STUDY PROTOCOL

Title: **Dietary Ketosis a Metabolic Sister to Calorie Restriction (CR): Fatty acids activate AMPK energy circuits modulating global methylation via the SAM/SAH axis**

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Title of Study: **Dietary Ketosis a Metabolic Sister to Calorie Restriction (CR): Fatty acids activate AMPK energy circuits modulating global methylation via the SAM/SAH axis**

Principle Investigator: Dr. Kelly J. Gibas, CFMP, LPCC

- Protocol Number: Bristlecone-001
- Protocol Date: 09/15/2017 – 05/15/2018
- Version #1
- Investigators: Dr. Kelly J. Gibas (Principle Investigator)

  Dr. Julie A. Gomer (Sub-investigator)

- Bristlecone Health, Inc.

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- Source of external funding: N/A
- Hypothesis or Study Synopsis:

Nutritional epigenetics denotes gene–diet interactions and highlights the modulatory role of nutrition in aging and age-related diseases such as cancer, CVD, diabetes and neurodegenerative disorders. Nutrients are a source of epigenetic modification; they are able to regulate the placement of histone modifiers distinguishing phenotype from genotype. The energy status of the cells (fed or fasted) modulates the regulation of global DNA methylation via the S-adenosylmethionine (SAM), the methyltransferase inhibitor, S-adenosylhomocysteine (SAH) axis and whole blood histamine levels. Insulin resistance and hyperinsulinemia dysregulate cellular signals leading to metabolic inflexibility. Chronic elevations in insulin with long-standing impairments in glucose delivery are associated with profound changes in epigenetic patterns due to over-activation of the mTOR kinase pathway and repression of AMPK. Dietary ketosis is known to change the metabolic status of the cells by increasing the AMP/ATP ratio. AMPK activation adapts rRNA synthesis away from growth/biosynthesis and toward ATP availability and utilization, thereby, attenuating the progression of hypo-metabolic diseases in the body and the brain at the level of the genome.

The study will explore whether early stage memory loss (SMC & MCI) and the comorbidity of Metabolic Syndrome are symptomatic of peripheral and cerebral cellular hypo-metabolism induced by chronic insulin resistance. We will attempt to show that consequential to systemic hyperinsulinimia, aberrant crosstalk between the mitochondria and nuclear genome results in the dysregulation of the regulatory kinases mediating metabolic state and intracellular/extracellular signaling: mTOR and AMPK. The suppression of AMPK signals with chronic overexpression of

mTOR signaling will adapt rRNA synthesis away from nutrient availability and toward ATP consuming processes: the biosynthesis of cholesterol, triglycerides, glycogen with inhibition of fatty acid oxidation, histone acetylation with a down-regulation of NAD+ and SAHH cofactors leading to global DNA hypo-methylation and local hyper-methylation, suppression of the SAM/SAH ratio, the inhibition of SIRT (sirtuin) expression and normalized whole blood histamine levels. These epigenetic shifts mediate global metabolic inflexibility by channeling fuel substrates toward cytosolic, substrate level phosphorylation (SLP) via over expression of the glycolytic enzymes including PDK (pyruvate dehydrogenase kinase) and away from mitochondrial oxidation mediated by the suppression of PDC (pyruvate dehydrogenase complex), the major regulatory gateway of metabolism between glycolysis and citric acid cycle. We will attempt to show that activation of the AMPK pathway via induced and controlled dietary ketosis will inhibit mTOR signaling away from the biosynthesis of energy and SLP toward the generation of ATP by increasing the cellular AMP/ATP ratio, thus regulating oxidative metabolic signals and attenuating global, cellular hypo-metabolism evidenced by marked reductions in lipid synthesis and LP-IR score (particle concentration and size), HgA1c, fasting insulin/HOMA-IR, blood ketones, fasting triglycerides together with epigenetic regulation of DNA methylation status including normalized whole blood histamine levels and homo-cysteine regulation. Improvement in cerebral glucose metabolism and corresponding diagnosis of SMC/MCI will be assessed by the objective changes in the outcome measures of MoCA, BVMT-R (Brief Visual Memory Test-Revised) and Rey Auditory Verbal Learning Task (RAVLT), administered at baseline and weeks 2/4/6/8/10/12.

Research Question: Are selective memory complaints (SMC), mild cognitive impairment (MCI) and comorbidity of Metabolic Syndrome symptomatic of peripheral and cerebral hypo-metabolism with corresponding epigenetic shifts in global DNA hypo-methylation/histone acetylation away from nutrient availability and toward biosynthesis with up-regulation of substrate level phosphorylation (SLP) initiated by sustained metabolic inflexibility (hyperinsulinemia), over-activation of the mTOR kinase pathway, and repression of mitochondrial oxidation via inhibition of pyruvate dehydrogenase complex (PDC)?

- Background:

  Dr. Kelly J. Gibas, CFMP, LPCC

  Doctor of Clinical Behavior Sciences

  Assistant Professor: Human Bioenergetics & Applied Health Science at Bethel University

  Bristlecone Health, Inc.: Founder/CEO/Clinical Director

  Study interests: Metabolome; the study of impaired glucose metabolism an cerebral/somatic epigenetic histone modulation leading to chronic degenerative diseases

  Recent publications:

M.K Gibas, K.J. Gibas, Induced and controlled dietary ketosis as a regulator of obesity

and metabolic syndrome pathologies, Diab Met Syndr: Clin Res Rev (2017), http://dx.doi.org/10.1016/j.dsx.2017.03.022 (RCT, March 2017) PMID:28433617

Gibas, K.J (2017). The Starving Brain: Overfed meets undernourished in the pathology of mild cognitive impairment (MCI) and Alzheimer's disease (AD). *Neurochemistry International,* https://doi.org/10.1016/j.neuint.2017.09.004. PMID: 28899812

Gibas, K.J. The Retrograde Signal: Glucose dependency marks the cancerous phenotype. *Austin Diabetes Research,* Austin Diabetes Res. 2017; 1(1): 1065.

Dahlgren K, Gibas KJ. Ketogenic diet, high intensity interval training (HIIT) and memory training in the treatment of mild cognitive impairment: A case study. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. 2018. doi:10.1016/j.dsx.2018.04.031.

Brown D, Gibas KJ. Metabolic syndrome marks early risk for cognitive decline with APOE4 gene variation: A case study. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. 2018. doi:10.1016/j.dsx.2018.04.030.

Gibas KJ, Halikas A. AMPK induced memory improvements in the diabetic population: A Case Study. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. 2018. doi:10.1016/j.dsx.2018.04.033.

Gibas, K. (December 2018). *Mouth breather? Enduring patterns of hypocapnia induce cerebral hypoxia and consequential cognitive impairments mediated by aberrant HIF-1 survival signals common to the cell danger response.* Poster presentation at Cell Symposia: Metabolites as Signaling Molecules Conference, 2018, Seattle, WA. http://www.cell-symposia.com/metabolites-2018/conference-program.asp

Morrill SJ, Gibas KJ. Ketogenic diet rescues cognition in ApoE4 patient with mild Alzheimer's disease: A case study. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. 2019;13(2):1187-1191. doi:10.1016/j.dsx.2019.01.035.

Cox N, Gibas S, Salisbury M, Gomer J, Gibas K. Ketogenic diets may reverse Type II diabetes and ameliorate clinical depression: A case study. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. 2019. doi:10.1016/j.dsx.2019.01.055.

Stoykovich S, Gibas K. APOE ε4, the door to insulin-resistant dyslipidemia and brain fog? A case study. *Alzheimers & Dementia: Diagnosis, Assessment & Disease Monitoring*. 2019;11:264-269. doi:10.1016/j.dadm.2019.01.009.

Gibas, K. & Stoykovich, S. (April, 2019). *ApoE4, the door to insulin-resistant dyslipidemia and brain fog? A case study.* Poster presentation at American Diabetes Association/WCITD – 2019. New York, NY. https://cmoffice.kenes.com/cmsearchableprogrammeV15/conferencemanager/programme/personid/anonymous/WCITD19/normal/b833d15f547f3cf698a5e922754684fa334885ed#!abstractdetails/0000394330

Study Goals and Objectives

Primary objectives:

1. Improve cerebral and peripheral substrate oxidation as measured by cognitive assessments (MoCA, BVMT-R & RAVLT) and improved blood lipids (reduction in small LDL particle size)
2. Normalizing the SAM/SAH ratio and whole blood histamine levels to restore global DNA methylation for disease prevention
3. Restoration and balancing of the AMPK/mTOR pathways for disease prevention
4. Alleviate insulin resistance in order to reverse T2DM
5. Restore cerebral glucose metabolism in order to prevent neuron death
6. Prevent cardiovascular disease by restoring metabolic flexibility through fatty acid oxidation

Secondary objectives:

1. Improved self-efficacy; empowerment to normalize blood lipids through nutritional interventions
2. Reduction in depression and anxiety associated with metabolic syndrome
3. Reduction in weight, BMI, body fat mass, HgA1c and normalized blood pressure

Subject Selection:

- The study subjects are adults (18-80) referred by their physician and previously diagnosed with MetS and/or T2DM as measured by possessing at least 2 of the following physiological measures: type II diabetes, BMI >30, HgA1c > 5.7, waist/height ratio >.6, fasting glucose > 125. All subjects will volunteer for the study after being referred by their healthcare provider; they will be randomly assigned to one of two groups: experimental group with induced and controlled dietary ketosis or control group with standard American diet. Participants in both groups will also be required to play PEAK brain training games on iPhone, iPad or Android devices for 120 minutes per week.
- The study will include at least 30 subjects (15 per group) but no more than 80 subjects (40 per group).
- Men and women only (ages 18-80)

Inclusion/Exclusion criteria:

Inclusion criteria:

- Male or Female (age 18-80)
- Previously diagnosed with MetS and/or T2DM as measured by possessing at least 2 of the following physiological measures: type II diabetes, BMI >30, HgA1c > 5.7, waist/height ratio >.6, fasting glucose > 125
- Subjective Memory Complaints (SCM) – Subjects score $\geq$ 3 'yes' answers on the Subjective Memory Complaints Questionnaire at the initial visit
- Previously diagnosed with Mild Cognitive Impairment (MCI)

Exclusion criteria:

- Previously diagnosed with Alzheimer's disease (AD), dementia or Parkinson's disease

Study procedures/Research method

- The duration of subjects' participation will be 12 weeks. They will be asked to meet with Dr. Gibas or Dr. Gomer each week at Bristlecone Health, Inc. for approximately 20-30 minutes. During that time, they will receive cognitive assessments (MoCA, BVMT-R, and Rey Auditory Verbal Learning Task), which are memory tests. They will be conducted at Bristlecone Health, Inc. on weeks 0 and 12. Blood ketones and fasting triglycerides will be measured at Bristlecone Health, Inc. on weeks 0, 3, 6, 9 and 12. Participants will also receive weekly weight and body fat measurements.
- Pre/post blood labs will be drawn to measure biomarker changes associated with MetS on weeks 0 and 12 at a North Memorial Reference Laboratory. The following blood labs will be assessed: HgA1c, fasting insulin, fasting glucose, NMR particle size testing and methylation profile, SAM/SAH ratio testing and whole blood histamine. There are 21 North Memorial Reference Laboratories throughout the metro area for convenience.
- Subjects will be randomly assigned to either an experimental or control group in the order of referral (e.g., subject number one assigned to experimental group; subject number two assigned to control group; subject number three assigned to control group, and so on). Regardless of group assignment, the subject will receive the aforementioned cognitive and blood lab assessments.
- Subjects in the experimental group will receive clinically regulated meal plans designed to facilitate prolonged benign dietary ketosis (BDK) in order to regulate glucose with restored insulin sensitivity focused at reversing the impaired capacity to switch between fat and carbohydrate oxidation. Subjects in the control group will follow their current dietary protocol (Standard American Diet-SAD). Both groups will play PEAK brain training game on iPhone, iPad or Android devices for 120 minutes per week.

  RESEARCH METHOD:

  Analyses of variance (ANOVA) will be run to assess differences between the experimental and control group. The ANOVA will be run on both groups measuring baseline and post program (week 0 and week 12) HgA1c, fasting insulin, fasting glucose, NMR particle size testing and a methylation profile, which includes: SAM/SAH ratio test and whole blood histamine.

  An ANOVA will be run on both groups measuring blood ketones and fasting triglycerides at weeks 0, 3, 6, 9 and 12. The

ANOVA will also be run on both groups measuring cognitive measures using the Rey Auditory Verbal Learning Task, BVMT-R and MoCA assessments at weeks 0 and12. Mean scores will be used to assess significant differences between the two groups. Further analyses of possible treatment effects will be examine within-group differences using a paired sampled *t*-test.

Risk / Safety Information

- There are few foreseeable risks associated with this study. No costs are associated with participation nor will the subjects' decision to participate affect their employment/medical care or insurance status. Discomfort associated with study participation may include short-term general malaise commonly associated with dietary changes (if the subject is in the experimental group). Discomfort during blood draws at North Memorial Reference Laboratories may also occur. If subjects experience any discomfort, they will be instructed to inform the phlebotomist immediately. This study is considered to have minimal risk.
- The benefits which may reasonably be expected to result from this study are improved memory as measured by cognitive assessments (MoCA, BVMT-R, and Rey Auditory Verbal Learning Task), reversal of MetS, improved blood lipid biomarkers, decreased HgA1c, increased blood ketone levels, and reduced weight and body fat mass.

Monitoring and reporting of Adverse Events/Serious Adverse Events

- Subjects will receive instructions to contact the principal investigator or sub-investigator for nutritional counsel if they experience general malaise or any discomfort when changing dietary protocol.
- Subjects will receive instructions to inform the phlebotomist at North Memorial Reference Laboratory if they feel any discomfort during blood collection.
- If a subject presents with an adverse effect from following the study protocol, they will be referred back to their primary care physician in order to determine if continued study participation is recommended. Subjects will only be allowed to continue with the study with written permission from their primary care provider.
- Any unanticipated problems will be reported to the IRB within (10) calendar days of being reported to the investigator.

Study Oversight

- The principle investigator (Dr. Kelly J. Gibas) will provide all oversight for the project and supervision all study activities.
- The only other person who will have access to subjects is sub-investigator (Dr. Julie A. Gomer).
- The Investigator will ensure that all study staff are adequately trained in the study protocol

- Both Dr. Gibas and Dr. Gomer have received NIH certification: "Protecting Human Research Participants"

Certificate of Completion

The National Institutes of Health (NIH) Office of Extramural Research certifies that

Kelly Gibas successfully completed the NIH Web-based training course

"Protecting Human Research Participants".

Date of completion: 07/25/2017.

Certification Number: 2440977.

Certificate of Completion

The National Institutes of Health (NIH) Office of Extramural Research certifies that

Julie Gomer successfully completed the NIH Web-based training course

"Protecting Human Research Participants".

Date of completion: 07/30/2017.

Certification Number: 2442927.

- Data Management
  - Who will be collecting, analyzing the data? Dr. Gibas & Dr. Gomer
  - Do you plan to have an independent data analysis? Yes, through the University of Minnesota (Aaron Rendahl, PhD and Yiwen Sun, PhD)
  - Data retention (i.e. how long so you plan to maintain records for this study) – 2 years post study
- IRB Review / Ethics / Informed Consent
  - Explain your informed consent process.

    1. Subjects will be given the informed consent during an individual intake meeting (after being referred by their primary care provider).
    2. Subjects will be provided with the opportunity to take the informed consent home to discuss with their families.
    3. Dr. Gibas or Dr. Gomer will explain the informed consent and answer any questions.
    4. Once the subject signs the consent, they will receive a copy of the signed consent for their records.
- Confidentiality
  - Discuss the procedures that will be used to maintain the **confidentiality of the research data**. Specifically, **how will data be stored to ensure that it is secure and remains confidential**?

- Research data will be used for the purposes of this study only. Copies of medical and clinical measures will be stored in a locked file at Bristlecone Health, Inc. and will only be accessed by Dr. Kelly J. Gibas and Dr. Julie A. Gomer (co-investigator). If a subject decides to withdraw from the study, his/her confidential medical data and clinical measures will be destroyed within 24 hours of receipt of the written request to withdraw from the study

- Data collection and reporting for the purposes of this study will remain confidential and subject identity will not be identifiable. Each subject will be given a unique ID code for the purposes of data analysis. Upon completion of the study, subjects have 8 weeks to obtain a copy of their clinical data measures. The data will be retained for 2 years post study.
- Data may be published in certain journals. Upon publication of the aforementioned study, all protected health information (PHI) will be protected and subjects' identities will not be disclosed in the publication or any other forum.