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Mucus Microbiome of Anastomotic Tissue During Surgery Has Predictive Value for Colorectal Anastomotic Leakage

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Abstract

AIMS: Anastomotic leakage (AL) remains the main cause for morbidity and mortality in colorectal surgery. Understanding the role of the gut microbiota in AL development is important, because it may allow the identification of specific microorganisms and/or their products that are associated with AL. The aim of this study was to evaluate the microbiota composition and the relationship between AL and the gut microbiota in colorectal surgery patients.

METHODS: We measured 16S rRNA gene abundance in colorectal anastomoses from patients who developed AL and from patients without AL. We used 16S rRNA sequencing to compare the microbiota composition of the specimens from patients who developed AL and the controls. From the data, we identified the most predictive features in AL.

RESULTS: The microbiota composition differed between patients who developed AL and those who did not. Patients without AL had a higher abundance of bacteria from the Bacteroidaceae family and a lower abundance of bacteria from the Lachnospiraceae family. A higher abundance of Faecalibacterium and Faecalibacterium prausnitzii was associated with AL. In addition, the microbiota composition was influenced by the use of a C-seal during surgery.

CONCLUSIONS: The gut microbiota plays a role in the development of AL, and specific microorganisms and their products may be involved in the pathogenesis of AL. The use of a C-seal during surgery may influence the microbiota composition and reduce the risk of AL.

Keywords: colorectal surgery, microbiota, anastomotic leakage, Lachnospiraceae, Bacteroidaceae

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Sample Collection

Bacterial DNA of the available proximal donuts was isolated and subsequently analyzed using MiSeq sequencing of the amplified 16S rRNA genes. The reason for studying 16S rRNA genes using MiSeq sequencing is because all bacteria have 16S rRNA genes, and the small differences in their 16S rRNA genes allow us to identify all the microbial groups present within a sample. Sequencing allows us to quantitatively analyze the relative abundance of all species, including species which we are yet unable to culture in the laboratory.

The often complex bacterial composition of a sample, including the analysis of more rare low-abundance bacteria, can hence be measured in a much more cost-effective and accurate fashion than was possible with previous microbiome classification methods such as fluorescent in situ hybridization microscopy counting techniques.

DNA Extraction and MiSeq Preparation

Total DNA was extracted, as described by de Goffau et al,\(^1^5\) from 0.25 g of a “donut.” Care was taken not to include any macroscopic traces of stool. The additional purification steps using columns were not needed after DNA precipitation. The V3 to V4 region of the 16S rRNA gene was amplified from the DNA by PCR using modified 341F and 806R primers with a 6-nucleotide barcode region of the 16S rRNA gene was amplified from the DNA by PCR. Sequences of 341F and 806R were primer aatgataccgcagacctgt

The sequence of the 341F primer and the 806R primer was aatgataccgcagacctgt.

using modified 341F and 806R primers with a 6-nucleotide barcode region of the 16S rRNA gene was amplified from the DNA by PCR


gagat

on the 806R primer. The sequence of the 341F primer and the 806R primer was aatgataccgcagacctgt.

The sequence of the 341F primer and the 806R primer was aatgataccgcagacctgt.

MiSeq Sequencing Pipeline and Statistical Analysis

Software that was used to analyze the data received from Illumina paired-end sequencing included PANDAseq,\(^1^8\) QIIME, and ARB.\(^1^9\) Reads with a quality score <0.9 were discarded by PAN-

DAsq. Statistical analyses were performed on the family, genus, and species level. QIIME identified sequences down to the family and genus level and was used to perform weighted alpha-diversity analyses, whereas ARB was used to identify sequences down to the species level. Principal component analysis (PCA) was performed to describing the variations in all the bacterial groups into a very limited amount of new relevant dimensions of variability to address the issue of multiple testing. The hierarchical clustering analysis was performed with the Hierarchical Clustering Explorer version 3.0 (http://www.cs.umd.edu/hcil/ multi-cluster/). Percentages (%) given of a microbial group in a group of patients indicate the average percentage of reads assigned to that group. The Simpson index was used as a measure of microbial diversity. Nonparametric tests were used, as microbial abundances are rarely normally distributed and are preferred as they are more conservative. Mann–Whitney

U or Spearman’s \(\rho\) tests were used as indicated. The use ± indicates that a standard deviation is given. All tests were two-tailed, and a \(P < 0.05\) was considered to indicate statistical significance. All statistical analyses were performed using IBM SPSS Statistics 20.0.

RESULTS

Of 123 samples in total, 122 were included in the PCA, as 1 sample had an insufficient amount of bacterial DNA (Table 1). However, 3 C-seal AL patients and 1 non-C-seal AL patient were excluded from subsequent statistical analyses, as clear nonmicrobiota-related reasons were found why these patients developed AL; 3 had necrosis of the proximal bowel loop and 1 had a technical failure of the C-seal.

Surprisingly almost no difference was found between AL and non-AL patients when the 118 samples were analyzed together; only the Blautia genus was more abundant among AL patients (\(P = 0.040\)). However, when we looked at the subgroup of non-C-seal patients, the microbiota of AL versus non-AL was different.

Sixty samples were from patients who were randomized to the C-seal group and 58 were from the group without a C-seal. Of the 60 C-seal patients, 15 developed AL, whereas this number was 10 of the 58 in the non-C-seal patients. In a comparison of the C-seal samples with all the non-C-seal samples, no statistically relevant differences were found in bacterial compositions either on the genus or family level. However, AL patients without a C-seal had a much lower microbial diversity (\(P = 0.006\)), more Bacteroides (\(P = 0.006\)), more Lachnospiraceae (\(P = 0.05\)), and less Prevotella (\(P = 0.05\)) and Streptococci (\(P = 0.03\)) than C-seal patients who developed AL. All other patient characteristics were, in comparison with the presence or absence of a C-seal, irrelevant. Striking differences were subsequently revealed between C-seal samples and non-C-seal samples in regard to AL and non-AL samples.

Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AL (n = 29)</th>
<th>No AL (n = 94)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-seal/no C-seal</td>
<td>18/11</td>
<td>45/49</td>
<td>0.207</td>
</tr>
<tr>
<td>Sex male/female</td>
<td>22/7</td>
<td>61/23</td>
<td>0.365</td>
</tr>
<tr>
<td>Age, y (SD)</td>
<td>63.4 (10.4)</td>
<td>63.4 (10.4)</td>
<td>0.510</td>
</tr>
<tr>
<td>Body mass index (SD), kg/m²</td>
<td>26.0 (3.9)</td>
<td>26.9 (4.0)</td>
<td>0.075</td>
</tr>
<tr>
<td>Charlson comorbidity index</td>
<td>0.415</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>16</td>
<td>56</td>
<td>0.830</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>23</td>
<td>0.462</td>
</tr>
<tr>
<td>2+</td>
<td>8</td>
<td>15</td>
<td>0.197</td>
</tr>
<tr>
<td>Indication for surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>26</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>Diverticular disease</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Preoperative treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>13</td>
<td>39</td>
<td>0.831</td>
</tr>
<tr>
<td>Short course</td>
<td>11</td>
<td>28</td>
<td>0.494</td>
</tr>
<tr>
<td>Long course</td>
<td>5</td>
<td>27</td>
<td>0.240</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>5</td>
<td>30</td>
<td>0.160</td>
</tr>
<tr>
<td>Corticosteroid use</td>
<td>1</td>
<td>3</td>
<td>0.999</td>
</tr>
<tr>
<td>Deviating ostomy present</td>
<td>1</td>
<td>9</td>
<td>0.449</td>
</tr>
<tr>
<td>ASA-score</td>
<td>1</td>
<td>7</td>
<td>0.795</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>64</td>
<td>0.376</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>11</td>
<td>0.528</td>
</tr>
</tbody>
</table>

All patients received mechanical oral bowel preparation and antibiotics prophylaxis.
FIGURE 1. Principal component analysis (PCA) plot of all 122 samples, divided over all 4 groups and relevant associations. PC1, represented by the x-axis, is associated with AL in non-C-seal patients and describes 58% of the variation in the data. PC3, represented by the y-axis, is positively associated with microbial diversity and negatively associated with AL and describes 6% of the variation in the data. Vectors in the upper right corner represent the correlation coefficients of the respective variables with PC1 and PC3. Colored vectors correspond to the AL and C-seal status as indicated in the legend. In non-C-seal patients, the AL cases nearly without exception found in the lower bottom corner, as indicated with a dashed red circle, which is indicative of a microbiota dominated by Lachnospiraceae and Bacteroidaceae of low diversity. Non-C-seal controls are more commonly found to have higher scores on PC3, which is associated with a higher microbial diversity, containing more Ruminococcaceae, more Prevotella copri, and/or more Streptococcaceae.

Principal Component and Hierarchical Clustering Analysis

A PCA plot showed all 122 samples, divided into 4 main groups based upon C-seal status and AL occurrence, combined with a correlation analysis of the main microbial groups and microbial diversity (Fig. 1). This highlights some of the main differences between C-seal patients and non-C-seal patients with respect to clinical outcome. The most striking aspect of Figure 1 is how nearly all non-C-seal AL samples cluster together in the lower right corner as indicated with a red dashed circle, whereas the other 3 groups have a seemingly identical distribution. Correlation analyses confirm that the clustering/scattering of C-seal AL samples and C-seal non-AL samples is almost identical, indicating that in the C-seal patients the dominant microbial composition of the samples is unlikely to be related to the development of AL. The distribution of non-C-seal non-AL samples at first glance seem to be similar to the distribution of C-seal patients, yet correlation analyses show that these samples tend to be more located to the upper left. Non-C-seal AL samples score higher on PC1 (P = 0.012) and lower on PC3 (P = 0.0006) than non-C-seal non-AL samples. The localization of samples in the lower right corner is associated with a bacterial composition that is strongly dominated by Lachnospiraceae and/or Bacteroidaceae and that is consequently low in microbial diversity.

A hierarchical clustering analysis on the C-seal samples in combination with a microbial profile at the family level yet again showed a homogeneous distribution in both AL and non-AL samples (data not shown). However, when this technique is applied to the non-C-seal samples, a cluster of samples is found within the hierarchical clustering in which AL samples are overrepresented (7/14 vs 3/44, P = 0.0002) and in which Lachnospiraceae are dominant (40%) followed by Bacteroidaceae (28%) (Supplemental Figure 1, http://links.lww.com/SLA/B359). Furthermore, a microbial profile of non-C-seal samples with a focus on both the microbial diversity, which is lower in the AL samples (P = 0.0002), and the combined prevalence of Lachnospiraceae and Bacteroidaceae underscores the strong association of these family groups (Fig. 2) with the occurrence of AL.

Taxonomic Analysis

Individual analyses of taxonomic groups on the phylum, family, genus, and species level in the case of C-seal samples again highlight the apparent irrelevance of the microbiota composition in regard to the occurrence of AL. Only the presence or absence of a few low-abundant opportunistic pathogenic groups was found to be almost exclusively (weakly) associated with AL in C-seal samples. These included the Tenericutes phylum (11/15 vs 19/45, P = 0.037, chi-square test), the Leptotrichia family (8/15 vs 9/45, P = 0.013), and Prevotella oralis (7/15 vs 6/45, P = 0.007). The abundance of Bacteroides uniformis and Bacteroides ovatus were found to be negatively associated with the occurrence of AL in C-seal patients (0.8% vs 2.5%, P = 0.001 and 0.9% vs 0.4%, P = 0.01). Of the different Bacteroides species, B. uniformis was also the most negatively correlated with P. oralis (P = 0.008).

Differences between AL and non-AL in the non-C-seal group were much more abundant. The Lachnospiraceae family is associated with AL (40% vs 27%, P = 0.010), and consists of multiple important genera of which the Blautia genus (8% vs 4%, P = 0.003), in particular Blautia obeum, is the most strongly associated with AL (7% vs 3%, P = 0.005). The Bacteroidaceae family is furthermore associated with AL (28% vs 17%, P = 0.008). On the contrary, Prevotella copri and the Streptococcus genus are both negatively associated with AL development in non-C-seal patients (Tables 2 and 3). P. copri was completely absent in 8/10 of the AL cases, whereas it was absent in only 11/48 of the non-AL cases (P = 0.0005, chi-square test).

Predictive Analyses

As Figure 1 and Supplemental Figure 1, http://links.lww.com/SLA/B359 show, AL cases of non-C-seal patients seem to be almost without exception dominated by Lachnospiraceae and Bacteroidaceae with correspondingly low microbial diversity scores. As a measure for future predictive analyses, we defined a set of criteria to describe a microbial composition that predisposes patients to developing AL after surgery. These criteria were chosen as such
that an approximately equal number of patients from the C-seal and the non-C-seal patient cohort would meet these criteria. Samples were prone to developing AL if the total sum of Lachnospiraceae and Bacteroidaceae in them was higher than 60% and when the Simpson diversity score on the family level was <0.75. Eight out of 14 samples from the non-C-seal group who met these criteria developed AL. For the C-seal group, this was only 3 out of 13. The odds ratio for developing AL when meeting the criteria as defined above was 0.9 for the C-seal group (P = 0.9), but for the non-C-seal group this was 28 (P = 0.00001).

DISCUSSION

This study shows a relation between the composition of the intestinal microbiota and the subsequent development of AL after stapled colorectal anastomoses, but only in patients who underwent surgery without the additional C-seal that covered the anastomoses. In a previous pilot study on AL, we analyzed 16 non-C-seal patients of whom 8 developed AL.13 The present study included an additional 63 C-seal and 44 non-C-seal patients, with 2 additional leakages in the non-C-seal group.

Non-C-seal

In this larger group of non-C-seal samples, the correlations with AL confirm most of the results we found in the pilot study, as a high abundance of Lachnospiraceae and Bacteroidaceae and a lower microbial diversity are still strongly associated with AL. A bacterial composition that consists of 60% or more of these 2 families seems predictive for AL.

The trophic network of species in intestinal microbiota with a low diversity may be more easily disturbed than in microbiota with a high diversity.20 This disturbance could be provoked by preoperative or surgical procedures, such as intravenous antibiotics, mechanical bowel preparation, the creation of deviating ostomies, opioids, or even the impact of the surgery itself.20–23 A disturbed microbial composition may affect the metabolic balance; a reduction of butyrate production might, for instance, initiate energy deprivation, causing impaired functioning of the colonic cells and their ability to heal. It has been found in rats that an intraluminal infusion of SCFAs resulted in significantly stronger colonic anastomoses.24 Rectal irrigation with SCFAs in humans with ulcerative colitis or diversion colitis has also shown promise.9,25 Furthermore, a disturbed microbiota of low diversity may lack colonization resistance to pathogenic bacteria that could play a role in the development of AL, for example, Enterococcus faecalis.26–28 It would be very interesting to compare the microbiota at the time of surgery with the microbiota at the time of AL.

In this study, the focus in the non-C-seal samples seems to be on the importance of microbial diversity and possibly on the role of mucin degradation, possibly with an important mediating role of the Ruminococcaceae family, which contains a high number of important butyrate-producing species such as F. prausnitzii. Of the 3 most dominant microbial families in the gut, Bacteroidaceae (19%), Lachnospiraceae (29%), and Ruminococcaceae (16%), the first 2 are strongly negatively correlated with microbial diversity. Both Bacteroides and Blautia (from the Lachnospiraceae family) are known mucin-degraders that mainly either produce acetate and propionate or propionate and propanol, but neither of them produces butyrate.29,30 Despite their high prevalence, Ruminococcaceae are

TABLE 2. Main Associations With AL in Non-C-seal Samples (MW-U Test)

<table>
<thead>
<tr>
<th>Reduced Risk</th>
<th>Average, %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevotella copri</td>
<td>1.0</td>
<td>0.007</td>
</tr>
<tr>
<td>Streptococcus genus</td>
<td>2.6</td>
<td>0.012</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>0.5</td>
<td>0.018</td>
</tr>
<tr>
<td>Eubacterium biforme</td>
<td>1.5</td>
<td>0.010</td>
</tr>
<tr>
<td>Lachnospiraceae</td>
<td>29</td>
<td>0.010</td>
</tr>
<tr>
<td>Blautia genus</td>
<td>4.9</td>
<td>0.004</td>
</tr>
<tr>
<td>Blautia obeum</td>
<td>3.8</td>
<td>0.005</td>
</tr>
<tr>
<td>Blautia glucerasei</td>
<td>0.7</td>
<td>0.014</td>
</tr>
<tr>
<td>Ruminococcus torques</td>
<td>1.4</td>
<td>0.029</td>
</tr>
<tr>
<td>Coprococcus</td>
<td>5.9</td>
<td>0.098</td>
</tr>
<tr>
<td>Roseburia</td>
<td>3.7</td>
<td>0.094</td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>19</td>
<td>0.008</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>19</td>
<td>0.028</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>3.2</td>
<td>0.013</td>
</tr>
</tbody>
</table>
strongly positively associated with microbial diversity, especially the metabolically highly important key­stone species F. prausnitzii (7%) and Ruminococcus bromii (3%).31

C-seal

In the C-seal trial, we found a trend to more AL in C-seal patients than in non-C-seal patients.32 However, the overall microbial composition in C-seal patients does not seem to play a role in the occurrence of AL at first. Our observations suggest that the C-seal influences the microbial composition after introduction. This may be due to the barrier it creates between the mucosa and the (fresh) luminal content, interrupting the supply of new resources. The subsequent reduced rate of metabolism (SCFA production), possibly reduces the rate of mucin synthesis by the human host, negatively affects wound healing.33 The C-seal may create a new ecosystem that benefits the patients than in non-C-seal patients.32 However, the overall microbial composition in patients that underwent standard colorectal surgery has a predictive value in regard to whether they develop AL or not. Patients seem to have a higher risk of developing AL when their microbial diversity is low, which in turn is harmful consequences of the dominant gut microbiota before surgery in regard to wound healing. Further studies should be conducted to elicit the possible mechanisms between the microbial composition and the development of AL.

CONCLUSIONS

The microbial composition in patients that underwent standard colorectal surgery has a predictive value in regard to whether they develop AL or not. Patients seem to have a higher risk of developing AL when their microbial diversity is low, which in turn is often associated with an overabundance of members from the mucin­degrading families Lachnospiraceae and Bacteroidaceae. The intro­duction of a C-seal, however, completely negates the protective or harmful consequences of the dominant gut microbiota before surgery in regard to wound healing. Further studies should be conducted to elicit the possible mechanisms between the microbial composition and the development of AL.

ACKNOWLEDGMENTS

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