Incidence and predictive biomarkers of _Clostridioides difficile_ infection in hospitalized patients receiving broad-spectrum antibiotics

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Trial enrichment using gut microbiota derived biomarkers by high-risk individuals can improve the feasibility of randomized controlled trials for prevention of _Clostridioides difficile_ infection (CDI). Here, we report in a prospective observational cohort study the incidence of CDI and assess potential clinical characteristics and biomarkers to predict CDI in 1,007 patients ≥ 50 years receiving newly initiated antibiotic treatment with penicillins plus a beta-lactamase inhibitor, 3rd/4th generation cephalosporins, carbapenems, fluoroquinolones or clindamycin from 34 European hospitals. The estimated 90-day cumulative incidences of a first CDI episode is 1.9% (95% CI 1.1-3.0). Carbapenem treatment (Hazard Ratio (95% CI): 5.3 (1.7-16.6)), toxigenic _C. difficile_ rectal carriage (10.3 (3.2-33.1)), high intestinal abundance of _Enterococcus_ spp. relative to _Ruminococcus_ spp. (5.4 (2.1-18.7)), and low Shannon alpha diversity index as determined by 16S rRNA gene profiling (9.7 (3.2-29.7)), but not nor- malized urinary 3-indoxyl sulfate levels, predicts an increased CDI risk.
Clostridioides difficile infections (CDI) are considered the leading cause of infectious healthcare-related diarrhea worldwide and substantial costs have been attributed to its management1-3. Different medical interventions, including vaccines, probiotics, and agents neutralizing antibiotic residuals in the colon, are being developed to prevent CDI4-7. These interventions, however, require data from pivotal trials demonstrating clinical efficacy prior to approval by regulatory authorities. Given the relatively low incidence of CDI per individual patient in target populations, trials require large numbers of patients to demonstrate efficacy of a preventive measure. For example, a trial assessing the efficacy of probiotics for the prevention of CDI included 2981 patients, assuming a 12-week incidence of 4%. As the incidence of CDI in the placebo group was only 1.2%, the trial failed to demonstrate efficacy, and would have required a much larger sample size to reach adequate power for a 50% reduction of CDI.8 The assumed incidence of the primary outcome is critical for successful execution of a trial and should be assessed in depth. Furthermore, the sample size can be reduced by enrichment of the RCT population with patients at a particularly high risk of CDI. Preclinical attempts to specify such a population identified advanced age, comorbidities, specific high-risk antibiotics (clindamycin, cefepime, fosfomycin, moxalactam, and trimethoprim/sulfonamides), and previous colonization with C. difficile as potentially important predictive factors.9-11 More recent findings suggest that the composition of the gut microbiota and their associated metabolites play an important role in the pathogenesis of CDI12,13. Therefore, further assessment of these potential biomarkers is warranted.

Here, we demonstrate that the 90-day incidence of CDI and antibiotic-associated diarrhea (AAD) in patients ≥50 years of age treated with predefined broad-spectrum antibiotic classes is 1.9% (95% CI: 1.1–3.0) and 14.1% (95% CI: 12.0–16.4), and that carbapenem treatment, toxigenic C. difficile carriage, and the composition and diversity of the gut microbiota predict CDI but not AAD.

Results
A total of 1007 evaluable participants were enrolled (Fig. 1). Median age was 70 years (IQR: 62–79) and 592 participants (58.8%) were male (Table 1). Of 172 (17.1%) subjects who did not complete follow-up, 86 died after a median of 20 (IQR: 8–40) days and 86 withdrew their consent or were lost-to-follow-up after a median of 5 (IQR: 3–11) days.

Antibiotic treatment at day 1 and overall is provided in Table 2. The first dose of one of the five antibiotic classes was given after a median of 1 (IQR: 0–2) hospitalization day. Most participants (93%) received only one of the five antibiotic classes on day 1, most frequently a penicillin with a beta-lactamase inhibitor, followed by a third or fourth generation cephalosporin (Table 2). Apart from these five classes, 19% of patients received other additional antibiotics on day 1.

In total, 135 participants reported 176 AAD episodes, of which 114 episodes in 100 participants occurred within 28 days. The cumulative incidence of AAD within 28 and 90 days was 10.5% (95% CI: 8.6–12.5) and 14.6% (95% CI: 12.4–17.0), respectively. In 48 AAD episodes, no fecal sample was obtained for diagnosis of CDI, mainly because episodes were reported too late. Ten other subjects had fecal samples collected that were not correctly tested. The remaining 118 were tested according to the ESCMID guidelines, of which 5 were tested in the hospital laboratory only. Overall, 17 CDI episodes were detected in 15 subjects. Of these, nine first episodes occurred within 28 days. The number of subjects and CDI episodes per country and site are provided in Supplementary Table 2. The median time to the first CDI episode was 18 days (IQR: 4–38; range: 1–78). The imputation model deviated from the planned analysis due to unforeseen imputation model overfit and indications for missing not at random (see Supplementary methods). After correction, the imputation model estimated that, among the non-tested AAD episodes, three CDI episodes were missed, of which two within 28 days. The estimated cumulative incidence of CDI, therefore, was 1.1% (95% CI: 0.6–2.1) within 28 days and 1.9% (95% CI: 1.1–3.0) within 90 days (Table 3).

Participants receiving carbapenems at day 1 had a higher incidence of CDI over the 90-days period than other patients (Table 3). Baseline toxigenic C. difficile carriage results were available in 983 (97.6%) participants, of which 35 were positive. The incidence of CDI was more than tenfold higher in participants with a positive baseline toxigenic C. difficile polymerase chain reaction (PCR) for the follow-up period of 28 and 90 days (Table 3). Time to first CDI was considerably shorter for participants with baseline toxigenic C. difficile carriage: 2 days (IQR: 1–3) vs. 30 days (IQR: 16–44) for those developing CDI within without baseline toxigenic C. difficile carriage (see Supplementary Table 4).

Baseline N3-IS levels were available for 938 (93.1%) participants. The distribution of N3-IS levels at baseline was comparable for patients developing or not developing AAD or CDI (Supplementary Table 3). None of the participants had a low baseline N3-IS level. No CDI episodes were identified in 28 participants with intermediate N3-3 levels. The AUC of baseline N3-IS for CDI within 90 days was 0.441 (95% CI: 0.323–0.559). No discriminative cut-off value for baseline N3-3 level for prediction of CDI was identified.

Day-6 N3-3 levels were available in 806 subjects of which 3 had CDI and 13 had AAD without known CDI status prior to the day-6 visit. As 6 subjects withdrew from the study at the day-6 visit, there were 784 subjects for this analysis. Day-6 N3-3 levels were lower in patients developing CDI compared to those not developing CDI (Supplementary Table 3). In 199 (25.4%) subjects with intermediate day-6 N3-3 levels, the cumulative incidence of CDI at day 90 was 4.1% (95% CI: 1.9–7.6), compared to 0.7% (95% CI: 0.2–2.0) in subjects with high day-6 N3-3 levels (SDHR: 5.8 (95% CI: 1.6–21.4)).

Baseline 16S rRNA gene profiling analysis results were available in 945 (93.8%) participants. A detailed description of the metagenomic analyses, including determination of alpha and beta diversity indices and LEfSe results, is reported in Berkell et al.14. For baseline alpha diversity, participants with a CDI episode had a median Shannon index of 2.19 (IQR: 1.87–3.37) and Inverse Simpson index of 5.53 (IQR: 3.12–17.33), while participants without a reported CDI episode had a median Shannon index of 3.38 (IQR: 2.91–3.76) and Inverse Simpson index of 13.87 (IQR: 8.74–21.26). Both indices were strongly predictive for CDI (Table 3) and moderately for AAD (Table 4).

The LEfSe analysis revealed two OTUs associated with CDI and 17 with no CDI (Supplementary Table 3 in Berkell et al.14), of which two and eight, respectively, had mean relative abundance of at least 0.5% and were selected for modeling. Based on model AICs and confirmation in the validation dataset, the best OTU-ratio model consisted of the relative abundance of Enterococcus (OTU1) divided by the relative abundance of Ruminococcus (OTU31), as in Table 3 (see Supplementary Table 5 for alternative models). Overall, 171 subjects (16.9%) were above the optimal breakpoint and had a predicted 90-day risk of CDI of 5.7%. For the OTU-abundance model, Enterococcus (OTU1) and Alistipes (OTU68) was selected as the best model (Table 3, Supplementary Table 6). 181 subjects (18.0%) had relative abundance of Enterococcus (OTU1) ≥ 0.087% and Alistipes (OTU68) < 0.087% and had a predicted 90-day risk of 5.8%. The SDHR of the selected OTU models was similar to.
the RR observed in the validation dataset: RR 4.6 (95% CI: 1.4–28.8) for the ratio of OTU1/OTU31 and RR 6.2 (95% CI: 2.0–34.8) for the relative abundance of OTU1 ≥ 0.087% and OTU68 < 0.087% (Supplementary Tables 5 and 6). Most but not all of the alternative OTU models were confirmed in the validation. For CDI within 28 days, predictive performance of 16S rRNA gene sequencing data could not be estimated due to the low number of CDI episodes within 28 days.

Different sensitivity analyses confirmed that rectal carriage of toxigenic C. difficile and the selected OTU models were predictive for CDI (Supplementary Tables 7–12). However, in the subset of countries with high testing rates, relative abundance of Porphyromonas (OTU69) turned out to be more predictive as compared to OTUs selected in the main analysis. Carbapenem use at baseline was confirmed in some sensitivity analyses, but lost statistical significance in the subset of countries with high testing rates. Further exploration of the data revealed that CDI testing was correctly performed in 81% of AAD episodes in patients with baseline carbapenem use, compared to 65% of other AAD episodes. Due to the low number of CDI episodes observed, the original plan to perform multivariable analysis for CDI prediction was abandoned.

The incidence of AAD was increased threefold in subjects receiving carbapenems at baseline compared to patients not

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**Fig. 1 Inclusion flowchart.** *Based on 28 hospitals that provided screening data. Hospitals were requested to complete on the screening log for all screened patients up to March 23, 2017, and all eligible patients for the entire study period. Of the enrolled patients, 897 (89.1%) were enrolled in hospitals that provided screening data. **Subjects who signed informed consent but met one of the exclusion criteria at baseline. This includes 33 subjects from one side that applied an early consent procedure for subjects at high risk of receiving antibiotics in the near future. Abbreviations: AB antibiotics, CDI C. difficile infection, ICU intensive care unit, MD medical doctor.
Table 1 Baseline characteristics.

| Baseline variable                          | Total population (n = 1007) |
|--------------------------------------------|-----------------------------|
| Age (median [IQR])                         | 70.0 [61.5, 79.0]           |
| Male gender (%)                            | 592 (58.8)                  |
| Charlson comorbidity index (median [IQR])  | 5.1 [3.8, 6.8]              |
| Karnofski score (median [IQR])             | 80 [70, 90]                 |
| Cardiovascular disease (%)                 | 321 (31.9)                  |
| Hematological/ oncological disease (%)     | 340 (33.8)                  |
| Diabetes mellitus (%)                      | 281 (27.9)                  |
| COPD (%)                                   | 160 (15.9)                  |
| Gastrointestinal disease (%)               | 156 (15.5)                  |
| Moderate to severe chronic kidney disease (%) | 132 (13.1)        |
| Connective tissue disease (%)              | 54 (5.4)                    |
| AIDS (%)                                   | 11 (1.1)                    |
| Has history of CDI (%)                     | 14 (1.5)                    |
| Years since last CDI episode (median [IQR])| 0.8 [0.3, 1.1]              |
| Used systemic antibiotics in past 3 months (%) | 405 (42.0)                |
| Used immunosuppressants in past 3 months (%) | 232 (23.2)                |
| Used proton pump inhibitors in past 3 months (%) | 497 (50.9)                |
| Hospitalized in past 12 months (%)        |                             |
| Not hospitalized                           | 463 (46.8)                  |
| Once                                       | 261 (26.4)                  |
| Twice                                      | 102 (10.3)                  |
| Three or more times                        | 163 (16.5)                  |
| Received outpatient care in past month (%) | 556 (55.5)                 |
| Received tube feeding during current admission prior to inclusion (%) | 8 (0.8)                    |
| ICU admission in past 3 months (%)         | 33 (3.3)                    |

Indication for antibiotic treatment

| Indication for antibiotic treatment | Total population (n = 1007) |
|------------------------------------|-----------------------------|
| Respiratory tract infection        | 340 (33.8%)                 |
| Urinary tract infection            | 171 (17.0%)                 |
| Abdominal infection                | 157 (15.6%)                 |
| Pharyngeal treatment               | 90 (8.9%)                   |
| Febrile neutropenia                | 85 (8.4%)                   |
| Skin and soft tissue infection     | 66 (6.6%)                   |
| Other indications                  | 122 (12.1%)                 |

*Totals add up to >100% because some patients had more than one indication. Other indications include: bacteruria (1), Bartonellosis (1), blood stream infection (15), cervical lymph node tuberculosis (1), central nervous system infection (7), endocarditis (13), infection with unknown focus (33), Lyme’s disease (2), ocular infection (4), orchitis (1), osteoarticular infection (32), pericarditis (3), perineal infection (3), surgical site infection (5), Trauma (1), Vasculitis (2).

Abbreviations: AIDS acquired immunodeficiency syndrome, CDI Clostridiods difficile infection, COPD chronic obstructive pulmonary disease, ICU intensive care unit, IQR interquartile range.

as compared to the OTU ratio model (Table 4, Supplementary Table 13). The complete case analysis confirmed the association of carbapenems and OTU models with AAD (Supplementary Tables 14 and 15).

Discussion

In this study of adults aged ≥50 years receiving broad-spectrum systemic antibiotic treatment during hospitalization, we observed a cumulative CDI incidence of 1.1% (95% CI: 0.6–2.1) within 28 days and 1.9% (95% CI: 1.1–3.0) within 90 days of antibiotic treatment initiation. Carbapenem prescription, rectal carriage of toxigenic C. difficile at baseline, a low alpha diversity of the intestinal microbiome, as well as a high relative abundance of Enterococcus (OTU1) and low relative abundance of Ruminooccus (OTU31) or Alistipes (OTU68) predicted an increased risk of CDI. Urinary N3-IS level at baseline was not associated with occurrence of CDI. AAD could not be predicted reliably with any of the studied parameters. The involvement of different infectious and non-infectious causes of AAD may hamper accurate biomarker-based prediction of AAD.

Although all evaluated antibiotic classes have been associated with CDI, in our study, only patients treated with carbapenems had a threefold increased risk of AAD and an almost fivefold increased risk of CDI within 90 days as compared to patients not receiving carbapenems at baseline. The higher incidence in patients receiving carbapenems may be due to the broader antimicrobial activity spectrum resulting in more disruption of the intestinal microbiota, or to differences in baseline risk factors in patients receiving carbapenems compared to those receiving other antibiotics. Independent of the mechanism, enrichment of a trial population with patients receiving carbapenems is likely to increase the incidence of CDI and hence make a trial more efficient. The expected efficiency gain will be driven by the proportion of patients receiving carbapenems, which was relatively low in our study, but varied substantially among hospitals. Few patients received clindamycin and CDI was observed in none, prohibiting incidence estimation for this antibiotic. Less than 2% of our population had a history of CDI, a risk factor for which we could not determine the incidence of CDI, due to the absence of outcomes in this small subgroup, even though CDI history has previously been found to predict a high CDI risk

We studied three distinct biomarker types. Previous studies have found associations between urinary N3-IS levels and the microbiota composition. In particular, the relative abundance of bacteria from the class Clostridia was positively associated with N3-IS, whereas Bacilli were negatively associated with N3-IS. As Clostridia have been associated with a decreased and Bacilli with an increased CDI risk, we hypothesized that patients with a low N3-IS level would be at an increased risk of CDI afterwards, confirming the direction of the hypothesized association. Altogether, we conclude that N3-IS is very unlikely to be a relevant baseline predictor of CDI risk in this patient population, but when measured after several days of antibiotic treatment it may predict the subsequent risk of CDI.

Toxigenic C. difficile carriage at baseline was detected in 3.6% of participants. In previous studies, the prevalence of toxigenic C. difficile carriage was very heterogeneous with a pooled prevalence of 8.1% (95% CI: 5.7–11.1%) According to the observed carriage prevalence may be due to a potentially lower sensitivity of rectal swabs compared to stool samples for detection of toxigenic C. difficile carriage. PCR testing based on swabs dipped in stool samples of CDI patients has yielded high detection rates, but this may be different for detection of colonization. On the other
Table 2 Antibiotic treatment at baseline and during follow-up.

| Variable                        | N (%)  | D1     | N (%) any time | DOT |
|---------------------------------|--------|--------|----------------|-----|
| One antibiotic class            | 939 (93.2%) | 607 (60.3%) |                |     |
| Two antibiotic classes          | 66 (6.6%)      | 319 (31.7%) |                |     |
| Three+ antibiotic classes       | 2 (0.2%)      | 81 (8.0%)  |                |     |
| One antibiotic class            | 758 (75.3%) | 412 (40.9%) |                |     |
| Two antibiotic classes          | 236 (23.4%) | 363 (36.0%) |                |     |
| Three+ antibiotic classes       | 13 (1.3%)      | 232 (23.0%) |                |     |
| Penicillin with beta-lactamase inhibitor | 409 (40.6%) | 530 (52.6%) | 8 (5-12) |     |
| Third or fourth generation cephalosporin | 394 (39.1%) | 457 (45.4%) | 7 (4-9) |     |
| Fluoroquinolone                 | 181 (18.0%) | 326 (32.4%) |                | 9   |
| Carbapenem                      | 64 (6.4%)      | 143 (14.2%) |                | 8   |
| Clindamycin                     | 29 (2.9%)      | 45 (4.5%)  |                | 8   |
| Other antibiotic classes        | 193 (19.2%) | 407 (40.4%) |                | 9   |
| Any antibiotic                  | 1007 (100.0%) | 1007 (100.0%) |            | 11  |

*Out of the five antibiotic classes given, not including other classes.

Table 3 Cumulative incidence of CDI within 28 and 90 days.

| Subgroup          | N     | Events | Incidence | SDHR |
|-------------------|-------|--------|-----------|------|
| CDI within 28 days|       |        |           |      |
| Total population  | 1007  | 11.0   | 1.1% (0.6-2.1) |     |
| Penicillin + BLI  | 409   | 4.4    | 1.1% (0.4-2.7)  | 1.0 (0.3-3.6) |
| Third/fourth gen cephalosporin | 394 | 3.5 | 0.9% (0.2-2.5) | 0.7 (0.2-2.8) |
| Fluoroquinolone   | 181   | 1.6    | 0.9% (0.1-4.1)  | 0.7 (0.1-5.1)  |
| Carbapenem        | 64    | 2.5    | 4.0% (0.8-11.8) | 4.2 (0.9-19.3) |
| Clindamycin       | 29    | 0.3    | -          | -       |
| History of CDI    | 31    | 0.5    | -          | -       |
| No history of CDI | 976   | 10.6   | 1.1% (0.6-2.1) |     |
| Toxicogenic C. difficile carriage (GeneXpert, Cepheid) | 45 | 4.6 | 10.6% (3.2-23.2) | 16.2 (4.3-60.8) |
| No toxicogenic C. difficile carriage | 962 | 6.5 | 0.7% (0.3-1.6) | - |
| Intermediate N3-IS level (≤–8 ≤ log2(N3-IS) ≤ 2.5) | 38 | 0.2 | - | - |
| High N3-IS level (log2(N3-IS) > 2.5) | 969 | 10.8 | 1.2% (0.6-2.1) | - |
| Shannon index ≤ 2.586 | 164 | 4.6 | 4.6% (1.9-9.1) | 10.2 (2.3-44.4) |
| Shannon index > 2.586 | 843 | 3.8 | 0.5% (0.1-1.4) | - |
| Inverse Simpson index ≤ 7.674 | 220 | 7.2 | 3.5% (1.5-6.9) | 7.4 (1.6-33.0) |
| Inverse Simpson index > 7.674 | 787 | 3.8 | 0.5% (0.1-1.5) | - |
| CDI within 90 days |       |        |           |      |
| Total population  | 1007  | 18.0   | 1.9% (1.1-3.0) |     |
| Penicillin + BLI  | 409   | 5.4    | 1.4% (0.5-3.1)  | 0.6 (0.2-1.8) |
| Third/fourth gen cephalosporin | 394 | 7.1 | 1.9% (0.8-3.9) | 1.0 (0.4-2.8) |
| Fluoroquinolone   | 181   | 2.7    | 1.6% (0.3-4.9)  | 0.8 (0.2-3.3)  |
| Carbapenem        | 64    | 5.1    | 8.3% (2.7-17.7) | 5.7 (1.9-17.4) |
| Clindamycin       | 29    | 0.3    | -          | -       |
| History of CDI    | 31    | 0.8    | -          | -       |
| No history of CDI | 976   | 17.1   | 1.9% (1.1-3.0) |     |
| Toxicogenic C. difficile carriage (GeneXpert, Cepheid) | 45 | 4.9 | 11.4% (3.4-24.8) | 8.6 (2.7-27.5) |
| No toxicogenic C. difficile carriage | 962 | 13.1 | 1.5% (0.8-2.5) | - |
| Intermediate N3-IS level (≤–8 ≤ log2(N3-IS) ≤ 2.5) | 38 | 0.3 | - | - |
| High N3-IS level (log2(N3-IS) > 2.5) | 969 | 17.7 | 1.9% (1.1-3.1) | - |
| Shannon index ≤ 2.586 | 164 | 11.6 | 7.5% (3.7-13.0) | 9.7 (3.2-29.7) |
| Shannon index > 2.586 | 843 | 6.4 | 0.8% (0.3-1.8) | - |
| Inverse Simpson index ≤ 7.674 | 220 | 11.6 | 5.7% (2.9-9.8) | 6.9 (2.3-21.3) |
| Inverse Simpson index > 7.674 | 787 | 6.4 | 0.9% (0.3-2.0) | - |
| Ratio OTU1/OTU3 ≥ 8.5a | 171 | 11.5 | 5.7% (1.8-13.5) | 5.4 (2.1-18.7) |
| Ratio OTU1/OTU3 < 8.5 | 836 | 6.7 | 1.1% (0.4-2.3) | - |
| OTU1 ≥ 0.087% or OTU68 < 0.087%b | 181 | 11.3 | 5.8% (2.3-19.8) | 5.4 (2.0-19.5) |

*The bias-adjusted sensitivity, specificity and C-statistic were 51.2%, 83.7%, and 0.675, respectively.
*bThe bias-adjusted sensitivity, specificity and C-statistic were 54.4%, 82.8% and 0.686, respectively.

Based on the competing events model using multiple imputed data. The number of events is averaged over the imputation datasets and can therefore have decimals. Cumulative incidence was calculated using competing events analysis. Abbreviations: BLI beta-lactamase inhibitor, CDI Clostridioides difficile infection, CI confidence interval, N3-IS normalized 3-indoxyl sulfate level in urine, OTU1 Enterococcus, OTU31 Ruminococcus, OTU68 Alistipes, SDHR subdistribution hazard ratio. Bold text denotes statistical significance. aBias-adjusted incidences and SDHRs are provided.

Abbreviations: DI day one of antibiotic treatment, DOT days on treatment given as Median (interquartile range); combination treatment is counted as one DOT.
hand, the relative risk of toxigenic C. difficile carriage for CDI is high compared to previous studies in which relative risks between 2.5 and 10 have been observed. The very short time to CDI occurrence in participants colonized with C. difficile was surprising. A recent study among hospitalized medical patients also using PCR testing on rectal swabs for detection of toxigenic C. difficile found a similar carriage rate of 3.4% and a relative risk for CDI among carriers of 16.6 (95% CI: 4.0–69.1), but no difference in time to occurrence of CDI (median [range] 23 [4–30] days for carriers vs. 11 [4–30] days for noncarriers). The low prevalence of C. difficile carriage would make this biomarker very inefficient for enrichment of prevention trials, as about 30 patients would need to be screened to enroll one high risk patient. Moreover, rapid point-of-care testing must be available with affordable cost.

Concerning the 16S rRNA analysis, Enterococcus (OTU1), Ruminococcus (OTU31), or Alstistes (OTU68) were most predictive of the risk of CDI. Participants with more or equal than 8.5 times higher relative abundance of Enterococcus compared to Ruminococcus had a fivefold higher risk to develop CDI. Patients with high relative abundance of Enterococcus and low relative abundance of Alstistes also had an increased risk of approximately 5 times. Several alternative models with similar performance were identified. There is large uncertainty around these estimates, with high risk of false positive findings, due to the low number of CDI episodes and substantial expected bias that needed to be corrected for. Therefore, we validated our findings in an independent dataset and selected from the best performing models those that were confirmed in the validation dataset.

High abundance of Enterococccae spp. has previously been associated with CDI risk. Ruminococcus spp. are a member of the class Clostridia, which have repeatedly been associated with a decreased risk of CDI. Alstistes spp. are a member of the phylum Bacteroidetes which have also been associated with CDI.

### Table 4 Cumulative incidence of AAD within 28 and 90 days.

| Subgroup                        | N   | Events | Incidence | SDHR |
|---------------------------------|-----|--------|-----------|------|
| **AAD within 28 days**          |     |        |           |      |
| Total population                | 1007| 101.7  | 10.5% (8.7–12.6) | 1.2 (0.8–1.8) |
| Penicillin + BLI                | 409 | 45.5   | 11.6% (8.6–15.0) | 1.2 (0.8–1.8) |
| Third/fourth gen cephalosporin  | 394 | 29.6   | 7.8% (5.3–10.8)  | **0.6 (0.4–1.0)** |
| Fluoroquinolone                | 181 | 18.6   | 10.7% (6.6–15.9) | 1.0 (0.6–1.7) |
| Carbapenem                     | 64  | 15.6   | 25.0% (14.9–36.4) | **2.8 (1.7–4.9)** |
| Clindamycin                    | 29  | 2.0    | 7.6% (1.3–21.8)  | 0.7 (0.2–2.6) |
| History of CDI                 | 31  | 3.8    | 13.0% (2.5–32.1) | 1.2 (0.3–4.6) |
| No history of CDI              | 976 | 97.9   | 10.4% (8.5–12.5) | - |
| Toxigenic C. difficile carriage | 45  | 7.8    | 18.1% (7.9–31.4) | 1.9 (0.9–4.0) |
| No toxigenic C. difficile carriage | 962 | 92.9   | 10.2% (8.3–12.2) | 1.0 (0.7–1.6) |
| Intermediate N3-1S level (log2(N3-1S) < 2.5) | 38 | 2.8 | 7.5% (1.5–20.1) | 0.7 (0.2–2.7) |
| High N3-1S level (log2(N3-1S) > 2.5) | 969 | 98.9 | 10.6% (8.7–12.7) | - |
| Shannon index ≤ 3.155           | 399 | 49.4   | 12.8% (9.6–16.5) | 1.5 (1.0–2.2) |
| Shannon index > 3.155           | 608 | 52.4   | 9.0% (6.8–11.5)  | - |
| Inverse Simpson index ≤ 14.339  | 529 | 59.2   | 11.6% (8.9–14.6) | 1.3 (0.8–1.9) |
| Inverse Simpson index > 14.339  | 478 | 42.5   | 9.3% (6.8–12.2)  | - |
| **AAD within 90 days**          |     |        |           |      |
| Total population                | 1007| 135.0  | 14.1% (12.0–16.4) | 0.9 (0.6–1.3) |
| Penicillin + BLI                | 409 | 51.0   | 13.1% (9.9–16.6) | 0.9 (0.6–1.3) |
| Third/fourth gen cephalosporin  | 394 | 47.0   | 12.6% (9.4–16.1) | 0.8 (0.6–1.1) |
| Fluoroquinolone                | 181 | 25.0   | 14.6% (9.8–20.3) | 1.0 (0.7–1.6) |
| Carbapenem                     | 64  | 21.0   | 33.8% (22.3–45.7) | **3.0 (1.9–4.7)** |
| Clindamycin                    | 29  | 3.0    | 11.4% (2.8–26.8) | 0.8 (0.2–2.3) |
| History of CDI                 | 31  | 3.9    | 13.4% (2.6–33.0) | 0.9 (0.2–3.6) |
| No history of CDI              | 976 | 131.1  | 14.1% (11.9–16.5) | - |
| Toxigenic C. difficile carriage | 45  | 8.3    | 19% (8.6–32.5) | 1.5 (0.7–3.1) |
| No toxigenic C. difficile carriage | 962 | 126.7  | 13.9% (11.7–16.2) | - |
| Intermediate N3-1S level (log2(N3-1S) < 2.5) | 38 | 4.1 | 11.0% (3.0–24.7) | 0.8 (0.2–2.3) |
| High N3-1S level (log2(N3-1S) > 2.5) | 969 | 130.9  | 14.2% (12.0–16.6) | - |
| Shannon index ≤ 3.155           | 399 | 67.2   | 17.7% (13.9–21.8) | **1.5 (1.1–2.2)** |
| Shannon index > 3.155           | 608 | 67.8   | 11.7% (9.2–14.5) | - |
| Inverse Simpson index ≤ 14.339  | 529 | 80.1   | 15.9% (12.8–19.3) | 1.3 (0.9–1.9) |
| Inverse Simpson index > 14.339  | 478 | 54.9   | 12.1% (9.3–15.4) | - |
| Ratio OTU21/OTU64 ≥ 6           | 227 | 51.0   | 20.0% (14.7–25.8) | **1.7 (1.3–2.4)** |
| Ratio OTU21/OTU64 < 6           | 780 | 84.0   | 12.3% (9.9–15.2) | - |
| At least two of OTU69 < 0.140% or OTU21 ≥ 0.013% or OTU648 < 0.006% | 448 | 87.2 | 18.4% (14.4–22.8) | **1.8 (1.4–2.4)** |
| Less than two of OTU69 < 0.140% or OTU21 ≥ 0.013% or OTU648 < 0.006% | 559 | 47.8 | 10.6% (8.2–13.4) | - |

*Based on the competing events model using multiple imputed data. The number of events is averaged over the imputation datasets and can therefore have decimals. Cumulative incidence was calculated using competing events analysis. Abbreviations: AAD-associated diarrhea, BLI beta-lactamase inhibitor, CDI Clostridioides difficile infection, CI confidence interval, N3-1S normalized 3-indoxyl sulfate level in urine, OTU21 uncultured Lactobacillus, OTU69 Porphyromonas, OTU648 Blautia, SDHR subdistribution hazard ratio. Bold text denotes statistical significance. 

### Notes

- The bias-adjusted sensitivity, specificity and C-statistic were 32.1%, 79.0%, and 0.555, respectively.
- The bias-adjusted sensitivity, specificity and C-statistic were 58.2%, 57.8% and 0.580, respectively.
Enterococcus/Ruminococcus could be translated into a duplex quantitative PCR that could serve as a rapid point-of-care screening test to identify patients at high risk of CDI and to enrich trial populations. The design of such a test would require further testing and optimization and independent confirmation of the predictive performance. Unfortunately this could not be performed in the current study due to lack of leftover material. The OTU-coefficients model may be more useful if relative abundances are generated, such as with 16S rRNA gene analysis or similar techniques. Recent advances in high-throughput sequencing, such as nanopore technology may allow the point-of-care application of this model or rapid estimation of alpha diversity in the future. At present, however, the costs associated with such analyses would probably not outweigh the benefits of more accurate risk prediction.

Several limitations of the study have to be discussed. First, we lacked a CDI test result in one third of AAD episodes, mostly due to delayed reporting of diarrhea by the participant, especially after hospital discharge. The apparent difference in testing between antibiotic groups may have influenced the association with carbapenem use and lack of association with other antibiotic classes. If CDI causes more severe diarrhea symptoms compared to non-CDI AAD, patients with CDI may have reported diarrhea more frequently, thus fewer CDI episodes might be missing. To our knowledge, however, there is no published data to support this and we did not collect data on diarrhea severity. Apparently, the collection of information through a daily paper diary was insufficient to keep participants aware of the requirement to report diarrheal episodes. For future studies, more active follow-up procedures should be considered, such as a smartphone app, repeated text messages, or regular telephone calls. We mitigated the problem of missing samples by performing multiple imputation and we performed different sensitivity analyses to test the robustness of the results, which yielded similar results. Second, the low number of CDI episodes in our study precluded multivariable analysis. Hence, whether carbapenem use, C. difficile carriage, and the selected OTUs are independent predictors remains to be determined. Third, 9% of included participants were lost for other reasons than death, of which most withdrawals occurred around day 6. The collection of another rectal swab and urine sample during the second visit apparently decreased the incidence of CDI within 90 days and incidence of AAD within 28 and 90 days. Sample size calculation. The primary endpoint was CDI within 28 days after initiation of antibiotic treatment. An episode of CDI was defined as a clinical picture compatible with CDI and microbiological evidence of free toxins and the presence of C. difficile in stools, using one of the two algorithms recommended in the 2016 European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guideline, without reasonable evidence of another cause of diarrhea, or pseudomembranous colitis as diagnosed during endoscopy, after colostomy or on autopsy. 24. Diagnosis of CDI was considered in stool samples that contained C. difficile spores, or C. difficile toxins A+B as diagnosed during endoscopy, after colostomy or on autopsy. 24. Diarrhea was defined as loose stools, i.e., taking the shape of the receptacle, corresponding to Bristol stool chart types 5–7, and a stool frequency of at least three in 24 consecutive hours. Secondary endpoints included incidence of CDI within 90 days and incidence of AAD within 28 and 90 days.
of any RCT targeting the prevention of CDI in this at-risk population. The sample size was chosen such that the impact of randomly over- or underestimating the incidence would be acceptably low. Assuming an event rate of approximately 2% in the total included population, 839 participants would be needed to accurately determine the event rate. Assuming an event rate of 5% in one of the subgroups, 335 participants would be needed in that subgroup to accurately determine the event rate in the high-risk group. If one third of participants were part of such a high-risk subgroup, this would require a total study population of 1000 patients. Therefore, we aimed to include 1000 participants into the study. This sample size was deemed sufficient to perform a robust sample size calculation for an RCT.

**Data analysis.** Data were analyzed in R version 3.5.1 (2018-07-02)25. Baseline characteristics, antibiotic use, and sample collection data are given for the total population as proportions, mean (SD), or median (IQR), as appropriate. Missing CDI test results from the central laboratory were complemented with the local laboratory results, if tested according to one of the ESCMID algorithms. For other missing data, we used the mice package for multiple imputation with 100 imputed datasets (see Supplementary methods). We used Rubin’s rule to derive confidence intervals.

16S rRNA gene sequencing was performed as described in Berkell et al.14. Potential predictive biomarkers that emerged from the linear discriminant analysis effect size (LEfSe) model performed for CDI vs. no CDI and AAD (including CDI) vs. no AAD at the level of the operational taxonomic unit (OTU) were utilized for further analysis here. OTUs were defined by a cut-off of 97% similarity of the reads which is supposed to resemble species boundaries, although imperfect26.

To calculate the incidences of CDI and AAD at day 28 and day 90, cumulative incidences were estimated from the imputed data using the cumulative incidence function (cif) competing event (compR package version 2.2–7)27. This method takes loss to follow-up and competing events (death) into account. Stratified cumulative incidences were estimated per subgroup and were compared using subdistribution hazard ratios (SDHR) from the competing events model. The SDHR is calculated as the ratio of the subdistribution hazard function, which is defined as the instantaneous rate or events of a certain type in subjects who have not yet experienced that event28 and can be interpreted as a relative risk if the incidence is low. Predefined subgroups were antibiotic class at baseline, history of CDI, baseline toxiogenic *C. difficile* carriage, and low, intermediate and high N3-IS levels at baseline.

Stratified incidences for baseline N3-IS levels were first analyzed using previously published breakpoints and categorized as low (log2(N3-IS) ≤ 8), intermediate (−8 ≤ log2(N3-IS) ≤ 2.5), or high (log2(N3-IS) > 2.5)26. Next, we calculated the area under the curve (AUC) of the receiver operating characteristic (ROC; pROC package version 1.13.0) and determined the optimal breakpoint based on the Youden index (OptimalCutpoints package version 1.1–3), assuming a lower value would be associated with a higher CDI risk. We also performed an unplanned analysis of CDI incidences at day 90 stratified for day-6 N3-IS levels (using the previously published breakpoints) in patients with no CDI prior to the day-6 visit and with day-6 N3-IS results available.

The microbiome-based risk strata were not prespecified. We defined low and high alpha diversity based on the Shannon index and Inverse Simpson index, for which we used the Youden index for dichotomization29. From the OTUs associated with the studied outcomes identified by LEfSe14, we further selected those with mean relative abundance of at least 0.5%. Based on these OTUs we de

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