Infect Dis Ther (2023) 12:209–225
https://doi.org/10.1007/s40121-022-00722-9

ORIGINAL RESEARCH

Risk Factors Associated with Severe *Clostridioides difficile* Infection in Patients with Cancer

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Received: July 14, 2022 / Accepted: October 31, 2022 / Published online: November 28, 2022

ABSTRACT

Introduction: Antibiotic use is a risk factor for *Clostridioides difficile* infection (CDI). Few studies have correlated use of prior antibiotic classes with CDI, microbiome composition, and disease severity in patients with cancer. We hypothesized that previous antibiotic exposure and fecal microbiome composition at time of presentation are risk factors for severe CDI in patients with cancer.

Methods: This non-interventional, prospective, cohort study examined 200 patients with cancer who had their first episode or first recurrence of CDI. *C. difficile* was identified using nucleic acid amplification testing. Univariate analysis was used to determine significant risk factors for severe CDI. Fecal microbiome composition was determined by sequencing the V3/V4 region of 16 s rDNA encoding gene. Differential abundance analyses were used to single out significant microbial features which differed across severity levels.

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Results: On univariate analysis, factors associated with severe CDI included the presence of toxin A/B in stools (odds ratio [OR] 2.14 [1.05–4.36] $p = 0.04$ and prior 90-day metronidazole use (OR 2.66 [1.09–6.50] $p = 0.03$). Although alpha and beta diversity was similar between disease severity groups and toxin A/B in stools, increased abundance of *Bacteroides uniformis*, *Ruminococcaceae*, and *Citrobacter koseri* were associated with protection from severe CDI ($p < 0.05$) and depletion of anaerobes was higher in patients with prior metronidazole exposure.

Conclusion: Use of metronidazole for non-CDI indications within 90 days prior to diagnosis and presence of toxin A/B in stools were associated with severe CDI. Findings provide valuable insights into risk factors for severe CDI in an underserved population with cancer that warrants further exploration.

Keywords: *Clostridioides difficile*; Metronidazole; Anaerobes; Microbiome; Cancer

Key Summary Points

Few studies have correlated use of prior antibiotic classes with CDI, microbiome composition, and disease severity in patients with cancer.

We hypothesized that previous antibiotic exposure and fecal microbiome composition at time of presentation are risk factors for severe CDI in patients with cancer.

Factors associated with severe *Clostridiodes difficile* infection in the oncologic population include the presence of toxin A/B in stools along with prior metronidazole use.

INTRODUCTION

Clostridioides difficile infection (CDI) is a major public health threat in the USA and affects 66 per 100,000 persons under the age of 65 and 667 per 100,000 of those 65 years of age or older [1, 2]. Acquisition of CDI results in increased length of stay, hospital readmissions, death, and economic burden [2–4], and recurrence due to relapse of infection or re-infection continues to be a major challenge.

CDI and its complications disproportionally affect patients with cancer, with an incidence ranging from 6% to 33%, which is higher than that in the general patient population (less than 1%) [5]. The reasons behind why there is a higher risk are multifactorial and include prolonged and frequent healthcare visits, exposure to chemotherapy [6] and immunosuppressant agents [7] that can weaken the immune system. Importantly, the use of broad-spectrum antibiotics can promote *C. difficile* growth by altering the intestinal microbiome. Antimicrobials, along with some forms of chemotherapy and radiation therapy, can cause structural and functional changes in the complex colon microbiome that usually provides resistance to *C. difficile* colonization [8] by inhibiting germination and toxin production by *C. difficile* [9]. Antibiotics can alter bile acid metabolism, the fermentation of carbohydrates, and the production of metabolites that favor *C. difficile* growth and toxin production [10, 11]. In order to assist physicians with their goal of reducing the risk for severe disease in patients with cancer, it is important to determine the association of prior antibiotic exposure and microbiome composition with CDI severity. We hypothesized that previous antibiotic exposure and microbiome composition at time of CDI presentation are risk factors for severe disease in patients with cancer.

METHODS

This was a prospective, observational, non-interventional, single-center cohort study conducted at MD Anderson Cancer Center in Houston, Texas that enrolled 200 sequential patients who provided informed consent and had their first episode or first recurrence of CDI between October 27, 2016 and July 1, 2019. *C. difficile* was identified by nucleic acid

amplification (BioFire, Salt Lake City, UT) testing (NAAT) and toxin production was assessed via enzyme immunoassay (EIA) for A/B toxins (Meridian Bioscience Immunocard®, Cincinnati, OH); both toxin positive and toxin negative patients were included in the study. We studied adult inpatients (age greater than 18 years) with a malignancy either active or in remission who had a first episode or first recurrence of CDI defined as presence of diarrhea (more than three unformed bowel movements or more than 200 mL unformed stool) within 24 h prior to therapy and a NAAT positive for *C. difficile* in stool within 48 h prior to CDI specific therapy. We excluded patients receiving antimotility agents at the time of consent without anticipated discontinuation and/or oral contrast within 48 h prior to symptoms. Patients concurrently participating in other CDI trials, receiving microbiota transplant, outpatients, or patients with an expected survival of less than 4 days were also excluded.

Figure 1 shows the study flowchart for study sample selection based on the previous inclusion and exclusion criteria.

Baseline demographics and clinical and laboratory findings were extracted from medical records and are shown on Tables 1 and 2. Neutropenia was defined as an absolute neutrophil count of less than 500 cells/μL and lymphopenia as an absolute lymphocyte count of less than 1000 cells/μL.

Compliance with Ethics Guidelines

This study was performed in accordance with the Helsinki Declaration of 1964 and its later amendments. The study was reviewed and approved by the University of Texas MD Anderson Cancer Center Institutional Review Board (OHRP IORG0000083). Written, informed consent was obtained from all study subjects prior to enrollment in the study and

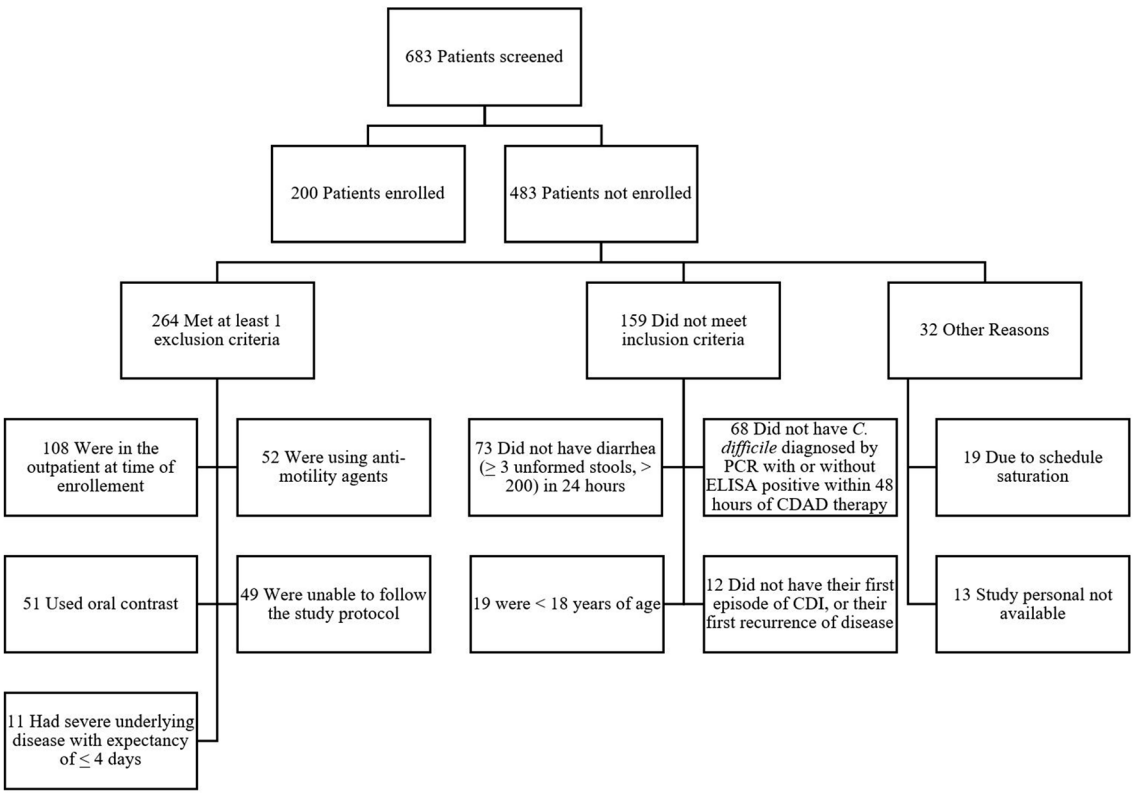

Fig. 1 Study and patient sample selection based on inclusion and exclusion criteria. *PCR* polymerase chain reaction, *ELISA* enzyme-linked immunosorbent assay

Table 1 Baseline demographic characteristics of patients with cancer and *C. difficile* infection (CDI) by disease severity

Variable	Overall $N = 200$	CDI severity[a]		*P* value[b]
		Non-severe $n = 158$	Severe or fulminant[c] $n = 42$	
Age (mean, SD)	60 (14)	60 (14)	61 (11)	0.43
Gender, *n* (%)				0.49
Male	95 (47)	73 (46)	22 (52)	
Female	105 (53)	85 (54)	20 (48)	
Race, *n* (%)				0.17
White	152 (76)	118 (75)	34 (81)	
African American	23 (12)	18 (11)	5 (12)	
Asian	3 (2)	3 (2)	0 (0)	
Other	22 (11)	19 (12)	3 (7)	
Ethnicity, *n* (%)				0.65
Hispanic	29 (15)	23 (15)	6 (14)	
Non-Hispanic	164 (82)	130 (83)	34 (81)	
Unknown	5 (3)	3 (2)	2 (5)	
Underlying malignancy, *n* (%)				0.38
Solid tumor	97 (49)	80 (50)	17 (40)	
Hematologic	64 (32)	50 (32)	14 (33)	
Stem cell transplant	39 (20)	28 (18)	11 (26)	

SD standard deviation

[a]Patients with CDI were categorized according to disease severity at the time of diagnosis as defined by the 2017 IDSA/SHEA (18) clinical practice guidelines for *C. difficile* infection in adults and children. Severe CDI was defined as WBC count of $> 15{,}000$ cell/mL and/or a serum creatinine of > 1.5 mg/dL. Fulminant disease is when cases present with hypotension, shock, ileus, or a megacolon

[b]Chi-square or Fisher's exact test when indicated to compare differences in proportions between severity categories

[c]Patients with severe and fulminant disease were grouped together given the low frequency of cases with fulminant disease ($n = 5$)

[d]"Unknown" ethnicity data are patients wherein no ethnicity data was denoted

included consent to publish data in aggregate and devoid of all identifiers.

Microbiome Studies

For microbiome studies bacterial DNA was extracted from unformed stools using the QIAamp DNA FFPE Tissue Kit (Qiagen). Using polymerase chain reaction, the 16S rRNA V4 region was amplified and sequenced using the MiSeq platform (Illumina). An average of 21,848 sequences were obtained from each sample (range 525 to 78,404). We rarefied the OTU (operational taxonomic unit) counts with a minimum of 500 read counts across samples when calculating the alpha diversity. But we used full unrarefied data for all the other downstream analyses, in order to not lose

Table 2 Baseline clinical characteristics of patients with cancer and *C. difficile* infection (CDI) by disease severity

Variables	Overall $N = 200$	CDI severity[a]		*P* value[b]
		Non-severe CDI $n = 158$	Severe or fulminant[c] $n = 42$	
Episode, *n* (%)				0.19
First episode	185 (93)	144 (92)	41 (98)	
First recurrence	15 (5)	14 (8)	1 (2)	
Presenting symptoms, *n* (%)				
Nausea	86 (43)	71 (45)	15 (36)	0.28
Vomiting	56 (28)	46 (29)	10 (24)	0.50
Abdominal pain	73 (37)	54 (34)	19 (45)	0.19
Cramping	31 (16)	23 (15)	8 (19)	0.48
Bloating	17 (9)	11 (7)	6 (14)	0.21
Bloody stools	11 (6)	10 (6)	1 (2)	0.46
Mucus in stools	4 (2)	2 (1)	2 (5)	0.20
Tenesmus	4 (2)	4 (3)	0 (0)	0.58
Urgency	5 (3)	4 (3)	1 (2)	1.00
Incontinence	9 (5)	8 (5)	1 (2)	0.69
Fever	63 (32)	50 (35)	13 (31)	0.93
Antimicrobial exposure[d] (past 90 days), *n* (%)	181 (91)	142 (90)	39 (93)	0.77
Aminoglycosides	6 (3)	6 (4)	0 (0)	0.35
Carbapenems	31 (16)	25 (16)	6 (14)	0.81
Cephalosporin	94 (47)	69 (44)	25 (60)	0.07
Daptomycin	6 (3)	5 (3)	1 (2)	1.00
Fidaxomicin	4 (2)	3 (2)	1 (2)	1.00
Lincosamides	6 (3)	6 (4)	0 (0)	0.35
Macrolides	2 (1)	1 (1)	1 (2)	0.38
Metronidazole	26 (13)	16 (10)	10 (24)	**0.02**
Oxazolidones	23 (12)	16 (10)	7 (17)	0.28
Penicillins	64 (32)	49 (31)	15 (36)	0.56
Quinolones	84 (42)	65 (41)	19 (45)	0.63
Sulfonamides	21 (11)	15 (10)	6 (14)	0.40
Tetracyclines	19 (10)	17 (11)	2 (5)	0.38
Tigecycline	1 (1)	1 (1)	0 (0)	1.00
Vancomycin	51 (26)	41 (26)	10 (24)	0.78

Table 2 continued

Variables	Overall $N = 200$	CDI severity[a]		P value[b]
		Non-severe CDI $n = 158$	Severe or fulminant[c] $n = 42$	
Antifungals	77 (39)	62 (39)	15 (36)	0.68
Antivirals	83 (42)	66 (42)	17 (40)	0.88
Other	9 (5)	7 (4)	2 (5)	1.00
Immunosuppressed (90 days), *n* (%)	113 (57)	87 (55)	26 (62)	0.43
Recent chemotherapy (90 days), *n* (%)	138 (69)	108 (68)	30 (71)	0.70
Proton pump inhibitor use (90 days), *n* (%)	130 (65)	104 (66)	26 (62)	0.64
Use of H2 blockers (90 days), *n* (%)	56 (28)	43 (27)	13 (31)	0.63
Use of GABA mimetics (90 days), *n* (%)	105 (53)	85 (54)	20 (48)	0.48
Zolpidem	14 (7)	10 (6)	4 (10)	0.50
Other benzodiazepines	48 (24)	41 (26)	7 (17)	0.21
Gabapentin or pregabalin	54 (27)	46 (29)	9 (21)	0.32
Opioid use (90 days), *n* (%)	158 (79)	125 (79)	33 (79)	0.94
Laxative use (90 days), *n* (%)	117 (59)	90 (57)	27 (64)	0.39
Antimotility use (90 days), *n* (%)	61 (31)	47 (30)	14 (33)	0.65
CDI acquisition, *n* (%)				0.85
Healthcare facility onset	80 (40)	64 (41)	16 (38)	
Healthcare facility associated	66 (33)	52 (33)	14 (33)	
Community onset/healthcare associated	32 (16)	26 (16)	6 (14)	
Community onset	21 (11)	15 (9)	6 (14)	
Other	1 (1)	1 (1)	0 (0)	
Charlson comorbidity score, mean (SD)	5.53 (2.68)	5.41 (2.52)	6.26 (2.96)	0.06
Horn's Index, *n* (%)				0.05
Medical management	193 (97)	154 (97)	39 (93)	
ICU stay, no invasive	2 (1)	0 (0)	2 (5)	
ICU stay, with invasive procedures	5 (3)	4 (3)	1 (2)	
Critically ill, shock	0 (0)	0 (0)	0 (0)	
Zar score, *n* (%)				**< 0.01**
Not severe (< 2)	157 (79)	132 (84)	24 (57)	
Severe (> 2)	43 (22)	26 (16)	18 (43)	
Laboratory parameters				
Neutropenia (< 500) ($N = 194$) (%)	48 (25)	42 (27)	7 (17)	0.17

Table 2 continued

Variables	Overall $N = 200$	CDI severity[a]		*P* value[b]
		Non-severe CDI $n = 158$	Severe or fulminant[c] $n = 42$	
Lymphopenia (< 1000) ($N = 192$) (%)	134 (70)	111 (70)	24 (57)	0.06
Serum albumin, mean (SD)	3.28 (0.68)	3.32 (0.66)	3.14 (0.75)	0.14
Diagnostic modality, *n* (%)				
C. difficile PCR positive	197 (99)	155 (98)	42 (100)	1.00
Toxin A/B positive	62 (31)	43 (27)	19 (45)	**0.03**
Co-pathogen present (any), *n* (%)	18 (16), $n = 111$	16 (19.05), $n = 84$	2 (7), $n = 27$	
Campylobacter	1 (1)	1 (1)	0 (0)	1.00
Salmonella	1 (1)	1 (1)	0 (0)	1.00
Vibrio spp.	2 (2)	2 (2)	0 (0)	1.00
Enteroaggregative *E. coli*	2 (2)	2 (2)	0 (0)	1.00
Enteropathogenic *E. coli*	5 (5)	4 (5)	1 (4)	1.00
Cryptosporidium	1 (1)	1 (1)	0 (0)	1.00
Giardia	2 (2)	2 (2)	0 (0)	1.00
Norovirus	1 (1)	1 (1)	0 (0)	1.00
Rotavirus	2 (2)	2 (2)	0 (0)	1.00
Sapovirus	1 (1)	0 (0)	1 (4)	0.24

SD standard deviation, *PCR* polymerase chain reaction

Significant *p* values < 0.05 are in bold

[a]Patients with CDI were categorized according to disease severity at the time of diagnosis as defined by the 2017 IDSA/SHEA (18) clinical practice guidelines for *C. difficile* infection in adults and children. Severe CDI was defined as WBC count of > 15,000 cell/mL and/or a serum creatinine of > 1.5 mg/dL. Fulminant disease is when cases present with hypotension, shock, ileus, or a megacolon

[b]Chi-square or Fisher's exact test when indicated to compare differences in proportions between severity categories

[c]Patients with severe and fulminant disease were grouped together given the low frequency of cases with fulminant disease ($n = 5$)

[d]Some antibiotics were used concurrently and were not mutually exclusive

important information from raw microbiome data. In order to account for differences in the depth of reads, we obtained the OTU relative abundances by scaling the OTU counts by their total counts in each sample. VSEARCH was used for analyzing nucleotide sequences [12]. Paired-end reads were merged, de-replicated, and sorted according to length and size. Sequences were subjected to quality control, error-corrected, and chimera-filtered using the UNOISE algorithm generating a preliminary list of OTUs. Both OTUs and presumed chimeras were assigned taxonomy in QIIME [13] using the Mothur method [14] with the Silva database version 128 [15]. In the case when sequences were rejected by the UNOISE algorithm, sequences matching a database entry with a perfect score were restored to generate the final list of OTUs.

Once an OTU table was created, the clustered phylogenetic tree and the assigned taxonomy were loaded into R 3.6.1 for additional quantitative analysis. The individual OTU counts were normalized by the total OTU counts in each sample; the scaled OTU abundance vector sums up to 1. Alpha diversity scores and UniFrac distance [16] between samples were determined using PhyloSeq [17] R packages.

All the other analyses, including testing procedures and visualization results, were performed and produced in R.

Outcome Definitions and Statistical Analysis

Patients were categorized as having severe CDI if they met 2017 IDSA/SHEA CDI guidelines definition that includes white blood cell (WBC) count of greater than 15,000 cell/mL and/or a serum creatinine of greater than 1.5 mg/dL and categorized as fulminant disease when cases present with hypotension, shock, ileus, or a megacolon [18]. As a result of the small number of fulminant cases, the severe CDI cases were combined with fulminant cases under the "Severe CDI" group. Data was collected via REDCap, a web-based application (Vanderbilt University) hosted by MD Anderson [19]. Analysis was performed using SPSS 24 (IBM SPSS Inc.). Descriptive statistics were used to present demographics, risk factors, and clinical outcomes. Using an exploratory approach to identify risk factors, we first performed chi-square or Fisher's exact test when indicated to compare differences in proportions between severity categories. Variables with $p < 0.25$ found in univariable analyses were included in a multivariable logistic regression analysis (backward selection) that assessed significant risk factors for severe CDI considering CDI severity as the dependent variable and clinical and microbiological parameters as the independent variables. For microbiome studies, stool samples were collected at baseline using the OMNIgene-GUT kit (DNA Genotek, Inc.) and then stored at − 80 °C. DNA extraction and bacterial 16S rDNA sequencing were performed as described previously [20]. And we adopted the same methodology to implement the microbiome analysis as described by Wang et al. [21].

RESULTS

Clinical and Microbiological Characteristics

The study enrolled 200 patients of which 53% were female, 76% White, and 82% non-Hispanic with a mean age of 60 years (Table 1). Patient distribution by malignancy type included solid tumors (49%, 97/200) of which 33% were gastrointestinal malignancies, hematologic malignancies (32%, 64/200) including leukemia, lymphoma, myeloma, and myelodysplasia, and hematopoietic stem cell transplantation (20%, 39/200) of which 13 were autologous.

Most CDI infections were primary (93%, 185/200), with 43% (86/200) of patients presenting with nausea, 37% (73/200) with abdominal pain, and 32% (63/200) with fever. As shown in Table 2, within 90 days before diagnosis, 91% (181/200) of patients received antimicrobials, 42% (84/200) received fluoroquinolones (a common choice for neutropenic prophylaxis), 57% (113/200) immunosuppressants, and 69% (138/200) chemotherapy. Other known risk factors for CDI such as proton pump inhibitors and H2 blockers were taken by 65% (130/200) and 28% (56/200) of patients, respectively.

Forty percent (80/200) of cases were identified as healthcare facility onset (occurring 48–72 h after admission). Healthcare facility-associated infection (onset in the community within 4 weeks of discharge) was identified in 33% (66/200) of the study participants. Community onset, but healthcare associated (defined as onset within 12 weeks after discharge from a healthcare facility) was identified in 16% (32/200) of the cases and only 11% (22/200) were community onset cases (no prior healthcare contact in 12 weeks). Following the 2017 IDSA/SHEA guidelines severity stratification, most patients presented with non-severe CDI (79%, 158/200) followed by severe (19%,

Table 3 Logistic regression analyses of risk factors for severe/ fulminant *C. difficile* infection in patients with cancer

Variable	Univariable analyses[a]		Multivariable analyses[b]	
	Crude OR (95% CI)	*p* value	Adjusted OR (95% CI)	*p* value
Episode		0.19		–
First episode	Reference			
Recurrent episode	0.25 (0.03, 1.97)			
Symptoms				
Abdominal pain	1.59 (0.80, 3.18)	0.19		–
Bloating	2.23 (0.77, 6.43)	0.14		–
Mucus in stools	3.90 (0.53, 28.55)	0.18		–
Antimicrobial exposure				
Cephalosporin	1.90 (0.95, 3.79)	0.07		–
Metronidazole	2.77 (1.15, 6.68)	**0.02**	2.66 (1.09, 6.50)	**0.03**
Use of GABA mimetics—other benzodiazepines	0.57 (0.24, 1.38)	0.21		–
Charlson comorbidity score	1.13 (0.99, 1.28)	0.07		–
Horn's Index		0.17		–
1—Medical management	Reference			
2,3,4—ICU stay or critically ill	2.96 (0.64, 13.78)			
Laboratory parameters				
Neutropenia	0.54 (0.22, 1.32)	0.18		–
Lymphopenia	0.51 (0.25, 1.04)	0.07		–
Serum albumin	0.67 (0.39, 1.15)	0.14		–
Presence of toxin A/B in stool	2.21 (1.10, 4.46)	**0.03**	2.14 (1.05, 4.36)	**0.04**
Co-pathogen present—Sapovirus	*	0.12		N/A

Severe/fulminant CDI disease severity was defined based on the 2017 IDSA/SHEA clinical practice guidelines (18) for *C. difficile* infection in adults and children. Severe CDI was defined as WBC count of > 15,000 cell/mL and/or a serum creatinine of > 1.5 mg/dL. Fulminant disease is when cases present with hypotension, shock, ileus, or a megacolon
Significant *p* values < 0.05 are in bold
[a]These are variables with $p < 0.25$ in univariable logistic regression analyses which were included in multivariable logistic regression analysis
[b]Backward selection was preferred for multivariable regression analysis because of the small sample size and the study not having enough events per variable. Multivariate model with all factors was not used because of overfitting
*Odds ratio was not able to be estimated due to the failure of model converge which was caused by 0 patient with sapovirus had non-severe CDI

37/200) and only 2% (5/200) experienced fulminant disease.

When variables were examined descriptively (Table 2), a significantly higher proportion of patients receiving metronidazole within 90 days of enrollment [10 of 42 (24%) vs 16 of 158 (10%), $p = 0.02$], as well as patients with Toxin A/B in stools [19 of 43 (44%) vs 43 of 158

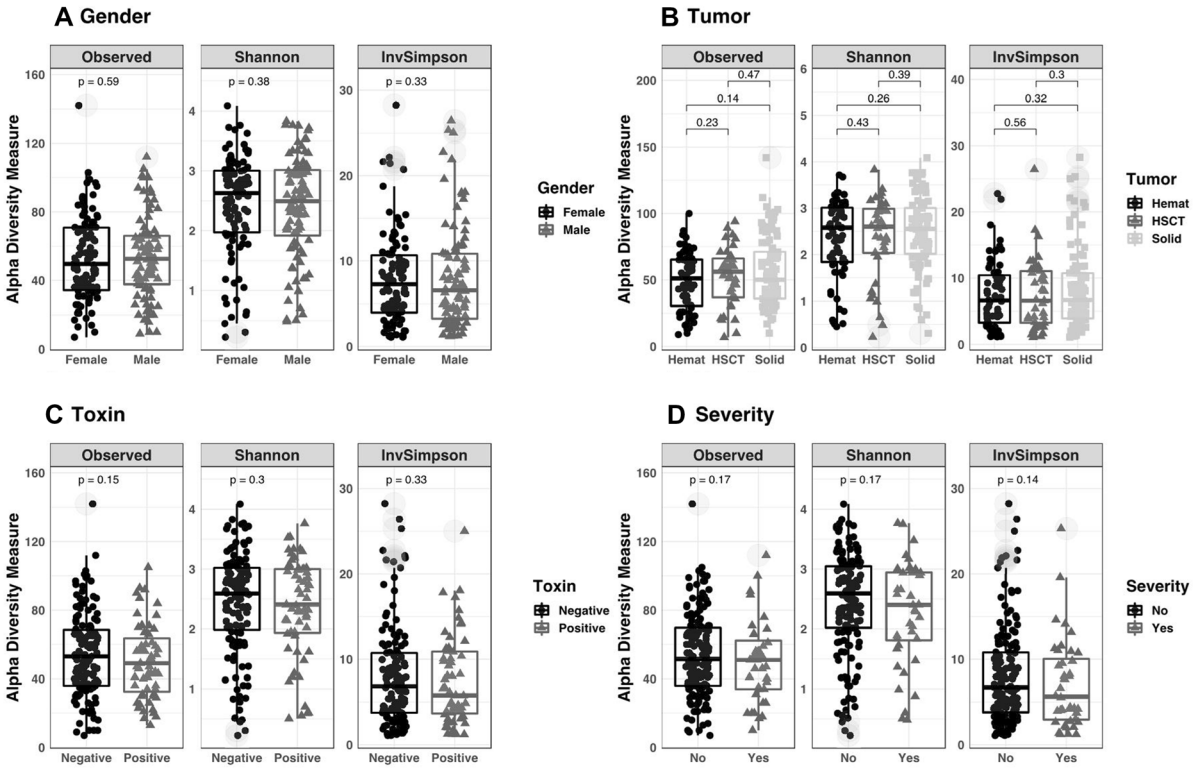

Fig. 2 Fecal microbiome richness, diversity, and evenness as determined by 16S rRNA sequencing performed in stools from patients with *C. difficile* infection. The observed number of species, and Shannon and Simpson indices are shown according to study participant gender (**a**), tumor type (**b**), presence of toxin A/B by EIA (**c**), and disease severity according to the 2017 IDSA/SHEA clinical practice guidelines (**d**). For **d** severe CDI is labeled as Yes. Severe CDI was defined as WBC count of > 15,000 cell/mL and/or a serum creatinine of > 1.5 mg/dL. Fulminant disease is when cases present with hypotension, shock, ileus, or a megacolon

(27%), $p = 0.03$] had severe CDI. As expected, (since part of the 2017 IDSA definition for severe disease), total WBC was higher in patients with severe CDI (median 9.6 ml^3) than in non-severe CDI [median 5.24 ml^3 (OR 1.14, CI 1.06–1.22, $p < 0.01$)]. Similarly, serum creatinine for CDI was higher in patients with severe CDI (median 2.44 mg/dL) than non-severe CDI [median 0.81, (OR 8.6, CI 3.9–18.75, $p < 0.01$)]. Further analysis using a multivariate model/approach that excluded total WBC and serum creatinine showed that the prior exposure to metronidazole within 90 days and the presence of toxins A/B in stool were significantly associated with severe CDI (Table 3).

In a post hoc analysis that examined the number of antibiotic classes the patients were exposed to prior to enrollment and stratified data according to disease severity, the mean ± SD of antibiotic classes the patients received was 3.0 ± 2.2. There were no differences in the mean number of antibiotic classes received by patients with severe CDI when compared to non-severe CDI (2.9 ± 2.0 vs 3.0 ± 2.6, P = NS).

Microbiome Analysis

Stool samples collected at baseline from 159 non-severe cases and 35 severe cases were studied for microbiome composition (stool samples could not be obtained for six patients). There was no statistically significant difference in alpha diversity (using the Simpson index) based on gender ($p = 0.33$), tumor type ($p = 0.29$; 0.23; $p = 0.53$), toxin positivity ($p = 0.41$), and severity of CDI based on 2017

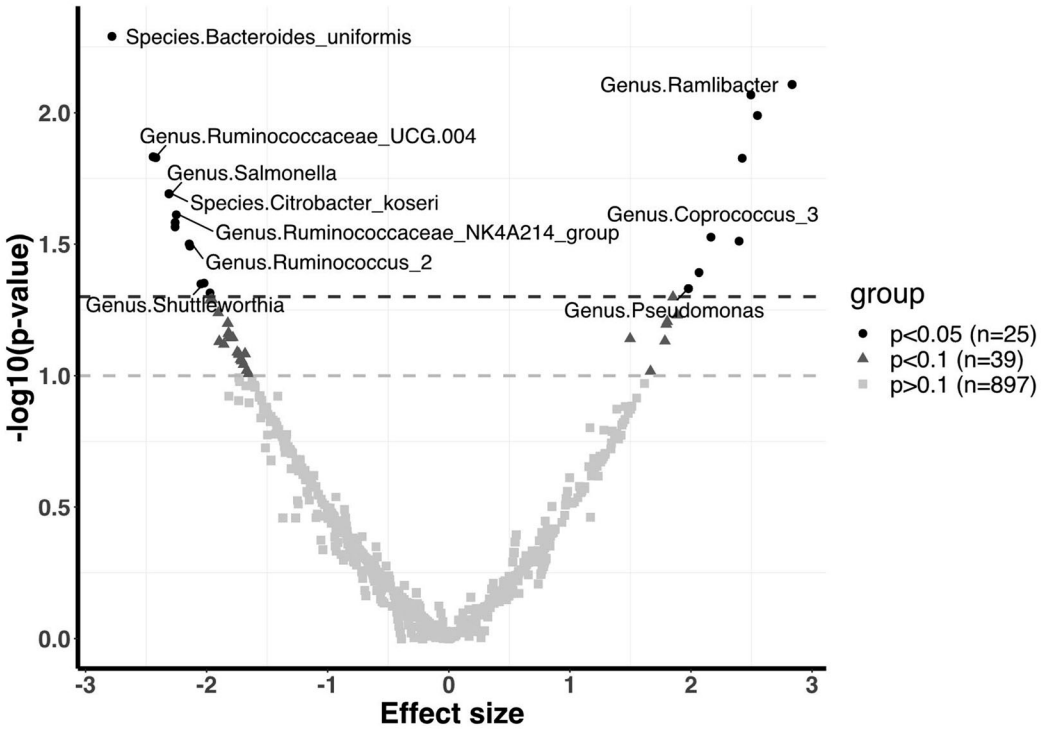

Fig. 3 Bacterial genus and species abundance associated with severe and non-severe *C. difficile* infection as determined by progressive permutation analysis of fecal 16S rRNA sequences. The strength of the statistical association is depicted on the *y*-axis and the effect size on the *x*-axis. Factors associated with increased severity are on the right side of the plot and those associated with protection are on the left side of the plot. Severe CDI was defined as WBC count of > 15,000 cell/mL and/or a serum creatinine of > 1.5 mg/dL. Fulminant disease is when cases present with hypotension, shock, ileus, or a megacolon

IDSA/SHEA guidelines ($p = 0.22$) (Fig. 2). Beta diversity was examined on the basis of CDI severity. Using a principal component analysis, we noted no differences between patients with non-severe versus severe disease on the basis of either weighted or unweighted Unifrac distances [16, 22].

Using differential abundance analysis, we searched for individual microbial features that were robustly associated with severe CDI and found that the abundance of *Bacteroides uniformis* and *Citrobacter koseri* at the species level and *Salmonella* and *Ruminococcaceae* at the genus level were associated with protection from severe CDI ($p < 0.05$). This was confirmed by differential abundance analysis when compared to non-severe cases (Fig. 2). To further examine the effect of metronidazole on the microbiome, we conducted a post hoc analysis on stools from patients who either received or did not receive metronidazole 90 days prior to diagnosis (Fig. 3). At the genus level, the use of metronidazole was associated with increased abundance of *Yersinia*, *Providencia*, and *Pseudoflavonifractor*, whereas patients not exposed to metronidazole had an increased abundance of *Prevotella*, *Muribaculaceae*, *Clostridiales, Candidatus*, and other anaerobes including *Bacteroides*.

DISCUSSION

In addition to traditional risk factors for CDI such as the use of antibiotics, histamine type 2 blockers, proton pump inhibitors [16, 17], GABA mimetics, opioids, laxatives, and antimotility agents [23, 24], patients with cancer are at risk of acquiring CDI as a result of chemotherapy [25, 25] and immunosuppressive

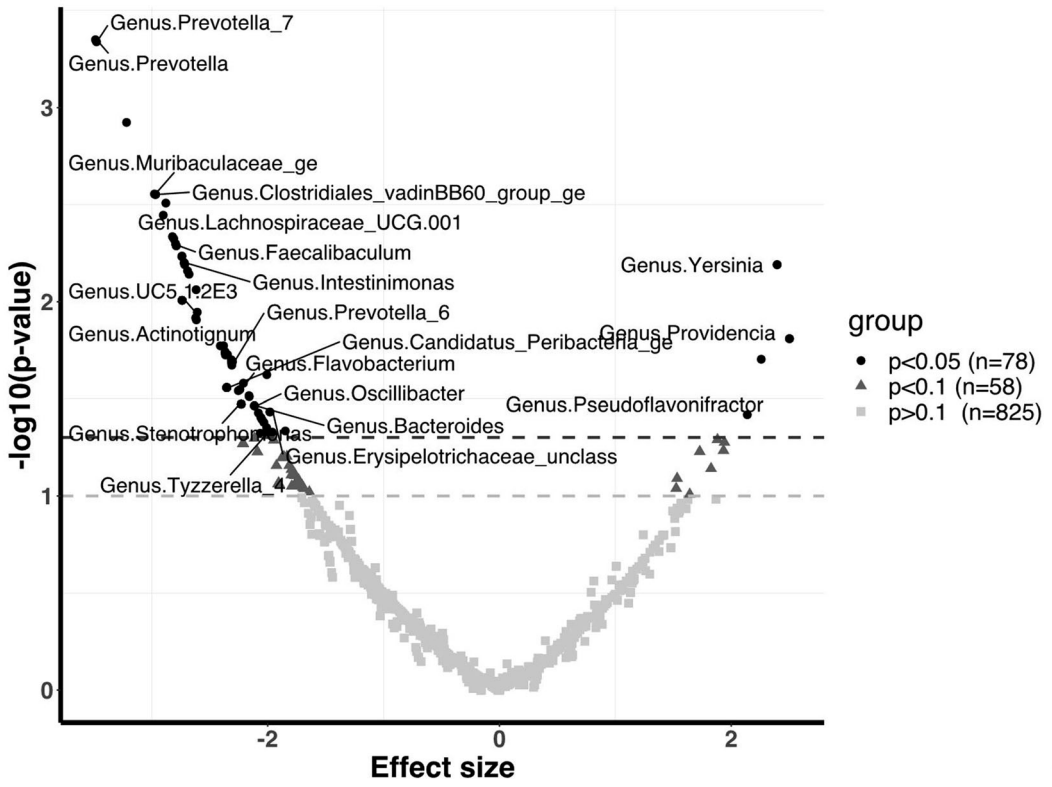

Fig. 4 Fecal bacterial genus and species abundance determined 16S rRNA sequencing done at the time of *C. difficile* infection diagnosis in patients with and without exposure to metronidazole 90 days prior to presentation. Associations were examined by differential abundance analysis. The strength of the statistical association is depicted in the *y*-axis and the effect size in the *x*-axis. Taxa associated with the use of metronidazole are seen to the right side of the plot and taxa in stools from patients not receiving metronidazole are depicted to the left

treatment [27]. Some of these risk factors are also associated with severe CDI [28]. Current guidelines for the diagnosis and treatment/management of CDI [18] stratify therapy on the basis of disease severity. However, these guidelines have limitations/limited applicability for patients that are immunosuppressed or with cancer in part because the guideline definition of severe CDI focuses solely on renal function and presence of leukocytosis and does not consider cancer-specific factors such as type of cancer, use of chemotherapy, immunosuppression, prior antibiotic therapy, hematopoietic stem cell transplantation status, the presence of *C. difficile* enterotoxins, and microbiome composition.

In this study conducted in patients with solid and hematologic malignancies including hematopoietic stem cell transplant recipients, we examined clinical features at presentation, use of antimicrobials, known risk factors, laboratory findings, microbiome composition at time of presentation, and risk of severe CDI. In univariate analysis, the use of metronidazole within 90 days prior to diagnosis for reasons other than CDI was associated with severe disease. Also, and consistent with prior reports, the presence of fecal toxin A/B by EIA was associated with severe CDI [29]. Following multivariate analysis, both factors were independently associated with severe CDI.

A recent meta-analysis reported that the use of each of clindamycin, fluoroquinolones, cephalosporins, monobactams, and carbapenems [30] was associated with CDI but no studies have examined the association of specific

antibiotics with CDI disease severity in patients with cancer. Metronidazole has intrinsic activity against *C. difficile* and an older study showed that the use of metronidazole for indications other than CDI was protective of subsequent CDI [31]. A study done in patients with cancer previously identified metronidazole use as a risk factor for CDI [32] but was confounded by the use of this agent to treat prior CDI episodes. Two other studies have associated metronidazole with CDI infection [21, 33]. However, in both studies, a history of prior CDI infection was noted. The reason behind the paradoxical association of prior metronidazole use and severe CDI in our study is complex and likely multifactorial. In our cohort, use of one or more antibiotic agents in the 90 days prior to CDI diagnosis was common (91%, 181/200). Prior use of metronidazole was more commonly observed in patients that later developed severe or fulminant CDI (24%, 10/42) compared to only 10% (16/158) of patients with non-severe CDI ($p = 0.02$). Of note, only 4 of 26 (15%) received metronidazole within 90 days for a previous diagnosis of CDI. In our study, we adjusted for potential confounding from prior CDI episodes treated with metronidazole by removing from the analysis patients with prior episodes of CDI in the 12 weeks prior to enrollment. A hypothesis that could explain this observation is that metronidazole-resistant strains are selected more easily in the background of dysbiosis caused by metronidazole itself, alone or in combination with other antibiotics. In addition, the efficacy of metronidazole has decreased over time and is no longer a recommended agent for the treatment of CDI as a first-line agent. The reasons for metronidazole failure likely relate to the drug's pharmacodynamics in the colon lumen. Metronidazole has high oral bioavailability leading to high serum concentrations but low concentrations in the lumen of the colon. In this environment, rapid transit and exposure to sub-inhibitory concentration of metronidazole can favor development of drug resistance. In fact, studies have shown that the minimum inhibitory concentrations (MICs) for metronidazole have increased over time leading to resistance in up to 18.3% of isolates [34]. The mechanisms behind metronidazole non-susceptibility are just beginning to be understood. Recently, a 7-kb plasmid (pCD-METRO) conferring 25-fold increases in MIC has been identified worldwide [35]. The effect of metronidazole as a risk factor for severe CDI could also be by indirect means such as eliminating competing anaerobes that antagonize *C. difficile*. This can be seen in the microbiome post hoc analysis (Fig. 3) showing that patients receiving metronidazole in the 90 days prior to diagnosis had fewer anaerobes in their stools at the time of CDI. Another hypothesis is that the use of metronidazole in the 90 days prior to CDI diagnosis could also be a surrogate for a higher Charlson comorbidity index and prior use of broad-spectrum antibiotics, thereby also increasing the patient's risk for more severe infection.

Probiotic use has been hypothesized as a possible intervention that can prevent CDI. A Cochrane review done in 2017 showed moderate certainty evidence that suggests that probiotics can be effective in preventing CDIs in patient who are *not* immunocompromised [36]. Studies focusing on probiotic use to prevent CDI in the immunocompromised population like the patients in this study have been scant, most of them are reviews and analyses of various case reports. No randomized control trial has been done yet that focuses on the safety and efficacy of probiotics in the immunocompromised population, especially patients with malignancies. Our institution does not employ an automatic order set for probiotic use in our patients, even for those who are receiving broad-spectrum antibiotics. This is in part due to various reviews [37] and case reports [38–40] showing the risk of invasive probiotic-related infections especially in an immunocompromised host because of their low leukocyte, especially neutropenic counts and their increased risk for gastrointestinal translocation.

Neutrophils are important in controlling CDI and it has been noted that the disease is common in patients receiving myelosuppressive chemotherapy, occurring in 7% of cases [41]. Enterotoxins A and B are potent inducers of interleukin (IL)-8, a chemokine important in recruiting neutrophils to the site of infection.

When analyzed independently of neutrophils, the presence of toxin A/B remained significant, suggesting that toxicity other than mediated by WBC is important in causing severe CDI, possibly as a result of mucosal injury and dehydration. While we did not observe more severe disease in patients with neutropenia, other studies have shown that CDI in such patients can result in atypical presentations [42], progress to severe colitis [41], and is associated with bacteremia and death [43]. The link between neutropenia and increased severity is explained in part by the fact that neutrophils are important in the early phases of CDI and are needed to enhance phagocytosis by macrophages [44]. In animal studies, neutropenia increases mortality by allowing mucosal injury [45] and translocation of other intestinal bacteria to deeper tissues and the bloodstream. However, when dysregulated and present in excess, neutrophils can also contribute to poor CDI outcomes [46]. The lack of an association could be explained by patient heterogeneity since this study considers patients with any type of malignancy with a wide range of white blood cell counts.

The occurrence of CDI is tightly linked to the composition of the host microbiome and the presence or loss of specific bacterial species [44]. Both chemotherapy and antibiotic use can cause microbiome disruption. Chemotherapy regimens can contain drugs with intrinsic antibiotic activity, can alter the microenvironment conditions by causing mucositis, or be associated with decreased caloric intake that can alter bacterial substrate availability favoring CDI. Antibiotics more commonly associated with CDI include those with anti-anaerobic activity such as clindamycin and beta-lactam/beta-lactam inhibitors, although fluoroquinolones and cephalosporins have also been implicated. The loss of anaerobic commensal bacteria leads to depletion of bacterial species that convert primary bile into secondary bile acids and consume sialic acid and succinate. This favors growth of vegetative *C. difficile* and toxin A/B production in the colon. This was seen in the post hoc analysis (Fig. 3) where it was noted that if a patient did not receive metronidazole in the last 90 days before CDI, there was an increased number of anaerobes including *Prevotella, Muribaculaceae*, *Clostridiales*, and *Bacteroides*.

While the alpha and beta diversity were similar across the CDI severity strata in this study, when using a differential abundance analysis, we noted that increased relative abundances of *B. uniformis*, *Ruminococcaceae*, *Citrobacter koseri*, and *Salmonella* were linked to protection from severe CDI (Fig. 2). *Bacteroides* are gut commensals that can digest complex polysaccharides and produce short chain free fatty acids and antagonize *C. difficile* multiplication [47]. Under the same genera, *Bacteroides fragilis* has been shown in mouse models to modulate gut microbiota, inhibit *C. difficile* adherence to colonic cells, and alleviate barrier destruction [48]. Various studies have also shown that *Ruminococcaceae* (which are butyrogenic) are diminished in CDI [49, 50].

The limitations of this study include exclusion of the severely ill (patients expected to survive less than 4 days); hence, we may underestimate the proportion of patients with fulminant CDI. This study also evaluated a relatively small sample size across a variety of malignancies from a single health care system that may limit the study's generalizability. Lastly, testing for metronidazole susceptibility was not done.

CONCLUSION

Severe CDI in patients with cancer was associated with metronidazole use in the 90 days prior to CDI diagnosis and presence of toxin A/B in stools. Furthermore, the impact of metronidazole on CDI severity may be linked to a fecal microbiome composition that is decreased in anaerobe richness that enables CDI development. These findings provide valuable insights into risk factors for severe CDI in the high-risk population of patients with cancer that warrants further exploration, including studies on possible *C. difficile*-associated resistance to metronidazole.

ACKNOWLEDGEMENTS

We thank the participants of this study.

Funding. This work is supported by Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA, who also funded the journal's Rapid Service Fee.

Author Contributions. All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Denise Marie Francisco, MD, Adilene Olvera, MPH MLS and Eduardo Yepez Guevara, MD. Statistical analysis was performed by Denise Marie Francisco, MD, Liangliang Zhang, Ying Jiang. The drafts of the manuscript were written by Denise Marie Francisco, MD with the guidance of Pablo C. Okhuysen, MD and all authors commented and added on previous versions of the manuscript. All authors read and approved the final manuscript.

Disclosures. Denise Marie Francisco, MD has no competing interests and she has changed her affiliation to the University of Illinois College of Medicine in Peoria. Samuel L. Aitken has also changed his affiliation to the University of Michigan. Ryan Dillon, MsC and Engels N. Obi, PhD are employed under Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Kenilworth, NJ, USA. Pablo C. Okhuysen, MD has faculty grant/research support from Merck Sharp and Dohme Corp, Deinove, Summit, Melinta and Napo. He is a paid consultant to Napo and is a consultant with Ferring Pharmaceuticals, Inc.

Compliance with Ethics Guidelines. This study was performed in accordance with the Helsinki Declaration of 1964 and its later amendments. The study was reviewed and approved by the University of Texas MD Anderson Cancer Center Institutional Review Board (OHRP IORG0000083). Written, informed consent was obtained from all study subjects prior to enrollment in the study and included consent to publish data in aggregate and devoid of all identifiers.

Prior Presentation. A portion of the data was presented during the Infectious Diseases Society of America – IDWeek 2020 (October 21–25, 2020 held as a virtual conference, online) as an abstract entitled "Metronidazole Exposure Prior to *Clostridiodes difficile* Infection (CDI) is a Risk Factor for Severe *C. difficile* Disease in Cancer Patients".

Data Availability. The de-identified datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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