Reviewer's report

Title: Growth of human gastric cancer cells in nude mice is delayed by a ketogenic diet supplemented with omega-3 fatty acids and medium-chain triglycerides

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Reviewer: Thomas Seyfried

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The authors have done a good job in addressing prior issues and have improved the clarity of the manuscript. There are, however, several continuing issues that must be addressed.

Continuing Issues

1. While the inhibitory effects of the MCT-fish oil diet in delaying tumor growth are significant, the experimental design does not show which component of the diet is responsible for the effect. Several previous studies in rodent tumor models have shown that unrestricted consumption of ketogenic diets (KD) does not reduce tumor growth and may actually increase tumor growth over that of standard diets (studies referenced in the manuscript). On the other hand, fish oil diets are anti-angiogenic/anti-inflammatory and can reduce tumor growth (these studies are also referenced in the manuscript). It is therefore unclear whether the positive effect on tumor growth seen by the authors using the MCT/Fish oil diet is due to the MCT diet or to the fish oil supplementation.

In order to distinguish these possibilities, the authors would need to include additional diet control groups such as a standard diet supplemented with fish oil and an MCT diet without fish oil supplementation. It is not possible to accurately interpret the data without these additional control groups. It is possible, for example, that the fish oil is preventing tumor growth by inhibiting the growth promoting effects of the unrestricted MCT diet. These control groups are needed to identify the role of ketone bodies in therapeutic efficacy. What would the authors conclude if the MCT diet elevated ketones without reducing tumor growth relative to the standard diet? Alternatively, what would the authors conclude if tumor growth was reduced and ketones elevated in the SD supplemented with fish oil? Without these control groups the conclusions of the study can be misleading.

2. The authors also cite studies in their rebuttal letter indicating that tumor cells may have some capacity for respiration. There are concerns with the authors' arguments. While Warburg mentioned that some respiration capacity might be retained in tumor cells, it was unclear from his studies whether this was from the tumor cells or from stromal cells in the tumor tissue. He recognized this reservation in his 1931 monograph (preface to the English edition, page ix). Warburg used tissue slices, not cultured cells, for many of his studies. Tumor
tissue contains varying numbers of host macrophages, which are glycolytic under hypoxic conditions but respire under oxygen. This could give the impression that some tumor cells may respire when tumor tissue is moved from an anaerobic to an aerobic environment. However, the authors are correct in stating that respiration may not be completely defective in all tumor cells.

The Zu and Guppy analysis is flawed, as they did not consider the influence of the Crabtree effect in their survey, i.e., the inhibition of respiration by glucose. Most normal cells become glycolytic when they are grown in culture due to the Crabtree effect. Culturing cells in high glucose (10 mmol or greater) will suppress respiration. The Crabtree effect will therefore reduce differences between normal cells and tumor cells for glycolytic ATP production, which is what the Zu and Guppy analysis shows. Warburg also recognized (1956) that tumor cells should be compared with the normal cells from which the tumor arose in order to make the most accurate comparisons. If this is done with data from the Zu and Guppy survey, it appears that glycolytic ATP production is greater in most of the hepatoma cells (Table 2) than in normal adult mouse liver that has normal respiration (Table 1).

There are also issues with the Rossignol et al study. These investigators compared the metabolism of HeLa cells grown in glucose medium with HeLa cells grown in galactose/glutamine medium. They assumed that the higher pH in the culture medium of the cells in the galactose/glutamine than in the glucose medium was due to lower lactate production and improved respiration due to changed substrate availability. They did not directly measure lactate in the medium. It is important to mention that galactose is converted to glucose by the epimerase reaction and that glutamine stimulates glycolysis while adding ammonia to the medium (Siu & Wood, JBC, 234:2223, 1959; DeBerardinis et al., PNAS, 104:19345-50, 2007). This would neutralize the pH thus appearing to reduce lactate. Moreover, glutaminolysis stimulates lactate production while enhancing protein synthesis (DeBerardinis et al., PNAS, 104:19345-50, 2007). This could account for some of the findings of the Rossignol et al study, despite the appearance of increased respiration, which could be uncoupled. Glutamine can provide reducing equivalents through the TCA cycle, but this would require a normal electron transport chain to convert this to useable ATP. No evidence was presented that the mitochondria in the Rossignol et al study were coupled or that the HeLa cells could survive under low sugar levels in the presence of fats or ketones. Consequently, the conclusions of the Rossignol et al must be viewed cautiously.

3. The mouse body weights should be presented as means rather than as medians.

4. An explanation is needed as to why HUVEC cells were used. It is not clear how endothelial cells relate to the tumor cells.

5. A representative H&E histological section should be shown to illustrate necrotic areas of the tumors.
What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests.