Low carbohydrate high fat diet feeding does not cause increased ketone body disposal in mice.

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# ABSTRACT

# INTRODUCTION

# METHODS

## Animal Husbandry and Rodent Diets

Mice were maintained in in ventilated cages at 70F at 40-60% humidity in a room with a 12-hour light/dark cycle (ZT0=6:00AM). Mice were provided *ad libitum* access to food and water unless otherwise noted. For Muscle *Tsc1* knockout mice, wild-type mice were generated by crossing XXX. are defined as homozygous floxed *Tsc1*, absent the *Ckmm*-Cre transgene, while Muscle *Tsc1* knockout mice are defined as homozygous floxed *Tsc1*, with one copy of the *Ckmm*-Cre transgene. A/J mice (RRID:IMSR\_JAX:000646) were purchased at 8 weeks of age from The Jackson Laboratories and at 10 weeks of age were fed control or ketogenic diets for four weeks. Diversity outbred mice (43rd generation, non-siblings RRID: IMSR\_JAX:009376) were purchased from the Jackson Laboratories and placed on ketogenic diet at 12 weeks of age for four weeks. Mice were maintained on a normal chow diet (Lab Diet 5L0D; 5% of calories from fat, 24% from protein, 36% carbohydrate) unless otherwise specified. For ketogenic diet interventions mice were placed on either a ketogenic diet (Research Diets D17053002, 85% fat, 15% protein, 0% carbohydrates) or a matched synthetic control diet (Research Diets D1053001 10% fat, 15% protein, 75% carbohydrates). Both diets were in meal, not pellet format and were provided in custom jars with holes to provide access. All procedures were approved by the University of Michigan Institutional Animal Use and Care Committee.

## BHB Tolerance Tests

Fed mice were intraperitoneally injected with 1 mg/kg (A/J ketogenic diet studies) or 1.5 mg/kg (muscle *Tsc1* knockout and diversity outbred studies) of beta-hydroxybutyrate dissolved in PBS at approximately ZT8. Prior to the injection, and then every 15 afterwards, a tail blood draw was collected and analyzed using a Precision Xtra Ketone Body Assay. To summarize these data we calculated area under the curve and baseline-adjusted area under the curve where we subtracted the contribution of the pre-injection ketone body levels to circulating amounts.

## mRNA Analysis

Quantitative real-time PCR was performed by extracting RNA from muscle lysates using PureLink mRNA kits (Thermo Scientific cat # 12183-018A) and synthesizing cDNA using a high capacity first strand cDNA synthesis kit (Thermo Scientific cat # 4368813). cDNA was amplified using SYBR Green (Thermo-Fisher 4367659) and the primers noted in Table 1 using a QuantStudio 5. Relative expression was determined us the Ct method. For RNAseq, data was re-analysed from GSE84312 [1]

## Cholesterol Analyses

## Statistical Analyses

All statistical analyses were performed using R version 4.2.2 [2]. We set statistical significance for this study at 0.05. For pairwise testing, we first tested for normality via Shapiro Wilk tests, and then equal variance using Levene’s test. On this basis either Mann-Whitney, Welch’s or Student’s *t* tests were performed as noted in the text and figure legends. For longitudinal data, including BHB tolerance tests we constructed mixed linear models using the lme4 package (version 3.1; [3]). The repeated measure (random intercept) was the animal, and Chi-squared tests were performed comparing models with and without diet terms. For experiments with both rodent sexes, we first tested for modification by sex using the interaction terms from a 2x2 ANOVA, and if significant reported this effect while also stratifying by sex and reporting those results.

# RESULTS

## Activation of mTORC1 promotes Ketone Disposal

To test whether activation of mTORC1 in muscle tissue alters disposal of ketone bodies, we performed a BHB tolerance test in *Tsc1* knockout mice. The *Ckmm*-Cre induced ablation of *Tsc1* causes activation of mTORC1 in muscle tissues. As shown in Figure 3A, both male and female knockout mice cleared the injected beta-hydroxybutyrate much more rapidly than their wild-type littermates. Using mixed-linear models and using sex as a covariate, and the animal as a random intercept, we found a significant reduction in BHB levels after the challenge (p=0.004). Similarly, when calculating the area under the curve from 0 to 60 minutes, there was a reduction in the knockouts, after adjusting for sex (25%; p=0.016). When stratifying by sex, knockouts had 41% lower AUC in males and 11% lower in females though this modification by did not reach statistical significance (p=0.20).

## mTORC1 regulates expression of Ketolysis genes

## KD Feeding Does not Improve BHB Disposal in A/J Mice

We hypothesized that prolonged exposure to elevated ketone body levels would result in physiological adaptations, resulting in improved disposal of ketone bodies. To test this hypothesis in male wild-type A/J mice we fed 10-week-old A/J mice a control or a ketogenic diet for three weeks and then performed a BHB tolerance test. As expected, baseline ketone body levels were elevated from 0.43 mg/dL to 0.75 mg/dL in this assay (p=0.017). Upon injection we were surprised to observe that ketone body levels remain elevated more-so in the ketogenic diet-fed mice than the control mice (Figure 2A), even after subtracting for baseline differences (Figure 2B-C). These data suggest that ketone disposal is not improved after three weeks of a ketogenic relative to a control diet, and may actually be somewhat worsened in A/J mice (p=0.274 via linear mixed models).

To understand this surprising lack of adaptation, we performed mRNA quantification of quadriceps from these A/J mice evaluating the expression of key transporters and enzymes involved in ketolysis. As shown in Figure 2D, most ketolytic gene expression was reduced. Among the MCT1 family transporters that conduct ketone bodies into cells, *Slc16a1* transcripts were modestly reduced in muscles from A/J mice fed ketogenic diets (17% reduced, pdiet from a 2x2 ANOVA =0.064). *Bdh1* encodes beta hydroxybutyrate dehydrogenase, the interconversion step between beta-hydroxybutyrate and acetoacetate. We found that this gene is downregulated in A/J muscles by 42% (pdiet from a 2x2 ANOVA =0.009). *Oxct1* encodes succinyl-CoA:3-ketoacid CoA transferase (also known as SCOT). This transcript was downregulated in both male and female mice by 52% (pdiet from a 2x2 ANOVA=8.4 x 10-9). While there was no significant sex x diet interaction in these analyses, we thought it was notable that male mice had higher expression of each of these genes than female mouse muscle lysates, with 15-30% increases (psex from a 2x2 ANOVA=4.7 x 10-3 for *Slc16a1*, 8.9 x 10-3 for *Bdh1* and 0.055 for *Oxct1*). This may suggest enhanced ketone body catabolism in male versus female mice, but this was not explored further. We think the key finding here is that the transcriptional downregulation of genes involved in ketone body disposal in muscle is consistent with reduced disposal of injected BHB. This brings up an interesting question though, if ketone bodies are at steady state in serum, but disposal is reduced, that would imply that production may also be reduced. To test this we also looked at three ketogenic enzymes in the livers of these mice, and indeed found downregulation of hepatic *Hmgcl, Hmgcs2,* and *Bdh1* in the livers of male mice, though only *Bdh1* was downregulated in female mouse livers (Supplementary Figure 1, psex x diet <0.01 for both *Hmgcl* and *Hmgcs1*).

## Evaluation of Diversity Outbred Mice Demonstrates Substantial Variation in BHB Disposal

To evaluate if A/J mice were atypical in their lack of adaptation to improved ketolysis, we performed BHB tolerance tests on diversity outbred mice before or after four weeks of a ketogenic diet. Diversity outbred (DO) mice are genetically unique, so represent the integrated genetic variability of the intercrossed eight founder strains [4]. As hypothesized by this level of genetic variability, DO mice had variable responses to BHB Tolerance Tests at both baseline and after four weeks of ketogenic diet (Figure 3A). In Figure 3B we describe the within-mouse effects of the diet, again showing substantial between-strain variability, likely due to genetic differences. There was more variability in the area under the curve post-diet than pre-diet (SDfollow-up/SDbaseline= 1.93; p<1 x 10-15 via Levene’s test) suggesting diet-induced variability. Consistent with our findings using inbred A/J mice the majority of DO mice had worsened ketone disposal after diet (35 mice), with a smaller number of mice having improved ketone disposal by our assay (10 mice), more than would be expected by chance (p=1.9 x 10-4 from a χ2 test). Taking the population together there was a significant decrease in ketone disposal (increase in baseline adjusted KTT) in these mice after four weeks of ketogenic diet (p=4.8 x 10-5 from a paired Wilcoxon test).

To better understand the physiological basis and repercussions of this variation we performed an exploratory analysis to identify potential correlations between baseline, follow-up and changes in ketone disposal and a variety of other outcomes (Figure 3C). While none of these associations met our threshold for statistical significance we three interesting potential relationships between i) baseline ketone disposal and baseline weight, ii) between follow up and changes in ketone disposal and weight gain on a ketogenic diet (STATS), and iii) baseline ketone disposal and changes in and follow up total cholesterol levels (STATS, Figure 3C-D). The relationships between ketone disposal and other physiological parameters warrant further investigation.

# DISCUSSION

Based on our pilot study of 43 mice we estimate we would have only been able to detect correlations or r>0.55 between two correlated but independent variables.

# ACKNOWLEGEMENTS

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# Figure Legends

**Figure 1: Knockout of Muscle *Tsc1* enhances BHB disposal.** BHB Tolerance tests in male and female wild-type and muscle *Tsc1* knockout mice. Mice were injected with 1 g/kg BHB and followed for 1h. A) Absolute values stratified by sex. B) Area under the curve. Asterisks indicate p<0.05 from Student’s *t*-tests (n=4-10/group).

**Figure 2: Ketone body disposal is reduced in male A/J mice after ketogenic diet feeding.** BHB Tolerance tests in male A/J mice fed a control or ketogenic diet for three weeks. Mice were injected with 1.5 g/kg BHB and followed for 1h. A) Absolute values. B) Baseline subtracted values. C) Area under the curve for baseline subtracted values. D) qRT-PCR analysis of muscle quadriceps mRNA expression in male and female A/J mice. Asterisks indicate p<0.05 from Welch’s *t*-tests (n=3-8 in A-B and Student’s *t*-tests in D).

# Supplementary Figures

**Supplementary Figure 1:** Downregulation of ketogenic gene mRNA levels after four weeks of ketogenic diet in A/J mice. Asterisks indicate pairwise p<0.05 by Student’s *t* test (n=7-8/group).