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

Jasper B. van Praagh, BSc,† Marcus C. de Goffau, PhD,†† Ilsalien S. Bakker, MD, PhD,‡§ Harry van Goor, PhD,|| Hermie J. M. Harmsen, PhD,† Peter Olinga, PhD,¶ and Klaas Havenga, MD, PhD♦

Objective: The aim of the present study is to investigate the association of gut microbiota, depending on treatment method, with the development of colorectal anastomotic leakage (AL).

Background: AL is a major cause for morbidity and mortality after colorectal surgery, but the mechanism behind this complication still is not fully understood.

Methods: Bacterial DNA was isolated from 123 “donuts” of patients where a stapled colorectal anastomosis was made and was analyzed using 16S MiSeq sequencing. In 63 patients, this anastomosis was covered with a C-seal, a bioresorbable sheath stapled to the anastomosis.

Results: In non-C-seal patients, AL development was associated with low microbial diversity (P = 0.002) and a high abundance of the dominant Bacteroidaceae and Lachnospiraceae families (P = 0.008 and 0.010, respectively). In C-seal samples, where AL rates were slightly higher (25% vs 17%), an association with the gut microbiota composition was almost undetectable. Only a few opportunistic pathogenic groups of low abundance were associated with AL in C-seal patients, in particular Prevotella oralis (P = 0.007).

Conclusions: AL in patients without a C-seal can be linked to the intestinal microbiota, in particular with a low microbial diversity and a higher abundance of especially mucin-degrading members of the Bacteroidaceae and Lachnospiraceae families. In C-seal patients, however, it seems that any potential protective benefits or harmful consequences of the gut microbiota composition in regard to wound healing are negated, as progression to AL is independent of the initially dominant bacterial composition.

Keywords: anastomotic leakage, colorectal, intraluminal sheet, microbiome, surgery


A nastomotic leakage (AL) remains the main cause for morbidity and mortality in colorectal resection surgery, leading to prolonged hospital stays and significant costs.1–3 Although surgical factors as perfusion of and tension on the anastomosis and patient-related factors as comorbidity and medication are known factors, in many cases no explanation can be given for the failure of anastomotic healing.

It is well recognized that the gut microbiota plays an important role in human health, and an expanding list of diseases has been associated with the microbial composition and/or their products.4–6 Intestinal diseases, in particular, have been associated with the intestinal microbiota.4–7 Products produced by bacteria, such as short-chain fatty acids (SCFAs), are important for colonic cells. Butyrate, for example, is the primary energy source for colonic cells.7 In addition to its importance in the defense against opportunistic pathogens, the glycoproteins in the mucus layer (mucin) also serve as a source of nutrients for commensals/symbionts such as the anti-inflammatory butyrate-producing bacterium Faecalibacterium prausnitzii.8 However, when the supply of butyrate to the colon is diminished or stopped, the colonic mucosa may enter a state of energy deprivation, leading to colitis and diarrhea.9,10 Furthermore, the colonic microbiota is also important in regard to wound healing.11 In addition, selective decontamination of the digestive tract reduces infections and seems to have a beneficial effect on AL in colorectal surgery.12

In a previous pilot study,13 we investigated the possible role of colonic microbiota in AL using samples from 8 patients who developed AL matched with 8 patients without AL who were included in the C-seal trial14 but who were not treated with a C-seal. We found that an overabundance of bacteria from Lachnospiraceae family and low microbial diversity were linked to AL development.

The aim of the present exploratory study is to investigate the role of the gut microbiota using 16S rRNA analysis, in the development of AL in greater detail using a larger group of patients, and to analyze whether the use of a C-seal during treatment, an intraluminal sheet originally designed for the protection of the anastomosis,14 influences the role between the gut microbiota composition and AL development.

METHODS

The methods used for this study are the same as described in the previously performed pilot study.13

Patients

Twenty-nine patients who developed AL were matched on sex, age, and preoperative chemotherapy and radiotherapy with 94 patients without AL. AL was defined as AL leading to a reintervention. All patients participated in the C-seal trial, a trial to evaluate the efficacy of the C-seal in the prevention of clinical AL in the stapled colorectal anastomosis. This multicenter trial was designed to evaluate the efficacy of the C-seal; the primary endpoint was AL requiring reintervention. This trial was open for inclusion from December 2011 until January 2014.

The study was approved by the Medical Ethics Committee of the University Medical Centre Groningen and all participating
centers. The trial was registered in The Netherlands National Trial Register under the number NTR3080. In total 539 patients were included; all patients provided written informed consent; and additional consent was asked to retrieve and store the circular stapler donuts. All data were collected anonymously, encoded, and saved in a database.

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-abundant 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,\textsuperscript{15} 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 PCF1 and 806R primers with a 6-nucleotide barcode on the 806R primer. The sequence of the 341F primer and the 806R region of the 16S rRNA gene was amplified from the DNA by PCR. The forward primer was aatgatacggcgaccaccgagatct and the reverse primer was caagcagaagacggcatacgt. The sequence of the primer was gatctNNNNCCTACGGGAGGCAGCAG and caagcagaagacggcatacgt. The sequence of the primer was gatctNNNNCCTACGGGAGGCAGCAG and caagcagaagacggcatacgt. The sequence of the primer was gatctNNNNCCTACGGGAGGCAGCAG and caagcagaagacggcatacgt.

MiSeq Sequencing Pipeline and Statistical Analysis

Software that was used to analyze the data received from Illumina paired-end sequencing included PANDAseq,\textsuperscript{18} QIIME, and ARB.\textsuperscript{19} Reads with a quality score <0.9 were discarded by PANDAseq. Statistical analyses were performed on the family, genus, and species level. QIIME identified sequences down to the family and genus 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.

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. Patient Characteristics**

<table>
<thead>
<tr>
<th></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>45/40</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>
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<td></td>
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</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>0.179</td>
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<tr>
<td>Indication for surgery</td>
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<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>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>Preoperative treatment</td>
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<td></td>
<td></td>
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<tr>
<td>Radiotherapy</td>
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<tr>
<td>Short course</td>
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<td>Chemotherapy</td>
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<td>30</td>
<td>0.160</td>
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<tr>
<td>Corticosteroid use</td>
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<td>3</td>
<td>0.999</td>
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<tr>
<td>Deviating ostomy present</td>
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<tr>
<td>ASA-score</td>
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<tr>
<td>3</td>
<td>5</td>
<td>11</td>
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All patients received mechanical oral bowel preparation and antibiotics prophylaxis.

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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 seems 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 homogenous 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 = 0.002), 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 processes, 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...
strongly positively associated with microbial diversity, especially the metabolically highly important keystone species \textit{F. prausnitzii} (7\%) and \textit{Ruminococcus bromii} (3\%).\textsuperscript{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.\textsuperscript{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.\textsuperscript{33} The C-seal may create a new ecosystem that benefits the growth of potential opportunistic pathogens as seen in our analysis, like \textit{P. oralis}, \textit{Fusobacteriaceae}, \textit{Leptotrichiaceae}, bacteria from the phylum \textit{Tenericutes}, and \textit{Enterococci} as seen by others,\textsuperscript{20} represent (if at all present) a very small minority, but could perhaps prosper and subsequently increase inflammation in this new situation.

Another ecological factor might be that shielding off the mucosa, and the subsequent lack of metabolism, makes the environment more aerobic. As the metabolism diminishes, oxygen diffusing from the blood into the lumen is utilized less rapidly,\textsuperscript{34} making life hard for commensal oxygen sensitive species while facilitating growth for opportunistic facultative pathogens, such as \textit{Enterococcus} species, which are excrete gelatinase GeIE causing degradation of the anastomotic tissue.\textsuperscript{29}

**Strengths and Limitations**

Most of the information that is available on the composition of the gut microbiota is derived from fecal samples. This reflects the composition present in the lumen of the distal colon and rectum, but is different from the composition in the mucosa.\textsuperscript{35} The bacterial DNA from the mucus layer was isolated in this study, giving a much better insight on the microbial composition around the anastomosis than a fecal sample could provide. Although all patients had oral mechanical bowel preparation and the intestine was checked on residual luminal content, we cannot guarantee that in some of the samples small traces of luminal content might have been present.

The method used to identify the bacterial DNA has its limitations because 16S rRNA sequencing can only detect relative abundance and not the absolute density of bacterial DNA present in the mucus. In addition, 16S analysis is only capable of identifying particular species based upon their 16S rRNA gene; genetic variation within species cannot be measured. Some species, like \textit{Escherichia coli}, have a huge genetic diversity, distinguishing pathogenic \textit{E. coli} from nonpathogenic \textit{E. coli} is impossible with 16S analysis. Furthermore, to confirm our hypotheses, our data should have included the mucosal microbiome of the patients after the surgery, and, ideally, after the development of AL. As this is very difficult, alternatively, fecal samples could be collected both before and after surgery, in addition to the "donut" sample taken during surgery.

Regarding the prevention of AL, we would recommend future research to be focused on altering the gut microbiota by diet before surgery into another stable yet healthy low-risk composition, favoring \textit{Ruminococcaceae}, \textit{P. copri}, and a high microbial diversity.

**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 mucin-degrading families \textit{Lachnospiraceae} and \textit{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.

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