nature portfolio

Peer Review File

A very low carbohydrate, ketogenic diet suppresses tumor insulin signaling and enhances immune infiltration in women with endometrial cancer: results from a randomized feasibility study.

Corresponding Author: Dr Marcus Goncalves

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Due to the limited sample size of this feasibility study, it seems that there is not much the authors can do in terms of statistical analysis. However, there are still some points that need to clarify.

1. The PCA plot shows that the samples are not separated by diets (Figure 3 A), which suggest that the diets do not have a significant effect on gene expression. How the authors interpret this outcome? Are there any genes differentially expressed genes between KD and SD?

2. It needs to carefully interpret the GSEA results (Figure 3B). Please note that GSEA analysis does not imply that the genes in the lists are differentially expressed. It just indicates how a gene list is ranked among all the genes.

3. Figure 3B is not very informative. It is not necessary to show the unsignificant results. It may be more informative to show the expression of genes in the leading edge between the groups of comparison plus the corresponding p-values. In addition, $p = 0$ is not correct, which needs to be corrected.

Reviewer #2

(Remarks to the Author)
The authors have addressed my concerns

Reviewer #3

(Remarks to the Author)
This is a revised manuscript from Dantas et al. looking at the efficacy of a very low carbohydrate ketogenic diet in obese women with endometrial cancer. The study was aimed at determining the dietary impact on tumor metabolism and tumor biology, especially anti-tumor immunity. There were several concerns raised by the reviewers, and based on the rebuttal and revised manuscript, few substantial changes have been made to address the comments.
Regarding novelty, the authors specifically state that their work is different from published work of the Gower group in that their “study specifically extends this research into the context of endometrial cancer biology…” The title of the study from the Gower group is: “A Ketogenic Diet Is Acceptable in Women with Ovarian and Endometrial Cancer and Has No Adverse Effects on Blood Lipids: A Randomized, Controlled Trial.” It remains unclear why this study is novel given the published work.

For comments raised by reviewer 2, points 4, 5, 6, 7 and 8 were not addressed in a satisfactory manner. The reviewer remains concerned that the study is under powered and therefore the authors do not reach meaningful conclusions. There is also inconsistency in the data as pointed out regarding pAKT levels in the study. This remains a concern, and no clear conclusion can be reached given that this is a small, feasibility study.

The authors state…" We recognize that the PCA results suggest that individual variation was a primary driver of differences, rather than the diet itself. However, relevant biological changes can still happen even without large changes in the PCA." This response does not at all address the concern raised by the reviewer.

Reviewer #4

(Remarks to the Author)
Thank you for allowing me to review this significant and important study. As noted by the authors, dietary patterns can impact tumor progression and development, however, few dietary intervention studies are attempted in patients with cancer. This study provides critical data to guide future research to determine dietary guidelines during cancer treatment. However, a few details should be addressed:

1) The paper consistently uses language to indicate how the dietary intervention impacts "obese" patients with endometrial cancer. However, at least 3 participants randomized at baseline had a BMI that would classify them as "overweight." The BMI and fat percentages of participants was considerably heterogenous, ranging from overweight to class 3 obesity. This distinction is important as the impact of the intervention may be more consequential in those with higher BMI. As noted by the authors, the intervention group had a significantly higher mean BMI which may have skewed the results when comparing groups.

2) The attrition rate for feasibility was set at 25%, however, attrition over 20% is noted to introduce significant bias. Was there a reason the attrition rate was set at 25% as opposed to the standard 20%?

3) Endometrial cancers can result in a hypermetabolism, thus, a predictive equation such as Mifflin St Joer or Harris Benedict would not be appropriate to estimate the needs of the study population. It is feasible that the meals provided to the VLCD reflected a caloric deficit in addition to a macronutrient shift which may be the underlying factor in the metabolic changes reported by the end of the intervention. Since the dietary pattern of the standard diet group was not evaluated, it is difficult to make a casual claim for the macronutrient shift as opposed to a calorie reduction resulting in weight loss.

4) Please change the spelling of "dietician" to "dietitian." The spelling is inconsistent in the manuscript.

Thank you again for allowing me to participate in the publication of this study.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
There are still some concerns regarding the presentation and interpretation of the results.
1. The title on Figure 2A. "Tumor transcriptomic changes with VLCD" could be misleading because the PCA plot is not designed to show transcriptomic changes. If the authors want to show transcriptomic changes, a heatmap or a volcano plot (e.g., log2 fold changes vs. adjusted p-values) may be more appropriate.

2. The p-values shown on Figure 2B and Figure 3A should be corrected for multiple comparisons. Conclusions should be redrawn correspondingly.

3. It is not clear how the samples were classified into the TCGA subtypes.

4. Besides CD8+ T-Cell signature, were there any other immune cell signatures tested?

Reviewer #4

(Remarks to the Author)
Thank you for the opportunity to review the amended and edited version of this manuscript. All of the recommendations I have provided have been appropriately addressed. As previously stated, I believe this study is noteworthy and adds critically important data to develop precision nutrition recommendations in the clinical setting for cancer patients.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
My concerns have been appropriately addressed by the authors. The response is satisfactory. I have no further questions.

REVIEWER COMMENTS

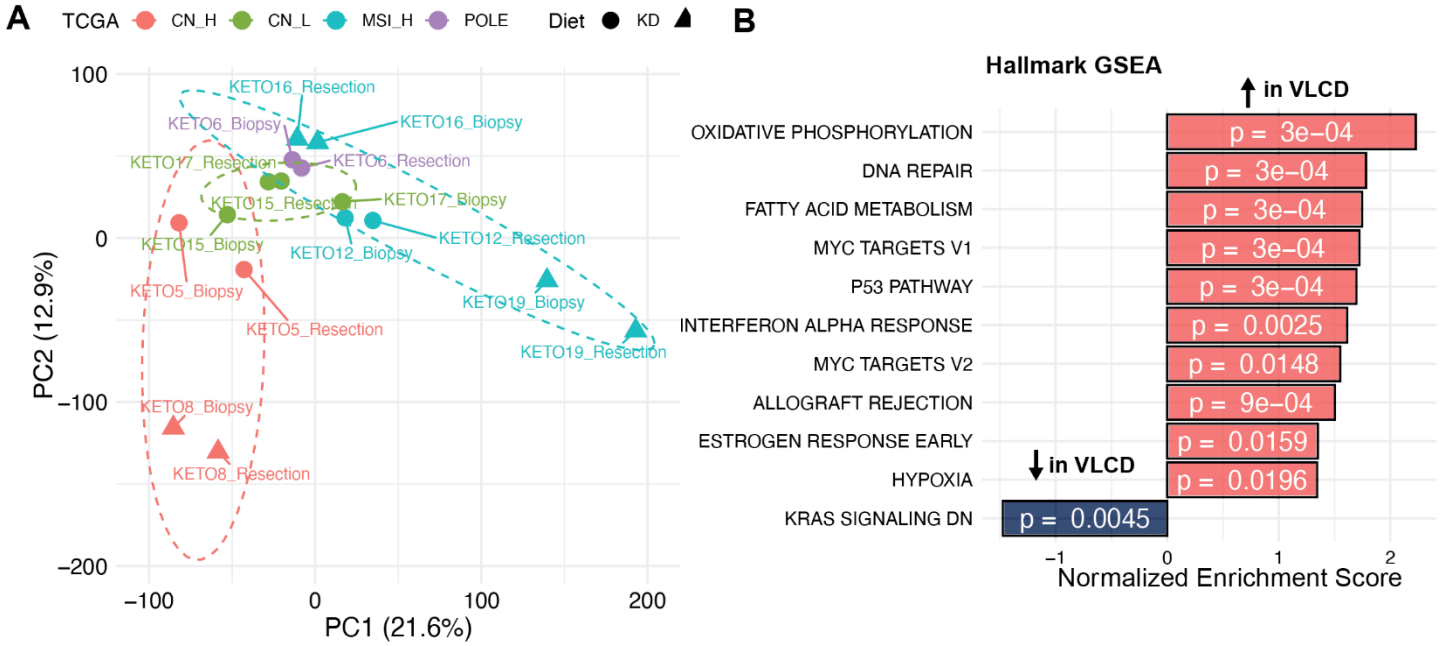
Reviewer #1 (Remarks to the Author):

Due to the limited sample size of this feasibility study, it seems that there is not much the authors can do in terms of statistical analysis. However, there are still some points that need to clarify.

1. The PCA plot shows that the samples are not separated by diets (Figure 3 A), which suggest that the diets do not have a significant effect on gene expression. How the authors interpret this outcome?
Response:

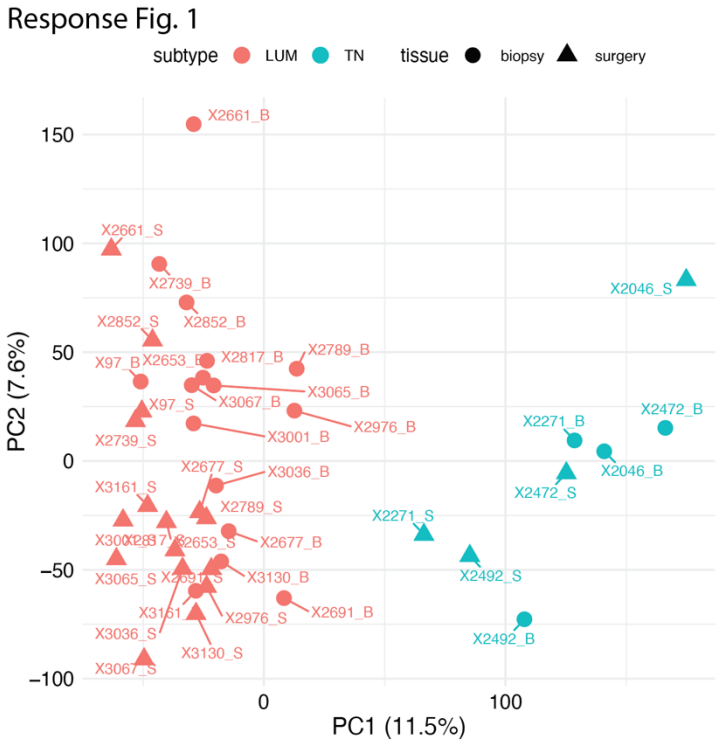
We thank the reviewer for this question. To address this issue, we performed a new PCA plot using variance stabilizing transformation (VST) from the Deseq2 R package to reduce variance and highlight distribution patterns associated with biology and not technical variability. We have now included this new PCA as Fig. 3A. Here, samples are unbiasedly clustered according to TCGA molecular classification, with MSI-High (hypermutated) samples clustering closely with POLE (ultramutated) samples. In contrast, copy number high (CN-H) samples form a distinct cluster from copy number low (CN-L) samples, which are closer to POLE and MSI-H samples. This separation accurately reflects the underlying molecular, clinical and histological differences. Here, the CN-H samples are from carcinosarcoma and serous histology, which have worse prognosis, and are categorized as Type II and high-grade, in the WHO and FIGO classifications. Conversely the rest of the patients have endometrial endometroid histology, with low grade, which are both associated with better prognosis. This analysis highlights that the TCGA molecular subtypes drive the dominant transcriptomic differences between the samples. Furthermore, close clustering of biopsies and resections from the same patient further supports the accuracy of this PCA in capturing true biological relationships.

Figure 3



However, in experiments involving heterogeneous samples, differences arising from factors such as tumor subtype, histology, or donor can be much greater than those caused by the treatment itself. As a result, these intrinsic biological differences may dominate the principal components in PCA, potentially obscuring the specific effects of the intervention.

To illustrate this point we analyzed a previously published RNA-Seq dataset from patients with breast cancer in which biopsies are compared to surgical pieces after neoadjuvant therapy with different type of chemotherapies (PMID: 35523804). In the Response Fig. 1, it can be seen that the differences in the PCA are mainly driven by tumor subtype (Lum, luminal, or TN, triple negative) rather than chemotherapy or tissue of origin (biopsy or surgical piece). Furthermore, in this particular experimental design, even with the additive effects of chemotherapy and comparing two different tissue types (biopsies vs surgical pieces) the dominating effect of tumor subtypes in the PCA is not overcome. Similarly to our study, here the biopsy and resection derived from the same patient are also found close to each other in the two-dimensional space of the PCA. This experiment highlights the issue of biological variance over experimental variables as a driver of differences in dimensionality reduction.



Are there any genes differentially expressed genes between KD and SD?

Yes, there are 1156 genes with a p-value adjusted for multiple testing (p.adj) < 0.05, which are differentially expressed when comparing KD to SD, accounting for the differences between biopsy and surgical resection in the design. We have now included an additional list with the expression of these genes (DEG_list.csv).

2. It needs to carefully interpret the GSEA results (Figure 3B). Please note that GSEA analysis does not imply that the genes in the lists are differentially expressed. It just indicates how a gene list is ranked among all the genes.

We completely agree, pathways analysis goes beyond changes in individual genes. GSEA can detect coordinated, modest changes across a set of functionally related genes, even when no single gene reaches statistical significance, thereby capturing pathway-level effects that might be missed by traditional differential expression analysis

3. Figure 3B is not very informative. It is not necessary to show the unsignificant results. It may be more informative to show the expression of genes in the leading edge between the groups of comparison plus the corresponding p-values. In addition, p = 0 is not correct, which needs to be corrected.

Thank you for this comment. GSEA analysis reporting pvalues = 0 is a known issue that arises when the resulting pvalues are smaller than the resolution allowed by the number of permutations (https://www.gsea-

msigdb.org/gsea/doc/GSEAUserGuideTEXT.htm, explained in the "Nominal P value" section). To address this issue, we repeated the analysis using fGSEA, which has algorithmic improvements that allow for a more precise calculation of p-values, and also increased permutations to 10000 (DOI:10.1101/060012). The results were almost identical to the previous analysis, but report exact p-values. fGSEA results and leadingEdge analysis are now reported in Supp. Table 6.

Reviewer #2 (Remarks to the Author):

The authors have addressed my concerns

Reviewer #3 (Remarks to the Author):

This is a revised manuscript from Dantas et al. looking at the efficacy of a very low carbohydrate ketogenic diet in obese women with endometrial cancer. The study was aimed at determining the dietary impact on tumor metabolism and tumor biology, especially anti-tumor immunity. There were several concerns raised by the reviewers, and based on the rebuttal and revised manuscript, few substantial changes have been made to address the comments.

Regarding novelty, the authors specifically state that their work is different from published work of the Gower group in that their "study specifically extends this research into the context of endometrial cancer biology…" The title of the study from the Gower group is: "A Ketogenic Diet Is Acceptable in Women with Ovarian and Endometrial Cancer and Has No Adverse Effects on Blood Lipids: A Randomized, Controlled Trial." It remains unclear why this study is novel given the published work.

We agree that both studies are randomized controlled trials evaluating VLCD in women with endometrial cancer. However, we would like to argue that both studies complement each other and that ours provides unique insights:

- **Dietary composition:** a key difference between the study of Cohen et al. is the macronutrient composition of the VLCD diets. While in their study 70% of the energy was derived from fat, 25% protein, and 5% carbohydrate, the one in our study was 87.2% of energy from fat, 8.7% from protein, and 6.2% from carbohydrate. It is not obvious to the field that this formulation would be tolerated by patients with endometrial cancer.
- Another essential difference is that in our study, **VLCDs were provided to the patients** on a weekly basis. This difference in design enhances compliance and addresses the non-trivial issues of diet preparation, logistics, distribution, and preservation, which are crucial for scaling up this type of study in the future.
- While Cohen's et al. provides data only at baseline and after 12 weeks of VLCD, our study reports changes in a **comprehensive metabolic panel weekly since baseline**. This design allows us to show that metabolic adaptations to VLCD in patients with endometrial cancer are evident even after the first week of VLCD.
- Our study also aimed to assess the effects of the diets directly on the tumor biology and immune microenvironment using a **biopsy -> intervention-> tumor assessment design**. This required the optimization of protocols for biopsy collection, tumor identification, purity estimation and RNA-sequencing so that the biopsy and tumor would be comparable. To our knowledge, no other study has attempted this design to test the effects of diets or other interventions on endometrial cancer.

For comments raised by reviewer 2, points 4, 5, 6, 7 and 8 were not addressed in a satisfactory manner. The

reviewer remains concerned that the study is under powered and therefore the authors do not reach meaningful conclusions.

I think the reviewer is referring to reviewer no.3 from the previous round of revisions, as it is the only one with 8 independent points. Please let us discuss these points further:

Point 4: "Though the authors performed RNA-seq and identified several potential pro-inflammatory pathways, the functional readout of these pathways by cytokine profiling for IFN, TNF and IL6 were unchanged. This is quite counterintuitive and does not support the RNA-seq data."

Our previous response: Changes in immune pathways within tumors (local inflammation) often do not correlate with changes in circulating cytokine levels (systemic inflammation). Therefore, the measurement of the circulating cytokines serves as a control for the effect of VLCD on systemic immunity.

New additions: We agree that, at first glance, upregulation of inflammatory pathways in tumor RNA-seq alongside unchanged circulating IFN/TNF/IL-6 might seem counterintuitive. However, there are several biological and technical points to consider:

1. Plasma Does Not Reliably Represent the Tumor Cytokine Milieu

- Peripheral blood cytokine profiles often fail to capture the heterogeneity and concentrations of cytokines present in the tumor microenvironment (TME). Cytokines in cancer typically act in an autocrine or paracrine manner within the TME and are often sequestered or consumed locally, rather than being released into circulation (https://doi.org/10.1016/j.ccell.2024.11.011).
- Studies have shown poor correlation between tumor cytokine levels and their matching plasma concentrations, indicating that circulating cytokines do not necessarily reflect the tumor cytokine environment (10.1038/s41598-022-17592-3). For example, Dranoff (Nat Rev Cancer, 2004) highlights that cytokines in the TME are tightly regulated and may not spill over into the bloodstream.
- Additionally, other research suggests that plasma cytokines may originate from tissues other than the tumor itself, further complicating their interpretation as a direct readout of tumor activity (doi: 10.1593/neo.10166).

2. Pathway Enrichment Reflects Transcriptional Programs, Not Circulating Cytokine Levels

- Pathway enrichment analyses, such as those identifying "IFN-γ response" or "TNF/NF-κB" pathways (e.g., MSigDB Hallmark gene sets), reflect downstream transcriptional programs rather than direct increases in circulating cytokine levels. Furthermore, different cytokines or stimuli can trigger the activation of the same pathways (doi.org/10.1084/jem.20241207).
- For example, the activation of the TNF/NF-κB pathway can occur through multiple upstream signals, and the downstream transcriptional response may persist even in the absence of detectable changes in circulating TNF levels (Liberzon et al., Cell Syst 2015).

Point 5: Tumor-infiltrating CD8+ T cells were not found by IHC, but only in the adjacent tumor-myometrium interface. If cytotoxic T cells are not infiltrating the tumor, this does not support the claim the authors are making that VLCD facilitates an antitumoral response. Based on the data presented, it is unclear that VLCD will mediate actual T cell infiltration and anti-tumor immunity.

Our previous response: This is an important conclusion from our manuscript that requires further studies. Increased infiltration of the tumor margin by CD8+ T cells suggests that VLCD enhances the antitumor immune response. However, this is insufficient to overcome the tumors' immune escape mechanisms. Further studies should address these escape mechanisms and the effectiveness of immune checkpoint blockade on them.

New additions: The spatial distribution of immune cells in the tumor bed is a crucial prognostic factor, predicting response to immunotherapy. Based on the distribution of immune cells in the tumor bead, tumors can be classified into three categories, which also have functional correlations (doi: 10.3389/fmcb. 2023.1084887):

Immune-inflamed tumors show dense lymphocyte infiltration within tumor nests, with immune cells adjacent to cancer cells. This indicates tumor-specific T cells were generated and trafficked; the bottleneck is effective function in the suppressive tumor bed.

Immune-desert tumors lack lymphocytes in the parenchyma and often at the periphery. This suggests minimal pre-existing antitumor immunity; the rate-limiting step is generation and initial recruitment of tumor-specific effector cells.

Immune-excluded tumors contain immune cells, but these remain in the stroma or invasive margin and do not enter tumor nests. Priming and recruitment occurred; the bottleneck is penetration across physical or biochemical barriers.

We believe that the increase of CD8+ T cells in the invasive margin shows a change from the **"immune-desert" category to the "immune-excluded"**. This change suggests a transition from a lack of tumor-specific T cells to an active priming and recruitment towards the tumor. The mechanisms by which tumors prevent effective infiltration (immune exclusion) are being actively studied (doi: 10.1007/978-3-030-38862-1_6). Among the proposed mechanisms, the tumor's metabolic activity is particularly relevant to our study. It is believed that through the Warburg effect, tumors acidify their environment, suppressing T cell function and antigen presentation (doi: 10.1126/science.1160809, doi.org/10.1155/2018/1218297). We could speculate that the VLCD might have reversed some of these conditions. Furthermore, other groups have shown similar effects of VLCD diets on CD8 T-Cells and anti-tumor immunity in pre-clinical models doi: 10.1172/jci.insight.145207). Future studies should investigate the impact of these changes in CD8 T cell distribution with the rate of response in combination with other therapies such as immunotherapy. With the recent FDA approval of pembrolizumab for advanced or recurrent endometrial cancer, we believe this finding will be of high medical interest.

Point 6: Several concerns exist with data interpretation. For instance, pAKT staining and quantification presented in Figure 2C illustrates that the majority of patients on both SD and VLCD exhibited a dramatic drop in pAKT levels. This does not appear to be unique to VLCD, though only the VLCD group is statistically significant. If the data set was larger, the interpretation of this data could be very different.

Our response was: We acknowledge that pAKT levels decreased in both the SD and VLCD groups, and only the VLCD group reached statistical significance, likely due to the small sample size and variability. The reduction in pAKT in the SD group was likely related to the extended fasting period prior to surgical resection, which lowers insulin levels. Patients were not told to fast for the biopsy procedure so there is uncontrolled confounding in this measurement. We hypothesize that the VLCD mimics this effect of fasting on tumors; however, this question will need to be addressed in studies that control for fasting duration for both biopsy and surgical resection.

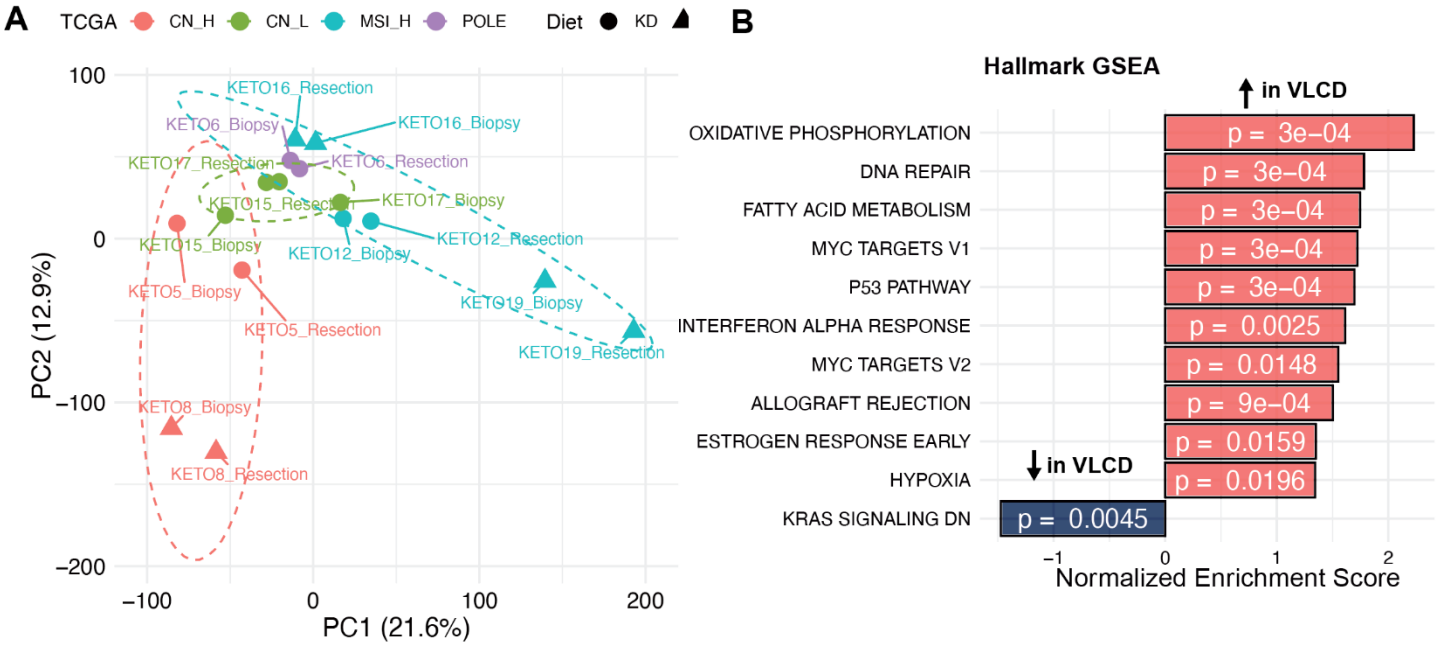
New addition: We cannot avoid the confounding factor that patients are fasted for the surgery, but are not required to fast for the biopsy collection. Because of this, we decided to move the panel to Supp. Fig. 3.

Point 7. Additional concerns exist with the RNA-seq that is severely underpowered, especially for clinical material. PCA analysis in Figure 3A showed that the "primary driver of differences between samples was the individual rather than diet…" This is concerning and suggests that the RNA-seq does not support major diet-dependent differences in these tumor samples.

Our previous response: We recognize that the PCA results suggest that individual variation was a primary driver of differences, rather than the diet itself. However, relevant biological changes can still happen even without large changes in the PCA.

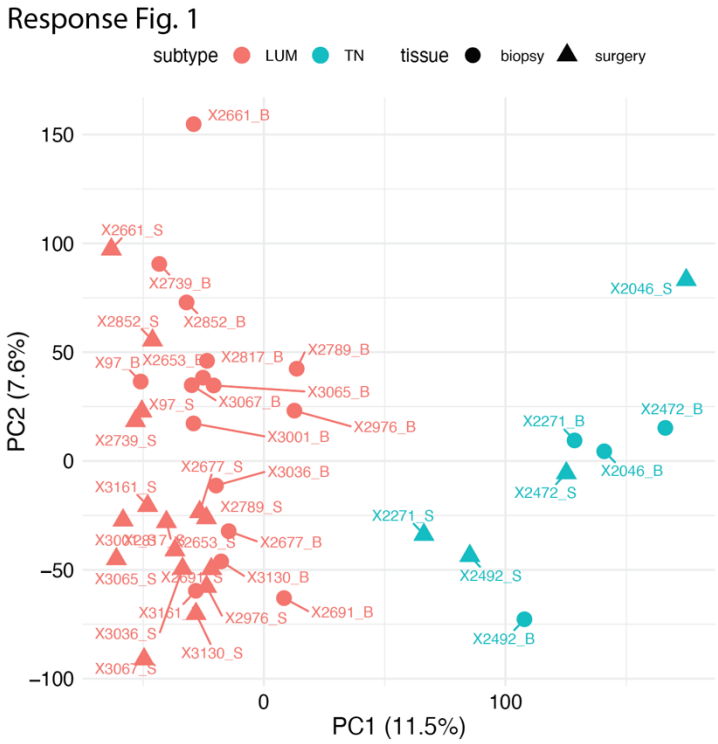
New Additions: To address this issue, we performed a new PCA plot using variance stabilizing transformation (VST) from the Deseq2 R package to reduce variance and highlight distribution patterns associated with biology and not technical variability. We have now included this new PCA as Fig. 3A. Here, samples are unbiasedly clustered according to TCGA molecular classification, with MSI-High (hypermutated) samples clustering closely with POLE (ultramutated) samples. In contrast, copy number high (CN-H) samples form a distinct cluster from copy number low (CN-L) samples, which are closer to POLE and MSI-H samples. This separation accurately reflects the underlying molecular, clinical and histological differences. Here, the CN-H samples are from carcinosarcoma and serous histology, which have worse prognosis, and are categorized as Type II and high-grade, in the WHO and FIGO classifications. Conversely the rest of the patients have endometrial endometroid histology, with low grade, which are both associated with better prognosis. This analysis highlights that the TCGA molecular subtypes drive the dominant transcriptomic differences between the samples. Furthermore, close clustering of biopsies and resections from the same patient further supports the accuracy of this PCA in capturing true biological relationships.

Figure 3



However, in experiments involving heterogeneous samples, differences arising from factors such as tumor subtype, histology, or donor can be much greater than those caused by the treatment itself. As a result, these intrinsic biological differences may dominate the principal components in PCA, potentially obscuring the specific effects of the intervention.

To illustrate this point we analyzed a previously published RNA-Seq dataset from patients with breast cancer in which biopsies are compared to surgical pieces after neoadjuvant therapy with different type of chemotherapies (PMID: 35523804). In the Response Fig. 1, it can be seen that the differences in the PCA are mainly driven by tumor subtype (Lum, luminal, or TN, triple negative) rather than chemotherapy or tissue of origin (biopsy or surgical piece). Furthermore, in this particular experimental design, even with the additive effects of chemotherapy and comparing two different tissue types (biopsies vs surgical pieces) the dominating effect of tumor subtypes in the PCA is not overcome. Similarly to our study, here the biopsy and resection derived from the same patient are also found close to each other in the two-dimensional space of the PCA. This experiment highlights the issue of biological variance over experimental variables as a driver of differences in dimensionality reduction.



Point 8. Another example of data without any clear conclusion: "The insulin-PI3K pathway promotes cell proliferation and suppresses apoptosis so we next performed IHC against Ki67 (proliferation) and cleaved caspase 3 (CC3, apoptosis), but no statistically significant differences were found." It would be expected that based on diet-dependent differences in circulating insulin and glucose levels found in Figure 1, some type of tumor-dependent response would be observed in the PI3K pathway.

Our previous response: The lack of significant differences in Ki67 and cleaved caspase 3 staining is consistent with the short study duration and small cohort size. While changes in circulating insulin and glucose were observed, it is possible that the short intervention duration did not allow sufficient time to translate into detectable changes in tumor proliferation or apoptosis. We will clarify in the manuscript that these findings should be viewed as preliminary and hypothesis-generating, warranting further investigation in longer-term studies.

New additions: The lack of effect on the PI3K pathway on participants on a VLCD as a single intervention for endometrial cancer is not surprising. Up to 80% of endometrial tumors have mutations in the PI3K pathway, rendering the pathway constitutively active. In our study, mutations in either PIK3CA and/or PTEN are highly prevalent. Additionally, several participants also have other driver mutations, such as in TP53, which could

further accentuate the lack of response to changes in insulin and glucose. This finding agrees with previous studies from our group, showing that ketogenic diet alone has minimal effects on tumor Ki67 and CC3 (doi.org/10.1038/s41586-018-0343-4) in human organoids xenografted in immunosuppressed mice.

There is also inconsistency in the data as pointed out regarding pAKT levels in the study. This remains a concern, and no clear conclusion can be reached given that this is a small, feasibility study

We cannot avoid the confounding factor that patients are fasted for the surgery, but are not required to fast for the biopsy collection. Because of this, we decided to move the panel to Supp. Fig. 3.

The authors state…" We recognize that the PCA results suggest that individual variation was a primary driver of differences, rather than the diet itself. However, relevant biological changes can still happen even without large changes in the PCA."
This response does not at all address the concern raised by the reviewer.

We addressed this point in the new response to point 7 above.

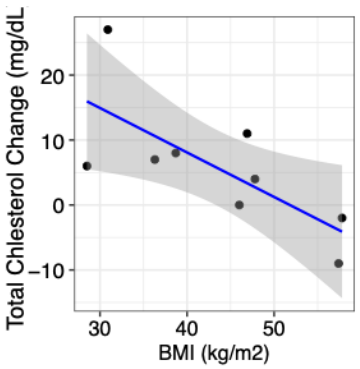
Reviewer #4 (Remarks to the Author):

Thank you for allowing me to review this significant and important study. As noted by the authors, dietary patterns can impact tumor progression and development, however, few dietary intervention studies are attempted in patients with cancer. This study provides critical data to guide future research to determine dietary guidelines during cancer treatment. However, a few details should be addressed:

We thank the reviewer for his kind words towards our work.

1) The paper consistently uses language to indicate how the dietary intervention impacts "obese" patients with endometrial cancer. However, at least 3 participants randomized at baseline had a BMI that would classify them as "overweight." The BMI and fat percentages of participants was considerably heterogenous, ranging from overweight to class 3 obesity. This distinction is important as the impact of the intervention may be more consequential in those with higher BMI. As noted by the authors, the intervention group had a significantly higher mean BMI which may have skewed the results when comparing groups.

We thank the reviewer for this comment. We have now made clear in the text that there were 3 participants with BMI <30 which would be considered overweight and not obese. We have also performed a correlation analysis to test whether baseline BMI correlates with changes between baseline and endpoint for the analytes shown in Figure 1 and those reported in the Supplement. Table 5 found that only changes in total cholesterol were inversely correlated with BMI. Patients with the highest BMI showed the most significant decreases in total cholesterol.



2) The attrition rate for feasibility was set at 25%, however, attrition over 20% is noted to introduce significant bias. Was there a reason the attrition rate was set at 25% as opposed to the standard 20%?

Our approach was informed by previous dietary intervention studies in comparable patient groups, which frequently report dropout rates exceeding 20% (reviewed in PMID: 35614234) owing to difficulties with tolerating dietary modifications. While our final dropout rate of 21% marginally surpassed the 20% threshold commonly considered as a potential source of bias, the causes of dropout were non-systematic, as they were related to ineligibility or personal scheduling conflicts, rather than intervention-related factors (Supp. Figure 1).

3) Endometrial cancers can result in a hypermetabolism, thus, a predictive equation such as Mifflin St Joer or Harris Benedict would not be appropriate to estimate the needs of the study population. It is feasible that the meals provided to the VLCD reflected a caloric deficit in addition to a macronutrient shift which may be the underlying factor in the metabolic changes reported by the end of the intervention. Since the dietary pattern of the standard diet group was not evaluated, it is difficult to make a casual claim for the macronutrient shift as opposed to a calorie reduction resulting in weight loss.

This is a thoughtful comment. The VLCD arm had an overall weight loss of 5.5 ± 0.8%. Weight loss occurs as a consequence of negative energy balance, which can be caused by a decrease in caloric intake or an increase in energy expenditure. It has been speculated that in cancer patients, tumors can lead to increased energy expenditure (hypermetabolism) and weight loss. We agree on the limitations of the predictive equations for determining caloric intake and used those initial estimates only as a guide for menu planning. Weight loss was not a goal of the study and when detected at the weekly assessments, participants were offered more calories in the subsequent week.
Unfortunately, indirect calorimetry or doubly labeled water were not performed on these patients to match calorie intake with total energy expenditure. Given the high rate of anorexia, a known effect of the VLCD (PMID: 25698989, 16129086), we suspect that the weight loss was given by a lack of appetite rather than caloric restriction.

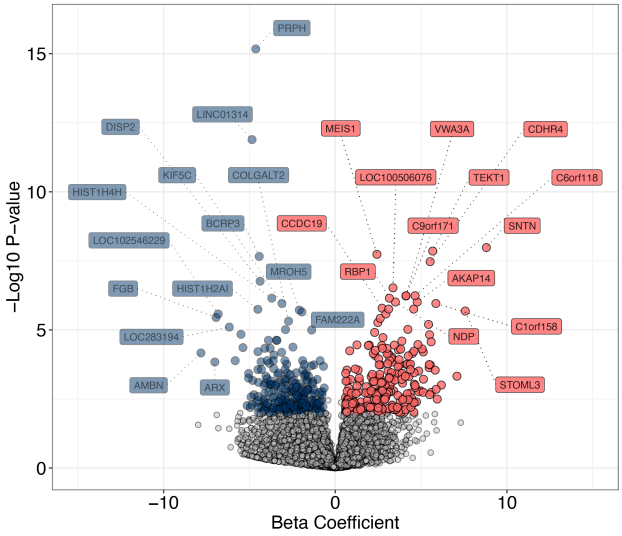
4) Please change the spelling of "dietician" to "dietitian." The spelling is inconsistent in the manuscript.
Done
Thank you again for allowing me to participate in the publication of this study.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

There are still some concerns regarding the presentation and interpretation of the results.
1. The title on Figure 2A. “Tumor transcriptomic changes with VLCD” could be misleading because the PCA plot is not designed to show transcriptomic changes. If the authors want to show transcriptomic changes, a heatmap or a volcano plot (e.g., log2 fold changes vs. adjusted p-values) may be more appropriate.

We have previously performed volcano plots with differentially expressed genes, like the one below. However, since the manuscript does not focus on any of these particular genes, we decided that showing the pathway analysis was more informative.



We have now changed the title of the Figure 2 to “Pathway enrichment analysis of endometrial cancer under VLCD”. To comply with the reviewer’s comment, we also removed the sentence “We used principal component analysis (PCA) to evidence transcriptional differences between subjects and changes with the diets” from the results section.

2. The p-values shown on Figure 2B and Figure 3A should be corrected for multiple comparisons. Conclusions should be redrawn correspondingly.

We have now added to Figure 2B and Figure 3A the FDR values reported from the analysis (Benjamini and Hochberg). We have also replaced p-values for q-values when reporting RNA-Seq results, like in the abstract section. It is important to note that the objective of this study and of this analysis in particular is hypothesis generation. As discussed in the GSEA documentation, an FDR <25% is appropriate for generating hypothesis (https://www.gsea-msigdb.org/gsea/doc/GSEAUserGuideTEXT.htm#_False_Discovery_Rate):

“The GSEA analysis report highlights enrichment gene sets with an FDR of less than 25% as those most likely to generate interesting hypotheses and drive further research, but provides analysis results for all analyzed gene sets. In general, given the lack of coherence in most expression datasets and the relatively small number of gene sets being analyzed, an FDR cutoff of 25% is appropriate.”

We have also mentioned in the discussion that the small sample size in the histology and transcriptomics analysis is a limitation of our study. In agreement, we also now state in the abstract and concluding sentence of the manuscript the data here should be interpreted as “proof of concept” and “hypothesis generating”.

3. It is not clear how the samples were classified into the TCGA subtypes.

Thank you for this comment, we have now added a more detailed description in the methods and their corresponding references:

MSK-IMPACT targeted sequencing

Mutations were assessed using Integrated Mutation Profiling of Actionable Cancer Targets (MSK- IMPACT) at Memorial Sloan Kettering (MSK)[1]. The MSK-IMPACT assay interrogates 505 cancer-associated genes and reports somatic mutation count, POLE mutation status, TP53 mutation status, MSIsensor score, tumor purity, fraction of genome altered (FGA), and tumor mutational burden (TMB)[1,2]. POLE exonuclease-domain hotspot mutations were defined according to Leon-Castillo et al[3]. MSIsensor scores were interpreted as follows: ≥10 as MSI-H, ≥3 and <10 as MSI-indeterminate, and <3 as stable (MSS), as described[4]. Molecular subtypes were assigned hierarchically: I) POLE subtype was defined by the presence of a recognized hotspot mutation in the POLE exonuclease domain[3]. II) MSI-H subtype was assigned when the MSIsensor score was ≥10[4,5]. III) CN-H subtype was called based on a TP53 homozygous deletion or a pathogenic driver mutation curated by OncoKB, CIViC, and/or Cancer Hotspots in cBioPortal[6-8]. IV) CN-L subtype was assigned to samples lacking any defining features of the other categories[2].

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4. Besides CD8+ T-Cell signature, were there any other immune cell signatures tested?

Yes, we performed pathway enrichment analysis using the other cell signatures from the Bagaev et.al. paper, also used for Fig. 2A (https://pubmed.ncbi.nlm.nih.gov/34019806/). However, only CD8 T cells were significantly enriched. Please see whole table below.

Bagaev. Et.al

Cells	SIZE	ES	NES	NOM p-val	FDR q-val
CD8_T_CELLS	21	0.6739727	1.7454808	0.00532531	0.03448314
ENDOTHELIUM	44	0.47423196	1.4451522	0.03754513	0.23777494
MACROPHAGES	44	0.37776914	1.1466607	0.23903818	0.7754444
NK_CELLS	30	0.40466443	1.1331402	0.2695858	0.61601275
DENDRITIC_CELLS	45	0.338012	1.0307409	0.4010516	0.73113674
MYELOID_CELLS	31	0.34741756	0.97710735	0.48807296	0.7287256
MONOCYTES	36	0.33010638	0.96117854	0.515435	0.6575557
CD4_T_CELLS	20	0.35554615	0.9085207	0.5814659	0.6768925
TREGS	26	0.2740041	0.7423473	0.85160524	0.8643015
B_CELLS	9	-0.3834125	-0.7893148	0.7431408	0.79491615
FIBROBLASTS	47	-0.3151397	-0.9276681	0.58615357	0.71910083
NEUTROPHILS	33	-0.3499701	-0.9646976	0.5211709	0.85758144
PLASMACYTOID_DENDRITIC_CELLS	40	-0.3402276	-0.9730729	0.498867	1
T_HELPERS	14	-0.5278374	-1.1992798	0.24154504	1

As a control, we also performed enrichment analysis using the cell signatures from Gavish et.al (https://www.nature.com/articles/s41586-023-06130-4), curated and available at (https://www.gsea-msigdb.org/gsea/msigdb/human/collections.jsp#C4). In this analysis, CD8 T cells with cytotoxic activity appear as the third most enriched pathway (q-val: 0.00011, NES: 2.176). Furthermore, signatures related to CD8 T cells appear four additional times among the top 30 pathways (total:148).

Gavish Et al.

NAME	pval	padj	ES	NES
GAVISH_3CA_METAPROGRAM_EPITHELIAL_CILIA	1.8023E-12	2.6675E-10	0.87161733	2.58169741
GAVISH_3CA_MALIGNANT_METAPROGRAM_24_CILIA	8.9033E-09	6.5884E-07	0.79441044	2.37670857
GAVISH_3CA_METAPROGRAM_CD8_T_CELLS_CYTOTOXIC	2.2855E-06	0.00011275	0.70741533	2.17580973
GAVISH_3CA_METAPROGRAM_CD4_T_CELLS_UNASSIGNED	4.4156E-06	0.00016338	0.699474	2.15138446
GAVISH_3CA_METAPROGRAM_CD8_T_CELLS_UNASSIGNED_2	1.7773E-05	0.00052609	0.66609075	2.08590109
GAVISH_3CA_METAPROGRAM_MACROPHAGES_STRESS_HSP	2.3295E-05	0.00057461	0.66193704	2.07289351
GAVISH_3CA_METAPROGRAM_FIBROBLASTS_STRESS	3.7109E-05	0.00078459	0.65437531	2.04921352
GAVISH_3CA_METAPROGRAM_CD4_T_CELLS_CYTOTOXIC	7.0607E-05	0.00116109	0.65569381	2.01672895
GAVISH_3CA_METAPROGRAM_CD8_T_CELLS_HEAT_SHOCK	6.4586E-05	0.00116109	0.63287611	1.99640663
GAVISH_3CA_MALIGNANT_METAPROGRAM_5_STRESS	0.00016672	0.00224311	0.62637117	1.96151695
GAVISH_3CA_METAPROGRAM_EPITHELIAL_STRESS	0.00012383	0.00183264	0.62935324	1.95435375
GAVISH_3CA_METAPROGRAM_MACROPHAGES_MES_GLYCOLYSIS	0.00020331	0.00250753	0.63515434	1.95355534
GAVISH_3CA_MALIGNANT_METAPROGRAM_21_RESPIRATION	0.00074297	0.00665251	0.63245895	1.89474177
GAVISH_3CA_MALIGNANT_METAPROGRAM_13_EMT_2	0.00042627	0.00450632	0.61590444	1.89434808
GAVISH_3CA_MALIGNANT_METAPROGRAM_3_CELL_CYLCE_HMG_RICH	0.00076414	0.00665251	0.62802554	1.89192403
GAVISH_3CA_METAPROGRAM_CD8_T_CELLS_MEMORY_NAIVE_1	0.00040785	0.00450632	0.59643201	1.8814438
GAVISH_3CA_MALIGNANT_METAPROGRAM_7_STRESS_IN_VITRO	0.00085747	0.00705029	0.61505027	1.86397392
GAVISH_3CA_METAPROGRAM_ENDOTHELIAL_STRESS	0.00069705	0.00665251	0.5837005	1.84128227
GAVISH_3CA_METAPROGRAM_MACROPHAGES_RESPIRATION	0.00134139	0.00945358	0.60090045	1.82986737
GAVISH_3CA_METAPROGRAM_FIBROBLASTS_HYPOXIA	0.00100536	0.00783126	0.58671028	1.82193302
GAVISH_3CA_MALIGNANT_METAPROGRAM_11_TRANSLATION_INITIATION	0.00148135	0.00996547	0.5975927	1.81979459
GAVISH_3CA_METAPROGRAM_CD4_T_CELLS_STRESS_HSP	0.00115442	0.00854271	0.5713909	1.80245165
GAVISH_3CA_MALIGNANT_METAPROGRAM_17_INTERFERON_MHC_II_1	0.00181698	0.01169187	0.57968833	1.78295758
GAVISH_3CA_METAPROGRAM_CD4_T_CELLS_T_REG	0.00264944	0.01633821	0.5699704	1.75306796
GAVISH_3CA_MALIGNANT_METAPROGRAM_14_EMT_3	0.00349786	0.01991087	0.55793816	1.7472151
GAVISH_3CA_METAPROGRAM_B_CELLS_HSP_STRESS	0.00322582	0.01909687	0.55489294	1.72312945
GAVISH_3CA_METAPROGRAM_CD4_T_CELLS_DYSFUNCTION	0.00666827	0.03524655	0.546837	1.68191616
GAVISH_3CA_METAPROGRAM_EPITHELIAL_EPI_1	0.00629693	0.03451651	0.53388876	1.65790439
GAVISH_3CA_METAPROGRAM_CD8_T_CELLS_DYSFUNCTION	0.0100665	0.05137387	0.52611488	1.64755869
GAVISH_3CA_METAPROGRAM_EPITHELIAL_EMT_LIKE_2	0.01819341	0.08414451	0.50912933	1.59436746