




# Evaluation of the Dynamiker<sup>®</sup> Fungus (1–3)- $\beta$ -D-Glucan Assay for the Diagnosis of Invasive Aspergillosis in High-Risk Patients with Hematologic Malignancies

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## ABSTRACT

**Introduction:** The Dynamiker<sup>®</sup> Fungus (1–3)- $\beta$ -D-glucan assay (DFA) allows the testing of samples in smaller batches compared to the well-established Fungitell<sup>®</sup> assay (FA) making the assay cost-effective in centers with small numbers of samples. Evaluations of its performance for the diagnosis of invasive aspergillosis (IA)

are limited. Therefore, we compared the two assays and evaluated their clinical performance in diagnosing IA.

**Methods:** A total of 60 adult hematology patients were screened for IA, 13 with probable IA, 19 with possible IA, and 28 with no IA. Serum specimens ( $n = 166$ ) were collected twice-weekly and tested for (1–3)- $\beta$ -D-glucan (BDG) using FA and DFA which were compared quantitatively with Spearman rank correlation analysis and qualitatively with the Chi-square test. Agreement and error rates were determined using FA as the reference method. Sensitivity, specificity, and positive predictive and negative predictive values in diagnosing IA were calculated.

**Results:** The performance of the DFA was highly consistent with that of the FA, both quantitatively ( $r_s = 0.913$ ) and qualitatively ( $\kappa = 0.725$ ). The agreement was 85% with 8% minor, no major, and 7% very major errors (FA+/DFA–). Using a cut-off value of 20 pg/mL for DFA, very major errors were reduced to 1%, although 5% major errors were detected. BDG levels were lower with DFA than FA (slope  $0.653 \pm 0.031$ ). Sensitivity, specificity, positive predictive value, and negative predictive value (NPV) was 67%, 53%, 44%, and 74% for FA, and 53%, 67%, 49%, and 71% for DFA, respectively. The optimal BDG positivity threshold calculated did not lead to significant test quality improvement for either assay. However, a

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higher % of patients with probable IA (62%) had  $\geq 2$  consecutive positive specimens compared to patients with no IA (FA-BDG 26%,  $p = 0.10$ , and DFA-BDG 10%,  $p = 0.01$ ) leading to improved sensitivity and NPV (71% and 85% for DFA, and 95% and 96% for FA, respectively). **Conclusion:** DFA could be a valuable alternative to the FA, particularly in laboratories with small numbers of samples. The results of the BDG testing should be carefully interpreted in the high-risk setting of patients with hematologic malignancies. Higher NPV was found using as criterion  $\geq 2$  consecutive positive samples for diagnosing IA.

**Keywords:** (1–3)- $\beta$ -D-glucan; Diagnosis; Dynamiker® Fungus assay; Invasive aspergillosis; Hematology patients

### Key Summary Points

The Dynamiker® Fungus (1–3)- $\beta$ -D-glucan assay (DFA) allows the testing of samples in smaller batches and therefore is more suitable for laboratories with small numbers of samples.

DFA provided comparable results with the well-established Fungitell® assay (FA) although (1–3)- $\beta$ -D-glucan (BDG) levels were lower than FA.

Lowering the cut-off value of DFA to 20 pg/mL, reduced very major errors although more major errors were detected.

Low positive predictive values (44–49%) and moderate negative predictive values (71–74%) in diagnosing invasive aspergillosis (IA) were found with both assays.

Higher negative predictive values (85–96%) were found using  $\geq 2$  consecutive positive samples for diagnosing IA with both assays.

## INTRODUCTION

Signs and symptoms of invasive aspergillosis (IA) can be subtle and non-specific, particularly in hematology patients, posing a considerable challenge for its clinical management [1]. Given that delayed initiation of targeted therapy is associated with increased mortality, early and accurate diagnosis is a critical priority for a successful outcome [2]. While conventional microbiological and radiological techniques are regarded as the cornerstone of IA diagnosis, they are not sufficiently sensitive and highly specific, respectively [3], especially in cases of high-risk patients at a severe stage of their hematologic malignancy [4–6]. Therefore, screening strategies using culture-independent methods, characterized by rapid turnaround times, enhanced sensitivity, and relative ease of use, are important adjunctive diagnostic tools [7]. Although screening for circulating galactomannan (GM) has been endorsed as a standard non-invasive tool for IA diagnosis [8–11], remarkable variability in GM sensitivity has been associated with the underlying disease [12] and the administration of mold-active antifungal therapy [13]. Given the challenges in the diagnostic process of IA arising from the fact that none of the current assays (serological or molecular) alone are able to confirm the infection, several studies have recommended the application of combined biomarker screening in high-risk hematology patients [14–17].

The polysaccharide (1–3)- $\beta$ -D-glucan (BDG), a major cell wall component of most pathogenic fungi, is released into the body fluids and surrounding tissues in the course of an invasive fungal disease (IFD). Consequently, BDG antigenemia has been incorporated in the definition of a probable IFD [9]. Although BDG is a pan-fungal biomarker, quantification of serum-circulating BDG titers has demonstrated wide utility in distinct clinical settings, including IA [18, 19]. In particular, its use is recommended for diagnosing IA and guiding pre-emptive antifungal therapy in hematology patients [11]. To date, several BDG assays, utilizing different standards (pachyman versus lentinan) and detection techniques (colorimetric versus

turbidimetric), are commercially available. Among them, the Fungitell® assay (FA; Associates of Cape Cod, MA, USA) is widely used in the Western Hemisphere (USA and Europe) as it was the first FDA-cleared and CE-marked in vitro diagnostic screening test for BDG. Nevertheless, FA performance is challenging, since 21 specimens per run have to be assessed in order to guarantee cost-efficiency [20]. On the other hand, the Dynamiker® Fungus BDG assay (DFA; Dynamiker Biotechnology (Tianjin), China) (CE-marked) contains detachable strip plates [21] providing technical flexibility as it allows the conducting of frequent testing, independent of the number of samples in a cost-effective manner, even in settings with few samples per run. Notably, FA and DFA performances rely on the same basic principles (protease zymogen-based colorimetric assays), and thus BDG measurements generated by them can be directly comparable. Currently, data to support the DFA as an alternative to the FA for IA diagnosis are limited [22]. Based on these grounds, we aimed to evaluate the performance of the DFA in serum samples of patients with hematologic malignancies at risk for IA, in terms of its qualitative and quantitative agreement with the FA as well as its diagnostic accuracy.

## METHODS

### Study Design and Population

A total of 60 adult hematology patients at risk for IA [23–25] according to the attending clinicians were screened for the detection of GM and BDG in serum samples collected during a 6-month period between 2013 and 2015 in each of four tertiary care hospitals in the area of Athens, Greece, namely Attikon University General Hospital (1 March–31 August 2013), Evangelismos General Hospital (1 January–30 June 2014), Hippokration General Hospital (1 June–30 November 2013), and Laiko General Hospital (1 April–30 September 2015). The participating centers attend cases of high complexity, including oncology wards (Hippokration), as well as hematology and bone

marrow transplantation units (Attikon, Evangelismos, and Laiko). Patient episodes (proven, probable, possible, or no evidence for IA) were stratified according to the 2020 definitions of the European Organization for Research and Treatment of Cancer–Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycosis Study Group Education and Research Consortium (EORTC/MSGERC) Consensus Group. Briefly, the designation of probable IA required the combination of at least one host factor, a clinical feature, and mycological evidence (all three criteria should be fulfilled). Cases that met the host and clinical criteria without supportive mycological findings were considered possible IA [9]. Patients' demographic (gender, age, underlying disease) and clinical characteristics during the survey period (current medications together with radiological, histological, and microbiological findings, and outcome during hospitalization) were obtained from computerized databases at each center.

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Attikon University General Hospital (358, 08/11/2012, chair Prof. C. Liapis), Evangelismos General Hospital (96, 27/02/2013, chair Prof. A. Skoutelis), Hippokration General Hospital (42, 06/06/2013, chair Prof. N. Alexandropoulos), and Laiko General Hospital (4635, 17/04/2013, chair Prof. A. Konstantinidis). Written informed consent for data collection, data analysis, and publication was obtained from each patient or relative.

### Clinical Samples and Biomarker Testing

The number of evaluable serum specimens for the detection of fungal biomarkers was 166, of which serial samples were collected from 44/60 (73%) patients. The obtained sera were divided into aliquots and were stored at  $-70^{\circ}\text{C}$  until analyzed. A commercially available sandwich enzyme-linked immunoassay (Platelia *Aspergillus* EIA; Bio-Rad Laboratories) was used to quantify the GM antigen in accordance with the manufacturer's instructions. A result was

considered positive when the index value was  $\geq 0.5$  [26]. BDG was initially detected with the FA, as recommended by the manufacturer. BDG levels of  $\geq 80$ , 79–60, and  $< 60$  pg/mL were considered positive, indeterminate, and negative results, respectively. Serum assays were performed in duplicate [20]. Samples were previously tested for GM and BDG with FA and archived at  $-70^\circ\text{C}$  [14]. The archived serum samples were retrospectively tested 2 years later for the presence of BDG using the DFA according to the manufacturer's specifications. BDG concentrations of  $> 95$ , 70–95, and  $< 70$  pg/mL were considered positive, inconclusive, and negative results, respectively [21]. Samples with BDG levels above the upper validation limit (500 pg/mL for the FA and 600 pg/mL for the DFA) were diluted and retested, whereas BDG concentrations below the lower validation limit (31 pg/mL for the FA and 37.5 pg/mL for the DFA) were calculated by extrapolation. To rule out that BDG was degraded over the years at  $-70^\circ\text{C}$ , we retested 15 serum samples using the FA. The mean difference of the BDG measurements was  $-23$  pg/mL (higher values over the years), showing a coefficient of variation of 12% (data not shown). Thus, there was no evidence for a bias due to BDG degradation, as previously described [27].

## Data Analysis

(1) *Descriptive statistics* Medians with interquartile ranges (IQR) or means  $\pm$  standard deviation (SD) were calculated for continuous variables, while numbers and percentages were calculated for categorical parameters. (2) *Analytical evaluation* The two BDG assays were compared quantitatively and qualitatively. For the quantitative analysis, the results of the assays were analyzed with paired Student's *t* test or Wilcoxon matched-pairs signed rank test, depending on the validity of the normality assumption. The Spearman's rank correlation coefficient ( $r_s$ ) was calculated, and linear regression analysis was conducted to determine whether the slope of the regression line differs significantly from zero. For the qualitative analysis, the categorical agreement between the test kits was estimated

using the FA as the reference, given that it is the one that has been studied most extensively and has been applied to most clinical studies for BDG detection. The Chi-square ( $X^2$ ) test ( $3 \times 3$  contingency table) was used to evaluate whether there was a significant association between the results of the two assays, while the strength of agreement was assessed by calculating the kappa coefficient ( $\kappa$ ). Errors were defined as minor (MiE), indicating that the result was intermediate/inconclusive in one assay and negative or positive in the other, major (MaE), indicating a false-positive result (negative by the FA and positive by the DFA), and very major (VmE), indicating a false-negative result (positive by the FA and negative by the DFA). Furthermore, considering that GM serves as a surrogate marker for IA [8–11], the DFA-BDG concentrations were also compared with the corresponding GM indices, after excluding patients who developed other fungal or bacterial infections associated with elevated BDG titers during the sampling period [28]. (3) *Diagnostic evaluation* The BDG results generated by the two assays were grouped according to the stratification of IA. The significance of the differences between groups was assessed with unpaired Student's *t* test/one-way analysis of variance or Mann–Whitney/Kruskal–Wallis tests, depending on the number of variables and the validity of the normality assumption. In order to determine the clinical accuracy of both assays, the sample/patient positivity rates for different patient populations defined as per the classification of IA were calculated and compared using  $X^2$  and Fisher's exact tests. Sensitivity/specificity rates and positive/negative predictive values (PPV/NPV) of each test kit were assessed using  $2 \times 2$  tables, and the 95% confidence interval (CI) was estimated. Receiver operating characteristic (ROC) analysis was performed to evaluate the ability of BDG to distinguish between patients, with probable versus no IA.

In any case, a two-tailed *p* value of  $< 0.05$  was considered to reveal a statistically significant difference. All data were analyzed using the statistics software package GraphPad Prism, v.8.0 for Windows (GraphPad Software, San Diego, CA, USA).



## RESULTS

### Patients' Characteristics and IA Episodes

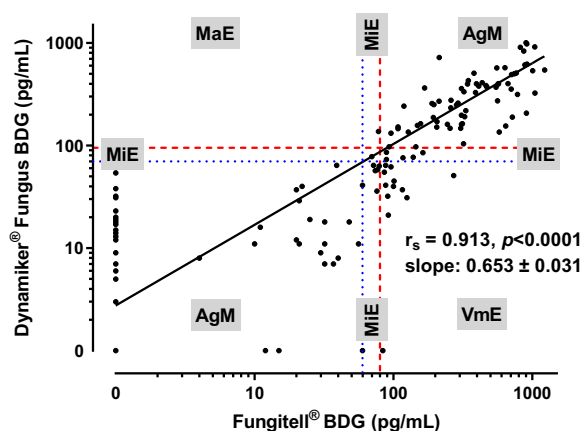
Overall, 30/60 (50%) patients enrolled in the study were men with median (range, IQR) age 53 (18–81, 25) years. The most common underlying hematological disorder was acute myelogenous leukaemia (AML) (40/60; 67%), followed by acute lymphoblastic leukemia (ALL) (8/60; 13%), non-Hodgkin's lymphoma (NHL) (3/60; 5%), chronic lymphocytic leukemia (2/60; 3%) and various other conditions, such as myelodysplastic syndrome, myeloma, chronic myeloid leukemia, Burkitt lymphoma, and Hodgkin disease (7/60; 12%). Among patients, 17/60 (28%) had undergone autologous hematopoietic stem cell transplantation (HSCT).

There were 13 (22%) patients with probable IA and 19 (32%) cases classified as possible IA, while no proven IA was documented. Most of the patients (43/60; 72%) had received  $\geq 2$  defined daily doses of antifungal drugs, with 20/60 (33%) being on mold-active treatment (16 voriconazole, 2 itraconazole, and 2 liposomal amphotericin B, of which 9 with probable IA, 7 with possible IA, and 4 with no evidence for IA) at the time serum samples were collected. During the sampling period, 3/60 (5%) patients developed candidemia due to *C. kefyr* (no IA receiving anidulafungin as empirical therapy), *C. parapsilosis* (probable IA receiving micafungin as empirical therapy), and *C. tropicalis* (no IA without receiving antifungal drugs). In addition, 3/60 (5%) patients developed bacteremia due to *E. coli* (probable IA), *S. aureus* (no IA), and *S. aureus* plus *K. pneumoniae* (no IA). The crude mortality rate within hospital stay was 13% (8/60; 6 AML (1/6 autologous HSCT), 1 ALL, and 1 NHL), either due to the underlying malignancy and/or due to multiple infections/sepsis.

### Comparative Performance of the BDG Assays

The median (range, IQR) FA-BDG and DFA-BDG concentrations were 85 (0–1229, 309) pg/mL

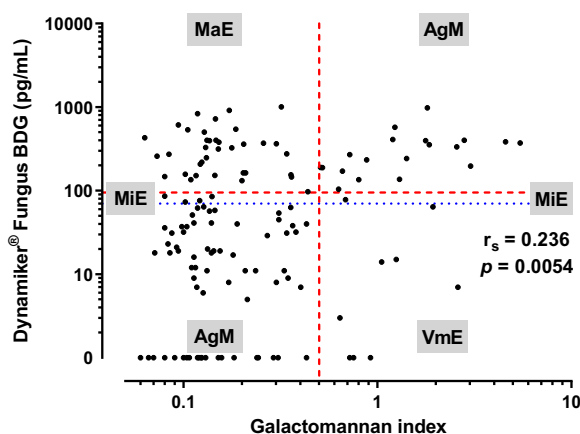
and 53 (0–1004, 233) pg/mL, respectively. The mean ( $\pm$  SD) absolute and relative difference between the BDG values of the assays tested was 51 ( $\pm$  149) pg/mL and 14 ( $\pm$  44)%, respectively, with FA-BDG levels being significantly higher ( $p < 0.0001$ ). BDG concentrations generated by the two assays were linearly correlated (slope  $0.653 \pm 0.031$ , Y-intercept 20.57 pg/mL,  $r^2 = 0.73$ ) with high degree of correlation [Spearman  $r_s$  (95% CI) = 0.913 (0.882–0.936),  $p < 0.0001$ ] (Fig. 1). A statistically significant relationship between the two assays was found [ $\chi^2(4) = 121.7$ ,  $p < 0.0001$ ] with 85% agreement ( $\kappa = 0.725$ , 95% CI 0.633–0.816) 8% MiE and no MaE. Twelve VmE (7%) were observed, with values reported from the FA being significantly higher than those generated by the DFA [median (range, IQR) 94 (84–272, 17) pg/mL versus 41 (0–64, 20) pg/mL, respectively,  $p = 0.0005$ ] (Fig. 1). By reducing the positive cut-off value of DFA to  $> 20$  pg/mL, agreement increased to 89% with 9 MiE (5%), 9 MaE (5%) and 1 VmE (1%).



**Fig. 1** Scatter plot of the (1–3)- $\beta$ -D-glucan (BDG) serum concentrations as determined by the Fungitell® and Dynamiker® Fungus assays. The red broken and blue dotted lines indicate the manufacturer's positive and negative cut-off value of each test, respectively ( $\geq 80$  and  $< 60$  pg/mL for Fungitell® and  $> 95$  and  $< 70$  pg/mL for Dynamiker® Fungus).  $r_s$ , Spearman's rank correlation coefficient with the  $p$ -value, slope of the regression line  $\pm$  95% confidence interval. AgM agreement, MiE minor errors, MaE major errors, VmE very major errors

### Comparison of the DFA-BGD Levels with the GM Index Values

The median (range, IQR) GM index values in patients with probable IA was 0.9 (0.1–5.5, 1.5). A weak but significant ( $p = 0.0054$ ) correlation between the BDG concentrations obtained by the DFA and the corresponding GM indices was observed, with a Spearman  $r_s$  (95% CI) of 0.236 (0.066–0.392) (Fig. 2). The overall percentage of agreement plus MiE was 68% (64% and 4%, respectively). The MaE were 26%, while the VmE were 6% with median (range, IQR) GM index value 1.0 (0.6–2.6, 0.7) and median (range, IQR) DFA-BGD concentration 5 (0–64, 14) pg/mL.



**Fig. 2** Correlation between the serum (1–3)- $\beta$ -D-glucan (BDG) concentrations and galactomannan indices as determined by the Dynamiker® Fungus and Platelia *Aspergillus* EIA assay, respectively, after excluding patients who developed other fungal ( $n = 3$ ) or bacterial ( $n = 3$ ) infections associated with elevated BDG titers. The red broken lines depict the manufacturer's positive cut-off value of each test ( $> 95$  pg/mL for Dynamiker® Fungus and  $\geq 0.5$  for Platelia *Aspergillus* EIA), while the blue dotted line indicates the negative cut-off value of the BDG assay ( $< 70$  pg/mL).  $r_s$ , Spearman's rank correlation coefficient with the  $p$  value. *AgM* agreement, *MiE* minor errors, *MaE* major errors, *VmE* very major errors

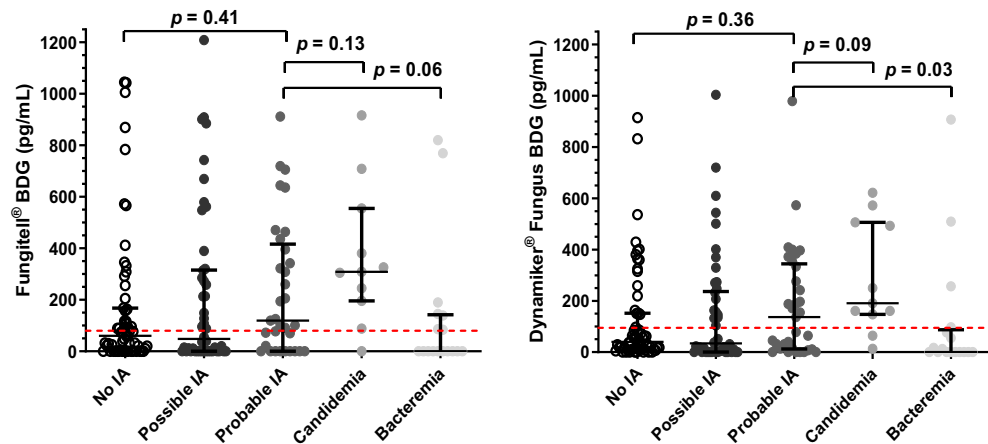
### Clinical Performance of the BDG Assays

The BDG results for patients with IA, candidemia, bacteremia, and no IFD are shown in Fig. 3.

(1) *Quantitative comparisons* The median (range, IQR) FA-BDG and DFA-BDG concentrations for cases of candidemia (3 patients, 11 samples) were 309 (0–916, 248) pg/mL and 191 (13–622, 346) pg/mL ( $p = 0.08$ ), respectively. In particular, 10 (91%) and 9 (82%) samples were FA-BDG and DFA-BDG positive, respectively. Serial serum specimens were available for 2/3 patients and both had four consecutive positive samples regardless of the BDG assay used. The median (range, IQR) FA-BDG and DFA-BDG levels for cases of bacteremia (3 patients, 17 samples) were 0 (0–820, 139) pg/mL and 0 (0–907, 77) pg/mL ( $p = 0.57$ ), respectively. Specifically, 7 (41%) and 4 (24%) samples were FA-BDG and DFA-BDG positive ( $p = 0.46$ ), respectively. One patient had two consecutive positive serum specimens irrespectively of the BDG assay used, whereas the remaining 2 patients had a single (DFA testing) or non-sequential positive samples (FA testing). The median (range, IQR) FA-BDG concentrations for cases of probable, possible, and no evidence for IA were 119 (0–912, 396) pg/mL, 48 (0–1229, 307) pg/mL, and 60 (0–1045, 166) pg/mL, respectively ( $p = 0.41$ ). The median (range, IQR) DFA-BDG concentrations for cases of probable, possible, and no evidence for IA were 137 (0–979, 320) pg/mL, 34 (0–1004, 227) pg/mL, and 40 (0–915, 141) pg/mL, respectively ( $p = 0.36$ ). Still, the median BDG concentration for cases of candidemia, although limited, and probable IA were not different ( $p = 0.13$  for FA and  $p = 0.09$  for DFA). On the other hand, the median BDG titer was statistically different between patients with bacteremia and probable IA when DFA testing was performed ( $p = 0.03$ ), but not in case of FA testing ( $p = 0.06$ ).

(2) *Qualitative comparisons* Sample/patient BDG distributions for patients with IA, excluding cases of candidemia and bacteremia ( $n = 6$ ), are summarized in Table 1.

Concerning the FA-BDG testing, 67 (49%) samples were positive with a median (range, IQR) BDG concentration of 309 (88–1229, 451)



**Fig. 3** Scatter plots of the (1–3)-β-D-glucan (BDG) serum levels measured by the Fungitell® and Dynamiker® Fungus assays for patient populations defined according to the EORTC/MSGERC classification of invasive aspergillosis (IA) [9] as well as patients with candidemia and bacteremia. The red broken line indicates the manufacturer’s cut-off value of each test ( $\geq 80$  pg/mL for Fungitell® and  $> 95$  pg/mL for Dynamiker® Fungus)

**Table 1** Positivity rates of (1–3)-β-D-glucan as determined by the Fungitell® and Dynamiker® Fungus assays in patients with probable, possible and no invasive aspergillosis (IA) with respective EORTC/MSGERC classification of patients (9)

Patient classification	No of samples/patients	No of positive samples/patients	
		Fungitell®	Dynamiker® Fungus
Probable IA	Samples ( $n = 33$ )	20 (61%)	17 (52%)
	Patients ( $n = 11$ )	7 (64%)	7 (64%)
Possible IA	Samples ( $n = 46$ )	22 (48%)	20 (44%)
	Patients ( $n = 19$ )	12 (63%)	12 (63%)
No evidence for IA	Samples ( $n = 59$ )	25 (42%)	18 (30%)
	Patients ( $n = 24$ )	10 (42%)	8 (33%)
Total	Samples ( $n = 138$ )	67 (49%)	55 (40%)
	Patients ( $n = 54$ )	29 (54%)	27 (50%)

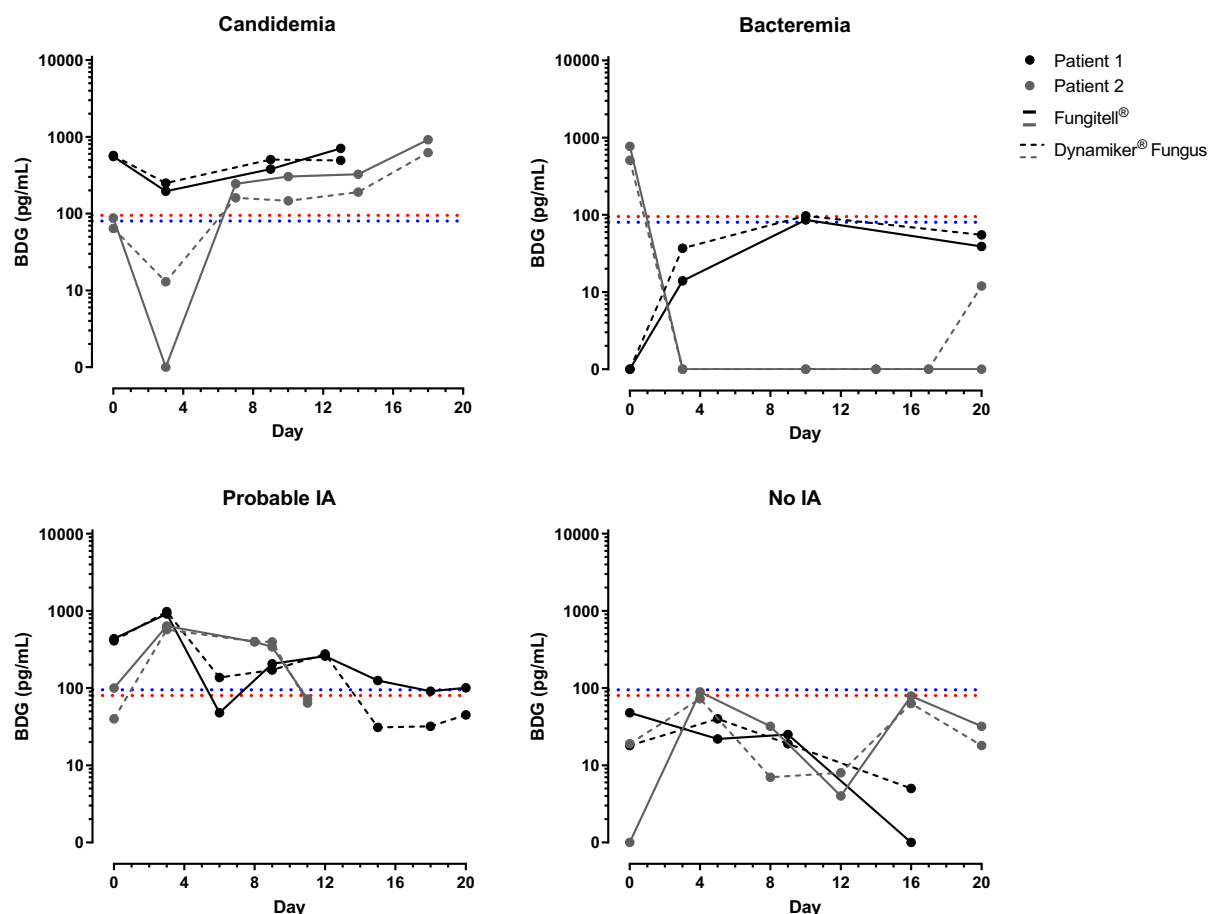
Patients with candidemia ( $n = 3$ ) and bacteremia ( $n = 3$ ) were excluded

pg/mL, 9 (6%) were indeterminate, and 62 were negative. For the DFA-BDG testing, 55 (40%) samples were positive with a median (range, IQR) BDG concentration of 315 (97–1004, 238) pg/mL, 5 (4%) were inconclusive, and 78 were negative. The sample positivity rate of FA-BDG was higher in patients with probable IA (61%) than in patients with possible IA (48%), and no evidence for IA (42%). Similarly, the positivity

rate of DFA-BDG was higher in patients with probable (52%) and possible IA (44%) than in patients with no evidence for IA (30%). Nevertheless, the true positivity rate for samples originating from the probable cases of IA and the false positivity rate in control (no IA) samples did not differ significantly [ $X^2(3) = 2.8$ ,  $p = 0.42$  for FA and  $X^2(3) = 4.4$ ,  $p = 0.22$  for DFA].

The 29 (54%) and 15 (28%) patients were FA-BDG positive in at least one and two samples, respectively. In particular, serial serum specimens were available for 8/11 patients with probable IA, of which 5 (62%) had at least two consecutive positive samples, 1 had a single positive sample, and 2 were FA-BDG negative. Of 19/24 patients with no evidence for IA for whom serial samples were available, 5 (26%) had at least two consecutive positive samples ( $p = 0.10$ ), 4 had a single positive sample, and 10 were FA-BDG negative. Similarly, 28 (52%) and 13 (24%) patients were DFA-BDG positive in at least one and two samples, respectively. Of 8/11 patients with probable IA, 5 (62%) had at least two consecutive positive samples, 1 had a

single positive sample, and 2 were DFA-BDG negative. However, only 2/19 (10%) patients with no IA had two consecutive positive samples ( $p = 0.01$ ), 5 had a single positive sample, and 12 were DFA-BDG negative. Representative FA- and DFA-BDG kinetic profiles over time for different patient populations are shown in Fig. 4. The positivity rates for patients with probable, possible, and no IA were 64%, 63%, and 42% for FA-BDG [ $X^2(2) = 2.5$ ,  $p = 0.28$ ], and 64%, 63%, and 33% for DFA-BDG [ $X^2(2) = 4.8$ ,  $p = 0.09$ ]. Even when the analysis was performed per patient, the difference between the true (patients with probable IA) and the false (patients with no IA) positivity rates was greater



**Fig. 4** Representative (1–3)- $\beta$ -D-glucan (BDG) kinetic profiles over time for patients with candidemia, bacteremia, probable invasive aspergillosis (IA), and no IA as determined by the Fungitell® and Dynamiker® Fungus

assays. The blue and red dotted lines indicate the manufacturer's positive cut-off value of Fungitell® ( $\geq 80$  pg/mL) and Dynamiker® Fungus ( $> 95$  pg/mL), respectively



but not significant ( $p = 0.30$  for FA and  $p = 0.14$  for DFA).

(3) *Diagnostic characteristics* The test characteristics for differentiating probable IA versus no evidence for IA considering one or at least two consecutive positive samples are shown in Table 2. Moderate sensitivity (53–67%) and specificity (53–67%), but higher NPV (71–74%), were found for both assays by relying on a single positive sample, while their PPV was low (44–49%). Nevertheless, improved BDG performance was observed by using  $\geq 2$  consecutive positive test results, as the sensitivity (71–95%) and NPV (85–96%) of both assays increased significantly, whereas specificity (51–66%) and PPV (43–47%) remained unchanged. ROC curve analysis did not reveal statistically significant results when patients with probable IA were compared with patients with no IA for both assays (area under the curve = 0.58–0.59,  $p = 0.16$ –0.23). Similar results were obtained for different patient population combinations, namely probable + possible IA versus no IA and probable IA versus possible + no IA (data not shown). The highest sensitivity (73%, 95% CI 54–87%) was observed with a cut-off value of 20 pg/mL for FA, but specificity was low (32%, 95% CI 21–46%). A 92% (95% CI 81–97%) specificity was found with BDG levels > 600 pg/mL, albeit with very low (15%, 95% CI 5–32%) sensitivity. The highest likelihood ratio (LR = 2) was found for 370 pg/mL with specificity 86% (75–94%) and sensitivity 27% (13–46%). Using a cut-off value of 10 pg/mL for DFA, sensitivity

reached its maximum (79%, 95% CI 61–91%), but specificity decreased (24%, 95% CI 14–37%). The highest specificity (97%, 95% CI 88–100%) was observed with BDG levels > 555 pg/mL, although with extremely low (6%, 95% CI 0.7–20%) sensitivity. The highest likelihood ratio (LR = 2) was found for 154 pg/mL with specificity 76% (63–86%) and sensitivity 48% (31–66%).

## DISCUSSION

The performance of the newer DFA was highly consistent with that of the well-established FA, both quantitatively (Spearman  $r_s = 0.913$ ) and qualitatively (85% agreement,  $\kappa = 0.725$ ), supporting the equivalence of the two BDG assays. BDG levels were lower with DFA than FA (slope  $0.653 \pm 0.031$ ). Although the false positivity rate of FA was higher (42% versus 33%), sensitivity and specificity rates of both BDG assays for the diagnosis of IA in hematology patients were only 53–67% resulting in low PPV (44–49%) but higher NPV (71–74%). Efforts to determine optimal cut-off values did not facilitate significant test quality improvement indicating that all the results should be interpreted in the context of the specific patient population. However, a higher percentage of patients with probable IA compared to no IA had  $\geq 2$  consecutive samples positive, which may be used as a diagnostic criterion for IA improving

**Table 2** Test characteristics (mean, 95% confidence interval) for differentiating probable invasive aspergillosis versus no evidence for invasive aspergillosis taking into account a single or at least two consecutive positive samples

	No of positive samples			
	Fungitell®		Dynamiker® Fungus	
	1	$\geq 2$	1	$\geq 2$
Sensitivity	67% (47–82%)	95% (72–100%)	53% (35–70%)	71% (48–88%)
Specificity	53% (39–66%)	51% (36–65%)	67% (53–79%)	66% (51–78%)
PPV	44% (30–60%)	43% (28–60%)	49% (32–66%)	47% (30–65%)
NPV	74% (57–86%)	96% (78–100%)	71% (57–82%)	85% (69–94%)

PPV positive predictive value, NPV negative predictive value

the sensitivity (71/95%) and NPV (85/96%) of both BDG assays (DFA/FA).

Until recently, the FA was the only available commercial BDG assay in the Western Hemisphere. Nevertheless, the need for testing the clinical specimens in batches might hamper its use, as the cost of the test per patient varies depending on the number of samples run per plate using one kit (each kit contains two plates). Therefore, if fewer than 42 duplicate tests are run, a whole plate must still be used [20], and consequently the cost per patient would significantly increase. Furthermore, the collection of a sufficient number of samples decreases the frequency of testing, leading to an unfavorable delay of results, and thereby the suitability of the FA for rapid diagnosis becomes questionable. In contrast, the DFA can be performed using 8-well strips (7 wells/run are filled with standards and controls) [21], and thus in terms of practicability it provides technical flexibility, permitting frequent, cost-effective testing, specifically in laboratories processing low sample numbers. Another benefit is that it contains four vials of the main reagent (1 vial/3 strips), in contrast to the manufacturer of the FA who provides 2 vials of Fungitell® reagent (1 vial/plate) [20, 21]. Notably, the DFA main reagent can be frozen and thawed at least once for up to 5 days after resuspension without affecting the assay's performance, allowing its post preparation and dispersion into single-use aliquots for testing of low sample numbers during the working week [22]. Moreover, the DFA includes a positive control, ensuring that the assay has performed properly, as opposed to the FA where the validity of the generated results are based solely on the correlation coefficient of the standard curve and the optical density rate of the negative control [20, 21].

So far, the two assays have been compared only once [22]. In particular, White et al. performed a retrospective case-control study evaluating the overall FA and DFA performances in patients with and without IFDs, as well as the specific clinical performances in IA, invasive candidiasis (IC), and *Pneumocystis pneumonia* (PCP) groups with respect to the non-IFD group. According to their findings, the median FA-BDG and DFA-BDG concentrations were 46 and

79 pg/mL, respectively, with no significant differences between them ( $p = 0.90$ ). There was a weak but significant correlation between the assays (Spearman  $r_s = 0.387$ ,  $p < 0.0001$ ) and their agreement was 76% ( $\kappa = 0.5$ ). On the other hand, the median FA-BDG and DFA-BDG levels in our patient population were 85 and 53 pg/mL, respectively, with FA-BDG levels being significantly higher ( $p < 0.0001$ ). BDG concentrations generated by the two assays exhibited a high degree of correlation (Spearman  $r_s = 0.913$ ,  $p < 0.0001$ ), and the FA-DFA agreement was 85% ( $\kappa = 0.725$ ). Concerning the clinical performance of DFA-BDG testing, White et al. reported higher positivity rate for samples originating from patients with probable IA compared to ours (76% versus 52%), as well as higher sensitivity and specificity for differentiating probable IA from no IA compared to our results (81% versus 53% and 78% versus 67%, respectively).

When comparing the performance of an assay with that reported in previous studies, differences in study design and interpretation of the results must be considered. Namely, White et al. used a mixed control group of patients (43/64; 67% hematology patients with no IFD) not highly representative of the actual clinical setting, which could skew the DFA-BDG diagnostic parameters and lead to overestimation. Similarly, a mixed group of patients (21 with probable IA, 15 with proven IC, 6 with probable PCP, 1 with probable IFD, 10 with possible IFD, and 4 with suspected PCP) was used for the determination of the FA-DFA agreement. In addition, IA was defined according to the 2008 EORTC/MSG consensus criteria, where at least one positive GM result (GM index  $\geq 0.5$ ) is required as mycological evidence [29]. In contrast, we applied the 2020 EORTC/MSGERC revised criteria, where a GM index  $\geq 1.0$  is used for IA classification [9]. Notably, a 33% reduction in the classification of probable IA has been observed using the GM as the only mycological criterion [30]. Furthermore, serial samples were available for a small proportion of patients (19%), and the optimal DFA-BDG positivity threshold defined (69 pg/mL) is not IA-specific, as opposed to our study where sequential serum specimens from 73% of patients were tested,

and disease-specific cut-off values were determined. Cut-offs to optimize the performance of a BDG assay may differ not only according to the pathogen but also the host [19, 31]. Of note, our results are consistent with data of Weinbergerova et al., revealing a weak but significant correlation between the BDG concentrations and the corresponding GM indices [32]. Both studies included similar-sized cohort of patients with probable IA (13 versus 15) and patients on antifungal therapy (72% versus 84%) that could have masked the actual GM levels [13, 33]. Moreover, it is well known that secondary metabolites are detected at different stages of IA and with a different duration. In contrast, White et al. reported a strong correlation and a good agreement between the DFA-BDG and GM index values [22].

The optimal use of BDG quantification in the serum of hematology patients at risk for IFD, particularly IA, is not well defined [18]. Previous studies on the diagnostic performance of BDG testing, restricted to patients with hematologic malignancies and proven/probable IA, have shown diverging results, with sensitivities ranging from 46 to 88% [14, 19, 32]. Indeed, regardless of the manufacturer, the assay's contribution in the diagnosis of IA in our population was modest given its moderate sensitivity (53–67%), which was not significantly improved when optimal cut-off values were applied (57–73%), which is in agreement with previous findings suggesting BDG's limited usefulness as a screening method for IA in hematology patients [14, 32], and supports the recent EORTC/MSGERC recommendation that the detection of BDG is not considered to provide mycological evidence of any invasive mold disease [9]. In fact, BDG is not an IA-specific marker, and false positive results have been related to several factors limiting specificity and PPV [28]. Among them, the assumption that concomitant or recent bacterial bloodstream infections may contribute to elevated circulating BDG seems to have been abandoned [18, 28], corroborating our finding.

Nonetheless, it has previously been suggested that  $\geq 2$  consecutive serum samples should be considered to increase the diagnostic accuracy of the BDG, since the results could be

translated to higher probability of an IFD [15, 34, 35], which is in line with our finding. In particular, in the present study, 62% of patients with probable IA had at least two consecutive positive specimens by both assays, as opposed to 26% and 10% of patients with no IA who had  $\geq 2$  consecutive FA-BDG and DFA-BDG positive samples, respectively. Indeed, the use of at least two consecutive positive test results improved BDG performance, since the sensitivity and NPV of both assays significantly increased (from 53–67% to 71–95%, and from 71–74% to 85–96%, respectively), while specificity and PPV remained unchanged. Interestingly, the median FA-BDG concentration for cases of candidemia, although limited, and probable IA were 309 and 119 pg/mL, respectively. Likewise, Azoulay et al. evaluated the accuracy of BDG for diagnosing IFDs in a large cohort of critically ill hematology patients (BDG was assayed at ICU admission), and reported higher FA-BDG levels in patients with candidemia ( $\sim 450$  pg/mL) than in patients with IA ( $\sim 110$  pg/mL) [36]. However, additional studies are needed to further validate an optimal absolute value that can be used to differentiate IA from candidemia in patients with hematologic malignancies.

On the other hand, although high NPV ( $> 90\%$ ) has been reported as one of the major advantages of BDG for non-hematological populations [37, 38], false-negative results can occur in the hematology setting [39]. Indeed, 4/11 (36%) patients with probable IA were BDG-negative by both assays, all being on mold-active treatment (voriconazole) at the time of blood sampling. Hence, a possible explanation could be that mold-active therapy might have negatively influenced the assay's performance, similarly to what has already been demonstrated for GM screening [13]. Although data supporting this hypothesis in the context of IA are lacking [39], lower BDG concentrations were reported in hematology patients with breakthrough candidemias than in non-breakthrough episodes [40]. Of note, 4/11 (36%) patients with probable IA receiving micafungin or caspofungin as empirical therapy had significantly higher FA-BDG and DFA-BDG levels (median concentrations 233 and 184 pg/mL,

respectively) compared to 7/11 patients with probable IA all receiving voriconazole as mold-active treatment (median FA-BDG and DFA-BDG concentrations 0 and 14 pg/mL, respectively,  $p = 0.008$ – $0.03$ ). It is possible that different classes of antifungal agents may affect BDG levels dissimilarly based on their mechanisms of action. Azoles and polyenes have fungicidal activity against most *Aspergillus* spp., whereas echinocandins are fungistatic.

## CONCLUSIONS

The DFA yielded similar results to the FA, while its cost-effective handling and technical flexibility may favor its use in daily routine, particularly in laboratories with a small sample throughput. However, there was no significant difference in diagnostic accuracy when performance was stratified according to the brand, as both assays revealed moderate sensitivity for diagnosing IA in hematology patients. Therefore, it is of critical importance that results of BDG testing be carefully interpreted in this specific high-risk setting, alongside other clinical and laboratory findings keeping the potential pitfalls for false-positive and false-negative results in mind. Nevertheless, the use of at least two consecutive positive serum samples may be used as a diagnostic criterion for IA.

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**Data Availability.** The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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