Prospective study in critically ill non-neutropenic patients: diagnostic potential of (1,3)-β-D-glucan assay and circulating galactomannan for the diagnosis of invasive fungal disease

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Abstract Diagnosis of invasive fungal disease (IFD) in patients under intensive care is challenging. Circulating biomarkers, (1,3)-β-D-glucan (BG) and galactomannan (GM), were prospectively assessed in 98 critically ill patients at risk of IFD. There were 11 cases of invasive aspergillosis (IA; 4 proven and 7 probable), 9 cases of proven invasive candidiasis (IC), 1 case of mixed proven IC and probable IA, 1 case of proven zygomycosis, and 1 case of mixed mycelial proven IFD. In all IA cases there was no significant difference when the area under the receiver operating characteristic curve (AUC) of GM (0.873 [95% CI, 0.75–0.99]) and BG (0.856 [95% CI, 0.71–0.99]) were compared ($p=0.871$). The AUC for BG in IC and for the rest of the IFD cases was 0.605 (95% CI, 0.39–0.82) and 0.768 (95% CI, 0.63–0.90) respectively. Positive BG (40%) predated blood culture ($n=3$) and abdominal pus ($n=1$) a mean of 3.25 days before *Candida* was grown. In patients with IFD caused by molds, BG appeared a mean of 5.65 days before culture results. For the diagnosis of patients at risk of IC, BG has shown a high NPV (94.5%), with positive results also predating blood cultures in 30% of patients. In conclusion, early BG results permit a

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This work is dedicated to the memory of Jose Ponton who passed away on 21 July 2010

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timely initiation of antifungal therapy in patients at risk of IFD.

Introduction

Invasive fungal diseases (IFD) in patients under intensive care are among the most difficult infectious diseases to recognize clinically and their diagnoses are frequently missed because the current diagnostic techniques are inadequate [1].

Yeasts of the genus *Candida* are the predominant pathogens in the intensive care setting, causing a broad spectrum of presentations, including catheter-related candidemia, disseminated candidiasis, endocarditis, thrombophlebitis, osteomyelitis, etc. [2]. *Candida* spp. are the first cause of bloodstream infections in the Intensive Care Unit (ICU). Other yeasts, such as *Cryptococcus*, are emerging, particularly in solid organ transplant recipients. Molds are also emerging as important pathogens in non-neutropenic ICU patients, including *Aspergillus* spp. [3] followed by *Fusarium*, *Scedosporium*, and Zygomycetes, although the latter are less common.

Diagnosis of IFD in critically ill patients is challenging because of non-specific clinical presentation, poor diagnostic yield of traditional microbiological techniques and non-specificity of radiological imaging [3, 4]. In ICU patients, because of their critical situation, the "gold standard" diagnostic procedures, such as histological examination and culture of deep tissue or fiberoptic bronchoscopy with bronchoalveolar lavage, require an aggressive approach that is often precluded owing to poor clinical status [5, 6].

Conventional diagnostic tests (cultures and direct examination) of respiratory tract samples have a low diagnostic yield [4]. With the advent of new non-invasive diagnostic tools such as Galactomannan (GM) and (1,3)-β-D-glucan (BG) testing, the diagnosis of IFD in adult neutropenic patients and hematopoietic stem cell transplant recipients has improved [6]. However, data on the performance of GM and BG in medical ICU patients are scarce [7–11].

In this context, we conducted a single center, case-controlled, prospective diagnostic study in order to evaluate and compare the diagnostic performance of serum GM and BG as diagnostic adjuncts in proven and probable IFD in a cohort of critically ill, non-neutropenic patients at a high risk of IFD.

Materials and methods

Selection of patients

Levels of BG and GM in serum samples were requested by the attending ICU physicians when judged clinically necessary: in those critically ill patients with several host factors and with a clinical syndrome compatible with IFD. Basically, there were two major infectious entities: invasive aspergillosis (IA) and other invasive mold diseases, and invasive candidiasis (IC).

Patients were identified as being at risk of IFD caused by *Aspergillus* or other molds (*Fusarium*, *Scedosporium*, and Zygomycetes) if they had a clinical syndrome compatible with pneumonia, sinonasal and/or central nervous system (CNS) disease or a subcutaneous tissue infection secondary to traumatic injury. The definition of pneumonia required at least two of the following criteria: fever, worsening gas exchange, pulmonary infiltrates or dyspnea. Sinonasal disease required imaging showing sinusitis and at least one of the following three signs: pain, nasal ulcer with black eschar, and extension across the bony barriers. CNS disease required one of the following: focal lesion or meningeal enhancement on radiological imaging. In addition, patients had at least one host factor namely: neutropenia (neutrophil count <500 neutrophils/mm^3), chronic obstructive pulmonary disease (COPD), liver cirrhosis, HIV infection, solid-organ cancer, patients with a hematological malignancy, steroid use, use of T cell immunosuppressant drugs, solid organ transplant recipients, prolonged stay in the ICU (>21 days), malnutrition or renal failure with renal replacement therapy. Patients with a clinical syndrome compatible with pneumonia were included in the study if bronchoalveolar lavage (an aggressive diagnostic procedure) could not be performed.

Patients were identified as being at risk of IC according to a strategy integrated into a risk-predictive model reported by Ostrosky-Zeichner et al. [12]. This model permits the identification of patients at risk after the first 4 days following admission to the ICU. These criteria are: systemic antibiotics (days 1–3), OR the presence of a central venous catheter (days 1–3), AND at least TWO of the following: total parenteral nutrition (days 1–3), any dialysis (days 1–3), pancreatitis (days −7–0), any use of steroids (days −7–3), or use of immunosuppressive agents (days −7–0), and any major surgery (−7–0). Patients at risk of IC required a clinically compatible syndrome; namely, the presence of signs and symptoms consistent with sepsis syndrome and/or radiological abnormality consistent with peritoneal abscesses. Antibiotic and antifungal treatments were started at the discretion of the attending physician.

The study was approved by the Ethics Committee.

Definition of IC and IA

Proven and probable IC, IA, and mold invasive disease were based on the modified definitions of de Pauw et al. [5], excluding the detection of GM and BG in serum samples. Patients with IA had compatible radiological images, as described elsewhere [13, 14].

A positive result for *Candida* spp. required a blood culture obtained via a central venous catheter to be accompanied by co-synchronous specimens (either via an arterial catheter or from a peripheral vein) that yielded positive results as well [15].

Microbiological assay

Aspergillus and other molds were identified following the guidelines of De Hoog et al. [16]. Blood cultures were incubated for 5 days in an automated blood culture system BacTAlert 3D (bioMérieux, Marcy l'Etoile, France). All *Candida* isolates were identified to the species level using the API ID 32 C (bioMérieux, Marcy l'Etoile, France).

GM and BG testing

Galactomannan (Platelia *Aspergillus*®, Bio-Rad Laboratories, Marnes La Coquette, France) and BG (Fungitell®, Associates of Cape Cod, Falmouth, MA, USA) were performed according to the manufacturer's recommendations for testing serum samples, as described elsewhere [8, 17]. BG assays were performed in duplicate. In patients with more than one sample, we chose only the first one for the analysis.

Statistical analysis

Measurable and categorical variables were respectively described with mean, standard deviation (SD), and range, or frequency distribution values. Continuous variables were compared using the Mann–Whitney U test. Relevant summary diagnostic parameters namely: sensitivity (S), specificity (SP), positive and negative predictive values (PPV, NPV), positive and negative likelihood ratio (PLR, NLR), and efficiency (E) were calculated.

Comparisons between areas under the curve (AUC) were performed using the method of Hanley and McNeil [18]. The optimal cut-offs were determined with receiver operating characteristic (ROC) curves and AUC were estimated to analyze the discriminatory power. The cut-offs for both GM and BG in serum were based on the AUC provided by the ROC curves and consequently the cut-offs chosen were those that offered the best test performance (discriminative power) in our population. All estimations were reported with 95% confidence intervals (CI).

Results

Demographics

Over a period of 24 months (between June 2008 and May 2010) 965 patients were admitted to the ICU. Of these, 149 (15.4%) had a compatible clinical syndrome and host factors of IFD, and 98 (10.2%) met the criteria for inclusion (23 case patients in the IFD group and 75 patients in the control group without evidence of IFD; Tables 1, 2 and 3). The overall prevalence of IFD in this cohort was 23.4% (10.2 and 13.2% for mycelial fungi and *Candida* spp. respectively; Table 3).

The mean disease severity at admission measured by the simplified Acute Physiology Score II (SAPS II) and by Acute Physiology and Chronic Health Evaluation II (APACHE II) of the 23 patients with IFD was 53.8 (range 15–93) and 27.1 (range 12–57) respectively, and for the control group it was 49.9 (range 12–83) and 24.4 (range 8–42) respectively. The ICU mortality in the IFD group was 19 out of 23 (82.6%) and 33 out of 75 (44%) in the control group (p=0.0012).

Twenty-six (26.53%) patients had two predisposing defined host and/or risk factors, 16 (16.32%) had three, and 8 (8.1%) had four or more. The reasons for ICU admission for IFD are shown in Table 1. The most prevalent syndromes were severe pneumonia with septic shock (52.0%) and abdominal/urological septic shock (34.6%).

The rate of necropsy was 9 out of 52 (17.3%). Diagnostic biopsies were obtained in 29 out of 98 deeply infected sites (29.59%). The characteristics of the 23 patients with IFD are detailed in Online Resource 1. There were 4 patients with proven IA, 7 with probable IA, 9 with proven IC, 1 with proven zygomycosis, 1 patient had mixed proven IC and probable IA, and finally, 1 other patient had mixed mold disease.

Patients at risk of IA

Serum GM levels

The serum GM diagnostic accuracy as given by AUC for all IA case patients was 0.873 (95%CI, 0.75–0.99) and for proven IA cases it was 0.862 (95%CI, 0.63–1.00), as shown in Fig. 1a and b). The median of GM levels for the control group (which included the IC and the 2 patients with proven mold disease) was 0.246 (interval quartile range [IQR]: 0.099–0.196). The mean GM level for the two neutropenic control patients was not different from the mean value for the 84 non-neutropenic control patients: 0.288 (SD: 0.27, IQR: 0.09–0.48) and 0.245 (SD: 0.76, IQR: 0.10–0.19) respectively (p=0.937).

Online Resource 2 is a table that shows the performance of GM for the diagnosis of all IA cases with the use of different GM cut-off values. In our population the best diagnostic performance of GM was obtained with a GM cut-off≥0.5. Four case patients (numbers 51, 56, 63, and 67, shown in Online Resource 1) had GM serum levels <0.5. Three of them had been previously treated empirically: 1

Table 1 Baseline characteristics of 98 critically ill patients at risk of invasive fungal disease

Syndrome on ICU admission, number (%)	Age, mean, years (range)	Sex (male/female)	APACHE, mean (± SD)	SAPSII, mean (± SD)	SOTR	ICU stay, median, days (range)	MODS, number (%)	Mortality in patients with MODS, number (%)	Total ICU mortality (%)	Hospital stay after ICU, mean, days (range)	Hospital mortality after ICU stay, number (%)
Group 1[a] 51 (52.0)	60.9 (21–83)	31/20	24.7 (7.2)	52.6 (13.2)	5[e]	11 (1–79)	18 (35.2)	14 (77.7)	26 (50.9)	11 (0–59)	1 (1.96)
Group 2[b] 34 (34.6)	64.4 (22–89)	22/12	49.7 (20.7)	26.9 (12.3)	5[f]	17.25 (1–61)	14 (41.2)	12 (85.7)	15 (44.1)	13 (0–176)	5 (14.7)
Group 3[c] 3 (3.1)	44.3 (32–61)	3/0	58.66 (11.05)	29.33 (8.96)	0	37 (36–58)	1 (33.3)	1 (100)	2 (66.6)	0	0
Group 4[d] 10 (10.2)	56.5 (39–78)	6/4	17.8 (2.0)	38.8 (9.5)	1[g]	19 (5–42)	1 (10)	1 (100)	2 (20)	7 (0–236)	1 (10)

IFD=invasive fungal disease, ICU=Intensive Care Unit, APACHE=Acute Physiology and Chronic Health Evaluation, SAPS II=Simplified Acute Physiology Score II, MODS=multiple organ dysfunction syndrome, SOTR=solid organ transplant recipients

[a] Severe pneumonia with septic shock

[b] Abdominal/urological septic shock and meningoencephalitis (n=1), fasciitis (n=1), phlebitis (n=1), and cellulitis (n=1) with septic shock

[c] Severe polytrauma

[d] This group included patients with diverse clinical situations: 4 patients had central nervous system (CNS) diseases: epilepsy (n=3), subarachnoid hemorrhage (n=1); 2 patients had myasthenic crisis; 2 patients had major vascular surgery; 1 patient had acute myocardial infarction, 1 patient had Ludwig's angina

[e] Renal transplant (n=5)

[f] Renal transplant (n=3), liver transplant (n=2)

[g] Liver transplant (n=1)

Table 2 Occurrence of IFD in 98 critically ill patients at risk

Group	Proven IA, number (%)	Probable IA, number (%)	Proven IC, number (%)	Mixed proven IC and probable IA, number (%)	Proven zygomycosis, number (%)	Mixed proven mold disease,[e] number (%)	Non-IFD, n (%)	Total n
Group 1[a]	0 (0)	4 (7.8)	2 (3.9)	0 (0)	0 (0)	0 (0)	45 (88.2)	51
Group 2[b]	3 (8.8)	3 (8.8)	6 (17.6)	1 (2.94)	0 (0)	0 (0)	21 (61.8)	32
Group 3[c]	0 (0)	0 (0)	0 (0)	0 (0)	1 (33.3)	1 (33.3)	1 (33.3)	3
Group 4[d]	1 (10.0)	0 (0)	1 (10.0)	0 (0)	0 (0)	0 (0)	8 (80.0)	10
Total number	4	7	9	1	1	1	75	98

IA=invasive aspergillosis, IC=invasive candidiasis, IFD=invasive fungal disease

[a] Severe pneumonia with septic shock

[b] Abdominal/urological septic shock and meningoencephalitis (*n*=1), fasciitis (*n*=1), phlebitis (*n*=1), and cellulitis (*n*=1) with septic shock

[c] Severe polytrauma

[d] This group included patients with diverse clinical situations: 4 patients had CNS diseases: epilepsy (*n*=3), subarachnoid hemorrhage (*n*=1); 2 patients had myasthenic crisis; 2 patients had major vascular surgery; 1 patients had acute myocardial infarction, 1 patient had Ludwig's angina

[e] *S. apiospermum, R. arrhizus, F. solani*

with caspofungin and liposomal amphotericin B and 2 with voriconazole. On the other hand, 3 patients (numbers 4, 8, and 101) in the control group presented values≥0.5: 1 had been treated with piperacillin/tazobactam and no known potential causes of false GM reactivity were identified in the 2 other patients.

Serum BG levels

The serum BG diagnostic accuracy as given by AUC for all IA case patients was 0.856 (95% CI: 0.714–0.998) and for proven IA cases it was 0.928 (95% CI: 0.874–0.982), as shown in Fig. 1a and b. The mean BG level for the control group (without the inclusion of the IC patients and the patient with mixed mold disease) was 179.6 (IQR: 40.52–132.98). The mean BG level for the two neutropenic control patients was not different from the mean value for the 74 non-neutropenic control patients: 630.2 (SD: 846.34; IQR: 31.69–1,228.60) and 167.4 (SD: 369.04; IQR: 40.52–132.98) respectively (*p*=0.719). Online Resource 3 is a table that shows the performance for the diagnosis for proven IA cases and for proven and probable IA cases with the use of different BG cut-off values. In our population the best diagnostic performance of BG (for proven and probable IA cases) was obtained with a cut-off ≥80 pg/mL.

One patient (number 63, shown in Online Resource 1) had BG levels >80 pg/mL and had been previously treated empirically with voriconazole. Fourteen out of the 24 patients in the control group that presented values ≥80 pg/mL had co-synchronous bacteremias: *Klebsiella pneumoniae* (*n*=3), *Streptococcus pneumoniae* (*n*=3), *Pseudomonas aeruginosa* (*n*=2), *Enterococcus faecium* (*n*=2), *Escherichia coli* (*n*=2), and *Staphylococcus simulans* (*n*=2). Two other patients had been treated with amoxicillin-clavulanic acid and 3 other patients had undergone major surgical procedures

2 days (*n*=1) and 6 days (*n*=2) predating the BG assay. No known potential causes of false BG reactivity were identified in 5 other patients. In all 24 patients, IFD was excluded after the careful assessment of clinical, microbiological records, and outcome.

Comparison of GM and BG for the diagnosis of IA

There was no significant difference when the AUC for GM and BG were compared in either the total number of cases of IA, proven and probable, or in the number of proven IA cases (*p*=0.8719 and *p*=0.588 respectively).

Combination of GM and BG levels

One proven IA case patient (number 56) and 2 probable IA case patients (numbers 51 and 67) with GM levels≤0.5, shown in Online Resource 1, had positive BG in serum samples and therefore upgraded the AUC, S, and NPV to: 0.942 (95%CI: 0.89–0.98), 83.33 (95%CI, 58.08–100.0), and 97.50 (95%CI, 93.45–100.0) respectively, although these differences were not statistically significant.

Comparison of culture results and GM and BG levels

In patients with IA, GM (41.6%), and BG (50%) a mean period of 6.5 days (range 4–11) passed before *Aspergillus* was grown.

Patients at high risk at IC

Serum BG levels

The diagnostic accuracy, as given by the AUC for all IC case patients, was 0.605 (95% CI, 0.387–0.823), as

Table 3 Characteristics of critically ill patients at risk of IFD, and microbiological and serological data

Patients	Proven IA (n=4)	Probable IA[a] (n=8)	Proven IC[a] (n=10)	Proven Zygo (n=1)	Mixed proven mold disease (n=1)	Non-IFD (n=75)	Total (n=98)
Mechanical ventilation mean time (days)	12.5	16.37	14.4	0	59	13.87	16.92
Systemic antibiotics/CVC	4/2	8/1	10/2	1/0	1/1	74/11	97/17
Acute renal failure with ERPT (%)	2 (50)	3 (37.5)	4 (40)	1(100)	1(100)	12 (16)	23 (23.46)
Steroids[b] (%)	2 (50)	6 (75.0)	3 (30)	0	0	11 (14.6)	21 (21.42)
Neutropenia (%)	0	2 (25.0)	1 (10)	0	0	2 (2.6)	4 (4.08)
Immunosuppressive agents (%)	0	3 (37.5)	4 (40)	0	0	12 (16)	18 (18.36)
TPN (%)	2 (50)	1 (12.5)	4 (40)	0	0	9 (12)	15 (15.30)
Major surgery (%)	2 (50)	2 (25)	7 (70)	1 (100)	1 (100)	18 (24)	31 (31.63)
Onco-hematological disease (%)	0	1 (12.5)	1 (10)	0	0	4 (5.3)	6 (6.12)
SCT autologous (%)	0	0	0	0	0	1 (1.33)	1 (1.02)
Non-hematological							
COPD (%)	1 (25)	4 (50)	1 (10)	0	0	13 (17.3)	19 (19.38)
SOT (%)	0	1 (12.5)	2 (20)	0	0	9 (12)	11 (11.22)
HIV (%)	0	0	0	0	0	5 (6.66)	5 (5.10)
Cirrhosis (%)	1 (25)	0	1 (10)	0	0	9 (12)	11 (11.22)
Systemic diseases (%)	1 (25)	0	0	0	0	7 (9.3)	8 (8.16)
Solid cancer (%)	1 (25)	2 (25)	4 (40)	0	0	12 (16)	19 (19.38)
Chronic renal failure (%)	1 (25)	2 (25)	2 (20)	0	0	12 (16)	16 (16.32)
Pancreatitis (%)	0	0	1 (10)	0	0	5 (6.6)	6 (6.12)
Clinical criteria							
Lung nodules	3[c]	6	0	0	0	3	12
Consolidation	1	2	1	0	0	20	23
Abdominal abscesses	0	0	3	0	0	2	5
Aspergillus / *Candida* (sterile site) positive cultures	4/0	8/1	1/10	0/0	0/0	6/0	18/10
Fungal structures on biopsies or necropsies	4	0	0	1	1	0	6
Serum GM index, mean (range)	2.50 (0.155–4.39)	1.32 (0.122–4.56)	0.40 (0.059–2.5)	0.1	0.115	0.25 (0.032–7.06)	0.423 (0.032–7.06)
Serum BG pg/ml, mean (range)	1,513 (639–1,747)	2,399.7 (24.34–8,009)	1,655.8 (18.36–7,836)	29	535	178.09 (16–2,625)	501.82 (16–7,836)

IFD=invasive fungal disease, IA=invasive aspergillosis, IC=invasive candidiasis, Zygo=Zygomycosis, CVC=central venous catheter, ERPT= extra-renal purification techniques, TPN=total parenteral nutrition, SCT=stem cell transplantation, COPD=chronic obstructive pulmonary disease, SOT=solid organ transplant, HIV=human immunodeficiency virus, GM=Galactomannan, BG=(1,3)-β-D-glucan

[a] One patient with proven IC and probable IA

[b] Minimum dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks

[c] One patient with halo sign

shown in Fig. 1c. The mean of the BG levels for all patients with IC was 1,655.8 pg/mL (SD: 2,970.45; IQR: 61.99–1,219.6).

Online Resource 3 shows the performance of BG in serum samples for the diagnosis of IC with the use of different BG cut-off values. The best diagnostic performance of BG for IC was obtained with a BG cut-off≥ 80 pg/mL. Three case patients (numbers 94, 166, and 171,

Online Resource 1) had BG serum levels <80 pg/mL. One of them had been treated with anidulafungin prior to diagnosis.

Comparison of culture results with BG levels

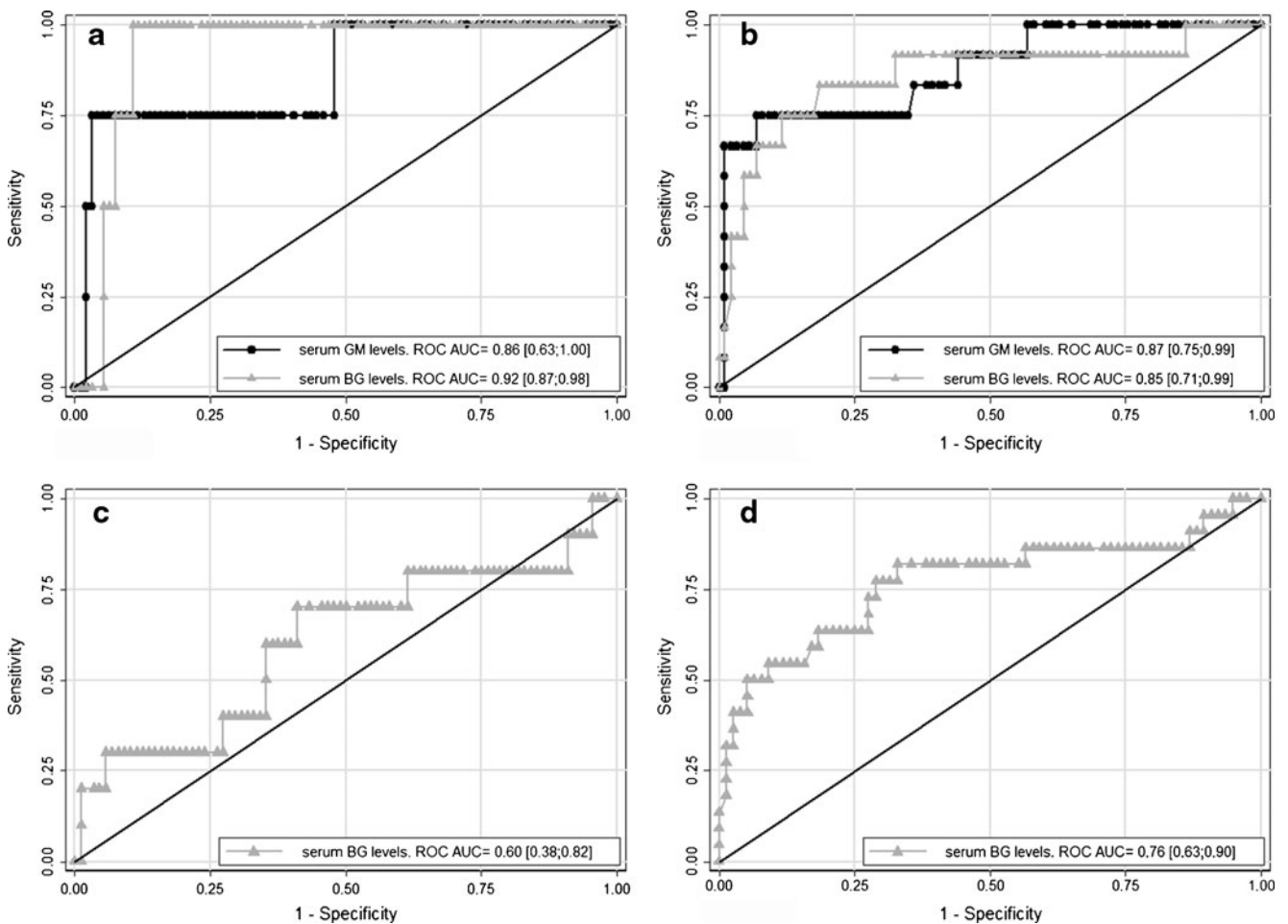In patients with IC, BG appeared a mean 3.25 days before *Candida* was grown in 40% of IC cases and 4.8 days

Fig. 1 Area under the curve (AUC) of the receiver-operating characteristic (ROC) curves of galactomannan (GM) and/or (1,3)-β-D-glucan (BG) determinations in serum for: **a** proven IA cases; **b** all invasive aspergillosis (IA) cases, proven and probable; **c** for invasive candidiasis (IC) cases, and **d** for all invasive fungal disease (IFD) cases (IA, IC, and mixed mold disease)

(range, 2–7) before *Candida* was identified to species level in 50% of cases.

Invasive fungal diseases

Serum BG levels

The diagnostic accuracy as given by AUC for all IFD case patients (all IA, IC, and the patient with mixed mold disease) was 0.768 (95% CI: 0.633–0.903), as shown in Fig. 1d. The mean BG level for all patients with IFD was 1,626.94 pg/ml (SD: 2,509.25; IQR: 117.34–1,701.78). The best diagnostic performance of BG for all IFD was obtained with a BG cut-off≥80 pg/mL , as shown in Online Resource 3.

The patient with proven zygomycosis (number 93, Online Resource 1) had negative BG antigenemia. Zygomycetes produce little or no BG and these invasive diseases cannot be detected with this biomarker.

Discussion

This single-center, prospective observational study investigated the diagnostic performance of BG and GM assays in the setting of critically ill non-neutropenic patients at high risk of, and with a clinical syndrome compatible with, IFD. Currently, inadequate data are available on the use of fungal biomarkers as diagnostic adjunct tools in ICU patients [7–9, 19–21]. This study has limitations, as it was physician- and not protocol-driven: the date of entry was not uniform, the work-up of IFD was not defined, nor was the timing of the sampling. It is, however, representative of the typical context in which the application of these diagnostic adjuncts is practiced.

Currently, different risk-identification strategies have been proposed and/or developed for identification of ICU patients at risk of IFD. For IA, Meersseman et al. [3] have proposed a risk stratification scheme, but prospective validation studies are not available for this proposal. For IC, which is the most

prevalent IFD in ICU patients, Eggimann and Ostrosky-Zeichner [22] discuss and summarize the development of different risk-identification strategies, targeting early identification of IC. Our study was based on one of these strategies, that of Ostrosky-Zeichner [12].

Our patients were at particularly high risk of IFD, as almost 50% of them had two or more host and/or risk factors, and this explains the high incidence of IFD, above 20% in our cohort. Nevertheless, it is important to stress that the true incidence of IFD is uncertain without necropsy studies [23–27]. A limitation of our study is the low rate of necropsies, because they are a critical means of checking the accuracy of new diagnostic tools such as GM and BG. In our study, there was a stark difference between the mortality rates in the IFD group compared with the control group, 82.6% and 44% respectively ($p=0.0012$). In the light of previous studies [28, 29] that highlighted the high mortality attributable to IFD, these data underscore the need for improved diagnosis of IFD.

Conventional diagnostic methods are insensitive and slow, and invasive diagnostic procedures, such as biopsies and bronchoalveolar lavage are problematic and not feasible in many critically ill patients. Non-invasive diagnostic methods independent of cultures for detecting circulating fungal antigens are increasingly introduced in clinical practice, but in the setting of ICU patients and solid organ transplant recipients the available current experience with GM and BG assays, as diagnostic adjuncts, is scarce [7–11, 19–21].

Positive assays of GM and BG are included as microbiological diagnostic criteria for probable IFD [5]; these revised definitions do not include critically ill patients in the ICU, other than those with proven IFD [3]. The detection of circulating GM has improved the diagnosis of IA in adult neutropenic patients and hematopoietic stem cell transplant recipients in seminal screening studies based on biopsies and a high rate of necropsies (≥90%) [6, 23, 24, 26].

In ICU patients, circulating GM was compared prospectively with the detection of GM in BAL fluid in a sound study with a 95% necropsy rate [8]. This important study showed that with a cut-off of 0.5 the S and SP in BAL were 88% and 87% respectively, whilst the S of serum GM was only 42% for proven IA.

The different diagnostic efficiency of circulating GM in adult onco-hematological patients and critically ill non-neutropenic patients may be understood with an experimental animal model in mice with IA, treated with either corticosteroids or chemotherapy [30]. In steroid-treated individuals and in patients with cirrhosis (equivalent to ICU patients), inflammation in the lung is more pronounced and fungal burden and angio-invasion is minimal compared with neutropenic individuals: in the latter case, the fungal burden and angio-invasion are pronounced. Of note, most

ICU patients at risk of IA are non-neutropenic patients (93.92% of the patients included in our study). It has been maintained that neutrophils clear GM from the blood through mannose-binding receptors [31]. This histopathological pattern has been widely confirmed in autopsies of patients with proven IA [8, 25].

In our patients the S, SP, PPV, and NPV of GM for proven and probable IA cases were 66.6, 97.6, 80.0, and 95.4% respectively, with a cut-off ≥0.5. The SP in our study and in the study of Meersseman et al. were concordant and high (97.6 and 96% respectively); however, the S was remarkably higher in our population (66.6 and 42% respectively). This could be due to the low rate (20%) of steroid-treated patients in our population compared with 48% in their study. In 3 out of 4 of our patients with false-negative results, these could be explained through the administration of systemic antifungals with *Aspergillus* activity, as has been described by Marr et al. [32].

(1,3)-β-D-glucan is a cell wall polysaccharide of most fungi, with the exception of the Zygomycetes. Its presence in blood can be used for the detection of IFD including IC, IA, and different mycelial invasive diseases. BG assay was first introduced in Japan almost two decades ago [9, 20]. Nowadays, there are four commercially available kits to detect BG with different cut-off values and different reactivity to different (1,3)-β-D-glucan standards [20]. The Fungitell BG assay was cleared in 2004 by the US Food and Drug Administration for the presumptive diagnosis of IFD.

The S and NPV of BG in our study were higher for proven IA and for proven and probable IA cases compared with GM, although these differences were not statistically significant ($p=0.5888$ and $p=0.871$ respectively; Online Resources 2 and 3). Interestingly, in patients with IA, both GM and BG appeared a mean of 6.5 days before *Aspergillus* was grown. The clinical impact of this fact is that early diagnosis of IA implies the possibility of prompt initiation of antifungal therapy, which is crucial for reducing mortality [33].

As assays with individual biomarkers have inherent limitations, it is possible that these could be avoided with the combined use of GM and BG, for the diagnosis of IA: this strategy has been studied before by our group [17]. In our patients the combination of GM and BG upgraded the S and NPV, but the differences were not statistically significant.

One patient treated with voriconazole (with probable IA) had false-negative BG results. Interestingly, Koo et al. [21] found no decrease in S in the presence of systemic antifungal therapy, even in patients receiving antifungal therapy for more than 7 days. This has been explained because of the slow clearance of BG in some fungal infections [17, 34–36].

In our study there were 24 control patients (24.4%) with high BG levels without IFD. Seven such patients had co-synchronous Gram-positive bacteremia and 7 others had Gram-negative bacteramia; a further 2 patients had been treated with amoxicillin-clavulanic acid and 3 had undergone major surgery during the week before the BG assay. All are recognized as potential sources of false-positive BG results [7, 17, 37–42]. Such conditions were not identified in any of the 5 remaining patients. In our patients *Candida* colonization was unlikely to be the cause of the false-positive BG antigenemia, as other patients with intense *Candida* colonization in the surveillance cultures had negative BG antigenemia (data not shown). In our population the rate of false-positive BG results was significantly higher ($p \leq 0.0001$) compared with circulating GM results. Our study shows that the discriminatory power of GM for the diagnosis of IA is significantly higher ($p = 0.0260$) when the AUC for GM and BG were compared.

Data on the performance of BG in ICU patients are scarce for the diagnosis of IC. Digby et al. [7] have questioned the clinical utility of BG in ICU patients with the use of the kit FungiTec G assay (cut-off: 20 pg/mL), and maintain that serum BG levels do not show a correlation with the presence of IFD and do not appear to be specific. Given their use of an inappropriately low cut-off, 20 pg/mL versus the usual 80 pg/mL, a high rate of false-positives is to be expected. An early retrospective study with Fungitell for the diagnosis of proven IC (candidemia) reported a 78% S with a cut-off ≥ 80 pg/mL [9]. León et al. [10] have recently conducted a prospective study including 240 non-neutropenic adult ICU patients to assess the usefulness of the BG assay for discriminating between *Candida* species colonization and IC. Using a cut-off of 75 pg/mL they reported a S and SP of 77.8% and 52.7% respectively. A current study in 106 high-risk surgical ICU patients has shown that two consecutive BG ≥ 150 pg/mL has the best diagnostic yield, with S and SP of 73 and 78% respectively, and preceded by 2 days the start of antifungal therapy based on microbiological documentation of IC in 73% of cases [11]; these results are in line with another recent study in surgical ICU patients [43]. The reasons for the need to use a high cut-off are likely to be related to the high rate of false-positives found. In our study the S was lower (70%) for proven IC and the SP was higher (59.09%) with a cut-off ≥ 80, compared with these studies. The basis of elevated serum BG values has a variety of potential causes. These include intestinal *Candida* overgrowth and translocation [44], cryptic pulmonary *Pneumocystis jiroveci* infection [45], BG production by concomitant bacterial infections [39], and iatrogenic contamination [38, 46].

Positive BG (40%) predated blood culture ($n=3$) and abdominal pus ($n=1$) a mean of 3.25 days (range 2–5) before *Candida* was grown. Early microbiological results permit a timely initiation of antifungal therapy after blood culture sampling, which decreases mortality [47, 48].

The NPV of BG in our cohort for all IFD cases was above 90% and thus our results are in line with the values reported in different mixed non-ICU populations [9, 17, 49, 50]. These high NPV values allow IFD to be reasonably excluded. It is also worth noting that of the 4 survivors in the proven and probable IFD group, 3 had negative BG findings. This is suggestive of the potential utility of the serum BG level, in the ICU setting, as a prognosis indicator.

Currently, another diagnostic possibility under investigation is the use of real-time PCR for detection of *Candida* in serum samples [15]. PCR is a promising diagnostic tool that requires the development of a commercial PCR that would facilitate a multicenter evaluation.

In conclusion, the data presented herein show that the prospective evaluation of circulating BG and GM in high-risk non-neutropenic critically ill patients generates positive results that predate culture results and improves the diagnosis of IA. For the diagnosis of patients at risk of IC, BG has shown a high NPV (94.5%) and positive results also predate blood culture results in 30% of IC cases. Evidence-based prospective studies identifying patients at high risk of having IFD are needed for further evaluation of these non-invasive diagnostic tools.

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