Peer Review File

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Reviewer A

General Comment: This paper about the Glucose-Ketone Index is an important contribution to the field of ketogenic diet and cancer research. However, there are some issues that need clarification before publication can be recommended.

General Reply: We would especially like to thank this reviewer for the important questions and suggestions given to improve our manuscript. As a result, we reevaluated some of our data using different statistical tests and a software package.

Comment 1. Page 6, line 152: You state that the SD was provided ad libitum throughout the study. This is confusing, since I assume that mice receiving the SD also were fasted for 18 hours as shown in Figure 1. Do you mean that all mice received the SD-U until the start of the study at day 0? Please clarify.

Reply 1. We removed the sentence "the SD was provided ad libitum throughout the study" at line 152. We added all mice were fasted on line 155.

Comment 2. Statistical tests: Why did you use the t-test for comparing blood parameters between groups? That assumes that parameters are normally distributed, which is a doubtful assumption for skewed parameters such as beta-hydroxybutyrate. I would suggest to use the U-test here, too. Which test was used to compare the Kaplan-Meier survival curves shown in Figure 5?

Reply 2. We reanalyzed the data using the U-test as suggested, and have now included this information in the statistical analysis section (lines 188-189). Accordingly, we modified the legend of Figure 2 on lines 369-372 to show the p values between the groups, as requested. We also reanalyzed the survival data using the log rank test and included this information in the statistical analysis section. We also reanalyzed the survival data in Figure 5 using the log-rank test. Accordingly, we modified the legend of Figure 5 (lines 194-197). It is also necessary to mention that we had 9-10 mice/diet group, not 8 mice, as was stated in the original manuscript. We made this change to the revised manuscript.

Comment 3. Linear regression analysis: From Figure 6 it is visible that regression on the GKI only explains about 30% of variation of the days of survival ($R^2=0.2966$). This means that there are other important variables missing. Please add a multivariable regression model using body weight change and gender (male vs. female mouse) as covariates besides the GKI. Also perform a multivariable regression analysis with glucose, BHB and the GKI, and report variance inflation factors for these three variables in order to judge their collinearity. My suspicion is that glucose is the major explanatory variable responsible for the "significance" of the GKI (see point 6 below), and that GKI is no longer significant if glucose is included in the model. Report the significance of each variable used in multivariable analysis. Please also report the exact p-values, not just $p<0.05$.

Reply 3. We agree with the reviewer that the regression explains only about 30% of variation of the days of survival. We know from our study, which included only males, that variation in blood glucose, ketones, and body weights can contribute to some of the variation, but we did not include enough mice in the study to identify the contribution of these additional variables using a multivariable regression model. It is our view that we would need to increase sample size to help quantify the contribution of other variables in the regression analysis. We chose a simple linear regression model to focus specifically on the association of the GKI value with survival. We agree with the reviewer that glucose is a major variable contributing to the linkage of the GKI to tumor growth and survival, but elevations in ketone bodies can also play a role, as we previously described (https://doi.org/10.3390/metabo11090572). Indeed, higher ketone body levels can indirectly reduce glucose levels (10.1007/978-0-387-85998-9_45), which would further link low glucose to a more favorable outcome. We prefer to use the GKI as a more predictive value than using either the glucose value alone or the ketone value alone due to the inherent variations in these values. Our findings in this mouse model are also consistent with the recent findings of the Hagihara et al group showing that low GKI values are linked to better survival and quality of life for patients with various cancers than are higher GKI values. We thank the reviewer for alerting us to the findings of the Hagihara group (mentioned below in comment 6). We have now added this statement to the revised manuscript (lines 294-296). It is also important to mention that a reanalysis of the data using Graph Pad Prizm software resulted in a slightly different r^2 and significance value. We have now reported the exact p value as suggested.

Comment 4. Page 7, Line 187: Experimental Design: Move to the Materials section!
Reply 4. We moved the Experimental Design to the Materials section – line 178, as suggested.

Comment 5. Page 7, Lines 190-191: "The experiment was terminated when mice in the control group showed signs of morbidity…". This is inconsistent with the Kaplan-Meier plot in Figure 5 which shows that all mice from all groups eventually died or had to be scarified. Please correct this statement.
Reply 5. We corrected the statement in the revised manuscript – line 181 now.

Comment 6. Page 11, Line 297: "The value of the GKI in predicting tumor growth and survival was also recently demonstrated in patients with glioblastoma." This statement is misleading, since both cited studies only were single case reports, so no statistical prediction was made in these studies. In contrast, the GKI apparently was not well correlated to clinical outcomes in a cohort of advanced cancer patients studied by Tan-Shalaby et al. (Nutr Metabol 13:52, 2016). Recently Voss et al. in their ERGO2 trial found that glucose, but not ketones alone, were significantly correlated with overall survival in glioblastoma patients (Int J Radiat Onocol Biol Phys 108:987-955, 2020). Finally, Hagihara et al. also found that glucose, but not the level of ketosis, was significantly correlated with overall survival. They also found significant correlations

for CRP and Albumin levels. Thus, there is the possibility that correlation of the GKI to outcome is mostly due to the effect of blood glucose. This should be discussed and tested in additional analysis (see point 3 above).

Reply 6. We thank the reviewer for these comments. We agree that our statement was misleading: "The value of the GKI in predicting tumor growth and survival was also recently demonstrated in patients with glioblastoma (13, 43).", We have therefore modified the statement to read: "The GKI was recently associated with improved survival in patients with glioblastoma, but further studies with larger patient numbers would be required to determine if the GKI could be predictive of improved survival in GBM patients." (lines 302-304). In none of the other patient studies referenced, however, (Tan-Shalaby et al. and Voss et al) were the total calories of the KDs sufficiently restricted. Ketone body elevation becomes more therapeutic when total calories become restricted. Therapeutic outcome is best under low glucose levels and elevated ketone levels, which also reduces systemic inflammation (doi:10.1371/journal.pone.0018085). The GKI captures the therapeutic value of both reduced glucose and elevated ketone bodies. We have now discussed this on lines 310-314).

Reviewer B

Comment 1. The paper submitted by Thomas N. Seyfried and co-workers presents a set of data, describing the prognostic potential of the glucose ketone index in successful managing systemic metastasis including spread to brain.

-The introduction is brief and coherent, well brings into the topic of the paper.

-The methods for validation the hypothesis are properly chosen and described in sufficient detail.

-The results are clearly presented. Statistical analysis of the data is well explained does not raise major comments.

-The discussion of the obtained data contains references relevant for the topic of the paper and properly indicates translational value of the performed studies.

Overall the article adds a significant knowledge to understanding the role of ketogenic diet in anticancer therapy. I recommend publication of the paper in the Longhua Chinese Medicine.

Reply 1. We thank the reviewer for the positive comments.

Reviewer C

Comment 1. The study by Akgoc et al describes the use of GKI to predict the survival and metastasis of Mouse tumor cells to visceral organs and the brain. The study is well written and well planned with appropriate statistical methods used. The authors conclude that GKI could predict tumor growth, metastasis, and survival. At this time, the data presented in limited but large studies or maybe pilot studies in humans should be considered.

Reply 1. We thank the reviewer for the positive comments and agree that our findings in this preclinical mouse model of metastasis should be validated in human pilot studies. This will be an important next step.