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Elevated Fecal Calprotectin Associates with Adverse Outcomes from *Clostridium difficile* Infection in Older Adults

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Abstract

Background—*Clostridium difficile* infection (CDI) causes a mild to moderate colitis in most patients, but some, especially older adults, develop severe, adverse outcomes. Biomarkers predicting outcomes are needed to optimize treatments. This study tested whether fecal calprotectin associated with a composite primary outcome of complicated CDI (intensive care unit admission, colectomy, or death due to CDI within 30 days of diagnosis) and/or 8-week recurrence.

Methods—Stool was collected in Cary-Blair media at the time of diagnosis from inpatients of age >60 years that tested positive for *C. difficile* (enzyme immunoassay [EIA] for toxin A/B or polymerase chain reaction for the *tcdB* gene). Fecal calprotectin was measured and normalized to solid stool weight. Analysis was performed using logistic regression. Variables were selected for the final model using likelihood ratio tests.

Results—Fifty patients were included with a mean age 72.8 ($\pm$ 7.5), and 13 (26%) developed the primary outcome. Clinical variables such as age, gender, and comorbid disease did not associate with complicated CDI/recurrence, nor did traditional biomarkers such as serum albumin or white blood cell count. A high normalized fecal calprotectin (>2000 μ g/g) associated with the primary outcome in the final model after adjustment for gender and detectable fecal toxin(s) by EIA (OR 24.9, 95% CI 2.4–257.9, P =.007) with a specificity of 91.9%.

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Declaration of Interest

All authors report no conflicts of interest to disclose.

Conclusion—This study provides evidence that fecal calprotectin level associates with complications from CDI in older adults. Further studies are required to validate these findings in larger cohorts and incorporate them into clinical prediction algorithms.

Keywords

Clostridium difficile; colitis; biomarkers; older adults

Introduction

Clostridium difficile infection (CDI) is caused by an anaerobic Gram-positive bacillus that produces cytotoxins TcdA and TcdB, causing symptomatic disease in the gastrointestinal tract [1]. The past decade has seen a significant increase in the incidence of CDI in the United States, now with at least 450,000 new cases and 29,000 deaths per year [2].

The manifestations of CDI can vary from a self-limited diarrheal illness to a fulminant, life-threatening colitis [1]. It is presently incompletely understood why certain patients experience severe disease and adverse outcomes while others do not, but advanced age is a major risk factor [3, 4]. Ninety-two percent of CDI-related deaths occur in adults of age 65 or older, where CDI is the 18th leading cause of mortality [5]. The risk of recurrent CDI is 2-fold higher with each decade of life [3].

Although risk factors for adverse outcomes following CDI have been identified, a highly accurate, validated risk-prediction model does not exist. In a previous study, we developed a predictive model for complicated CDI based on clinical variables alone [6], and this performed well with an area under the receiver operator characteristic curve (AUROC) of 0.83, but this is still less than desirable for widespread clinical deployment. As a result, biomarkers that augment clinical data and predict outcomes of CDI to aid in clinical decision-making have been sought. Calprotectin, a protein found in the cytoplasm of neutrophils and measured in stool, has been demonstrated to associate with infectious diarrhea and inflammatory bowel disease (IBD) [7, 8], and has been shown to be highly correlated with the severity of colonic inflammation measured by endoscopic scoring in ulcerative colitis [9]. However, an association with outcomes of CDI has previously been little studied [10]. This study tests the hypothesis that fecal calprotectin levels associate with complicated CDI and/or recurrence.

Materials and Methods

Human subjects and stool sample collection

This study was approved by the University of Michigan Institutional Review Board. Stool samples from hospitalized patients at the University of Michigan Health System submitted to the microbiology laboratory for *C. difficile* testing in patients of age ≥ 60 were consecutively evaluated for inclusion between November 2010 and November 2012. Testing was performed on stools via using the C. DIFF QUIK CHEK COMPLETE[®] test for *C. difficile* glutamate dehydrogenase (GDH) and toxins A or B (Techlab, Inc., Blacksburg, VA) by enzyme immunoassay. All GDH⁺/toxin⁻ stool tests were subjected to analysis for the

tcdB gene by real-time PCR using the GeneOhm™ Cdiff Assay (BD, Franklin Lakes, NJ) run on a Cepheid SmartCycler® System (Cepheid, Sunnyvale, CA). This testing algorithm is illustrated in Figure 1. All included samples tested positive for presence of toxigenic *C. difficile* and represented CDI episodes that were primary and non-recurrent, i.e. no episode of CDI in the preceding 8 weeks, a criterion promoted by the Centers for Disease Control and Prevention surveillance definition [11]. All laboratory testing of inpatients was performed at the discretion of the inpatient care team, which ordered *C. difficile* testing per guidelines recommending testing only symptomatic patients with suspected CDI [12, 13]. Samples were sent to the lab in Cary-Blair transport medium, per hospital policy, and immediately frozen at –80°C until analysis. Confirmation of all positive *C. difficile* tests was attempted by anaerobic culture on taurocholate-cycloserine-cefoxitin-fructose agar at 37°C. Cultured isolates were ribotyped using a high-throughput, fluorescent PCR-ribotyping scheme described elsewhere [14, 15].

Measurement of calprotectin

Frozen aliquots of collected stool were thawed and homogenized by vortexing. Calprotectin was measured from a portion of the sample, while the remaining mixture was centrifuged and the solid stool pellet was weighed. Measurement of calprotectin was performed with the BÜHLMANN Quantum Blue® lower range (30–300 µg/g) rapid calprotectin quantitative lateral flow assay and the resulting values were normalized to the weight of the stool pellet. Normalization to stool weight was performed due to the variable degree of dilution that resulted from initial collection of the clinical samples in a sufficient volume of Cary-Blair transport medium to cover the stool in the transport container.

Clinical epidemiology and statistical analysis

Clinical data on demographics (as summarized by the Charlson-Deyo comorbidity index [16]), vitals and laboratory results within 48 hours of diagnosis, medications, and the composite primary outcome of complicated CDI (intensive care unit admission, colectomy, or death due to CDI within 30 days of diagnosis [11]) and/or recurrence (recurrent symptoms and positive testing within eight weeks of initial onset, but after the original symptoms resolved [11]) were extracted from the medical record. This composite outcome was utilized due to the low prevalence of complicated CDI (n=5) and recurrent CDI (n=8) alone; furthermore, use of a composite outcome that includes recurrence is supported by prior studies, including those evaluating the use of fidaxomicin in CDI [17]. Variable constructions such as log transformation or dichotomization were explored and utilized if they better fit the data. In particular, normalized fecal calprotectin levels showed a bimodal distribution, thus they were dichotomized with a cutoff of 2000 µg/g.

All analyses were conducted in R version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria) and a two-tailed *P* value of <.05 was considered significant. After generation of descriptive statistics and examination of relationships between fecal calprotectin and other variables, simple logistic regression was used to model individual risk factors for the primary outcome of complicated/recurrent CDI. Variables were selected for inclusion in the final multivariable model using backwards elimination, guided by likelihood ratio tests with a cutoff α <0.1. Care was taken to avoid multicollinearity and inclusion of

other variables that could be in the causal pathway between acquisition of CDI and the development of complicated disease, such as vital signs and laboratory parameters. Interactions between terms in the final model were evaluated and included if significant.

Results

Descriptive statistics

A total of 50 patients were included in the study for analysis and their characteristics are summarized in Table I. Since a high percentage of patients were diagnosed by *tcdB* PCR (60%), we also examined the breakdown of these characteristics by method of diagnosis (Table I). The average age was 72.8 (standard deviation [SD] $\pm$ 7.5 years) with 54% female subjects. Nine (18%) patients had CDI previously and 31 (62%) were healthcare-associated CDI cases. Use of proton pump inhibitors (PPIs) and concurrent non-CDI antibiotics was high, at 74% and 80%, respectively. The primary outcome of complicated/recurrent CDI occurred in 13 (26%) patients. Normalized calprotectin values were higher in patients with the primary outcome (Figure 2), though they did not substantially differ by method of CDI diagnosis (Figure 3).

Analytical statistics

In the unadjusted analysis of risk factors for complicated/recurrent CDI, other than calprotectin, no other variables had a significant association, though detectable stool toxin(s) by EIA approached significance (Table II). A high normalized fecal calprotectin was associated with complicated/recurrent CDI (odds ratio [OR] 7.08, 95% confidence interval [CI] 1.39–36, $P=.016$). The final multivariable model for complicated CDI is shown in Table III. The association between high normalized fecal calprotectin and complicated CDI remains significant in this model, after adjustment for gender and detectable fecal toxin(s) (OR 24.9, 95% CI 2.4–257.9, $P=.007$). Detectable stool toxin(s) by EIA was also associated with complicated/recurrent CDI in this model (OR 9.92, 95% CI 1.55–63.65, $P=.016$). If modeled as a diagnostic test, a high normalized fecal calprotectin was 38.5% sensitive and 91.9% specific for complicated/recurrent CDI.

Discussion

This study suggests an association between high fecal calprotectin and complicated/recurrent CDI in a cohort of older adults. This association was strong, with an estimated OR of 26, and this held up after adjustment for gender and detectable stool toxin(s) by EIA, which itself was also associated with complicated/recurrent CDI. When used as a diagnostic test, despite a low sensitivity of only 38.5%, the specificity of a high calprotectin for complicated/recurrent CDI was 91.9%, suggesting the test may allow clinicians to justifiably intensify treatment when elevated. It may perform well in this role to confirm, or rule-in, adverse outcomes as part of a two-step algorithm that uses a more sensitive test initially to rule-out complicated/recurrent CDI.

This finding comes at a time when rates of CDI are increasing and older adults are bearing the brunt of this epidemic [5], but clinicians lack reliable, prospectively-validated tools to help aid in decision-making. This is of special importance given that the increasing

availability of effective treatments for severe CDI that could improve outcomes such as fidaxomicin [17], monoclonal antibodies [18, 19], fecal microbiota transplantation [20–24], and diverting loop-ileostomy [25], and that broad use of these treatments in all patients is limited due to cost [26], uncharacterized safety profile [27], and/or their invasive nature.

A biomarker-based method for risk stratification is appealing due to its objective and readily measureable nature, and it may provide means to better allocate resources. Other biomarkers such as fecal IL-8 [28, 29] and serum procalcitonin [30] have been studied in relation to severe and complicated CDI, but none have been incorporated yet into prediction algorithms. This study suggests that fecal calprotectin may also have a role in such an algorithm.

It is intriguing that fecal Clostridial toxin entered the final model as an important covariate. It has been previously demonstrated that fecal lactoferrin levels are higher in patients with detectable stool Clostridial toxin(s) [31], and that there is a high concordance between lactoferrin and calprotectin levels [10]. This suggests that there is an association between the degree of toxin production and clinical severity that also influences lactoferrin/calprotectin levels, but prior studies regarding this have reached contradictory conclusions [32–36].

Some of our results contrast with prior work. In a previous study with a cohort of 1144 cases of CDI, we identified independent risk factors for complicated CDI such as age, gender, and elevated WBC, which we failed to validate in the current study [6]. This may have been due to the smaller cohort and insufficient power; also, we focused on older adults in this study and that could shift which risk factors are most important. Alternately, this could indicate that calprotectin, which was associated with adverse outcomes despite the small sample size, is a stronger predictor than clinical variables. Furthermore, Swale et al. [10] previously studied fecal calprotectin in patients with CDI and levels were higher in those with severe CDI vs. non-severe CDI (969.3 vs. 512.7 mg/kg, respectively), based on parameters present at diagnosis such as WBC, fever, acute kidney injury, and severe colitis on imaging/exam. However, this result approached but did not reach significance ($P = .09$). The investigators did not assess the outcome of complicated CDI but did evaluate all-cause mortality and recurrence and no difference was observed ($P = .5$ and $.53$, respectively). Their study was not focused on older adults, as ours was. The overall prevalence of mortality in their cohort was low at 9% and, thus, there may have been insufficient power to detect a true difference. Given the lack of reporting of the complicated CDI outcome, it is not possible to determine if our results differ from those from Swale et al. with regards to calprotectin.

This study has several limitations. It is notable that this is a single center study with a small cohort, and this could affect the generalizability of the results. Due to our center's practice of collecting samples in Cary-Blair media, we also needed to normalize to the solid stool pellet weight, and this may have been an imperfect solution to the problem. The calprotectin assay used also had a limited range (30–300 µg/g) and “ceiling/floor” effects could have affected the results, but this is mitigated by the decision to dichotomize the variable. Our center also performs multistep testing (Figure 1), with reflex to *tcdB* PCR for discordant EIA results for GDH/toxins. We noted a particularly high prevalence of diagnosis by PCR vs. EIA (60% vs. 40%, respectively), and this could lead one to question our case definition for CDI since diagnosis by PCR could lead to false positives. However, this is mitigated by

our review of each patient's chart confirming they met clinical criteria for CDI [12] and by our clinical microbiology laboratory's validation of its multistep algorithm demonstrating excellent sensitivity/specificity against a reference standard [37]. Furthermore, the clinical characteristics did not differ significantly between these two groups (Table I), nor did the fecal calprotectin levels (Figure 3). Finally, the population of older adults in this study was more homogenous than what might be expected at other centers with respect to certain risk factors such as concurrent non-CDI antibiotic use and PPI use (Table I), which were highly prevalent, limiting the ability to study the impact of these other factors on complicated CDI.

In summary, this study provides evidence that fecal calprotectin is a useful biomarker for complicated and recurrent CDI in older adults. However, given the limitations acknowledged above, future studies are needed to validate this finding in a larger cohort and at other centers, to better understand the interplay between fecal Clostridial toxin levels and calprotectin, and to determine the role of fecal calprotectin in biomarker-based risk prediction algorithms for complicated and recurrent CDI.

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Figure 1. Clinical microbiology laboratory testing algorithm for *C. difficile* infection at the University of Michigan

Footnote. Adapted from Rao et al., 2014 [38]. Used with permission under the Creative Commons licence.

Abbreviations. CDI, *Clostridium difficile* infection; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; PCR, polymerase chain reaction.

Figure 2. Fecal calprotectin levels in patients with complicated/recurrent CDI and uncomplicated, non-recurrent CDI

These boxplots show that patients with complicated/recurrent CDI (adverse outcomes) have higher normalized fecal calprotectin levels (box represents the interquartile range [IQR] and whiskers extend to 1.5 times the IQR).

Abbreviations: CDI, *Clostridium difficile* infection.

Figure 3. Fecal calprotectin levels in patients with CDI diagnosed by a positive toxin EIA vs. those diagnosed by a positive PCR for *tcdB*

These boxplots show that patients have comparable normalized fecal calprotectin levels, irrespective of the method of CDI diagnosis, though there is greater variability in those diagnosed via *tcdB* PCR (box represents the interquartile range [IQR] and whiskers extend to 1.5 times the IQR).

Abbreviations: CDI, *Clostridium difficile* infection; EIA, enzyme immunoassay; PCR, polymerase chain reaction.

Table I

Characteristics of the study population.

Variable	mean ($\pm$ SD) or n (%)		
	Toxin EIA+ (n=20, 40%)	Toxin EIA– (n=30, 60%)	All Patients (n=50)
Age (years)	74.2 ($\pm$ 8.1)	71.9 ($\pm$ 7.1)	72.8 ($\pm$ 7.5)
Female gender	9 (45%)	18 (60%)	27 (54%)
White race	19 (95%)	28 (93.3%)	47 (94%)
Charlson-Deyo score, unweighted	2.4 ($\pm$ 2.16)	2.03 ($\pm$ 1.45)	2.18 ($\pm$ 1.76)
Prior CDI	3 (15%)	6 (20%)	9 (18%)
Healthcare-Associated CDI	8 (40%)	23 (76.7%)	31 (62%)
Immunosuppression	10 (50%)	15 (50%)	25 (50%)
Concurrent non-CDI antibiotic use	15 (75%)	25 (83.3%)	40 (80%)
PPI use	17 (85%)	20 (66.7%)	37 (74%)
Fever	5 (25%)	7 (23.3%)	12 (24%)
Systolic blood pressure (mm Hg)	102.1 ($\pm$ 14.5)	100 ($\pm$ 27.3)	100 ($\pm$ 22.7)
Serum creatinine (mg/dL)	1.7 ($\pm$ 1.6)	1.8 ($\pm$ 1.7)	1.8 ($\pm$ 1.7)
Serum albumin (g/dL)	2.9 ($\pm$ 0.6)	3.2 ($\pm$ 0.6)	3.1 ($\pm$ 0.58)
Circulating WBC (1000 cells/mm ³)	23 ($\pm$ 28.2)	11.3 ($\pm$ 5.7)	16.1 ($\pm$ 19.2)
Infection with <i>C. difficile</i> ribotype 027	7 (35%)	4 (13.3%)	11 (22%)
Normalized fecal calprotectin (μ g/g)	533.7 ($\pm$ 1056)	1287 ($\pm$ 2272)	985 ($\pm$ 1904)
30-day ICU admission	4 (20%)	4 (13.3%)	8 (16%)
Attributable 30-day ICU admission	3 (15%)	1 (3.3%)	4 (8%)
30-day Colectomy	1 (5%)	0	1 (2%)
Attributable 30-day Colectomy	1 (5%)	0	1 (2%)
30-day mortality	2 (10%)	3 (10%)	5 (10%)
Attributable 30-day mortality	1 (5%)	1 (3.3%)	2 (4%)
Complicated CDI	3 (15%)	2 (6.7%)	5 (10%)
Recurrent CDI	5 (25%)	3 (10%)	8 (16%)
Complicated/Recurrent CDI	8 (40%)	5 (16.7%)	13 (26%)

Abbreviations: CDI, *Clostridium difficile* infection; EIA, enzyme immunoassay; PPI, proton pump inhibitor; SD, standard deviation; WBC, white blood cell count.

Table IIUnadjusted analysis of risk factors for complicated/recurrent CDI (n=50).^I

Variable	OR (95% CI)	P
Age (years)	1.04 (0.96–1.13)	.983
Female gender	2.38 (0.62–9.09)	.246
Charlson-Deyo score	0.78 (0.51–1.19)	.067
Prior CDI	0.3 (0.03–2.69)	.994
Healthcare-Associated CDI	0.17 (0.04–0.66)	.074
Immunosuppression	1.88 (0.52–6.84)	.639
Concurrent non-CDI antibiotic use	3.86 (0.44–33.9)	.994
PPI use	1.23 (0.28–5.42)	.748
Fever	2.55 (0.62–10.6)	.915
Systolic blood pressure (mmHg)	1.01 (0.98–1.05)	.617
Serum creatinine (mg/dL)	0.53 (0.12–2.28)	.713
Serum albumin (g/dL)	0.42 (0.14–1.28)	.237
Circulating WBC (1000 cells/mm ³)	1 (0.97–1.04)	.777
Detectable stool toxin(s) by EIA	3.33 (0.9–12.4)	.072
Ribotype 027	3.23 (0.78–13.3)	.32
Normalized fecal calprotectin >2000 µg/g	7.08 (1.39–36)	.016

^IThe race variable was not significant and not reported here as the model did not converge to give precise estimates.

Abbreviations: CDI, *Clostridium difficile* infection; CI, confidence interval; EIA, enzyme immunoassay; OR, odds ratio; PPI, proton pump inhibitor; WBC, white blood cell count.

Table III

Explanatory model for the relationship between normalized fecal calprotectin and complicated CDI (n=50).

Variable	OR (95% CI)	P
Gender	4.72 (0.9–24.9)	.067
Detectable stool toxin(s) by EIA	9.92 (1.55–63.6)	.016
Normalized fecal calprotectin >2000 µg/g	24.9 (2.4–257.9)	.007

Abbreviations: CDI, *Clostridium difficile* infection; CI, confidence interval; EIA, enzyme immunoassay; OR, odds ratio.