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

Study 3

Principal Investigator

Jane F. Ferguson, PhD

Division of Cardiovascular Medicine
Vanderbilt University Medical Center

2220 Pierce Ave, PRB 354
Nashville TN 37232

Co-Investigator

Jonathan D. Mosley, MD PhD

Division of Clinical Pharmacology
Vanderbilt University Medical Center

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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), 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].

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 1**). 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].

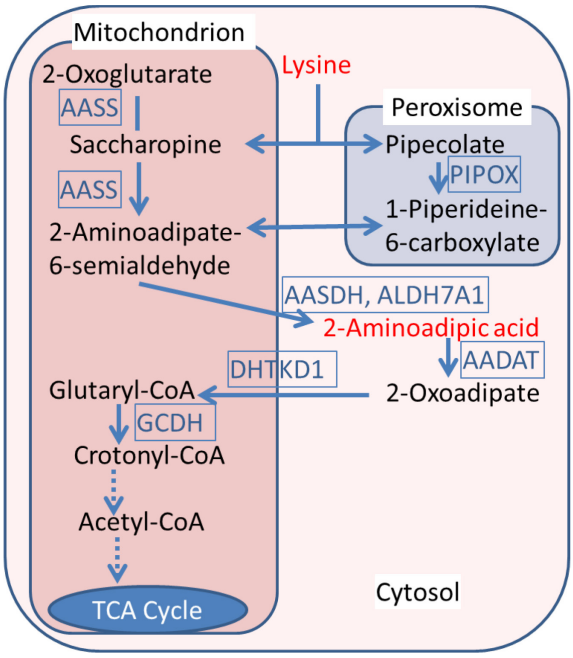

Figure 1. Multicompartmental model of L-lysine degradation (Adapted from Danhauser et al. 2012)

Genetics: Variation in genes encoding enzymes in the lysine degradation pathway (indicated in boxes in **Figure 1**) 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 Aim

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) 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 ~25% of distribution). The aim of the second phase of the study was to invite individuals with high or low 2-AAA (N=80) to participate in a dietary lysine modification study, to assess the effect of controlled lysine intake on plasma and urine 2-AAA. In this third phase of the study, we will invite a subset of individuals (N=20) from phase 2 to participate in a lysine metabolism study. We hypothesize that individuals differ in their rate of metabolism or excretion of lysine to 2-AAA. This will be assessed by the rate of conversion of ^{13}C lysine to ^{13}C 2-AAA in plasma, and the rate of excretion of ^{13}C lysine and ^{13}C 2-AAA in urine. We expect that individuals with high plasma 2-AAA have an increased rate of metabolism of lysine to 2-AAA, and a reduced rate of urinary excretion compared with low-2-AAA individuals.

Specific Aim: To determine whether acute lysine administration leads to variability in increased plasma 2-AAA in humans. Catabolism of lysine leads to generation of 2-AAA. To address the hypothesis that acute lysine ingestion leads to differences in the increased circulating and excreted 2-AAA in humans *in vivo*, we will administer isotope-labeled lysine (5g orally) to healthy volunteers (N=20) and measure the level of 2-AAA in plasma and urine at baseline and serially post-ingestion. Carbon-13 is a stable naturally occurring heavy isotope of carbon. Inclusion of a ^{13}C label on lysine allows for subsequent differentiation between endogenous and exogenous lysine and 2-AAA for calculation of clearance and excretion of the lysine bolus.

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].

Human clinical: We tested our ability to use lysine to modulate 2-AAA in the acute setting (human pilot study, N=10, NCT02756117). The rationale to use lysine as a probe to increase 2-AAA was based in our hypothesis that exogenous lysine is a contributor to 2-AAA levels, and that individuals with altered 2-AAA have alterations in the lysine-2-AAA metabolic pathway. We demonstrated that acute oral lysine (10g) resulted in an increase in plasma lysine (P<0.001, **Fig.2A**) and

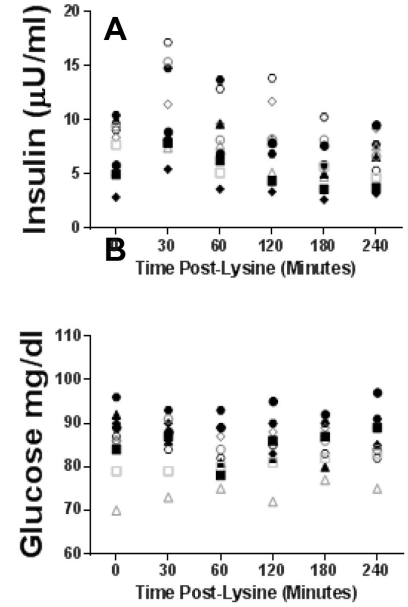

Figure 3. The effect of an oral lysine bolus (10g) on plasma insulin (A); and glucose (B) in 10 healthy subjects. Closed shapes denote male, open shapes denote female subjects.

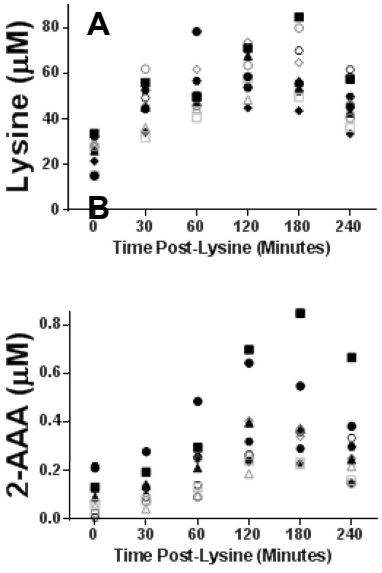

Figure 2. The effect of an oral lysine bolus (10g) on plasma lysine (A); and 2-AAA (B) in 10 healthy subjects. Closed shapes denote male, open shapes denote female subjects.

2-AAA (P<0.0001, **Fig.2B**). There was an increase in plasma insulin (P<0.0001, **Fig.3A**) with no change in glucose (**Fig.3B**). Amino acids are known to stimulate insulin secretion, so the effect on insulin is not necessarily attributable to 2-AAA. There were no changes in other amino acids. We detected a significant increase in urinary lysine and 2-AAA, and importantly, observed variation in the fractions excreted as lysine (**Fig.4A**), or as the 2-AAA metabolite (**Fig.4B**). We thus confirmed the hypothesis that plasma 2-AAA is modulated in part by oral lysine intake. However, we demonstrated that there is considerable inter-individual variability in the 2-AAA response to lysine (**Fig.2B**), supporting the premise that factors other than diet (e.g. genetics) contribute to modulation of 2-AAA.

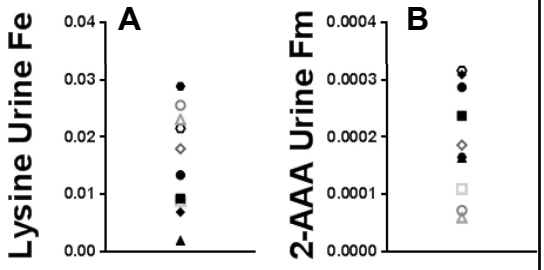

Figure 4. Estimates of urinary lysine fraction excreted (Fe) and 2-AAA fraction metabolized (Fm) over 4 hours post lysine bolus.

Human lysine tracer study: The stable isotope tracer ^{13}C-lysine replaces ^{12}C with ^{13}C on the lysine molecule. Previous studies have used isotope labeled lysine to calculate amino acid flux[28,29]. We invited two subjects from the pilot study to return for a labeled lysine challenge to distinguish between endogenous and exogenous metabolism (NCT03063476). We administered 5g of stable isotope-labeled lysine (U-^{13}C lysine, Cambridge Isotope Laboratories) as an oral bolus, with repeated sampling of blood and urine over 6 hours for measurement of ^{12}C and ^{13}C lysine and 2-AAA. We observed the expected increase in ^{13}C isotope-labeled lysine and 2-AAA in plasma in both subjects (**Figure 5**). Interestingly, we also observed a marked increase in endogenous (unlabeled) lysine and 2-AAA in Subject 02, not observed in Subject 01, suggesting secretion of stored intracellular lysine and 2-AAA into the circulation, potentially as a result of catabolism of lysine from endogenous proteins[26].

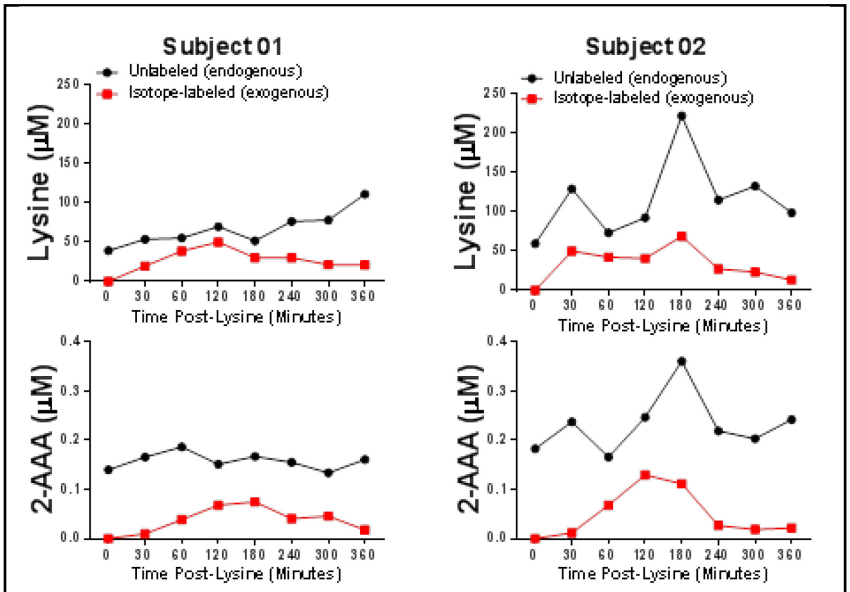

Figure 5. The effect of an oral isotope-labeled ^{13}C lysine bolus (5g) on plasma lysine and 2-AAA in 2 healthy subjects.

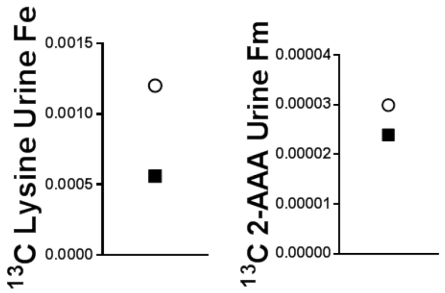

Figure 6. Estimates of urinary lysine fraction excreted (Fe) and 2-AAA fraction metabolized (Fm) over 6 hours post ^{13}C lysine bolus. Closed shapes denote male, open shapes denote female subject.

The rate of urinary clearance of ^{13}C lysine and excretion of metabolized ^{13}C 2-AAA also differed between the 2 subjects (**Figure 6**). Subject 01 excreted a greater proportion of the lysine and 2-AAA, with concurrently lower plasma levels of both. While the goal of this pilot study was to demonstrate feasibility, and was not powered to detect differences, these data further highlight inter-individual differences in the metabolic response to an identical lysine load. We hypothesize that there are genetic differences, between individuals with high or low 2-AAA, which control the rate of lysine and 2-AAA metabolism.

4.0 Inclusion/Exclusion Criteria

Inclusion Criteria:

- Prior participant in 2-AAA study.

Exclusion Criteria:

- Newly diagnosed disease, including cardiovascular, renal, liver disease, bleeding disorder, or Diabetes mellitus.
- Individuals who are pregnant or lactating.
- Inability to provide electronic informed consent.
- Inability to fast for 8 hours.

5.0 Enrollment

Our goal is to obtain study visit data for 20 individuals. To allow for expected drop-outs, we will enroll up to 30 participants who meet the inclusion and exclusion criteria. We will identify potential participants based on their participation in the previous phases of the study. We will contact eligible participants directly, by email or phone, and invite them to participate.

6.0 Study Procedures

Screening

Potential participants will be identified based on their participation in the previous phases of the study. Informed consent will be obtained electronically. Inclusion/exclusion criteria will be reviewed to confirm that the individual 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.

Study Visit

Participants will be requested to arrive at the Vanderbilt Clinical Research Center on the morning of the study visit (~8:00 AM) in a fasting state (at least 8 hours, with no food or drink, excluding water). After informed consent has been reviewed and documented, inclusion/exclusion criteria will be reviewed to confirm that the subject meets study eligibility requirements. The participant'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

participant's vital signs, height, weight, waist and hip circumference prior to study interventions. All participants will be asked to provide a urine sample for baseline 2-AAA measurement and will undergo a urine pregnancy test (if the woman is of child-bearing potential).

Following the baseline urine sample, an intravenous line will be inserted into a superficial peripheral vein to perform blood draws. Participants will be asked to remain in a seated position for the duration of the study, but will be permitted to walk for brief periods throughout the study (e.g. to use the restroom). A baseline blood draw will be taken (time = 0 hr) for measurement of baseline plasma 2-AAA levels and related biomarkers. Immediately following the baseline blood draw, subjects will be given an oral bolus of ^{13}C L-lysine (5g) (Cambridge Isotope Laboratories, Tewksbury, MA) in 50ml water. This is an amount of lysine equivalent to that which is found in a 5oz serving of beef. A similar dose of lysine has previously been shown to alter the response to glucose[17]. Blood samples will be taken at time = 30 minutes 1, 2, 3, 4, 5 and 6 hrs post-lysine administration. Normal (0.9%) Saline (NS) will be infused at a rate of 10 ml/hr to flush the canula prior to each blood draw, and a 3-5ml blood discard will be performed prior to each collection of samples. Each blood draw (including baseline) will collect about 20cc of blood, for a total collection of 160cc, or approximately 11 tablespoons of blood. Blood will be collected into one tube for serum (5ml) and 2 tubes for plasma (4ml each). Urine samples will be collected throughout the visit, in 2-hour increments (0-2, 2-4 hrs and 4-6 post-lysine). Peak post-prandial plasma 2-AAA levels are expected to be observed ~3hours post-lysine[27]. Plasma and serum will be prepared from blood samples, and both plasma and urine aliquots will be frozen at -80°C and stored prior to analysis. 2-AAA and other metabolites and biomarkers (e.g. lysine, creatinine) will be measured at Vanderbilt core facilities (e.g. Mass Spectrometry core).

Compensation
Participants will receive $150 following successful completion of the study visit.

Biomarkers
Blood and urine samples will be coded for subject confidentiality. Plasma, serum 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. 2-AAA and other relevant metabolites and biomarkers will be measured at Vanderbilt core facilities (e.g. Mass Spectrometry core, Hormone Assay core). For possible future investigations, excess blood, cell, and urine samples will be frozen and stored in Dr. Ferguson's laboratory.

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.

Lysine Administration: 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. There are no additional risks associated with the use of stable isotope tracer ^{13}C lysine. The proposed dose of lysine may cause mild gastrointestinal upset in some participants. This is expected to be minor and transient.

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

Based on our preliminary study, we expect that some individuals will have modest increases in ^{13}C 2-AAA post-lysine, estimated at 0.075μM at peak. We expect that other individuals will have greater response, estimated at 0.13μM peak ^{13}C 2-AAA. Assuming a SD of 0.04μM, we would require a total of 16 individuals to detect significant differences in response by group, at $\alpha=0.05$ and 80% power. We aim to recruit 20 individuals to allow for adequate power in the event of attrition or lower than expected separation between groups.

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. Participants 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. Data will be de-identified to protect study participants' identities.

13.0 References

1. CDC. National Diabetes Statistics Report: Estimates of Diabetes and Its Burden in the United States, 2014. Atlanta, GA: US Department of Health and Human Services: 2014.
2. Stokes A et al. Deaths Attributable to Diabetes in the United States: Comparison of Data Sources and Estimation Approaches. *PLoS ONE*. 2017;12:e0170219.
3. Carmody D et al. GCK-MODY in the US National Monogenic Diabetes Registry: frequently misdiagnosed and unnecessarily treated. *Acta Diabetol*. 2016;53:703–8.
4. Naylor RN et al. Cost-effectiveness of MODY genetic testing: translating genomic advances into practical health applications. *Diabetes Care*. 2014;37:202–9.
5. Wang TJ et al. 2-Aminoadipic acid is a biomarker for diabetes risk. *The Journal of Clinical Investigation*. 2013;123:4309–17.
6. Chang YF. Pipecolic acid pathway: the major lysine metabolic route in the rat brain. *Biochemical and biophysical research communications*. 1976;69:174–80.
7. Posset R et al. Understanding cerebral L-lysine metabolism: the role of L-pipecolate metabolism in Gcdh-deficient mice as a model for glutaric aciduria type I. *Journal of inherited metabolic disease*. 2015;38:265–72.
8. Danhauser K et al. DHTKD1 mutations cause 2-aminoadipic and 2-oxoadipic aciduria. *American journal of human genetics*. 2012;91:1082–7.
9. Joint WHOFAOUNUEC. Protein and amino acid requirements in human nutrition. *World Health Organ Tech Rep Ser*. 2007;1–265, back cover.
10. Neis EP et al. The role of microbial amino acid metabolism in host metabolism. *Nutrients*. 2015;7:2930–46.
11. Stiles AR et al. New Cases of DHTKD1 Mutations in Patients with 2-Ketoadipic Aciduria. *JIMD Rep*. 2015;
12. Xu WY et al. A nonsense mutation in DHTKD1 causes Charcot-Marie-Tooth disease type 2 in a large Chinese pedigree. *American journal of human genetics*. 2012;91:1088–94.
13. Xu W et al. DHTKD1 is essential for mitochondrial biogenesis and function maintenance. *FEBS letters*. 2013;587:3587–92.
14. Wu Y et al. Multilayered genetic and omics dissection of mitochondrial activity in a mouse reference population. *Cell*. 2014;158:1415–30.

15. Yuan W et al. Amine metabolomics of hyperglycemic endothelial cells using capillary LC-MS with isobaric tagging. *Journal of proteome research*. 2011;10:5242–50.
16. Zeitoun-Ghandour S et al. C. elegans metallothioneins: response to and defence against ROS toxicity. *Molecular bioSystems*. 2011;7:2397–406.
17. Lin H et al. Myeloperoxidase-mediated protein lysine oxidation generates 2-aminoadipic acid and lysine nitrile in vivo. *Free radical biology & medicine*. 2017;104:20–31.
18. Wu HQ et al. L-alpha-aminoadipic acid as a regulator of kynurenic acid production in the hippocampus: a microdialysis study in freely moving rats. *Eur J Pharmacol*. 1995;281:55–61.
19. Irving BA et al. Effect of insulin sensitizer therapy on amino acids and their metabolites. *Metabolism*. 2015;64:720–8.
20. Sell DR et al. Aging, diabetes, and renal failure catalyze the oxidation of lysyl residues to 2-aminoadipic acid in human skin collagen: evidence for metal-catalyzed oxidation mediated by alpha-dicarbonyls. *Annals of the New York Academy of Sciences*. 2008;1126:205–9.
21. Barazzoni R et al. Insulin fails to enhance mTOR phosphorylation, mitochondrial protein synthesis, and ATP production in human skeletal muscle without amino acid replacement. *American journal of physiology*. 2012;303:E1117-25.
22. Vallat C et al. Treatment with vigabatrin may mimic alpha-aminoadipic aciduria. *Epilepsia*. 1996;37:803–5.
23. Saremi A et al. Advanced Glycation End Products, Oxidation Products, and the Extent of Atherosclerosis During the VA Diabetes Trial and Follow-up Study. *Diabetes Care*. 2017;40:591–598.
24. Feldman A et al. Clinical and Metabolic Characterization of Lean Caucasian Subjects With Non-alcoholic Fatty Liver. *Am J Gastroenterol*. 2017;112:102–110.
25. Chang AY et al. Combining a nontargeted and targeted metabolomics approach to identify metabolic pathways significantly altered in polycystic ovary syndrome. *Metabolism*. 2017;71:52–63.
26. Robinson MM et al. High insulin combined with essential amino acids stimulates skeletal muscle mitochondrial protein synthesis while decreasing insulin sensitivity in healthy humans. *J Clin Endocrinol Metab*. 2014;99:E2574-83.
27. Lanza IR et al. Quantitative metabolomics by H-NMR and LC-MS/MS confirms altered metabolic pathways in diabetes. *PLoS ONE*. 2010;5:e10538.
28. El-Khoury AE et al. Twenty-four-hour intravenous and oral tracer studies with L-[1-13C]-2-aminoadipic acid and L-[1-13C]lysine as tracers at generous nitrogen and lysine intakes in healthy adults. *The American journal of clinical nutrition*. 1998;68:827–39.
29. Metges CC et al. Availability of intestinal microbial lysine for whole body lysine homeostasis in human subjects. *Am J Physiol*. 1999;277:E597-607.