Response to “Letter to the Editor for Mucus Microbiome Composition as a Predictor for Colorectal Anastomotic Leakage”

Reply:

This letter is in response to the letter by Lamendella and Stewart, which argued the used statistics in our article. We thank the editors for the opportunity to rebut the issues raised.

The first point made by the authors is that we selected 2 cohorts of patients, with no difference in anastomotic leakage between the groups, and that we made an inappropriate subgroup analysis as is common in the genre of microbiome causing leaks'. Aside from the fact that literature that focuses on the microbiome causing anastomotic leaks is still sparse, the point itself is incorrect.

The microbiome analyses we performed were done on circular stapler donuts that were collected during the C-seal trial. This trial was designed to evaluate the efficacy of the C-seal, a biodegradable intraluminal sheath, in avoiding clinical relevant anastomotic leakage. Unfortunately, this trial was discontinued after interim analysis due to futility. A subset of patients consented to use the donut for further analyses. Based on availability, we could use the donut of 29 patients who developed anastomotic leakage, which we matched with 94 patients who did not develop anastomotic leakage based on sex, age, and preoperative chemotherapy and radiotherapy, all stated in our article. Although we did not match on the use of the C-seal, it was to be expected that the percentage of C-seal patients in the selected group is roughly 50% as the original randomization was done on a one to one basis.

Interestingly, when we compared the microbiome of the 29 patients with a leak with the 94 without a leak, there was only a slight difference—the Blautia genus was more abundant among AL patients (P = 0.040), see also our article—contrary to our pilot study of 16 patients (all without the C-seal). A subsequent analysis of C-seal and no C-seal patients was not more than logical, with the additional benefit that the microbiome might help explain the results of the C-seal trial (see Discussion).

Since there were no truly convincing differences found between C-seal AL-cases and controls, whereas the opposite was found for the non-C-seal cohort (and thus the regular procedure), one can conclude that the presence of a C-seal might have influenced the microbiota severely after application. However, this then occurred after our sampling and is not seen in our analysis.

We accept that improvements can indeed be made to the 16S data processing pipelines, yet these would not have altered the main findings of this work. A stricter quality score filter would indeed cause fewer low-quality reads to inflate the apparent diversity or the total number of observed species. We have as such specifically chosen not to use rarefaction curves; we furthermore mainly focus on the dominant species present within the samples as they determine most of the metabolic (and other) activity. We do this by utilizing the Simpson diversity index and Principal Component Analysis to analyze our data, both techniques that focus on the abundant groups. Our main findings, in which we have confidence, are in regards to the more prevalent bacterial groups, not the rare ones (which might have been missed).

Species-level identification might have been thought to be difficult in 2009 (see reference from authors Letter to the Editor) but it can be done for most species now, using 16S reads covering multiple variable regions (V3 and V4), as reference libraries have increased in size and quality. We would now however recommend a different method of taxonomic assignment as utilized in this study, which is called oligotyping. Instead of allocating reads to the closest previously defined taxonomic box of a particular species, oligotyping instead combines all the reads of a study into its own set of taxonomic boxes (oligotypes). A representative sequence of each oligotype can then be compared to the reference library, frequently even allowing for the identification of different strains/oligotypes of the same species (Supplemental Fig. 4 of Salter et al, 2017). Aside from this, the main findings in our study were on the family level, using the species level just as additional detail.

Regarding the Lachnospiraceae family and butyrate production; it indeed contains various important butyrate producers such as the Roseburia and Coprococcus genus. This family, however, also contains dozens of non-butyrate-producing species. This is one of the reasons why the genus and species level were also analyzed. It was found that within the Lachnospiraceae family that the non-butyrate-producing species accounted for most of the difference between non-C-seal cases and controls. Somewhat elevated levels of Roseburia and Coprococcus were indeed observed in cases, but not in a significant manner. The Ruminococcaceae family, which includes the (more) important butyrate producer Faecalibacterium prausnitzii, was associated with an increased diversity of the gut microbiome.

Principal component analysis, an unsupervised dimension reduction method (SPSS standard settings, no rotation), is used to show both visually and statistically that a significant difference does exist between cases and controls of a particular multidimensional dataset. Principal component 1, which describes more than half of all the variation in the data (58%), clearly demonstrates that non-C-seal AL cases score significantly higher on PC1 than non-C-seal controls. This shows that this gut microbiota composition is significantly associated with anastomotic leakage. The next step is to ascertain what this difference, as described by PC1, exactly is. PC3, which describes 6% of the variation was also associated with AL-status in non-C-seal cases and was similarly of interest. Correlation analyses with these particular principal components can subsequently be used to identify which combination of species is driving the mucus microbiome to be more prone to AL development.

In order to use PCA in such a fashion, the data indeed need to be normalized in such a way that sampling depth is simply irrelevant (at least for the somewhat abundant bacterial groups). To do so all bacterial groups are normalized into a percentage of reads per sample, as for example shown in Figure 2, before being further analyzed. We assumed (apparently incorrectly) that this was self-evident. We acknowledge that the predictive analysis has an element of ad hoc semblance, yet any set of parameters focusing on diversity and the two main bacterial families, without the focus on equal numbers, would have resulted in the same conclusions.

The main weakness of this set of parameters is not the statistical aspect but the potential universal applicability aspect. If samples, for example, in future studies are stored in slightly different conditions, for a longer or shorter time, being processed with different reagents (different DNA isolation kit), etc then the percentages of different bacterial groups after sequencing may differ. Furthermore, the gut/mucus microbiome differs between inhabitants from different countries. These parameters can thus not be used yet to make decisions on whether to treat a patient or not. They do, however, possibly...
provide us with a “direction” in regards where to steer the gut microbiota composition toward in patients. Basically, eat more fiber and vitamins (vegetables) and less sugar, (animal) protein, and (saturated) fats.

As discussed above, we still support our study and defend its conclusions. We believe our statistics and methods for an exploratory study of a limited and unique set of samples were appropriate and therefore academically meaningful. We hope the academic world will engage in more studies into the relation of the mucus microbiome and anastomotic leakage, as the etiology of anastomotic leakage is still unclear in many cases.

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REFERENCES