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Comparison of Exogenous Ketone Administration versus Dietary Carbohydrate Restriction on Myocardial Glucose Suppression: A Crossover Clinical Trial

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ABSTRACT

The ketogenic diet (KD) is standard-of-care to achieve myocardial glucose suppression (MGS) for assessing inflammation using 18F-fluorodeoxyglucose (FDG)-positron emission tomography (PET). However, failure to suppress physiologic glucose uptake remains a significant diagnostic barrier. While extending the duration of KD may be effective, exogenously delivered ketones may provide a convenient, reliable, and same-day alternative. The aims of our study were to determine 1) whether exogenous ketone administration is non-inferior to the KD to achieve MGS, and 2) whether serum beta-hydroxybutyrate [BHB] levels can predict MGS.

Methods: KEETO-CROSS is a crossover, non-inferiority trial of the KD (endogenous ketosis) versus ketone ester (KE, exogenous ketosis) drink. Twenty healthy participants were enrolled into three arms: 1) weight-based KE drink 2) 24-hour KD, and 3) 72-hour KD (N=18 completed all arms). The primary outcome was achievement of complete MGS on PET (non-inferiority margin 5%). The area under receiver operating characteristics (AUROC) of endogenous BHB levels (analyzed in a laboratory and by point-of-care device) for predicting MGS was analyzed in 37 scans completed on the KD.

Results: Mean age was 30$\pm$7 years, 50% were female, 45% were non-white. Median (25^{th}-75^{th} percentile) achieved BHB levels (mmol/L) were 3.82 (2.55-4.97) (KE drink), 0.77 (0.58-1.02) (24-hour KD), and 1.30 (0.80-2.24) (72-hour KD). The primary outcome was achieved in 44% (KE drink), 78% (24-hour KD), and 83% (72-hour KD) of participants (non-inferiority p=0.97 and 0.98 for KE vs. 24-hour and 72-hour KD). Endogenous BHB levels robustly predicted MGS (AUROC 0.88, 95%CI 0.71, 1.00). BHB $\geq$0.58 correctly classified 92% of scans. A point-of-care device provided comparable predictive value.

Conclusions: In healthy volunteers, KE was inferior to KD for achieving MGS. Serum BHB is a highly predictive biomarker for MGS and can be clinically implemented upstream of FDG-PET, with rapid facilitation by point-of-care testing, to reduce false positive scans.

Keywords: ketogenic diet, ketone ester, FDG, PET, myocardial glucose uptake

INTRODUCTION

Detecting inflammation is clinically relevant for diagnosing several cardiovascular diseases, though remains challenging to achieve by current techniques. Non-invasive diagnosis of a growing number of inflammatory pathologies, as well as malignant cardiac masses, relies on visualizing glucose uptake by abnormal cells using fluorine-18 fluorodeoxyglucose (FDG) positron emission tomography (PET). However, since normal myocardium can also utilize glucose *(1)*, distinguishing physiologic from pathologic uptake can be particularly problematic and remains the 'Achilles' heel' of employing FDG-PET for such diagnostic testing *(2)*.

Short-term dietary modification through a low-carbohydrate, high fat ketogenic diet (KD) to suppress physiologic glucose uptake is standard-of-care for evaluating myocardial inflammation using radiolabeled markers of glucose utilization. The KD accomplishes this metabolic switch by reducing insulin-dependent myocardial glucose uptake, while inflammatory cells continue to consume glucose through non-insulin dependent glucose transporters. However, myocardial FDG suppression rates remain suboptimal and retrospective data suggest myocardial glucose suppression (MGS) is achieved in 81-84% of subjects even following strict, highly supervised dietary restrictions *(3, 4)*. Non-diagnostic and false positive scans lead to misdiagnosis, inappropriate immunotherapy, repeat scans with excess radiation exposure, and unnecessary costs to the patient and healthcare system.

One strategy to improve appropriate MGS is to increase the length of dietary modification *(5)*. However, adherence to the KD may not always be feasible, is often challenging, and requires advanced patient preparation. The negative correlation between carbohydrate and ketone utilization by the heart has been known for years *(6)*, and exogenous ketones significantly reduce myocardial glucose uptake through substrate competition and

inhibition of intracellular glucose phosphorylation *(7)*. A ketone ester (KE) compound can rapidly and safely achieve high levels of ketosis and may efficiently and effectively prepare patients for same-day evaluation of myocardial inflammation by FDG-PET *(8)*. However, no studies have directly compared a KE versus a KD strategy for achieving MGS. Additionally, given the inverse relationship between ketosis and MGS, ketone biomarkers may logically predict MGS. Despite promising data, neither hypothesis has been evaluated.

Ketogenic Endogenous versus Exogenous Therapies for myoCaRdial glucOse SuppresSion (KEETO-CROSS) is a crossover, non-inferiority trial of a novel MGS strategy comparing KE with the gold standard 24-hour KD, as well as 72-hour KD, in participants free of cardiovascular disease. We sought to determine whether a nutritional ketosis strategy would provide non-inferior diagnostic value compared with short term KD to achieve MGS on FDG-PET. We secondarily assessed the value of serum ketone levels (beta-hydroxybutyrate [BHB]) as an upstream biomarker of MGS on FDG-PET to reduce false positive and non-diagnostic scans.

METHODS

KEETO-CROSS Study Design

We conducted a crossover, open-label, non-inferiority trial comparing exogenous ketosis (KE) with endogenous ketosis (KD) (NCT04275453). Participants were randomly first assigned to the KE arm (1 visit) or KD arm (including two visits occurring at 24 and 72 hours) with at least a 1-week washout period in between arms. Participants aged 18-60 were enrolled at the University of Pennsylvania between January 2020 and January 2021. To ensure that any myocardial glucose uptake would be physiologic rather than indicative of underlying pathology,

we excluded individuals with any reported history of cardiovascular disease (including hypertension, hyperlipidemia, and diabetes mellitus). We also excluded pregnant/breast feeding women. Recruitment was continued until a total of 18 participants attended all three visits (KE, 24-hour KD, and 72-hour KD) to adequately power the trial. The CONSORT diagram for KEETO-CROSS is depicted in Supplementary Figure 1. The study was approved by an institutional review board and informed consent was obtained.

Patient Preparation Methods

Ketone Ester Arm

Participants during the KE arm were permitted to eat ad-lib during the day before their study visit and started fasting at least since midnight prior to FDG-PET scan (Figure 1). During the visit, participants received weight-based dosing (714 mg/kg) of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (H.V.M.N., Miami, Florida), which has been extensively studied for the purposes of achieving ketosis *(8-10)*. Briefly, this KE undergoes rapid enzymatic hydrolysis ultimately to form BHB (and other ketone bodies), achieving high BHB levels in less than 1 hour with a short compound half-life (0.8-3.1 hours). This high dose was selected to engender robust ketosis and substrate competition. Participants were injected with FDG 1 hour after KE ingestion since the peak ketotic effect of the KE occurs around this time.

Ketogenic Diet Arm

During the KD arm, participants presented for two visits: one after 24 hours of KD and then again after 72 hours of KD (Figure 1). The day before the 24-hour KD visit, participants began a low carbohydrate, high fat diet intended to achieve less than 20 grams of carbohydrate intake per day (Supplementary Methods) and fasted from at least midnight until FDG-PET scan the next day. Thereafter, they continued the KD for two additional days, fasting again from at

least midnight before the 72-hour KD visit. A detailed dietary log was maintained by study participants during the dietary arm and reviewed by a nuclear cardiologist prior to FDG injection. All participants were deemed to have adequate dietary adherence by dietary log review.

Laboratory Testing and Echocardiography

Details of laboratory testing (for BHB, insulin, glucagon, non-esterified fatty acid (NEFA), and glucose levels) and echocardiography are available in the Supplement.

FDG-PET Protocol and Study Outcomes

Participants underwent FDG-PET using a PET/CT scanner (Philips Ingenuity TF, Cleveland, OH). At study visits, FDG (~185 MBq [~5.0 mCi]) was injected and PET images of the chest were acquired 1 hour later. Low-dose CT images were also acquired for attenuation correction purposes and to aid in distinguishing FDG activity in the myocardium and blood pool on fused PET/CT images. Images were analyzed using MIM software (Cleveland, OH) by a board-certified nuclear cardiologist blinded to participant characteristics. The primary outcome of trial was complete MGS, defined by FDG activity in all segments of the myocardium lower than the blood pool *(2)*. The secondary trial outcome was the ratio of the average myocardial to blood pool standardized uptake value (SUV) in the septal and lateral walls.

Statistical Analysis

Baseline characteristics were described using means ± SD and medians and 25^{th}-75^{th} percentiles or percentages as appropriate for the levels of measurement and distributions of the

variables. Biomarker levels were compared using non-parametric testing since they were right-skewed, and the false discovery rate method was used for multiple testing correction.

We pre-specified a modified-intention-to-treat analysis among participants that completed all 3 visits. A sample size of 18 participants provided 80% power to detect a 10% difference between the KE and 24-hour KD group with a 5% non-inferiority bound at an alpha level of 5% and standard deviation of paired differences is 0.36, assuming a correlation of 0.62 between studies. The primary (dichotomous) outcome of the trial was assessed using the exact non-inferiority test of the difference between paired binomial proportions. As non-inferiority was only specified for the primary outcome, the secondary (continuous) outcome was analyzed using Wilcoxon Sign-Rank test since data were right skewed. To limit multiple testing, the primary analysis compared KE and the 24-hour KD (gold standard), while other comparisons are considered exploratory. To understand reasons for MGS failure with KE drink, we used logistic regression to assess the relationship between log-transformed, post-KE drink biomarker levels with MGS, displaying the odds ratio per standard deviation increase for ease of comparison between biomarkers.

In a pre-specified analysis, we assessed receiver operating characteristics of biomarkers using c-statistics to predict achievement of MGS, accounting for clustering at the participant level when applicable. For ease of comparison between biomarkers, c-statistics for insulin and glucose levels were displayed modeling the risk for MGS failure since these biomarkers are theoretically inversely related to MGS, while other biomarkers are directly related. We used continuous splines with 4 knots after confirming non-linearity to model endogenous BHB levels and the myocardium to blood pool SUV ratio. Logistic splines were also employed to model the relationship between MGS and endogenous ketone levels. We further assessed the diagnostic

value of point-of-care BHB values after the protocol modification. Bland-Altman plot and Spearman's Rho were used to compare ketone levels by laboratory and point-of-care analysis. Analyses were performed separately for endogenous and exogenous ketone levels since each mode of ketosis reflects differing cardiovascular physiology and mechanisms of action *(1)*.

Analyses were performed using STATA version 14 (STATA Corp, College Station, TX) and StatXact-12 (Cytel, Waltham, MA). For the primary outcome assessing non-inferiority, a one-sided p-value<0.05 was considered significant, while other analyses used a two-sided p-value < 0.05.

RESULTS

The baseline characteristics of the 20 KEETO-CROSS heathy participants who completed 57 FDG-PET scans are shown in Supplementary Table 1. Mean age was 30±7 years, 50% were female, and 45% were non-white. Average duration of fasting (hours) prior to FDG injection was 15.9±1.3 (KE drink arm), 16.7±1.6 (24-hour KD arm), and 15.2±2.0 (72-hour KD arm).

From a hemodynamic perspective, systolic blood pressure (119±12 to 124±11 mmHg; P=0.023) and heart rate (62±12 to 71±13 beats per minute; P<0.001) increased significantly ~ 30 minutes after consumption of the KE drink. Heart rate also showed a trend for increment (68±13 and 76±12 bpm; P=0.14), whereas systolic blood pressure (117±15 and 117±11 mmHg; P=0.90) did not change with diet-induced ketosis at 24 and 72 hours respectively.

Metabolite and Hormones Levels by Study Arm

BHB, NEFA, insulin, and glucagon levels by study visit are displayed in Figure 2 for the 18 participants in the modified intention-to-treat analysis. BHB levels (presented as median, 25th-75th percentile) increased rapidly and significantly from immediately prior (0.12, 0.10-0.26 mmol/L) to 30 minutes after KE drink ingestion (3.82, 2.55-4.97 mmol/L, p<0.001). Post-KE drink levels were significantly higher than that achieved by 24-hour KD (0.77, 0.58-1.02 mmol/L, p<0.001) and 72-hour KD (1.30, 0.80-2.24 mmol/L, p=0.029). A similar pattern was observed when comparing the post-KE drink insulin levels with other times points. Glucagon and NEFA levels were lower post-KE drink compared with the 24 and 72-hour KD arms (p<0.05 for all comparisons), though were not different from before KE drink levels (p>0.20 for both comparisons). As expected, glucose levels were lower following the 24-KD and 72-KD groups compared with overnight fasting, reflected by the pre-KE drink levels (Supplementary Table 2).

KEETO-CROSS Primary and Secondary Outcomes

Using the modified intention-to-treat protocol, we assessed the primary outcome (complete MGS) in the 18 participants that completed all three study visits (Figure 3). Complete MGS was achieved in 8/18 (44%), 14/18 (78%), and 15/18 (83%) participants in the KE, 24-hour KD, and 72-hour KD arms, respectively. KE failed to meet the non-inferiority bound compared with the KD arms (non-inferiority p=0.97 and 0.98 for KE vs. 24-hour and 72-hour KD, respectively).

Among the 18 participants completing all three visits, the secondary outcome (average SUV uptake myocardium/blood pool, presented as median [25th-75th percentile]) was 1.01 (0.72-1.70), 0.67 (0.61-0.79), and 0.63 (0.56-0.66) in the KE, 24-hour KD, and 72-hour KD arms, respectively (p=0.008 for KE vs. 24-hour KD, and p=0.004 for KE vs. 72-hour KD) (Figure 3).

Individual-level responses to MGS strategies are shown in Supplementary Figure 2. There were two participants that failed at least 1 dietary strategy and suppressed with the KE (Supplementary Figure 3).

Utility of Biomarkers to Predict Myocardial Glucose Suppression

Figure 4 shows area under the receiver operating characteristics curve (AUROC) for endogenous serum BHB levels to predict MGS in the KD arms (n=37 scans). Serum BHB levels robustly predicted MGS (c-statistic 0.88, 95% CI 0.71-1.00). A threshold serum BHB level <0.34 mmol/L correctly classified failure to achieve MGS, while BHB levels $\geq$1.09 mmol/L correctly classified MGS success, in these FDG-PET scans. As a single threshold, using a BHB level $\geq$0.58 mmol/L for predicting MGS resulted in the highest correct classification of any value (92%), including 6/7 (86%) scans that were correctly classified as likely failure, and 28/30 (93%) studies that were correctly classified as likely MGS based on BHB results. Glucose (c-statistic 0.78, 95%C CI 0.61-0.95), glucagon (0.75, 95% CI 0.56-0.94), insulin (0.69, 95% CI 0.44-0.92) and NEFA (0.66, 95% CI 0.42-0.90) were less predictive of MGS, in comparison with BHB.

Representative images further illustrating the correlation between common myocardial FDG uptake patterns and their respective BHB levels are shown in Figure 5. The relationships between BHB levels and glucose uptake using spline analyses are shown in Figures 6 and 7.

We also assessed the relationships between pre-KE drink BHB levels, post-KE drink BHB levels, and the difference between these levels with MGS. All three measurements were lower among those that failed MGS than those that appropriately suppressed ($p<0.05$ for all comparisons) (Supplementary Table 3). All three measurements also significantly predicted

MGS (AUROC 0.85, 95% CI 0.62-1.00; 0.85, 95% CI 0.67-1.00; 0.82, 95% CI 0.60-1.00, respectively).

Log BHB levels post-KE drink were significantly associated with MGS (standardized odds ratio 4.92, 95%CI 1.25-18.43, p=0.022). Alternatively, log-transformed NEFA levels (standardized odds ratio 2.07, p=0.16), insulin levels (standardized odds ratio 1.02, p=0.97), and glucagon levels (standardized odds ratio 1.37, p=0.51) were not predictive of MGS post-KE drink.

Utility of POC Ketone Testing to Predict MGS

To enhance clinical utility and provide a rapid assessment of ketosis prior to FDG-PET, we also investigated POC ketone testing (paired testing available in 59 samples). Bland-Altman and scatter plots are shown in Supplementary Figures 4 and 5 comparing laboratory-derived BHB values and POC BHB values. The mean difference of laboratory and POC derived BHB levels was 0.04 (95%CI -0.88, 0.97 mmol/L), and correlation was strong (Spearman's rho 0.96, $p<0.001$).

Since agreement was worst at the highest BHB levels which were obtained after KE administration, we also assessed BHB levels obtained during the KD visits separately (as these lower values are encountered clinically with the KD preparation, N=28 samples). The mean difference of laboratory and POC derived BHB levels was -0.02 (95%CI -0.29, 0.25 mmol/L), and correlation was strong (Spearman's rho 0.98, $p<0.001$) (Supplementary Figures 6 and 7).

POC BHB levels during the KD significantly predicted MGS (c-statistic 0.87, 95% CI 0.66-1.00) (Supplementary Figures 8 and 9). Using POC BHB levels $\geq$0.6 mmol/L for predicting MGS correctly classified 89% of scans.

Adverse Events

Mild (grade 1) adverse events occurred in nine participants in the KE drink arm which were mostly gastrointestinal (nausea or heartburn, N=7) including one participant who experienced emesis, and two participants reported a headache. In the KD arm, one participant reported presyncope (grade 1), while another participant reported back pain (grade 2) which led to study discontinuation.

DISCUSSION

FDG-PET plays a critical role in assessing cardiovascular inflammation, infection, and tumors, yet adequate myocardial preparation to suppress physiologic glucose uptake remains a substantial diagnostic barrier, particularly as certain patterns of incomplete suppression (Figure 5) can be indistinguishable from pathology. Such challenges may ultimately lead to misdiagnosis, inappropriate therapy along with potentially harmful side effects, extra-radiation exposure for repeat testing, and unnecessary costs to the patient and healthcare system.

In KEETO-CROSS, an open-label, crossover trial of healthy participants, we found that a generalized strategy of KE was inferior to 24 and 72-hour KD to achieve MGS. However, a few individuals who failed the KD were able to suppress with the KE drink. Importantly, however, we found that serum BHB levels during the KD robustly predicted MGS and identified potential thresholds that can be used upstream of FDG-PET to predict MGS, thereby reducing false positive or indeterminate scans. To facilitate clinical implementation, we tested the utility of a POC ketone meter that showed similar ability to predict MGS to laboratory-based analysis. Thus, our findings 1) support the continued use of the dietary modification through the KD rather than

KE to achieve MGS, 2) provide strong evidence for assessing BHB levels prior to FDG-PET to ensure adequate dietary preparation, and 3) demonstrate how a POC ketone device can be implemented to aid in rapid-decision making.

Despite several strategies to facilitate the myocardial "metabolic switch" to suppress physiological glucose uptake, current MGS rates vary substantially, though much of these data are derived retrospectively and from convenience cohorts referred for clinical indications *(2)*. The current trial was designed to address these challenges by investigating the use of an oral KE, which could facilitate same-day scans without patient preparation, in healthy volunteers. Endogenous and exogenous ketosis suppress myocardial glucose uptake in different ways (Figure 7). While endogenous ketosis has been standard-of-care for MGS, exogenous ketones also reduce myocardial glucose uptake, as shown in pre-clinical and clinical work *(11, 12)*. However, our results demonstrate that despite significant acute ketosis achieved, rates of suppression were inferior to the KD. We hypothesize several mechanisms for failure of the KE to uniformly suppress myocardial glucose uptake in these healthy volunteers. Post-translational protein acetylation and attenuation of insulin signaling are necessary for the efficient inhibition of the glucose transporter member 4 (GLUT4) translocation,*(13)* and thus FDG accumulation in myocytes. In this sense, acute exogenous ketone delivery may not engender effective substrate competition and a "Randle Effect" in the healthy heart given normal mitochondrial function *(7, 14)*. For example, post-KE drink NEFA levels were significantly lower than that achieved by KD. In this way, the KD may be more effective since it feeds both ketones and fatty acids for substrate competition leading to increased fatty acid oxidation and protein acetylation. Second, failure of exogenous ketones could relate to increased insulin levels and, thus, signaling, an effect that can be counterproductive to achieving MGS. Although, we did not find a relationship

between post-KE insulin levels and MGS. Third, the hemodynamic effect of exogenous ketones could theoretically contribute to increased myocardial glucose uptake, consistent with the vital sign changes in our study *(11)*. Finally, the metabolic effect of exogenous ketone administration may be more dependent on the duration, rather than the peak levels, of ketosis before the "metabolic switch" occurs. In this sense, it remains to be proven whether extending the delay between KE and FDG administration would have led to improved suppression rates.

Notably, we found that ketone levels strongly predicted MGS. Broadly speaking, ketogenesis is a complex process that is influenced by hormonal signaling (including insulin and glucagon levels), transcriptional regulation, and post-transcriptional modification *(1)*. As the end result of several biological processes that reflect the milieu of glucose regulation, it is not surprising that ketone levels significantly relate to MGS. In our study, endogenous BHB levels had an AUROC of 0.88 for predicting MGS, and a cutoff of 0.58 mmol/L accurately classified MGS 92% of the time. This predictive ability of BHB was stronger than those with insulin, glucagon, glucose, and NEFA, consistent with other studies that have found modest predictive ability for these biomarkers *(15)*, and clarifies that ketosis is a more potent driver, or predictive biomarker, of MGS than these other metabolic processes. Such potent prediction has clinical implications, as BHB levels can be routinely assessed prior to FDG-PET to ensure adequate patient preparation. For instance, we found that 86% of subjects with BHB levels <0.58 mmol/L failed to make the metabolic switch, and oftentimes, the patterns of incomplete suppression were indistinguishable from inflammation (Figure 5). Consequently, if BHB levels are below this threshold, a longer duration of the KD (e.g. 72 hours) can be undertaken with the visit rescheduled. Such an approach could considerably minimize false positive or non-diagnostic scans, decreasing diagnostic uncertainty in clinical reading, inappropriate diagnoses, unnecessary

costs, and radiation exposure from repeated scans. Alternatively, KE could be theoretically combined with the KD in participants below the threshold, since higher levels of ketosis post KE significantly predicted MGS. Moreover, POC ketone testing allows for rapid triage of patients and has already been in implemented in other clinical settings *(16)*. Such testing showed high agreement with laboratory-based analyses at levels encountered during KD and similarly predicted MGS. Given the reasonable costs of ketone meters, it may be possible for patients to monitor ketone levels remotely prior to FDG-PET, potentially avoiding unnecessary travel and improving clinical throughput if levels predict inadequate preparation. Importantly, however, further studies in cohorts of patients with heart failure will be needed given relative myocardial glucose avidity in the failing heart *(1)*.

There are some limitations. While we chose to study KE against an active comparator (KD, which is standard-of-care), a placebo (fasting-only) arm would have provided firm conclusions on whether KE (and not just prolonged fasting) did suppress myocardial glucose uptake in some participants. However, an additional arm would have engendered greater radiation exposure against a weak comparator, and historical data on MGS are available for fasting only patient preparations *(2)*. In addition, our sample size is modest. Finally, our study design is open-label, which was limited by practicality of the dietary intervention, though endpoint adjudication is blinded. Strengths include a strict definition of complete MGS, crossover design, inclusion of extended duration KD in addition to the gold standard 24-hour KD preparation, analysis of several relevant hormones and metabolites, and assessment of a POC device.

In summary, a general strategy of exogenously administered KE was inferior to KD to achieve MGS. BHB levels strongly predicted MGS in healthy participants and can be clinically

implemented prior to FDG-PET to minimize false positive or non-diagnostic scans, potentially decreasing inappropriate diagnoses, radiation exposure, and healthcare system costs. POC ketone testing can provide rapid and accurate triage for adequate patient preparation for FDG-PET. Further study to validate the diagnostic value of BHB levels in other patient cohorts, including older individuals, diabetics, and subjects with cardiomyopathies, is warranted.

DISCLOSURES

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KEY POINTS

Questions: 1) Is exogenous ketone administration non-inferior to the standard ketogenic diet to achieve myocardial glucose suppression? 2) Can a serum biomarker predict myocardial glucose uptake status?

Pertinent Findings: 1) Exogenous ketone administration was inferior to the ketogenic diet to achieve myocardial glucose suppression. 2) Beta-hydroxybutyrate (BHB) levels strongly predicted the metabolic switch of glucose uptake in the heart of healthy volunteers.

Implications for patient care: BHB testing can provide rapid and accurate triage for adequate patient preparation for cardiovascular inflammation imaging with FDG-PET.

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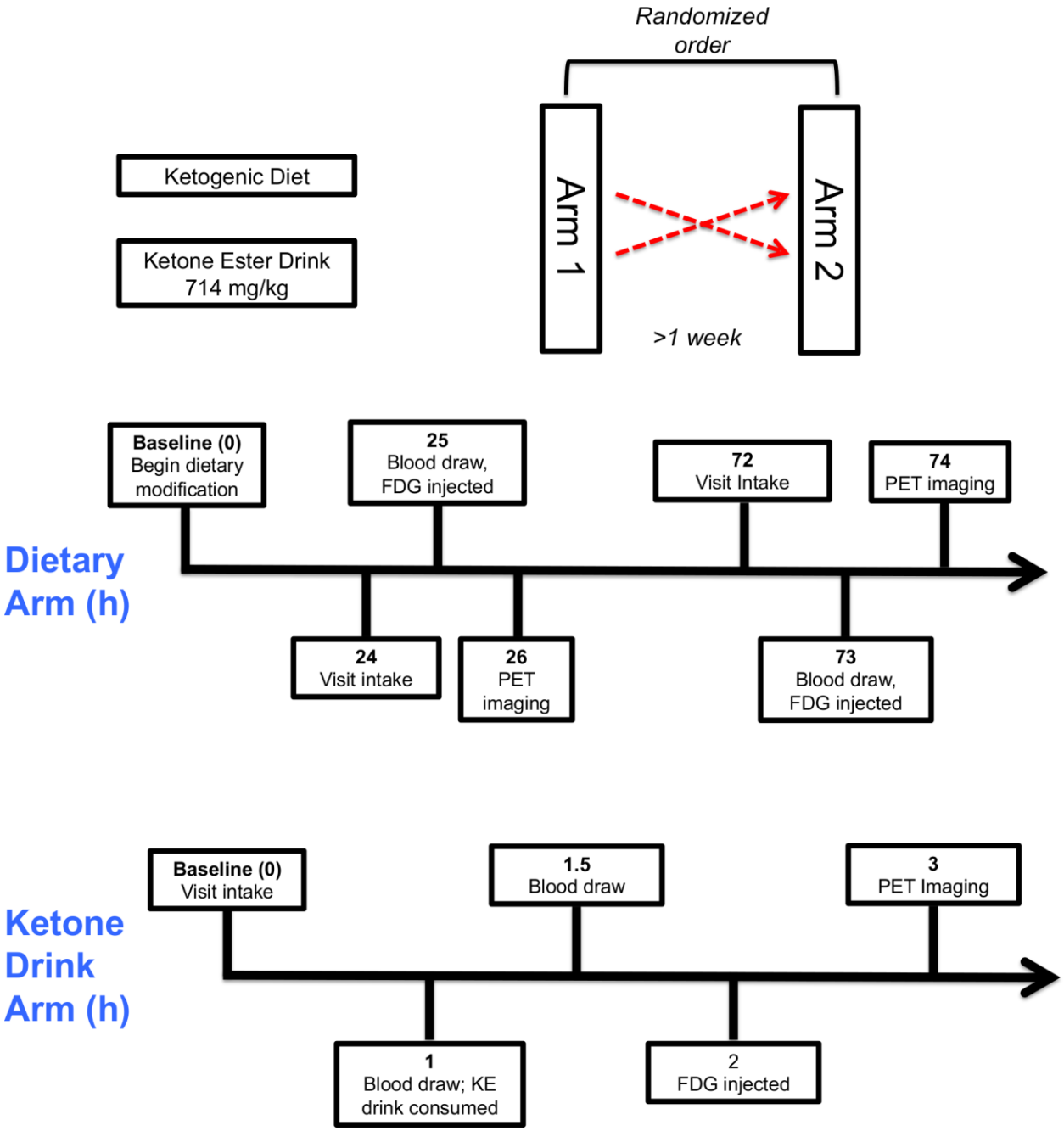


Figure 1: KEETO-CROSS Study Design

Participants were assigned in random order to KE and 24/72-hour KD arms, and after at least a 1-week washout period, participants returned for the remaining arm. Visit timepoints in hours depicted. ^{18}F-FDG, fluorine-18 fluorodeoxyglucose; KD, ketogenic diet; KE, ketone ester; PET, positron emission tomography.

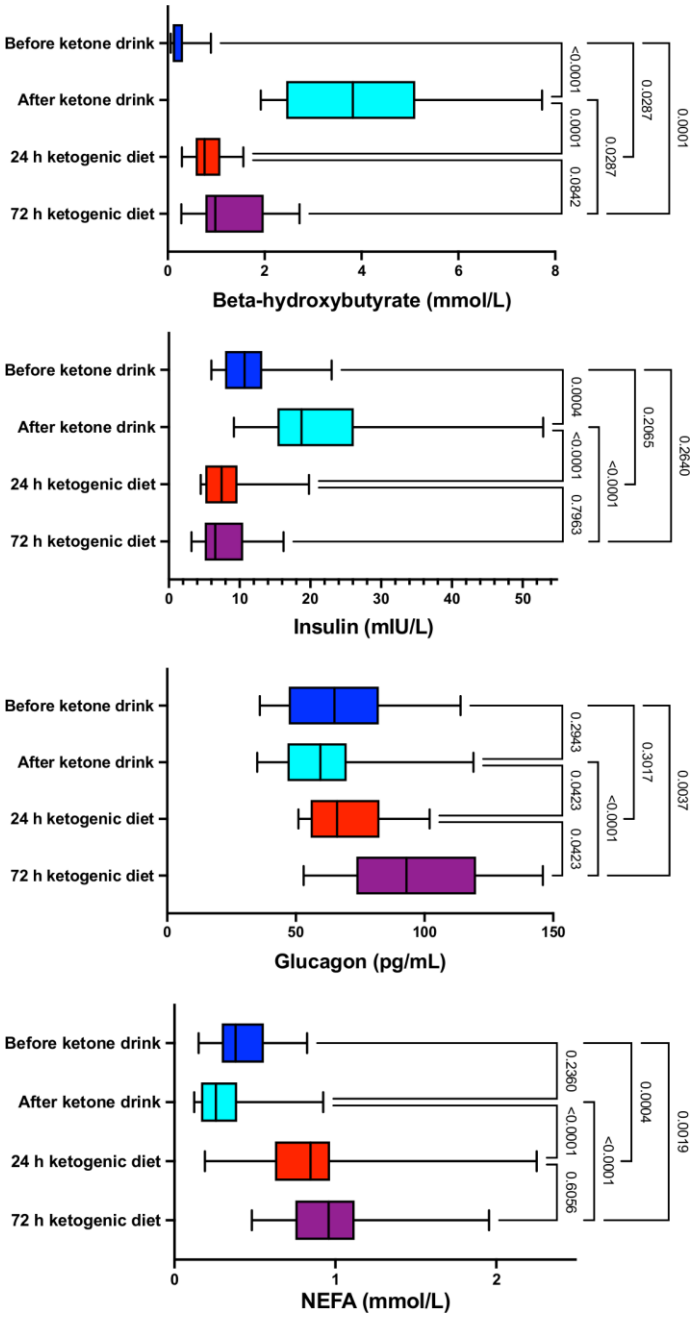


Figure 2: Biomarkers Levels by Study Arm

Box-and-whisker plots for ketone, insulin, glucagon, and NEFA levels are displayed by study visit for the 18 participants included in the modified intention-to-treat analysis. Whiskers depict minimum and maximum values. P-values corrected for multiple testing. NEFA, non-esterified fatty acid.

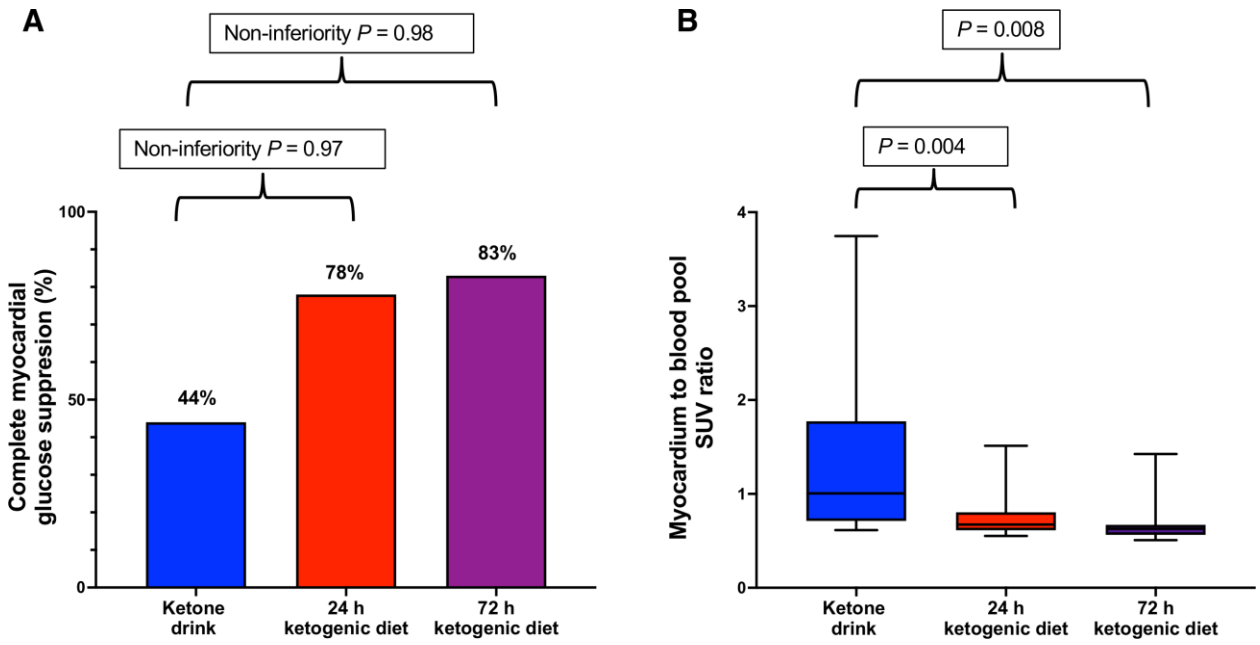


Figure 3: Primary and Secondary Endpoints of KEETO-CROSS

A.) Percentages of primary endpoint achievement (complete myocardial glucose suppression, left) and B.) box-and-whisker plots of secondary outcome (average SUV uptake myocardium to blood pool, right) shown among 18 participants who completed all study visits. SUV, standardized uptake value.

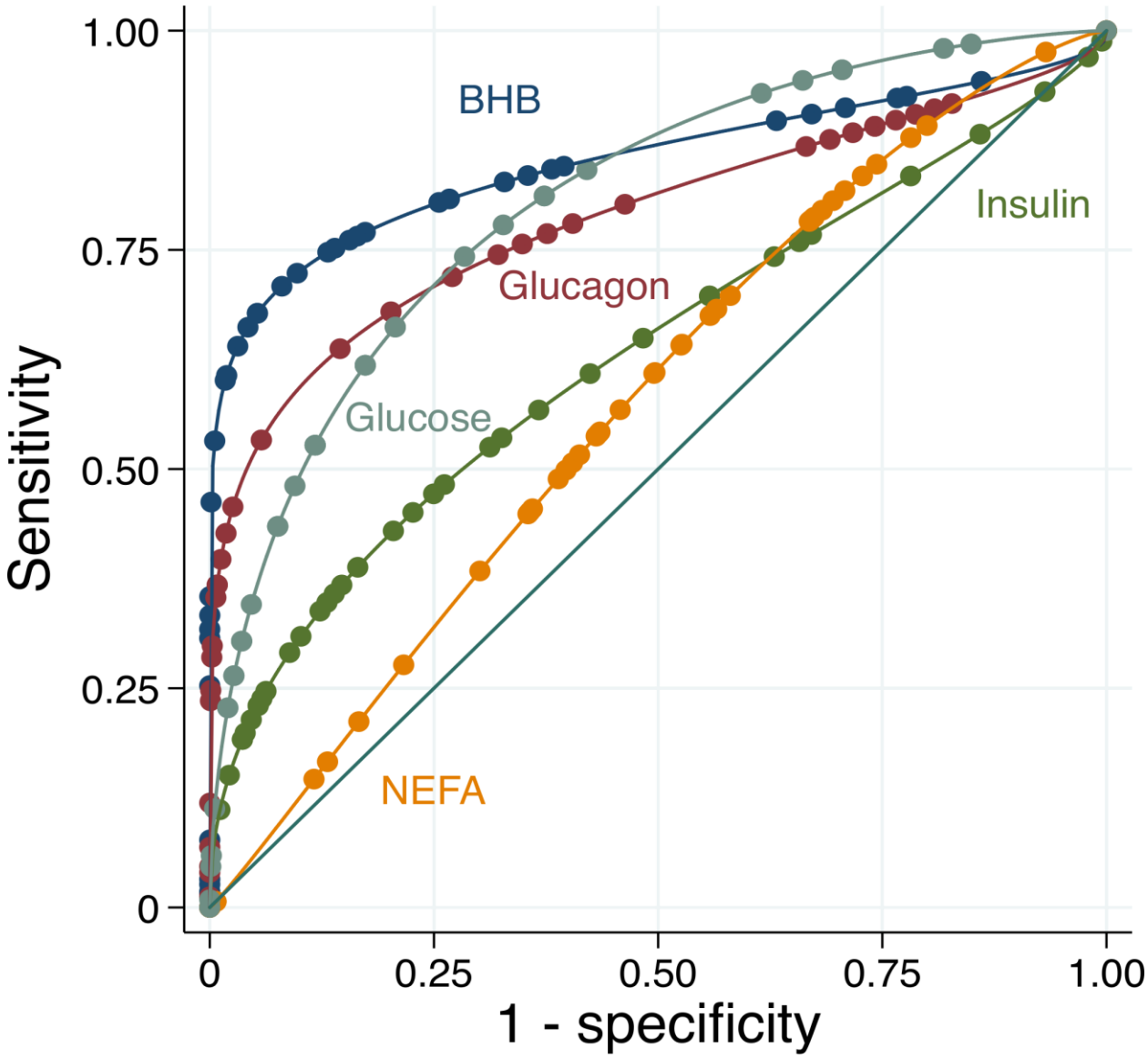


Figure 4: Receiver Operating Characteristics Curve Analysis to Predict Myocardial Glucose Suppression During Ketogenic Diet

Receiver operating characteristics curve shown for BHB, insulin, glucagon, NEFA, and glucose obtained during the ketogenic diet to predict the primary outcome in all participants. BHB, beta-hydroxybutyrate; NEFA, non-esterified fatty acids.

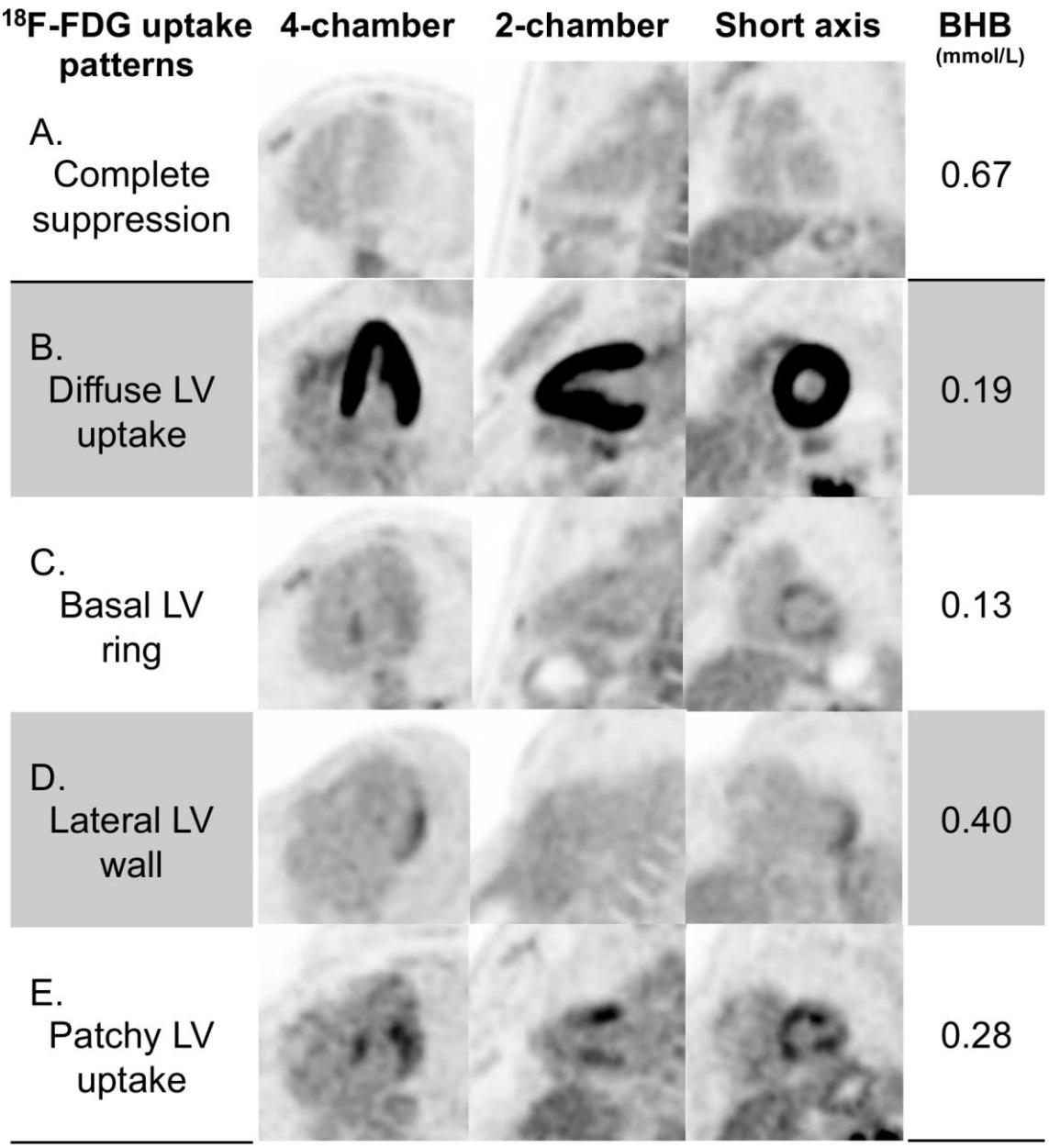


Figure 5: Myocardial FDG Uptake Patterns in Healthy Volunteers

Representative images (displayed using the same window width 0-5) of the most common myocardial FDG uptake patterns encountered in our healthy cohort with their corresponding BHB levels. Please notice that patterns C-E can be potentially mistaken as myocardial inflammation, however, the accompanying BHB levels should raise concern for incomplete suppression. BHB, beta-hydroxybutyrate, LV, left ventricular.

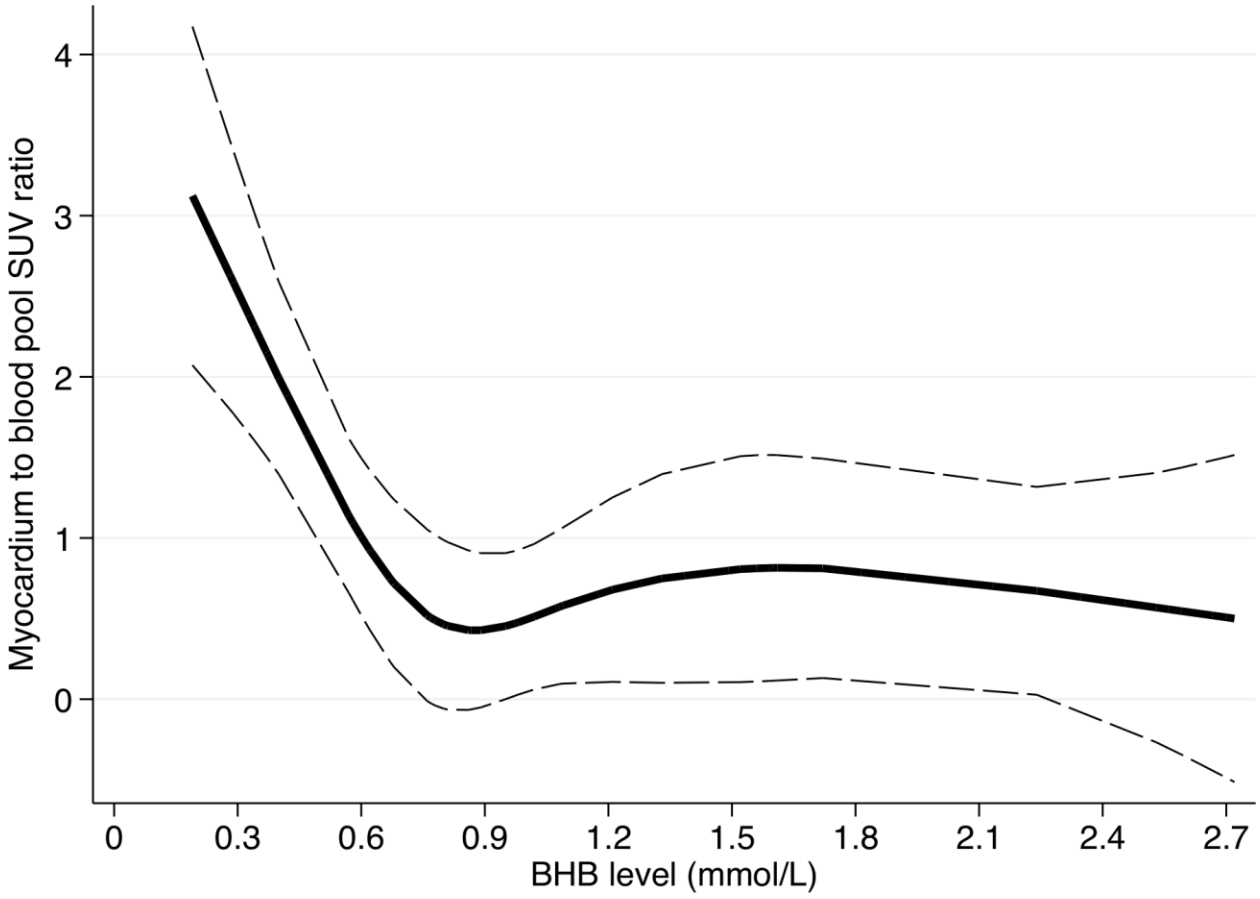


Figure 6: Relationship between Endogenous Ketone Levels and Myocardial Glucose Uptake

Continuous spline analysis with 4 knots depicts the relationship between endogenous ketone levels (BHB) and the secondary outcome (SUV ratio of the myocardium to blood pool). BHB, beta-hydroxybutyrate.

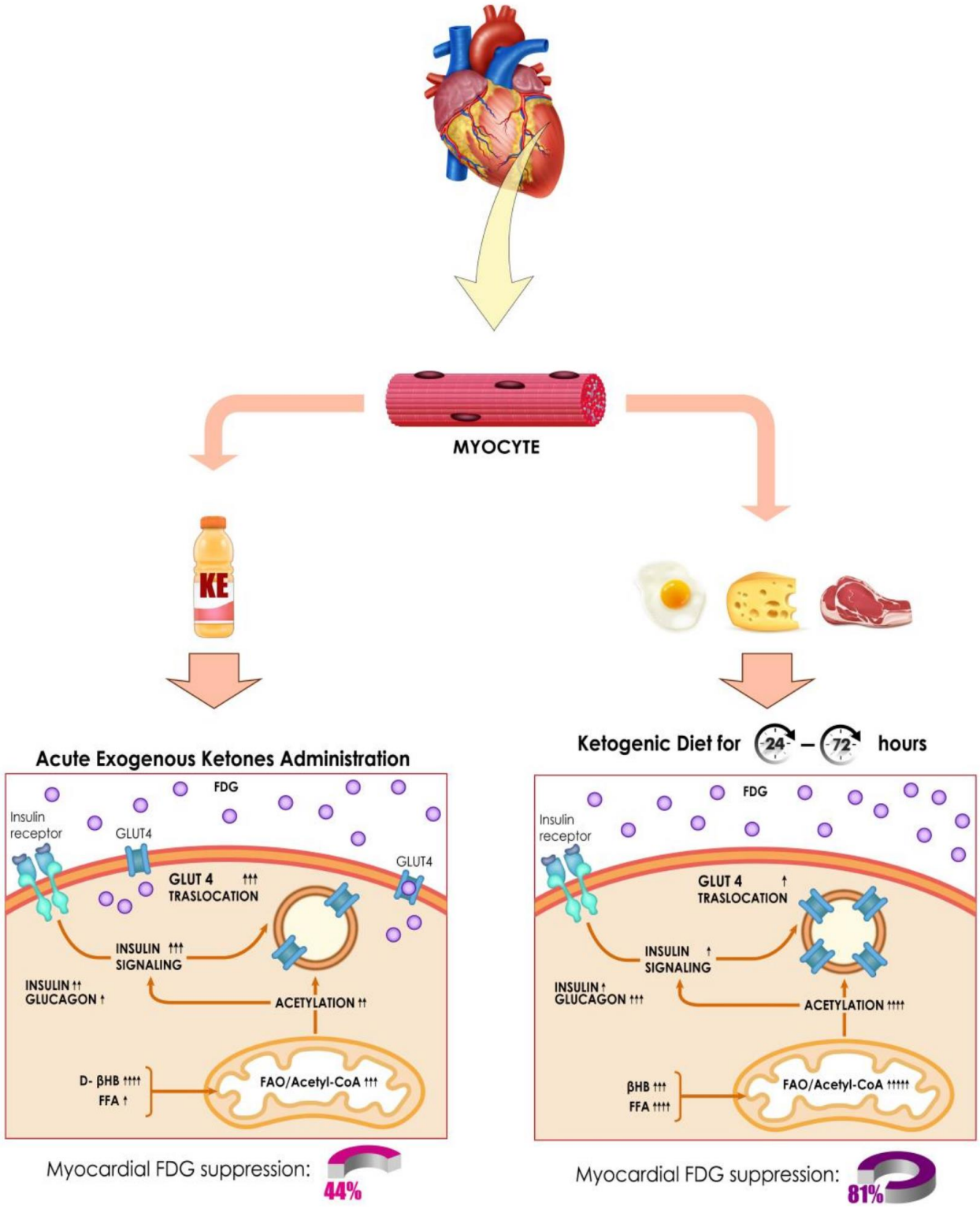
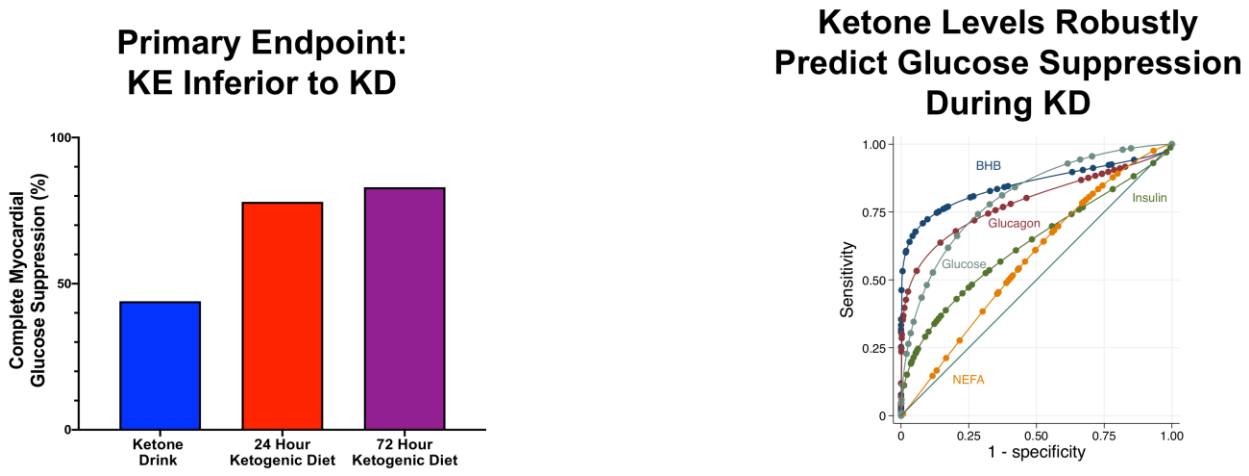


Figure 7: Proposed molecular mechanisms associated with myocardial FDG suppression between acute exogenous ketones administration (A) and endogenous ketosis induced by dietary

modification (B). Post-translational protein acetylation and attenuation of insulin signaling are both required mechanisms for successful inhibition of the glucose transporter member 4 (GLUT4) translocation. Acute administration of the ketone ester (KE) drink leads to a several-fold increment in D-Beta-Hydroxybutyrate (D-βHB) levels, but also appears to decrease free fatty acid (FFA) levels, which may ultimately impact the modulation of insulin signaling and the process of acetylation. In contrast, while the ketogenic diet (KD) yields lower βHB levels after 24 to 72 hours, it actually increases FFA and glucagon and lowers insulin levels on a sustained fashion, eventually, augmenting fatty acid oxidation (FAO) over glucose oxidation ("Randle effect"), increasing protein acetylation, and limiting insulin signaling. This may explain why myocardial FDG suppression rates were 44% for KE and 81% for KD. Adapted from "Insulin Mechanism", by BioRender.com (2021).

KEETO-CROSS

A Randomized, Crossover, Open-Label, Non-Inferiority Trial of Endogenous versus Exogenous Ketosis to Suppress Myocardial Glucose Uptake on ^{18}F-FDG PET



Graphical Abstract

SUPPLEMENTARY METHODS

Dietary guidance provided to KEETO-CROSS study participants.

Sample Meal Plan

Eating the following meals is recommended. This will ensure that you are limiting your carbohydrate and sugar intake and eating the proper, high fat foods.

Breakfast: Scrambled eggs and plain breakfast sausage cooked in butter; black coffee and/or water (no limit on how much)
Lunch: Grilled chicken with olive oil and red wine vinegar dressing (no limit on how much), cheddar cheese (2-oz limit)
Dinner: Grilled or broiled Steak, pork chop, hamburger; 1/4 cup broccoli
Snacks: Hard-Boiled eggs (no limit on how many)
If you choose to eat something other than the previous meals listed, please follow the guidelines below.

Permitted Foods

Fish including: Flounder, Herring, Salmon, Sardines, Sole, Tuna, Trout are ALL OKAY as long as they are not breaded.
Unprocessed Meats/Poultry including: Chicken, Turkey, Duck, Goose, Beef, Pork, Plain Bacon, Plain Sausage, Veal, Lamb, are ALL OKAY as long as they are not breaded or honeyed.
Shellfish including: Clams, Crab, Shrimp, Squid/Calamari are ALL OKAY as long as they are not breaded.
Vegetables raw or cooked including: Alfalfa, Bamboo Shoots, Bok Choy, Broccoli, Cauliflower, Celery, Chard, Cucumber, Eggplant, Garlic, Ginger, Palm, Leeks, Lettuce (including: Romaine, Iceberg, Spinach, Arugula, Endive), Mushrooms, Radishes are Artichoke, Avocado, Asparagus, Beets, Brussel Sprouts, Carrots, Cabbage, Collard Greens, Fennel, Jicama, Kale, Okra, Olives, Onion Peppers (Green, Red, Yellow, Jalapeno), Turnips, Water Chestnuts, Zucchini ALL OKAY if you are only eating cup TOTAL for the entire day- do NOT exceed.
Eggs: Hard-boiled, Fried, Over-Easy and Omelets are ALL OKAY as long as milk or vegetables have not been added.
Cheese: hard, natural, unprocessed cheese such as cheddar and mozzarella are ALL OKAY as long as the label reads “0 g carbohydrates”.
Herbs and Spices are fine. But please read the label and be sure they do not contain any sugar / carbs.
Salad Dressings: Olive Oil and Vinaigrette are the only dressings allowed.
Beverages including: Water, Non-Flavored Club Soda/Seltzer, Coffee and Tea (with no added, sugar, flavors or cream) are ALL OKAY.

Forbidden Foods

Lunchmeat as well as Sweetened or cured meats, such as flavored/sweetened bacon, hams and/or sausages are NOT ALLOWED.
Oysters and Mussels are NOT ALLOWED.
Milk, yogurt, cottage cheese, and processed cheese are NOT ALLOWED.

Breads, Toast, Rice, Pastas, Noodles of any kind (including: Egg Noodles, Rice Noodles) are NOT ALLOWED.
All Cereals, Oatmeal, Bagels, Toast, Crackers, Doughnuts, Muffins are NOT ALLOWED.
Grains including: Oats, Barley, Quinoa, Granola, Corn are all NOT ALLOWED.
All Nuts (including: Peanuts, Almonds, Cashews, Walnuts, Macadamia, Pistachio, Hazel) are NOT ALLOWED. Seeds including Sunflower, Pumpkin, Chia are NOT ALLOWED.
Vegetables, raw or cooked, including: Corn, Peas, Potatoes, Pumpkin, Squash, Tomato, are NOT ALLOWED.
Beans including: lentils, baked beans, cannellini, kidney, lima beans, black-eyed peas are NOT ALLOWED.
Fruits are also high in natural sugars and carbohydrates. Fruits are NOT ALLOWED.
Drinks including: Sports Drinks, Juices, Alcoholic Beverages (all Beer, Wine and Spirits), Lattes, Coffee with creamers or sugars, Flavored Waters, Sodas, Diet Sodas, Iced Tea, Teas, Milkshakes are NOT ALLOWED.
Soda, Diet Soda and Sweetened Iced Tea are NOT ALLOWED.
Artificial sweeteners (e.g. Splenda, Sweet'N Low, Equal) are NOT ALLOWED.
Condiments including: Ketchup, Mayonnaise, Tartar Sauce, Mustard, Relish, Barbeque Sauce are NOT ALLOWED.
Most marinades are all very high in sugar and carbohydrates; they are NOT ALLOWED.
Sauces including: Barbeque, Hot Sauce, Sweet and Sour, Cheese, Chicken Wing Sauce, Pasta Sauces (including: Tomato, Pesto, White Wine and Lemon, Vodka, Marinara, Pomodoro, Bolognese, Alfredo, Carbonara, Ragu), Marsala, Soy Sauce, Tikka Masala, Peanut Sauce, Béchamel, Hollandaise, Pico de Gallo, Guacamole, Aiolis, Steak Sauces (A1, Worcestershire), Hummus are all NOT ALLOWED.
Oils and Spreads including Cream Cheese, Cottage Cheese, Yogurt, Sour Cream, Nutella, Peanut Butter, Jellies, Jams, Canned Cheese and Cheese Dips are NOT ALLOWED.

SUPPLEMENTARY METHODS

Metabolite and Hormone Level Analysis

Venous blood was collected via peripheral phlebotomy prior to ketone drink and near the time of FDG injection at each study visit (**Figure 1**); serum BHB levels were then determined on freshly processed samples using a Beckman-Coulter analyzer (AU5800 or AU680). The reportable range for BHB is 0.02-13.5 mmol/L, and the coefficient of variation through multilevel, internal quality control ranges from 0.8%-2.6%. Starting in September 2020, the study protocol was modified to additionally include paired point-of-care (POC) capillary BHB testing using a commercially available device that has been previously studied in other clinical settings (Abbott, Precision Xtra),(*11*) and POC BHB samples were obtained in 44/57 visits (59 individual sampling timepoints).

Insulin, glucagon, and non-esterified fatty acid (NEFA) were batch analyzed at the Penn Radioimmunoassay and Biomarkers Core. Blood samples were collected on ice into tubes on ice containing EDTA and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Samples were centrifuged at 4°C, separated, and frozen at -80°C for subsequent analysis. NEFA levels (range 0.125-2.9 mM) were measured in duplicate using enzymatic colorimetrics (Wako Chemicals, Richmond, VA). Plasma insulin (range 2-100 mIU/mL) and glucagon (range 20-400 pg/mL) were measured in duplicate by double-antibody radioimmunoassay (Millipore, Billerica, MA). Glucose levels were measured using a point-of-care device near the time of FDG injection for the KD visits and before KE administration.

Echocardiography

To confirm the absence of structural heart disease, comprehensive 2D/Doppler echocardiograms were performed prior to KE administration by board-certified sonographers

using guideline-based protocols (*11*). Left ventricular (LV) ejection fraction (EF) was assessed using the biplane method of disks. All echocardiographic assessments were performed offline using Syngo Dynamics (Siemens Medical Solutions, Malvern, PA) by a cardiologist board-certified in echocardiography who was blinded to participant clinical characteristics.

SUPPLEMENARY TABLES

Supplementary Table 1. Baseline Clinical Characteristics of KEETO-CROSS Study Participants

	All Study Participants N=20
Age, years	30±7
Women, n (%)	10 (50%)
Race, n (%)	
• White	11 (55%)
• Black	4 (20%)
• Asian	4 (20%)
• Other	1 (5%)
Average number of alcohol drinks (per week)	6.7±3.3
Current smoker	1 (5%)
Physical Characteristics	
• Systolic blood pressure (mmHg)	119±12
• Diastolic blood pressure (mmHg)	75±10
• Heart rate (beats/min)	71±12
• Body mass index (kg/m^2)	24.3±3.1
Laboratory Testing	
• Creatinine (mg/dL)	0.8±0.1
• Glucose (mg/dL)	81±10
• Hemoglobin (g/dL)	13.2±1.6
• Low density lipoprotein cholesterol (mg/dL)	102±22
• High density lipoprotein cholesterol (mg/dL)	55±12
• Triglycerides (mg/dL)	78±24
Echocardiography	
• LV end-diastolic diameter (cm)	4.78±0.37
• LV septal wall thickness (cm)	0.77±0.10
• LV ejection fraction (%)	59±4
• Septal e' velocity (cm/s)	11.9±2.3
FDG-PET	
• Fasting time before FDG injection at ketone drink visit (hr)	15.9±1.3
• Fasting time before FDG injection at 24-hour ketogenic diet visit (hr)	16.7±1.6
• Fasting time before FDG injection at 72-hour ketogenic diet visit (hr)	15.2±2.0

Supplementary Table 2. Glucose Levels by Study Arm

	Glucose level (mg/dL) (N=18)
• Pre-ketone ester	92 (91-101)
• 24-hour ketogenic diet*	86 (81-90)
• 72-hour ketogenic diet*	83 (76–95)

Presented as median (25^{th}-75^{th} percentile)

*$p<0.05$ for comparison against pre-ketone ester levels

Supplementary Table 3. Ketone levels Before and After Ketone Drink, Stratified by Achievement of Myocardial Glucose Suppression

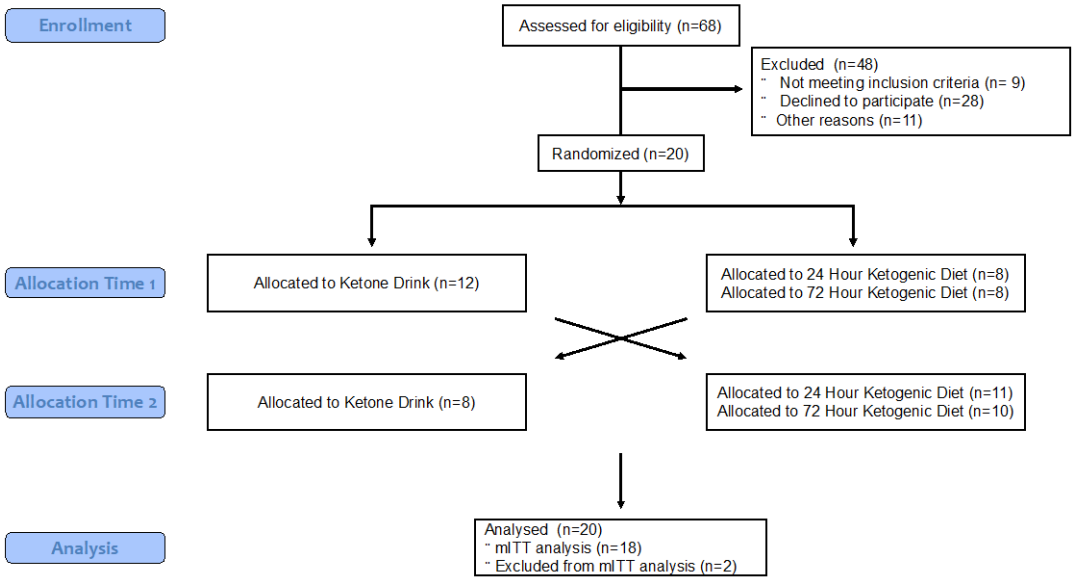
	Myocardial Glucose Suppression Failure (N=11)	**Myocardial Glucose Suppression Success (N=9)**	**P-value**
Beta-hydroxybutyrate level (mmol/L)			
• Pre-ketone ester	0.10 (0.10, 0.11)	0.26 (0.15, 0.57)	0.008
• Post-ketone ester	2.58 (2.20-3.23)	4.97 (3.82, 5.35)	0.01
• Difference between pre- and post-ketone ester levels	2.49 (2.10-3.13)	4.71 (3.22, 5.19)	0.018

Presented as median (25^{th}-75^{th} percentile)

SUPPLEMENTARY FIGURES

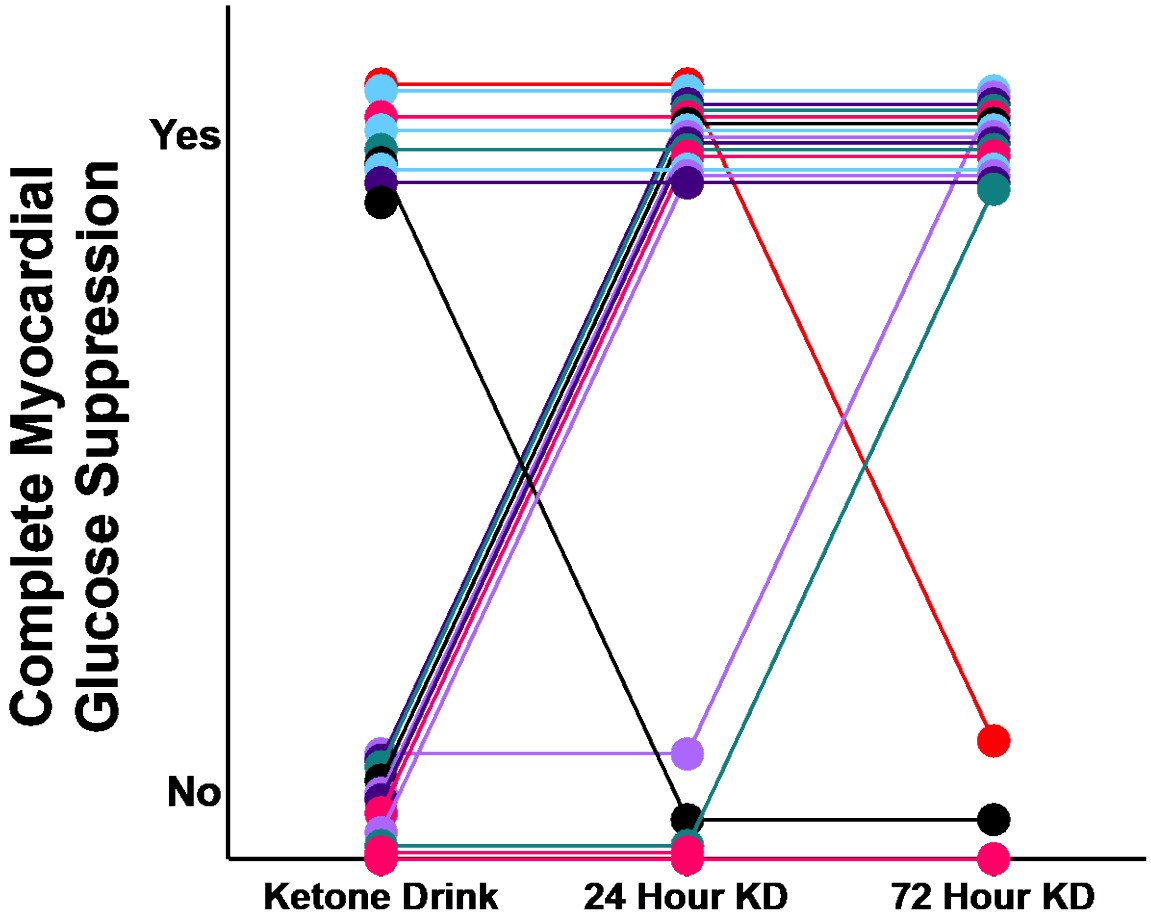
Supplementary Figure 1: KEETO-CROSS CONSORT Diagram

68 participants were initially assessed for eligibility, with 20 participants ultimately randomized to either ketone ester or ketogenic diet arms first. A total of 18 participants completed all 3 study visits, included in the mITT analysis. mITT, modified intention-to-treat.



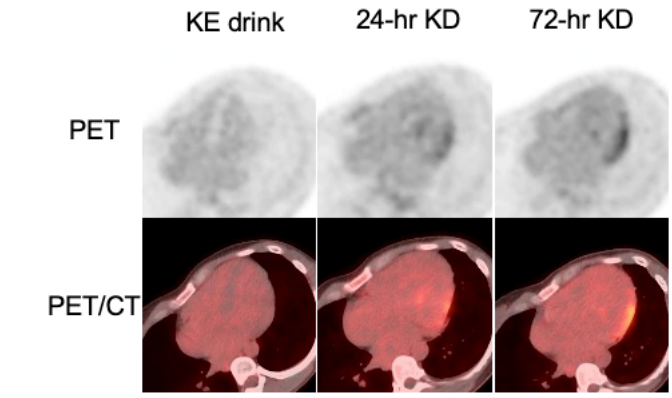
Supplementary Figure 2: Individual Responses to Modes of Preparation for Myocardial Glucose Suppression

The two most common combinations of outcomes were participants who failed the ketone ester drink and suppressed with both diet durations (N=7), and participants who suppressed in all 3 arms (N=6). Two participants failed at least 1 dietary preparation while suppressing with the ketone ester drink.



Supplementary Figure 3: Case of subject who suppressed after the ketone ester drink but failed all dietary strategies

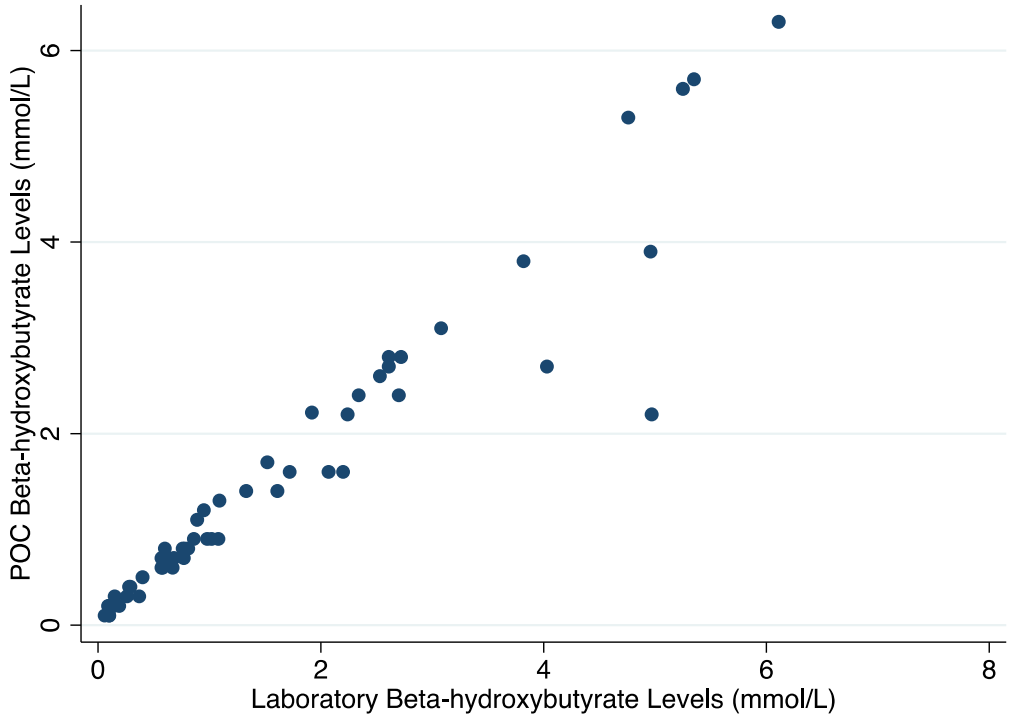
Cardiac FDG-PET images of a healthy volunteer who only achieved complete myocardial glucose suppression after the ketone ester (KE) drink and failed to do so following the ketogenic diet for 24 and 72 hours. Please notice that using a cutoff of ≥ 0.58 mmol/L, beta-hydroxybutyrate (BHB) levels of 0.29 and 0.40 would have predicted failure at 24 and 72 hours respectively.



	KE drink	24-hr KD	72-hr KD
Fast time (hours)	14.6	16.2	13.1
BHB-pre (mmol/L)	0.19	0.29	0.40
BHB-post (mmol/L)	2.61	N/A	N/A

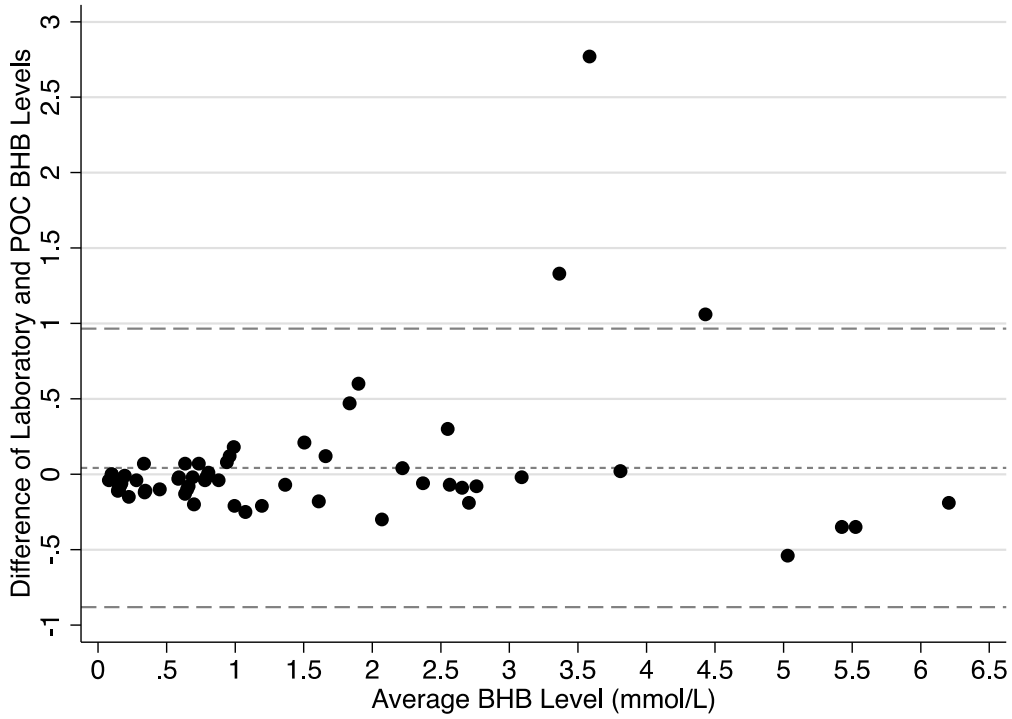
Supplementary Figure 4: Scatter Plots showing Relationship Between Point-of-Care and Laboratory Derived Beta-hydroxybutyrate Levels.

Scatterplot of beta-hydroxybutyrate levels with both analytic techniques, with Spearman's Rho 0.96 (N=59 paired samples). POC, point-of-care.



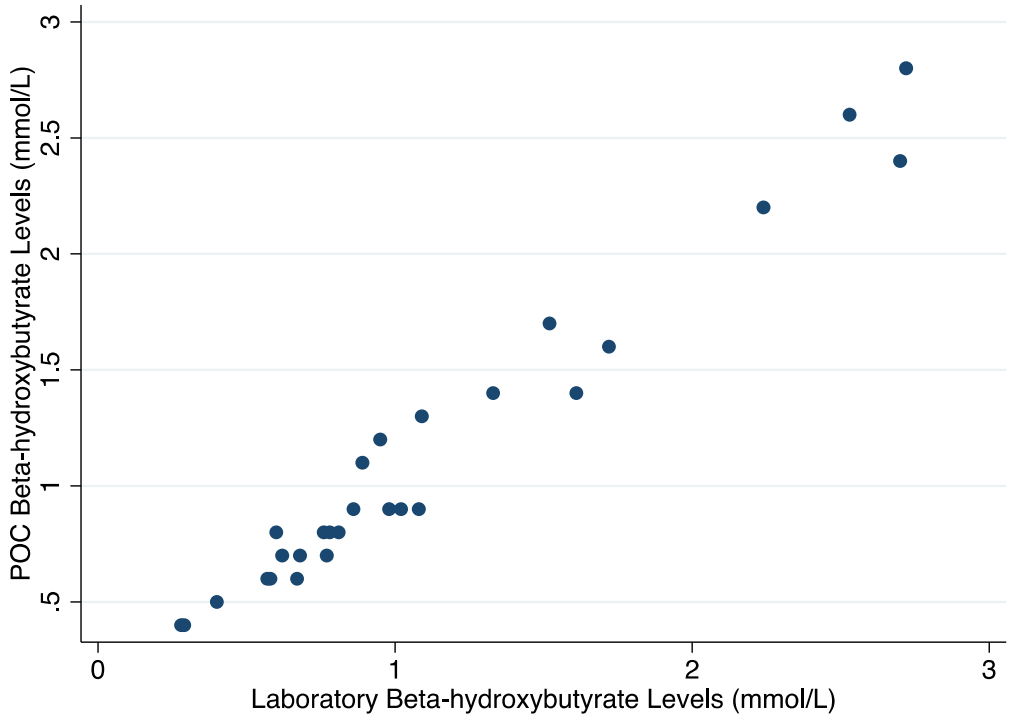
Supplementary Figure 5: Bland-Altman Plots showing Relationship Between Point-of-Care and Laboratory Derived Beta-hydroxybutyrate Levels.

Bland-Altman plots showing mean agreement 0.04 (95%CI -0.88, 0.97 mmol/L) (N=59 paired samples). POC, point-of-care.



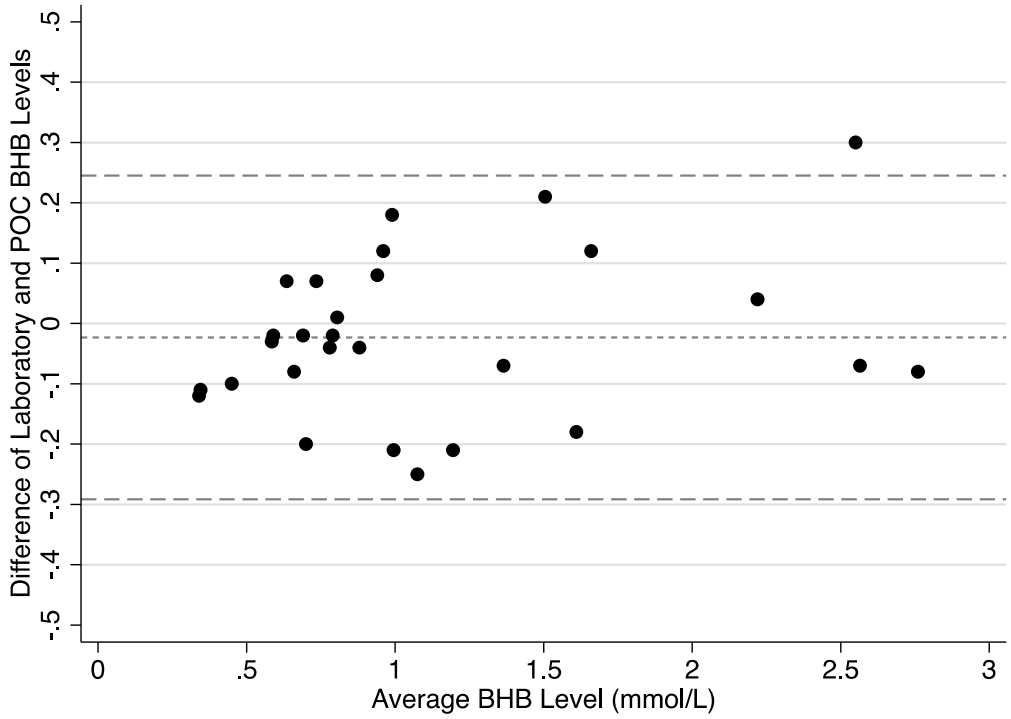
Supplementary Figure 6: Scatter Plots showing Relationship Between Point-of-Care and Laboratory Derived Beta-hydroxybutyrate Levels Achieved during Ketogenic Diet.

Scatterplot shown with both ketone analytic techniques during ketogenic diet visits only (N=28 paired samples), with Spearman's Rho 0.98. POC, point-of-care.



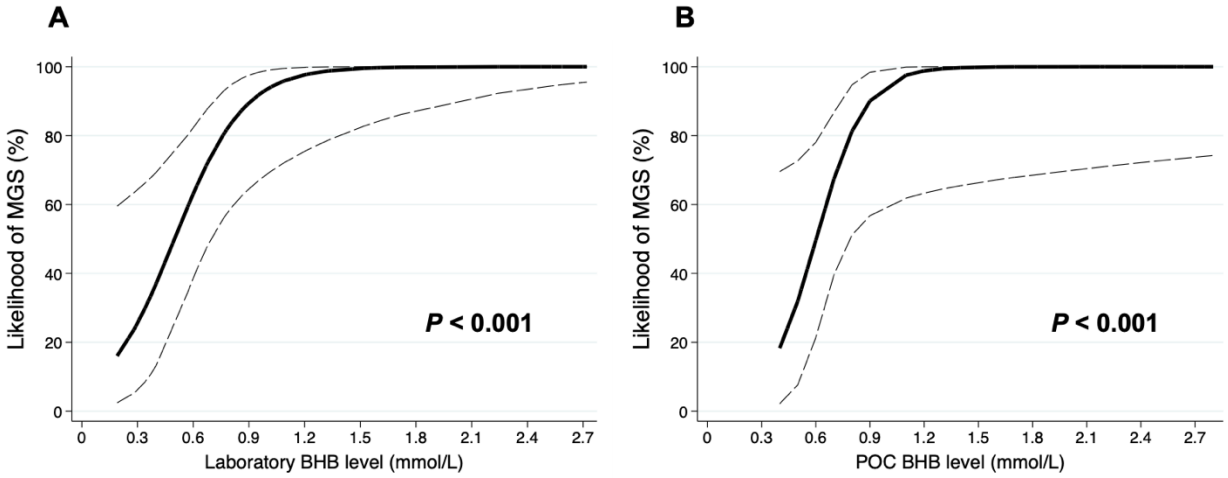
Supplementary Figure 7: Bland-Altman Plots showing Relationship Between Point-of-Care and Laboratory Derived Beta-hydroxybutyrate Levels during Ketogenic Diet.

Bland-Altman plots showing mean agreement -0.02 (95%CI -0.29, 0.25 mmol/L) during ketogenic diet visits only. POC, point-of-care.



Supplementary Figure 8: Logistic Spline of Ketone Levels to Predict Myocardial Glucose Suppression During Ketogenic Diet.

Logistic splines showing relationship between beta-hydroxybutyrate (BHB) levels and probability of achieving myocardial glucose suppression for the laboratory (A) and point-of-care (B) BHB assays.



Supplementary Figure 9: Area Under Receiver Operating Characteristics Curve for Point-of-Care Ketone Levels to Predict Myocardial Glucose Suppression During Ketogenic Diet

Receiver operating characteristics curve shown for point-of-care beta-hydroxybutyrate levels obtained during the ketogenic diet to predict the primary outcome in all participants. AUROC, area under receiver operating characteristics curve.