Dear Editor,

We would like to thank you for the opportunity to revise and resubmit our manuscript (Ms. No.: 30159) entitled "High throughput RNA Sequencing Utility for Diagnosis and Prognosis in Colon Diseases." The suggestion presented by the reviewer was constructive and instrumental in improving our project conclusions. We hope our modifications, edits and additions in the manuscript help alleviate the concerns posed by the reviewers. The following summarizes our responses to individual reviewer comments as well as highlighted excerpts of new information added to the manuscript. Once again, we greatly appreciate your consideration and earnestly await your response.

Sincerely,

Mamie Gao, Allen Zhong, Neil Patel, Anjali Garg, Chiraag Alur, Dinesh Vyas

Listed below are the individual reviewer comments as well as our responses and subsequent changes in the manuscript. A highlighted version of the manuscript has also been uploaded.

Reviewer # 00503512:

"Gao et al. review the use of RNA Seq for the discovery of new biomarkers and therapeutic targets in colorectal diseases. The review is generally well written and the topic is novel and relevant. I was just puzzled by the last 2 paragraph: there seem to be some undue overlapping between the "technical limitations" and the "treatment" discussion. The authors should revise this part carefully."

Response:

The reviewer raises a valid point about the clarity of the arguments made in the final 2 paragraphs in our original manuscript, specifically the separation between "technical limitations" and "treatment". To better organize the argument that there are current limitations to the therapeutic uses of RNA sequencing applications in the treatment of colorectal disease, we have listed short-comings in the literature that have prevented use of research towards RNA therapeutics in colorectal disease, as well as attempted therapeutics and technical reasons for their failures. We hope these changes better organize this argument and provide a more logical flow.

We placed the small sample size argument earlier and separated it from the other therapeutic limitations arguments.

As seen in the previous studies described above, small sample sizes were used for most research conducted. While some studies used 400 samples, many had sample sizes that ranged from 6 to 30. Larger samples sizes can help to yield more significant results[53]. Another study with promising results that are not significant due to sample size is Cohen et al.'s study on the predictive value of Target Now. Target Now uses immunostaining and RNA expression on tumor samples to identify potentially beneficial or ineffective drugs. The results of this study were not statistically significant due to its small sample size of 19 patients[54]. Despite the promising results of the Target Now study, the small sample size exemplifies the limitations of much of the RNA Sequencing literature and its application in colorectal diseases.

IT IS INCORPORATED at page 12 and paragraph 2

We also separated many of the arguments in the final 2 paragraphs into separate paragraphs about distinct limitations of using siRNA for therapeutic uses. A conclusion paragraph was also created.

Complications and side effects have been seen when siRNA has been used as a therapeutic agent, further limiting the usage of RNA sequencing in colorectal disease. A phase 1 drug candidate that targeted apoB was withdrawn because of the immune response elicited by its cationic lipid-based formulation that delivers siRNA into endosomes where immune receptors are most dense. This caused one patient to have severe flu-like symptoms typical of an immune response[55].

In response, dual targeting siRNA is being studied to reduce the potential for off-target gene silencing. Theoretically, fewer strands compete for RISC entry which helps avoid the innate immune response. However, more research needs to be conducted in this area[56].

The biodistribution of siRNA *in vivo* has also been a limitation of siRNA application. Van de Water found that intravenous siRNA accumulates in the kidney of rats rather than being absorbed in the GI tract. There, it acts to suppress the gene function in proximal tubules[57], limiting the application of siRNA in colorectal disease. Further research is needed to manipulate the localization of siRNA for therapeutic colorectal applications.

To conclude, despite the large amount of research dedicated to using RNA sequencing to diagnose and screen for colorectal diseases, further studies need to be conducted on using these techniques for treatment of these colorectal diseases. With more research, RNA sequencing could be the next novel treatment for colorectal diseases.

It is INCORPORATED at page 12 and paragraph 3 and ends at page 13, paragraph 2