Predicting recurrence of Clostridium difficile infection by on-site profiling of faecal volatile organic compounds.

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ABSTRACT

Background
Stool emanates odors caused by specific profiles of volatile organic compounds (VOCs). These VOC profiles may be different for healthy and diseased individuals. We have previously shown that VOC profiling accurately diagnoses *C. difficile* infection (CDI). Whether VOC profiling at the time of diagnosis can also predict CDI recurrence risk after treatment is unknown.

Methods
We examined the clinical outcome of CDI patients whose fecal samples were analyzed by Field Asymmetric Ion Mobility Spectroscopy (FAIMS). We used the CDI-VOC score generated by FAIMS (scale 0-1) at the time of initial CDI diagnosis and compared this score between patients who developed a CDI recurrence during in-hospital follow-up and those who did not.

Results
17 of 46 patients were discharged while still on CDI therapy. The other 29 patients were included in the analysis; 16 of those developed a recurrence. The crude, unadjusted hazard ratio (HR) for developing recurrent CDI was 1.2 (95% CI: 1.0-1.5; p= 0.04) for every 0.10 increment in the CDI-VOC score. After adjustment for known risk factors for CDI recurrence (age, PCR-ribotype 027, the use of antibiotics or proton pump inhibitor after CDI treatment, and whether the index episode was a first episode or a first recurrence) this finding was maintained (HR 1.4; 95% CI 1.1-1.8; p<0.01). The HR for developing recurrent CDI was 4.1 (95% CI: 1.3-13.1; p<0.01) for patients with a CDI-VOC score above the optimal discriminating threshold value.

Conclusion
Fecal VOC profiling identifies patients with high CDI recurrence risk. This could help clinicians to select appropriate treatment.
INTRODUCTION

*Clostridium difficile* is a common and resilient cause of nosocomial diarrhoea. *C. difficile* infections (CDI) are a major concern in hospitals. The spore-forming ability of *C. difficile* enhances transmission; recurrence of CDI occurs frequently, leading to increased morbidity, length of stay and healthcare costs.\(^1\) The overall risk of at least one recurrence ranges from 12% to 64%.\(^3;4\) The most important risk factors for development of recurrent disease are older age, use of antibiotics after CDI diagnosis, use of proton pump inhibitors, and infection with ribotype 027 strain.\(^3;4\)

Patients with a high risk of recurrence might benefit from a different treatment strategy at an earlier stage, for instance with specific antibiotics (e.g. fidaxomicin), immunotherapy, or faecal microbiota transfer.\(^5\) However, the ‘clinical prediction rule’, a risk assessment based on risk factors (age >65 years, severe or fulminant underlying illness and additional antibiotic use after CDI therapy), has only limited accuracy for predicting recurrent disease; and since it requires an estimation on whether a CDI patient will need additional antibiotics in the near future, it has limited practical use.\(^6\) An additional diagnostic test, available at the time of CDI diagnosis, to predict the risk of recurrence could better enable clinicians to select the most appropriate treatment.

Recently, we established that the profile of volatile organic compounds (VOCs) emanating from unprocessed stool samples can differentiate between *C. difficile*-positive and -negative samples with high diagnostic accuracy.\(^7\) Faecal VOC composition is importantly influenced by the intestinal microbiota.\(^8\) In patients with recurrent CDI this microbiota appears less diverse and over time less variable than in patients with a first episode of CDI.\(^5;9-11\) In the initial diagnostic study we assessed the accuracy of faecal VOC profiling for diagnosing CDI;\(^7\) in this study we evaluated whether faecal VOC profiling can also be used to predict recurrence risk.

METHODS

**Samples, patients, and follow-up**

The original diagnostic study included 71 consecutive *C. difficile* positive samples collected from patients in two hospitals.\(^7\) A stool sample was considered positive if it had both a positive result in the direct toxin enzyme immunoassay (VIDAS EIA *Clostridium difficile* A and B; Marcy l’Etoile, France) and a positive anaerobic culture with *C. difficile*. Samples were stored at 4° C until VOC analysis, the majority of samples was analysed within two weeks after collection. Out of these 71 samples, 14 samples were discarded because they were repeat samples from the same patient. Another 11 samples were discarded because follow-up information was not available. We reviewed the medical records of the remaining 46 CDI patients, and determined whether they developed a CDI recurrence during in-hospital follow-up. The faecal samples included in this study represented either a first CDI episode or a first CDI recurrence (the ‘index
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episode’); no samples obtained during subsequent recurrences were included. Recurrent CDI was defined as a second episode of CDI during in-hospital follow-up after finishing treatment for the index CDI episode, provided the initial symptoms fully resolved. We collected follow-up data between end of CDI treatment and death or hospital discharge. Follow-up data included data on treatment of CDI, resolution or recurrence of CDI, use of antibiotics after CDI resolution, and use of PPI.

VOC analysis

Ion mobility spectrometry (Field Asymmetric Ion Mobility Spectrometry (FAIMS); Lonestar, Owlstone, Cambridge, UK) was used to analyse the chemical composition of gases and volatile organic compounds emanating from the stool samples. A more in-depth description of FAIMS technology, the sample handling, and the FAIMS data analyses was published previously.\(^7\) In summary, this technique separates volatile organic compound molecules based on their size, mass and mobility in different electric fields. Rather than identifying individual molecules in the gas, it creates a profile of the chemical components (“chemical fingerprint”).\(^{12}\) FAIMS analysis acquires a very large amount of data (i.e. over 50,000 measurements per analysed sample). A compression algorithm (wavelet transform plus subsequent univariate feature selection via Wilcoxon rank-sum test) was used to pre-process the data. After data reduction, a machine learning algorithm (random forest classifier) was applied, using cross validation to translate the data into a ‘CDI-VOC score’ between 0 and 1, indicating the probability of CDI based on the VOC profile. In the initial diagnostic study we assessed the accuracy of this CDI-VOC score for diagnosing CDI\(^7\); in this study we evaluated whether this CDI-VOC score can also be used to predict recurrence risk.

Statistical analysis

We compared the CDI-VOC score of patients who did develop CDI recurrence with the CDI score of patients who did not. To account for different follow-up lengths, results were analyzed by means of survival analysis. Association between CDI-VOC score and time to CDI recurrence were first tested in a univariable Cox-regression giving a crude hazard ratio (HR) estimate. An HR adjusted for known risk factors was calculated using multivariable Cox regression. Survival was calculated from end of initial CDI treatment. Patients without reported recurrence were censored at the date of discharge from the hospital. An optimal cut-off for CDI-VOC score for prediction of recurrence was determined using ROC analysis. Kaplan-Meier analysis is used to plot survival curves for subgroups with CDI-VOC score below and above this threshold. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM corp.

Ethics approval

The study was approved by the ethical review board of the VU University medical centre.
RESULTS

Seventeen of 46 patients were discharged while still on CDI therapy. Sixteen of 29 remaining patients (55%) developed a recurrence of CDI; the median time between end of initial CDI treatment and recurrence was 13 days (range 4-66 days). Patient characteristics, split by recurrence, are shown in table 1. In accordance with existing literature, those with recurrent disease were older and more often infected with the ribotype 027 strain. For those who developed recurrent disease, the index CDI episode was more often a first recurrence than a first episode. They more often used antibiotics (not for treatment of CDI) and they had a longer length of hospital stay after the index episode.

VOC analysis

In Cox regression analyses, the hazard ratio (HR) for developing recurrent CDI was 1.2 (95%CI: 1.0-1.5; p=0.04) for every 0.10 (or 10%) rise in CDI-VOC score. We subsequently accounted for other predictors of recurrence by multivariate Cox regression analysis (table 2). After adjusting for ribotype 027, which was the only other significant variable (p <0.10) in univariate analysis, the HR was 1.3 (95%CI: 1.0-1.5; p=0.02). After adjustment for age, ribotype 027, the use of antibiotics or proton pump inhibitors after the index CDI episode, and whether the index episode was a first episode or a first recurrence, a 0.10 (or 10%) increase in CDI-VOC score remained a significant predictor of recurrence, with a HR of 1.4 (95%CI 1.1-1.8; p<0.01).

Based on the receiver operating characteristics curve, we defined the optimal cut-off value between high and low CDI-VOC score at 0.93. Kaplan-Meier analysis

Table 1: patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Total (n=29)</th>
<th>With recurrence (n=16)</th>
<th>Without recurrence (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>21 (72%)</td>
<td>10 (63%)</td>
<td>11 (85%)</td>
</tr>
<tr>
<td>Median age in years (range)</td>
<td>67 (17-85)</td>
<td>70 (17-85)</td>
<td>64 (23-78)</td>
</tr>
<tr>
<td>Index episode = 1st episode</td>
<td>22 (76%)</td>
<td>11 (69%)</td>
<td>11 (85%)</td>
</tr>
<tr>
<td>Index episode = 1st recurrence</td>
<td>7 (24%)</td>
<td>5 (31%)</td>
<td>2 (15%)</td>
</tr>
<tr>
<td>Ribotype 027</td>
<td>13 (45%)</td>
<td>8 (50%)</td>
<td>5 (38%)</td>
</tr>
<tr>
<td>CDI treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Metronidazole</td>
<td>13 (45%)</td>
<td>8 (50%)</td>
<td>5 (38%)</td>
</tr>
<tr>
<td>- Metronidazole + vancomycin</td>
<td>16 (55%)</td>
<td>8 (50%)</td>
<td>8 (62%)</td>
</tr>
<tr>
<td>Use of (non CDI) antibiotics during FU</td>
<td>12 (41%)</td>
<td>9 (56%)</td>
<td>3 (23%)</td>
</tr>
<tr>
<td>Use of PPI during FU</td>
<td>14 (48%)</td>
<td>6 (38%)</td>
<td>8 (62%)</td>
</tr>
<tr>
<td>Death during FU</td>
<td>6 (21%)</td>
<td>3 (19%)</td>
<td>3 (23%)</td>
</tr>
<tr>
<td>Median days till death or discharge (range)</td>
<td>25 (3-241)</td>
<td>33 (11-98)</td>
<td>13 (3-241)</td>
</tr>
<tr>
<td>Median days till recurrence (range)</td>
<td>NA</td>
<td>13 (4-66)</td>
<td>NA</td>
</tr>
</tbody>
</table>

CDI: C. difficile infection. FU: in-hospital follow-up from end of CDI treatment until discharge or death. PPI: proton pump inhibitor. NA: not applicable.
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(figure 1) compared time to recurrent CDI between patients with a CDI-VOC score above and below this value. The proportion of patients who developed recurrent CDI was significantly higher amongst those with a CDI-VOC score >0.93 (p<0.01 by log-rank test).

In Cox regression analyses, the HR for developing recurrent CDI was 4.1 (95%CI: 1.3-13.1) for patients with a CDI-VOC score >0.93 compared to those with a CDI-VOC score ≤0.93. Adjustment for age, ribotype 027, the use of antibiotics or proton pump inhibitors after the index CDI episode, and whether the index episode was a first episode or a first recurrence, the HR associated with a high CDI-VOC score increased to 20.8 (95% CI 3.4-125).

DISCUSSION

The results of our study suggest that the faecal VOC profile from CDI patients is a significant and promising predictor of recurrent CDI. Previous studies indicate that the composition of the intestinal microbiome is an important determinant of recurrent CDI disease. Given that the faecal VOC profile largely reflects microbiome composition, this lends biological plausibility to our results.

The recurrence rate in our study population was remarkably high (55%). A probable explanation is that 17 of 46 patients (37%) were discharged while still on CDI therapy. It is likely that patients who were discharged relatively early after CDI diagnosis were in a better condition, and those remaining in hospital had
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more comorbidity or were more severely ill, and were therefore more at risk for recurrent disease. Furthermore, the prevalence of *C. difficile* ribotype 027 was relatively high, and a significant proportion of patients had a first CDI recurrence as index episode.

The device we used for VOC analysis is relatively low-cost, mobile and compact; the analysis is quick and does not require expensive equipment or extensively trained personnel. Faecal VOC profiling could therefore be readily available in the clinic. Better identification of patients at high risk of recurrence could improve treatment strategy for these CDI patients, for instance by considering faecal microbiota transfer at an early stage.

This study has limitations. Although the results were statistically significant, they reflect a limited number of patients with variable, relatively short follow-up. The uncertainty around the estimated hazard ratios is considerable (due to the small event rate observed in this study), which is especially the case in the multivariate analysis (where guidelines recommend a number of 10 events per variable in the model). This study clearly calls for reproduction with a larger number of patients and the cut-off found here needs to be validated in an external cohort.

These limitations aside, in conclusion this study suggests that fecal VOC profiling is a promising independent predictor of CDI recurrence.

### Table 2: Hazard ratios (95% Confidence Intervals) for developing recurrent *C. difficile* infection as computed by Cox regression analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Crude HR</th>
<th>p</th>
<th>mutually adjusted HR*</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (per decade)</td>
<td>1.1 (0.8-1.5)</td>
<td>0.60</td>
<td>1.1 (0.8-1.5)</td>
<td>0.39</td>
</tr>
<tr>
<td>Use of antibiotics during FU</td>
<td>1.3 (0.5-3.9)</td>
<td>0.59</td>
<td>2.7 (0.6-11.0)</td>
<td>0.18</td>
</tr>
<tr>
<td>Use of PPI during FU</td>
<td>0.5 (0.2-1.5)</td>
<td>0.25</td>
<td>0.9 (0.3-3.3)</td>
<td>0.90</td>
</tr>
<tr>
<td>Index episode was first recurrence</td>
<td>2.2 (0.7-7.5)</td>
<td>0.20</td>
<td>2.7 (0.5-15.8)</td>
<td>0.27</td>
</tr>
<tr>
<td>Ribotype 027</td>
<td>2.8 (1.0-8.1)</td>
<td>0.06</td>
<td>2.9 (0.6-13.2)</td>
<td>0.18</td>
</tr>
<tr>
<td>0.1 (10%) increase in CDI-VOC score</td>
<td>1.2 (1.0-1.5)</td>
<td>0.04</td>
<td>1.4 (1.1-1.8)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

HR: Hazard ratio. FU: in-hospital follow-up after end of CDI treatment. PPI: proton pump inhibitor. CDI: *C. difficile* infection. CDI-VOC score: CDI volatile organic compound (VOC) score as generated by faecal VOC profiling. *Multivariate model adjusted for age, use of antibiotics after completing CDI therapy, use of PPI after completing CDI therapy, index episode being first recurrence, ribotype, and 0.10 (or 10%) increase in CDI-VOC score.
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REFERENCE LIST

(9) Seekatz AM, Rao AM, Santhosh K, Young VB. Dynamics of the fecal microbiome in patients with recurrent and nonrecurrent *Clostridium difficile* infection. Genome Med 2016;8:47.