Author's response to reviews

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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Author's response to reviews: see over
Cover letter with a point-by-point description of the changes made

First, we would like to thank the reviewers for their comments and suggestions that will improve the quality of the manuscript.

Reviewer 1 - Thomas Seyfried

Major compulsory revisions:

1.1 The authors showed that glucose metabolism was greater in the carcinoma cells than in the control PMBC. It would be interesting to determine if the lactate production in the cultured tumor cells changes if beta-hydroxybutyrate is added to the culture medium. Please see comments to question 4.

1.2 Tumor cell survival for at least 72 hrs in a low glucose/high ketone culture medium would suggest normal respiration in these cells. Such findings would also indicate that these cells do not show the Warburg effect...

Please see comments to question 4.

2.1 The authors mention that reduced body weight was not a factor in the smaller size of the tumors in the KD group compared to the SD group. However, it is interesting that body weights were smaller in the KD group than in the SD group over the first 20 days of the experiment.

The mean body weight of KD animals was slightly smaller than that of the SD animals at the start of experiment (27.4 ± 3.3 g KD vs. 28.5 ± 2.2 g SD). The difference, however, is not statistically significant (P=0.62, Mann-Whitney U test). In addition, the body weights of KD and SD animals did not significantly differ during the experiment as well as at its end. The P levels for different time points are between 0.62 (d6) and 0.72 (d27) (Mann-Whitney U test).

2.2 The authors should conduct a linear regression analysis (using body weight as the independent variable and tumor volume as the dependent variable) over the first 20 days of the experiment to determine the relationship of body weight to tumor growth. The regression should also be conducted after the first 20 days. These analyses were done by a professional statistician. She found no correlation between body weight and tumour volume at different time points within the 20-days period and after day 20; the P values are: 0.84 (d6), 0.56 (d20) 0.70 (d21), and 0.89 (D27, nonparametric ranc variance test of Puri and Sen). The Fig. shows the body weight versus tumour volume on day 20. The linear regression is not significant (P=0.9).

2.3 It is unusual to find a cancer therapy that extends mouse survival, which does not also reduce tumor weight and volume. Further comment is required to address this apparent discrepancy.

In our model, animals with tumours reaching a target volume of 600-700 mm$^3$ were sacrificed. Thus, tumours of both groups revealed nearly the same target volume (658 ± 32.6 mm$^3$ KD vs. 622 ± 35.1 mm$^3$ SD) at the end of experiment, but the time needed to reach the target volume differs significantly. Tumours of SD animals reached this target volume at 23.9 ± 8.5 days and tumours of KD animals at 34.2 ± 3.9 days. The reduced tumour growth in KD animals during the experiment is also statistically highly significant in comparison to SD animals. The P values are between 0.0005 (d6) and 0.0098 (d20, Mann-Whitney U test). In addition, it should be noted that the tumours of SD and KD animals had the same volume, but
KD tumours revealed significantly larger necrotic areas than tumours of the SD group (Fig. 5 of the revised manuscript).

3.1 The authors show that the KD increased ketosis, but did not reduce glucose levels. In this regard, the authors should reference the previous work of Fearon et al. Brit J Cancer 1985; Amer J Clinical Nut 1988. These investigators considered that the inability of their KD to reduce tumor growth was due to persistently high glucose levels. Also, glucose levels were reduced in the patients from the Nebeling study. We referenced the previous work of Fearon et al. in the revised discussion. In addition, the observation from the Nebeling study is mentioned.

3.2 What would the authors expect with regard to mouse survival if glucose levels were reduced in their study? The authors should also recognize that insulin resistance and gluconeogenesis could account in part for the elevated glucose levels under ketogenic diet feeding.

KD and SD animals did not demonstrate significant differences in the blood glucose levels, but the ketogenic diet induced significantly a mild ketosis in the KD animals (Tab. 2 of the revised manuscript). Fearon et al. (Pubmed-ID: 2861842) described a ketogenic diet inducing significantly higher blood glucose concentrations in ketotic, tumour bearing rats than in ketotic non-tumour bearing rats. The authors explain the failure of the ketogenic diet to inhibit tumour growth with permanently increased blood glucose levels. The observation that the animals of the KD did not demonstrate reduced blood glucose levels may really indicate the powerful generation of glucose from non-sugar carbon substrates (gluconeogenesis). However, different carbohydrate-free diets lead to significantly lower circulating glucose levels than carbohydrate-enriched diets (e.g. Morris KL et al., 2003; Pubmed-ID: 12208192). Nebeling et al. (Pubmed-ID: 7790697) described for her two patients that “within 7 days of initiating the ketogenic diet, blood glucose levels declined to low/normal levels and blood ketones were elevated twenty to thirty fold”. Given a tumour’s high demand for glucose, we expect that reduced glucose levels should inhibit tumour growth. Nebeling et al. calculated from results of PET scans a 21.8% average decrease in glucose uptake at the tumour site in both subjects.

3.3 The authors will need to measure serum insulin levels to support their claim that reduced insulin levels contribute to the anti-tumor effect of the unrestricted KD. As suggested, we measured the insulin levels of KD and SD animals at the end of experiment. The means are slightly different, but not significantly. See revised manuscript.

4.1 The glucolytic behaviour of tumors persists even in the presence of oxygen. This is due to defective respiration according to Warburg’s extensive data on tumors. The authors mention on page 19 that oxygen and ketone bodies might sustain tumor cells in viable tumor zones. This possibility would refute Warburg’s hypothesis. Ketone bodies can only be metabolized for energy with near normal mitochondria and respiration... The ability to reactive respiration in tumor cells has not been clearly demonstrated in any previous study despite claims to the contrary.

In his pioneering work on cancer metabolism, Warburg described that cancer cells show a dramatically increased contribution by glycolytic ATP turnover to total ATP production under air-saturated conditions. He found that up to 55% of total ATP is generated by glycolysis and at least 45% of total ATP comes from aerobic processes (Warburg O, ed. Über den Stoffwechsel der Tumoren, Berlin, Julius Springer 1926; Warburg O, 1956, Science 123). Tumour cells - in contrast to benign cells - use intensively the glycolytic pathway for energy production and are more dependent on an unrestricted glucose supply than benign cells.
However, this does not mean that tumour cells are unable to use their mitochondria. Warburg never said that tumour cells exclusively generate ATP via glycolysis. He noted that “all tumour cells which have contact to vascularisation obtain the oxygen they need for respiration and can survive without glucose” (Warburg O, Wind F, Negelein E. Klinische Wochenschrift 1926 (5): 829). In addition, Zu and Guppy reviewed that the contribution of glycolytic ATP differs dramatically (from 0.65% to 65%) in tumour cells of different cell lines (Pubmed-ID: 14697210). Rossignol et al. clearly demonstrated elevated oxidative phosphorylation in HeLa cervical cancer cells subsequent to a change in substrate availability (Pubmed-ID: 14871829). The authors did not use ketone bodies in their study, however, they exchanged glucose for glutamine and subsequently observed an altered mitochondrial structure as well as an elevated rate of oxidative phosphorylation in these cells: “Our data show that the defective mitochondrial system described in cancer cells can be dramatically improved by solely changing substrate availability and that HeLa cells can adapt their mitochondrial network structurally and functionally to derive energy by glutaminolysis only.” In another study, Boren et al. analysed metabolic adaptations of different cell lines incubated with increased doses of butyrate (Pubmed-ID 12750369). They found decreased glucose uptake and utilization as well as increased oxidation of butyrate; this correlates with differentiation. In contrast, under equal conditions MIA pancreatic carcinoma cells sustained high rates of glycolysis; these cells did not show differentiation. All these data may not be representative or comprehensive, but they demonstrate a substantial variability of different cancer cells to variation of substrate availability. Since we have no own data that confirm our statements on p. 19 we revised the paragraph.

4.2 It would therefore be important to show that survival for 3-4 days is similar in the 23132/87 tumor cells grown in either high glucose medium or in low glucose medium (less than 0.02 mmol) supplemented with high ketone bodies (5-10 mmol).... The authors should present these data if they are available.

As suggested, we did this experiment (with the MultiTox-Fluor Multiplex Cytotoxicity Assay from Promega measuring the number of live and dead cells in a single well). The figure shows the results for 23132/87 tumour cells incubated with 10 mmol/l β-OHB in the presence of indicated glucose concentrations for 72 h. The results are: (1) With decreased glucose levels the lactate production drops; (2) Decreased blood glucose levels influenced slightly the cell viability (up to 20% are dead cells), whereas the cytotoxicity increased (up to 40%). The same results were observed in control experiments with same glucose levels but without β-OHB. In addition, we also tested different levels of β-OHB (1.25-10 mmol/l) in glucose-free medium and we did not find an obvious effect on cell viability. Reduced levels of glucose and β-OHB, respectively, decreased cell proliferation, but β-OHB reduced more strongly proliferation (but without increasing cell death). In summary, these results may indicate that proliferation of 23132/87 tumour cells correlates with glycolytic degradation of glucose, whereas non-proliferation correlates with aerobic degradation of β-OHB. However, these preliminary results do not really support our statement on p. 19 (old version), and therefore we revised the paragraph.

Minor essential revisions:
5. Data presentation can be improved by using means with either SD, SEM, or 95% CI.
The data presentation was improved by statistical analyses in the revised manuscript.
6. Data in figure 6, 7, and 11 are confusing and could be better summarized rather than presenting data point for each mouse. Fig. 6 and 11 were revised (see Fig. 5 and 6 in the revised manuscript); Fig. 7 was removed. The results are summarized in the manuscript.

Reviewer 2 - Robert Gillies

Major concerns:

1.1 The major concern is that the experiment describes results from only 24 mice (12 control and 12 experimental). While this likely has enough power for some of the endpoints, it is extremely limited.

Different researchers have published results they obtained with the same number of animals per group or smaller; for example: Kato et al. (Pubmed-ID: 17640164); Zhou et al. (Pubmed-ID: 17313687); Raghunand et al. (Pubmed-ID: 11494116); Jennings et al. (Pubmed-ID: 11988845). For our experiments, the number of animals necessary for statistical analysis was calculated by a professional statistician.

1.2 It is certainly underpowered if animals are split into long- and short-term survivors and statistics of these groups should be discounted.

Indeed it is inappropriate to split the animals of the KD group into “long-term” and “short-term” survivors because the number of animals is too low for statistical analyses. However, the number of animals of the undivided KD group is sufficient for non-parametric statistics.

1.3 The overall experiment should have been reproduced at least once.

The number of animals is sufficient for non-parametric statistics. This was proved by a statistician. The hypothesis that the ketogenic diet prolongs animal survival and inhibits tumour growth was successfully proved by statistical analysis. In addition, the European and national guidelines for animal care demand a biometric calculation about the amount of animals needed to ensure statistical relevant data.

2. Similarly, this only used a single tumor type. In the Methods, this was described containing the TKTL1 gene product. Is the claim being implied that this gene product required for the observed responses? If so, a matched tumor without TKTL1 should have been used for comparison.

TKTL1 is an enzyme of the non-oxidative pentose phosphate pathway. It has been shown that TKTL1 correlates with a poor prognosis in a variety of carcinomas (Langbein S et al., 2006; Volker HU et al., 2007). We checked different tumour cell lines for TKTL1 expression and found none negative for TKTL1. Presently, we have no information that the TKTL1 gene product is really required for the observed responses.

3. The reduction in tumor growth for the KD group was attributed mainly to an increased lag period. Would the same be true if the cells were pre-acclimated to a low glucose ketogenic media prior to injection? It is possible that the lag occurred simply because the metabolic landscape changed?

We agree with the reviewer that the reduced tumour growth can be explained with an increased adaption needed by the tumour cells with the ketogenic diet. However, it is frequently described in literature that animals were switched directly from normal feed to the diet following tumour cell injection (e.g. Kato et al.; Pubmed-ID: 17640164). In contrast, Zhou et al. (Pubmed-ID: 17313687) started with ketogenic feeding 3 days after tumour cell injection. However, the authors did not explain whether the tumour cells have really adapted to the in vivo conditions within these 3 days. Freedland et al. (Pubmed-ID 17999389) even fed their animals for 24 days with a carbohydrate-negative diet prior to tumour cell injection. In
Further experiments we will compare the effects of immediate and delayed ketogenic diet feeding following tumour cell inoculation. The possible influence of tumour take by ketogenic diet was discussed in the revised manuscript.

**Minor concerns:**

4. **It is also concern that these tumors were grown heterotopically. While orthotopic models of gastric cancers may not be available, they are available for other types of cancer.**

   The heterotopic transplantation of human cancer cells into immunodeficient mice is a commonly used xenograft model. For example, Hardmann et al. (PubMed-ID: 17571951) used cells of a breast cancer cell line injected subcutaneously between the scapulae; Venkateswaran et al. (PubMed-ID: 18042933) injected cells of a human prostate cancer cell line into the flanks of mice (together with matrigel). Ngo et al. (PubMed-ID: 12855654), Kato et al. (PubMed-ID: 17640164), and Zhou et al. (PubMed-ID: 17313687) inoculated cancer cells subcutaneously into the lateral flank. This short list of published papers demonstrates that the heterotopic xenograft model is frequently used by many groups.

5. **Personal experience has showed us that the NBDG assay does not work as advertised...**

   The 2-NBDG assay works very well in our hands (Fig. 1 in the revised manuscript). For the revised manuscript we used endothelial (HUVEC) cells instead of PMBC. We are able to demonstrate a dose-dependent and time-dependent uptake of 2-NBDG by tumour cells (Fig. 1 in the revised manuscript). In addition, it is possible to measure and quantify the uptake of 2-NBDG. This is also described in different journals, e.g. Cytometry. The addition of D-glucose to the media markedly reduced 2-NBDG uptake (not shown). In contrast, L-glucose does not influence 2-NBDG uptake as shown by Aller et al., Cytometry 1996. Since Glut-1 is the only isoform of the glucose transporter expressed by astrocytes 2-NBDG should be transported by Glut-1 (Vannucci SJ et al., Glia 1997).

**Reviewer 3 - W. Elaine Hardman**

**General:**

The revised manuscript was shortened and the number of Tables and Figures was reduced. All statements are now supported with statistical analysis.

**Major compulsory revisions:**

*The diet was changed immediately after injecting the tumor cells. The main difference was in the first 20 days. I would interpret this as a difference in tumor cell take than a difference in tumor growth...*

The reviewer is right if she interprets the data as a difference in tumour cell take. We introduced this explanation in the revised manuscript. For further comments please see the answers to question 4.

*I also have difficulty with discarding the results from half of the tumors...*

Subcutaneously inoculation of human cancer cells into immunodeficient mice is a commonly used xenograft model. With our strategy to inoculate subcutaneously tumour cells in both hind flanks, we obtain tumour growth in 100% of the animals. In contrast, the subcutaneous inoculation in one hind flank reduces the tumour growth rates to approximately 80%. The reason for this observation is unknown, but a possible cause is the microenvironment. Tumour cells located near small blood vessels may have an advantage in growth. To overcome this problem we decided to inoculate subcutaneously the same cell number in both hind flanks and selected the larger of the two tumour nodules between day 8 and day 10 following cell injections (and not at the end of experiment). The data of these selected tumours are analysed
and presented in the manuscript. The sentence in Methods on p. 10 “The larger of the two tumour nodules per animal was selected to determine the final target volume and wet weight” is misunderstanding, and therefore the paragraph was revised.

1.1 There is no mention of vitamin or mineral mix in the diets. This is standard in most animal diets.

The SD diet is a nutritionally balanced diet for nude mice provided by the special animal feed manufacture Altromin, Germany. Further information is available on the company’s homepage (www.altromin.de). The KD consists of a mixture of absolute fresh and high quality food, and therefore no vitamin or mineral mix was added. The quality of the paste is routinely controlled by the Chemical Laboratory of Drs. Kaiser and Woldmann, Germany. Prior to starting with our feeding experiments, the local ethical committee reviewed the experimental setup in accordance to European and national guidelines for animal care. These reviewers permitted the feeding experiments because of the limited administration of the KD diet. However, in following experiments we shall analyse the diet for vitamins.

1.2 The composition of the last three diets in table 1 is unnecessary...

As recommend by the reviewer, we removed the last three diets from table 1.

2. Figure 2 is not very helpful - there are no differences between groups in the IHC results and the results are not unexpected. Statements in the results are adequate.

We present Fig. 2 as additional file 1 in the revised manuscript.

3. There should be a statistical analysis of the body weights to support (or not) the statements (page 14) made about differences... Statistical analysis can also determine whether the weight gains were really different or not.

The data about the body weight was checked by a professional statistician and she found no significant differences between the mean body weights of both groups; the P values are: d0, 0.62; d6, 0.20; d8, 0.79; d12, 0.45; d16, 0.62; d20, 0.18; d21, 0.50; d23, 0.85; d26, 0.62; d27, 0.72 (Mann-Whitney U test). In addition, the paragraph “Course of body weights” was revised because the statements were misunderstanding.

4.1 Tumor growth - of course the end tumor weights were not different, the end was determined by tumor size. The slopes of the tumor growth can be statistically analyzed and should be if the authors want to make statements about differences.

We statistically analysed the slopes of the mean tumour volumes in the revised manuscript.

4.2 The descriptions of tumor growth analyses on p. 15 are strange. It is most unusual to split the groups into subgroups so that one can refer to subgroups that “fit a hypothesis”. Statistic can also determine if 8 of 12 is different from 2 of 12 or not.

The reviewer is right. The number of animals in the subgroups is too small for statistical analyses. We revised the complete paragraph on p. 15 (see also our statement to question 12).

4.3 The shift in tumor growth rates, indicates that “take” was delayed, not tumor growth slowed. This is also indicated in Fig. 6 A. Five tumor did not grow from day 7 to day 20, indicating lack of take. It has to be assumed that after day 20, these began to grow, these probably are most of the “long-term survivors”. This is not trivial since there could be clinical signifiance for prevention of metatsatic tumor take.

We agree with the reviewer’s interpretation that the take of tumour cells was possibly delayed and not tumour growth was slowed. The main differences are really observed in the first 20
days, but after day 20 the differences in tumour volume between SD and KD animals are statistically different, too.

5-6. Most of table 2 is not needed; Table 3 is not needed.
We removed Tab. 2 and 3.

7. Survival analyses should be statistically analyzed and there should be a section in the results.
The survival was statistically analysed in the revised manuscript.

8. Figure 5 for tumor volume is not needed.
As recommended, we removed Fig. 5.

9. Figure 7 is not needed. Perhaps a correlation analyses of $\beta$-OHB with survival should be used to support the statement on page 16.
As recommended, Fig. 7 was removed and the paragraph “The ketogenic diet influenced plasma OHB levels but not glucose levels” was revised.

10. Table 4 - are there any (statistical) differences?
The data in Tab. 4 was statistically analysed. We found that the differences are significant after day 6 (P=0.004, Mann-Whitney U test).

11. Figure 8 - unnecessary. Figure 9 and 10 - one sample would be enough.
Fig. 8 was removed. Fig. 9 and 10 was modified; see Fig. 6 in the revised manuscript.

12. Figure 11 - C and D not necessary. B may be necessary.
We revised Figure 11. See Fig. 6 in the revised manuscript.

13. Figure 12 not necessary unless some statistical analyses is done, this would require assessing vessels in multiple tumors. If a firm statement cannot be supported, then a statement should not be made.
We agree with the reviewer that statistical analyses are necessary, but these results are very important and we like to present them in an additional file. However, since we cannot give a statement supported by statistic analyses, we do not mention “neovascularisation” in the conclusions of the abstract, in the discussion, and in the conclusions of the discussion.

14. Discussion should be shortened.
We revised the discussion.