CHAPTER

The non-selective effect of selective decontamination of the digestive tract on the intestinal microbiota

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ABSTRACT

Background
Selective decontamination of the digestive tract (SDD) is selectively aimed at removal of potential pathogenic microorganisms. Few studies have addressed the effect of SDD on overall gut microbiota composition. In this study, the effect of SDD on gut microbiota was evaluated, in a large homogenous group of elective colorectal cancer surgery patients.

Methods
Rectal swabs were taken from 118 patients undergoing colorectal surgery for colorectal carcinoma. These patients were randomly assigned to receive or not to receive perioperative SDD. Rectal swabs were taken prior to surgery, three days after commencing administration of SDD. Gut microbial profiles were obtained with the IS-pro technique. Differences in abundance for different taxonomical groups and Shannon diversity between the groups were assessed. Unsupervised and supervised classification techniques were used to assess the difference in microbial signatures between the two groups.

Findings
SDD samples showed different microbial signatures than control samples. *Escherichia coli*, *Sutterella* spp., *Faecalibacterium praunzitii* and *Streptococcus* spp. were the most discriminatory species. The SDD group showed clustering into two subgroups. In one subgroup a decrease in Proteobacteria was observed, whereas the other subgroup showed a shift in Proteobacteria, which was characterized by a decrease in *E. coli* and *Sutterella* spp. and an increase in *Desulfovibrio* spp. and *Hafnia alvei*.

Interpretation
SDD is nonselective and also impacts potentially beneficial species in the community. Additional studies are needed to evaluate if we can predict which patients benefit from SDD, in order to administer SDD in a select group of patients.

Funding
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INTRODUCTION

On many intensive care units (ICU’s) in the Netherlands, selective decontamination of the digestive tract (SDD) has been administered to patients for many decades. SDD decreases the rate of ventilator-associated pneumonia\(^1\) and ICU related mortality.\(^2\) ICU related infections are mainly caused by aerobic Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Pseudomonas aeruginosa* and *Enterobacter* spp., *Staphylococcus aureus* and yeasts.\(^3\) These potentially pathogenic microorganisms (PPM's) colonize the oropharynx and gastrointestinal tract of patients. To prevent or eliminate colonization and subsequent infection with PPM's, SDD is administered in the oropharynx and gastrointestinal (GI) tract, which consists of a mixture of nonabsorbable tobramycin, colistin and amphotericin B. SDD is supposed to selectively eliminate PPM's while leaving the anaerobic intestinal microbiota intact.\(^4\) There have however been few studies that addressed the effect of SDD on gut microbiota. Studies based on microbial culture show that administering SDD leads to a decrease in the number of patients that are colonized by Gram-negative bacteria in the gastrointestinal tract.\(^1\) However, the vast majority of our gut microbiota is uncultivable, and culture based studies may give biased results on the effect of SDD on gut microbiota.\(^5,6\) A limited amount of studies based on molecular techniques has been performed on the effect of SDD on gut microbiota. These molecular studies are, however, based on small study groups, and have mainly evaluated gut microbiota composition with techniques targeted at specific species, such as QPCR or FISH. Moreover, in both the culture and molecular based studies, patient groups were small and heterogeneous. ICU patients are patients with different comorbidities and confounding factors, which may influence the gut microbiota composition, such as feeding through a nasogastric tube.\(^7\) Importantly, a variety of other antibiotics are commonly administered to these patients besides SDD, which can severely confound results when studying the effect of SDD on the composition of gut microbiota. Therefore, here we evaluate the effect of SDD on gut microbiota, in a large homogenous group of elective colorectal cancer surgery patients, who were randomized to receive perioperative SDD or no perioperative SDD.
METHODS

Design:
This study was accessory to the SELECT trial: ‘Perioperative Selective Decontamination of the Digestive Tract (SDD) in Elective Colorectal Cancer Patients: a Multicenter Randomized Clinical Trial.’ The SELECT trial is registered at ClinicalTrials.gov, identifier: NCT01740947. This descriptive, prospective accessory study was initiated by the VU University Medical Center. Study subjects were randomly assigned to the intervention or control group. The intervention group (hereafter referred to as SDD group) received SDD four times daily, starting three days before surgery. SDD was orally administered and consisted of a 10 ml suspension containing 5 ml amphotericin B (500 mg) and 5 ml of a mixture of colistin (1000 mg) and tobramycin (80 mg). In addition, a single preoperative parenteral dose of 1000 mg of cefazolin and 500 mg of metronidazole was given. The control group (hereafter referred to as control group) routinely received a single preoperative parenteral dose of 1000 mg of cefazolin and 500 mg of metronidazole. A preoperative rectal swab was taken in the operating theatre from all subjects, before the administration of cefazolin and metronidazole.

Subjects and clinical data collection:
Colorectal carcinoma patients undergoing elective colorectal surgery (formation of an anastomosis) with curative intent, were recruited by the participating hospitals of the SELECT trial group. Inclusion and exclusion criteria have been extensively described in the study protocol. Written informed consent was obtained from all study subjects. Furthermore, the study was approved by the Medical Ethical Review Committee of the VU University Medical Center and the Central Committee on Research Involving Human Subjects.
Baseline characteristics including age, gender, smoking habits, alcohol intake, body mass index (BMI), surgical history and preoperative radio (chemo) therapy were obtained.

Rectal swabs:
Rectal swabs provide a good method to produce highly reproducible microbiota profiles. The rectal swabs (FLOQSwabs 552C, Copan, CA, USA) were taken preoperatively. Swab tips were transported in a sterile container which contained 500 µl Reduced Transport Fluid (RTF) buffer. Within half an hour of transportation they were stored at a temperature of –20 ºC, prior to sample handling.

IS-profiling of the intestinal microbiota:
Analysis on the intestinal microbiota was performed with the IS-profiling (IS-pro) technique, as described previously. With IS-pro, bacterial species are discriminated
based on the length of the 16S-23S rDNA interspace region. The IS-pro technique consists of 3 separate steps, described more in detail below.

**DNA isolation:**
DNA from the samples was isolated with the IVD-labeled, automated NucliSENS easyMag extraction system (EasyMag, Biomerieux). One ml of lysis buffer including guanidine thiocyanate was added to each Eppendorf tube containing a swab. Tubes were vortexed for 5 minutes at 1400 rpm (Thermomixer comfort, Eppendorf, Hamburg, Germany). Hereafter tubes were centrifuged for 2 minutes at 14000 rpm. Of the supernatant, 200 µl was added to the easyMag container, together with 2 ml of lysis buffer. After the mixture was incubated for at least 10 minutes at room temperature, DNA extraction was performed on the easyMag machine with the specific A protocol, as described by the manufacturer. The DNA was eluted in 110 µl buffer and stored at 4 ºC prior to polymerase chain reaction (PCR) amplification.

**IS-fragment amplification:**
IS-fragments were amplified in 2 separate PCR reactions, with phylum specific, fluorescent primers. In the first PCR reaction IS-fragments of bacteria belonging to the phyla Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria and Verrucomicrobia were amplified. In the second PCR reaction IS-fragments of bacteria belonging to the phylum Proteobacteria were amplified. Amplifications were performed on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Cycling conditions for PCR were 35 cycles of 94 ºC for 30 s, 56 ºC for 45 s and 72 ºC for 1 min; and a final extension at 72 ºC for 11 min.

**IS-fragment analysis:**
After the PCR reactions were completed, 5 µl of PCR product was mixed with 19.5 µl formamide and 0.5 µl Mapmaker 1500 ROXlabeled size marker (BioVentures, Murfreesboro, TN, USA). Subsequently, DNA fragment analysis was performed on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems). All data were preprocessed with the proprietary software suite (IS-Diagnostics, Amsterdam, the Netherlands) and further analyzed with the Spotfire software package (TIBCO, Palo Alto, CA, USA).

**Statistical analysis:**
After processing the data, each sample resulted in a unique interspace profile. Each profile consisted of a set of peaks. On the X-axis the length of the peak was depicted, measured in nucleotides. On the Y-axis the height of the peak was depicted, measured in relative fluorescence units (RFU), reflecting quantity of PCR product. Each peak in a
profile was considered as an operational taxonomic unit (OTU) and its corresponding intensity as its abundance. For correlations, all RFU intensities were log2 transformed.

**Phylum and species abundance:**
A boxplot was made for abundance at a phylum level for Proteobacteria, Bacteroidetes and Firmicutes/Actinobacteria/Fusobacteria/Verrucomicrobia (FAFV group). P-values for differences in abundance of the SDD group versus the control group were calculated for the phyla, by performing a two-sample t-test. A p-value of < 0·05 was considered statistically significant.

**UPGMA clustering analysis:**
A clustered heat map analysis was made by generating a correlation matrix of all log2 transformed profile data, followed by unsupervised clustering with the unweighted pair group method with arithmetic mean (UPGMA).

**Diversity analysis:**
To characterize species diversity in a sample, Shannon diversity index was calculated. Shannon’s diversity index accounts for both abundance and evenness of a species present in a sample by calculating the proportion of species relative to the total number of species. Diversity was calculated for the overall composition and per phylum, based on the resulting profiles using the R 2.15.2 software package. P-values for difference in Shannon’s diversity index of the SDD group versus the control group were calculated, by performing a two-sample t-test. A p-value of < 0·05 was considered statistically significant.

**Partial least squares discriminant analysis (PLS-DA):**
PLS-DA is a supervised pattern recognition technique used in the case where the number of independent variables (species in microbiota analysis) is larger than the number of data points (samples). It aims to identify patterns in complex, high dimensional data by rotating PCoA (Principal Components Analysis) components, such that a maximum separation among classes is obtained, and to understand which variables carry the class separating information. PLS-DA was performed to provide a quantitative estimate of the discriminatory power of each OTU. The discriminatory power of each OTU is expressed as a variable importance value, which we used to define the OTU’s that were the most discriminant between the SDD group and the control group. A list of the four most discriminant species was made.

**Role of the funding source**
The sponsor of this study had no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report.
RESULTS

One hundred and eighteen patients underwent a rectal swab for microbiota analysis. Fifty-six patients received SDD treatment and sixty-two patients were allocated to the control group. The patients’ baseline characteristics are shown in table 1. There was no statistically significant difference in age and BMI between the SDD and the control group (P= 0.1 and P= 0.2, respectively).

| Study and patient characteristics | SDD group (n=56)
<table>
<thead>
<tr>
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<tr>
<td>Age (years)</td>
<td>66.3 (64.0-68.5)</td>
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<tr>
<td>Sex (M)</td>
<td>34 (60.7%)</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>26.9 (25.2-28.7)</td>
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<tr>
<td>Active smoker</td>
<td>6 (10.7%)</td>
</tr>
</tbody>
</table>

Table 1 Study and patient characteristics

| Study and patient characteristics | Control group (n=62)
<table>
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<tr>
<td>Age (years)</td>
<td>68.8 (66.3-71.2)</td>
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<tr>
<td>Sex (M)</td>
<td>40 (64.5%)</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>25.6 (24.6-26.7)</td>
</tr>
<tr>
<td>Active smoker</td>
<td>4 (6.6%)</td>
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1 Data expressed as mean with 95% Confidence Interval (CI)
2 One patient did not provide BMI and data on smoking habit

Total abundance of the phylum Proteobacteria (P = 0.0002) and the FAFV group (P < 0.0001) was significantly decreased in the SDD group, compared to the control group (figure 1). Total abundance of the phylum Bacteroidetes was equal between the SDD group and the control group (P = 0.37).

Figure 1 Phylum abundance analysis
Boxplot of the phylum abundance analysis. Total Proteobacteria and FAFV group abundance was significantly reduced in the SDD group versus the control group (P = 0.0002, respectively P<0.0001).
Shannon diversity was significantly decreased in the SDD group, compared to the control group. This held true for all phyla combined (P= 0.0081), but was mainly defined by a decreased diversity of the phyla Proteobacteria (P= 0.047) and FAFV group (P< 0.0001). A heat map was generated from all IS-profiles, based on clustering by Proteobacteria. There was no clear clustering based on Bacteroidetes or the FAFV group. Based on Proteobacteria, there was a clear separation of the SDD group from the control group. However, the SDD group clustered into two separate groups. One group with a reduction in Proteobacteria abundance and diversity (hereafter referred to as SDD no Proteobacteria group) and one group without a clear reduction in Proteobacteria, but a shift in Proteobacteria composition (hereafter referred to as SDD shift Proteobacteria group) (figure 2). We performed a Random Forest supervised classification analysis on 21 samples of the SDD shift Proteobacteria group and 22 samples of the control group, in order to assess the most discriminant Proteobacteria between these groups to determine which species determine the shift. We found that in the SDD shift Proteobacteria group, the shift was defined by a decrease in *Escherichia coli* and *Sutterella* spp., and an increase in *Desulfovibrio* spp. and *Hafnia alvei*, compared to the control group.

![Figure 2 UPGMA clustering analysis](image-url)

Unsupervised clustering based on Proteobacteria, depicted as a heat map of all profiles colored by phylum. A clear subclustering into two subgroups for SDD is shown. One SDD subgroup shows reduction of Proteobacteria, compared to controls. And one SDD subgroup showed no clear reduction in Proteobacteria, but a shift in Proteobacteria composition.

Supervised classification with PLSDA showed a clear separation between the SDD group and the control group (figure 3). The clearest separation was found when all phyla were taken together (sensitivity, specificity: 92%, 91%), with an accuracy of 92%. Samples of the SDD group were associated with a decreased abundance of *E. coli*, *Sutterella* spp., *Faecalibacterium prausnitzii* and *Streptococcus* spp (figure 4), as defined by PLS-DA.
Figure 3 Partial least squares discriminant analysis for all phyla, Proteobacteria, Bacteroidetes, and the FAFV group
PLS-DA showed clear separation of SDD samples versus control samples, based on total microbiota analysis

Figure 4 Abundance bar chart of the 4 species, discriminating most between SDD and control samples
Samples of the SDD group were associated with a decreased abundance of E. coli, F. prausnitzii, Sutterella spp., and Streptococcus spp.
DISCUSSION

This study shows that SDD not only decreases colonization of the gastrointestinal tract with potential pathogenic Gram-negative microorganisms, but also reduces the abundance of normal colonizers of our gastrointestinal system, and leads to a shift in total microbiota composition. Remarkably, administering SDD does not invariably lead to the desired decrease in potential pathogenic Gram-negative bacteria in every patient. On the phylum level, we found that SDD led to a significant decrease in abundance and Shannon diversity of the phylum Proteobacteria. In addition, we found that administering SDD resulted in a decrease in abundance of *E. coli*, a well-known Gram-negative potential pathogen and also a representative of the phylum Proteobacteria. This is in line with previous studies, showing a decrease in abundance of Gram-negative bacteria including *E. coli*. However, also the abundance and Shannon diversity of the FAFV group was significantly decreased in the SDD group, compared to the control group. Within the FAFV group, *F. prausnitzii* abundance was most significantly diminished in the SDD group, compared to the control group. Previous studies have also shown that SDD affects the abundance of *F. prausnitzii*. Benus et al. have shown that *F. prausnitzii*, *Eubacterium rectale* and *Roseburia intestinalis* abundance is significantly decreased in SDD patients. These bacteria belong to the Clostridium cluster IV and XIVa group, which were reduced in studies performed by Buelow et al. Not only are specific taxonomic groups affected by SDD, but the microbiota composition as a whole is affected by SDD. Based on supervised microbiota analysis with PLS-DA, there was a clear separation between the SDD group and the control group, based on all phyla taken together. Buelow et al. also found that microbiota profiles from healthy patients were clearly distinct from microbiota profiles from patients receiving SDD during ICU stay. To our knowledge, no supervised classification technique has yet been used to obtain the discriminatory power of each OTU in defining the microbial difference between patients treated with SDD and controls. The most discriminatory species between the SDD group and the control group were *E. coli*, *Sutterella* spp., *F. prausnitzii* and *Streptococcus* spp. With unsupervised UPGMA clustering, there was a separation of control samples from SDD samples. Remarkably, the SDD samples were divided into two separate subgroups. One subgroup had an expected decrease in Proteobacteria, but the second subgroup showed a shift in composition of Proteobacteria. By means of the RF technique, we determined the species, belonging to the phylum of Proteobacteria, that discriminated most between the SDD group and the SDD subgroup with a shift in Proteobacteria composition. The SDD subgroup with a shift in Proteobacteria showed a decrease in *E. coli* and *Sutterella* spp. but an increase in *Desulfovibrio* spp. and *H. alveii*. This contrasting effect of SDD, dividing the SDD group into two subgroups, has never been shown in previous studies.
Our study has limitations. Patients in this study were colorectal cancer patients. Colorectal cancer is associated with a change in gut microbiota composition, which could have influenced the gut microbiota composition in our patient group. However, our results extend previous results and offer novel insights into the sheer effects of the (oral component of) SDD on gut microbiota composition. Our study is unique because it is randomized and controlled, in a large sized homogenous patient group. This gives a better and detailed insight in the effect of SDD, while omitting other concomitant factors, influencing gut microbiota composition. Moreover, as a first, we have used rectal swabs to analyze the effect of SDD on gut microbiota composition. It has been previously shown that microbiota composition, derived from rectal swab samples, is similar to that from fecal samples.9,13

SDD is designed to selectively target Gram-negative bacteria, yeasts and \textit{Staphylococcus aureus}. The decrease in abundance and Shannon diversity of Proteobacteria is in line with expectations. This also holds true for \textit{E. coli} and \textit{Sutterella} spp., two of the four species, discriminating SDD patients from control patients. Colistin and tobramycin, two of the three components of SDD are effective against potential pathogenic Gram-negative bacteria, belonging to the phylum Proteobacteria. What we can conclude is that decontamination of potential pathogenic Gram-negative bacteria by SDD was adequate.14 Remarkably, abundance and Shannon diversity of FAFV group was also affected by SDD. The only pathogen in this group that SDD is aimed to target is \textit{Staphylococcus aureus}. Although Bacteroidetes and Firmicutes constitute the vast majority of gut microbiota, staphylococci do not belong to the 30 most abundant genera.15 Hence, the significant decrease in the abundance of the FAFV group cannot be explained merely by a decrease in \textit{S. aureus}. \textit{Faecalibacterium} is one the most abundant genera in the gut.15 The decrease in \textit{Faecalibacterium prausnitzii} explains the decrease in the FAFV group for a significant part. \textit{Faecalibacterium prausnitzii} is difficult to culture and is extremely sensitive to oxygen.16 Very little is known about susceptibility to antibiotics of this species, and no clinical breakpoints for antibiotics have been set. Nevertheless, Benus et al. have performed a susceptibility test by an E-test for colistin and tobramycin on \textit{F. prausnitzii}. They have found that the MIC of tobramycin for \textit{F. prausnitzii} was 4 µg/ml. In SDD, per gift, 60 mg of tobramycin is administered, suspended in 10 ml. About eight liters of fluid enter the bowel each day, which consists of water from diet, saliva, gastric juice, bile, pancreatic juice and intestinal secretions. SDD is administered four times daily. With each administration, 60 mg of tobramycin is distributed in 2 liter of fluid, so the concentration of tobramycin will be around 30 µg/ml. The MIC of 4 µg/ml should easily be attained. Theoretically, the administration of tobramycin could affect \textit{F. prausnitzii} abundance. Contrastingly, we have found that SDD significantly decreases \textit{Streptococcus} spp. abundance, despite the fact that colistin and tobramycin
have no in vitro activity against *Streptococcus* spp. Previous studies have characterized the interspecies interaction between Gram-negative bacteria and streptococci. Gram-negative bacteria have been shown to promote streptococcal colonization in the airway of Cystic fibrosis patients, and to promote streptococcal biofilm formation.\textsuperscript{17,18} To conclude, in vitro antibiotic susceptibility of a bacterium may differ from the antibiotic susceptibility in vivo, because the micro-organism is part of a complex polymicrobial environment and interaction.\textsuperscript{19} These complex microbial interactions may partly explain why the whole microbial community is affected by SDD, as shown in our study. Remarkably, SDD did not lead to a decrease in Proteobacteria in every SDD patient. There was a clear clustering into two separate groups. One SDD subgroup showed a decrease in Proteobacteria. And the other did not show a clear decrease in Proteobacteria, but a shift in species, mainly attributed to a decrease in *E. coli* and *Sutterella* spp. and an increase of *Desulfovibrio* spp. and *H. alvei*. *H. alvei* is a Gram-negative rod with a naturally occurring resistance to colistin.\textsuperscript{20} *Desulfovibrio* spp. are strictly anaerobic Gram-negative rods that are resistant to colistin.\textsuperscript{21,22} To exert effect, aminoglycosides needs to be taken up by the bacterial cell. This process is an O2 demanding process.\textsuperscript{23} Because of this, anaerobic bacteria are not susceptible to tobramycin. The increase of *Desulfovibrio* spp. and *H. alvei* in the SDD Proteobacteria subgroup might be a reflection of selection of SDD resistant bacteria. *Desulfovibrio* spp. have been associated with a variety of chronic inflammatory diseases, such as periodontitis and inflammatory bowel disease\textsuperscript{24}, and they have been shown to induce apoptosis of gastrointestinal epithelial cells.\textsuperscript{25} Both *H. alvei* and *Desulfovibrio* are bacteria that can cause infections.\textsuperscript{26–31} Abis et al. have shown that administering perioperative SDD in colorectal cancer patients, undergoing surgery, decreased the number of postoperative infectious complications.\textsuperscript{14} Our findings show that not all patients benefit from the advantage of SDD, namely the eradication of potential pathogenic Gram-negative bacteria. The results also provide a first indication that it might be feasible to predict in which patients SDD will exert its desired effect. More research, in the form of fecal sampling and microbial profiling before administering SDD, is needed to address this question. *F. prausnitzii* is a prominent resident of the gut and well known for its beneficial properties.\textsuperscript{32–34} *F. prausnitzii* has anti-inflammatory properties and produces butyrate, which serves as an energy source for gut epithelial cells and thus is important in maintaining bowel health. Moreover a decrease in *F. prausnitzii* is associated with several disease states, such as IBD and obesity. In contrast, small bowel microbial composition is predominated by *Veillonella* spp. and streptococci such as *S. parasanguinis*.\textsuperscript{35} Van den Bogert et al. have shown that stimulation of dendritic cells with a specific *S. parasanguinis* strain results in higher DC maturation and subsequently higher activation marker
expression. In conclusion *Streptococcus* spp. have immunomodulatory properties and play an important role in shaping the immune system and immune homeostasis. Although administering SDD has been proven to reduce ICU related mortality, the effects of SDD on other health outcome measures have never been investigated. SDD leads to a nonselective change in gut microbiota composition. Additional longitudinal studies are needed to evaluate the effect of SDD on long-term health and to evaluate if we can predict which patients benefit from SDD.

Firstly, administering SDD decreases ICU related mortality, but is not as selective as meant to be. The term selective should better be adapted to decontamination of the digestive tract (DD), so that clinicians are aware of the broad antimicrobial effect against both potential pathogenic as well as beneficial bacteria, at which SDD is not targeted. Secondly, personalizing SDD administration, based on assessing intestinal microbiota composition and predicting what patient subgroups may benefit from administering SDD, holds great promise for the future and might prevent harmful and unnecessary administering of SDD in a subgroup of patients.

**Declaration of interests**

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**Contributors**

AEB, GSA, SJO and MVE designed the SELECT trial and invented the accessory study design. LP performed the IS pro technique on all 118 samples. MVD reviewed the literature, wrote the manuscript and designed the figures. MVD, GSA, AEB, SJO, MVE, LP and PHS revised the manuscript. All authors read and approved the final manuscript.

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