Determinants of Alpha-aminoadipic Acid (2-AAA) and Relationship to Diabetes: Study 2

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Determinants of alpha-aminoadipic acid (2-AAA) and relationship to diabetes:

Study 2

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1.0 Background

Diabetes is a major global health concern, associated with significantly increased mortality and high incidence of co-morbidities[1]. Given the high rate of cardiovascular and other diseases in individuals with diabetes, diabetes-related mortality may be under-estimated and is estimated to be the third largest cause of death in the US[2]. Treatment efficacy and successful disease management varies between patients. This is in part attributable to the fact that diabetes is a heterogeneous disease, with multiple underlying causes. In a small proportion of cases of monogenic diabetes, the defects have been identified by genetic testing, sometimes leading to vastly improved treatment strategies[3,4]. However, the vast majority of cases of diabetes are polygenic and multifactorial. Despite some understanding of disease pathophysiology, and a variety of treatment options, the underlying causes of diabetes are unknown in most cases. A broader understanding of the variety of paths that lead to diabetes could identify alternative therapeutic targets. This may be crucial to preventing or managing disease in specific subsets of patients. Large-scale metabolomics studies have successfully identified novel candidates for diabetes, including a novel diabetes biomarker, α-aminoadipic acid (2-AAA, **Figure 1**), which predicts the development of diabetes in humans[5]. In matched diabetes cases and controls from the Framingham Heart Study (FHS), higher plasma 2-AAA (at baseline) was associated with future diabetes, independent of fasting glucose and other known risk markers (age, sex, BMI, family history, diet)[5].

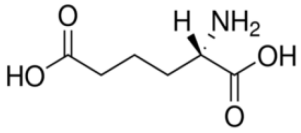

Figure 1. L-α-aminoadipic acid

Source of 2-AAA: 2-AAA is generated from the catabolism of the amino acid lysine through the saccharopine (mitochondrial) and/or the pipecolic acid (peroxisomal) pathways[6,7] (**Figure 2**). Both pathways converge in the generation of 2-aminoadipate-6-semialdehyde, which is then further metabolized to 2-AAA in the cytosol[8]. 2-AAA is subsequently metabolized to 2-oxoadipate (α-ketoadipic acid), and further to acetyl-coA, entering the TCA cycle. Lysine is an essential amino acid which is acquired from dietary sources, with a recommended daily intake of 30mg/kg/day[9]. A portion of lysine in humans may be derived from the microbiome, with bacteria and fungi capable of de novo synthesis, in addition to modulation of bioavailability of dietary sources[10].

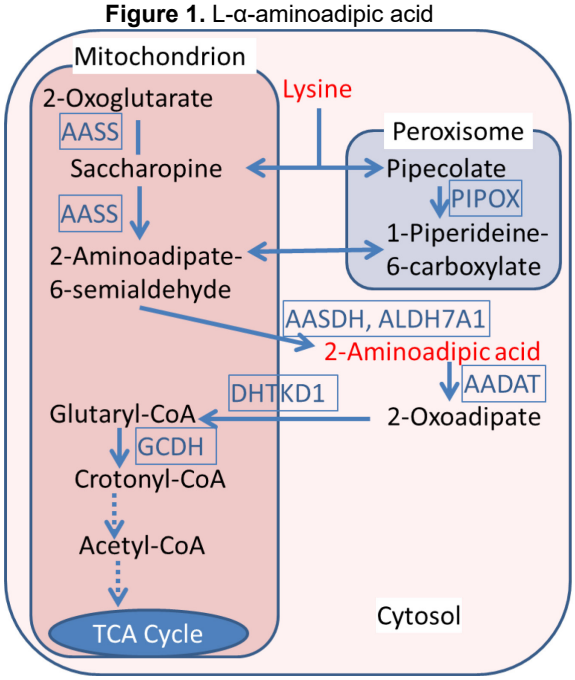

Figure 2. Multicompartmental model of L-lysine degradation (Adapted from Danhauser et al. 2012)

Genetics: Variation in genes encoding enzymes in the lysine degradation pathway (indicated in boxes in **Figure 2**) may influence 2-AAA levels and downstream function. The strongest evidence thus far exists for *DHTKD1*, variation in which has been associated with 2-Ketoadipic, 2-Aminoadipic and 2-Oxoadipic Aciduria[8,11], and Charcot-Marie-Tooth

Disease[12]. *DHTKD1* expression correlates with ATP production in mitochondria *in vitro*, and siRNA knockdown of *DHTKD1* leads to impaired mitochondrial biogenesis and increased reactive oxygen species production, resulting in apoptosis and reduced cell growth[13]. Variation in mouse *Dhtkd1* has been found to associate with expression of the gene (eQTL) and levels of protein (pQTL) in liver, as well as with serum 2-AAA levels[14]. Liver *Dhtkd1* expression correlated significantly with serum 2-AAA in these mice. Further, liver 2-AAA was negatively associated with liver mass, fasting glucose and serum cholesterol while higher liver 2-AAA was associated with higher insulin sensitivity.

2.0 Rationale and Specific Aims

Diabetes is a significant contributor to mortality and morbidity. The lysine-derived metabolite α-aminoadipic acid (2-AAA) has been identified as a novel predictor of diabetes development in humans, potentially identifying at-risk individuals before development of other known risk markers. Little is known about the function of 2-AAA.Our preliminary investigation of human clinical, human genetic, animal and cell experimental data has revealed multiple lines of evidence supporting 2-AAA and its pathway as biologically relevant to diabetes pathophysiology. However, it is not yet clear whether 2-AAA is itself causal in diabetes development, or is a biomarker for altered metabolic processes, and many questions remain as to mechanisms of action. The determinants of variation in 2-AAA levels between individuals are not known. The aim of the first phase of this study (2-AAA Screening study, ongoing) was to measure plasma 2-AAA levels from healthy individuals from the general population, to identify subjects with extremely high or low 2-AAA (defined as top and bottom 10%-25% of distribution). In this second phase of the study, individuals with high or low 2-AAA will be invited to participate in a dietary lysine modification study, to assess the effect of controlled lysine intake on plasma and urine 2-AAA.

Specific Aim: Determine whether plasma 2-AAA levels are responsive to dietary intervention within individuals in the extremes of 2-AAA distribution in the general population. We hypothesize that plasma 2-AAA levels in a well-nourished population are determined by genetics and gene-diet interactions and are not simply a reflection of dietary lysine exposure. Based on plasma 2-AAA measurements in our Phase 1 Screening study (ongoing, planned N=400), we will invite subjects who fall in the top and bottom ~10% of circulating 2-AAA (up to N=80) to return for future study. We will implement a dietary intervention to assess whether 2-AAA is a biomarker of lysine intake, or if 2-AAA levels are determined by genetic or gene-diet effects. Subjects will consume two different diets ("normal" and "high" lysine) in a randomized order (one week each), separated by a 2-week habitual diet washout period.

3.0 Animal Studies and Previous Human Studies

2-AAA has been identified as a marker of oxidative stress in C. *elegans*, and in endothelial cells in response to hyperglycemia[15], highlighting a potential mitochondrial phenotype, and relationship between 2-AAA and reactive oxygen species (ROS)[16,17]. In

animal models, administration of 2-AAA resulted in increased insulin levels and lower fasting plasma glucose, but no change in peripheral insulin sensitivity as measured by insulin tolerance test[5]. *In vitro* 2-AAA treatment has been shown to enhance insulin secretion in pancreatic β cells and islets. 2-AAA is thought to have important functions in the brain[18], as well as in other organs, including liver and pancreas.

High baseline plasma 2-AAA in healthy individuals was associated with increased future risk of diabetes (12-year follow-up) in FHS participants and validation samples (N~2,000)[5]. Insulin sensitizing therapy leads to reduction in 2-AAA, but not other putative diabetes-biomarkers, including branched-chain amino acids (BCAAs)[19]. Increased 2-AAA is found in skin in the setting of diabetes, as well as in chronic renal failure and sepsis, and increases with age[20]. Further, decreased urine 2-AAA has been reported in diabetes patients compared with non-diabetic[14], highlighting a likely complex feedback relationship between 2-AAA levels and early diabetes development vs. the setting of established disease. Treatment of overweight/obese adults with impaired fasting glucose or untreated diabetes with pioglitazone (45 mg/day) and metformin (1000 mg twice/day) led to a significant reduction in plasma 2-AAA and lysine, concurrent with an increase in insulin sensitivity[19]. Acute insulin infusion (7 hrs) in these subjects also led to a decrease in both lysine and 2-AAA[19,21]. The epilepsy drug vigabatrin, which acts through GABA transaminase inhibition, leads to increased 2-AAA in plasma and urine, to levels similar to those seen in alpha-aminoadipic aciduria[22]. Apart from diabetes, 2-AAA has been found to be elevated in the setting of other cardiometabolic or inflammatory diseases including atherosclerosis[23], chronic renal failure and sepsis[20], non-alcoholic fatty liver disease (NAFLD)[24] and polycystic ovary syndrome (PCOS)[25]. There is likely some reciprocal regulation between insulin and 2-AAA: insulin infusion in humans leads to decreased 2-AAA[19,21,26], while insulin deprivation and hyperglycemia in the setting of type 1 diabetes leads to increased 2-AAA[27].

Observational data in humans does not support a relationship between dietary intake of protein or lysine and 2-AAA (as assessed in FHS[5], and our own preliminary data). There are limited data on 2-AAA in response to dietary intervention. There was no change in 2-AAA in obese subjects following 16 weeks of caloric restriction, despite weight loss[28]. However, dietary protein was not altered in this study. In a multi-diet comparison, 2-AAA decreased on a low fat diet, and increased on a very-low carbohydrate diet[29]. The relationship between dietary lysine and 2-AAA has never been assessed through a randomized intervention trial. We will address this in the current study. One week of diet is appropriate to identify short-medium term dietary biomarkers[30], but not long enough to affect other variables (which could alter 2-AAA through reverse causation).

We have already analyzed plasma samples for the first 122 participants of phase 1 of the study. As expected, there was variability in plasma 2-AAA, with some evidence for differences by sex (**Figure 3**). We will select initial participants from the extremes (by sex) of this group. Analysis of additional samples is pending and will continue to be batched based on sample accrual.

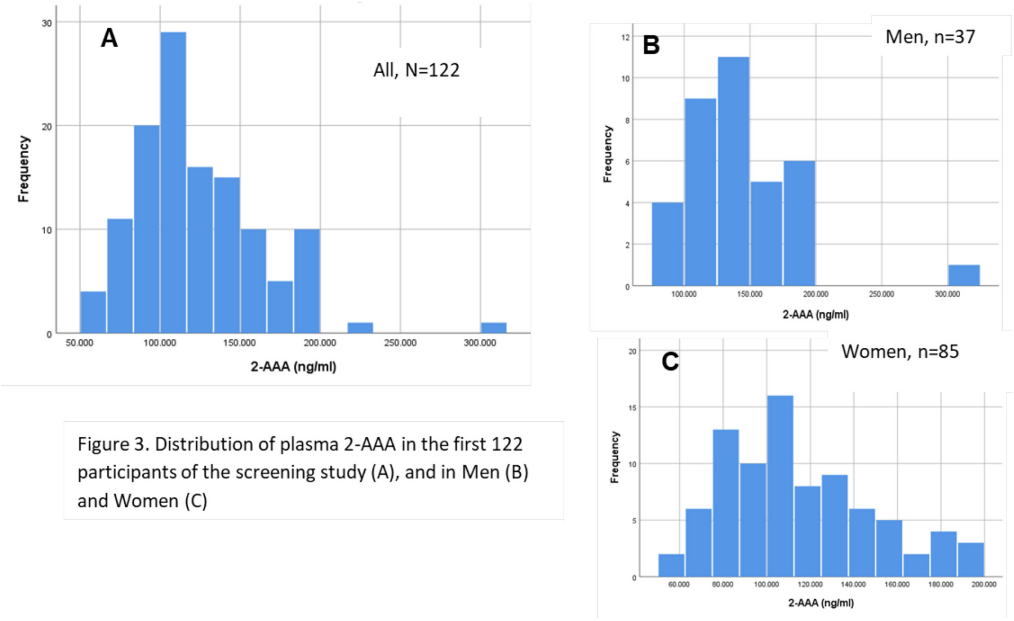

Figure 3. Distribution of plasma 2-AAA in the first 122 participants of the screening study (A), and in Men (B) and Women (C)

4.0 Inclusion/Exclusion Criteria

Inclusion Criteria:

- Prior participant in 2-AAA screening study.
- Identified as eligible due to high or low plasma 2-AAA, in the absence of hyperglycemia, as defined by study team.

Exclusion Criteria:

- Individuals who currently use tobacco products.
- Use of prescription or over-the-counter medications or dietary supplements which could modulate levels of 2-AAA and unwilling to discontinue use (from 24 hours prior to first study visit until completion of study). Hormonal birth control is acceptable.
- Follow a severely restricted diet or have food allergies, which would preclude adherence to study diet.
- Newly diagnosed disease (since screening visit), including cardiovascular, renal, or liver disease, or Diabetes mellitus.
- Individuals who are pregnant or lactating
- Inability to provide written or electronic informed consent
- Inability to fast for 8 hours

5.0 Enrollment/Randomization

We will enroll up to 80 subjects who meet the inclusion and exclusion criteria. We will identify potential participants based on their results from the screening study. We will contact eligible participants directly, by email or phone, and invite them to participate.

6.0 Study Procedures

The study will be conducted as a randomized crossover dietary intervention, with 4 separate study visits (Figure 4). Individuals will be randomized to normal or high lysine diet for 1 week, followed by a 2-week washout, where participants return to their habitual diet. They will then complete the second 1-week dietary intervention. The entire study period encompasses 4 weeks.

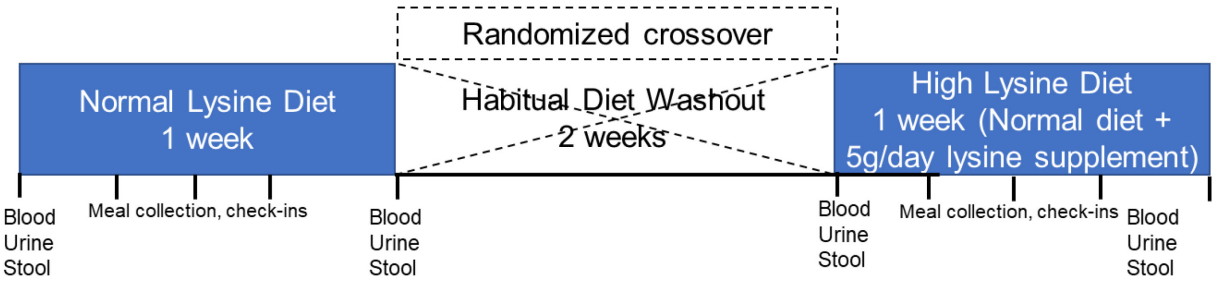

Figure 4. Overview of the proposed dietary intervention study.

Screening

Subjects will be screened based on their 2-AAA levels and other data obtained during phase 1 of the study. Inclusion/exclusion criteria will be reviewed to confirm that the subject meets study eligibility requirements. Individuals who meet the inclusion criteria, and do not meet any of the exclusion criteria will be invited to participate in the study. Informed consent will be obtained via telephone with a phone consent witness or electronically. All participants will be instructed to discontinue use of over-the-counter medications and supplements on the day prior to the study visit. Participants will be asked about any major changes to their diet since their screening visit, and if any changes are reported, they will be asked to complete an online food frequency questionnaire (DHQ III) at any time before their visit (Food Frequency Questionnaire Instructions, **Appendix A**). In addition, participants will be asked to complete a 24-hour diet recall (ASA24) around the time of each study visit (Diet Recall Instructions, **Appendix A**). As part of the screening questionnaire, participants will be asked about their physical activity to ascertain their energy requirements for the dietary intervention. Participants will be asked to pick up a stool collection kit from the study coordinator (from the Preston Research Building, or another convenient location) prior to their first visit. If you are a woman and are able to become pregnant, you will have a urine pregnancy test to make sure that you are not pregnant before you receive treatment in this study.

Study Visits

There will be four separate study visits. All study visits will follow similar procedures. Subjects will be requested to arrive at a research room in the Preston Cancer Research building or the Vanderbilt Clinical Research Center on the morning of each study visit (~7:00 - 11:00 AM) in a fasting state (at least 8 hours, with no food or drink, excluding water). At the first visit, after informed consent has been reviewed and documented, inclusion/exclusion criteria will be reviewed to confirm that the subject meets study eligibility requirements. Subject's medical history and medications will be discussed and documented by study personnel to assess for any changes that may potentially exclude the subject from participating. Study personnel will collect the subject's blood pressure, heart rate, height, weight, waist and hip circumference. All subjects will be asked to provide a urine sample. Subjects will be asked to provide a drop of blood via fingerprick for measurement of fasting glucose. Venipuncture will be performed to obtain a blood sample (~50ml). All subjects will be asked to bring a stool sample (collected in the 24 hours prior to each study visit) and will be given additional collection kits for subsequent samples at their study visits. Participants will be asked to fill out a questionnaire to assess general wellbeing and mental health (PHQ).

Diet

Subjects will meet with a dietician and be provided a menu, shopping list, and a gift card to purchase foods to prepare 3 meals and 1-2 snacks per day during the study period. All subjects will consume the same foods for both dietary periods (normal lysine and high lysine, 1 week each), with lysine supplements (5g/day) included for the high lysine diet period. Menus will be developed by the study dietitian (Dr. Heidi Silver, Co-I). Energy content of meals will be personalized for each participant based on the individual's estimated daily energy requirement using the Harris Benedict Equation with an activity factor of 1.2.

Compensation

Subjects will receive $250 following successful completion of the study visit.

Biomarkers

Blood, urine and stool samples will be coded for subject confidentiality. Plasma, serum, buffy coat, stool and urine aliquots will be frozen at -80°C and stored for subsequent measurements. Peripheral blood mononuclear cells (PBMCs) may be isolated from blood for analysis. We are already storing buffy coat and extracting DNA in the first phase of this study. While we do not expect the DNA to change between visits, we will store additional buffy coat samples to allow for back-up samples for DNA extraction in the event of any sample failures from phase 1, or for any follow-up analyses. Samples will be prepared for metabolite measurement by methanol extraction and centrifugation in Dr. Ferguson's laboratory using standard protocols[31]. 2-AAA and other metabolites and biomarkers (e.g. lysine, creatinine, insulin) will be measured at Vanderbilt core facilities (e.g. Mass Spectrometry core, Hormone Assay core). DNA from buffy coat will be extracted and submitted for genotyping at the Vanderbilt Technologies for Advanced Genomics (VANTAGE) core. Microbial DNA will be extracted from stool, prepared for sequencing (16S V4 or metagenomic), and sequenced at VANTAGE. For possible future

investigations, excess blood, urine and stool samples will be frozen and stored in Dr. Ferguson's laboratory.

Genomic Data**:** Genomic data will be deposited in dbGap, a controlled-access database, and will be made available as part of the Policy for Sharing of Data Obtained in NIH Supported or Conducted Genomic Studies. All deposited data and metadata will be documented, per NIH guidelines. All data will be de-identified to protect study participants' identities.

7.0 Risks

Venous Blood draw: This is a routine procedure that is considered standard of care in clinical medicine. At the study visit, subjects will undergo venous blood draws. All blood draws will be performed by trained personnel using universal precautions to protect both the subject and personnel. The risks to subjects are minimal, but may include pain, bruising, allergic reaction, infection or bleeding at the needle stick site. A small number of individuals may experience dizziness, lightheadedness or fainting. These usually resolve without any specific medical therapy over the course of minutes to days.

Fingerprick: This is a routine procedure that is considered standard of care in clinical medicine. Each subject will undergo a fingerprick using a sterile, single-use lancet to pierce the skin on the pad of fingertip and extract a single drop of blood. The risks to subjects are minimal, but may include pain, bruising at the puncture site, and/or infection or bleeding at the puncture site. These reactions usually resolve without any specific medical therapy over the course of minutes to days.

Dietary Intervention and Lysine Supplementation: There are no known risks associated with dietary intervention. Lysine is expected to be well-tolerated by all participants. Lysine is used as a dietary supplement, available over the counter, with no known long-term risks. The proposed dose of lysine may cause mild gastrointestinal upset in some subjects. This is expected to be minor and transient.

Stool Sample Collection: There are no known risks associated with stool sample collection. Sample collection may be inconvenient for some individuals.

Private Health Information: This information will be collected during the course of the study. However, only key study personnel will have access to this information, which will be stored in a HIPAA compliant, password protected REDCap database. No protected health information will be shared with employers, insurers, or non-research personnel.

8.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

Adverse events will be reported to the IRB per Vanderbilt University IRB policy.

9.0 Study Withdrawal/Discontinuation

Subjects may withdraw from the study at any time and should notify study personnel if they wish to withdraw from the study. Subjects may request their biological samples to be destroyed at any time. However, any data or biological samples that have already been used for research cannot be destroyed. Subjects may be discontinued from the study at the discretion of the investigator (possible reasons listed below). Subjects will receive financial compensation for completion of the visit.

Possible reasons for withdrawal/discontinuation from study include, but are not limited to:

- Noncompliance with treatment or procedures
- Decision by participant/participant withdraws consent
- Significant adverse event deemed by investigator to preclude continued participation

10.0 Statistical Considerations

The primary goal is to measure changes in 2-AAA in plasma and urine following dietary intervention study. At 80% power and α=0.05 we require 39 subjects to detect a diet-induced change of 0.5 SD in 2-AAA. Although we expect that a true diet effect would be greater, we have chosen a low threshold to avoid Type 2 error. We expect that individuals in our high and low groups have a significantly different risk of future diabetes. To detect significant differences in disease risk by 2-AAA status at 80% power and α=0.05 (based on 1.6-fold increased risk of diabetes per SD of 2-AAA) we require 76 subjects. These considerations informed our choice to include up to 80 subjects (40 high and 40 low) for the dietary intervention study.

11.0 Privacy/Confidentiality Issues

Strict confidentiality will be maintained to the fullest extent by the research team, including keeping all data in a secure location. All specimens will be coded anonymously to remain confidential and identifiers will be kept in a separate, secure location. Samples may be shared with third parties outside of Vanderbilt for future testing but will remain anonymous to the recipient. Subjects may contact the principal investigator at any time to request that samples be destroyed.

12.0 Follow-up and Record Retention

Anticipated study duration is 5 years. Research data will be maintained by the PI after study closure. After study closure, research data will be maintained for a minimum of 6 years and possibly indefinitely. Data will be stored on the Vanderbilt University computer network in a password-protected database. Only members of the study team will have

access. Pertinent paper documentation will be kept in locked office and only study personnel will have access. Only personnel directly involved with the study will have access to source data and the electronic database. Genomic data will be deposited in dbGap, a controlled-access database, and will be made available as part of the Policy for Sharing of Data Obtained in NIH Supported or Conducted Genomic Studies. All deposited data and metadata will be documented, per NIH guidelines. All data will be de-identified to protect study participants' identities.

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14.0 Appendices

Appendix A

Diet Questionnaire and Recall Instructions

Food Frequency Questionnaire (NCI Diet History Questionnaire (DHQ) III))
At any time following consent, but before your study visit, please complete a food frequency questionnaire. You will be provided with a unique URL to access your questionnaire.
Alternatively, we will provide you with a username and password, and the Study Access Code, which you may use to log in at: http://www.dhq3.org/respondent-login/
You will be given the option to download a Nutrition Report following completion of the questionnaire.

This survey may take you 30-60 minutes to complete.

24-Hour diet recall (NCI Automated Self-Administered 24-Hour (ASA24®) Dietary Assessment Tool)
You will be asked to recall all of the foods and drinks consumed on the day before your study visit, starting at midnight on the day before your visit, and ending at midnight on the day of your study visit. You may complete the assessment in the morning before your study visit, or immediately following your study visit.
The recall can be accessed at: https://asa24.nci.nih.gov/
Please log in with the username and password provided to you by the study coordinator.
You will be given the option to download a Nutrition Report following completion of the questionnaire.

This survey may take you 20-30 minutes to complete.