

# *Clostridioides difficile* infection: history, epidemiology, risk factors, prevention, clinical manifestations, treatment, and future options

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**SUMMARY** *Clostridioides difficile* infection (CDI) is one of the major issues in nosocomial infections. This bacterium is constantly evolving and poses complex challenges for clinicians, often encountered in real-life scenarios. In the face of CDI, we are increasingly equipped with new therapeutic strategies, such as monoclonal antibodies and live biotherapeutic products, which need to be thoroughly understood to fully harness their benefits. Moreover, interesting options are currently under study for the future, including bacteriophages, vaccines, and antibiotic inhibitors. Surveillance and prevention strategies continue to play a pivotal role in limiting the spread of the infection. In this review, we aim to provide the reader with a comprehensive overview of epidemiological aspects, predisposing factors, clinical manifestations, diagnostic tools, and current and future prophylactic and therapeutic options for *C. difficile* infection.

**KEYWORDS** *Clostridioides difficile*, *Clostridium* infections, bacteriophages, antibacterial agents, bacteria, antibodies, monoclonal, prevention, surveillance

## INTRODUCTION

*Clostridioides difficile* infection (CDI) is a disease primarily involving the colon. It typically occurs in patients whose gut microflora has been disrupted by antibiotic therapy. The most common clinical manifestation of CDI is diarrhea, and the typical patient is hospitalized, comes into contact with spores of *C. difficile* which vegetate, multiply, and secrete toxins capable of causing pathogenic damage that can manifest in clinical conditions ranging from mild colitis to fulminant colitis and even death.

Over the last three decades, a profound shift in the epidemiology of *C. difficile* infection has been observed. This change began in the Western world and has since spread globally. First, CDI episodes, recurrences, and severity have increased, with a consequent increase in mortality (1). The epidemiological change appears to have started in Quebec, Canada, where researchers noticed outsized increasing rates of CDI and its associated 30-day mortality between 1991 and 2003. During this period, incidence increased from 35 to 156 per 100,000 population, and mortality increased from 5% to 14% (2). The following year, McDonald et al. in the United States (US) identified a new strain (the same one that caused the Quebec outbreak) with increased fluoroquinolone resistance that was referred to as NAP1/027 (3). From that moment on, the NAP1/027 strain spread to Europe and then worldwide (4) significantly impacting health and costs (5, 6). From 1999 to 2004, a fourfold increase in mortality was observed in the US (from 5.7 to 23.7 yearly *C. difficile* mortality rates per million population) (7).

Elderly and fragile populations are those who paid the highest price, with a mortality of 13.5% in CDI patients over 80 years (8). In 2014, a multistate point prevalence study in the US (183 hospitals—data referring to 2011) reported *C. difficile* as the most commonly reported nosocomial pathogen, causing 12% of healthcare-associated infections (9). From an epidemiological perspective, this was a paradigm-changing study: for the first time, *C. difficile* surpassed *Staphylococcus* as the prevalent agent of nosocomial infections. Similar data, referring to 2015, were reported by the same authors in 2018 (10). However, when adjusting data to account for increased diagnostic sensitivity due to the increased use of nucleic acid amplification testing, the data appear to indicate a decrease in healthcare-associated CDI (HA-CDI) episodes from 2011 onward, although the numbers remain significantly elevated (11). Likely, the reduction of fluoroquinolone use, combined with the decline in NAP1/027 epidemiology, contributed to this downward trend (12).

Regarding management, metronidazole and vancomycin have long remained the mainstay for CDI treatment. In 2013, we had the first robust evidence from a randomized trial on the effectiveness of fecal microbiota transplantation (FMT) in recurrent CDI (rCDI) (13). In 2017, the first trial of a monoclonal antibody (bezlotoxumab) against CDI demonstrated a reduction in recurrences (14). In the same year, fidaxomicin replaced metronidazole as the first-line therapy for initial CDI episodes (15). In the meantime, surgical techniques have also been implemented (16).

Currently, CDI is an extremely complex problem that attracts significant scientific and research interest, along with relevant funds. Aside from studies on antibiotics, different other therapeutic strategies are being investigated, including vaccines, oral spores, antitoxin compounds, small molecules, natural products, and many others. Below, we will review the current main evidence on CDI focusing on prevention, clinical manifestations, and treatment options.

## HISTORY

The first description of human pseudomembranous colitis (PMC) was reported by Finney in 1893 (17). The patient was a 22-year-old debilitated woman who underwent surgical resection of a gastric tumor. She later developed severe diarrhea and died on the 15th post-operative day. The autopsy described a “diphtheritic membrane” in her small bowel. Several cases of PMC have been reported in the pre-antibiotic era, often as a complication of surgery or severely debilitating diseases (18). Certainly, PMC became more common with the introduction of antibiotics. In the 1950s, the suspected causing

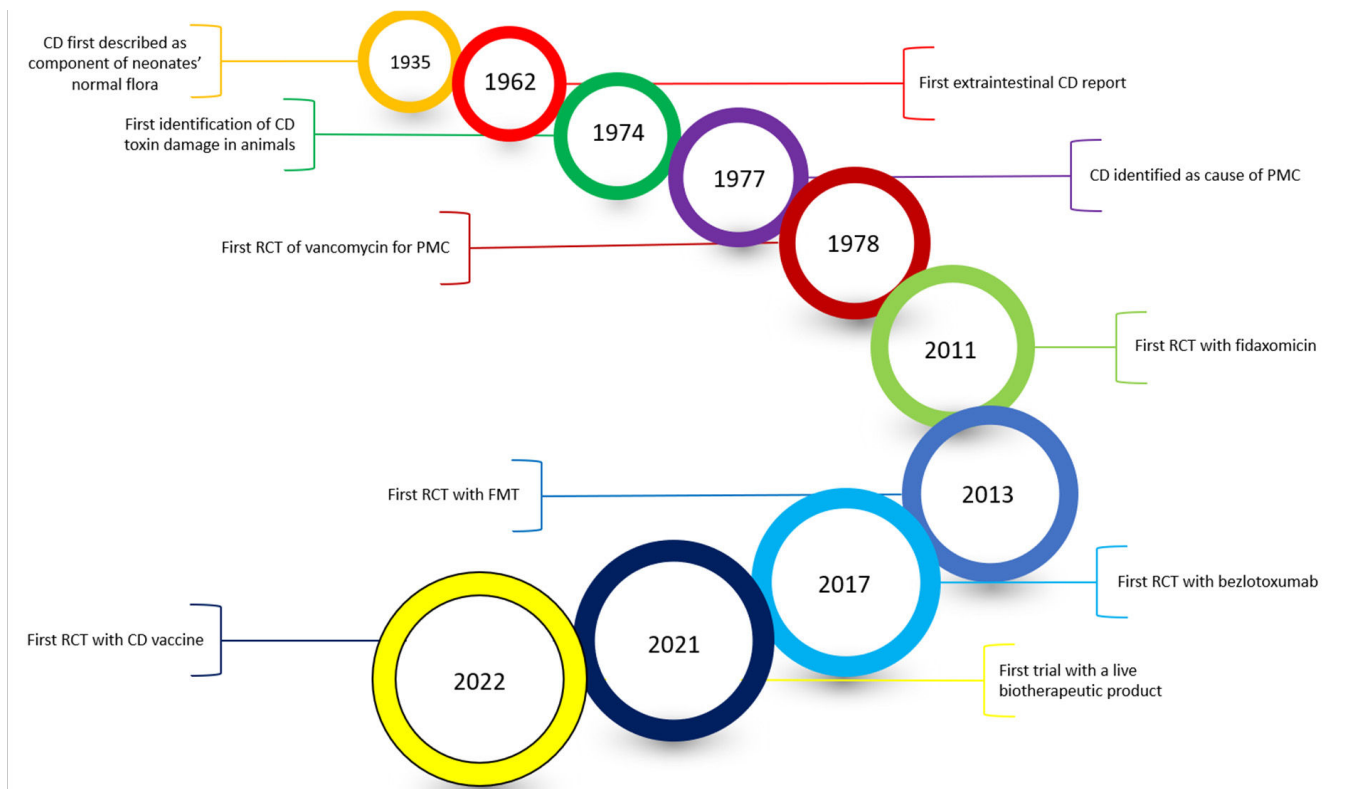
pathogen of PMC was *Staphylococcus aureus*. This hypothesis was based on the frequent detection of this organism in stools, leading to oral vancomycin becoming the standard treatment for this disease (19, 20). In 1974, Tedesco et al. published a decisive study that laid the foundation for the definitive cause of PMC (21). In this prospective study, among 200 patients receiving clindamycin, 42 patients developed diarrhea, and 20 (10%) showed proctoscopic findings of PMC. Staphylococci and other pathogens were not recovered, but subsequent investigations on stored stool specimens from eight patients revealed *C. difficile* on culture (22). *C. difficile*, named *Bacillus difficilis* at that time, was reported for the first time by Hall and O'Toole in 1935 as a colonizing organism of the neonatal intestinal flora (23). In 1962, Smith and King documented the occurrence of *C. difficile* in extraintestinal sites of eight patients, including blood, soft tissue, peritoneal and pleural fluids, and vaginal vault (24). Three of these patients eventually died. The authors concluded that *C. difficile* did not regularly produce its characteristic toxin in the human body, or alternatively, the human body was not considerably sensitive to the lethal action of this toxin.

The original description of *C. difficile* cytotoxin might be attributable to Green who found a cytopathic toxin in the stools of guinea pigs treated with penicillin. At the time, this finding suggested a viral pathogen; however, in retrospect, it was likely the first description of the cytopathic toxin produced by *C. difficile* (25). In 1977, Rifkin et al. demonstrated the presence of a toxin in the stools of two patients with antibiotic-associated colitis that could be neutralized by *Clostridium sordellii* antitoxin (26). The following month, similar findings were reported by Larson and Price in 11 patients affected by PMC or antibiotic-associated colitis (27). Lastly, in 1978, George et al. demonstrated the isolation of *C. difficile* and the presence of a preformed fecal toxin in the feces of a patient with clindamycin-associated PMC (28). This finding was, almost simultaneously, confirmed by Bartlett et al. who showed that toxin-producing clostridia were responsible for antibiotic-associated pseudomembranous colitis through testing human stools both in tissue cultures and hamster models (29). The "hamster model" has been shown to be central in discoveries about PMC. Bartlett et al. demonstrated that clindamycin-associated colitis in hamsters is due to a clindamycin-resistant, toxin-producing strain of *Clostridium* (30, 31). Furthermore, the protective effect of vancomycin on clindamycin-induced colitis has been shown in hamsters before *C. difficile* was found to be the cause of fatal hamster colitis (32). In 1978, the first randomized controlled trial (RCT) on patients with PMC suggested that oral vancomycin therapy is associated with rapid clinical and histological improvement (30). The "hamster model" turned out to be crucial even in detecting toxin A which represents an important factor in the pathogenesis of PMC resulting in differing from toxin B, the previously described cytopathic toxin (33). Both these toxins produced by *C. difficile* are responsible for human PMC symptomatology causing cytotoxicity, inflammation, and cellular detachment from the intestinal epithelium. In 1988, a *C. difficile* strain from a patient suffering from PMC was shown to produce a binary toxin that might be thought to be an additional virulence factor (34), as subsequently confirmed in the 2000s (35). Milestones in *C. difficile* history are briefly illustrated in Fig. 1.

In 2016, Lawson et al. proposed the reclassification of *Clostridium difficile* as *Clostridioides difficile* when it was necessary to classify *C. difficile* and the related *C. mangenotii* into a new genus following the restriction of the genus to *Clostridium butyricum* and related species in 2015 (36). Recent studies using molecular methods (DNA-rRNA pairing and 16S rRNA) have shown the diversity of organisms that were previously included in the single genus "*Clostridium*." In order to minimize confusion when renaming a new genus resembling the genus *Clostridium*, the term "*Clostridioides*" was coined, retaining the species name "*difficile*" due to the unusual difficulty in its isolation and study (37).

## DIFFERENCES IN EPIDEMIOLOGY

Since March 2003, an increasing incidence of CDI caused by the hypervirulent *C. difficile* PCR ribotype (RT) 027/toxinotype III was first recognized in Canada, the US, and,



**FIG 1** Milestones in *Clostridioides difficile* history. CD, *Clostridioides difficile*.

thereafter, in Europe and in several other countries. More recently, a post hoc analysis on 1,501 clinical isolates from MODIFY I and II studies (clinical trials comparing bezlotoxumab vs placebo) showed a significant continent distribution preference among five *C. difficile* clades (38). In detail, a predominance of clade 1 (including the non-toxicogenic RT009, RT010, and RT039) was found in Europe with the exception of Poland where clade 2 predominated. On the other hand, this study demonstrated the prevalence of RT027, belonging to the hypervirulent clade 2, as well as a prevalence of clade 1 in the US (38).

Comparing CDI epidemiological differences between countries and territories is challenging because of various approaches to diagnosis, definition, and data reporting. A systematic review and meta-analysis showed the heterogeneity of HA-CDI incidence, between 2006 and 2016, between territories, reporting high rates in North America, particularly among the elderly from 2006 to 2016. The authors of this study attribute such differences to various testing policies and methodologies, under-ascertainment of cases, and reporting requirements (39). For instance, a European, multicenter, prospective, biannual point prevalence study showed a wide variety of testing approaches for *C. difficile* infection across Europe reporting the use of optimum testing methods only in two-fifths of hospitals (39). A recent systematic review reported a substantial global incidence, with the highest incidence rates occurring in hospitalized patients and marked heterogeneity between countries (40). More specifically, incidence rates of HA-CDI and community-associated CDI (CA-CDI) in the US were 8.00 and 2.00 per 10,000 patient days, respectively. Canada reported a rate of 4.3 per 10,000 patient days for HA-CDI, whereas CA-CDI data were unavailable. On the other hand, the highest incidence in Europe was reported in Poland (HA-CDI and CA-CDI 6.18 and 1.4 per 10,000 patient days, respectively), and the lowest incidence in the United Kingdom (UK) (HA-CDI and CA-CDI 1.99 and 0.56 per 10,000 patient days, respectively). In Australia, rates were 3.19 and 1.19 per 10,000 patient days for HA-CDI and CA-CDI, respectively. Over 2009–2019, no clear incidence trend emerged, with most countries showing stable rates. A point prevalence survey of healthcare-associated infections in 25 US hospitals found that *C.*

*difficile* remained the most frequent pathogen and confirmed that the prevalence of CDI remained stable between 2011 and 2015 (15% of all infections) (10). On the other hand, in Europe, the frequency of *C. difficile* causing healthcare-associated infections in acute care hospitals appeared to increase from 5.4% to 7.3% according to two point prevalence surveys, respectively, in 2011–2012 and 2016–2017 (41).

Considering all these findings, epidemiological differences in CDI frequency in different countries might be explained by different burdens of well-defined or conflicting risk factors. Established risk factors for both initial and recurrent HA-CDI are antibiotic use, recent hospitalization, proton pump inhibitor (PPI) use, and increasing age. The latter risk factor might be of particular interest, as the proportion of the elderly population is increasing worldwide. According to the latest figures from Eurostat (the statistical bureau for the European Union), the proportion of individuals over 65 years old accounted for 28% of the population in Japan, 20.3% in European countries, and 16% in the US (42). However, an epidemiologic review made in Japan (2006–2017) showed a lower incidence of CDI (0.8–4.71 episodes per 10,000 patient days) than that reported in Europe and the US (43). Considering different antibiotic use as risk factors for CDI, the highest incidence rates of CDI found in the US and Poland might be explained by the different antimicrobial use of prescribed medicines and by the high prevalence of 027 ribotypes. A report on antibiotic consumption in 76 countries over 16 years (2000–2015) showed that the US alongside France and Spain had the highest antibiotic consumption rates among the Western high-income countries (HIC) in 2000. These figures were essentially confirmed in 2015 when the leading HIC consumers of antibiotics were again the US and France, and also Italy (44). According to the European point prevalence surveys (2016–2017) (41), antimicrobial use in Poland was relatively low [36.7 defined daily dose (DDD) per 100 patient days] and much lower compared to the UK (64.2 DDD per 100 patient days) who had the lowest incidence of CDI in Europe (40). A recent review and meta-analysis confirmed PPI use to be associated with CDI, including recurrent CDI, both in adult and pediatric patients (45). A systematic review of data from 23 countries indicated that nearly one-quarter of adults used PPIs. Over the study period (1988–2022), the prevalence rates of PPI utilization continued to rise and were found to be quite similar between the US (6.7%) and some European countries (UK 7.7% and Denmark 7.4%) but not other (Spain 18.7%) (46).

Regarding controversial issues, the type of diet is a possible host-related risk factor for CDI. The Mediterranean diet was found to promote changes in the human gut microbiota, lowering the Firmicutes/Bacteroidetes ratio and increasing the presence of Bacteroidetes due to lower animal protein intake. In addition, higher Bifidobacteria counts and higher total short-chain fatty acids (SCFA) were found to be associated with greater consumption of plant-based nutrients (47). In mice experimental studies, a high-fat/high-protein diet may enhance CDI risk and severity during antibiotic treatment, whereas a high-carbohydrate diet may be protective despite high levels of refined carbohydrates and low levels of fiber (48).

According to this study, these findings might be due to the synergistic effect of a loss of microorganisms that normally inhibits *C. difficile* overgrowth and the abundance of an amino acid that promotes *C. difficile* overgrowth. On the other hand, a high-carbohydrate diet might be protective despite other authors finding that simple carbohydrates, specifically trehalose, are implicated in the proliferation of RTs 027 and 078 epidemic *C. difficile* strains (49). These epidemic ribotypes have been shown to acquire unique mechanisms for metabolizing low concentrations of the disaccharide trehalose. Nevertheless, increases in total dietary trehalose were likely minimal particularly in the US and Europe during the period 2000–2006 when the incidence of CDI rose substantially in the US and England because of the spread of hypervirulent RT027 (50). Therefore, the increase in CDI incidence due to epidemic RTs should be attributed to other causes rather than trehalose consumption.

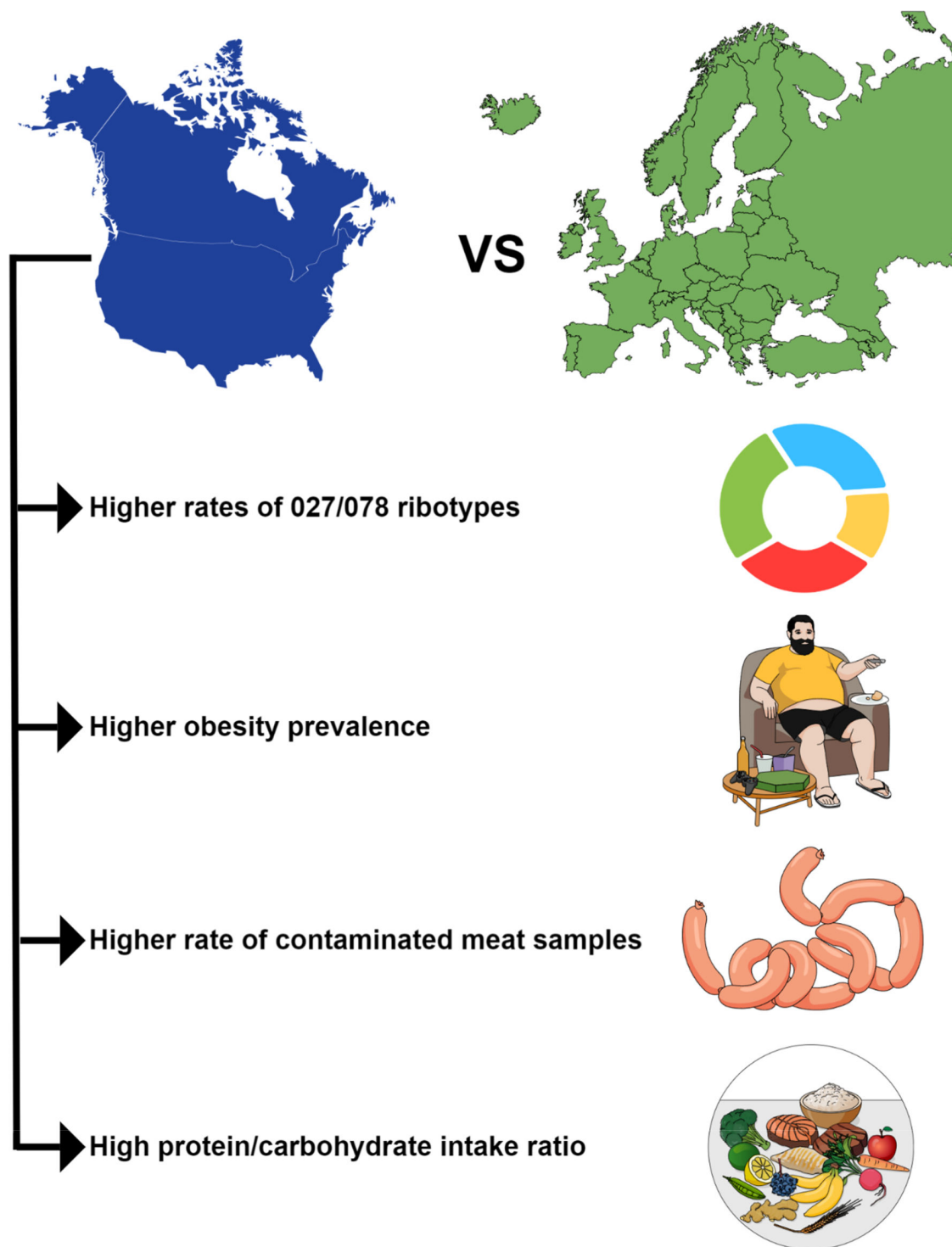
Another possible host-related risk factor for CDI is body mass index (BMI). The composition of intestinal microbiota has been shown to differ in lean vs obese animals

and human volunteers, with a decreased proportion of Bacteroidetes to Firmicutes in obese individuals (51). While a case-control study including 148 patients with CDI showed that a high BMI value was significantly associated with CDI (52), a recent systematic review and meta-analysis, which included studies published up to February 2021, demonstrated a significant negative association between BMI and CDI. Possible explanations of this paradoxical decrease in risk of CDI among obese individuals might include altered gut microbiota, underdosing of antibiotics due to high volume of drug distribution, concomitant medications exerting a protective effect against CDI (i.e. metformin), and comparators with increased risk of CDI (53). In a pooled analysis of BMI trends in adults from 1975 to 2014, Western countries showed different frequencies of obesity, with the highest and the lowest rates in the US and Poland, respectively (54). However, these countries showed the highest incidence of CDI according to the recent meta-analysis of Finn et al. (40), suggesting that the impact of BMI on the CDI frequency differences is questionable.

Although *C. difficile* is primarily a nosocomial pathogen, the prevalence of CDI in the community appears to be increasing in the US and, to a lesser extent, in Europe and other territories (11, 39). Moreover, patients with CA-CDI are generally younger, healthier individuals who lack well-known risk factors for CDI (55). *C. difficile* can be found worldwide in various environments including water, soil, and, as commensal or pathogen, in the digestive tracts of most mammals, birds, and reptiles. Furthermore, food products such as meat, fish, and vegetables could be potential sources of exposure to *C. difficile* by the fecal-oral route. Direct transmission of *C. difficile* from animals, foods, or the environment to humans has not been demonstrated, although overlapping PCR ribotypes from animals, foods, and human sources have been found. However, the ubiquitous nature of *C. difficile* and its capacity to persist for prolonged periods as an endospore make it very difficult, if not impossible, to ascertain the foodborne transmission of this organism. In a previous review, European studies have reported lower prevalence rates (up to 3% of meat samples), while in the US and Canada, *C. difficile* is generally reported at much higher rates, in up to 42% of meat samples. Additionally, RTs 078 and 027 have not been found in meat samples in Europe but represent the main RT in food in North America (56). A more recent review and meta-analysis (2009–2019) showed that the overall prevalence of *C. difficile* in all food samples was 6.3% (from 0.1% to 66.7%), but differences in different territories, especially Central/North America, Europe, and Asia, were not significant. Prevalence rates were similar in vegetables, meat, salad, and poultry (from 5.5% to 6.2%) but much higher in seafood samples (10.3%). Toxin genes were identified in 61.7% of *C. difficile* strains (57). Seafoods, including oysters, are well-known carriers of *C. difficile* with a high consumption of these foods in Asia and Europe, which might explain the similar prevalence of *C. difficile* in food in these two continents (58).

Another potential risk factor for CDI is climate. CDI appeared more frequent in cold climates, although this finding may be influenced by higher awareness in some studies (59). In detail, a clear seasonality for CDI with higher numbers of affected patients occurring in the winter months was observed in various countries such as Germany, Canada, and the Northeastern region of the US (60). Brown et al. hypothesized that this marked CDI incidence associated with the co-seasonality of influenza and pneumonia might be due to a combination of factors, including hospital crowding and increased use of certain antibiotics during wintertime (61). If climate influences CDI incidence, community-acquired *C. difficile* might play a role in explaining CDI peak incidence in cold climates. For instance, a Canadian study showed the highest prevalence of *C. difficile* in retail meats in winter, raising questions about the possible seasonality of this infection (62).

In conclusion, several factors likely interplay to lead to different incidences of CDI among various countries and territories. In particular, the main potential factors contributing to the higher CDI prevalence in North America compared to Europe are illustrated in Fig. 2. Besides well-known risk factors, diet, especially the Mediterranean



**FIG 2** Potential factors contributing to the higher CDI prevalence in North America compared to Europe.

diet, with low animal protein and high carbohydrate intake, appears to play a primary role in reducing CDI risk in countries where this diet is more widespread.

**RISK FACTORS**

When considering risk factors for CDI, it is important to keep in mind that they partly differ in terms of predisposing to a single CDI episode, recurrent CDI, severe/complicated



CDI, and/or fatal CDI. Genetics, resident microbiota, age, immunity, and comorbidities play a role in modulating the risk for hosts. Antibiotics and older age are the two most relevant risk factors for developing CDI. In the following section, we describe the main risk factors associated with CDI.

## Antibiotics

In relation to antibiotics, different classes confer different levels of risk, and there is also an intra-class stratification difference. It is important to understand that these differences are not fixed but rather evolve according to changing epidemiology (different strains and different susceptibilities).

In particular, the risk of antibiotic-associated CDI is increased if *C. difficile* is resistant to the antibiotic to which the patient has been exposed (63). In 1999, Johnson et al. demonstrated that CDI outbreaks occurring in the US between 1989 and 1992 were linked to the appearance of an epidemic *C. difficile* strain with high resistance to clindamycin [minimal inhibitory concentration (MIC) >256 µg/mL] (63). Similarly, in 2005, McDonald et al. demonstrated that outbreaks in the US between 2000 and 2003 were associated with the epidemic emergence of the RT027, which had a 100% resistance rate to moxifloxacin (3).

Usually, cephalosporins are among the antibiotics more commonly associated with CDI development. These drugs have been and still are widely used because of their favorable pharmacokinetic/pharmacodynamic properties, but current stewardship efforts are focused on reducing their use. *C. difficile* strains are intrinsically resistant to third-generation cephalosporins (64). The risk associated with cephalosporins decreases from third/fourth, to second, to first generation. In a recent longitudinal case-cohort study on nursing home residents in the US, cefixime, clindamycin, and moxifloxacin showed higher adjusted relative risk for CDI development: 4.26, 4.04, and 3.39, respectively (65). Within the same class, fecal excretion also matters; for example, cefotaxime is considered less impactful on gut flora compared to ceftriaxone since the former has no biliary excretion (66).

Apart from the specific antibiotic molecule, the duration of antibiotic exposure has a significant impact. A longer therapy causes a more prolonged disruption of the gut flora; a 14-day course of antibiotics confers an adjusted relative CDI risk of 27% compared to 7-day courses (65). CDI risk is higher during antibiotic courses within the first month after antibiotics [odds ratio (OR) 6.7–10.4] but remains elevated for 3 months after antibiotic cessation (odds ratio 2.7; 95% CI: 1.20–6.15) (67).

Moreover, adding a beta-lactamase inhibitor to a beta-lactam antibiotic confers an increased risk for CDI development (e.g., co-amoxiclav vs amoxicillin) because this combination causes a stronger disruption of the gut microflora, especially with increased activity against anaerobes (e.g., *Bacteroides*) (68–71). In this regard, aztreonam and aminoglycosides are weak inducers of CDI, likely due to no activity against anaerobes (68–70). Tetracyclines are another antibiotic class regarded as a weak inducer of CDI. Multiple data support the evidence that tetracyclines carry a low risk of CDI (or even a protective role); this should be taken into account for cases where discontinuing antibiotics is not possible (65, 72).

In Fig. 3, we categorized antibiotics according to their risk of CDI induction.

## Age

Age is an independent factor associated with CDI, recurrent CDI, severe CDI, and fatal CDI. Resident colonic microflora changes with age, with a decrease in inter-species diversity, therefore lowering resistance to colonization by pathogenic bacteria (78). A clear example is the progressive decrease of bifidobacteria with age. Bifidobacteria are associated with the production of health-promoting metabolites, including short-chain fatty acids, conjugated linoleic acid, and bacteriocins. It has been demonstrated that the relative abundance of bifidobacteria is 60%–70% in early life, 30%–40% in

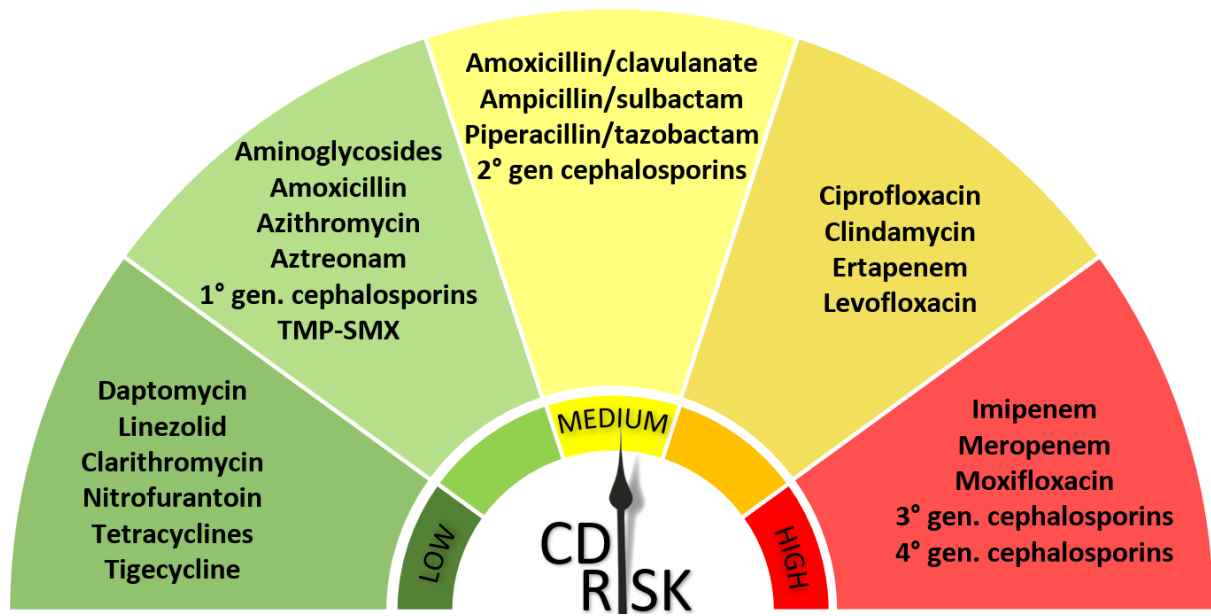


FIG 3 Approximate risk of CDI development according to different antimicrobials (65, 73–77).

early adulthood, 10% in late adulthood, and 0%–5% in the elderly (79). Furthermore, immunosenescence contributes to increased recurrence rates of CDI and correlates with worse clinical outcomes (80).

From an immunological point, both cellular and humoral immunity are impaired in elderly patients. Naive T cells, especially the CD8<sup>+</sup> subset, sharply decrease with age (81). This decrease in naive T cell numbers is believed to be a result of thymic involution in combination with ongoing differentiation of naive T cells into memory cells or effector cells. B cell response is also affected by age, and the quality of secretory IgA response may be altered (82). Additionally, phagocytosis and intracellular killing from polymorphonuclear cells (PMNs) are deficient in the elderly (83); this has specifically been demonstrated against *C. difficile* more than 30 years ago by Bassaris et al. They showed that polymorphonuclear cells from elderly healthy subjects exhibited a marked reduction in their ability to ingest *C. difficile* as compared to PMNs of young healthy subjects ( $P < 0.001$ ) (84).

In terms of epidemiology, large data on US inpatients provide us with CDI prevalence rates that we can assume are comparable with Western countries. CDI prevalence rates in inpatients were 0.14% for those aged 0–18, 0.31% for those aged 19–44, 0.84% for those aged 45–64, 1.35% for those aged 65–79, and 1.85% for those  $\geq 80$  years (85). As expected, attributable mortality also increases with age. In a review analysis conducted on 10975 CDI cases, Karas et al. reported pooled attributable mortality rates according to age groups: mortality was 2.5% for those  $< 60$  years old, 4.3% for the age group 61–70, 9.4% for the age group 71–80, and 13.5% for those  $> 80$  years old (8). These data clearly prove the impact of age on both disease incidence and outcome.

## Obesity

Obesity has been identified as a risk factor for CDI in many clinical reports. Studies on the gut microbiota of obese patients have demonstrated an increased Firmicutes-to-Bacteroidetes ratio, the same finding has been observed in CDI patients. Recent studies have confirmed an association between BMI and immunological and inflammatory molecules such as serum complement component 3 and C-reactive protein (86).

In a retrospective case-control study conducted in Israel, researchers found that patients with CDI had a significantly higher BMI compared to controls (33.6 vs 28.9,  $P = 0.001$ ), and the association remained significant also in the multivariable analysis with an

OR of 1.196 per one-unit increase in the BMI scale (52). Similarly, a study conducted in the US on 196 hospitalized CDI patients found that those with BMI >35 kg/m<sup>2</sup> were 1.7-fold more likely to be associated with severe CDI compared to those with a BMI 20–35 kg/m<sup>2</sup> ( $P < 0.005$ ), and BMI >35 kg/m<sup>2</sup> was an independent predictor of severe CDI ( $P = 0.038$ ) (87).

However, a meta-analysis published in 2022 reported that individuals with high BMI had significantly decreased odds of CDI with the pooled OR of 0.88 (95% CI 0.80–0.97) (53). How should we interpret these findings? It is likely that there is a U-shaped relationship between BMI and CDI, with both low and high BMI carrying an increased risk of developing CDI and its severe forms. A paper from Nathanson et al. supports this hypothesis. The authors analyzed 22,937 patients with CDI visited at the emergency department, finding that being underweight (BMI <19) or morbidly obese (BMI >40) was associated with an increased risk of in-hospital mortality in CDI patients (88).

## Hypoalbuminemia

Hypoalbuminemia is regularly recognized as a risk factor for CDI, rCDI, severe/complicated CDI, and fatal CDI. Hypoalbuminemia correlates with high inflammatory conditions and is commonly seen in critically ill patients independently of nutritional status (89). Indeed, hypoalbuminemia is observed in ~100% of patients admitted to the intensive care unit (ICU) after 48 hours, and the lower the albumin the worse the prognosis (90). There is evidence showing that during inflammatory phases, there is an increased “escape” of albumin into the extravascular compartment through the continuous capillaries. Moreover, the rate of albumin synthesis in the critically ill may be significantly altered (91), making replacement of losses insufficient. In addition, patients with CDI commonly experience protein-losing enteropathy which can further contribute to the “negative balance” of albumin metabolism in these patients (92). Albumin works as a buffer in maintaining acid-base homeostasis (91) and has a great chelating activity (93). Its role as a scavenger has also been tested against *C. difficile* toxins. It has been shown that human serum albumin can bind to toxin A and toxin B, preventing their internalization into host cells thus reducing the toxin-dependent glucosylation of Rho proteins necessary for toxin-induced cellular damage (94, 95). When human serum albumin was added to zebrafish embryos exposed to toxin B, their mortality decreased from 50% to 30% ( $P < 0.0001$ ) (95). In this context, it seems possible that human serum albumin could serve as a protein with an “immunity role” in CDI. However, human experimental studies are currently lacking.

## Sex

Female sex is more commonly associated with CDI (96). This is an exception compared to the majority of bacterial infectious diseases. In fact, females typically present stronger immune responses to self and foreign antigens, therefore being more prone to autoimmune diseases and, at the same time, less prone to infections. Several factors are implicated in this evidence: genetic, anatomic, immunological, and hormonal. From an immunological perspective, females display stronger innate and adaptive immune responses. Concerning *C. difficile*, adult females have greater antibody responses, higher B cell numbers (96), and higher immunoglobulin (Ig)M and IgG levels but lower IgA levels (97). CDI is a unique case where the disease is more common in females, accounting for 55%–60% of clinical episodes, with even higher percentages in community-acquired cases (98). In addition to the aforementioned substrates which contribute to the difference between sexes, behavioral factors could play an important role: females are usually more exposed to antibiotics in the community and are more often in contact with children, who are a well-known source of *C. difficile*. Despite higher CDI incidence in females, associated mortality is greater in males (99).

## Impaired humoral immunity

Several studies confirm the importance of humoral immunity in protecting humans from CDI pathogenesis. As early as 1985, Perlmutter et al. demonstrated that *C. difficile* was found in 24% of children with chronic diarrhea and hypogammaglobulinemia (100). In 2001, Kyne et al. demonstrated that rCDI was by far more common in patients unable to mount an adequate IgG response against toxin A (OR 48; 95% CI: 3.5–663) (101). In the following years, studies confirmed that low serum levels of IgG directed against both toxin A and toxin B were associated with recurrences (102).

From a real-world point of view, there is a substantial prevalence of hypogammaglobulinemia in transplant recipients (both hematopoietic stem cell and solid organ transplanted patients). In a study on 235 heart-transplanted patients, Munoz et al. found that severe hypogammaglobulinemia (<400 mg/dL) was more common among those who developed CDI compared to those who did not (71.4% vs 29.5%,  $P = 0.03$ ). In the multivariate analysis, the only independent risk factor for CDI after heart transplant was severe hypogammaglobulinemia (relative risk 5.8; 95% CI: 1.05–32.1;  $P = 0.04$ ) (103). Similarly, a case-control study on 41 kidney-transplant recipients indicated that patients who presented with a first CDI episode beyond the first month were more likely to have hypogammaglobulinemia ( $P = 0.002$ ). Poor outcome (graft loss and/or all-cause mortality) was more common among CDI cases [adjusted hazard ratio (HR) 5.69;  $P = 0.001$ ] (104). Low serum immunoglobulins are usually present in HIV-infected individuals. A case-control (1:2) study investigated risk factors for CDI among hospitalized HIV patients. Cases (HIV that developed CDI) had significantly lower gammaglobulin levels on admission compared to controls (HIV who did not develop CDI) (OR 0.68; 95% CI: 0.48–0.96) (105).

It is crucial to include gammaglobulin level measurement in the global assessment of CDI patients because secondary hypogammaglobulinemia is commonly detected in several populations/conditions such as transplanted patients, HIV, chemotherapy exposed, steroids and/or rituximab exposed, and chimeric antigen receptor-T therapy-treated patients.

## Gastric acid suppressants

Gastric acid suppressants have long been identified as predisposing to CDI. Even when H<sub>2</sub> antagonists were still in use, there was evidence of their role in predisposition in both colonization and infection (106, 107). It is important to note that clostridial spores are able to survive the acidic environment of the stomach; therefore, the relationship between gastric acid suppressants and CDI cannot be merely explained by the lack of acid barrier effect. So why do gastric acid suppressants predispose to CDI? Antacids are associated with intestinal dysbiosis: the long-term reduction of gastric acid secretion increases the risk of imbalances in the gut microbiota composition.

A meta-analysis of controlled observational studies published in 2018 assessed the relationship between PPI and the risk of CDI. It included 50 studies with a total of 342,532 individuals. The pooled analysis showed a significant association between PPI use and the risk of developing CDI (OR 1.26; 95% CI: 1.12–1.39;  $P < 0.001$ ) (108). In 2021, a systematic review and meta-analysis by Mehta et al. focused on the relationship between acid suppressants and recurrent CDI: they included 9 studies involving 5,668 inpatients, of whom 1,003 (17.7%) developed recurrent CDI. They found that those who received acid suppressants during the hospitalization were 64% more likely to develop recurrent CDI (OR 1.64; 95% CI, 1.13–2.38;  $P = 0.009$ ) (109).

Acid suppressants are not only associated with developing CDI (and recurrent CDI) but also with worse outcomes. In 2013, Shivashankar et al. analyzed data from 1446 inpatients with CDI (median age 62.5). Patients with severe-complicated CDI ( $n = 487$ ) were defined as those who required ICU admission, colectomy, or died within 30 days of CDI diagnosis. Multivariate analysis demonstrated that H<sub>2</sub>-blockers/PPI use was associated with an increased risk of severe-complicated CDI (OR 1.8; 95% CI: 1.3–2.6,  $P = 0.0002$ ) (110).

Another interesting study on 240 patients with CDI (mean age 69.1) with a 180-day follow-up included 91 patients who died within the follow-up period. Not only was daily use of PPI independently associated with mortality, but cumulative analysis confirmed the association of duration-dependent PPI usage with a high mortality rate. In fact, those who received PPI for 1–14 days had an HR for mortality of 1.91 (95% CI: 1.14–3.20), while those who received PPI for 15–28 days had an HR for mortality of 2.79 (95% CI: 1.69–4.59). The authors clearly demonstrated significant differences in the gut microbiota of CDI patients exposed and non-exposed to PPI (111).

A recent study conducted by Chinese colleagues found that patients receiving omeprazole were infected with *C. difficile* exhibiting higher fluoroquinolone MICs. Moreover, this study demonstrated that omeprazole facilitated *C. difficile* sporulation and germination by blocking the pathway of purine metabolism and promoting cell motility and toxin production by activation of the flagella (112).

While the Infectious Diseases Society of America (IDSA) guidelines state that there is insufficient evidence for discontinuation of PPIs as a measure for preventing CDI, they also state that stewardship activities to discontinue PPIs are indicated (15). Indeed, antacid overuse is a common and growing problem, with some papers reporting that more than 50% of CDI patients who were prescribed PPI lacked a valid indication (113). In that respect, CDI diagnosis may be a good opportunity to review patients' chronic medical therapy, discontinuing PPI use if no longer necessary.

## Renal impairment

Renal failure is a well-recognized risk factor for CDI. Understanding the extent to which this risk is borne by the underlying disease itself, or by higher rates of hospitalization, increased exposure to antimicrobials, or immune abnormalities in chronic kidney disease (CKD) patients is challenging. (114). From a purely "intestinal" point of view, dysbiosis is common among CKD patients. In these patients, a reduction in the number of *Bifidobacteria* and *Lactobacillus* has been demonstrated (115). In addition, the increased plasma concentration of urea and uric acid leads to greater excretion in the gut lumen, triggering a process that culminates in damage to the intestinal barrier (114).

Data from more than 150 million hospitalizations between 2005 and 2009 in the US showed an almost twofold increase in CDI incidence in patients with CKD compared to patients without CKD (1.5% vs 0.7%) (116). Moreover, CKD patients on dialysis were more likely to develop CDI compared to CKD patients not on dialysis (OR 1.33; 95% CI 1.32–1.35;  $P < 0.001$ ). When analyzed for CKD severity, it appeared clear that CDI incidence increases in more severe CKD (116). CKD is not only associated with CDI but also with recurrent CDI and severe CDI. In 2015, a meta-analysis on the risk of incident and recurrent CDI in CKD and end-stage kidney disease patients was published, including a total of 162,218,041 patients. The pooled risk ratio (RR) of CDI in patients with CKD was 1.95 (95% CI 1.81–2.10), the pooled RR of CDI in patients with end-stage renal disease (ESRD) was 2.63 (95% CI 2.04–3.38), and the pooled RR or recurrent CDI in patients with CKD was 2.61 (95% CI 1.53–4.44) and in patients with ESRD was 2.23 (95% CI 0.59–8.37) (117). A meta-analysis assessing clinical outcomes of CDI in patients with CKD and ESRD was performed by Thongprayoon et al., including 116,875 patients. The pooled RR of mortality risk of CDI in CKD/ESRD patients was 1.76 (95% CI 1.32–2.34) (118).

## PATHOGENIC DETERMINANTS

### Ribotypes

PCR ribotyping is the gold standard method used for typing *C. difficile* isolates and investigating CDI epidemiology (119). PCR ribotyping amplifies 16S-23S intergenic spacer regions, which vary substantially in size between strains, thus allowing typing with high discriminatory power, generating a pattern of bands in PCR amplifications that is unique for a specific PCR-RT (120, 121). The most prevalent RTs are 001, 002, 014/020, 017, 018, 027, and 078.

RT001 (often grouped with RT072) is one of the most frequent RTs in European countries. This strain is commonly identified in HA-CDI cases, but there are also some reports of CA-CDI (122, 123). Moreover, different reservoirs have been described for this strain, including goats and poultry, and seafood (124). RT001 has been reported as the cause of severe CDI in up to 70% of cases and has been strongly associated with resistance to moxifloxacin and levofloxacin (125). High-level resistance to erythromycin and ceftriaxone has also been reported (126).

RT002 is an emerging cause of severe HA-CDI but can also be a cause of CA-CDI. It has been considered an emerging ribotype since 2008, as a principal circulating ribotype in Asia (127, 128). RT002 is associated with fluoroquinolone and clindamycin resistance (129).

RT014 (frequently listed together with RT020 because they are difficult to distinguish) is among the 10 most commonly reported RTs across Europe, the US, and Australia (130–133). RT014/020 has been reported as one of the most predominant RTs among symptomatic (HA- and CA-CDI) and asymptomatic patients (134). Isolates of both animal and human origin were susceptible to first-line human CDI therapies and were resistant to clindamycin, erythromycin, and tetracycline (135). RT017 does not express toxin A due to a non-sense mutation at amino acid 47 (136). Moreover, it has been linked to severe disease, being mostly associated with higher 30-day mortality than the other RTs (137) and with resistance to clindamycin and rifampicin. Resistance to erythromycin and fluoroquinolones has been reported from Europe, while tetracycline-resistant isolates were reported from China (138, 139).

RT018 (ST-17, clade 1) has been reported as the predominant strain in Italian (140) and Japanese (127, 128) hospitals, mainly recovered from HA-CDI. RT018 has been associated not only with fluoroquinolone resistance (140) but also with resistance to macrolides and rifampicin (141).

RT027 (binary toxin positive) has been named the hypervirulent strain due to an increase in associated infections at the beginning of the new millennium. Whole-genome sequencing and phylogenetic analysis demonstrated the existence of two genetically distinct lineages (named FQR1 and FQR2) that originated in Canada and in the US and disseminated with distinct patterns: FQR1 was extensively reported in the US but later spread to South Korea and Switzerland; the FQR2 lineage spread more widely in the UK, continental Europe, and Australia (142). The typical fluoroquinolone resistance in these strains is thought to be the result of the widespread use of these antibiotics in the US during the late 1990s and early 2000s. This strain has been associated with higher virulence, mostly due to enhanced sporulation and toxin production. Additionally, the production of binary toxin has been linked to more severe disease (143, 144). Moreover, RT027 has been associated with a higher production of toxin (145) and with the production of a more lethal toxin B that is antigenically different from toxin B produced by other RTs (146).

RT078 (binary toxin positive) is a recently described hypervirulent ribotype that emerged in the Netherlands, mostly associated with a younger population and with CA-CDI (147). Moreover, it was isolated from environmental sources and animals (piglets) in Korea (148), Taiwan (149), and Japan (150).

## Adhesins

*C. difficile* surface proteins represent important virulence factors that facilitate colonization through adherence to the gut epithelium and activation of the host immune response. Among these, the S-layer is a conserved array of protein that envelops the cell and is composed of two subunits: the low molecular weight and high molecular weight S-layer proteins. There are 28 other cell wall proteins (CWP), which constitute 5%–20% of the S-layer and provide a range of additional functions (151). The S-layer provides a strong specific binding to human gastrointestinal tissue specimens, especially to the surface epithelium lining of the lumen and to HEp-2 cell lines (152, 153). In addition, the S-S-layer is implicated in sporulation and resistance to innate immune effectors,

including lysozyme and LL-37 (154). Many CWP are associated with pathogenesis and are often highly immunogenic. In particular, Cwp2 is the most expressed constitutive CWP. Since knockout experiments have demonstrated a significant reduction in adherence to Caco-2 cells, it has been suggested an important function as adhesin (155). Cwp66 was the first *C. difficile*-classified adhesin, as antibodies to Cwp66 reduced cellular adherence (156). Cwp22 is an L,D-transpeptidase, a peptidoglycan cross-linking enzyme implicated in toxin production, cell permeability, and autolysis, as well as reduced cellular adherence (157). Moreover, the CwpV can contribute to autoaggregative cell-cell interactions involved in colonization and biofilm-like growth (158). *C. difficile* produces two important collagen proteins: CD2831 and CbpA. CD2831 is a collagen-binding protein, which further promotes adhesion and biofilm formation while also modulating the classical immune response and promoting immune evasion (159). CbpA has been demonstrated to enhance collagen interaction and extracellular matrix adherence (160).

### Human receptors and cytokines

Combined repetitive oligopeptide sequences are believed to mediate toxin attachment to the cell surface via glycan-binding interactions. Two glycoproteins, sucrase-isomaltase (SI) and soluble glycoprotein 96 (gp96), have been reported as toxin A receptors. Moreover, additional receptor targets have been identified, including sulfated glycosaminoglycans and members of the low-density lipoprotein receptor family (161, 162). For toxin B, three classes of protein receptors have been reported: chondroitin sulfate proteoglycan 4, Frizzled 1 (FZD1), FZD2, FZD7, and Nectin 3. These proteins undergo constitutive endocytosis and recycling through clathrin-dependent pathways and thus could promote toxin entry (163, 164). The extracellular domain of lipolysis-stimulated lipoprotein receptor represents the host cell receptor of the *C. difficile* binary toxin (165).

The cytokine response to CDI has been investigated in humans. A case-control study revealed that patients with CDI produced significantly higher levels (at least twofold) of interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, IL-15, IL-16, IL-17A, and tumor necrosis factor alpha (166). Specifically, IL-1 $\beta$ , IL-6, IL-8, IL-17A, and IL-16 were the most upregulated, while IL-7 was lower in CDI patients. The authors also assessed the prognostic value of cytokine measurements and found that elevated serum levels of IL-2 and IL-15 were associated with a poor prognosis in CDI patients, whereas high levels of IL-5 and gamma interferon (IFN- $\gamma$ ) were linked to less severe disease (166). Similarly, Abhyankar et al. demonstrated a higher mortality in CDI patients in the top 25th percentile for TNF- $\alpha$  (HR = 8.35,  $P$  = 0.005) and IL-8 (HR = 4.45,  $P$  = 0.01) (167). In a mouse model, another group of researchers demonstrated that IL-33 drives activation of colonic group 2 innate lymphoid cells during infection, preventing *C. difficile*-associated mortality (168). Animal studies investigating the potential of cytokine inhibitors are currently being published (169, 170), and we will see if there is a possibility to translate the potential of these molecules into humans.

### Sporulation

*C. difficile* spore formation represents a transmission route for direct patient-to-patient spread and infection from contaminated surfaces in the environment. Furthermore, sporulation is responsible for the persistence and recurrence of *C. difficile* in patients. Environmental stimuli (e.g., nutrient deprivation or quorum sensing, stress factors, pH) could trigger *C. difficile* sporulation, with principal molecular regulators represented by CodY and CcpA, which are nutritional sensor proteins working as negative regulators (171). Moreover, Spo0A functions as a critical regulator for sporulation by regulating sporulation-specific RNA polymerase sigma factors, especially for  $\sigma$ E and  $\sigma$ K (mother cell specific),  $\sigma$ F and  $\sigma$ G (forespore specific) (172). Spores provide resistance to oxygen, UV, desiccation, heat, many disinfectants, and antibiotics. Efficiency in sporulation can vary between strains. High efficiency provides *C. difficile*-enhanced transmission in the hospital setting (173). Sporulation begins with an asymmetric septation that separates the larger portion of the mother cell from the smaller forespore portion. Afterward,

the forespore is engulfed by an intracytoplasmic double-membraned prespore, in a phagocytosis-like event. At this point, the spore cortex and coat layers are assembled, and the mother cell lyses, and later, the mature spore is released into the surrounding environment. Spores present a multi-layered structure, with each layer contributing to the overall resilience. The core represents the inner layer of the spore and contains the genomic DNA, mRNA, ribosomes, and proteins. The dense core is dehydrated due to the presence of up to 25% dipicolinic acid conjugated to calcium ions (Ca-DPA), and the DNA is compacted and bound to the small acid-soluble proteins that protect it from UV damage. The core protects the DNA from heat-induced damage. An extremely impermeable inner membrane and the primordial cell wall surround the core. A thick layer of cortex peptidoglycan is then synthesized exterior to the primordial layer. This layer is composed of approximately 25% of the N-acetylmuramic acid moieties that are modified to muramic- $\delta$ -lactam, with few crosslinks between adjacent N-acetylmuramic acid N-acetylglucosamine polymers that confer a more flexible structure (174). A second membrane, derived from the mother cell, envelops the cortex. The coat and the exosporium constitute the outermost protein layers of the spore.

### Germination

*C. difficile* spore germination is triggered in response to certain host-derived bile salt germinants (e.g., cholic acid derivatives) and amino acids (e.g., glycine or alanine), using the subtilisin-like, CspC pseudoprotease as the bile acid germinant receptor (175, 176). Interestingly, cholic acid and chenodeoxycholic acid derivatives from the gut microbiome could impact the *C. difficile* life cycle (177). SleC hydrolase is activated by CspB protease, which cleaves the N-terminal pro-sequence from the protein. Activated SleC degrades the cortex leading to hydration and to CaDPA release from the spore core in response to osmotic swelling. The mechanosensing protein SpoVAC allows CaDPA release from the core (178). GerG and GerS proteins have a pivotal role in *C. difficile* spore germination. Particularly, isolates with modifications in these determinants result in spores with germination defects, reduced responsiveness to bile salt germinants, and cortex degradation defects (179, 180). Ca<sup>2+</sup> represents an essential cofactor for *C. difficile* spore germination. Indeed, Ca<sup>2+</sup> works with glycine to stimulate germination, and it may play a role in the activity of the CspB serine protease, the CspC germinant receptor, the CspA pseudoprotease, or in the activity of the cortex hydrolase. Some cortex-degrading enzymes also require Ca<sup>2+</sup> (181).

### Toxins

Many *C. difficile* strains contain genes that encode up to three different toxins, which have been linked to the onset of clinical symptoms. Toxins are considered the major virulence factor in CDI pathogenesis. The genes encoding toxin A and toxin B are found in a 19.6-kb DNA region that also contains genes for four additional proteins (TcdR, TcdE, TcdL, and TcdC), with an important role in the regulation of expression and secretion (161). In particular, the activity of TcdR is modulated by activators and repressors and is influenced by environmental changes such as changes in temperature and nutrient-availability (182). Subsequently, toxin A and toxin B are secreted through TcdE, a protein predicted to adopt a holin-like function (183). Toxin A and toxin B are 308 and 270 kDa in size, respectively, and share 47% sequence identity. Their toxic action is related to the glucosyltransferase activity. More specifically, toxin A and toxin B bind the host receptors, followed by pore formation and translocation. Then, toxins mediated their action through glucosylation of host GTPases. Inactivation of the Rho-family GTPases leads to changes in the actin cytoskeletal structure, the secretion of cytokines, cell cycle arrest, and, ultimately, cell death (184). Disruption of focal adhesions and tight junctions in epithelial cells are thought to contribute to a reduction of barrier function and the onset of diarrhea. Additionally, the secretion of pro-inflammatory chemokines and cytokines further amplifies the intestinal damage associated with CDI. In epithelial



cells, the toxins induce caspase 3/7-mediated apoptosis. The intoxication leads to cell death, which may contribute to the formation of pseudomembranes and/or necrotic lesions in the colon (185–188).

The binary toxin is an ADP-ribosylating toxin, composed of two proteins: an ADP-ribosyltransferase enzymatic component involved in modifying host cell actin and a second component involved in binding to host cells and translocating CDTa to the cytosol (189). Binary toxin ADP-ribosylates cellular actin at Arg177, producing ADP-ribose and nicotinamide, prevents actin from further polymerization. This leads to complete depolymerization of the actin cytoskeleton, resulting in loss-of-barrier function and disruption of tight junctions (190). In addition, actin polymerization results in the redistribution of the microtubule network, affecting intracellular transport, cell division, and cilia formation while also providing long cellular protrusions that increase *C. difficile* adherence to host cells (191). Binary toxin represents a putative factor associated with increased virulence of the hypervirulent RT027. Its substantial contribution to *C. difficile* pathogenesis has become apparent in the rare events of infections sustained by strains that lacked toxin A and toxin B production but were positive for binary toxin production. These strains caused symptomatic infections (192). Binary toxin contributes to increased virulence and disease severity by activating the inflammatory response, with elevated IL-6 cytokine levels, inducing inflammation via the Toll-like receptor 2-dependent pathway, and suppressing the protective host eosinophil response (193).

### TOXINOGENIC CLOSTRIDIODES DIFFICILE CARRIAGE

An infectious disease might be defined as the result of the multiplication of a diffusive pathogen in host tissue and inflammation or the host tissue response to the pathogen. In fact, the mere carriage of a pathogen (for instance, *Candida albicans*), although necessary, is not alone sufficient to cause an infectious disease. Many pathogens are part of the resident tissue flora, being involved in a complex interplay between the host's immune system and the remaining species of the resident flora. For instance, *C. albicans* is part of the healthy gut microbiome, but the transition to tissue invasion and damage might lead to clinical diseases such as bloodstream infections (BSIs) (194). CDI, as a human infectious disease, is not an exception. In fact, the acquisition of toxigenic *C. difficile* frequently results in carriage without clinical illness. Conversely, a successful treatment of a CDI episode might lead to asymptomatic carriage with prolonged spore shedding. Moreover, asymptomatic carriage of toxigenic *C. difficile* is relatively common in healthcare facilities, with prevalence or incidence rates ranging from 7% to 18% in hospitals and up to 51% in long-term care facilities (195).

For instance, in a recent multicentric study on the natural history of *C. difficile* carriage, in a cohort of 1,432 patients negative at baseline, 9.9% acquired asymptomatic carriage of toxigenic *C. difficile*, and 13.4% of these patients were subsequently diagnosed with CDI within 12 weeks of enrollment. Only 39 patients (2.7%) developed CDI without prior carriage detection. Additionally, asymptomatic carriage was frequently a transient phenomenon, while in 39% of asymptomatic carriers, persistently positive cultures lasted for months, with 77 days as median time of colonization clearance (196). These findings might suggest that exposure to *C. difficile* spores is more likely to result in transient carriage or “pass-through” with positive stool cultures rather than stable colonization.

It has been suggested that asymptomatic *C. difficile* carriers might be protected against CDI and disease progression (197). Serum IgG levels were higher in asymptomatic patients compared to patients who subsequently developed CDI, and patients with mild manifestations of the disease have been shown to have increased IgM levels (against toxin and non-toxin antigens) compared to patients with recurrent disease (101, 198). On the other hand, colonization with non-toxigenic strains of *C. difficile* also has a protective role against CDI due to competition in the same ecological niche, but non-toxigenic strains might acquire the pathogenicity locus from toxigenic strains by transconjugation (199).

However, despite the potential resilience against CDI, *C. difficile* asymptomatic carriers might act as an infection reservoir and may pose a risk to others. Nevertheless, transmission potential is lower compared to patients with symptomatic CDI, and there is no conclusive evidence supporting the extension of contact precautions to *C. difficile* asymptomatic carriers during their hospital stay (199).

## CLINICAL FEATURES

Clinically evident CDI can range from mild diarrhea to fulminant colitis with toxic megacolon. Patients usually present with general malaise, diarrhea (~100%), fever (30%), and abdominal pain (20%) (71). Abdominal pain, when present, usually is localized in the lower abdominal quadrants. Hospitalized patients with diarrhea occurring >72 hours after admission who are receiving antibiotics are much more likely to have CDI than an infection caused by an alternative enteric pathogen (200). Usually, diarrhea begins during or shortly after receiving a course of antibiotic therapy, it may arise after days or even weeks after therapy termination (71). Stools are typically watery, with a characteristic foul odor; hematochezia is very uncommon (71). Diarrhea could persist for days despite the clinical improvement; therefore, the subjective evaluation and biohumoral improvement are indicative of a favorable trend even in the absence of a normalization of the bowel movements. In a few cases, diarrhea may be absent (e.g., in postoperative patients or those receiving opioids). On the other hand, severe CDI may cause paralytic ileus that can evolve into toxic megacolon with significant systemic involvement and a high mortality risk (71). Hypoalbuminemia is very frequently observed among CDI patients (201). The basic clinical features of CDI patients are illustrated in Fig. 4.

Depending on pre-existing conditions (e.g., heart disease) and the severity of hypoalbuminemia, the patient could paradoxically appear edematous, although being in intravascular water volume deficit. Hematochezia is uncommon and should suggest an underlying inflammatory bowel disease (IBD) or an occurring complication. Sweating is also uncommon among these patients. Cardiac function needs to be carefully evaluated since usually metabolic acidosis and hypokalemia (arrhythmia risk) coexist. Atrial fibrillation is detected in 39% of hospitalized patients with CDI diarrhea vs 17% of hospitalized patients with non-CDI diarrhea ( $P < 0.01$ ) (202). It is important to keep in mind that, in animal studies, *C. difficile* toxins were demonstrated to induce cardiotoxicity (203, 204). Mucous membranes could be dry, reflecting different levels of dehydration, and ultrasound measurements of the inferior vena cava could be useful in assessing the volemic state of the patients, especially in non-intensive settings (205). Tachycardia and hypotension are relatively common signs, with a prevalence of 71% and 46%, respectively (206). Severe CDI episodes may be further complicated by mental state change (207), reflecting hypoperfusion and toxemic state. Dehydration, combined with hypoalbuminemia (and toxemia), is very common and frequently leads to tissue damage potentially resulting in acute kidney injury, a defining criterion of a "severe" CDI episode (208). Importantly, *C. difficile* toxins can directly damage parenchymal organs (e.g., heart, kidneys, and brain) (209). For all the above reasons, in most severe cases, continuous ECG, arterial blood pressure, and urine output monitoring should be started, and a careful fluid balance calculation should be considered.

From a biochemical perspective, white cell count, serum creatinine, C-reactive protein, and erythrocyte sedimentation rate are commonly elevated, and their trend should be monitored. Serum potassium can be low due to fecal loss or elevated because of acute kidney injury and/or metabolic acidosis. When hypokalemia is present, checking serum magnesium level is important, and if low, magnesium correction is very helpful for potassium replacement to be effective. Procalcitonin is usually normal in mild CDI, but when it is high, it can indicate a moderate/severe CDI (210) or even a bacterial superinfection through gut translocation (211). Another important consideration, especially if sepsis or septic shock develops, is the possibility of candidemia (translocation from a damaged gut). In this context, blood cultures and serum beta-D-glucan measurement can be useful, but clinical suspicion remains fundamental, especially in critically ill

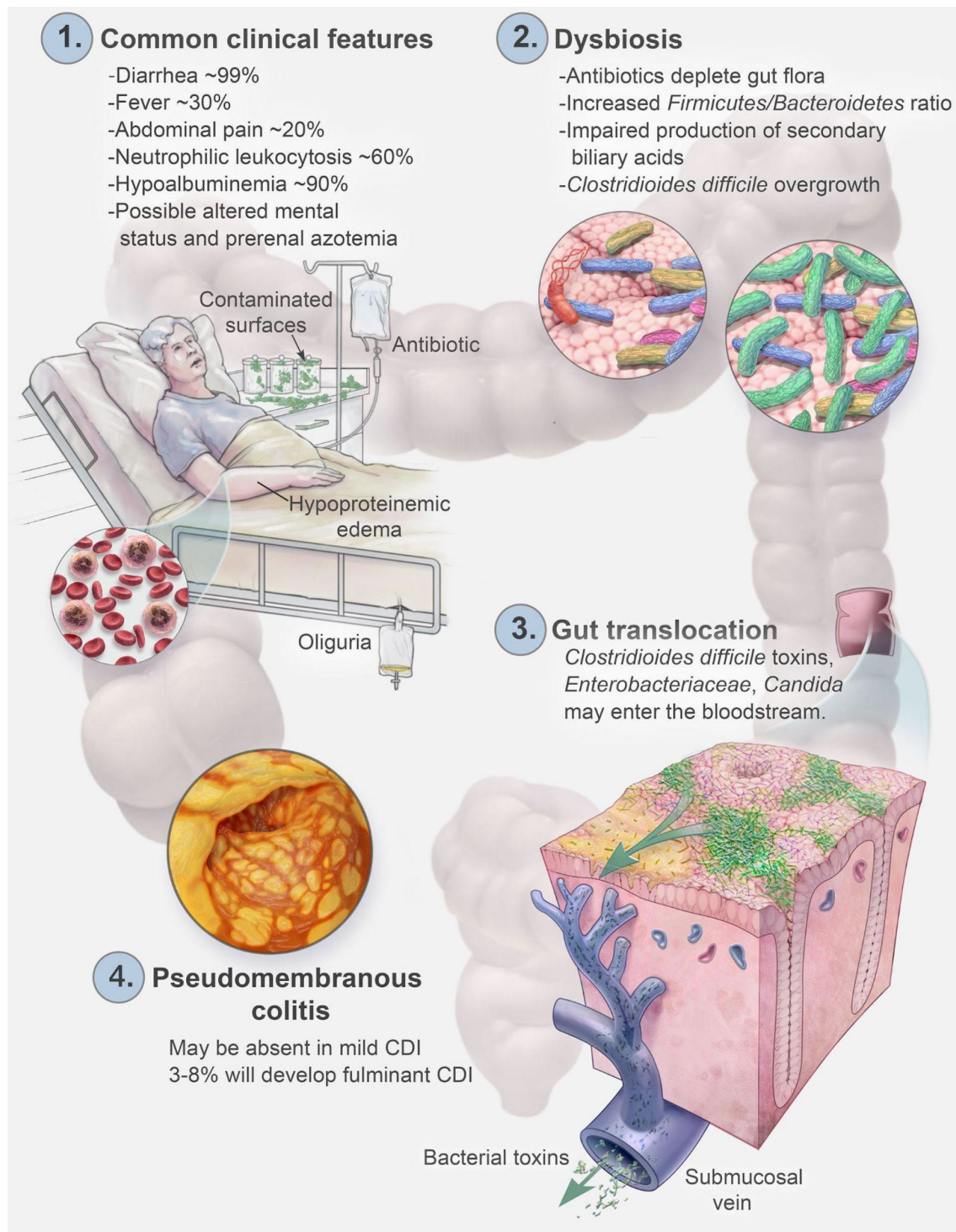


FIG 4 Main clinical features of CDI.

patients (211). Lactate measurement (and its trend) is also important and was mentioned in past guidelines as a marker to decide when to request surgical consultation for colectomy (207). The finding of eosinopenia on complete blood cell count can also be useful since it is associated with infections and can be used to identify more severe episodes but also when a differential diagnosis of diarrhea is needed (e.g., patients with inflammatory bowel disease and *C. difficile* toxin detection) (206). A low absolute eosinophil count in CDI has also been associated with an increased mortality risk (111).

Imaging in CDI is of limited usefulness because it almost always relies on the ability to identify possible complications (e.g., perforations) or concomitant conditions (e.g., diverticulosis and diverticulitis). Imaging studies can be useful in providing information on disease extension (e.g., megacolon and ileum involvement) or can be useful before a surgical evaluation for eventual colectomy. Characteristic computed tomography (CT) scan features in CDI include colonic-wall thickening, pericolic stranding, the “accordion sign,” the “double-halo sign,” and the presence of ascites (71). Detailed diagnostic features will be discussed in detail in the “Diagnosis” paragraph.

Recurrent CDI is common. Approximately 15%–25% of patients with an initial CDI episode will develop recurrent infections despite the lack of additional risk factors (15). In theory, subsequent episodes of CDI are usually less severe compared to the first episode; however, if they are temporarily close or if the patient is very frail, they may contribute to unfavorable outcomes. It is also important to note that loose stools may persist for quite a long time after an episode and that premature re-testing may lead to false-positive results; therefore, a careful clinical assessment is needed. In some cases, serial determinations of fecal calprotectin (FC) may be helpful in identifying a trend toward amelioration in terms of intestinal neutrophil recruitment/activation (212). Similarly, stool IL-1 $\beta$  has been used to differentiate CDI from non-CDI diarrheas (213). A deficient humoral immunity is strongly related to experiencing multiple CDI recurrences (refer to *Clostridioides difficile* and Immunity).

## EXTRAIESTINAL CLINICAL MANIFESTATIONS

Although *C. difficile* has no particular tropism for extraintestinal locations, in the literature, there are several reports of *C. difficile* isolated from different extraintestinal sites. Extraintestinal *C. difficile* spread is common in patients experiencing severe episodes when hematogenous dissemination occurs due to a significant gut disruption. However, these cases are really uncommon. Instead, what is more common is the occurrence of toxemia. It is important to distinguish between extraintestinal localization of *C. difficile* (microorganism in tissues) and extraintestinal effects of *C. difficile* (toxin-mediated damage).

Regarding the former, evidence of *C. difficile* presence in extraintestinal sites is found approximately in 0.17% of CDI cases: small bowel involvement, bacteremia, visceral or soft tissue abscess formation, infection of implanted prosthetic devices, wound infections, and osteomyelitis are the main reported extraintestinal localizations (214, 215). As expected, *C. difficile* bacteremia is more common among patients with gastrointestinal disruption caused by malignancies (216).

In terms of “toxemia,” growing evidence shows that *C. difficile* toxins reach the bloodstream more commonly than previously thought, probably due to an increased detection capability of testing tools. Toxins can contribute to extraintestinal organ damage. Almost all organs (kidneys, heart, liver, thymus, etc.) can be interested in this process. Granata et al. reported a new semi-quantitative diagnostic method to measure serum *C. difficile* toxin levels, capable of detecting picograms of toxins per microliter. Using this assay on 35 CDI patients, the authors found detectable toxemia in 94% of the patients (33 out of 35), thus demonstrating that the detection of toxemia is mainly a matter of sensitivity of the diagnostic tool (217).

From the perspective of organ damage, heart, kidneys, and brain have evidence of toxin-induced injury, mainly deriving from *in vitro* or animal studies. In particular, toxin B has been demonstrated to have cardiac tropism in zebrafish studies more than 15 years ago (203). More recently, Tonon et al. demonstrated that *C. difficile* toxin A and toxin B induced cardiovascular damage in an animal model through four mechanisms: (i) direct toxicity, (ii) hormonal stimulation, (iii) vascular endothelium alteration, and (iv) pro-inflammatory stimuli (204). Zebrafish exposed to *C. difficile* toxins experienced a decrease in heart rate as a sign of depressed cardiac function (204). In a recent interesting animal study conducted on mice, Mileto et al. demonstrated that bezlotoxumab was able to block systemic disease complications, thymic atrophy, and kidney inflammation

but did not prevent gut damage (218). Regarding the brain, it is well known that CDI patients can experience mental status alterations, a phenomenon that is currently being investigated (219–222). Di Bella hypothesized that, similarly to what happens with *Clostridium botulinum* and *Clostridium tetani*, *C. difficile* toxins may pass the blood-brain barrier, therefore exerting direct damage on human neuronal cells. Mice experiments on this topic are ongoing.

## COMPLICATIONS

CDI complications have a significant impact on clinical outcomes, including mortality. In this section, we will focus on complications specific to CDI and not those shared by other infectious processes.

Intestinal perforations are undoubtedly among the most dreaded complications, although rare. It is important to note that perforations can involve both the colon and/or the terminal ileum (223). Perforations are more likely to occur in an ectatic colon, which is defined as megacolon when the diameter of the transverse colon is >6 cm, with loss of haustration on radiologic examination (224, 225). The prevalence of toxic megacolon among CDI patients is between 0.4% and 3% (226), with higher rates occurring among patients older than 80 years (227). In a Canadian paper published in 2015 with follow-up data on 1,367 hospitalized CDI patients, toxic megacolon occurred in 1.1% of cases (228), having 2 patients experienced gut perforation and 16 underwent hemicolectomy.

Since CDI affects the colon in the vast majority of cases, the risk of bacterial and fungal gut translocations is high, with the colon acting as the port of entry. In a retrospective study on 393 cases of CDI, 79 developed a primary nosocomial BSI, while 321 did not. Etiologic agents of BSI were *Candida* species (47.3%), *Enterobacteriaceae* (19.4%), enterococci (13.9%), and mixed infections (19.4%). In-hospital mortality was 76.3% in CDI patients with BSI and 21.8% in CDI patients without BSI (229), as evidence of the significant impact of BSI on the outcome.

Concerning *Candida*, its link with *C. difficile* seems to be a two-way relationship: gastrointestinal colonization from *Candida* promotes CDI, and a CDI predisposes to a *Candida* translocation (230–232). Additionally, *Candida* and *C. difficile* share the same risk factors such as antibiotic therapy, impaired immune system, old age, and antacids. Longitudinal population-based surveillance reported that candidemia developed in 0.8% of CDI cases within 120 days (233).

Neurological complications should also be considered in CDI patients. It is well known that metronidazole, especially when administered for prolonged periods, can manifest its neurotoxicity, presenting as cerebellar syndrome, encephalopathy, seizures, and autonomic, optic, and peripheral neuropathies (234). Hypomagnesemia, often resulting from diarrhea, may lead to posterior reversible encephalopathy (235). The appearance of an altered mental status is relatively common in severe cases in elderly patients.

Lastly, CDI patients may become seriously ill and require ICU admission. In the Western world, approximately 3% of hospitalized CDI patients require ICU admission for CDI management (228). Hospitalized CDI patients experience a 12% 30-day mortality rate and a 22% 90-day mortality rate (228). Age strongly correlates with mortality (8), and immunosuppressive therapy is associated with a nearly 70% increased risk of both ICU admission and death (236).

## CLOSTRIDIODES DIFFICILE AND IMMUNITY

The human host interacts with *C. difficile* through several defense mechanisms of innate and adaptive immunity. Innate immunity defense mechanisms influence the primary response to CDI and may be summarized as follows: physical barriers, chemical barriers, microbiological barriers, and cellular immunity. Physical barriers are represented by the intestinal epithelium, which physically segregates the gut flora from the circulatory system (237).

Chemical barriers include antimicrobial peptides secreted by the Paneth cells. These cells, localized in the crypts of Lieberkühn, secrete several oligopeptides, such as defensins, with several immunological functions, both as antimicrobial peptides with broad antimicrobial spectrum and as immunomodulators. For instance,  $\alpha$ -defensin 5 has a direct antimicrobial effect, and it is also a potent lectin that binds bacterial exotoxins (238). Microbiological barriers are represented by the gut microbiota which competes and interacts with *C. difficile* populations. The intestinal microbiota is composed of a collection of bacteria, archaea, viruses, phages, fungi, and eukaryotic microorganisms that live in the human gut. This microbiological barrier interacts with *C. difficile* through various mechanisms, including (i) competition for available nutrients; (ii) stimulation of gut immunity; (iii) increased synthesis of short-chain fatty acids such as butyrate—that indirectly inhibits *in vitro* *C. difficile* growth and enhances gut defense barriers; (iv) regulation of metabolism of bile salts from primary bile acids to secondary bile acids, such as chenodeoxycholic acid, which inhibits *C. difficile* germination (239).

Regarding cellular immunity, enterocyte death and loss of epithelial integrity by the action of toxin A and toxin B result in the translocation of intestinal microbiota and subsequent secretion of pro-inflammatory cytokines, chemokines, and reactive oxygen and nitrogen species by the resident immune cells and intestinal epithelial cells (237). Among the secreted interleukins (such as IL-1, IL-8, IL-10, IL-12, etc.), it was recently found that the IL-27/human cathelicidin antimicrobial peptide (LL-37) axis might have a pivotal role in the innate immunity against CDI (240). IL-27 induces the expression of the human cathelicidin LL-37 (an antimicrobial peptide) by human colonocytes. A murine study conducted on IL-27-receptor-deficient mice and their impaired expression of cathelicidin-related antimicrobial peptide (CRAMP—mouse homolog for LL-37) has shown that restoration of CRAMP improved *C. difficile* clearance and reduced mice mortality caused by CDI. In clinical samples from 119 CDI patients, elevated levels of IL-27 were positively correlated with LL-37 in the sera and stools (240). Moreover, inflamed enteric glial cells, the intestinal equivalent of microglia in the central nervous system, overexpress S100B (a neurotrophin with trophic function at nanomolar concentrations and pro-inflammatory function at micromolar concentrations), which initiates the deleterious gliotic reaction with the maintenance of the inflammation in the gut (241). Increased S100B levels were found in colonic biopsies from CDI patients and colon tissues from *C. difficile*-infected mice (242). Besides, toxin A and toxin B were shown to upregulate S100B-mediated IL-6 expression, and inhibition of S100B activity was shown to ameliorate the intestinal injury and diarrhea caused by *C. difficile* toxins (242).

As mentioned earlier, neutropenia poses a risk factor for CDI as neutrophils are important in the immune response to CDI. While murine studies have shown an increased mortality in *C. difficile*-infected mice following the depletion of GR1+ (Ly6G) cells or an increase in mice mortality following the knock-out formation of inflammasome and neutrophils recruitment, other studies suggest that neutrophils contribute to tissue damage, and predominantly, neutrophilic inflammation is the main histological characteristic of CDI (237, 243). For instance, antibody-mediated inhibition of neutrophil recruitment in rabbits and rats was correlated with a reduction in toxin A-mediated enterotoxicity (237). Concerning the immune response to *C. difficile* toxins, it was found that the binary toxin acts as a priming signal for inflammasome formation and reduces the activity of host eosinophils as the eosinophil count was found to positively correlate with enhanced epithelial integrity in a murine model. Colonic eosinophilia is considered to be protective against CDI (193, 244).

The adaptive immunity is also of great importance in defending the host against *C. difficile*. Generally, the antibody response against *C. difficile* involves specific antitoxin antibodies and antibodies against non-toxin antigens. Regarding antitoxin antibodies, seroprevalence (i.e., serum IgG and IgA) against toxin A and toxin B is relatively widespread in the healthy population (around 60% of adults and older children) even in the absence of colonization or active infection, possibly reflecting a persistent long-life exposure to *C. difficile* or other clostridial species (243, 245). Following primary CDI,

high-affinity IgA and IgG both neutralize toxins, while IgM generally characterizes the early phase of the immune response and usually manifests a lower affinity for the antigen. Dimeric IgA are secreted across mucosal surfaces, likely mediating immunity against toxin A and toxin B.

Immunity against toxin A and toxin B (elevated levels—vs low level or undetectability—of specific serum IgA, IgM, IgG, and fecal IgA) was generally shown to be protective against primary CDI and its recurrences (243, 245). Moreover, elevated serum antibodies (IgA, IgM, and IgG) correlate with *C. difficile* colonization compared to lower serum antibody levels demonstrated in CDI patients, suggesting that colonized hosts are protected by serum antibodies against the transition to infection (198, 243).

Lastly, immunotherapy with a humanized monoclonal IgG against toxin B (bezlotoxumab) was shown to significantly reduce recurrences of CDI, in two randomized trials, while the addition of a humanized monoclonal IgG against toxin A (actoxumab) did not improve efficacy (14). Even if these results confirm the protective role of antitoxin B serum IgG against recurrences, these results contradict a previous study that suggested a protective role of antitoxin A serum antibodies (101).

Immunity against non-toxin *C. difficile* antigens and cellular adaptive immune response (such as T-cell response) in *C. difficile* pathogenesis are less explored fields. For instance, patients with recurrent CDI fail to mount an IgM immune response to *C. difficile* surface-layer proteins (proteins with a role in bacterial adhesion) compared to patients with a single episode of CDI (245, 246). It was also suggested that recurrent CDI patients have different T-cell immune responses compared to controls, in terms of flow cytometry markers: recurring patients had a greater number of circulating CD3(+) lymphocytes skewed toward a Th1/Th17 inflammatory population as well as possible immune plasticity (Th17/Treg) (247).

## CLOSTRIDIODES DIFFICILE INFECTION IN SPECIAL POPULATIONS

### Pregnant women

Pregnant women are generally considered at low risk of CDI. The first case of obstetric CDI was described in 1985 in the US (248). Since the early 2000s, an increased incidence of CDI in pregnancy, peri-, and postpartum has been documented in North America (249–252). Canadian data reported that the incidence rate of obstetric CDI doubled from 1999 to 2013 (from 15 admissions per 100,000 deliveries in 1999 to 30 in 2013) (250). In the US, Saha et al. reported a 3.4-fold increase in the incidence of obstetric CDI from 1997 to 2017 (252). Epidemiological data on obstetric CDI in Europe are scarce and consist of few published case reports or case series (253–256).

Pregnancy, peri-, and the postpartum period put women at an increased risk of developing CDI. Main risk factors are prior antibiotic therapy and hospitalization (221, 250, 256, 256). Additionally, other recognized risk factors are gestational diabetes, cesarean delivery (250, 251, 255), age  $\geq 35$  years old, inflammatory bowel diseases, smoking, multiple pregnancies, presence of co-infections during pregnancy (251), chronic steroid therapy, obstetric complications (255), and prior surgery (257). Two other additional factors contribute to an increased risk of obstetric CDI. The first one is the unavoidable exposure of these women to toxigenic *C. difficile* strains of asymptomatic neonate carriers. The second is the shift from Th1 toward Th2 response of the immune system during pregnancy, reducing the production of antibodies against *C. difficile* toxins A and B (258).

Regarding the timing of CDI, the available data report conflicting results. According to Unger et al., half of the cases were described in the postpartum (250), while in the observational study of Saha et al., 51.5% of cases were reported during pregnancy, with the second trimester as the most affected (257). Ruitter-Ligeti et al. reported cases that occurred only in the peripartum (251). Only a few retrospective studies compared maternal and neonatal outcomes of obstetric CDI with healthy pregnant controls (250, 251, 257). Women with CDI during pregnancy are generally more likely to undergo cesarean delivery than controls (250, 257) and to face obstetric complications such

as postpartum endometritis, and chorioamnionitis (250). Furthermore, Ruiter-Ligeti et al. observed a significant increase in maternal mortality, sepsis, paralytic ileus, venous thromboembolism, and longer hospital stay in women with peripartum CDI as compared to controls (251). Only one study focused on neonatal outcomes (birth weight and neonatal complications), reporting no differences between cases and controls (257).

No specific recommendations about the management and treatment of obstetric CDI are provided in the IDSA guidelines (15). The American College of Gastroenterology (ACG) recommends using oral vancomycin to treat pregnant, peripartum, and, with caution, breastfeeding patients with CDI (259). The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) advises oral vancomycin during pregnancy (208, 260). Oral vancomycin crosses the placenta, and it is not secreted into the milk (261, 262). As for metronidazole, it should be used during pregnancy only if the benefits outweigh the risks. Its use during breastfeeding is controversial; some authorities advise to discontinue breastfeeding during the treatment (259, 263). There are no data on the safety and efficacy of both fidaxomicin and bezlotoxumab during pregnancy and breastfeeding (264). FMT has been safely performed also during pregnancy (265) and in the postpartum (257). However, the ACG guidelines recommend avoiding FMT in pregnant patients due to potential procedural risks (259).

## Children

The incidence of CDI in the pediatric population has increased over time in the US but not in Europe (266). While in adults CDI is mainly healthcare associated, in children it is mainly a community associated (267, 268). The predominant RTs in the pediatric population in Europe are 014/020 (269–271) and 265 (272), while in the US, 014/020 and 106 are predominant (273).

During the first months of life, colonization by *C. difficile* is common, reaching its peak by the end of the first year of age (274). According to a recent systematic review and meta-analysis, the prevalence of *C. difficile* colonization among children is 15% during the first week of life, 41% at 6–12 months of age, and 12% among children aged 5 or older. The prevalence of toxigenic *C. difficile* colonization peaked at 14% among infants aged 6–12 months and decreased to 6% among children aged 5 years or older (274). Some specific pediatric populations show higher rates of colonization. Among these are patients with IBD (275), cystic fibrosis (276), or cancer (277), transplant recipients (278, 279), and preterm neonates (280).

Risk factors for pediatric CDI are the same as those described in “Risk Factors” for adults, except for advanced age (266). Some special pediatric populations with higher rates of colonization are also at higher risk of CDI. Among these are patients with IBD (275), cystic fibrosis (281), cancer, and transplant recipients (282). Patients with Hirschsprung’s disease have a higher risk of CDI (283, 284). The transmission among asymptomatic carriers in healthcare settings occurs less in children than adults (285, 286).

Despite high rates of colonization, children appear to be less affected by active infection compared to adults. The reasons are not yet well understood, but some hypotheses have been proposed over the years. The first hypothesis concerns the absence of *C. difficile* toxin receptors in infants, based on studies conducted on newborn rabbits (287). Additionally, age-dependent changes in intestinal microbiota have been proposed as protective factors against active infection (288), as well as the transplacental transfer of maternal antitoxin antibodies to newborns (289). Finally, Kocielek et al. recently demonstrated the association between *C. difficile* colonization and the production of IgA and IgG against toxins A and B in children aged 9–12 months. This humoral immune response could be the reason for the “protection” against active infection in infants (290).

CDI is relatively rare in neonates. Most children have mild or moderate CDI, with severe cases being less common than in adults (291). Some patients experience a more severe course of the disease (e.g., patients with IBD, cystic fibrosis, cancer, or



transplantation) (275, 278, 281, 291, 292). The rate of recurrence is 20%–30% after the first episode of CDI (290). Children with cancer (293–295) and IBD (296) are at high risk of experiencing recurrence of CDI. Other risk factors for recurrent CDI in children are recent surgery, prior antibiotic therapy (293, 295), transplantation (278), and tracheostomy tube dependence (294).

Distinguishing colonization from active infection can be challenging. When interpreting the results, it is essential to remember that young children and some special pediatric patients have high rates of colonization. It is also important to know when it is appropriate to test for *C. difficile*. The IDSA guidelines categorize the pediatric population into three age groups. For neonates and infants  $\leq 12$  months with diarrhea, testing for CDI is never routinely recommended, while for children aged 1–2 years, testing is recommended only when other infectious or non-infectious causes have been excluded. In contrast, for children  $\geq 2$  years, the testing is recommended for patients with prolonged or worsening diarrhea and risk factors or relevant exposures. Testing for *C. difficile* is recommended, regardless of children's age, in case of PMC, toxic megacolon, or clinically significant diarrhea (15). Similarly, the American Academy of Pediatrics suggests that testing infants  $\leq 12$  months should be limited to those with Hirschsprung's disease or other severe motility disorders or during outbreaks (297).

Oral vancomycin, metronidazole, and fidaxomicin are approved for use in the pediatric population. For non-severe pediatric CDI, discontinuation of antibiotics and appropriate rehydration could be sufficient without any antibiotic treatment (298). According to IDSA, for the first non-severe CDI episode, either metronidazole or vancomycin can be prescribed. For children with an initial episode of severe or recurrent CDI, oral vancomycin is recommended over metronidazole. For the initial non-severe episode, metronidazole dosage is 7.5 mg/kg three or four times daily for 10 days, and vancomycin dosage is 10 mg/kg four times per day for 10 days (15). Although no RCT comparing metronidazole with oral vancomycin in pediatric CDI exists, Yin et al. conducted an observational study in a propensity-score-weighted cohort of children with non-severe CDI, demonstrating that patients receiving oral vancomycin were more likely to experience clinical resolution by day 5 compared with those receiving metronidazole (299). Fidaxomicin is also considered safe and effective in pediatric CDI. The dosage of fidaxomicin for an initial CDI episode is 200 mg twice daily for 10 days, with adjustment needed in children weighing 12.5 kg or less. The Food and Drug Administration (FDA) approved fidaxomicin for children older than 6 months, while the European Medicines Agency (EMA) recommends caution in using it in this age group (300–302). The recent RCT SUNSHINE confirmed that the global cure without less recurrences was higher in patients undergoing fidaxomicin when compared to those taking oral vancomycin (68.4% vs 50%) (303). Thus fidaxomicin may be the preferred treatment for initial non-severe CDI episodes also in the pediatric population, according to current expert opinions (266). No RCTs are available comparing oral vancomycin with fidaxomicin for severe CDI treatment.

Regarding recurrent CDI, for second or subsequent recurrences, tapered regimens with vancomycin are suggested by IDSA (15). Bezlotoxumab and FMT are two other important tools for reducing CDI recurrences in adults. The MODIFY III trial recently proved that a single intravenous infusion of bezlotoxumab at the dosage of 10 mg/kg had PK and safety profiles similar to those observed in adults. This trial supports the use of bezlotoxumab also in children (304). FMT could be safely performed also in children, showing high efficacy (260). IDSA guidelines consider FMT in case of multiple recurrences of CDI following standard antibiotic treatments (15). Subsequently, the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition published specific guidance on FMT in pediatric CDI (305).

## Transplanted patients

Transplanted patients are at higher risk of experiencing CDI compared to the general population (306, 307). Diarrhea can be a very common complication in both solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) recipients. In addition to infectious causes, such as *C. difficile* or cytomegalovirus (CMV), we mention massive use of antibiotics (mainly for treatments but also for prophylaxis), long hospital stays, surgery, immunosuppressants, chemotherapy, and graft-vs-host disease (GVHD) (308, 309).

We now analyze separately the main features of CDI in SOT and HSCT populations. Table 1 summarizes the characteristics of CDI in transplanted patients.

### Solid organ transplant

CDI is a common problem for SOT recipients. In 2015, a meta-analysis estimated a prevalence of 7.4% of CDI in peri-transplant period, defined as “the time of transplant to the first discharge from the hospital” (310). In the same meta-analysis, pancreas (3.2%) and kidney (4.7%) transplant had the lowest prevalence. Heart (5.2%), intestine (8%), liver (9.1%), and lung (10.8%) followed. However, the highest prevalence was reported in patients with multiple transplants (12.7%) (310). Subsequent studies have confirmed this trend with patients, with kidney transplant recipients being the least affected and lung transplant recipients the most affected (311–313). CDI rates vary among transplanted organs in the studies due to different diagnostic methods and different follow-up periods after transplantation (306). SOT recipients have *C. difficile* colonization rates higher than non-transplanted patients (314). Keegan et al. first studied the association between *C. difficile* colonization and active infection. In their cohort (mostly kidney transplant recipients), the rate of toxigenic *C. difficile* colonization was 9.5%. In the multivariate analysis, only *C. difficile* colonization and hospital length of stay were independently associated with CDI (315).

Besides the classical risk factors for CDI, SOT recipients have additional factors, with more than 30 identified (306). Among these, we highlight hypogammaglobulinemia (see “Impaired humoral immunity”) (103), immunosuppressive therapy (316) and consequent low levels of *C. difficile* antitoxin antibodies (317), acute rejection (318), re-transplantation (319), Model for End-Stage Liver Disease score, and end-stage disease for liver transplant (320–322). Regarding immunosuppressants, Varma et al. retrospectively evaluated the relationship between the use of different classes of drugs and the risk of CA-CDI, finding the highest risk in patients using multiple classes and those taking calcineurin inhibitors (e.g., tacrolimus). Calcineurin inhibitors lead to attenuated IL-2 and IL-6, impairing humoral immune response and predisposing to CDI (316). Other previous studies found an association between corticosteroid therapy and the risk of CDI in SOT recipients (323–325).

The highest incidence of CDI in SOT recipients occurs within the first weeks, while late-onset CDI appears months or years after transplant (326). Recently, Hosseini-Moghaddam et al. conducted a population-based cohort study from 2003 to 2017 on 10724 SOT patients, finding that 60% of CDI were late onset (kidney transplant: median 2.2, IQR 0.4–6.0 years; non-kidney transplant: median 0.9, IQR 0.0–4.6 years) (313). In SOT patients, CDI shows a high rate of recurrence (327, 328) and severe course (310). The mortality rate in SOT recipients with CDI is higher when compared to non-transplanted patients (313, 329). CDI also is associated with an increased risk of loss of graft (311).

The management of the first episode of CDI, recurrences, and severe cases does not differ from that of the general population (326). AST guidelines indicate bezlotoxumab, in addition to the standard of care, for SOT recipients at risk for recurrent CDI (326). This recommendation was formulated on the basis of studies conducted in non-transplanted patients. Thus, Johnson et al. retrospectively evaluated the safety and the efficacy of bezlotoxumab plus standard of care in SOT and HSCT patients vs standard of care alone. In this study, bezlotoxumab significantly reduced the incidence of recurrent CDI in the transplanted population (330). FMT in SOT recipients is considered safe and

TABLE 1 Features of CDI in transplanted patients (SOT vs HSCT)<sup>a</sup>

CDI features	SOT	HSCT
Specific risk factor	Hypogammaglobulinemia, immunosuppressive therapy, low levels of <i>C. difficile</i> antitoxin antibodies, acute rejection, re-transplantation, MELD score, and end-stage disease	Low levels of <i>C. difficile</i> antitoxin antibodies, CMV and Herpesviridae reactivations, mucositis, bacterial infections, GVHD, previous chemotherapy, VRE colonization
Transplant with higher risk	Lung	Allogeneic
Most affected timing	First weeks after transplant	First weeks after transplant
Recurrence rate	Higher than general population	Similar to the general population
Outcomes (comparing transplanted to non-transplanted patients)	Loss of graft Higher mortality rate	Higher BSI incidence Higher mortality rate
Primary prophylaxis	Oral vancomycin 125 mg daily (not recommended by official guidelines)	Fidaxomicin 200 mg daily Oral vancomycin (not recommended by official guidelines)

<sup>a</sup>MELD, Model for End-Stage Liver Disease; VRE, vancomycin-resistant *Enterococcus*.

effective (331–333). AST guidelines recommend it in cases of multiple recurrences of CDI (323). ACG guidelines recommend appropriate donor screening before FMT (259). Currently, there is no evidence supporting the use of antibiotics in CDI prophylaxis in SOT recipients (15, 326).

### Hematopoietic stem cell transplant

HSCT recipients are nine times more affected by CDI than the general population (307). Furthermore, HSCT recipients have *C. difficile* colonization rates higher than the general population. Toxigenic *C. difficile* colonization was found to be a risk factor for CDI in this population (334–338).

Specific risk factors for CDI have been reported for HSCT patients: low levels of *C. difficile* antitoxin antibodies (317), CMV and Herpesviridae reactivations (339), mucositis (340) (339), bacterial infections within 100 days after transplant (341), GVHD (342) (312), previous chemotherapy (311, 341–343), and vancomycin-resistant *Enterococcus faecium* colonization (342). GVHD, in particular, could act both as a risk factor of CDI and a consequence of CDI and is also associated with recurrent CDI (342, 344). GVHD usually presents with symptoms similar to CDI and could be misdiagnosed. Endoscopy and colonic biopsies often are performed to establish the correct diagnosis, as the treatment of GVHD significantly differs from that of CDI. The colonic mucosa in GVHD presents a tortoiseshell-like pattern (345).

CDI is more common in patients undergoing allogeneic transplant compared with autologous (307, 346). It is most frequent in the first weeks after HSCT (347). CDI is associated with higher mortality in HSCT recipients when compared to non-HSCT patients (312), while recurrent CDI shows similar prevalence (341, 342). HSCT recipients with CDI experience BSI more commonly (348).

No specific guidelines are present for CDI treatment in HSCT recipients. The use of oral vancomycin in primary and secondary prophylaxis of CDI has been studied in this patient category. Four retrospective studies evaluated vancomycin as effective as primary (349, 350) and secondary (351, 352) prophylaxis, respectively. All studies except one (352) included only patients who underwent allogeneic stem cell transplantation. An RCT demonstrated the efficacy of once-daily oral fidaxomicin (200 mg) vs placebo in primary prophylaxis of CDI in HSCT patients (both autologous and allogeneic) undergoing fluoroquinolone prophylaxis for neutropenia (353). Despite these findings, ESCMID does not recommend routine prophylaxis, except in selected patients (204). Bezlotoxumab has also been found as safe and effective in HSCT recipients (330). Similar to SOT recipients, FMT is safe and effective also in HSCT patients (354). Current evidence is provided by case reports and series, so additional and more powerful studies are needed (355). ACG guidelines recommend appropriate donor screening before FMT (259). An

RCT (NCT02269150) on autologous fecal microbiota transplantation for prophylaxis of CDI in recipients of allogeneic hematopoietic stem cell transplantation is still ongoing (356).

### Inflammatory bowel disease patients

In the late 1970s, individuals with IBD were found to have an increased risk of colonization with toxin-producing *C. difficile*, sparking debate on whether these toxins could cause IBD or IBD flares (357). The incidence and severity of CDI have risen, especially in IBD patients (358, 359). Between 2004 and 2005, CDI cases diagnosed in IBD patients increased from 7% to 16% (360). The increasing CDI incidence primarily affects patients with ulcerative colitis, rising from 2.4% of admissions in 1998 to 3.9% in 2004, with lower rates observed in Crohn's disease patients (0.8%–2%) (361). Another study found overall CDI rates to be higher in ulcerative colitis patients than in Crohn's disease patients and nearly eight times higher in IBD patients compared to non-IBD patients (362). This disparity may be due to the lower incidence of colitis in Crohn's disease and less widespread colonic dysbiosis.

CDI and IBD coexistence increases risks for adverse outcomes compared to either condition alone. Patients with both conditions have longer hospital stays, poorer response to medical therapy, frequent IBD flares, higher likelihood of intensified therapy, and increased colectomy or gastrointestinal surgery rates (360, 361, 363). Mortality rates are four times higher, and healthcare costs are greater (364). Clinical presentations of CDI and IBD with colitis share many overlapping symptoms, such as diarrhea, abdominal discomfort, and fever. Differentiating between an acute IBD flare and CDI-complicating IBD is challenging. IBD patients presenting with worsening diarrhea or colitis symptoms should be tested for toxigenic *C. difficile* using nucleic acid amplification tests (NAATs) or enzyme immunoassays (EIAs) (207, 365, 366). Asymptomatic carriage of toxigenic *C. difficile* and potential overdiagnosis with NAAT complicate diagnosis (199, 367, 368). A two-step testing modality, including EIA for glutamate dehydrogenase (GDH) and *C. difficile* toxins, followed by NAAT for discordant results, may provide a more accurate diagnosis in IBD patients (369). Colonoscopy is more frequently used for diagnosing CDI in IBD patients, but differentiating histopathologic changes is difficult (360). No validated biomarkers distinguish CDI-induced colitis from IBD. In light of these diagnostic challenges, clinicians should initially treat symptomatic IBD patients with positive toxigenic *C. difficile* tests for CDI and later intensify IBD therapy if no clinical response is observed.

Managing CDI in IBD patients is complex due to challenges in differentiating symptoms, selecting antibiotic therapy, and adjusting immunosuppressants (370). FMT is an emerging treatment option for CDI in IBD. General management principles include fluid and electrolyte balance, laboratory data assessment, and infection control measures (370). Antibiotic treatment options include metronidazole, vancomycin, and fidaxomicin, with vancomycin or fidaxomicin preferred for IBD patients. Recurrent CDI management is particularly challenging in IBD patients, with FMT being a potential treatment (370). However, FMT's long-term effects remain unknown, and further research is needed to establish its efficacy and safety in IBD patients with CDI (370).

Differentiating between IBD flare symptoms and concurrent CDI poses a significant challenge in CDI management in IBD patients, especially in those with ongoing immunosuppressant therapy. Immunosuppression can exacerbate infections but may be necessary to treat IBD flares. Deciding to increase immunosuppressive therapy demands careful consideration. Studies show mixed results regarding adverse outcomes in patients treated with antibiotics and immunomodulators (371–373). Until more data are available, clinicians should cautiously escalate immunosuppressive therapy alongside antibiotics and closely monitor patients for symptom worsening and complications.

The ACG guidelines recommend a longer therapy duration for IBD patients (vancomycin for a minimum of 14 days), but this recommendation is based on their clinical experience (259).

In conclusion, *C. difficile* is a prevalent issue for IBD patients. All IBD flare-ups should be tested for CDI, with recurrent testing if symptoms persist. Vancomycin is preferred over metronidazole for treatment. Hospitalization may be necessary for severe cases, and immunosuppression agents should be cautiously managed. FMT is recommended for IBD patients with recurrent CDI.

## DIAGNOSIS

CDI diagnosis is primarily clinical but should be supported by laboratory tests and, when necessary, imaging and endoscopic findings (374). A prompt diagnosis is crucial both for correct patient management and implementation of infection control measures, especially in healthcare settings.

### Stool examination

Stool examination for CDI diagnosis requires both appropriate indication and accurate tests. Regarding the indication, *C. difficile* testing is recommended in cases of symptoms suggestive of CDI, thus—as stated in the IDSA guidelines—in cases of “unexplained and new onset  $\geq 3$  unformed stools in 24 hours” (15, 259, 375). Repeating *C. difficile* testing during the same diarrheal episode is not recommended, except in cases of outbreaks or high clinical suspicion (15, 375). Formed stool samples should not be tested for CDI. In case of paralytic ileus, ESCMID guidelines suggest a rectal swab for *C. difficile* testing (toxigenic culture, nucleic acid amplification tests, or glutamate dehydrogenase assays) (375). Testing on the same day of stool collection is highly recommended (376), if not possible, stool samples should be stored at 4°C, for a maximum of 72 hours (377). Storing stools at  $-20^{\circ}\text{C}$  alters *C. difficile* toxins (378), while this is not true for  $-80^{\circ}\text{C}$  (379). Stool samples should be collected before starting CDI treatment, in order to avoid false-negative tests (380). Tests of cure are not recommended (375), as in most patients, toxins can be detected on stools even 6 weeks after treatment and resolution of diarrhea (381).

A single test combining high sensitivity and specificity, low cost, and fast turnaround time for CDI diagnosis is still missing. Reference methods are cell cytotoxicity neutralization assay (CCNA) and toxigenic culture (TC). CCNA is the reference test for the detection of *C. difficile* toxins in stools. It consists of an inoculation of a stool sample filtrate onto a monolayer of a cell line. After 24–48 hours of incubation, cells are observed for toxin-induced cytopathic effect. If the cytopathic effect is observed, a neutralization assay is performed for confirmation. TC is the reference test for the detection of toxigenic *C. difficile* in stools. Once *C. difficile* is isolated in selective media, it aims at determining if the identified isolate is a toxin-producing strain or not (374, 382). CCNA and TC are accurate but no longer used in routine practice because they are not advantageous in terms of time and resources (259, 377). TC remains highly recommended in case of outbreaks (375). The other diagnostic tests for *C. difficile* on stool samples are EIAs for toxins, GDH, and nucleic acid amplification test. Currently, available enzyme immunoassays use monoclonal or polyclonal antibodies to detect *C. difficile* toxins A and B. They show low sensitivity and moderate specificity. Better accuracy is described for ultrasensitive toxin EIA assays (383); however, they are not yet commercially available. GDH is a metabolic enzyme expressed at high levels by all strains of *C. difficile*, both toxigenic and non-toxigenic strains. It shows high sensitivity and a favorable negative predictive value. It is frequently used as a screening test for CDI (370). NAATs include polymerase chain reaction, loop-mediated isothermal amplification, and helicase-dependent amplification assays. They detect targeted toxin genes directly (e.g., *tcdB*, *tcdA*, *cdt*). As GDH, they have high sensitivity and high negative predictive value (377).

In order to achieve optimal diagnostic accuracy (375) and reduce inappropriate CDI treatment (384), different algorithms including both a highly sensitive and a highly specific test have been proposed. European guidelines recommend a two-step algorithm based on a sensitive screening method (NAAT or GDH EIA) followed, in the case of positive result, by a toxin A/B EIA. An alternative algorithm is to screen samples with

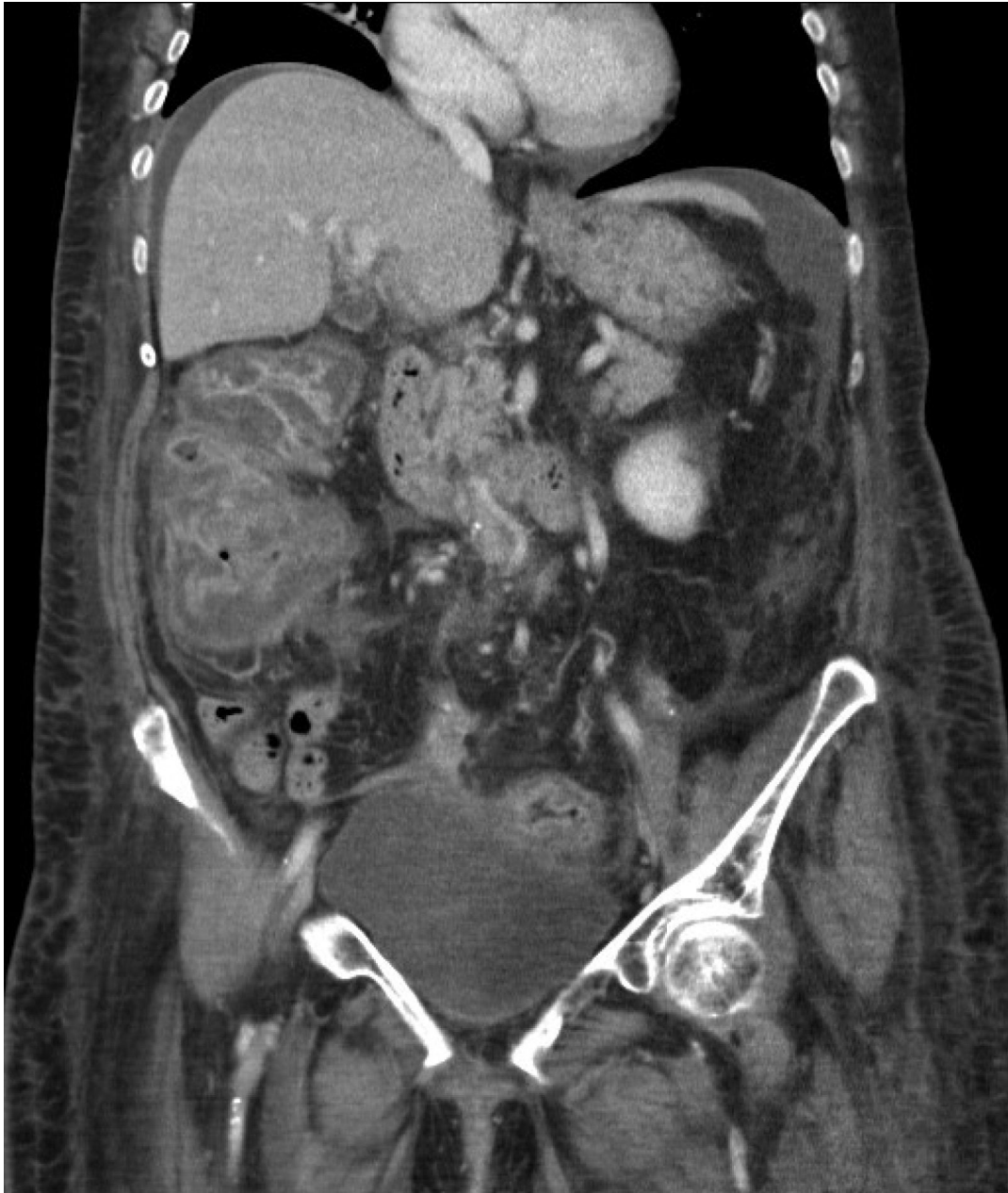
both a GDH and toxin A/B EIA. Samples with concordant positive or negative results can be reported as such. Samples with a negative GDH result but positive for toxin need to be retested as this is an invalid result. Samples with a positive first test result and negative second test result and samples with a GDH-positive test but negative toxin A/B test may represent samples with CDI or *C. difficile* carriage. In these cases, TC or NAAT can be performed optionally (375). AGC guidelines recommend a two-step testing algorithm too (259). IDSA guidelines propose the same diagnostic approach with the exception that NAATs alone can be considered if appropriate stool selection is guaranteed by laboratories (15). Kraft et al. conducted a systematic review in order to assess the diagnostic accuracy of NAAT alone or following GDH EIAs or GDH EIAs plus *C. difficile* toxin EIAs. This meta-analysis shows how there is insufficient evidence to recommend against repeat testing of the sample using NAAT after an initial negative result and supports the GDH/toxin/NAAT algorithm for CDI diagnosis (385). More recently, Prosty et al. conducted a systematic review and meta-analysis finding similar to all-cause mortality between patients NAAT+/Toxin + and NAAT+/Toxin– (386).

## Imaging

Imaging is crucial when suspecting a complication of CDI. Abdominal X-ray can be as normal as detecting abnormalities typical of CDI. The classic X-ray findings of CDI are colon dilatation, nodular haustral thickening, thumbprinting, and ascites (387, 388). Moreover, in severe/fulminant cases, toxic megacolon with colon distention and perforations with evidence of free air may be present (389).

Contrast-enhanced computed tomography (CECT) plays an important role in the diagnosis of CDI complications, especially when the patient has severe abdominal pain (390, 391). The most typical CECT finding of *C. difficile* colitis is colon wall thickening (388) (Fig. 5). In its classic form, it presents as a pancolitis (392). According to studies conducted in the 1990s, in 30%–40% of the cases, only the right side of the colon is involved. In other cases, isolated segments of the colon and rectum have been described (387, 393). Other reported common colonic signs are colon wall nodularity, dilatation, the “accordion sign” (indicating trapped positive contrast between the inflamed mucosal folds), and the “double-halo sign, target sign” (that indicates the multilayered appearance of high attenuation surrounding a central area of decreased attenuation representing edema, after intravenous contrast administration) (393–397). These findings have been reported also in the pediatric population (398). Extra-intestinal findings usually detected by CECT are pericolonic stranding and ascites (395–397). CT scan can be of great help in the diagnosis of CDI, but it lacks sensitivity, so it should not be used for screening purposes (390). Interestingly, in a retrospective study by Kirkpatrick and Greenberg, CT diagnosis of CDI had a sensitivity of 52% and a specificity of 93%, while the positive and negative predictive values were 88% and 67%, respectively. Sensitivity increased up to 70% using colonic wall thickness of greater than 4 mm as diagnostic criteria in addition to one of the following: colon wall nodularity, accordion sign, pericolonic stranding, or ascites (396). In 2016, Palau-Davila et al. developed a five-parameter CT radiological scale that predicted the need for surgery. The five parameters evaluated were increased cecum wall thickness >3 mm (four points), increased transverse colon wall thickness >3 mm (three points), increased sigmoid colon wall thickness >3 mm (six points), pancolitis (three points), and bowel dilation (eight points). The scale was considered positive if it was greater or equal to 6 (399).

Ultrasound could be useful in case of critically ill patients who cannot be transported to a CT scan room (390). Typical, but non-specifically, ultrasound findings of *C. difficile* colitis are colonic wall thickening, effacement of the lumen, diminution of large bowel content, the so-called “internal ring” and the hypoechoic “external ring,” that corresponds to the edematous mucosa and muscularis propria, respectively, and ascites (388, 400). Wiener-Well et al. prospectively evaluated patients with suspected CDI who underwent ultrasound and found out that colonic wall thickening had high positive and negative predictive values (0.80 and 0.90, respectively) (401).



**FIG 5** Pseudomembranous colitis with characteristic colon wall thickening and pancolonic involvement. (Image courtesy of Marco Cavallaro, reproduced with permission.)

The role of nuclear imaging in CDI is quite limited. Most of the time, *C. difficile* colitis is an incidental finding in scintigraphic examinations performed for other reasons (402–404). A colonic activity is commonly detected. As regard positron emission tomography, a few case reports (405–408) and a preclinical study on mouse model (409) have been published, showing that colon 18F-fluorodeoxyglucose uptake could predict the severity of the CDI episode.

### Endoscopy

Gastrointestinal endoscopy is not routinely performed and should be used sparingly to confirm the diagnosis of CDI (390). However, it could be considered when the suspicion is high and stool examination is negative (410), when other coexisting diseases have to

be ruled out, or if the patient is severely ill (391, 411). When gastrointestinal endoscopy is performed, an elevated risk of perforation and bleeding must be taken into account, particularly in cases of fulminant CDI (389, 412).

The typical endoscopic CDI presentation is PMC. Pseudomembranes are lesions of approximately up to 2 cm in diameter, usually yellow or white, irregularly distributed in the colon, and attached to the mucosa (Fig. 6). Ulcers can also be described in CDI (412, 413). These macroscopic findings are not specific to CDI. Colonic pseudomembranes could be found also in other bacterial infections (e.g., *Klebsiella oxytoca*, *Escherichia coli* O157:H7) (414, 415), in CMV colitis (416, 417), and, rarely, in parasitic infections (e.g., *Entamoeba histolytica*, *Schistosoma mansoni*) (418, 419). Also non-infectious diseases could present with colonic pseudomembranes. Among these, we report Behçet disease, ischemic colitis, IBD, and iatrogenic colitis (420).

Furthermore, pseudomembranes are not always present but are found in about 40%–60% of CDI cases (411, 421). Colonoscopy is generally preferred to sigmoidoscopy and flexible sigmoidoscopy because in one-third of patients only the right colon is involved (71). Pseudomembranes are less common among patients with IBD (422, 423) and those undergoing immunosuppressant therapy (424). The amount of pseudomembranes detected with endoscopy is not necessarily related to the severity of the disease, especially in the abovementioned special populations (425).

In patients with suspicion of *C. difficile* colitis undergoing colonoscopy and negative stool examinations or when a co-infection has to be ruled out, biopsies of the intestinal mucosa must be taken.

Endoscopy can also aid in the treatment of CDI. In fact, it can be used to carry out colonic decompression when significant distention is present or to deliver intracolonic drugs (e.g., vancomycin) (426–429) and FMT (430).

## Histologic features

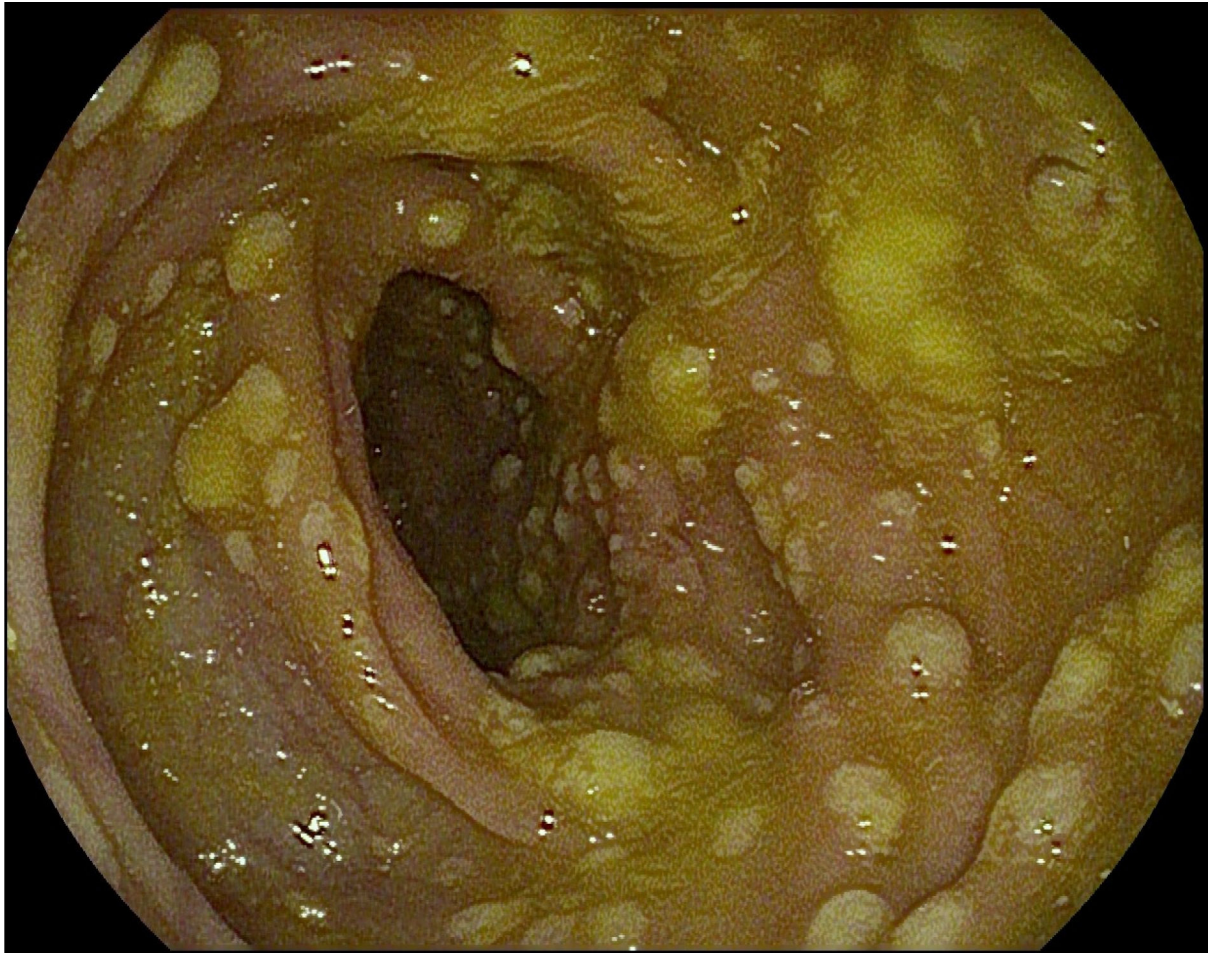
The histologic features of *C. difficile* colitis may range from mild to severe inflammation of the colonic mucosa. The typical pseudomembranes contain fibrin, mucin, inflammatory cells, and mucosal epithelial cells (389). In severe cases, cryptitis, crypt abscesses, gland dilation, and confluence of pseudomembranes could be detected (431), as well as complete necrosis of the mucosa (making differential diagnosis with ischemic colitis difficult) (387, 431), and massive edema which extends through the submucosa into the muscularis propria of the colon (389). Vasculitis and microthrombi are not common findings (387). Signet ring-like cells can be found because of the degeneration of the mucosa sometimes leading to misdiagnosis of carcinoma (432).

In 1977, Price classified PMC into three types. Type I lesions are characterized by patchy epithelial necrosis of the interglandular surface accompanied by an exudation of fibrin and neutrophils into the colonic lumen (Fig. 7). Type II lesions have more extended focal surface epithelial necrosis and more prominent exudate above (Fig. 8). Type III lesions are characterized by complete necrosis of the colonic mucosa, which is entirely covered by confluent pseudomembranes (433).

As mentioned in “Inflammatory bowel disease patients,” differentiating between IBD flares and concomitant CDI is quite challenging. Pseudomembranes could be absent in *C. difficile* colitis in IBD patients (360). In 2022, Sweeney et al. retrospectively evaluated colonic biopsies of IBD patients with diarrhea, including a group of patients with CDI and a group without. They reported that samples from patients with CDI showed more likely neutrophil-rich inflammation in the lamina propria distant from cryptitis, cryptitis, and neutrophilic intra-epithelial infiltrates without plasma cells nearby (434). These findings may help pathologists in the diagnosis of CDI in IBD patients.

When *C. difficile* colitis resolves, usually, the mucosa heals and returns to normal (435).





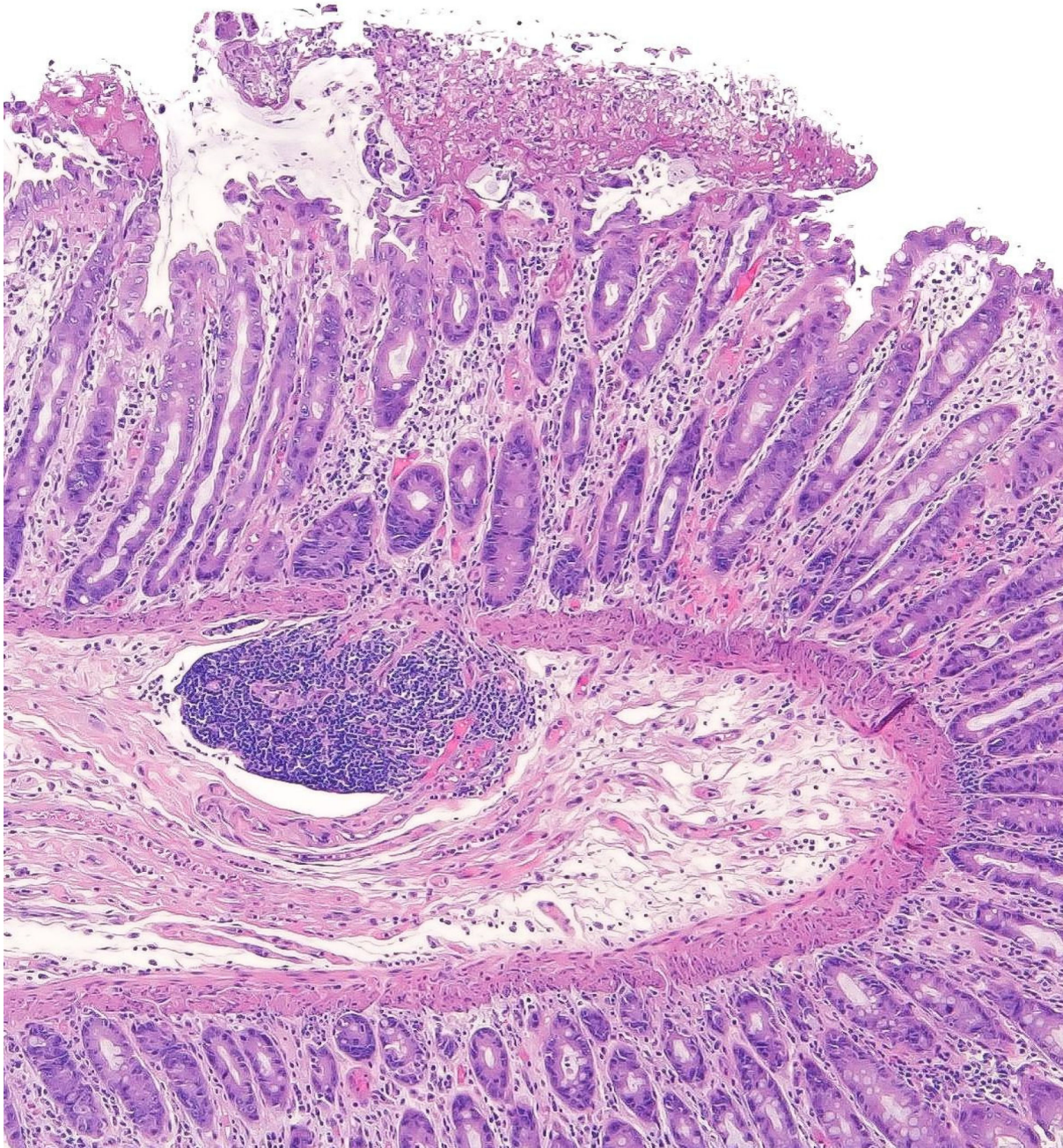
**FIG 6** Yellow-white pseudomembranes irregularly distributed and strongly adhering to the colonic mucosa. (Image courtesy of Lisa Fusaro, reproduced with permission.)

## Biomarkers

Over the years, several biomarkers have been proposed for the diagnosis of CDI. Among them, fecal calprotectin and fecal lactoferrin (FL) are the most studied.

Calprotectin is a protein with antimicrobial effects mostly released from neutrophils, after their activation or death within the innate immune response. Fecal calprotectin is a well-known non-specific marker of gastrointestinal inflammation (436), so it has been studied in the CDI setting (212). Many potential roles have been found for FC in CDI diagnosis and management. First of all, FC is higher in patients with CDI when compared to healthy subjects (437) and to patients with non-*C. difficile* diarrhea (438). In addition, in a case-control study performed by Barbut et al., FC and fecal lactoferrin were higher in CDI patients with detectable toxins in feces (439). Moreover, few studies found out that FC is a predictive marker to assess CDI severity in adults (437, 440–443). Furthermore, FC could play a role as a prognostic factor in CDI. Adults (444) and children (445) with recurrent CDI tend to have higher levels of FC when compared to patients who experience an isolated episode of CDI. Finally, FC levels could predict the outcome of patients after FMT, showing higher levels in those with recurrent CDI, when compared with those without recurrence (444).

Lactoferrin is an iron-binding glycoprotein with antimicrobial effects usually released by neutrophils after their activation during inflammation. During gastrointestinal inflammation, neutrophils infiltrate the mucosa, leading to an increase of lactoferrin's concentration in feces. Fecal lactoferrin is a marker of gastrointestinal inflammation (446).

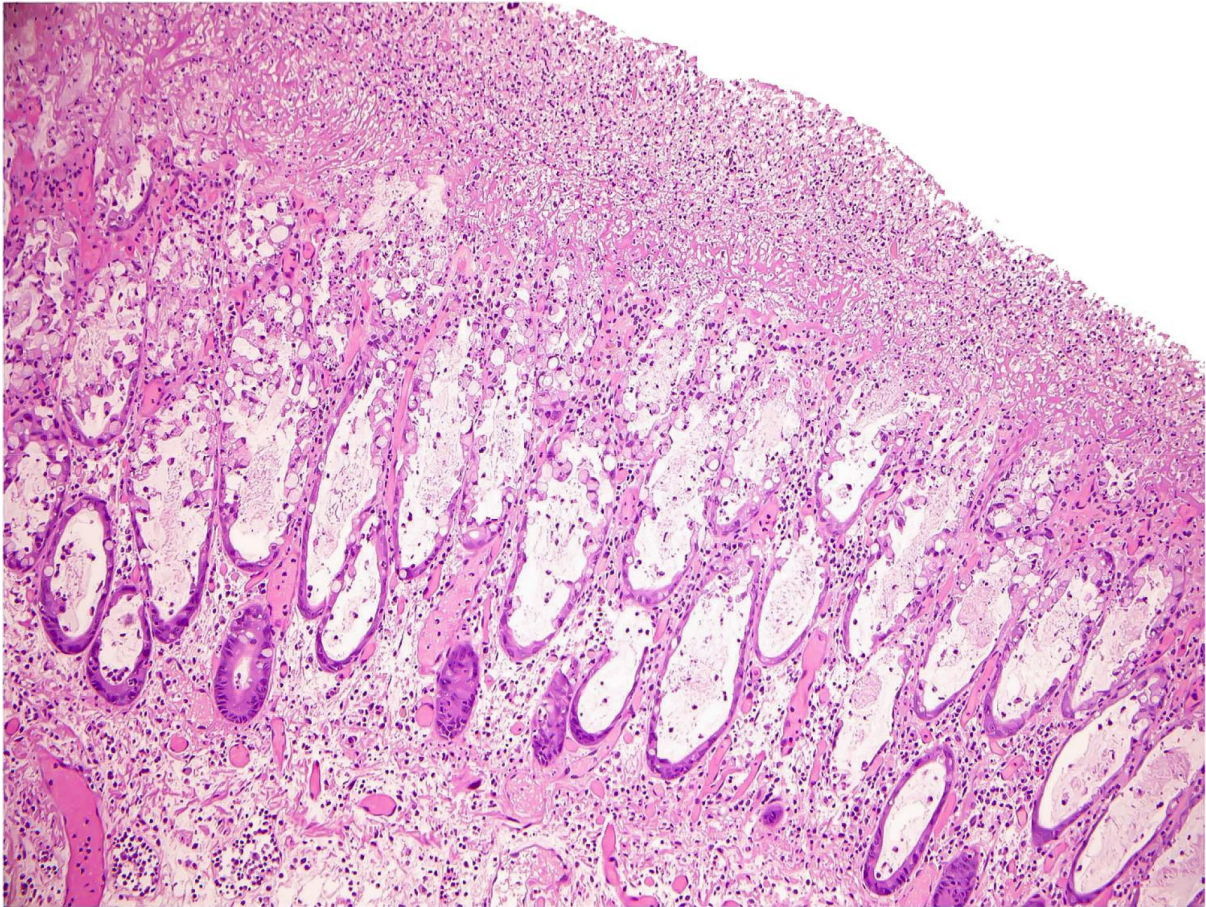


**FIG 7** PMC type I pattern with epithelial necrosis of the interglandular surface accompanied by an exudation of fibrin and neutrophils above (hematoxylin and eosin, 20 $\times$ ). (Image courtesy of Iacopo Ghini, reproduced with permission.)

In the setting of CDI, FL was found to be useful in screening for CDI in patients with diarrhea (447), as a predictor of severity (448–450) and recurrence (445).

Measurement of pro-inflammatory cytokines, such as IL-1 $\beta$  and IL-8, on feces has been proposed as possible tools in CDI diagnosis (448) and in assessing the severity of CDI (451). In particular, fecal IL-1 $\beta$  concentrations can differentiate CDI from non-CDI diarrheas (213). Animal models also proposed IL-23 as a potential marker of CDI (244, 452).

However, the IDSA guidelines do not recommend the use of adjunctive biomarkers in diagnosing CDI (15). ESCMID (208, 260) and ACG (259) guidelines do not mention their use in CDI diagnosis and management. The abovementioned studies are promising,



**FIG 8** Pseudomembranous colitis type II pattern with more extended focal surface epithelial necrosis and more prominent exudate above (hematoxylin and eosin, 20 $\times$ ). (Image courtesy of Iacopo Ghini, reproduced with permission.)

but they do not provide sufficient evidence to apply these markers in CDI screening, diagnosis, nor in assessing prognosis.

## MANAGEMENT

### General approach: fluid balance, electrolytes

We will focus on anti-infective treatment of CDI; however, a brief mention of the importance of proper management of dehydration, hypokalemia, and hypomagnesemia in these patients is mandatory. In addition, particular attention should be paid to heart failure development in CDI since it is not uncommon among these patients (453), with metabolic acidosis likely contributing to the insult and with a possible cardiotoxic role of toxins in worsening cardiac function (203, 454). With these assumptions, clinicians should be very careful with hydroelectrolytic balance because metabolic acidosis and hypoalbuminemia often coexist, and these patients tend to accumulate fluids in the third space, with a higher risk of pulmonary edema. It is also important to consider arrhythmias secondary to electrolyte disturbances (i.e., hypokalemia) and avoid unnecessary systemic antibiotics, antiperistaltic agents, and gastric suppressants (455).

### Antibiotic therapy

Despite the widely acknowledged superiority of FMT-based treatments, as of today, antibiotic therapy remains the most commonly used anti-CDI treatment. Historically, the most commonly used drugs for CDI have been metronidazole, vancomycin, and

fidaxomicin. Until 2014, metronidazole was considered an acceptable choice for a first mild CDI episode (365). However, guidelines have evolved over time and gradually replaced metronidazole with vancomycin, in both severe and mild/moderate CDI (456, 457). A habit not always explicitly mentioned in guidelines but commonly practiced by experienced clinicians, especially in mild cases, is discontinuation of concomitant antibiotics to observe the patient's response.

In the following sections, we will discuss the main treatments for CDI.

### Fidaxomicin vs vancomycin

Vancomycin has been the mainstay in CDI treatment for decades. Its efficacy is beyond dispute, and its superiority of vancomycin vs the "historical opponent metronidazole" has been demonstrated for severe CDI in 2007 by Zar et al. (456). Seven years later, superiority of vancomycin over metronidazole in mild cases was also demonstrated (457). In the past decade, the primacy of vancomycin was undermined by fidaxomicin, a macrocyclic antibiotic with similar clinical efficacy but with a lower impact on the gut flora due to more selectivity.

Fidaxomicin showed superiority over vancomycin in reducing recurrent CDI episodes (458). This evidence is solid, while data comparing vancomycin and fidaxomicin for initial CDI episodes are less clear. IDSA guidelines did change their strength of recommendation for fidaxomicin in 2018, where vancomycin and fidaxomicin were equated for the first CDI episode (strong recommendation and high quality of evidence) (15). In a focused update from 2021, it is stated "For patients with an initial CDI episode, we suggest using fidaxomicin rather than a standard course of vancomycin" (conditional recommendation and moderate certainty of evidence) (459). Conversely, ACG guidelines pose a distinction for an initial CDI episode: when non-severe both vancomycin and fidaxomicin have a strong recommendation, but if severe vancomycin has a strong recommendation, while fidaxomicin has a conditional recommendation (259).

A network meta-analysis published in 2018 evaluated 24 trials and found that fidaxomicin was superior to vancomycin in sustained symptomatic cure of mild to moderate CDI (OR 0.47; 95% CI 0.33–0.66), while this superiority was not statistically significant in severe cases (OR 0.57; 95% CI 0.30–1.11) (460). Several pre-clinical and clinical studies suggest that fidaxomicin is similar to vancomycin for an acute episode but is superior for sustained response. A meta-analysis published in 2022 by Tashiro et al. confirms these remarks: the authors evaluated six randomized controlled trials concluding that, compared to vancomycin, fidaxomicin was significantly associated with higher global cure rates (the ratio of the number of patients without recurrence after the achievement of clinical cure to the number of mITT patients) (RR = 1.18,  $P < 0.00001$ ), while clinical cure rates (the ratio of the number of patients with resolution of diarrhea and no further need for treatment of CDI to the number of mITT patients) were comparable ( $P = 0.31$ ). Fidaxomicin was indeed associated with significantly lower recurrence rates (RR = 0.59,  $P < 0.0001$ ). Adverse event rates were similar between the two drugs (461). When not considering trials and only including observational studies with both pros and cons of the type of study (pro: no selected populations; cons: no randomization), results can be overturned. Dai et al. in a meta-analysis of 10 real-world studies reported fidaxomicin having an OR of 2.81 (95% CI 1.08–7.29) for treatment failure compared to vancomycin (462). In the registrative study, researchers also evaluated the time to resolution of diarrhea, and this was shorter in the fidaxomicin group than in the vancomycin group (58 vs 78 hours in the modified intention-to-treat population and 55 vs 69 hours in the per-protocol population), although these differences were not statistically significant (458). In our opinion, evidence for the superiority of vancomycin or fidaxomicin for initial CDI episodes still shows some gaps.

In addition to data on direct clinical outcomes, data on microbiota disruption must be considered. Unlike vancomycin, fidaxomicin has a narrower spectrum with limited activity against enteric commensal bacteria. Indeed, it has been demonstrated that fidaxomicin use results in significantly reduced acquisition of vancomycin-resistant

enterococci and *Candida* species compared to vancomycin (7% vs 31% and 19% vs 29%, respectively) (463). It is well known that approximately one-fifth of hospitalized CDI patients will develop a bloodstream infection for which *Enterobacterales* and *Candida* are among the most commonly responsible agents (229), and the benefit of a narrow spectrum anti-CDI molecule may be useful for infective episodes other than CDI.

Moreover, fidaxomicin has shown a capability to reduce *C. difficile* sporulation and toxin production (464–467). On the other hand, evidence of sporulation inhibition and inhibition of toxin production by vancomycin is lacking (468). Both fidaxomicin and vancomycin inhibit the outgrowth of a vegetative cell from the germinating spore (469). Evidence from human studies showed that patients treated with fidaxomicin had lower detection of post-treatment toxins (470). The main differences between vancomycin and fidaxomicin are reported in Table 2.

From a perspective of bacterial killing which is reflected in clinical practice, vancomycin and fidaxomicin have different mechanisms of action. Vancomycin inhibits cell wall biosynthesis, while fidaxomicin inhibits RNA polymerase. Former guidelines recommended higher doses of vancomycin (i.e., 500 mg every 6 hours) in severe/complicated cases (366), even though the benefit of this higher dosage vs the standard dosage of 125 mg every 6 hours has never been demonstrated (478, 479). Despite the fact that the dosage of 125 mg every 6 hours results in high intraluminal levels (480), it is possible that another mechanism exists. Studies demonstrate that, at higher level concentrations up to 64× MIC, vancomycin displays an Eagle effect (more-drug-kill-less) against *C. difficile*. A study published in 2018 demonstrated that *C. difficile* survived clinically relevant high concentrations of vancomycin (up to 2,048 mg/L) while it did not survive such high concentrations of fidaxomicin (481).

*C. difficile* resistance to both vancomycin and fidaxomicin is rare. A US susceptibility testing and genomic surveillance spanning from 2012 to 2017 reported that 98.5% of the strains displayed a vancomycin MIC  $\leq$  2 mg/L (susceptible according to EUCAST breakpoints) (482). Reduced susceptibility to fidaxomicin has been seldom reported, a single patient with a strain with MIC of 16 mg/L at the time of recurrent CDI in the registration trial cohort was reported (483). Similarly, Marchandin et al. recently reported a case of *in vivo* emergence of fidaxomicin resistance in a patient with underlying diseases and three CDI episodes, with fidaxomicin MIC increasing from 0.063 mg/L in the first episode to 16 mg/L in the second and third episodes. The patient had received fidaxomicin for the first CDI episode (484).

In patients who concomitantly receive antibiotics, the superiority of fidaxomicin over vancomycin is evident not only in reducing recurrent CDI but also in improving clinical cure rates. A post hoc analysis of phase II and phase III studies published in 2011 showed that in subjects who received concomitant antibiotics with CDI treatment, the cure rate was 90.0% for fidaxomicin and 79.4% for vancomycin ( $P = 0.04$ ). In subjects receiving concomitant antibiotics during treatment and/or follow-up, treatment with fidaxomicin compared to vancomycin was associated with 12.3% fewer recurrences (16.9% vs 29.2%;  $P = 0.048$ ) (485). Similarly, the results of a recent open-label randomized clinical trial comparing standard dosage fidaxomicin vs standard dosage vancomycin in CDI patients receiving concurrent antibiotics for other infections have been published. The primary endpoint of clinical cure was reported in 73% (54/74) and 62.9% (44/70) of patients in the fidaxomicin and vancomycin arms, respectively (486). Regarding safety, fidaxomicin and vancomycin are both well tolerated and present similar safety profiles (487).

Further randomized clinical trials are ongoing (488) and may aid in clarifying the comparison of the two molecules.

### Teicoplanin as vancomycin?

Teicoplanin has long been considered the “European vancomycin.” It belongs to the glycopeptide class and was introduced into clinical use in 1988 in Italy (489). Teicoplanin has the same spectra as vancomycin except for VanB and VanC staphylococcal and enterococcal strains which are susceptible to teicoplanin but resistant to vancomycin

TABLE 2 Comparison: vancomycin and fidaxomicin<sup>a</sup>

Antibiotic	Fidaxomicin	Vancomycin
Biofilm inhibition	Yes (471, 472)	No
<i>C. difficile</i> MIC <sub>90</sub>	0.125 mg/L (473)	2 mg/L (473)
Category	Macrocyclic antibiotic	Glycopeptide
Dosage	200 mg PO q12h	125 mg PO q6h
Eagle effect	No	Yes
Efficacy in mild CDI	+++	+++
Efficacy in severe CDI	++	+++
Formulations	Tablets	Powder, tablets
Gut flora disruption	Low	Moderate/high
Mechanism of action	Inhibits RNA polymerase	Inhibits cell wall synthesis
MIC breakpoint	1 mg/L (474)	2 mg/L (475)
Molecular weight	1,058 Da (476)	1,449 Da (477)
PK/PD index	AUC/MIC (474)	AUC/MIC (474)
Post antibiotic effect	Long (474)	Short (474)
Prevention of rCDI	+++	+
Pulsed/tapered regimens	Evidence existing	Evidence existing
Risk of VRE emergence	Low	Moderate/high
Systemic absorption	Minimal	Minimal
Sporulation reduction	++ (464)	– (468)
Toxin reduction	++	– <sup>b</sup>

<sup>a</sup>AUC, area under the curve; Da, dalton; PK/PD, pharmacokinetic/pharmacodynamic; PO, per os; MIC, minimum inhibitory concentration.

<sup>b</sup>–, low.

(489). Teicoplanin is also active *in vitro* and *in vivo* against *C. difficile*. Clinical studies on teicoplanin are significantly fewer compared to vancomycin; however, results are very interesting. Already in the '90s, some clinical trials (although enrolling relatively small populations) demonstrated the non-inferiority of teicoplanin compared to vancomycin for CDI clinical cure (490, 491). When compared, teicoplanin MIC was lower than vancomycin MIC against *C. difficile* (0.5 vs 2 mg/L, respectively) (492). In 2013, teicoplanin obtained a licensed indication for CDI (in Europe) and was available for oral administration.

An interesting prospective observational study on hospitalized patients with CDI was conducted from 2013 to 2015 by a Serbian team (493). Although not classified as a trial, the researchers allocated drugs alternately (first patient vancomycin, second teicoplanin, and so on); however, during the study period, they had a shortage of one of the two drugs. Dosages were 100 mg every 12 hours for teicoplanin and 125 mg every 6 hours for vancomycin, both administered for 10 days. In the end, 107 patients received teicoplanin, and 180 received vancomycin. Clinical characteristics of patients were similar, and there was no statistically significant difference in time to resolution of diarrhea between the two treatment arms; however, those who received teicoplanin showed a significantly higher clinical cure rate compared to vancomycin (90.7% vs 79.4%,  $P = 0.013$ ; OR 2.51; 95% CI 1.19–5.28). Additionally, recurrence rates were significantly lower in patients treated with teicoplanin (9.3% vs 34.3%;  $P < 0.001$ ; OR 0.20; 95% CI 0.09–0.42). However, overall mortality rates were not different between the two groups (493).

In 2018, the network meta-analysis by Beinortas et al. reported that, for sustained symptomatic cure, teicoplanin was superior to vancomycin (OR 0.37; CI 95% 0.14–0.94) and metronidazole (OR 0.27; 95% CI 0.10–0.70) (460).

In conclusion, although teicoplanin is more expensive and less used than vancomycin, it is likely that it can have some advantages and should not be abandoned.

## Intravenous metronidazole: when?

The addition of intravenous metronidazole to the standard of care is commonly employed by clinicians, especially in severe CDI cases at high risk of progression/complications, although no clinical trial has explored the potential advantages of this strategy. The more conspicuous source of evidence dates back to a 10-year surveillance (1982–1991) conducted at the Minneapolis Veterans Affairs Medical Center and published in 1994. Out of 908 CDI patients, 52 (6%) were diagnosed with ileus, and 15 (29%) of them received intravenous metronidazole in addition to the standard oral treatment. Among those with severe ileus, seven out of eight were treated with intravenous metronidazole in addition to the oral medication, and six had a clinical response while the remaining two died. These results were at that time considered encouraging (494). From that time on, metronidazole was always included in CDI therapy which was extensively used by clinicians facing CDI.

In the last 10 years, moderate evidence was gathered mostly from three retrospective observational studies (206, 495).

Rokas *et al.* evaluated critically ill patients (ICU) with CDI who received oral vancomycin (monotherapy) or oral vancomycin with intravenous metronidazole (combination therapy). All patients with three or more of the following criteria were included: albumin <2.5 g/dL, heart rate >90 bpm, mean arterial pressure <60 mm Hg, white blood cell (WBC) count  $\geq$ 15,000 cells/mL, age >60 years, serum creatinine  $\geq$ 1.5 times baseline, or temperature  $\geq$ 100.4°F (38°C). Forty-four patients per group were included, with similar patient characteristics except for renal disease which was more prevalent in the combination group. They found that mortality was 36.4% in the monotherapy and 15.9% in the combination group ( $P = 0.03$ ) (496). However, given the difference in baseline characteristics between these populations, the comparison was prone to bias. To control this bias, Wang *et al.* retrospectively analyzed 2,114 CDI patients of which 993 received dual therapy with oral vancomycin and intravenous metronidazole, and 1,121 received monotherapy with oral vancomycin alone. Their results, after adjusting for CDI severity, indicated that the addition of intravenous metronidazole was not associated with death or colectomy within 90 days (aOR, 1.07; 95% CI, 0.79–1.45), which proved statistically significant when the analysis was restricted to patients with fulminant CDI (aOR, 1.17; 95% CI, 0.65–2.10) (206). Similarly, Vega *et al.* retrospectively compared outcomes of 138 severe CDI patients treated with vancomycin alone ( $n = 60$ ) or with oral vancomycin plus intravenous metronidazole ( $n = 78$ ). According to these results, the addition of intravenous metronidazole did not reduce 30-day mortality both in the overall population (12.8% monotherapy vs 18.3% combination,  $P = 0.371$ ) and in a 96-patient APACHE-II matched subgroup (14.6% monotherapy vs 18.8% combination,  $P = 0.785$ ) (495).

In real-life practice, several clinicians usually add intravenous metronidazole to a first-line anti-CDI agent in severe/complicated cases at risk for progression, in fulminant cases, or in refractory CDI (497). We prefer to add tigecycline to vancomycin or fidaxomicin in refractory CDI since it can be difficult to distinguish a clinical deterioration due to CDI progression or the occurrence of bacterial translocation. Moreover, in the rare case of a pharmacologic failure, tigecycline has a very low MIC for *C. difficile*, as explained in section “Tigecycline.” Metronidazole remains a viable option for patients with constipation or ileum.

## Tigecycline

Tigecycline is a broad-spectrum protein synthesis inhibitor of the glycylglycine class, active against gram-positive, gram-negative bacteria and anaerobes—including *C. difficile*, *Fusobacterium* spp., *Prevotella* spp., *Porphyromonas* spp., and *Bacteroides fragilis* group (498). Its *in vitro* activity against *C. difficile* was demonstrated more than 20 years ago (499). In subsequent years, many papers—although with small sample sizes—reported favorable outcomes when tigecycline was added to the standard anti-CDI treatment (498).

In 2016, Gergely Szabo et al. published an interesting retrospective study comparing 45 CDI patients who received tigecycline monotherapy vs 45 CDI patients receiving standard therapy (vancomycin plus metronidazole). They reported a better clinical cure (75.6% vs 53.3%;  $P = 0.02$ ), less complicated disease course (28.9% vs 53.3%;  $P = 0.02$ ), and less CDI sepsis (15.6% vs 40.0%;  $P = 0.009$ ) in the tigecycline group (500). However, the groups were not well matched, with a potential bias induced by a lower clinical severity of patients treated with tigecycline. To address this bias, in a subsequent study, Phillips et al. selected 168 CDI patients after a propensity score matching, where 140 received tigecycline and 28 did not. Adjusting for ATLAS score (age, treatment with systemic antibiotics, leukocyte count, albumin and serum creatinine as a measure of renal function), hypotension, treatment time period, and serum lactate, tigecycline did not significantly improve 30-day mortality (OR: 0.89; 95% CI 0.25–3.12;  $P = 0.853$ ) (501). In the absence of data based on robust studies (very low evidence), the ESCMID guidelines only weakly recommend tigecycline in patients who are deteriorating or progressing to severe-complicated disease (208).

Regarding microbiology study, tigecycline showed good *in vitro* activity against 606 toxigenic *C. difficile* strains collected over 14 years. The authors found that tigecycline MIC<sub>90</sub> was 0.064 mg/L, with no resistant strain (502). However, it is important to note that tigecycline, like other antimicrobials, is subject to changing CDI epidemiology. Specifically, tigecycline shows higher MIC against *C. difficile* RT 078. Hung et al. tested tigecycline on 1,112 *C. difficile* isolates in Taiwan and demonstrated that reduced tigecycline susceptibility was quite common among RT-078 strains (12/58; 20.7%) (503); therefore, this should be taken into account when tailoring therapy to local epidemiology. Nonetheless, large data confirm that tigecycline still maintains very low resistance rates (1%) against *C. difficile* (504). Aside from direct action, tigecycline has been demonstrated dose-dependent reduction in *C. difficile* spore production (505).

To summarize, tigecycline is a good companion drug in severe/complicated CDI cases, especially when bacterial translocation is suspected. Tigecycline retains good MIC on *C. difficile*, activity against other anaerobes, gram negatives, and gram positives, including enterococci and vancomycin-resistant strains (506). It is possible that the same potential could be shared by eravacycline and omadacycline (507, 508); however, clinical evidence on these agents is still lacking.

### Tapered/pulsed schemes

Considering that the recovery from CDI benefits from gut repopulation by “good bacteria,” regimens with progressively decreased amounts of drugs have been attempted during the years. These regimens (most of the literature focuses on vancomycin) allowed time for recovery of the flora, with tapered and pulsed schemes. The main expected advantage of these schemes consists in the reduction of recurrent episodes, not in resolving the single episode. A recent systematic review and meta-analysis showed that resolution rates were 83% for taper and pulse, 68% for taper alone, and 54% for pulse-alone regimens, with taper and pulse being found superior to taper alone ( $P < 0.0001$ ) and pulse alone ( $P < 0.0004$ ), while no significant difference was found comparing tapered and pulsed alone (509). Tapered/pulsed vancomycin is among the recommended options in ACG, ESCMID, and IDSA guidelines from the first recurrence onward (208, 259, 459).

Recent data on pulsed fidaxomicin have been available in the last few years. A randomized controlled trial published in 2017 compared extended pulsed fidaxomicin (200 mg twice daily on days 1–5, then once daily on alternate days on days 7–25) vs standard vancomycin (125 mg four times daily on days 1–10). The primary endpoint had sustained clinical cure 30 days after the end of treatment (day 55 for extended pulsed fidaxomicin and day 40 for vancomycin). Seventy percent of patients who received extended pulsed fidaxomicin achieved sustained clinical cure compared with 59% of those receiving vancomycin ( $P = 0.03$ ; OR 1.63; 95% CI 0.04–2.54) (510).



In 2021, Skinner et al. reported a case series of 46 patients with multiple recurrent CDI treated using a tapered-pulsed fidaxomicin regimen, the majority of whom (75%) had failed prior tapered-pulsed vancomycin treatment. The tapered-pulsed fidaxomicin regimen included 20 tablets of fidaxomicin administered as 200 mg once daily for 7 days followed by 200 mg every other day for the remaining 13 doses. Sustained clinical response (SCR) rates at 30 and 90 days were 74% and 61%, respectively (511).

More recently, a Spanish study on 254 CDI episodes compared the recurrence rate of fidaxomicin conventional dosing and fidaxomicin in extended pulsed dosing in clinical practice. Propensity score matching was performed to evaluate patients with a similar recurrence risk. No differences were observed in CDI recurrence rate in patients receiving extended pulsed vs conventional fidaxomicin dosing (OR 0.74; 95% CI 0.27–2.04) (512).

Studies from over a decade ago tested the so-called “chaser” regimens with rifaximin and fidaxomicin administered immediately after vancomycin treatment to reduce recurrence rates. Results were encouraging (513–515), though the number of patients was low and evidence is lacking.

### Antibiotics by rectal or trans-stoma enema

In severe or fulminant cases, or when megacolon is present, ileum is not an uncommon complication. Guidelines take this possibility into consideration and recommend, in such cases, the administration of vancomycin also via the rectum through a retention enema of 500 mg in 100 mL normal saline every 6 hours (15).

One year after the publication of the IDSA guidelines, Fawley et al. conducted a review of studies on vancomycin enema in CDI. Their findings indicated that case series with higher vancomycin doses and larger enema volumes showed greater efficacy. Therefore, they suggested a revision of the guidelines, proposing to administer vancomycin rectally as 500 mg in a volume of 500 mL every 6 hours as a retention enema. They utilized an 18F Foley catheter with a 30mL balloon inserted into the rectum, inflated the balloon, instilled the solution, and clamped the catheter for 60 minutes (516).

The World Society of Emergency Surgery (WSES) guidelines published in 2019 state that “in patients in whom oral antibiotics cannot reach the colon, vancomycin may be administered as a retention enema via a large rectal tube or catheter” (390). The possibility of antegrade instillation of vancomycin flushes via ileostomy in patients with a diverting loop ileostomy has been described, although specific methods are not detailed (390).

### Antibiotics via nasogastric tube

In critically ill patients (e.g., in ICU), the problem of impaired oral intake is common. In addition, enterally fed patients are more prone to develop CDI; therefore, the issue of having to administer anti-CDI treatment via nasogastric tube is common. For vancomycin, this issue is usually bypassed by using powder for injection (517). More recently, some literature reported experiences of administering crushed tablets of fidaxomicin via nasogastric tube. The first case report was published in 2013 by Maseda et al. and reported the efficacy of fidaxomicin 200 mg every 12 hours crushed and mixed with 20 mL water in a critically ill patient (518). In 2014, Tousseeva et al. assessed the stability of crushed fidaxomicin tablets dispersed in water, applesauce, or Ensure brand liquid nutritional supplement. They found that the recovery of the drug after crushing and dispersing in any of the three intermediates studied ranged from 95% to 108%, which is within the normal range of individual tablet variability (519). Subsequently, other reports of crushed fidaxomicin efficacy via nasogastric tube were published (520, 521).

### Recurrent CDI

Recurrent CDI is defined as the reappearance of symptoms of CDI in conjunction with positive laboratory testing for *C. difficile* in a patient who has had an episode of CDI in the preceding 2–8 weeks (15). Recurrent CDI is a common clinical challenge, as a first

recurrence is experienced in 20% of CDI patients (522, 523), among which a second recurrence is expected in 45% of cases (524). A recent survey conducted in the US showed that the burden of first recurrences did not vary significantly from 2011 to 2017 (11). In patients with suspected recurrent CDI, clinical approach should first consider the most frequently reported risk factors for a relapse, such as: (i) age >65–70 years; (ii) previous recurrence of CDI (<3 months); (iii) healthcare-associated CDI; (iv) prior hospitalization (<3 months); and (v) PPIs started during/after CDI diagnosis (525).

From a therapeutic point of view, the main international guidelines agree in favoring fidaxomicin over vancomycin for recurrent CDI (208, 259, 459). Standard or extended pulsed regimens of fidaxomicin are recommended with similar strength by ESCMID and IDSA guidelines (208, 259, 459), while ACG guidelines contemplate fidaxomicin only as standard regimen (259). Tapered/pulsed vancomycin is unanimously recommended as an option for recurrent CDI by all the three main guidelines (208, 259, 459). According to IDSA guidelines, bezlotoxumab may be added to the standard of care from the first CDI recurrence (459), while ESCMID and ACG agree to add it irrespectively from the episode but provided that the patient is at high risk of recurrence (208, 259).

FMT strategies for recurrent CDI will be discussed in the following section “FMT.”

## FMT

Oral administration of a suspension of human feces is an ancient practice. Evidence of its use in Chinese traditional medicine has been found since the 4th century as a remedy for food poisoning or severe diarrhea (526) and, in the 16th century, as the use of stool products (“yellow soup”) for the treatment of several gastrointestinal disorders (526). In the 17th century, the Italian anatomist Fabricius Aquapendente described the “transfaunation,” the transplantation of chewed material from a ruminant to another one, to solve digestive issues (527), a practice used in veterinary medicine in Europe. In Western medicine, the first use of FMT in humans has been described in 1958 by Eiseman et al., who tested this practice on four patients with PMC, unaware that *C. difficile* was the etiological agent (528). Despite encouraging results sporadically described in case records and in a case series (529), FMT was first tested only in 2013 in a randomized controlled trial, demonstrating the superiority of vancomycin followed by FMT over vancomycin alone in patients with recurrent CDI, thus opening the way to the use of FMT in clinical practice. In 2022, Aby et al. reported that the probability of FMT cost-effectiveness for first and subsequent CDI episodes was 90% (530).

For decades, it has been believed that the effectiveness of FMT was derived from the transfer of “good bacteria” from a “microbiologically healthy” donor to a patient with disrupted gut flora. However, a groundbreaking study by Ott et al. demonstrated that FMT was effective even when sterile fecal filtrate material was transferred to patients with CDI (531). This study, from an etiopathogenic perspective, opened up new hypotheses, such as the potential role of bacteriophages (531) (which will be further discussed in section “Phage therapy”).

FMT is recommended, with different emphasis, as a rescue therapy for patients with severe and fulminant CDI refractory to antibiotic treatment and not eligible for surgery by ESCMID (weak recommendation subjected to a preliminary risk-benefit analysis) (208, 260) and ACG (strong recommendation) (255) guidelines. In patients with multiple episodes of CDI, FMT is recommended as a preventive strategy against further recurrences by ESCMID (after standard-of-care antibiotic pre-treatment, weak, moderate recommendation) (208, 260), ACG, and IDSA (both strong recommendation) guidelines (208, 259, 459).

FMT is not a straightforward process, firstly for donor screening and secondly because delivering feces is challenging. FMT can be performed through different procedures, including colonoscopy, nasogastric/nasoduodenal tube, or enema, while recent studies have proposed a delivery via laboratory-designed frozen oral capsulized preparations (260). A recent meta-analysis has concluded that FMT performed with colonoscopy is superior to enema and nasogastric administration in successfully resolving CDI

symptoms, with a comparable efficacy of colonoscopy and capsule (430). Accordingly, IDSA guidelines gave a strong recommendation for delivering FMT through colonoscopy or capsules, reserving delivery by enema when the aforementioned methods are unavailable (208, 259, 459). However, it is important to consider that delivery of FMT via colonoscopy or nasogastric tube may expose the patient to the risk of perforation.

Furthermore, multiple factors related to patient characteristics (i.e., presence of inflammatory bowel diseases, CDI-related hospitalization before FMT, inpatient location of FMT, and administration of non-CDI antibiotics), procedural pitfalls (i.e., poor quality of bowel preparation before colonoscopy), and the severity of the CDI may increase the risk of failure up to fourfold after FMT (497). Other potential issues are related to the unavailability of healthy donors and to the fact that the donor may be a carrier for other infections (532). This risk is significantly reduced by donor screening, albeit it may be expensive: approximately 500\$–1,000\$ for stool screening and preparation) (533). Recent research demonstrated that the safety extends even to patients aged  $\geq 85$  years (534).

In a recently published randomized clinical trial comparing FMT vs vancomycin in patients with first or second CDI episodes, statistical superiority of FMT, both in first and second CDI episodes, was found (535). It is likely that this trial will serve as the basis for the recommendation of FMT as a first-line treatment for initial episodes of CDI in the upcoming guidelines.

## Bezlotoxumab

Starting with the assumption that CDI pathogenesis is mediated by toxins, over the years, efforts have been made to find a therapeutic solution capable of acting not only on the bacterium itself but on the effectors of damage (i.e., toxins). Studies on passive or active immunization against *C. difficile* toxins A and B have already been demonstrated to be effective in animals challenged with toxigenic *C. difficile* (536–538). Based on these premises and according to additional studies on rodents and piglets, the decision was made to develop fully human monoclonal antibodies against toxin A (actoxumab) and toxin B (bezlotoxumab). Contrary to expectations, these drugs did not show to be effective for CDI episodes in terms of clinical cure; however, bezlotoxumab proved to be effective (administered alongside standard therapy) in reducing recurrence (bezlotoxumab vs placebo in MODIFY I: 17% vs 28%,  $P < 0.001$ ; in MODIFY II: 16% vs 26%,  $P < 0.001$ ) (14). In this registration study, a subgroup analysis was performed on primary CDI patients, 75 out of 556 (13.5%) receiving bezlotoxumab plus standard-of-care treatment had an rCDI, while 114 out of 545 (20.9%) in the placebo group had rCDI at the 12 weeks follow-up (absolute difference: 7.4;  $P < 0.05$ ) (14). Today bezlotoxumab is recommended also in the first CDI episode (alongside standard of care) in patients at high risk of recurrence, who did not receive fidaxomicin according to the ESCMID guidelines (208) or from the first recurrence onward in combination with standard of care according to IDSA guidelines (459).

It is important to clarify that the protective benefit of bezlotoxumab on recurrent CDI appears to be mitigated in CDI episodes sustained by RT027. In fact, both the trial by Wilcox et al. (14) and a post hoc analysis of MODIFY I and II trials did not find statistical differences in the 027 subgroup (539, 540). Similarly, in the post hoc analysis by Prabhu et al., which included only patients at high risk of rCDI, 9 out of 67 (13.4%) in the bezlotoxumab group and 14 out of 81 (17.3%) in the placebo group experienced hospital readmission within 30 days, without statistical difference, leaving the benefit unproven or at least not demonstrated.

Nevertheless, a cost-effectiveness analysis (bezlotoxumab vs placebo) performed from data of the registration study demonstrated that bezlotoxumab was associated with 0.12 quality-adjusted life-years (QALYs) gained and was cost-effective in preventing CDI recurrences in the entire trial population, with an incremental cost-effectiveness ratios (ICER) of \$19824/QALY gained. Bezlotoxumab was also cost-effective in the subgroups of patients aged  $\geq 65$  years (ICER of \$15298/QALY), immunocompromised

patients (ICER of \$12597/QALY), and patients with severe CDI (ICER of \$21430/QALY) (541).

Regarding side effects, an issue arises in cardiopathic patients. In phase III clinical trials, heart failure has been more commonly reported in bezlotoxumab-treated patients compared to placebo-treated patients (14). These adverse reactions were observed primarily in patients with underlying congestive heart failure (CHF). In patients with a history of CHF, 12.7% of bezlotoxumab-treated patients and 4.8% of placebo-treated patients had a serious adverse reaction with worsening of heart failure during the 12-week study period. Additionally, in these patients, there were more deaths in the bezlotoxumab-treated group (19.5%), vs in the placebo-treated group (12.5%). Causes of death varied and included cardiac failure, infections, and respiratory failure. According to the product data leaflet in patients with a history of CHF, bezlotoxumab should be reserved in cases where the benefit outweighs the risk (542).

### Fulminant/refractory CDI

Complicated (or “fulminant”) CDI is a rather uncommon (up to 3%–5% of all CDI cases) but serious condition (543). There is no agreement on the very definition of “fulminant.” IDSA and ACG guidelines define a CDI episode as fulminant when the patient experiences hypotension, shock, ileus, or megacolon (259, 459). ESCMID guidelines define “fulminant” by the presence of one of the following factors that needs to be attributed to CDI: hypotension, septic shock, elevated serum lactate, ileus, toxic megacolon, bowel perforation, or any fulminant course of disease (i.e., rapid deterioration of the patient) (208).

No consensus exists regarding the management of fulminant CDI. IDSA guidelines do not include fidaxomicin for fulminant CDI since solid evidence on its use in these cases is lacking, thus recommending increasing the vancomycin dosage to 500 mg every 4 hours orally or, if not possible, rectally (259, 459). Similarly, ACG guidelines recommend vancomycin at 500 mg every 6 hours and suggest considering the addition of parenteral metronidazole (low quality of evidence) (208, 259, 459). Conversely, ESCMID guidelines do consider the use of fidaxomicin even in fulminant cases. However, they do not recommend adding metronidazole but suggest evaluating the addition of tigecycline on a case-by-case basis.

Increasing, although weak, evidence on the role of FMT for fulminant CDI has been published. Nevertheless, the ACG guidelines strongly recommend considering FMT for fulminant CDI refractory to antibiotics, especially in patients who are poor surgical candidates, noting that “careful donor selection and screening can mitigate the risk of infection transmission” (208, 259, 459). An RCT by Ianiro *et al.* compared single administration of FMT to multiple fecal infusions in severe, antibiotic-refractory CDI cases. The data suggested that multiple infusions of FMT may be more effective than a single infusion. However, a control group is lacking (544).

Early surgical treatment could be lifesaving in selected patients with fulminant CDI (545). ESCMID guidelines recommend consulting a surgeon for any severe complicated case (208, 259, 459). According to WSES guidelines, surgery should be promptly considered in patients who present organ failure, including increased serum lactate or vasopressor requirements (390). When the surgical route is feasible, the treatment of choice is total abdominal colectomy (542). Indications and techniques for surgery to treat CDI are discussed in depth in section “Surgery.”

Our opinion aligns more with ESCMID guidelines since we believe that gut translocation from bacteria other than *C. difficile* may precipitate the progression of the disease; therefore, tigecycline would have a double effect, but most importantly, a prompt surgical consultation should be requested in fulminant/refractory cases.

## Surgery

Apart from fulminant cases, an important consideration in the remaining “gray” cases is the timing of intervention because acting too early could lead to resection of an unresectable bowel intestine, but delaying intervention could be fatal. Although there is no common consensus, some authors have identified criteria that may be helpful in promptly recognizing patients who require surgical consultation. An abdominal CT is strongly recommended prior to surgery (15, 205, 287).

In 2018 guidelines, IDSA suggested surgical treatment for severely ill patients with fulminant CDI, particularly in those with WBC count  $\geq 25,000$  cells/mL and rising lactate level  $\geq 5$  mmol/L (15). WSES, in its 2019 guidelines, suggested a surgical approach in CDI patients with rapid toxemic progression despite medical therapy (390). ESCMID, in the 2021 guidelines, suggested surgical consultation for patients whose clinical condition is deteriorating and those not responding to CDI treatment (208). Lastly, ACG in its 2021 guidelines suggested a surgical approach for refractory severe or fulminant CDI (i.e., toxic megacolon, ischemia, and perforation) (259).

Patients with megacolon, colonic perforation, acute abdomen, septic shock, or organ failure usually benefit from prompt surgical evaluation. Another concerning category is that of patients with a reduction in the number of loose stools but with increasing leukocytes and inflammation indexes because reducing stools could be the preamble of an ileus/megacolon). Other alarming features are alteration of mental status which could reflect toxemia (546–549) and a rise in serum lactate or WBC which could reflect toxemic and/or bacteremic translocation from the gut (550).

The optimal timing for surgery is still debated, each guideline recommends different timing for surgical evaluation, but some authors suggest that the optimal window for surgical management could be 3–4 days (551). Some authors suggest surgical consultation when WBC  $\geq 20,000$  cells/mL and lactate  $\leq 4.9$  mmol/L since waiting longer is associated with higher mortality (550). Indeed, patients with higher mortality are those for whom surgery is delayed: as shown by some authors, more critical patients (e.g., with acute renal failure, multiorgan failure, requiring vasopressor or mechanical ventilation) have worse outcomes despite surgery (552, 553).

To date, two surgical approaches are employed, namely subtotal colectomy and diverting loop ileostomy with colonic antegrade lavage (15, 208, 390). Subtotal colectomy (surgical removal of colon, with or without sigma, anastomosis between ileum and rectum), the most commonly used surgical approach worldwide, has been shown to reduce mortality when compared to medical therapy (552–555). Although less common than subtotal colectomy, diverting loop ileostomy has increasingly become a solid alternative to colectomy for fulminant CDI (556). According to this approach, a loop ileostomy is used for colonic lavage with a warmed polyethylene glycol solution or electrolyte solution, followed by antegrade instillation of vancomycin through the ileostomy. At present, literature data showed no statistically significant difference between the two techniques in terms of outcomes. A large retrospective study published in 2019 by Juo et al. included 3,021 adult patients in the US from 2011 to 2015 who underwent surgery for CDI (2,408 subtotal colectomies and 613 loop ileostomies). The authors found that the annual proportion of patients undergoing diversion loop ileostomy increased from 11% in 2011 to 25% in 2015, and there was no difference in in-hospital mortality between subtotal colectomy and loop ileostomy group (26% vs 31%;  $P = 0.28$ ) (16). Another national US database review (2011–2016), including a total of 457 patients, showed no statistically significant difference in mortality between diverting loop and colectomy (26% vs 31%, respectively) (557). Furthermore, a retrospective multicentric study showed lower mortality in patients treated with diverting loop compared to colectomy (respectively, 17% vs 40%,  $P = 0.002$ ) (558).

To conclude, surgical therapy can be lifesaving, but timing is critical. Patients with signs of shock or ileum should be promptly evaluated by a surgeon. Additionally, “at-risk” patients whose clinical conditions are deteriorating, or who have worsening blood tests

indicating a fulminant form (such as lactate >2.2, WBC >20,000, creatinine >1.5, and albumin <2.4), could benefit from surgical management.

### Orally administered microbiome

As described before, FMT is a rescue therapy for rCDI. However, the heterogeneity of stool donors and the risk of acquiring MDR bacteria may pose a limit. Thus, in recent years microbiome-based drugs have been developed and others are in development.

Currently, only two fecal microbiota products have been approved by the FDA: Rebyota and Vowst.

Rebyota (RBX2660) is a novel filtered fecal microbiota suspension from qualified donors, for rectal administration, useful in reducing recurrence of CDI (559). It showed an overall 8.8% reduced risk of rCDI compared to placebo among the overall population. Results are non-statistically significant, but subgroup analysis showed a higher success rate in patients between the ages of 65 and 74, in the RBX2660 group (560). RBX2660 was approved by the FDA in 2022 (Rebyota) for patients  $\geq 18$  years old, after anti-CDI antibiotics, as a pre-packaged 150 mL suspension for one-dose rectal enema (561). The first real-life experiences with this product are being reported (562).

Vowst (SER-109) has been evaluated in a phase III trial conducted on 182 patients (563). SER-109 capsules containing Firmicutes spores are obtained through donation, purified, and administered after standard-of-care antibiotics, in four capsules each day for a total of 3 days. SER-109 showed a statistically significant superiority to placebo in reducing recurrence rate, respectively, 11/89 (12%) vs 37/93 (40%), with a relative risk of 0.32 (95% CI, 0.18–0.58,  $P < 0.001$ ) (564).

These products will be discussed in more detail in section “Live biotherapeutic products.”

### Other active compounds

Oral microbiome is a new paradigm, but several other compounds have demonstrated activity against *C. difficile*. In these sections, we will discuss the most studied.

#### Auranofin

Auranofin, an oral drug approved for rheumatoid arthritis, is known for its *in vitro* inhibition of *C. difficile* cell growth, sporulation, and toxin production (565). In a PK/PD point of view, oral auranofin was mostly excreted in feces, leading to high concentrations in the gut, and carried out its activity by causing an impaired synthesis of the selenoprotein in *C. difficile* resulting in alteration of protein synthesis (566, 567). Auranofin is also active against few gram-positive bacteria, such as methicillin-susceptible *S. aureus*, methicillin-resistant *S. aureus*, and *Enterococcus* spp., but lacks activity against gram-negative bacteria (567, 568).

#### Bacitracin

Bacitracin, an oral antibiotic, inhibits cell wall synthesis, and its activity in the inhibition of *C. difficile* toxin was, recently, discovered. (569). Two less recent clinical trials compared bacitracin to vancomycin for the treatment of CDI, reporting a clinical efficacy similar to vancomycin (570, 571). However, a network meta-analysis by Beinortas et al. showed inferiority of bacitracin compared to metronidazole, vancomycin, and fidaxomicin in terms of sustained clinical cure (460).

#### Berberine

Berberine chloride has potential activity against *C. difficile*. Its mechanism of action is still being evaluated; some authors found metabolic inhibition by ion secretion and reduction of bacterial enterotoxin formation in mice models (572). Berberine in combination with vancomycin can also inhibit *C. difficile* growth, biofilm formation, and

motility. Furthermore, berberine has a synergistic effect in reducing vancomycin MIC for CDI (573).

### **Butyrate**

Butyric acid is an important short-chain fatty acid produced during anaerobic fermentation by the intestinal microbiome. It contributes to a normal gut barrier integrity and function, and its reduction could be associated with intestinal epithelial apoptosis and *C. difficile* proliferation (574–576). Conversely, in a pig model, a high-fiber diet results in higher butyric acid concentration and inhibition of *C. difficile* growth (577). Butyrate-producing bacteria are being used as experimental anti-CDI therapy.

### **Ebselen**

Ebselen, an organoselenium compound, is a drug originally studied in clinical trials for psychiatric disorders. It demonstrated its activity on *C. difficile* in covalently binding to the cysteine protease domains of both toxin B and toxin A (578, 579). Furthermore, ebselen can kill *C. difficile* cells altering the redox homeostasis by changing cellular NAD and NADH concentration (578). Ultimately, it is observed that ebselen reduces rCDI and decreases inflammatory markers in a hamster model (580).

### **Manuka honey**

Manuka honey is common in New Zealand and Australia, and it is derived from the nectar of the Manuka tree. This honey is known to have antimicrobial properties against *S. aureus*, *E. coli*, and *Pseudomonas aeruginosa* due to its significant content of methylglyoxal. *In vitro*, Manuka honey demonstrated sporicidal, bactericidal, and antibiofilm effects against *C. difficile* (581–583). Furthermore, Manuka honey was used in an experimental therapy in four patients with rCDI and was administered via rectal enema in a 300-mL solution at 15%, after a 3-day course of fidaxomicin. A decrease in the *C. difficile* load was found in their gut microbiota, with a partial restoration of microbiota diversity and cessation of watery stools (584).

### **Nitazoxanide**

Nitazoxanide is an antiparasitic drug, with antibacterial activity against *C. difficile* and other infectious diarrhea (585, 586). Two randomized double-blind clinical trials comparing the use of nitazoxanide for CDI, both by Musher et al., are available. The first clinical trial, from 2006, compared the efficacy of nitazoxanide vs metronidazole in patients with CDI. It showed no statistically significant difference among nitazoxanide and metronidazole groups in terms of clinical response rate (85% vs 82%, respectively) and recurrence rate (respectively, 14% vs 24%) (587). The second clinical trial, published in 2009, compared the efficacy of nitazoxanide vs vancomycin in patients with CDI. Fifty patients were randomized to receive a 10-day treatment of nitazoxanide or vancomycin, showing no statistically significant difference among nitazoxanide and metronidazole groups in terms of clinical response rate (77% vs 74%, respectively) and recurrence rate (5% vs 7%, respectively) (588).

### **Pomegranate**

Pomegranate juice has *in vitro* antibacterial activity against *C. difficile* due to polyphenols, such as gallic acid (589, 590) and punicalagin (591). To date, pomegranate juice appears to have bactericidal activity against *C. difficile* (590) as well as the case of the hypervirulent strain NAP1/027/BI. Additionally, pomegranate can reduce *C. difficile* toxin B production, and it appears that it does not affect the growth of *Lactococcus lactis*, *Lactobacillus casei*, and *Bifidobacterium animalis* (591).

## Ramoplanin

Ramoplanin is a glycolipodepsipeptide oral antibiotic, with activity against gram-positive bacteria, both aerobic and anaerobic, reaching high concentrations in the colon.

Ramoplanin inhibits the transglycosylases responsible for peptidoglycan biosynthesis, preventing cell wall synthesis leading to cellular killing (592, 593). Ramoplanin can inhibit *C. difficile* growth and sporulation (594–598).

In a phase II clinical trial, ramoplanin showed similar clinical cure rate in comparison to vancomycin despite a higher rate of adverse events (599).

## Rifaximin

Rifaximin is a rifamicin antibiotic that acts in inhibition of bacterial RNA synthesis and commonly used for traveler's diarrhea. When administered orally, it achieves high colonic concentrations. In literature, a three-case series showed the usefulness of rifaximin in preventing *C. difficile* recurrence (513, 600, 601).

A phase II, double-blind, randomized clinical trial showed rifaximin use after a standard course of metronidazole or vancomycin for CDI can reduce recurrence rate (602). A different clinical trial showed similarity of rifaximin vs vancomycin in terms of clinical cure and recurrence rates (603).

Another single-blind randomized clinical trial by Gawronska et al. compared the use of rifaximin vs metronidazole for the treatment of CDI in pediatric patients with inflammatory bowel disease. Authors found a similar cure rate in both metronidazole and rifaximin groups (70.6% vs 78.6%, respectively,  $P = 0.5$ ), without difference in recurrence rates (17% vs 0%, respectively,  $P = 0.3$ ) (604). A network meta-analysis by Benoitras et al. showed inferiority of rifaximin compared to vancomycin and fidaxomicin but superiority to metronidazole, in terms of sustained clinical cure (460).

## Probiotics, prebiotics, and postbiotics

Probiotics are "live" microorganisms normally found in the intestinal tract, such as bacteria (i.e., Bifidobacteria and Lactobacilli) and yeasts (i.e., *Saccharomyces boulardii*). There are dozens of different species that have the ability of surviving the digestive action of gastric acid, intestinal enzymes, and bile salts. They are able to adhere to intestinal cells and begin to colonize them, without giving immune reactions. Probiotics have beneficial effects: antagonistic action against pathogenic microorganisms, production of antimicrobial substances, and protection of the gut against antibiotics-associated diarrhea.

Prebiotics, mainly represented by carbohydrates and oligosaccharides, are also essential elements of the microbiota, whose action aids in the expansion and activity of probiotics. These can be used to counteract the side effects of antibiotics. Prebiotics are found in certain foods such as whole grains, legumes, and vegetables (asparagus, artichokes, chicory, onion, and garlic) but also in bananas or honey. Prebiotics are also present in yogurt and fermented milk, often defined as symbiotic foods, due to the supply of both prebiotics and probiotics.

Postbiotics, defined as inactivated microbial cells that confer health benefits to the host, are released during the fermentation processes of food matrices by bacteria, and they have an intestinal modulation activity on the microbiome (605).

There are many studies concerning the role of probiotics on CDI. In 1994, a double-blind randomized controlled trial by McFarland et al. (606) evaluated the efficacy of *Saccharomyces boulardii* in combination with antibiotics for preventing *C. difficile* infection. A total of 124 patients were included, divided into the interventional group (*S. boulardii* 1 g/day) and control group (placebo). Patients treated with *S. boulardii* had lower recurrence rate of CDI (RR 0.43; 95% CI 0.20–0.97) compared to placebo. In the subgroup of patients with previous CDI episodes, recurrence rate was lower in the probiotic group compared to placebo (34.6% vs 64.7% respectively;  $P = 0.04$ ) but



not in the subgroup of patients with an initial CDI episode (19.3% vs 24.2% respectively;  $P = 0.86$ ). Another prospective double-blind trial by Surawicz et al. included 180 patients showing the efficacy of *S. boulardii* during antibiotic treatment in reducing antibiotic-related diarrhea. Among patients on placebo, 14/64 (21.8%) developed diarrhea compared to 11/116 (9.5%) patients treated with *S. boulardii*, with a statistically significant difference ( $P = 0.038$ ) (607). A multicentric study by Heil et al. evaluated probiotics use, at the time of antibiotic prescription, for primary prevention of CDI using a computerized clinical decision support tool. Propensity score-matched analysis showed that patients who received probiotics had similar rates of CDI compared to those who did not receive probiotics (OR 1.46; 95% CI 0.87–2.45) (608).

A systematic review by Maddoff and colleagues evaluated the effect of probiotics, prebiotics, and other polymers compared to placebo on the prevention of rCDI (609). The authors included eight RCTs, five of which showed no statistically significant benefits. Three out of eight studies showed benefits in the prevention of rCDI of *S. boulardii* (606), oligofructose (RR 0.24, 95% CI 0.11–0.56) (610), and the non-toxigenic *C. difficile* strain M3 (RR 0.11; 95% CI 0.02–0.54) (539). A systematic review with meta-regression analysis showed that the administration of probiotics during antibiotic courses in hospitalized adults reduces the risk of CDI by >50% (611, 612).

On the other hand, the main international guidelines do not recommend usage of probiotics for CDI prevention. More specifically, ACG recommends against the use of probiotics for the prevention of CDI in patients treated with antibiotics (primary prevention) (conditional recommendation, moderate quality of evidence) and also in the prevention of CDI recurrence (secondary prevention) (strong recommendation, very low quality of evidence) (208, 259, 459); ESCMID does not recommend routine administration of probiotics to prevent CDI when on antibiotic treatment (strong recommendation, low quality of evidence) (208, 259, 459), and IDSA states that there are insufficient data to recommend administration of probiotics for primary prevention of CDI outside of clinical trials (no recommendation) (208, 259, 459).

## ANTIMICROBIAL RESISTANCE

When it comes to antimicrobial resistance, one of the principal characteristics of *C. difficile* is that most antimicrobial compounds have limited to no activity against dormant cells, such as spores. This intrinsic resistance conferred by spores ensures that *C. difficile* can persist in the presence of antibiotics. Moreover, *C. difficile* demonstrates several acquired resistance mechanisms. In fact, there is a global increase in resistance to antibiotics in *C. difficile*, with the emergence of novel strains that are often more virulent and with multidrug resistance profiles. As seen in other species, common antimicrobial resistance mechanisms involve the alteration of the antibiotic, the modification of the antibiotic target site, and the extrusion of the drugs via efflux pumps.

Macrolides and lincosamides are often associated with the development of CDI (612). Erythromycin ribosomal methylase genes (*erm*) are considered to mediate the resistance of *C. difficile* to clindamycin and erythromycin. Other genes such as *cfb*, *cfrc*, and *cfre* encoding a 23S rRNA methyltransferase have been implicated in the resistance of *C. difficile* to macrolides and lincosamides (613). Efflux pumps may also play a role in resistance to these antibiotics (614).

Among beta-lactams, cephalosporins are considered the antibiotic mostly associated with CDI development (75). *C. difficile* strains are fully resistant to most cephalosporins (64). Penicillin-binding protein 2 (PBP2) is essential for *C. difficile* vegetative growth and serves as the primary bactericidal target for beta-lactams in *C. difficile*. PBP2 is insensitive to cephalosporin inhibition, appearing as the main basis for cephalosporin resistance in this organism (615). Furthermore, some *C. difficile* strains are known to encode beta-lactamase enzymes and efflux pumps that could also play a role in *C. difficile* resistance to cephalosporins (616).

Susceptibility of *C. difficile* to fluoroquinolones has always been low. The widespread development of fluoroquinolone resistance is associated with the emergence

of epidemic RT027 strains (617). Mutations in the quinolone resistance-determining regions of *gyrA* and/or *gyrB* genes result in several amino acid substitutions, conferring resistance to fluoroquinolones. Different types of efflux pumps have also been implicated in resistance to fluoroquinolones (618, 619).

Tetracycline-resistant *C. difficile* strains produce ribosomal protectant proteins such as Tet(M), Tet(W), and Tet (44), preventing binding of the antibiotics to the ribosome (620). To date, no resistance to newer tetracyclines such as tigecycline and omadacycline in *C. difficile* has been reported.

Resistance to chloramphenicol is mediated by two copies of the *catD* gene, which encodes the chloramphenicol acetyltransferase enzyme. This provides resistance based on modification of the antibiotic, by the insertion of an acetyl group from acetyl CoA to the primary hydroxyl group of chloramphenicol, rendering it unable to bind to the ribosome (621).

Genes that encode the rRNA methyltransferase *cfr* (*cfrB*, *cfrC*, and *cfrE*) have been detected among clinical isolates of *C. difficile* and are responsible for resistance to linezolid. The Cfr protein catalyzes the methylation of 8-methyladenosine at A2503, positioned in 23S rRNA of the large ribosomal subunit, inhibiting the interaction with the antibiotic (613).

Emerging resistances are considered those to first-line antibiotics. Resistance to vancomycin could arise due to alterations of the vancomycin-binding site in peptidoglycan precursors mediated by mutations in *vanG* operon enzymes (622). A *vanG* operon-like gene cluster has been detected in about 85% of *C. difficile* clinical isolates; however, it is not always associated with resistance. In particular, substitutions Ser313Phe and Thr349Ile in VanS and Thr115Ala in VanR have been associated with resistance to vancomycin in *C. difficile*. This type of resistance has been recently described in *C. difficile* clinical isolates in Israel and in the US, and also associated with RT027 (623, 624). More concerning are recent reports of *C. difficile* isolates carrying *vanB* and *vanA* genes that mediate high-level vancomycin resistance, described in Australia and Iran, respectively (625, 626).

Resistance to fidaxomicin is not widely known, although a single *C. difficile* strain isolated from a patient with CDI recurrence showed reduced susceptibility (483). Induced and genetically engineered mutations in RNA polymerase subunit *rpoB* and putative transcriptional regulator MarR caused resistance to fidaxomicin in experimental studies (627, 628).

While no longer recommended as first-line therapy, metronidazole is still used by clinicians. Metronidazole treatment failures have been increasingly recognized in recent years mainly attributed to suboptimal pharmacokinetics rather than resistance (629). Low levels of resistance of *C. difficile* to metronidazole have been reported in many countries (630). Metronidazole resistance in *C. difficile* may involve multi-genetic mechanisms that are possibly involved in oxidoreductive and iron-dependent metabolic pathways (631). Mutations in genes involved in electron transport such as the glycerol-3-phosphate dehydrogenase-encoding gene *glyC* (Ala229Thr) and the pyruvate-flavodoxin oxidoreductase (PFOR)-encoding gene *nifJ* (Gly423Glu) have been linked to resistance to metronidazole (632, 633). Impairment of intracellular iron content has been implicated in *C. difficile* resistance to metronidazole (631). Also, an efflux pump system has been molecularly confirmed in metronidazole resistance (634). High rates of metronidazole resistance have been observed in *C. difficile* isolates carrying the 7-kb plasmid pCD-METRO, in particular for isolates belonging to RT010 and RT020 (clade 1) and the epidemic strain RT027 (clade 2). However, the specific sequences responsible for metronidazole resistance in pCD-METRO have not been identified yet (630).

Rifamycins, such as rifaximin and rifampicin, were the most active agents *in vitro*, inhibiting *C. difficile* strains at very low concentrations. Mutations in the rifamycin resistance-determining region of RpoB found in clinical isolates of *C. difficile* are associated with rifamycin resistance (617, 635).

## INFECTION CONTROL

Surveillance of CDI on both the hospital and ward level associated with a timely feedback of infection rates is recommended as this strategy may lead to a reduction in the incidence of infection (636). The rate of hospital-onset CDI should be documented as the number of cases per 10,000 patient-days while the community-onset health-care facility-associated CDI prevalence rate as the number of cases per 1,000 patient admissions (15).

### Hand hygiene and disposable gloves

CDI is mainly related to the care practices of healthcare workers' hands (637). Implementing strict hand hygiene policies for healthcare workers is a key strategy to prevent *C. difficile* cross-infections in every clinical facility (638). Nevertheless, studies on healthcare workers have documented compliance rates from 9% to no more than 60% (639, 640). The implementation of hand hygiene programs in healthcare settings may have a highly positive impact on improving healthcare workers' adherence to hand hygiene. Additional policies to promote and facilitate hand hygiene include the installation of new sinks, posting dedicated signage and providing interventions to increase hand hygiene compliance (641, 642). Unfortunately, multimodal interventions that include either only some or all of the strategies recommended in the World Health Organization (WHO) guidelines (643), as well as the recommended strategies plus additional ones (e.g., performance feedback, education, cues such as signs or scent, placement of alcohol-based hand rub products close to point of use) may only slightly improve hand hygiene compliance (644). No strong recommendations exist regarding the most effective technique/product for the removal of *C. difficile* and its spores (636). *C. difficile* spores are highly resistant to alcohol; therefore, spores may simply be transferred onto the skin instead of being killed by the product. This was the reason why mechanical washing with soap and water, chlorhexidine-based antiseptic, is recommended as more effective at removing *C. difficile* spores than an alcohol hand rub (645).

Interestingly, despite both the Centers for Diseases Control and Prevention and WHO stating that, besides representing a useless waste of resources, inappropriate glove use may result in missed opportunities for hand hygiene (646), a "universal gloving" approach preceded and followed by hand washing for all healthcare workers having contact with CDI patient (wash-glove-wash policy) has been recognized as able to increase hand hygiene compliance by threefold (647). Furthermore, the introduction of automated hand hygiene technologies (e.g., providing healthcare workers with a "badge" to wear, that beeps when hand hygiene has not been performed by measuring hands alcohol concentration or hands washing time, may significantly and rapidly increase hands hygiene compliance by more than 90% while decreasing CDI incidence (648, 649). However, questions related to the high cost in the face of poor literature evidence, as well as workers' control and privacy issues, make their adoption still questionable.

### Patient isolation, cohorting interventions, and contact precautions

Patients may be exposed to CDI not only as a result of direct contact with an infected patient but also indirectly by contact with a contaminated environment. Consequently, CDI control policies are particularly complex. On one hand, this is because of the bacterial characteristics (e.g., ubiquitous environmental and human reservoirs, spore-forming bacterium with high environmental persistence). On the other hand, hospital facilities are promiscuous and often chaotic environments, characterized by high patient turnover. Consequently, each new patient is exposed to the risks of being infected with the same organism when admitted to the hospital, especially if located in a hospital room where an infected or carrier patient was previously hospitalized (650). Cohorting of staff, or patients, or both is thus a strategy of routine adoption to enhance infection control efficacy (651).

At the same time, maintaining consistently high levels of attention to diffusion control strategies among patients, caregivers, visitors, and healthcare providers is extremely complex. For example, after caring for patients with CDI, almost one-quarter of them have hands contaminated with *C. difficile* spores, whether or not they use gloves (652). Each hospital facility should provide protocols that nurses can automatically implement (nurse-driven protocols) to ensure prompt isolation of patients with suspected (patients with clinically significant unexplained diarrhea) or confirmed CDI (653). This includes following rigorous recommended hand hygiene practices (see above); moving the patient to a single-patient room with a dedicated toilet (if unavailable, putting patients with confirmed CDI in the same room); ensuring rigorous contact precautions including wearing a clean, non-sterile gown and gloves before entering the patient's room, and changing between patient contacts, while the diagnostic workup is ongoing and after CDI confirmation; providing the patient with a bath or shower with soap and water daily; avoiding patient transport from the isolation room, unless strictly necessary; maintaining all the above measures for at least 48 hours after diarrhea termination for asymptomatic patients. These measures should be maintained up to hospital discharge or patient's transfer to a different hospital or community facility. Furthermore, blood pressure cuffs, stethoscopes, glucometers, thermometers, bedpans, and any other portable medical equipment should be dedicated to every single patient and must not travel between patients and rooms (654, 655). In addition, the use of launderable cover for a patient's mattress and bed deck should be considered as has been described as associated with a decreased rate of healthcare-associated CDI (656).

## Environmental interventions

Studies reported that feces of CDI patients may contain up to 10 million bacteria per gram, while *C. difficile* spores may remain viable on contaminated fomites for months or years despite routine cleaning (657). However, the transmission of *C. difficile* does not only occur by contact. Indeed, airborne dissemination of *C. difficile* spores from infected and symptomatic patients has been demonstrated in multiple studies. Furthermore, a number of routine, daily practices (e.g., bed making, bedpan washing, toilet flushing, healthcare workers or visitors' movement) have been found as able to potentially generate aerosols embedding *C. difficile* spores (658).

Similar to hand hygiene, environmental cleaning procedures are among the interventions with the highest theoretical rationale for the control of CDI. Basic rules, such as starting to clean from the cleanest toward the dirtiest zones of the room (e.g., toilets), should be followed. Moreover, the reliability of cleaning practices should be routinely checked either through direct observation or, when possible, by adopting fluorescent markers (654).

The most effective interventions, resulting in a 45% to 85% reduction in CDI, include a daily to twice-daily sporicidal disinfection and the terminal cleaning of patients' rooms, including "high-touch" surfaces like bed rails, door handles, and side tables (636, 659). Chlorine-based products (e.g., sodium hypochlorite, sodium dichloro-s-triazinetrione, sodium dichloroisocyanurate), as well as hydrogen peroxide and peroxyacetic acid, have demonstrated a greater killing action toward *C. difficile* spores (660).

No-touch disinfection systems such as continuous ultraviolet germicidal irradiation (UV-C) at the room level have been shown to significantly reduce airborne bacteria, with the potential to lower the incidence of CDI and other healthcare-associated infections caused by contact pathogens (661). This strategy may be effective in reducing transmission/incidence of CDI, especially in clinical facilities with high CDI rates, to control deficiencies in routine cleaning and disinfection (e.g., human error or substantial contaminations) but must be intended as adjunctive, not substitutive, to standard cleaning and disinfection strategies (654, 662–664).

## The issue of cross-transmission from asymptomatic carriers

A large number of patients or healthcare workers carry *C. difficile* without clinical symptoms (asymptomatic carriers) and act as reservoirs of *C. difficile*. Although routine screening is not recommended (636), the risk of infection transmission from asymptomatic carriers may be high, as no strict contact precautions or cleaning procedures are adopted for these subjects. A recent US study screened daily the patients admitted to an ICU by sequencing whole genome on all isolates (665). They found that, despite almost 10% of patients detected as asymptomatic carriers of toxigenic *C. difficile*, only 1% of patients negative on admission acquired *C. difficile* via cross-transmission. The authors concluded that current infection prevention practices to prevent nosocomial cross-transmission of *C. difficile* are very effective. It should be noted, however, that the study was carried out in an ICU, where more strict adherence to prevention practice is expected, the nurse-to-patient ratio is very low (e.g., 1:1 or 1:2), and visitor access to the unit is very limited and controlled. The authors of the above study found that patients who carried toxigenic *C. difficile* on admission had 24 times greater risk for developing CDI compared to non-carriers, hoping for the development of interventions to prevent the transition from asymptomatic to symptomatic CDI.

## PRIMARY AND SECONDARY PROPHYLAXIS

In addition to prevention strategies and medication stewardship programs, the fight against CDI can benefit from prophylactic interventions tailored to individual patients, aimed either at preventing CDI in at-risk populations (primary prophylaxis) or at reducing the risk for recurrent CDI (secondary prophylaxis) (666). In detail, these prophylactic interventions may include active and passive immunization, antibiotic prophylaxis, and microbiota-targeted therapy to prevent or restore gut dysbiosis (666).

### Antibiotic prophylaxis

Although antibiotic prophylaxis may decrease CDI rates in at-risk populations, it can deeply impact the gut microbiome, increasing the risk of recurrent CDI, and induce antimicrobial resistance (e.g., vancomycin-resistant enterococci) (463, 667). Older studies showed that primary prophylaxis with metronidazole in asymptomatic *C. difficile* carrier patients was not effective, while oral vancomycin led to a “rebound effect,” characterized by a temporary positive effect followed by a paradoxical higher rate of both colonization and infection (668).

Oral vancomycin is the most studied strategy for primary and secondary prevention of CDI in patients receiving systemic antibiotics. However, risks exist that this drug, besides promoting drug-resistant organisms, has a relevant disruptive effect on the gut microbiota, theoretically leading to CDI recurrence and increasing the risk of treatment failure (669). This, along with the support of only low-quality evidence, has led to controversial and cautious recommendations by international guidelines regarding its use.

ACG guidelines conditionally recommend using low-dose vancomycin (i.e., 125 mg once daily, to be stopped 5 days after completion of antibiotic treatments) for secondary CDI prevention in high-risk patients undergoing systemic antibiotic therapy to prevent further recurrence (low-quality evidence) (259). The ESCMID guidelines recommend against routinely using anti-CDI antibiotic prophylaxis for patients on systemic antibiotic treatment, except for selected patients with a history of multiple recurrences of CDI hastened by systemic antibiotic use, who could be considered for this prophylaxis after consultation with Infectious Diseases or Clinical Microbiology specialists and after balancing risks and benefits (208). More recently, an updated meta-analysis stated that oral vancomycin “appears to be an efficacious option for prevention of CDI in high-risk subjects undergoing systemic antibiotic treatment,” claiming, however, for additional data from RCTs before recommending this option as a good clinical practice, also for establishing its optimal dosage and duration (670). At present, only very few studies

have explored the potential of prophylaxis with antibiotics different from vancomycin. An RCT reported a lower 30-day incidence of CDI (confirmed by toxin immunoassay or nucleic acid amplification test) in neutropenic patients undergoing hematopoietic stem cell transplantation with fluoroquinolone prophylaxis treated with fidaxomicin (200 mg once daily) vs placebo (353). Nevertheless, at present, only poor evidence supporting prophylaxis with vancomycin or fidaxomicin during antibiotic treatments or other procedures implying a CDI risk is available, so neither the optimal dosing strategy nor the long-term safety (e.g., selection of multidrug-resistant microorganisms) has been established (666). A trial aimed at assessing the effectiveness and safety of oral vancomycin vs placebo in the prevention of rCDI infection in patients under systemic antibiotic therapy has been planned (671).

### Microbiota-targeted therapy: dysbiosis prevention

A novel preventive strategy consists of administering molecules able to degrade some commonly used intravenous antibiotics in the gastrointestinal tract, thus protecting the colonic microbiota from disruption. At least two molecules have completed phase II trials. Ribaxamase (a class A serine enzyme) was shown to reduce the incidence of CDI in patients receiving ceftriaxone without affecting antibiotic efficacy. DAV-132, a colon-targeted adsorbent, showed potential against beta-lactams (penicillins, cephalosporins, and carbapenems), fluoroquinolones, and lincosamides (666). However, the above medications are not yet available in clinical practice and are discussed in detail in section "Vaccine."

### Proton pump inhibitor stewardship programs

The pathophysiological mechanism linking PPIs to CDI seems to be multifactorial. On one side, gastric pH increase induced by PPIs would promote bacterial overgrowth and spore survival. On the other side, PPIs might induce impairment in neutrophil bactericidal activity or boost *C. difficile* toxin expression (672).

It is well documented that PPIs use leads to an increased risk of recurrent CDI, particularly in immunocompromised patients. These results support stronger recommendations for PPIs stewardship upon CDI diagnosis (673). However, a clear association between PPIs and CDI has been demonstrated only through observational studies, so, at present, a cause-effect relationship has not been proven. Thus, a mandatory withdrawal of PPIs in patients at risk for CDI seems not justifiable (259, 674). However, the adoption of PPI stewardship programs discouraging unnecessary PPI use would be desirable (675).

## ANTIMICROBIAL STEWARDSHIP

Antimicrobial stewardship can be defined as the coherent set of actions that promotes using antimicrobials responsibly (676). Over the past decade, an increasing interest in this topic has been observed. Narrowing the spectrum, when possible, avoiding anaerobic coverage when not necessary, and optimizing the duration of antimicrobials are some of the basic principles of antimicrobial stewardship. CDI is strongly associated with antibiotic exposure, so it is not surprising that an interconnection between antimicrobial stewardship and CDI exists. In fact, CDI incidence has been used as an indirect index of the effectiveness of antimicrobial stewardship programs (677). In 2017, an important paper on this topic was published, detailing a stewardship intervention conducted in a region of Scotland serving 11% of the Scottish population. This intervention consisted of a mixed persuasive-restrictive intervention on the so-called 4C antibiotics: fluoroquinolones, clindamycin, co-amoxiclav, and cephalosporins. The authors identified 4,885 cases of hospital-onset CDI and 1,625 cases of community-onset CDI. *C. difficile* infection prevalence density fell by 68% in hospitals and 45% in the community, during antibiotic stewardship (678).

From a global perspective, regarding healthcare facility-associated CDI, carbapenems and third- and fourth-generation cephalosporins are the most commonly prescribed

antibiotics (74). Fluoroquinolones are also under surveillance, particularly in settings with high prevalence of O27 strains, which are commonly fully resistant to all tested fluoroquinolones (679). A retrospective case-control study conducted during an outbreak between 2000 and 2001 in the US on 253 nosocomial CDI cases showed that clindamycin (OR 4.8; 95% CI 1.9–12.0), ceftriaxone (OR 5.4; 95% CI 1.8–15.8), and levofloxacin use (OR 2.0; 95% CI 1.2–3.3) were independently associated with infection. Interestingly, the etiologic fractions for these three agents were 10.0%, 6.7%, and 30.8%, respectively (680, 681). An Austrian study published in 2014 demonstrated that a dramatic interventional reduction of moxifloxacin use in hospitalized patients was followed by a 46% reduction ( $P = 0.0044$ ) in CDI cases (682). Large data from the UK showed that significant reductions in fluoroquinolone use in hospitals and in the community were associated with a significant decrease in CDI caused by fluoroquinolone-resistant isolates (50). A systematic review and meta-analysis published in 2014 support this statement after evaluating 16 articles that used quasi-experimental or observational (case-control) study designs. Results showed that implementation of antibiotic stewardship programs had an overall protective benefit on CDI incidence (pooled risk ratio 0.48; 95% CI 0.38–0.62), indicating a risk reduction for CDI of 52%, with the most significant protective effect being observed among the geriatric populations (pooled risk ratio 0.44; 95% CI 0.35–0.56). Authors also found that restrictive antibiotic stewardship programs had a statistically significant protective effect on CDI incidence (pooled risk ratio 0.46; 95% CI 0.38–0.56), while persuasive antibiotic stewardship programs did not (pooled risk ratio 0.49; 95% CI 0.24–1.01). When stratifying per class, they found that cephalosporins restriction (14 studies) was associated with a CDI risk reduction of 50%, and fluoroquinolones restriction (six studies) was associated with a 55% CDI risk reduction (683).

Generally, tetracyclines, daptomycin, linezolid, and nitrofurantoin are associated with a lower risk of CDI compared to other classes of antibiotics (65, 76, 684, 685). In particular, tetracyclines and daptomycin have been associated with protective odds ratios (685). Experimental evidence supports this finding. A study conducted on mice showed that doxycycline and azithromycin treatment did not promote *C. difficile* colonization when compared to saline controls. Moreover, the authors found a significantly lower disruption of mice intestinal flora in those exposed to doxycycline and azithromycin compared to those exposed to ceftriaxone (686).

Regarding strategies to improve antimicrobial stewardship, nursing-based actions have been advocated due to growing recognition of the importance of engaging nurses in hospital stewardship efforts. Given their constant close proximity to patients, adaptive ability, and scientific understanding of care processes across the continuum of care, the nursing profession has the potential to play a special role in infection prevention programs. Particularly, nurses can inform decisions about the need for diagnostic tests based on assessed patient symptoms, ensure timely microbiology tests are performed before antibiotics are started, and promote discussions regarding antibiotic treatment, indication, and duration (687–689).

## FUTURE PERSPECTIVES

### Main anti-CDI drugs with ongoing or completed phase III studies

#### *Antibiotics*

Over the years, vancomycin and metronidazole were predominantly recommended for most CDI patients. Yet, following its FDA approval in 2011, fidaxomicin emerged as a contender, frequently viewed as on par with or even superior to oral vancomycin. Alongside these primary three antibiotics, other medications like bacitracin, nitazoxanide, rifaximin, and tigecycline have been used in an off-label context to address CDI. Furthermore, fusidic acid and teicoplanin, which are not available in the US, have undergone scrutiny as potential CDI treatments. Some candidates, such as cadazolid, LFF571, ramoplanin, and surotomycin, did not succeed in entering the market.

The journey toward creating antibiotics specifically for CDI continues, as six promising drugs are under active clinical investigation: ridinilazole (now in phase III), MGBBP3 (completed phase II), CRS3123, DNV3837/DNV3681, and ibezapolstat (all three are navigating phase II) (690). Ridinilazole, the only phase III trial antibiotic, will be discussed further.

Ridinilazole, a narrow-spectrum antibiotic developed by Summit Therapeutics Inc., has completed two phase II studies (691) and has been compared to vancomycin in two phase III trials: Ri-CoDIFy 1 (692) and Ri-CoDIFy 2 (692). The focal phase II investigation involved CDI patients aged between 18 and 90. These participants were allocated into two groups: one group was administered 200 mg of ridinilazole twice a day, while the counterpart received 125 mg of vancomycin, administered four times daily over a 10-day span (691). The primary target of effectiveness was sustained clinical response, defined as clinical remission without CDI recurrence within 30 days of the conclusion of antibiotic treatment. SCR was achieved in 66.7% of patients treated with ridinilazole vs 42.4% of patients treated with vancomycin, with a reported treatment difference of 21.1% (90% CI 3.1–39.1;  $P = 0.0004$ ) (691). In addition, further analyses of the phase II study specimens showed that ridinilazole had a reduced tendency for gut dysbiosis than vancomycin. The phase III study was conducted on 759 adult individuals with the same treatment regimen as the phase II studies (693), revealing statistically significant lower recurrence rates for ridinilazole (8.1%) when compared to vancomycin (17.3%;  $P = 0.0002$ ). However, the data did not confirm the supposed primary outcome for ridinilazole SCR superiority when compared to vancomycin (693). Gastrointestinal symptoms were the most common treatment-emergent side effects, with termination of study medication being uncommon in both treatment groups (546–548). In addition, ridinilazole and vancomycin are currently being evaluated in a clinical study (Ri-CoDIFy 3) involving adolescents aged 12–17 years with confirmed CDI (694). In conclusion, given the unmet SCR superiority over vancomycin, it is unlikely that a ridinilazole will enter the global market in the next 2 years.

### **Live biotherapeutic products**

The growing understanding and interest in the potential role of gut microbiome in the development and healing from CDI has driven the research in microbiome-based treatments. Live biotherapeutic products (LBPs) are classified by the FDA as non-vaccine biological products containing live organisms for the prevention, treatment, or cure of human illness or condition (695). LBPs for the prevention of CDI recurrence vary from prior microbiome-based treatments, such as probiotics and FMT.

Probiotics are bacteria that, when taken in appropriate amounts, provide health advantages (696). Typically, they are uncontrolled, resulting in variable product quality and efficacy (697, 698). Consequently, results from research on probiotics for the prevention of CDI are frequently inconsistent, thus resulting in the absence of clinical recommendations toward probiotic therapeutic strategies for CDI (15, 208, 259).

The FDA regulates LBPs, requiring strict standardization, acceptable manufacturing processes, and clinical studies to demonstrate safety and effectiveness (679, 699–702). Currently, three LBPs are participating in or have completed phase III CDI therapy studies. A substantial amount of information concerning these agents remains confidential and inaccessible for our review. These LBPs try to solve gut dysbiosis during antibiotic therapy in order to avoid further recurrence of CDI, which occurs in 15% and 25% of patients. We will discuss these LBPs below, and we will mention in Table 3 also an LBP that is ready to enter in phase III (VE303) (703).

### **CP101**

The oral capsule CP101, produced by Finch Therapeutics, contains freeze-dried healthy donor stool (704, 705). After feces are collected and screened, they are processed,



TABLE 3 FDA-approved and late-stage live biotherapeutic products for CDI

Product	Content	Phase	Route	Posology	Clinical cure (8 weeks)	Safety
RBX2660	Suspension of microbiota (from HD)	III	Enema	One enema once	71%–79%	Good
SER109	Pure Firmicutes bacterial spores (from HD)	III	Oral	4 cp Daily for 3 days	87%–91%	Good
VE303	Consortium of eight non-pathogenic Clostridia (from HD)	II	Oral	10 cp Daily for 14 days	>95%	Good

<sup>a</sup>cp, capsules; HD, healthy donors.

lyophilized, and encapsulated (705). The treatment regimen consists of the administration of a single pill, without prior gastrointestinal preparation. CP101 has concluded two phase II trials: PRISM3 (706, 707) and PRISM-EXT, an extension study for PRISM3 individuals with recurrence (708, 709). In 2021, recruitment for a phase III study (PRISM4) was initiated but was delayed due to new donor screening and manufacturing criteria for possible SARS-CoV-2 infection (710, 711).

The initial CP101 trials focused on improving the formulation and determining the appropriate dosage for individuals with at least four to five CDI relapses (704, 705), and, interestingly, the proof-of-concept trial revealed that 87.8% of patients did not develop CDI recurrence within 8 weeks from the first administration. In phase II clinical studies, a single capsule containing  $6 \times 10^{11}$  bacterial cells was used (712), resulting in 74.5% of individuals treated with CP101 achieving SCR at 8 weeks, compared to 61.5% of those who were in the placebo group (708, 709). In the PRISM-EXT open-label extension, the 8-week success rate achieved 80.3%. Participants in PRISM3 did not report any safety problems, and persistent clinical recovery was documented for up to 24 weeks (708, 709).

In January 2023 Finch Therapeutics announced its decision to discontinue the PRISM4 phase III trial of CP101 in recurrent CDI because of slower than anticipated enrollment, the impact of unauthorized use of intellectual property, and broader sector trends. The company will also reduce its workforce by 95% (713).

### RBX2660 (*Rebyota*)

RBX2660, created by Ferring Pharmaceuticals and Rebiotix, is a suspension of healthy donor feces in polyethylene glycol 3350 and administered through enema (714–716). No intestinal preparation is required prior to the enema, although a washout period of 24–72 hours is required following the final CDI antibiotic dosage. RBX2660 has completed a phase III study (PUNCH CD3) (717) and received FDA clearance in November 2022 for the prevention of CDI recurrence in patients over the age of 18 who have been treated with antibiotics for recurrent CDI (718). A phase III open-label experiment (PUNCH CD3-OLS) is now recruiting a more diversified group of patients (719, 720).

Three phase II studies have been conducted for RBX2660: PUNCH CD (713), PUNCH Open Label (715), and PUNCH CD2 (721). In the PUNCH-CD2 (open-label, non-comparative) trial, RBX2660 was effective in preventing CDI recurrence in 87.1% of patients who received two doses. The results were confirmed in the PUNCH Open Label study, with 78.9% of patients undergoing RBX2660 administration achieved 8-week SCR when compared to 30% of the historical control group (715). Regarding the appropriate dosage, the PUNCH CD2 (double-blind/placebo-controlled) trial confirmed that one dosage of RBX2660 was sufficient to achieve SCR when compared to placebo (721), which was further confirmed by the phase III study (PUNCH CD3), where a single dose was sufficient to determine 8-week SCR in 71.2% of patients (717). A secondary Bayesian analysis indicated a treatment superiority probability of 99.1% if compared to placebo to achieve 8-week SCR (717). Results from PUNCH CD3-OLS study showed that 75% of patients receiving RBX2660 achieved 8-week SCR, without severe adverse events attributable to RBX2660, thus demonstrating its safety in a real-world population (719, 720).

Multiple secondary analysis of phase II and phase III trial data uncovered additional effects related to RBX2660 administration. For example, patients exhibited beneficial

microbiome modifications toward post-treatment predominance of *Bacteroides* and *Clostridia* classes (722–724) despite both RBX2660 and the placebo increased microbiome alpha diversity without relation to CDI recurrence. However, in the PUNCH CD3 study, RBX2660 was associated with improved bile acid composition (722–724), and reduction in antimicrobial resistance for at least 6 months after administration, with 72.7% of patients with previous colonization by vancomycin-resistant *Enterococcus* in their stool resulting negative following RBX2660 treatment (725).

### Vowst (SER-109)

SER109 (Vowst), developed by Seres Therapeutics, is an oral capsule that includes live, pure Firmicutes bacterial spores collected from healthy donor feces, incorporating ethanol spore purification to reduce the potential risk of pathogen transmission. Firmicutes were chosen for their capacity to compete with *C. difficile* for vital nutrients and/or modify bile acid profiles, therefore reestablishing resistance to colonization (563, 726, 727). Prior to administration, bowel preparation with magnesium citrate is required to reduce the intestinal concentration of CDI-active antibiotics (563). Upon administration, four capsules daily over a 3-day course produce around  $3 \times 10^7$  spore colony-forming units. SER109 has completed phase II (ECOSPOR) (727) and phase III (ECOSPOR III) (563) studies and has early data from an open-label study (ECOSPOR IV) for patients who suffered recurrence after therapy with SER109 in ECOSPOR III (728, 729). In September 2022, the FDA received a Biologics License Application, and the product was approved on 26 April 2023 (564, 730).

During the phase Ib study, patients having at least three CDI episodes during 3 months previous to enrollment were administered up to 15 SER109 capsules per day (726), with 86.7% of patients not showing recurrence within the 8-week follow-up period. As expected, 16S rRNA sequencing demonstrated a persistent increase in microbiome diversity, with predominant growth of Firmicutes and amplification of potentially beneficial phyla not present in SER109 composition, such as Bacteroidetes, for up to 24 weeks after delivery. Of notice, SER109 failed to exhibit superiority over placebo in avoiding 8-week CDI recurrence without age stratification prior to analysis. In fact, subgroup analysis of patients aged 65 and older showed a significant benefit if treated with SER109 when compared to the placebo group (727). To address these initial concerns, the ECOSPOR III trial was conducted administering higher dosage of SER109, adding a positive toxin assay to the inclusion criteria. However, the COVID-19 pandemic resulted in the early termination of the trial, despite showing that 88.6% of patients treated with SER109 (vs 60.2% in the placebo group) achieved clinical remission 8 weeks after administration (563), data which were later confirmed by an open-label ECOSPOR IV extension study that reported clinical for 24-week remission in 91.3% of patients treated with SER109, with no remarkable safety concerns (563, 726, 727, 731).

A recent post hoc analysis on ECOSPOR III assessed the rate of rCDI for subgroups, including Charlson categories, baseline creatinine clearance, number of CDI episodes, exposure to non-CDI targeted antibiotics after dosing, and acid-suppressant medication use at baseline. Across all subgroups analyzed, SER109-treated subjects had a lower RR of rCDI compared with placebo (732).

### Phage therapy

Bacteriophages (or phages) are viruses that infect and replicate in bacterial cells. Their host range is usually narrow and limited to a single bacterial species or a specific strain within a species. Their clinical use as antibacterial agents predates the advent of penicillin; however, their variable activity and impractical time-consuming production have limited their use until the advent and worldwide diffusion of multidrug-resistant organisms, usually as “last chance” agents (733). Moreover, as the disruption of the gut “microbiological barrier” by antibiotics is pivotal in the pathogenesis of CDI, their narrow host range makes phages an interesting option as anti-*C. difficile* agents, preventing further dysbiosis. Indeed, a recent *in vitro* colonic model recently confirmed that specific

phages do not alter the microbiota composition (734). Phage therapy against CDI might have further advantages over antibiotic therapy. As infective particles, phages amplify their effective dose replicating in the site of infection and are able to penetrate the complex biofilm found in *C. difficile*-associated pseudomembranous plaques (735). Furthermore, it was also suggested that the transfer of gut phage communities (gut virome) might play a yet unrecognized role in the efficacy of fecal microbiota transplant in CDI therapy (736). *C. difficile* is susceptible to multiple genetically diverse phages (i.e., siphophages and myophages, belonging to the Caudovirales family) (737, 738).

However, despite being intriguing, the therapeutic use of phages against CDI is hindered by several drawbacks. Some drawbacks are determined by technical difficulties in phage “delivery” (i.e., the phage pharmacokinetic) and phage “action”: lack of knowledge on the fundamental relationship between virus and its host, and lack of clinical trials.

For instance, molecular characterization of phage entry in *C. difficile* cells is not completely understood. Recently, the role of *C. difficile* surface layer protein A as one of the most important phage receptors was studied, which was previously only hypothesized (737). Moreover, in the case of phages, there is a general lack of knowledge regarding efficient processes of drug formulation, encapsulation, storage, and delivery (738). Regarding clinical efficacy, to our knowledge, phages have not yet been studied *in vivo* in humans, but some experimental data *in vitro* and *in vivo* models have been collected. A therapeutic model involves the use of a single specific phage (“single-phage therapy”) (739). Phage CD140 has shown promising clinical efficacy in a hamster model and in other hosts (mice, *Galleria mellonella* wax worm), even though this therapy has not been shown to be protective against CDI relapses as single-phage therapy is prone to “phage resistance” (739, 740). CRISPR-engineered phages are a novel potential therapy, but their clinical efficacy has yet to be tested. Another therapeutic strategy is the use of “phage cocktails.” For instance, an *in vitro* study has shown that phage cocktails reduce *C. difficile* cell count without harming the other commensal bacteria, potentially preventing CDI relapses (741). However, the “optimal” phages combination is yet unclear, and human clinical studies have yet to be published.

## Butyrate-producing bacteria

Butyrate is the most abundant SCFA in the human gut (along with acetate and propionate), with a luminal concentration of 10–20 mM and a pivotal role in the metabolism of colonocytes and enterocytes, which use butyrate as their dominant energy source via  $\beta$ -oxidation and the tricarboxylic acid cycle, while enterocytes use mainly glucose and glutamate but also butyrate (742). Butyrate is synthesized by several species of commensal gram-positive bacteria from dietary starch and fiber, and its systemic absorption is low, as butyrate is readily consumed by the epithelial cells via a carrier-mediated cell entry (742). Butyrate plays an important role in the modulation of colonocyte proliferation and epithelial inflammation, and its role in the pathogenesis and treatment of intestinal neoplasms, inflammatory bowel diseases, and malabsorptive states is increasingly being studied (742).

Butyrate inhibits *C. difficile* proliferation with a complex mechanism. While in a recent study, it was shown that butyrate might directly inhibit *C. difficile* proliferation (743), its direct antimicrobial effect remains controversial. Butyrate’s main protective mechanism against CDI is thought to be the reduction of epithelial permeability and bacterial translocation, the stabilization of pro-inflammatory cytokines levels, and the modulation of regulatory T cells and neutrophils in the colonic lamina propria (744, 745).

Butyrate as a therapeutic agent has been studied in several formulations: as tributyrin, dietary supplementation of SCFA, or as butyrate-enriched microbiota (745–748). In recent years, butyrate-enriching bacterium *C. butyricum*, known as a probiotic used as anti-diarrheal treatment in Asia, was recently studied as a potential alternative as trophic agent vs antibiotic growth promoters in agriculture (749, 750). Although exceptionally described as a potential pathogen (751), *C. butyricum* MIYAIRI 588 strain (CBM 588)

was able to reduce antibiotic-induced gut epithelial damage in a murine *in vivo* study, the epithelial necrosis, and the presence of inflammatory cells (752). In particular, in a murine CDI model, mice treated with a combination of fidaxomicin and CBM 588 acquired enhanced resistance to *C. difficile* colonization and attenuated gut inflammation compared to mice where fidaxomicin was used as monotherapy. In this study, CBM 588 modulation of the gut microbiome (i.e., increasing *Lactobacillus* spp. and *Lactococcus* spp. composition) resulted in decreased gut succinate concentrations, with suppression of *C. difficile* proliferation. Moreover, CBM 588 enhanced the synthesis of pathogen-specific IgA by upregulating IL-17A-producing CD4+ cells in the colonic lamina propria (752). Despite its widespread consumption, especially in Asia, the clinical efficacy of *C. butyricum* against human CDI is understudied. For instance, in a recent Taiwanese retrospective study in medical wards, in 99 mild-moderate CDI-affected patients, the addition of *C. butyricum* to metronidazole resulted in a non-significant decrease in diarrhea duration in comparison to monotherapy with metronidazole alone ( $3.5 \pm 2.4$  vs  $4.2 \pm 3.5$  days;  $P = 0.71$ ) (753). In another study, available in the Japanese language literature and cited by the English language literature, in 71 patients affected by CDI, the co-administration of CBM 588 and vancomycin reduced stool frequency in comparison to vancomycin alone (3.9 vs 2.6 times/day;  $P < 0.05$ ), shortened the treatment periods, and this effect was not confirmed when vancomycin was co-administered with products containing *E. faecium* (754, 755).

### Ribaxamase and other antibiotic inhibitors

In general, beta-lactamase are known to be among the main causes of antibiotic resistance as these naturally occurring enzymes degrade beta-lactam antibiotics. Overcoming this obstacle has been the goal of the development of beta-lactam/beta-lactamase inhibitor combinations, such as, for instance, amoxicillin/clavulanate, ampicillin/sulbactam, piperacillin/tazobactam, ceftolozane/tazobactam, etc. However, beta-lactams, one of the main classes of antibiotic used in the treatment of human diseases promote gut dysbiosis, one of the major risk factors in the pathogenesis of CDI. Moreover, parenteral beta-lactams might be secreted through the bile in the small intestine. To reduce the collateral damage of intravenous antibiotic therapy, the possibility of using a synthetic beta-lactamase had already been investigated in 2003: an oral penicillinase purified from *Bacillus licheniformis* was used in combination with intravenous ampicillin, decreasing the gut concentration of ampicillin to an undetectable level while preserving serum concentration (756). Ribaxamase was developed as an extended spectrum beta-lactamase able to degrade both penicillins and cephalosporins. Formulated as a pH dependent for better release in the small intestine, derivative of the original modified *B. licheniformis* penicillinase (P1A), SYN-004, later referred as ribaxamase, was originally studied in *in vivo* animal models, where it degraded ceftriaxone in the gut of dogs and protected the microbiome of pigs from ceftriaxone-induced dysbiosis (757). Its spectrum of activity includes inhibition of penicillins, ceftriaxone, cefazolin, cefuroxime, cefoperazone, cefepime, and, at a higher concentration, cefotaxime and ceftazidime (757). Phase I clinical studies demonstrated ribaxamase safety, tolerability, and sporadic and negligible serum concentration (758). Phase IIa clinical studies confirmed the degradation, below quantification threshold, of luminal (measured in the participants' chyme) ceftriaxone co-administered with oral ribaxamase, independently by the assumption of the proton pump inhibitor esomeprazole but with highly variable chyme concentration of ribaxamase (759). In a subsequent phase IIb randomized placebo-controlled multicenter trial (NCT02563106) on 413 lower respiratory tract-infected patients, ribaxamase administered during, and for 72 hours after, treatment with ceftriaxone significantly reduced (from 3.4% to 1%; 95% CI  $-0.6$  to  $5.9$ ; one-sided  $P = 0.045$ ) the risk of CDI up to 4 weeks after treatment (760). However, in this study, mortality was higher in the ribaxamase group (11 deaths vs 5 deaths in the placebo group) and attributed by the authors to an imbalance in the underlying comorbidities of the patients. A subsequent study on fecal samples collected during the previous

clinical phase IIb study, analyzing the presence of antimicrobial resistance genes using whole-genome shotgun sequencing, revealed that ribaxamase reduced changes to the gut resistome, with significantly lesser genes encoding for beta-lactamase or vancomycin resistance in the ribaxamase vs the placebo arm (761). Ribaxamase has yet to be studied in a phase III clinical study as CDI prevention agent. However, a phase Ib/IIa trial is seeking to repurpose the drug as microbiome-protecting agent in allogeneic hematopoietic cell-transplanted patients who developed fever after conditioning therapy and are treated with intravenous beta-lactam antibiotics (meropenem, piperacillin/tazobactam, or cefepime) (NCT04692181), but the results are not yet available (762). It is important to remark that the ribaxamase activity spectrum does not include carbapenems. In order to expand the activity of orally administered beta-lactamase, a *Bacillus cereus*-derived metallo-beta-lactamase (SYN-006) is currently being studied as a potential gut microbiome protective therapy, but clinical studies on human subjects are not available (763).

Another recent strategy of beta-lactamase delivery was employed using engineered LBP. A strain of *Lactococcus lactis* was engineered in order to secrete a heterodimeric beta-lactamase, encoded via a genetically unlinked two-gene biosynthesis strategy that is thought to not be susceptible to dissemination by horizontal gene transfer, preventing both the increase in antimicrobial resistance genes and the loss of colonization resistance against *C. difficile* in a mouse model treated with ampicillin (764).

Regarding other antibiotic inhibitors, although it does not have enzymatic activity but rather presents as an activated-charcoal-based product, DAV132 has been studied in absorption of fluoroquinolones (moxifloxacin, levofloxacin, and ciprofloxacin) showing promising results (765, 766).

## Vaccines

The advent of bezlotoxumab as the first monoclonal antibody marketed against CDI recurrences has highlighted the importance of drugs designed to enhance immunity against *C. difficile*. As passive immunity wanes weeks after exposure to the specific therapeutic antibody, a more promising approach is focused on active immunity. Protective response to vaccination against human diseases was shown to last longer than passive immunization, or even lifelong.

Reflecting the pathogenesis of CDI, vaccines against *C. difficile* are categorized as toxoid based and non-toxoid based. In general, the rationale behind the development of a toxoid-based vaccine candidate is that high serum antitoxin-IgG (in particular, antibodies against toxin B) are inversely correlated with the severity and recurrence of CDI (767). However, antibodies against *C. difficile* toxins are not able to clear gut colonization. Moreover, toxin B-based vaccines are impaired by the variety of toxin B isoforms, as up to 12 subtypes of toxin B have been identified (767).

As toxoid-based vaccine candidates might protect against *C. difficile* disease severity and rCDI, and non-toxoid-based vaccine candidates theoretically might protect against gut colonization by *C. difficile*, the ideal vaccine candidate needs to have both these functions. Therefore, apart from stimulating a robust and lasting immune response, a theoretically ideal vaccine candidate might be bivalent or might require multiple combinations of antibodies.

Pfizer Inc.'s bivalent toxoid vaccine PF06425090 contains detoxified forms of *C. difficile* toxin A and B. It is the first vaccine to have completed both phase II (NCT02561195) and phase III (NCT03090191) (768, 769). In the phase II study, 855 healthy people between the ages of 65 and 85 were randomized to receive either one of two doses of PF06425090 or a placebo (768, 769). The primary effectiveness outcome was the measurement of neutralizing serum toxin A and B antibodies at certain time periods. The predetermined threshold for toxin A neutralizing antibody was attained in 95.6% of individuals in the 200 mcg dose regimen used for phase III trials (0, 1, and 6 months), compared to 1.9% of participants in the placebo group. In addition, 87.3% of those who received the vaccination and 7.5% of those who received the placebo met the

predetermined threshold for toxin B-neutralizing antibodies. In the phase III CLOVER Trial, PF06425090 was compared to a placebo in individuals aged 50 or older who had taken systemic antibiotics during the preceding 12 weeks or were at a higher risk of healthcare system interaction (768, 769). The unpublished results of the phase III CLOVER Trial (NCT03090191) have preliminarily suggested that vaccine efficacy (compared to placebo) within 3 years following dose 2 and dose 3 was 28.6% (96.4% CI 28.4%–61.0%) and 31% (96.4% CI 38.7%–66.6%), respectively (770). In this study, 7,707 participants were inoculated with the three doses of vaccine, and 17 developed a primary episode of CDI, while 25 out of 7,805 participants who had been inoculated with the placebo developed a primary episode of CDI. However, none of 17 patients in the vaccine arm sought medical attention for CDI, while 11 out of 25 patients sought medical attention in the placebo group. Moreover, median CDI duration was shorter in the vaccine arm (1 vs 4 days), and no unanticipated safety issues were reported. Although unpublished, the results of this study are encouraging.

Another vaccine has been tested in a phase III study. This unnamed vaccine candidate by Sanofi Pasteur is another bivalent-based toxoid vaccine comprising formalin-inactivated *C. difficile* toxin A and toxin B, tested in a phase II study (NCT01230957). Six hundred and sixty-one individuals aged 40–75 who were hospitalized within 60 days of enrollment or who resided in long-term care or rehabilitation institutions were enrolled. In three unique dosing regimens, they were randomly assigned to receive two different doses of the vaccine or a placebo, with or without an Al(OH)<sub>3</sub> (aluminum hydroxide) adjuvant (769). The primary effectiveness goal was the rate of patients who achieved a predetermined serum toxin A and B neutralizing antibody threshold 60 days after the initial vaccination dose. Patients who were randomized to receive the dose regimen used for phase III studies (100 mcg with an Al(OH)<sub>3</sub> adjuvant at 0, 7, and 30 days) had seroconversion rates of 97% and 92% for toxin A and B, respectively, compared to 7.9% and 13.0% in the placebo group. The phase III “Cdiffense study” compared the vaccination vs placebo in patients aged 50 years or older, with at least two previous hospital visits and systemic antibiotic usage during the previous 12 months, or who were anticipating hospitalization for pre-specified elective procedures within 60 days of enrollment. Researchers randomized participants 2:1 to receive either the vaccination ( $n = 6,201$ ) or a placebo ( $n = 3,101$ ) and compared the incidence of CDI within 3 years of the third dose (771). Among the mITT population, 34 out of 6,173 participants who received at least one vaccine injection were diagnosed within 3 years following dose 3 with CDI (by stool PCR or by endoscopic confirmation of PMC). On the other hand, only 16 out of 3,085 participants inoculated with the placebo were diagnosed with CDI, and the estimated vaccine efficacy was –5.2% (95% CI 10.4%–43.5%) (772), prompting the independent data monitoring committee to suggest that this research be terminated due to futility.

Another toxoid-based vaccine candidate, VLA84, has been developed by Valneva Austria GmbH and has completed a phase II trial (NCT02316470). This vaccine candidate consists of an adjuvant-free genetic fusion of the cell-binding domains from toxin A and toxin B (773), but phase III trials have yet to be started. Lastly, a non-toxoid-based vaccine candidate is GSK2904545A (GlaxoSmithKline), which is based on the F2 antigen, a cleavage fragment of toxin A (774). This vaccine candidate is still in phase I clinical trial (NCT04026009): participant recruitment is completed, but results are not yet available (775).

Although promising in reducing the frequency of CDI relapses, toxoid-based vaccines are not likely to prevent colonization of the host and prevent infection at an early stage. Non-toxoid-based strategies are currently being studied in preclinical investigations. A detailed list of potential non-toxoid-based vaccines has been widely reviewed elsewhere (767, 776); the candidate antigens are categorized as spore surface proteins, vegetative surface proteins, surface glycopolymeric targets, flagellar proteins, and intracellular proteins. As the spore surface protein is present before *C. difficile* starts to synthesize toxin A and toxin B, eliciting an immune response against *C. difficile* spores appears

to be the most promising strategy. However, only a fraction of the spore surface proteins is highly immunogenic, and, as shown by the human anthrax vaccines, it is unlikely that immunity against spore surface proteins alone is enough to protect against primary episodes of CDI (777). Another potential strategy, to target both *C. difficile* toxins and its colonization/adhesion factors, is the employment of genetically modified non-toxic anaerobes. For instance, a non-toxic *C. difficile* strain was genetically modified to express a chimeric protein (mTcd138), comprising the glucosyltransferase and cysteine proteinase domains of toxin B and the receptor-binding domain of toxin A (778). In this study, oral immunization with spores of non-toxic *C. difficile*-expressing mTcd138 provided mice with full protection against infection with the hypervirulent *C. difficile* RT027 and significantly decreased the lethality of CDI in a hamster model. Another example is the employment of genetically modified toxin A-expressing *Bacillus subtilis* in order to generate an immune cross-reaction to the coat of *C. difficile* spores and the cell surface of vegetative cells (779).

Overall, cost-effectiveness of a theoretical *C. difficile* vaccine candidate has been studied using two decision analytic Monte Carlo computer simulation models (780), and results suggest that a *C. difficile* vaccine could be cost-effective over a wide range of *C. difficile* risk, vaccine costs, and vaccine efficacies, especially when being used post-CDI treatment to prevent recurrent disease. Strikingly, even at a cost of \$1,600, a vaccine efficacy of 75% in reducing recurrences was estimated to be cost-effective. However, vaccination strategies with a rational resource allocation approach might include the vaccination of certain at-risk populations, such as people with multiple hospitalizations or anticipated exposures to broad-spectrum antibiotics, candidates to elective surgical procedures, or patients who might be admitted into long-term care facilities and nursing homes (773).

## CONCLUSIONS

The infection caused by *C. difficile* has become increasingly complex, partly due to intrinsic factors (O27 strain with reduced susceptibility) and partly due to the growing population of vulnerable individuals (transplant recipients, chemotherapy patients, IBD patients, etc.). In recent decades, there has been a growing interest in demonstrating the distant damages induced by toxins (extraintestinal manifestations) and in finding ways to address them (e.g., bezlotoxumab). The “bacteria-free” fecal transplantation has made us realize that there is still much to discover about this disease, particularly in the areas of phages, antimicrobial peptides, lesser-known metabolites, and fatty acids. It remains a challenging infection from both a clinical and research perspective. Complications can be formidable and need to be promptly anticipated and addressed through a profound understanding of the clinical aspects of this disease. Hopefully, this treatise will be helpful for those seeking a broad view of the microbiological and clinical aspects of this pathology.

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