magnetic field gradient sequences to obtain spectra from ventromedial prefrontal cortex and hippocampus.

Accelerometry: Physical activity will be monitored using the Actigraph GT3X-BT (Actigraph Corporation) accelerometer. The Actigraph is a 1 x 1 x 3/8 inch device worn as a wristband on the non-dominate hand. The Actigraph GT3X-BT measures accelerations in vertical, horizontal, and anterior-posterior directions. We will sample at 30 Hz per second over period of 96 hours at a time on two occasions during the study. Participants will be given a diary to record times for bed and times waking up daily, and time that the accelerometer was removed daily for extended periods of time.

Gene expression array: Peripheral blood cells will be collected using the PAXgene system,[98] to preserve transcript expression levels as they would be in vivo. Total RNA will be extracted using the PAXgene Blood mRNA kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Whole genome expression profiling of the samples will be conducted using the Illumina Human HT-12 microarray (Illumina, San Diego, USA) as previously described.[99]

Methylation arrays*:* One microgram of genomic DNA will be extracted from buffy coat samples was bisulfite converted using Zymo EZ-96 DNA Methylation Kit (Zymo Research Corp., Irvine, CA) per the manufacturer's protocol. CpG DNAm at individual sites will be assayed using the Illumina Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA) per the manufacturer's protocol*.*

White blood cell differential counts: The RNA and DNA used for the gene expression and methylation assay will be isolated from mixed population cells. The standard complete

blood count provides percentages of neutrophils, lymphocytes, monocytes, eosinophils and basophils. We will require further separation of lymphocytes to get the counts of T cells, B cells and natural killer cells. The distribution of white blood cells will be an important covariate in the analysis of gene expression and methylation to ensure that the associations that we observe is specific to 5-2 CR rather than changes in any of the cells during the dietary intervention. We will use cell specific monoclonal antibodies and flow cytometry to count the each cell population within the lymphocytes. The flow cytometry methods will require 5-10cc of whole blood.

4.e. End of participation

We will share with participants any new information that may relate to their willingness to continue to participate in this study. Within 3 days after the Week 8 Outcome Visit, either one of the investigators or nursing staff will contact the participant to assess whether there have been any problems since the visit. In addition, at the end of the Outcome visit, participants will be given an accelerometer to wear for 96 hours. They will be given instructions on how to wear the device and to mail it back to the unit via a pre-paid envelope. Completion of accelerometry and the final telephone or email communication mark the end of participation to the study.

Transfer of care or medical care offered at study completion is not applicable to this protocol, as the participants remain under the care of their primary health care providers.

We will discuss the results of physical examination, clinical lab tests, and structural MRI with participants and provide them relevant reports. We will encourage subjects to share any clinically significant information with their health care providers.

5. Management of Data and Samples

5.a. Storage

Samples and data collected under this protocol may be used to study physiology related to brain function, endocrinology and metabolism. The subject's stored samples will be labeled with a code (such as letters and numbers) that only the study team can link to the subject. Any identifying information about the subject will be kept confidential to the extent permitted by law. Samples acquired during this study will be tracked using the NIA Biological Sample Inventory system following NIH guidelines.

Samples of the subject's blood and cerebrospinal fluid will be kept in the National Institutes on Aging, NIH Core Lab or one of our contract facilities. The subject's samples may be tested immediately, or they may be frozen and used later. The subject's samples will be stored with a confidential code. Samples may be kept until no cells remain or until the investigators decide to destroy them. If the subject gives us permission some

samples may be released to other doctors and scientists who are not associated with this institute. The Principal Investigator on this protocol will decide which co-investigators and collaborating researchers may receive samples. The subject's samples may be used in their research only if the research has been approved by an Institutional Review Board (IRB) and is related to the original research questions association with this protocol or for other research purposes as indicated below.

We will code the blood, cerebrospinal fluid and urine samples that will keep the subject's identity confidential. We will retain a code list that enables us to link the clinical information and results on each sample to the subject. DNA extraction from PBMCs will take place for APOE genotyping (only at Baseline Visit) and methylation studies and RNA extraction will take place for gene expression studies. DNA samples will be stored at the NIA Core Lab until they are released to the appropriate NIA lab.

Access to research will be limited using either a locked room or a locked freezer. Samples and data will be stored using codes assigned by the investigators or their assigned designee(s). Data will be kept password-protected computers. Only investigators or their designee(s) will have access to the samples and data.

At the completion of the protocol (termination), samples and data will either be destroyed, or after IRB approval, transferred to another existing protocol or a repository protocol.

5.b. Data

Data and samples may be shared with collaborating laboratories at NIH or outside of NIH if consent for sharing was obtained in the original consent form. Samples/data that we may share in the future include biological specimens (blood, CSF), MRI images, MRS data, cognitive test results, and genomic data.

Samples and data will be stripped of identifiers and may be coded ("de-identified") or unlinked from an identifying code ("anonymized"). When coded data is shared, the key to the code will not be provided to collaborators, but will remain at NIH. Data and samples may be shared with investigators and institutions with an FWA or operating under the Declaration of Helsinki (DoH) and reported at the time of continuing review. Sharing with investigators without an FWA or not operating under the DoH will be submitted for prospective IRB approval. Submissions to NIH-sponsored or supported databases and repositories will be reported at the time of Continuing Review. Submission to non-NIH sponsored or supported databases and repositories will be submitted for prospective IRB approval.

Required approvals from the collaborating institution will be obtained and materials will be shipped in accordance with NIH and federal regulations.

Whenever appropriate, the subjects will be provided with additional pertinent information after participation. For unlinked data, the identities of the participants will not be traceable and no later information can be provided. For coded samples, participants will be provided with additional pertinent information after participation whenever possible. Data and samples may also be shared with collaborating laboratories at NIH or outside of NIH and/or submitted to NIH-designated repositories and databases if consent for sharing was obtained in the original consent form.

6. Additional Considerations

6.a. Research with investigational drugs or devices

This protocol does not include investigational drugs or devices.

6.b. Gene Therapy

Gene therapy is not used in this protocol.

7. Risks and Discomforts

7.a. Medical history and physical exam: There are minimal risks to completing medical history and performing a physical exam.

7.b. Anthropometric measurements: There are minimal risks in performing anthropometric measurements

7.c. PANAS: There is minimal risk to completing the PANAS questionnaire

7.d. Symptom Review Questionnaire: There is minimal risk to completing the symptom review questionnaire

7.e. RAND SF-36: There is minimal risk to completing the RAND-SF 36 questionnaire

7.f. Screening blood test: There is a slight risk of pain and bruising around the site where blood is drawn; bleeding and infection are rare side effects. To minimize this risk, the routine blood-drawing protocol will be followed and pressure will be applied to the site. Some people experience feelings of light-headedness or dizziness after having blood drawn. To reduce the risk of falling, we will monitor the subject closely and ask about these symptoms before we allow them to stand. The samples collected will be used to monitor the subject's medical condition and for research purposes.

7.g. Screening urine toxicology screen: A urine toxicology screen will be performed during the screening visit. If the drug test is positive the results of the drug testing will be noted in participants' NIH medical record. Participants who do not want this

information in their medical records should not participate in the study. The medical records can only be released with written agreement by the participant. However, insurance companies may require individuals to release these records and may not provide insurance if they refuse.

7.h. Urine pregnancy test: There is minimal risk to giving a urine specimen for pregnancy testing. However, due to the unknown risk of MRI or fluoroscopic LP on pregnant women or fetal development, women who are able to become pregnant will have a pregnancy test prior to these procedures. Participants will not be able to participate in the study if the pregnancy test is positive.

7.i. 4-hour mixed meal tolerance test: Drinking the Ensure Plus® shake may lead to a temporary sense of abdominal fullness and discomfort, nausea, vomiting, or diarrhea, but it is a rare occurrence. Needle insertion of the catheter will in rare cases produce blood at the site of insertion and bruising.

7.j.Lumbar Puncture (LP): A lumbar puncture has a very small risk of local or CNS infection, local or epidural hematoma formation or CSF leak and hypotension. A minimum of about 5% of participants who have an LP may experience post-LP headache, which is often mild and responds to hydration and regular analgesics, but rarely may be severe and require hospitalization. In severe cases, such headache may require a blood-patch.

To minimize the risk of infection, standard procedures for local anesthesia and antisepsis will be followed. To minimize the risk of post-LP headache, participants will be encouraged to present well-hydrated and only 15 mL of CSF will be collected. Moreover, an atraumatic needle will be used and the stylus will be reinserted prior to needle withdrawal as per current guidelines. Participants will be asked to remain in a reclining position for one hour and remain on the unit for three hours after the LP for observation. The LP may be aborted at any time at the participant's request or after a limited number of unsuccessful attempts at the clinical judgment of the investigator performing the procedure.

7.k. Radiation risk from fluoroscopy-guided lumbar puncture: We classify fluoroscopy-guided LP as greater than minimal risk. This research study involves exposure to radiation from the above-mentioned procedure, which may be conducted up to two times (during Baseline and Outcome Visits), depending on whether a landmark-guided LP at the bedside is deemed technically difficult by the medical staff or fails. In addition, an ultrasound-guided LP may be attempted prior to the fluoroscopy-guided LP, at the discretion of the interventional radiologist who will perform the procedure. This radiation exposure is not required for medical care and is for research purposes only. The effective dose of radiation the participant will receive in this study is 0.74 rem, which is below the guidelines of 5 rem per year allowed for research subjects by the NIH radiation Safety Committee. The average person in the United States receives a

radiation exposure of 0.3 rem per year from natural sources, such as the sun, outer space, and the earth's air and soil. While there is no direct evidence that the amount of exposure received from participating in this study is harmful, there is indirect evidence it may not be completely safe. There may be a very slight increase in the risk of cancer.

The participant will be screened by the clinical research coordinator or study physician or NP for radiation exposure in the past year, either from other research studies or from medical test or care, to ensure they are within the guidelines. This will be done using a radiation exposure checklist screening form.

7.l. Brain MRI: The Food and Drug Administration (FDA) has recently established safety criteria for human exposure to MRI studies. The MRI scanner used in this study satisfies these safety criteria. People are at risk for injury from the MRI magnet if they have pacemakers or other implanted electrical devices, brain stimulators, dental implants, aneurysm clips (metal clips on the wall of a large artery), metallic prostheses (including metal pins and rods, heart valves, and cochlear implants), permanent eyeliner, implanted delivery pump, or shrapnel fragments. Welders and metal workers are also at risk for injury because of possible small metal fragments in the eye of which they may be unaware. Participants will be asked to complete an MRI screening form for each MRI. If any participant is found not to be eligible for the MRI or refuse the MRI, they will be disqualified from the study. In addition, all magnetic objects (for example, watches, coins, jewelry, and credit cards) will be removed before entering the MRI scan room. Participants with history of surgeries, implanted devices, etc. will be asked to provide us with surgical reports mentioning the type of the clips, prostheses, etc. used. Every effort will be made to establish the MR compatibility or incompatibility of these implants, according to the safety standards of the NIA 3T MRI center, including obtaining reports by manufacturing companies for MR compatibility. The research staff will evaluate each person with a less severe history of claustrophobia on a case-by-case basis.

Regarding minor discomforts, radiofrequency pulses emitted by the MRI machine can induce mild muscle twitching in some participants. If the sensation becomes overly uncomfortable, the scan will be stopped. The loud sounds (> 120 dB) emitted by the MRI machine during scanning require earplugs but may cause mild acoustic discomfort.

7.m. Cognitive Testing: Occasionally, cognitive tests may be tiring or stressful. Psychological testing may cause some people to feel anxious.

7.n. Genetic testing (APOE genotyping and DNA methylation studies): The DNA analysis that will be done as part of this study is done for research purposes only.

Genetic testing can provide information about heritability of diseases and risk factors. Knowledge that participants or their children are at increased risk of disease may cause anxiety or depression or make them reevaluate their life. This is the case of the APOE ε4 gene, for which we test in this study and which increases the risk of Alzheimer's disease

and cardiovascular disease, although it does not mean that whomever carries that gene will develop Alzheimer's disease or coronary artery disease.

As a result of genetic testing performed on a participant, her/his biological relatives may find out that they are at risk for a disease, such as Alzheimer's disease, which might affect the participant's relationships with them, cause stress, anxiety, or depression.

Genetic counseling is available at the National Institutes of Health to help participants understand the nature and implications of genetic findings for them and their families.

Because of the emotional risk, some people who participate in research do not want to know the results of genetic testing. It is our policy to not disclose the results of genetic testing unless it may have direct medical or reproductive implications for participants or their families. Participants may choose to receive their information or they may choose not to receive the information. Whether they choose to receive the information or not, by agreeing to participate in this study, participants do not waive any rights that they may have regarding access to and disclosure of your records.

Results of genetic testing obtained at NIH are often preliminary and difficult to interpret because the testing is being done for research purposes only and the laboratories are not clinically certified. This is the case of the testing for the APOE ε4 gene that will take place in this study. In addition, testing for the APOE ε4 gene will take place after the end of the participants' study participation and will not be immediately available for review. If participants decide that they wish to be informed about the results of APOE ε4 genetic testing as conducted in this study and this result has become available, they will meet with the principal investigator, who will refer them to a CLIA-certified lab for confirmation. In addition, the principal investigator will discuss the significance of their particular genotype on AD and cardiovascular risk for participants and their families based on the literature.

Participants' genetic information will be kept confidential to the extent possible. The results of participants' genetic testing will be kept in a locked and secured manner at the NIH. Genetic information will not be part of participants' medical record. Genetic information about participants will not be revealed to others, including their relatives, without participants' written permission. Similarly, participants will not receive information about other family members.

Problems, such as with insurance or employment discrimination, may occur if participants disclose information about themselves or agree to have their research records released. We will not release any information about participants or their families to any physician, insurance company or employer unless you sign a document allowing release of the information.

Further discussion can be found under Section "20. Confidentiality" on pages 51-52.

7.o. Accelerometry: The accelerometer has no risks associated with its use and causes minimal inconvenience.

7.p. 5-2 CR/Control Diet: Overall, a minority of participants (9-15%) is expected to develop minor physical and psychological AEs during the first several weeks of 5-2 CR. Such AEs include lethargy, feeling cold, difficulty concentrating, and irritability. Participants may experience minor psychological symptoms such as feeling hungry (leading to preoccupation with food), lack of concentration, bad temper and irritability. The Harvie et al. study is especially important because our study design is similar to it. In the Harvie et al. study, a small number of participants on the 5-2 CR regimen (8%) reported physical symptoms including lack of energy, headaches, feeling cold, and constipation. Minor psychological symptoms included lack of concentration, bad temper, and a preoccupation with food among 15% of the 5-2 CR group. These adverse effects abated after cessation of CR. Conversely, 6% of 5-2 CR participants reported better health and increased energy.[40] Furthermore, 32% of 5-2 CR had increased self-confidence and positive mood. To minimize these risks, participants will be told ahead of time about potential side effects. During Compliance Visits, we will give participants the opportunity to discuss and will offer encouragement that may ameliorate minor psychological symptoms.

No major adverse effects have been associated with mild to moderate intermittent calorie restriction.[39,40,94] Two of our previous CR studies did not have a reported serious AE incident.[39,40] More severe caloric restriction in humans relative to our studies also produced no serious AEs [94]. We classify the 5-2 CR and control diet as minimal risk.

8. Subject safety monitoring

8.a. Monitoring of Subjects

The investigator or clinical staff performing a study procedure will monitor subject safety. This includes physicians, licensed practitioners (NPs) and RNs during LPs, blood draws, mixed meal testing, and History and Physical sessions; investigators administering questionnaires and cognitive testing, MRI techs and investigators during MRIs.

The PI will make determinations regarding the presence and grading of on adverse events (AEs) and unanticipated problems (UPs). Whenever study investigators contact subjects throughout the study, they will seek information on AEs and UPs. AE's and UPs will also be elicited during study visits, by subjects filling in the symptoms review questionnaire or during history and physical examination. Participants will be asked to use electronic mail (Secure Email & File Transfer Service: https://secureemail.nih.gov/bds/Login.do) or telephone to inform the investigators about any AE's or UPs experienced. AEs and UPs may be observed by the Investigator and/or study staff, elicited from the subject and/or family member, or volunteered by the study subject. AEs and UP's that had previously been reported by study subject will also be reassessed for duration, intensity and

possible reoccurrence. Assessment of safety will include vital signs, history and physical sessions, questionnaires on physical and psychological symptoms and clinical labs as indicated. Any AE or UP that occurs between the times a study participant signs the informed consent form and the end of participation will be assessed and recorded under NIH HRPP SOP # 16 and 16 A.

In particular, we will monitor the weight loss of the participant. If a participant on 5-2 CR loses 4-5% of their body weight within a 2-week period between visits, additional safety visits will be scheduled every week to monitor body weight more closely. If weight loss in that range or greater continues to occur, participants will be removed from the study.

A study participant will also be discontinued from the study if:

- Any adverse event occurs Grade 2 or greater and possibly related to the study.
- The participant develops any of the exclusion criteria.
- There is persistent non-compliance with study requirements (keeping follow-up visits).

Participants will be informed about new information from this or other studies that may affect their health, welfare, or willingness to stay in this study. Participants may decide to stop participating at any time. Any data or blood collected until that point in time will remain part of the study and are the property of the National Institute on Aging.

9. Outcome measures

All secondary outcome variables in this pilot study are exploratory.

9. a. Primary outcome measures

- Exosomal p-S312-IRS-1/p-panY-IRS-1 ratio

9. b. Secondary outcome measures

- Other exosomal protein and mRNA biomarkers of AD;
- S_I and HOMA-IR, indices of insulin resistance, derived from insulin and glucose values taken from a fasting blood draw;
- fMRI activation to appetitive food versus non-appetitive food stimuli;
- Resting state fMRI intrinsic functional connectivity of DMN;
- Behavioral performance and fMRI activation during a Color-Word Stroop task;
- Brain metabolites assessed with Magnetic Resonance spectroscopy and regional perfusion assessed with arterial spin labeling;
- Memory and executive function scores.
- Anthropometric measures (weight, waist circumference, sagittal diameter);

- Clinical Labs: lipid panel, uric acid;
- Plasma ketones, such as β-hydroxybutyrate and other metabolites.
- Plasma metabolites and hormones (GLP-1, insulin) during a mixed meal test;
- CSF and plasma AD biomarkers, including $A\beta_{1-42}$ and p_{181}Tau;
- Plasma and CSF neurotrophic factors, such as BDNF;
- Plasma and CSF reactive oxygen species, advanced glycation end-products and advanced oxidation protein products;
- Plasma and CSF pro-inflammatory cytokines, such as IL-6, IL-8, IL-12 and IL-23;
- Ratings of symptoms that can occur with 5-2 CR, mood and quality of life changes, recorded during both the 5-2 CR and control diet phases;
- Physical activity measured using an accelerometer.
- Global gene expression pattern, mitochondria gene expression and expression of other gene pathways responding to CR
- Genome-wide methylation in peripheral blood mononuclear cells

10. Statistical Analysis

10. a. Analysis of data/study outcomes

This is a pilot study that is exploratory in nature and one of its goals is to collect preliminary data for multiple outcomes that may be used in future studies on the brain effects of CR.

For the primary and each secondary scalar measure, a separate repeated measures mixed models analysis will be conducted to examine the effects of the between-subjects factor "Group" (5-2 CR group vs. "Healthy living" control group), the within-subjects factor "Time" (Baseline vs. Outcome) and their Interaction. An Auto-Regressive (AR)1 covariance matrix and Restricted Maximum Likelihood Estimation (REML) will be used. We will assess fit to the model using the AIC and BIC criteria (lower values indicate better fit). These parameters were chosen because they are recommended for repeated measures analyses, including fMRI task analyses.[100] Regarding within-subjects effects, we will assess post-hoc differences between the 8-week outcome visit and baseline for the 5-2 CR and control groups and for each group separately (i.e. post-hoc tests for the Group * Time interaction). This analytic approach is common in the CR literature.[101] Mixed model post-hoc tests will employ the Least Significant Difference (LSD) statistic. We will use one-tailed t-tests.

Criteria for significance will be set at alpha of 0.05 for all non-MRI analyses, and 0.005 uncorrected at the voxel level for fMRI analyses. Given that this pilot study is exploratory, we will not conduct correction of multiple comparisons for the various secondary outcomes.

Analysis of fMRI data will use a mixed model design to assess the effect of Group on

brain activation associated with viewing appetitive vs. non-appetitive food, the Stroop effect in the Stroop task, and DMN intrinsic functional connectivity. Analyses will be conducted at the whole brain level and within ROIs (vmPFC, OFC and insula for the fMRI food task, lateral and dmPFC for the Stroop task, and in DMN for resting state. The fMRI analyses will be corrected for type 1 error as the cluster level using Monte Carlo simulations.

For brain MRS measures, a repeated measures analysis will be separately used for each of the brain regions of interest: the hippocampus and the prefrontal cortex.

APOE genotype is a common covariate used when assessing populations with AD or at risk for AD. We intend to perform exploratory analyses including APOE, which may reduce error variance and improve our statistical models.

Plasma ketones are strongly affected by alternate day CR acutely but not chronically;[39] our main objective for measuring them is to assess compliance with 5-2 CR.

We do not anticipate a change in physical activity in 5-2 CR versus "healthy living" control groups, because no change has been noted in our previous studies. Nonetheless, this is a common analysis requested by reviewers, to make sure that exercise effects did not contaminate diet effects.

The data from the gene array and DNA methylation array will be analyzed using residuals after adjustment for white blood cell differential count in linear regression models. We will take two approaches to analyze the data. First we will focus on candidate genes approach where we select specific expression probe or CpG sites; DNA for these studies will be collected during Baseline, Compliance and Outcome Visits. Second, we will run genome-wide association analysis for differences in cross sectional and longitudinal changes in gene expression or DNA methylation between the control and 5-2 CR group at each gene array probe or CpG site. DNA for genome-wide association analyses will only be collected during Baseline and Outcome Visits. For the cross sectional analyses, we will use a paired t-test at each visit, and mixed models for longitudinal analysis. In addition, we will look at overall patterns of gene expression and methylation using principle components, DIANE and hierarchical clustering tools to determine whether there are specific gene expression and methylation signature in association with 5-2 CR. As secondary analyses, we will also determine whether gene expression and DNA methylation patterns can predict the changes in metabolic, cognitive and neurochemical variables collected in this study. Within the 5-2 CR group, we will test the association between baseline levels of gene expression or DNA methylations at each probe/CpG site with longitudinal changes in the main outcome variables using mixed models.

10. b. Power analysis

The accrual number request is 100 participants. Our target sample size for completing the study is N = 40. There will be 20 participants in each group. We anticipate up to 60 participants will not meet inclusion criteria during the screening visit or may withdraw from the study. Enrollment will continue and withdrawals will be replaced until we have acquired complete data from 40 participants.

For power calculations, we used G*Power (http://www.gpower.hhu.de/en.html).

First, we calculated power for the primary outcome (exosomal p-S312-IRS-1/p-panY-IRS-1 ratio, which is a measure of brain IR). Effect size (f) was determined based on studies with comparable sample sizes examining moderate CR (20-40%) over a 2-3 month period in humans.

Insulin Sensitivity (Peripheral and Brain): It is important to re-emphasize that reductions in fat mass will increase peripheral insulin sensitivity.[12,21,22] To determine effect size, we examined a study by Weiss and colleagues[101] that had a design similar to our own. They examined sedentary men and women aged 50-60 with a BMI of 23.5 to 29.9. They randomly assigned participants to moderate CR (n = 18) or a healthy lifestyle control group (n = 10) for 12 months. The CR group showed a significantly reduced BMI from 27.1 to 24.8, with a corresponding increase in S_i from 4.5 to 7.4. The healthy living control group showed a non-significantly reduced BMI from 27.9 to 27.4, with a non-significant increase in S_i from 4.2 to 4.5. The effect size was f = 0.440. Our most recent study on intermittent CR[40] showed a reduction of body fat from 33.6kg to 30.6kg by 3 mo., but a relatively smaller decrease (29.1kg) by 6 mo., suggesting that most of the weight loss occurred in the first three months. Based on these findings, we anticipate a comparable effect size (0.3) from our 8 weeks of 5-2 CR.

Regarding our primary outcome (exosomal p-Ser312-IRS-1/p-panY-IRS-1), in our published study, we examined differences between preclinical samples from participants who eventually developed AD (92.1 ± 7.7) and controls (14.7 ± 0.9)[51]. We base our power analysis on the assumptions that the our subjects at high risk for AD had intermediate high values for p-Ser312-IRS-1/p-panY-IRS-1 ratio at baseline (ratio = 46), that the 5-2 CR intervention had a similar modest effect size as for peripheral IR in decreasing the ratio (0.3), whereas the control intervention induced no change. We used the following input parameters to determine power for ANOVA repeated measures, between-within interactions: $\alpha = .05$ (one tailed); 2 groups (5-2 CR vs. Healthy Living diet); 2 repeated measures (Baselive vs. Outcome); correlation between repeated measures = 0.4. Based on these parameters, we achieve over 90% power with a total 40 participants (see Figure 4 below for post-hoc power calculation (achieved power, given certain N and effect size)). These calculations are conservative (e.g. the effect size may be greater than 0.3); therefore, it is likely that we will actually achieve greater power in this study.

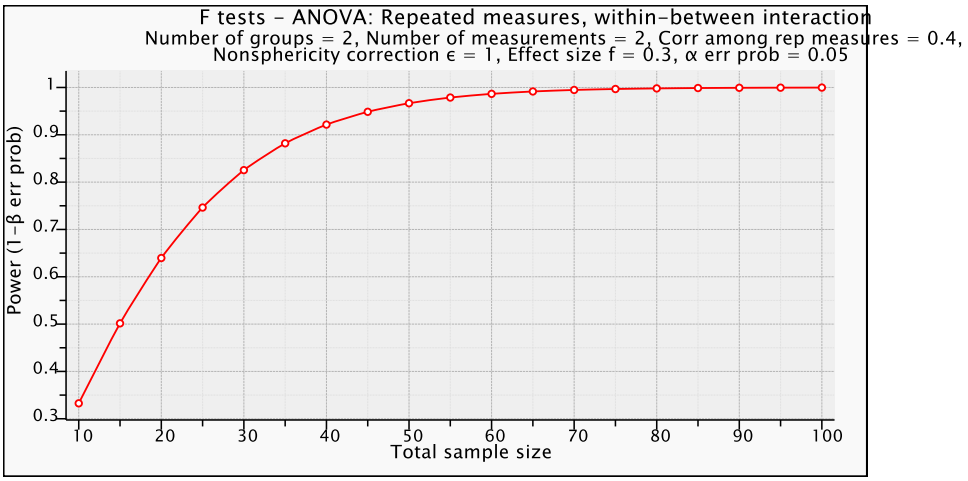

Figure 4. Post-hoc power calculation for primary outcome (exosomal p-Ser312-IRS-1/p-panY-IRS-1) analyzed with ANOVA for repeated-measures for within-between interaction. The x-axis depicts total sample size and the y-axis depicts Power.

In addition, we performed power calculations for several key secondary outcomes of interest.

Regarding fMRI, we performed power calculations based on a series of equations by Mumford and Nichols[100] that take into account the unique nature of fMRI data. We use an alpha = .005 for fMRI analyses, as recommended by Mumford and Nichols.

fMRI task 1 (appetitive foods): A recent study by Bruce et al.[102] examined young to middle-aged obese participants that either underwent 3 mo. of moderate CR (n=16) or bariatric surgery (n=15). They assessed fMRI activation to pictures of food versus pictures of animals at baseline and 3 mo. They found that CR participants showed a large cluster-wide increase in medial prefrontal cortex versus bariatric participants (t = 3.72, Cohen's d = 1.34 and f = 0.8), a brain region that they argue is involved in regulating appetite and suppressing cravings. To achieve at least 80% power with 20 subjects per group in a between subjects contrast, we would require a scan time of 6 min. for the fMRI task. Our scan time of 24 min. well exceeds this estimate and would achieve power of roughly 85-90%.

fMRI task 3 (resting state fMRI): Zhang et al.[103] recently published a proof-of-concept study on the acute effect of intranasal insulin (40 IU) on 14 subjects with type 2 diabetes and 14 healthy participants. Intranasal insulin, like CR, increases insulin availability in the brain.[104] They in part examined changes in resting state fMRI brain activity during placebo (day 1) and intranasal insulin (day 2 or 3), collapsed across groups. They found

robust activation in several brain networks, including a portion of DMN in a medial PFC area (t = 3.25, Cohen's d = 1.25 and f = 0.626) similar to what is described above for Bruce et al.[102] To the best of our knowledge, this is the only published study examining the effect of a therapeutic intervention manipulating brain insulin on resting state activity. We anticipate a similar activation pattern and effect size in our data. According to calculations based on Mumford and Nichols[100] for power estimation in fMRI, groups with n=20 each would require a 6-minute resting state scan to achieve 80% power. Our resting state scan lasts for approximately 7.5 minutes.

Memory and Executive Function: Witte and colleagues[65] examined aged participants divided into groups that either engaged in 3 months of moderate (30%) CR (n=20), received no intervention (n=10), or received unsaturated fatty acid supplementation (n=20). For the Rey Auditory Verbal Learning Test, which is very similar to our CVLT, CR participants showed a significant 130% gain (Cohen's d = 0.799) in a composite memory score after the intervention versus non-significant changes in the other groups. Our own intervention only differs in the length of time spent on CR and the intermittent nature of the CR, and so we anticipate a comparable effect size. Given that executive function is more consistently impacted by obesity and by extension IR across the lifespan,[105,106] we also anticipate a similar or greater effect size for executive function, although there are currently no studies in the literature directly comparable to our design. In summary, with an anticipated effect size f = 0.4, we will have power = 0.81 to detect an effect on cognition.

Plasma biomarkers: For plasma biomarkers, such as proinflammatory cytokines, ROS, BDNF, and affect measures, we note that our previous within-subjects study on intermittent CR in humans[39] produced robust differences in significant or near-significant trends in these measures by 3 mo. with n = 42 at alpha = .05. We anticipate being able to detect such effects and more subtle differences, because our between-subjects design is more powerful. In particular, informal power calculations suggest that we will have at least 80% power to detect differences in cytokines, ROS, and BDNF.

Global gene expression and genome-wide methylation: With sample size of 20 for each of the two groups, using the Illumina HT12 expression chip with 25,000 annotated genes, there is 80% power at alpha (two-tailed) = 0.05 to detect a 1.5-fold difference in expression. Similarly, with the sample size of the proposed study, we have 90% power to detect methylation differences of 40% between groups at alpha (two-tailed) = 0.05 for 45,000 CpG sites.

11. Human subjects protection

11. a. Subject selection

Statement of equitability

Participants will be recruited and enrolled equitably including, but not limited to,

without regard to race, ethnicity, sex, sexual orientation, unless explained below.

It is National Institute on Aging (NIA) policy that the regulations of the Department of Health and Human Services (HHS), set forth in 45 CFR Part 46, are applicable to all research involving human subjects, as defined by these regulations, for which the NIA is responsible, regardless of the source of funding or whether the research is funded. In the case of conflict between regulations of the funding or regulatory agency and HHS, the more restrictive regulations shall prevail. The NIA is also obligated by law to adhere to the regulations of the Food and Drug Administration (21 CFR Parts 50 and 56) governing projects involving investigational new drugs [within the meaning of 21 U.S.C. sections 355(i) or 357(d)], or investigational new devices [within the meaning of 21 U.S.C. section 360(g)].

Rationale for non-equitable selection

This study imposes certain age and BMI limits to define a suitable population of older overweight adults with IR, which is suitable for addressing the study hypotheses, as explained in the introduction. The main reason we study participants aged 55-70 years who are overweight and have IR is to examine brain IR and other AD-associated biomarkers at individuals at higher risk for disease during the preclinical stage of AD. We impose a lower limit of BMI to ensure that we will recruit a population with high probability of IR. IR is present in many middle-aged[23] and aged[24,25] adults with a BMI above 27.[26] Specifically, higher visceral adiposity due to being overweight or obese corresponds to higher IR in 25-40% of late middle-aged to geriatric Caucasian[42] and Asian[27] populations. Overweight to obese participants (BMI ≥ 27) aged 55-70 years have insulin sensitivity values of approximately 7.6 $min^{-1}/(\mu U\ mL^{-1}) \times 10^{-4}$.[24,25] In addition, we impose the upper limit of 350 pounds for body weight, because this is the limit of the scanner. In addition, we exclude pregnant women, given the fact that we employ brain MRI and XRays for fluoroscopy-guided LPs, which may pose a higher risk to the unborn fetus. We exclude nursing women because they have higher caloric needs.

11. b. Justification for exclusion of children

The goal of this study is to provide proof of concept that 5-2 CR compared to dietary counseling can lower IR and ameliorate any detrimental effects on middle to older age cognitive performance, brain metabolism and function and normalize any biomarker abnormalities associated with higher risk of AD. Children do not develop AD, and there are no data to suggest that IR during childhood may impact future AD risk. In addition, 5-2 CR in juvenile animals permanently stunts their physical growth. Children are, therefore, not an appropriate population to examine for the purposes of our study.

11. c. Justification for exclusion of other vulnerable participants

If the present study provides proof of concept that 5-2 CR compared to dietary

counseling improves cognitive performance, brain metabolism and function and normalizes biomarker abnormalities associated with higher risk of AD, it would "pave the way" for testing it in cohorts with age-related cognitive impairment or AD. The study requires following strict instructions for 5-2 CR diet, which participants with cognitive impairment would find hard to follow. In addition, this is a proof of concept study that involves more than minimal risk without direct benefit. The study involves some minor but real discomfort (e.g. feeling hungry) and invasive procedures (such as LP), which are not appropriate for those without consent capacity in this study.

11. d. Justification of sensitive procedures

The experimental paradigm does not involve the use of deception, or other sensitive procedures.

11. e. Safeguards for vulnerable populations

We will perform pregnancy tests and exclude pregnant (and nursing) women.

11. f. Qualifications of investigators

The Principal Investigator has verified that all individuals working on this protocol required to take HRPP training under OHSRP SOP 25 (Training requirements for the NIH Human Research Protections Program) have completed all required training.

Dimitrios Kapogiannis, M.D. (LCI/NIA)

Dr. Kapogiannis is a Board-certified neurologist with subspecialty training on dementia and Experimental Therapeutics in Neurology. He will be principal investigator and medically responsible investigator of the protocol. He has been primarily responsible for the design of the study and will oversee the project, ensure quality assurance of the data and analyze the data. He will obtain consent, conduct History and Physicals and perform LPs.

Mark Mattson, Ph.D. (LNS/NIA)

Dr. Mattson is the Chief of the Laboratory of Neurosciences. He will be an associate investigator in this protocol. Dr. Mattson has conducted animal studies examining the neurobiology of CR. More recently, he has collaborated with several groups to look at the long-term effects of continuous and intermittent CR on CSF biomarkers, oxidative stress, psychological wellbeing, and other factors.[39,40] He has also been primarily responsible for the design of the study. In addition, his lab will perform measurements of biomarkers in plasma and CSF and analyze data.

Josephine Egan, M.D. (LCI/NIA)

Dr. Egan is the Clinical Director of the NIA and the Chief of the Laboratory of Clinical Investigation. Dr. Egan will be an associate investigator on this protocol. Dr. Egan is an endocrinologist with extensive experience conducting and overseeing clinical research studies. She has contributed to the design of the study and will oversee its conduct to ensure participants' safety. Her lab will perform measurements of biomarkers in plasma and CSF and analyze data. She will obtain consent, and conduct History and Physicals.

Tamara Harris, M.D. (LEDB/NIA)

Dr. Harris is the Chief of the Geriatric Epidemiology Section at the NIA. She has contributed to the design of the study. She will analyze accelerometry data.

Toshiko Tanaka, Ph.D (TGB/NIA)

Dr. Tanaka is associate investigator of the study and has experience performing genetic analyses in large populations. She will analyze DNA methylation and gene expression data.

Luigi Ferrucci, M.D., Ph.D. (TGB/NIA)

Dr. Ferrucci is the NIA Scientific Director. Dr. Ferrucci has lead efforts to investigate the gene expression and DNA methylation changes that occur with age in observational studies of aging. He will analyze DNA methylation and gene expression data.

Onyinye Erondu, M.S., R.N.
Mrs. Onyinye is the Clinical Research Coordinator for the study; she has more than 14 years of clinical research experience. She will acquire consent, implement study procedures, including performing blood draws, mixed meal tolerance test, and assisting with the LP.

Seema Gulyani, Ph.D., CRNP. (LNS, NIA/NIH)

Dr. Gulyani is a Senior Research Fellow in the Laboratory of Neuroscience. She has completed her post-doctoral training in Neurosciences at University of California Los Angeles. She is also a licensed Nurse Practitioner and has practiced as a NP in the Johns Hopkins Neurology. Dr. Gulyani will be lead investigator on the protocol. She will be responsible for multiple clinical duties along with PI including overseeing participants' safety. She will obtain consent, conduct History and Physicals and perform LPs.

Roger Mullins, MA, Ph.D. (LNS/NIA)

Dr. Mullins is a Postdoctoral Fellow in the Human Neuroscience Unit under the training of Dr. Kapogiannis. He will be responsible for recruitment and contact with participants,

cognitive testing, MRI/fMRI/MRS, and data analysis. Dr. Mullins has over 5 years of experience with neuropsych assessment and MRI research on patients with neurodegenerative diseases

12. Anticipated benefit

This pilot study of two short-term diets is not expected to generate direct benefits for the participant. While the diets are likely to decrease body weight and increase insulin sensitivity (i.e., decrease HOMA-IR), changes which may be desirable to the participants and their physicians, the changes are likely to be temporary. This is a short-term study and, therefore, the magnitude and duration of these effects and their impact on long-term health is unclear. The study will generate generalizable knowledge on the effects of short-term implementation of 5-2 CR.

13. Classification of Risk

13. a. For adults

Overall, we classify the study as greater than minimal risk.

13. b. For adults without consent capacity

Not applicable.

13. c. For children

Not applicable.

13. d. Overall risk and benefit consideration

The risks are reasonable in relation to anticipated benefit.

14. Consent Documents and Process

14. a. Designation of those obtaining consent

The principal investigator, clinical research coordinator (nurse) and NIA research nurses and nurse practitioners will be authorized to obtain consent. Investigators identified as being authorized to obtain consent in section 11.f. above will be allowed to obtain consent. In addition, NIA research nurses and nurse practitioners are qualified to obtain consent by their training and will also be authorized to obtain consent. All staff obtaining informed consent has completed the NIMH HSPU “Elements of Successful Informed Consent” training.

14. b. Consent procedures

All participants will receive a verbal explanation in terms suited to their comprehension of the purposes, procedures and potential risk 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 regarding this study prior to signing.
When the participant fully understands her responsibilities and possible risks and discomforts and agrees to participate in the current study they will be provided with the consent to sign. A signed copy will be provided to each participant, as well as placed in her medical record. No procedures will begin until the informed consent has been properly obtained.

In particular, the summary study design will be detailed. Furthermore, participants will be fully informed about the data and safety monitoring procedures.

14. c. Consent documents

Consent forms contain all required elements specific to the CNS IRB protocol template (rev 1.27.14). As stated, a consent document for healthy volunteers is submitted with this protocol. No special documents are needed.

15. Data and Safety Monitoring

15. a. Data and safety monitor

The Principal Investigator (PI) will be the data and safety monitor.

15. b. Data and safety monitoring plan

The PI will conduct periodic review of the data every at least 3 months to ensure that data are collected, saved and stored in a consistent manner and according to the Protocol. The PI will conduct periodic review of safety data (clinically significant lab results, H&P sessions, procedure notes, etc.) every at least 3 months, including review of expected AE's occurring more frequently than expected or at greater severity than expected.

15. c. Criteria for stopping the study or suspending enrollment or procedures

Temporary suspension of enrollment will occur if any related AE of grade 3 or higher occurs, in anticipation of its review by the NIA Clinical Director and the IRB. Study procedures for participants already enrolled may continue, unless otherwise instructed by the NIA Clinical Director and/or the IRB. The study may resume enrollment after the NIA Clinical Director and the IRB have given us permission to do so.

16. Quality assurance

16. a. Quality assurance monitor

The PI will be responsible for QA monitoring of this study. The PI will address any issues discovered. In addition the NIA Clinical Director may randomly review charts for Quality assurance.

16. b. Quality assurance plan

The PI will acquaint the research team with the basic principles of the ICH-GCP guidelines. The Pi will ensure that the research team is following the protocol, complying with regulatory policies, and collecting and reporting data according to the protocol. The PI will delegate to an experienced research professional to conduct audit on 10-20% of participant's research charts once the participant has completed the study. In addition the Clinical Director may randomly review charts for quality assurance. The Quality Assurance designee will assess adherence to study procedures and timelines; review roles/responsibilities of research team members; clinical and research data collection; any AE or UPs. The PI will address any issues discovered.

17. Reporting of Unanticipated Problems, Adverse Events and Protocol Deviations

The Principal Investigator is responsible for detecting, documenting, and reporting unanticipated problems, adverse events (AEs), including serious adverse events (SAEs), and deviations in accordance with NIH policy, IRB requirements, and federal regulations. Relatedness to the research of all serious adverse events will be determined by the PI in consultation with the Clinical Director.

Serious unanticipated problems, serious adverse events (including deaths) that are not unanticipated problems, and serious protocol deviations will be reported to the IRB and CD as soon as possible and in writing not more than 7 days after the PI first learns of the event, unless immediate reporting is waived for specific serious adverse events as noted below. Not serious unanticipated problems and not serious deviations will be reported to the IRB and CD as soon as possible and in writing not more than 14 days after the PI first learns of the event. Written reports will be submitted in PTMS.

All adverse events, deviations, and unanticipated problems will be summarized and reported at the time of Continuing Review. The following expected adverse events will not be reported at the time of Continuing Review unless they occur at a severity or frequency greater than expected. Adverse events occurring at a greater severity or frequency than expected will be reported as Unanticipated Problems. These events include syncope or presyncope that occur during or after blood draws that may occur up

to 5% of the participants, laboratory values outside the normal range with no potential clinical significance that may occur up to 5% of the participants, or incidental findings on MRI that are non-life threatening and are not requiring further medically-indicated diagnostic testing or treatment that may occur up to 5% of the participants.

18. Alternatives to Participation

Subjects do not receive any treatment in this study or forego any treatment in order to participate in this study. The alternative, therefore, is not to participate.

19. Privacy

All research activities will be conducted in as private a setting as possible. All interactions will take place in private settings, limiting personnel present.

20. Confidentiality

20. a. For research data and investigator medical records

We will use hard copy case report forms to record clinical and research data, which will be kept in the participants' charts.

All hard copy research data and investigator medical records will be held at the nurse's station during the participant's visit and stored in a locked cabinet within a locked room when unoccupied. Biomarker levels, cognitive test results and MRS/fMRI images will be saved electronically on a secure NIA/NIH intranet with access limited to authorized NIA staff members and secure password protected NIA computers. All NIA members who have access to these databases have the proper training on participant confidentiality as well as the required Human Subject Protection Training.

When results of an NIA/NIH research study are reported in medical journals or at scientific meetings, the people who take part are not named and identified. The NIA/NIH will not release any information about the participant's research involvement without their written permission. However, if they sign a release form, for example, for an insurance company, the NIA/NIH will give the insurance company information from their medical record. This information might affect (either favorably or unfavorably) the willingness of the insurance company to sell them insurance.

In research collaborations all participants will be identified by a code rather than by their name. The results are research material and are strictly confidential. Information will not be released without the participant's consent, but may be reviewed by other researchers for scientific purposes.

All of the tests in this protocol are done for research purposes only. It is not our policy

to return research results to participants, however, if we find information that is important to the participant's health or well-being, we will return the results to them. In this event, participants will be referred to their private physician. If the participant requests a copy of lab results, those done under CLIA certification will be provided to the participant. For tests not done in a CLIA certified lab, including genetic results, referrals to CLIA certified labs in the participant's area will be provided. We are unable to pay for CLIA testing. The results or the scan from the MRI study will be provided to their physician with participants' written permission.

The genetic studies in this protocol are not done in a CLIA certified lab. We test for the APOE ε4 gene in this study, which increases the risk of Alzheimer's disease and cardiovascular disease, although it does not mean that whomever carries that gene will develop Alzheimer's disease or coronary artery disease. Participants who wish to know these results will meet with the principal investigator, who will consult them on the on the need for confirmation by a CLIA certified lab, and the significance of their particular genotype on AD and cardiovascular risk based on the literature. Genetic counseling will be available.

20. b. For stored samples

Biological samples (whole blood, plasma, serum, CSF, extracted DNA and RNA) will be stored at the NIA Core Lab or one of our licensed facilities and released to study investigators and collaborators by the Principal Investigator for analysis. Participants' names and identifying information will not be on the samples; samples will be identified by a code. The key to the code will be kept in a separate, secure area.

If a participant withdraws from this research project before it is complete, any samples obtained before she withdraws will be kept and her privacy will be protected.

20. c. Special precautions

Hard copy data/records will be locked within a cabinet inside of a locked room when occupied. Samples without identifiers will be in a single lock container. Electronic data will be stored on an encrypted, password protected NIH computer or encrypted NIH servers. Only study investigators will have access to the samples and data, as well as the NIA Clinical Director and IRB monitors or auditors as part of their supervisory duties.

Finally, de-identified results from clinical trials will be posted on clintrials.gov.

21. Conflict of interest

21. a. Distribution of NIH guidelines

NIH guidelines on conflict of interest have been distributed to all investigators.

21. b. Conflict of interest

There are no conflicts of interest to report.

21. c. Role of a commercial company or sponsor

There is no commercial company or sponsor for the study.

22. Technology transfer

The authors entered into a technology transfer agreement with Dr. Joel Kramer of the University of California – San Francisco for the utilization of the software EXAMINER.

23. Research and Travel Compensation

All participants will be compensated for their time and research-related inconveniences via NIA debit cards. Payments will be given as stated below. Compensation will typically be prorated for parts completed if the participants do not complete the study. Participants will be compensated for each visit as follows:

Screening	$50
Baseline	$350
Week # 2 Visit	$150
Week # 4 Visit	$150
Week # 6 Visit	$150
Week # 8 Visit	$500

Total compensation will be $1,350 for participants that complete the study. If they do not complete the study or if the study physician needs to extend their participation due to a missed visit, they will be paid for those parts completed; blood draw $20, Mixed Meal Test (MMT) $50, MRI $50, lumbar puncture $150, cognitive testing $20, calorie restriction for one week $50, accelerometer $10/day to wear.

Participants will be offered a meal on the Screening Visit, Baseline Visit, and Outcome Visit.

No travel or lodging compensation is provided, because all subjects will be recruited from the greater Baltimore area. No escort fee will be provided.

24. References

1. Kapogiannis, D. & Mattson, M.P. Disrupted energy metabolism and neuronal circuit dysfunction in cognitive impairment and Alzheimer's disease. *Lancet neurology* **10**, 187-198 (2011).
2. Goldstein, B.J. Insulin resistance as the core defect in type 2 diabetes mellitus. *Am J Cardiol* **90**, 3G-10G (2002).
3. Schrijvers, E.M.*, et al.* Insulin metabolism and the risk of Alzheimer disease: the Rotterdam Study. *Neurology* **75**, 1982-1987 (2010).
4. Cersosimo, E. & DeFronzo, R.A. Insulin resistance and endothelial dysfunction: the road map to cardiovascular diseases. *Diabetes Metab Res Rev* **22**, 423-436 (2006).
5. Gasparini, L.*, et al.* Stimulation of beta-amyloid precursor protein trafficking by insulin reduces intraneuronal beta-amyloid and requires mitogen-activated protein kinase signaling. *J Neurosci* **21**, 2561-2570 (2001).
6. Watson, G.S.*, et al.* Insulin increases CSF Abeta42 levels in normal older adults. *Neurology* **60**, 1899-1903 (2003).
7. Zhao, W.Q.*, et al.* Insulin receptor dysfunction impairs cellular clearance of neurotoxic oligomeric a-beta. *The Journal of biological chemistry* **284**, 18742-18753 (2009).
8. Planel, E.*, et al.* Insulin dysfunction induces in vivo tau hyperphosphorylation through distinct mechanisms. *J Neurosci* **27**, 13635-13648 (2007).
9. Messier, C. & Teutenberg, K. The role of insulin, insulin growth factor, and insulin-degrading enzyme in brain aging and Alzheimer's disease. *Neural Plast* **12**, 311-328 (2005).
10. Baker, L.D.*, et al.* Insulin resistance and Alzheimer-like reductions in regional cerebral glucose metabolism for cognitively normal adults with prediabetes or early type 2 diabetes. *Arch Neurol* **68**, 51-57 (2011).
11. Talbot, K.*, et al.* Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J Clin Invest* **122**, 1316-1338 (2012).
12. Craft, S. & Watson, G.S. Insulin and neurodegenerative disease: shared and specific mechanisms. *Lancet Neurol* **3**, 169-178 (2004).
13. Henneberg, N. & Hoyer, S. Desensitization of the neuronal insulin receptor: a new approach in the etiopathogenesis of late-onset sporadic dementia of the Alzheimer type (SDAT)? *Archives of gerontology and geriatrics* **21**, 63-74 (1995).
14. Zhao, W.Q., Chen, H., Quon, M.J. & Alkon, D.L. Insulin and the insulin receptor in experimental models of learning and memory. *European journal of pharmacology* **490**, 71-81 (2004).
15. Blalock, E.M.*, et al.* Aging-related gene expression in hippocampus proper compared with dentate gyrus is selectively associated with metabolic syndrome variables in rhesus monkeys. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**, 6058-6071 (2010).

16. Willette, A.A., *et al.* Calorie restriction reduces the influence of glucoregulatory dysfunction on regional brain volume in aged rhesus monkeys. *Diabetes* **61**, 1036-1042 (2012).
17. Raji, C.A., *et al.* Brain structure and obesity. *Human brain mapping* **31**, 353-364 (2010).
18. Rasgon, N.L., *et al.* Insulin resistance and hippocampal volume in women at risk for Alzheimer's disease. *Neurobiology of aging* **32**, 1942-1948 (2011).
19. Garcia-Casares, N., *et al.* Structural and functional brain changes in middle-aged type 2 diabetic patients: a cross-sectional study. *J Alzheimers Dis* **40**, 375-386 (2014).
20. Francis, G.J., *et al.* Intranasal insulin prevents cognitive decline, cerebral atrophy and white matter changes in murine type I diabetic encephalopathy. *Brain : a journal of neurology* **131**, 3311-3334 (2008).
21. Burns, S.F., Kelsey, S.F. & Arslanian, S.A. Effects of an intravenous lipid challenge and free fatty acid elevation on in vivo insulin sensitivity in African American versus Caucasian adolescents. *Diabetes Care* **32**, 355-360 (2009).
22. Convit, A., Wolf, O.T., Tarshish, C. & de Leon, M.J. Reduced glucose tolerance is associated with poor memory performance and hippocampal atrophy among normal elderly. *Proc Natl Acad Sci U S A* **100**, 2019-2022 (2003).
23. Wilson, P.W. & Kannel, W.B. Obesity, diabetes, and risk of cardiovascular disease in the elderly. *The American journal of geriatric cardiology* **11**, 119-123,125 (2002).
24. Brun, J.F., *et al.* Assessment of insulin sensitivity (S (I)) and glucose effectiveness (S (G)) from a standardized hyperglucidic breakfast test in type 2 diabetics exhibiting various levels of insulin resistance. *Acta diabetologica* (2010).
25. Malita, F.M., *et al.* Comparison between several insulin sensitivity indices and metabolic risk factors in overweight and obese postmenopausal women: a MONET study. *Nutrition, metabolism, and cardiovascular diseases : NMCD* **20**, 173-179 (2010).
26. Sainaghi, P.P., Castello, L., Bergamasco, L., Carnevale Schianca, G.P. & Bartoli, E. Metabolic characteristics of glucose intolerance: the critical role of obesity. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association* **116**, 86-93 (2008).
27. Matsuzawa, Y., Funahashi, T. & Nakamura, T. The concept of metabolic syndrome: contribution of visceral fat accumulation and its molecular mechanism. *Journal of atherosclerosis and thrombosis* **18**, 629-639 (2011).
28. Colman, R.J., *et al.* Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science* **325**, 201-204 (2009).
29. Martin, C.K., O'Neil, P.M. & Pawlow, L. Changes in food cravings during low-calorie and very-low-calorie diets. *Obesity (Silver Spring)* **14**, 115-121 (2006).
30. Weindruch, R. & Sohal, R.S. Seminars in medicine of the Beth Israel Deaconess Medical Center. Caloric intake and aging. *N Engl J Med* **337**, 986-994 (1997).
31. Mattison, J.A., *et al.* Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. *Nature* **489**, 318-321 (2012).

32. Anson, R.M., *et al.* Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 6216-6220 (2003).
33. Wan, R., Camandola, S. & Mattson, M.P. Intermittent fasting and dietary supplementation with 2-deoxy-D-glucose improve functional and metabolic cardiovascular risk factors in rats. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **17**, 1133-1134 (2003).
34. Wan, R., Camandola, S. & Mattson, M.P. Dietary supplementation with 2-deoxy-D-glucose improves cardiovascular and neuroendocrine stress adaptation in rats. *American journal of physiology. Heart and circulatory physiology* **287**, H1186-1193 (2004).
35. Arumugam, T.V., *et al.* Age and energy intake interact to modify cell stress pathways and stroke outcome. *Annals of neurology* **67**, 41-52 (2010).
36. Craft, S. The role of metabolic disorders in Alzheimer disease and vascular dementia: two roads converged. *Archives of neurology* **66**, 300-305 (2009).
37. Stranahan, A.M., *et al.* Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats. *Hippocampus* **18**, 1085-1088 (2008).
38. Willette, A.A., *et al.* Age-related changes in neural volume and microstructure associated with interleukin-6 are ameliorated by a calorie-restricted diet in old rhesus monkeys. *NeuroImage* **51**, 987-994 (2010).
39. Johnson, J.B., *et al.* Alternate day calorie restriction improves clinical findings and reduces markers of oxidative stress and inflammation in overweight adults with moderate asthma. *Free Radic Biol Med* **42**, 665-674 (2007).
40. Harvie, M.N., *et al.* The effects of intermittent or continuous energy restriction on weight loss and metabolic disease risk markers: a randomized trial in young overweight women. *Int J Obes (Lond)* **35**, 714-727 (2011).
41. Snijder, M.B., *et al.* The prediction of visceral fat by dual-energy X-ray absorptiometry in the elderly: a comparison with computed tomography and anthropometry. *Int J Obes Relat Metab Disord* **26**, 984-993 (2002).
42. Wilson, P.W.F. & Kannel, W.B. Obesity, Diabetes, and Risk of Cardiovascular Disease in the Elderly. *The American Journal of Geriatric Cardiology* **11**, 119-124 (2002).
43. Bookheimer, S. & Burggren, A. APOE-4 Genotype and Neurophysiological Vulnerability to Alzheimer's and Cognitive Aging. *Annu Rev Clin Psycho* **5**, 343-362 (2009).
44. Carmelli, D., *et al.* Midlife cardiovascular risk factors, ApoE, and cognitive decline in elderly male twins. *Neurology* **50**, 1580-1585 (1998).
45. Kim, H.J., *et al.* Effects of APOE epsilon4 on brain amyloid, lacunar infarcts, and white matter lesions: a study among patients with subcortical vascular cognitive impairment. *Neurobiol Aging* **34**, 2482-2487 (2013).
46. Protas, H.D., *et al.* Posterior cingulate glucose metabolism, hippocampal glucose metabolism, and hippocampal volume in cognitively normal, late-middle-aged

persons at 3 levels of genetic risk for Alzheimer disease. *JAMA Neurol* **70**, 320-325 (2013).
47. Yi, D.*, et al.* Beta-Amyloid Associated Differential Effects of APOE epsilon4 on Brain Metabolism in Cognitively Normal Elderly. *Am J Geriatr Psychiatry* (2014).
48. Cholerton, B.*, et al.* Insulin and sex interactions in older adults with mild cognitive impairment. *J Alzheimers Dis* **31**, 401-410 (2012).
49. Schmid, R.S. & Maness, P.F. L1 and NCAM adhesion molecules as signaling coreceptors in neuronal migration and process outgrowth. *Current opinion in neurobiology* **18**, 245-250 (2008).
50. Fiandaca, M.S.*, et al.* Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study. *Alzheimers Dement* (2014).
51. Kapogiannis, D.*, et al.* Dysfunctionally phosphorylated type 1 insulin receptor substrate in neural-derived blood exosomes of preclinical Alzheimer's disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* (2014).
52. Matthews, D.R.*, et al.* Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28**, 412-419 (1985).
53. Pacini, G. & Mari, A. Methods for clinical assessment of insulin sensitivity and beta-cell function. *Best practice & research. Clinical endocrinology & metabolism* **17**, 305-322 (2003).
54. Buss, R.W., Kansal, P.C., Roddam, R.F., Pino, J. & Boshell, B.R. Mixed meal tolerance test and reactive hypoglycemia. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* **14**, 281-283 (1982).
55. Gilhooly, C.H.*, et al.* Food cravings and energy regulation: the characteristics of craved foods and their relationship with eating behaviors and weight change during 6 months of dietary energy restriction. *Int J Obes (Lond)* **31**, 1849-1858 (2007).
56. Harvey, J., Wing, R.R. & Mullen, M. Effects on food cravings of a very low calorie diet or a balanced, low calorie diet. *Appetite* **21**, 105-115 (1993).
57. Kober, H.*, et al.* Prefrontal-striatal pathway underlies cognitive regulation of craving. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 14811-14816 (2010).
58. Siep, N.*, et al.* Fighting food temptations: the modulating effects of short-term cognitive reappraisal, suppression and up-regulation on mesocorticolimbic activity related to appetitive motivation. *NeuroImage* **60**, 213-220 (2012).
59. Beason-Held, L.L. Dementia and the default mode. *Current Alzheimer research* **8**, 361-365 (2011).
60. Wang, L.*, et al.* Cerebrospinal fluid Abeta42, phosphorylated Tau181, and resting-state functional connectivity. *JAMA Neurol* **70**, 1242-1248 (2013).
61. Nestor, L.J., Ghahremani, D.G., Monterosso, J. & London, E.D. Prefrontal hypoactivation during cognitive control in early abstinent methamphetamine-dependent subjects. *Psychiatry research* **194**, 287-295 (2011).

62. Stroop, J. Studies of interference in serial verbal reactions. *Journal of Experimental Psychology* **18**, 643-662 (1935).
63. Kapogiannis, D., Reiter, D.A., Willette, A.A. & Mattson, M.P. Posteromedial cortex glutamate and GABA predict intrinsic functional connectivity of the default mode network. *NeuroImage* **64**, 112-119 (2013).
64. Rupsingh, R., Borrie, M., Smith, M., Wells, J.L. & Bartha, R. Reduced hippocampal glutamate in Alzheimer disease. *Neurobiol Aging* **32**, 802-810 (2011).
65. Witte, A.V., Fobker, M., Gellner, R., Knecht, S. & Floel, A. Caloric restriction improves memory in elderly humans. *Proc Natl Acad Sci U S A* **106**, 1255-1260 (2009).
66. Verhaeghen, P. & Salthouse, T.A. Meta-analyses of age-cognition relations in adulthood: estimates of linear and nonlinear age effects and structural models. *Psychol Bull* **122**, 231-249 (1997).
67. Willette, A.A.*, et al.* Insulin Resistance, Brain Atrophy, and Cognitive Performance in Late Middle-Aged Adults. *Diabetes care* (2012).
68. Kerti, L.*, et al.* Higher glucose levels associated with lower memory and reduced hippocampal microstructure. *Neurology* **81**, 1746-1752 (2013).
69. Tan, Z.S.*, et al.* Association of metabolic dysregulation with volumetric brain magnetic resonance imaging and cognitive markers of subclinical brain aging in middle-aged adults: the Framingham Offspring Study. *Diabetes Care* **34**, 1766-1770 (2011).
70. Valcour, V.G.*, et al.* Insulin resistance is associated with cognition among HIV-1-infected patients: the Hawaii Aging With HIV cohort. *J Acquir Immune Defic Syndr* **43**, 405-410 (2006).
71. Crawford, J.R. & Henry, J.D. The positive and negative affect schedule (PANAS): construct validity, measurement properties and normative data in a large non-clinical sample. *The British journal of clinical psychology / the British Psychological Society* **43**, 245-265 (2004).
72. Dumurgier, J.*, et al.* CSF Abeta(1)(-)(4)(2) levels and glucose metabolism in Alzheimer's disease. *Journal of Alzheimer's disease : JAD* **27**, 845-851 (2011).
73. Weiner, M.W.*, et al.* The Alzheimer's disease neuroimaging initiative: progress report and future plans. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **6**, 202-211 e207 (2010).
74. Bosco, D., Fava, A., Plastino, M., Montalcini, T. & Pujia, A. Possible implications of insulin resistance and glucose metabolism in Alzheimer's disease pathogenesis. *Journal of cellular and molecular medicine* **15**, 1807-1821 (2011).
75. Diniz, B.S. & Teixeira, A.L. Brain-derived neurotrophic factor and Alzheimer's disease: physiopathology and beyond. *Neuromolecular medicine* **13**, 217-222 (2011).
76. Hickman, S.E., Allison, E.K. & El Khoury, J. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 8354-8360 (2008).

77. Vom Berg, J.*, et al.* Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline. *Nature medicine* **18**, 1812-1819 (2012).
78. Kahn, M.S.*, et al.* Prolonged elevation in hippocampal Abeta and cognitive deficits following repeated endotoxin exposure in the mouse. *Behavioural brain research* **229**, 176-184 (2012).
79. Cowley, T.R.*, et al.* Rosiglitazone attenuates the age-related changes in astrocytosis and the deficit in LTP. *Neurobiology of aging* **33**, 162-175 (2012).
80. Moreira, P.I.*, et al.* Oxidative stress and neurodegeneration. *Annals of the New York Academy of Sciences* **1043**, 545-552 (2005).
81. Mitsuhashi, M.*, et al.* Aging enhances release of exosomal cytokine mRNAs by Abeta1-42-stimulated macrophages. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **27**, 5141-5150 (2013).
82. Plank, M., Wuttke, D., van Dam, S., Clarke, S.A. & de Magalhaes, J.P. A meta-analysis of caloric restriction gene expression profiles to infer common signatures and regulatory mechanisms. *Mol Biosyst* **8**, 1339-1349 (2012).
83. Civitarese, A.E.*, et al.* Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. *PLoS Med* **4**, e76 (2007).
84. Capel, F.*, et al.* Macrophages and adipocytes in human obesity: adipose tissue gene expression and insulin sensitivity during calorie restriction and weight stabilization. *Diabetes* **58**, 1558-1567 (2009).
85. Bouchard, L.*, et al.* Differential epigenomic and transcriptomic responses in subcutaneous adipose tissue between low and high responders to caloric restriction. *Am. J. Clin. Nutr.* **91**, 309-320 (2010).
86. Marquez-Quinones, A.*, et al.* Adipose tissue transcriptome reflects variations between subjects with continued weight loss and subjects regaining weight 6 mo after caloric restriction independent of energy intake. *Am. J. Clin. Nutr.* **92**, 975-984 (2010).
87. Milagro, F.I.*, et al.* A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. *FASEB J.* **25**, 1378-1389 (2011).
88. Dixon, J.B. & O'Brien, P.E. Obesity and the white blood cell count: changes with sustained weight loss. *Obes. Surg.* **16**, 251-257 (2006).
89. de la Monte, S.M. & Wands, J.R. Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. *Journal of Alzheimer's disease : JAD* **7**, 45-61 (2005).
90. Heilbronn, L.K.*, et al.* Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. *JAMA : the journal of the American Medical Association* **295**, 1539-1548 (2006).
91. Noda, K.*, et al.* Lifestyle changes through the use of delivered meals and dietary counseling in a single-blind study. The STYLIST study. *Circulation journal : official journal of the Japanese Circulation Society* **76**, 1335-1344 (2012).

92. Allen, J.K., Stephens, J., Dennison Himmelfarb, C.R., Stewart, K.J. & Hauck, S. Randomized controlled pilot study testing use of smartphone technology for obesity treatment. *Journal of obesity* **2013**, 151597 (2013).
93. Macfarlane, D.J. & Thomas, G.N. Exercise and diet in weight management: updating what works. *British journal of sports medicine* **44**, 1197-1201 (2010).
94. Williams, K.V., Mullen, M.L., Kelley, D.E. & Wing, R.R. The effect of short periods of caloric restriction on weight loss and glycemic control in type 2 diabetes. *Diabetes care* **21**, 2-8 (1998).
95. Schulte, R.F., Lange, T., Beck, J., Meier, D. & Boesiger, P. Improved two-dimensional J-resolved spectroscopy. *NMR Biomed* **19**, 264-270 (2006).
96. Schulte, R.F. & Boesiger, P. ProFit: two-dimensional prior-knowledge fitting of J-resolved spectra. *Nmr in Biomedicine* **19**, 255-263 (2006).
97. Bolliger, C.S., Boesch, C. & Kreis, R. On the use of Cramer-Rao minimum variance bounds for the design of magnetic resonance spectroscopy experiments. *Neuroimage* **83**, 1031-1040 (2013).
98. Debey-Pascher, S., Eggle, D. & Schultze, J.L. RNA stabilization of peripheral blood and profiling by bead chip analysis. *Methods Mol Biol* **496**, 175-210 (2009).
99. (!!! INVALID CITATION !!!).
100. Mumford, J.A. & Nichols, T.E. Power calculation for group fMRI studies accounting for arbitrary design and temporal autocorrelation. *NeuroImage* **39**, 261-268 (2008).
101. Weiss, E.P.*, et al.* Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial. *The American journal of clinical nutrition* **84**, 1033-1042 (2006).
102. Bruce, A.S.*, et al.* A comparison of functional brain changes associated with surgical versus behavioral weight loss. *Obesity (Silver Spring)* **22**, 337-343 (2014).
103. Zhang, H.*, et al.* Intranasal Insulin Enhanced Resting-State Functional Connectivity of Hippocampal Regions in Type 2 Diabetes. *Diabetes* (2014).
104. Craft, S.*, et al.* Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. *Archives of neurology* **69**, 29-38 (2012).
105. Elias, M.F., Goodell, A.L. & Waldstein, S.R. Obesity, cognitive functioning and dementia: back to the future. *Journal of Alzheimer's disease : JAD* **30 Suppl 2**, S113-125 (2012).
106. Reinert, K.R., Po'e, E.K. & Barkin, S.L. The relationship between executive function and obesity in children and adolescents: a systematic literature review. *Journal of obesity* **2013**, 820956 (2013).

25. Attachments/Appendices

Please see subsequent pages for attachments and appendices.

ATTACHMENT A. Pre-Screen Checklist

Calorie Restriction Checklist – Inclusion and Exclusion Criteria (partial)

INCLUSION CRITERIA

☐ BMI ≥ 27; weight ≤ 350 lbs
☐ Age of 55-70 years

EXCLUSION CRITERIA

☐ History of cardiovascular disease
☐ History of cerebrovascular or other neurological diseases
☐ History of substance abuse in the past 6 months or positive urine drug screen
☐ History of clinically significant endocrine disorders (e.g. hypothyroidism)
☐ History of eating disorders, significant GI disorders or malabsorption disorders
☐ History of type 2 diabetes and/or use of anti-diabetes medications or insulin;
☐ History of hypoglycemia
☐ Current use of systemic corticosteroids
☐ Chronic HIV, HCV or HBV infections
☐ Current use of immunosuppressive agents (any)
☐ Contraindications for MRI (pacemakers, ferrous metal implants or shrapnel in or around the head, etc.)
☐ Contraindications to LP, such as Coumadin, known coagulopathy. Aspirin 81 mg qd is allowed. Aspirin up to 325 mg qd is allowed, if withheld for 7 days prior to the LP.
☐ Pregnancy or nursing.

ATTACHMENT B. ADVERTISEMENT BROCHURE AND FLIER

Recruitment materials are available as separate documents.

ATTACHMENT C. Activity Log

Activity Log (Week X)

Instructions: For each day you wear the physical activity bracelet, please write down the times when you woke up, bathed, exercised, and went to bed. If one of these activities did not occur, you can leave it blank.

Day 1

Woke up: _____
Bathed: _____
Exercised: _____
Went to bed: _____

Day 2

Woke up: _____
Bathed: _____
Exercised: _____
Went to bed: _____

Day 3

Woke up: _____
Bathed: _____
Exercised: _____
Went to bed: _____

Day 4

Woke up: _____
Bathed: _____
Exercised: _____
Went to bed: _____

ATTACHMENT D. PANAS

Positive and Negative Affective Schedule (PANAS)

Intermittent Calorie Restriction

Instruction: "Do you feel this way right now?"

	Very slighty or not at all	A little	Moderately	Quite a Bit	Extremely
Interested					
Distressed					
Excited					
Upset					
Strong					
Guilty					
Scared					
Hostile					
Enthusiastic					
Proud					
Irritable					
Alert					
Ashamed					
Inspired					
Nervous					
Determined					
Attentive					
Jittery					
Active					
Afraid					

Participant ID: _____

Investigator/Staff Name: _____

Symptoms Review Questionnaire

Intermittent Calorie Restriction

Since **last week**, on a 1-10 scale, please rate how you felt on **NON-CR DAYS for:**

	Less → More
Amount of Energy	0 1 2 3 4 5 6 7 8 9 10
Ability to Concentrate	0 1 2 3 4 5 6 7 8 9 10
Constipation	0 1 2 3 4 5 6 7 8 9 10
Headache Severity (if any)	0 1 2 3 4 5 6 7 8 9 10
Feeling Cold	0 1 2 3 4 5 6 7 8 9 10
Feeling Sad/Depressed	0 1 2 3 4 5 6 7 8 9 10
Feeling Irritable	0 1 2 3 4 5 6 7 8 9 10
Feeling Anxious	0 1 2 3 4 5 6 7 8 9 10
Feeling Hungry	0 1 2 3 4 5 6 7 8 9 10
Preoccupation with Food	0 1 2 3 4 5 6 7 8 9 10

Since **last week**, on a scale from 1-10, please rate how you felt on **CR DAYS for:**

	Less → More
Amount of Energy	0 1 2 3 4 5 6 7 8 9 10
Ability to Concentrate	0 1 2 3 4 5 6 7 8 9 10
Constipation	0 1 2 3 4 5 6 7 8 9 10
Headache Severity (if any)	0 1 2 3 4 5 6 7 8 9 10
Feeling Cold	0 1 2 3 4 5 6 7 8 9 10
Feeling Sad/Depressed	0 1 2 3 4 5 6 7 8 9 10
Feeling Irritable	0 1 2 3 4 5 6 7 8 9 10
Feeling Anxious	0 1 2 3 4 5 6 7 8 9 10
Feeling Hungry	0 1 2 3 4 5 6 7 8 9 10
Preoccupation with Food	0 1 2 3 4 5 6 7 8 9 10

Have you experienced any other symptoms that you did not have **before going on the diet**? If so, please write down those symptoms here:

Participant ID: _____
Investigator/Staff Name: _____
Date: _____

ATTACHMENT E. Symptoms Review Questionnaire for "Healthy Living Diet" participants

Symptoms Review Questionnaire

Healthy Living Diet

Since **last week**, on a scale from 1-10, please rate these items for how you felt

	Less → More
Amount of Energy	0 1 2 3 4 5 6 7 8 9 10
Ability to Concentrate	0 1 2 3 4 5 6 7 8 9 10
Constipation	0 1 2 3 4 5 6 7 8 9 10
Headache Severity (if any)	0 1 2 3 4 5 6 7 8 9 10
Feeling Cold	0 1 2 3 4 5 6 7 8 9 10
Feeling Sad/Depressed	0 1 2 3 4 5 6 7 8 9 10
Feeling Irritable	0 1 2 3 4 5 6 7 8 9 10
Feeling Anxious	0 1 2 3 4 5 6 7 8 9 10
Feeling Hungry	0 1 2 3 4 5 6 7 8 9 10
Preoccupation with Food	0 1 2 3 4 5 6 7 8 9 10

Have you experienced any other symptoms that you did not have **before going on the diet**? If so, please write down those symptoms here:

Participant ID: _____

Investigator/Staff Name: _____

Date: _____

26. Consent forms

Please see attached consent forms for healthy adult volunteers.