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Original Article

Biomarkers for the diagnosis of invasive candidiasis in immunocompetent and immunocompromised patients

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ABSTRACT

Blood culture methods show low sensitivity, so reliable non−culture diagnostic tests are needed to help clinicians with the introduction, de-escalation, and discontinuation of antifungal therapy in patients with suspected invasive candidiasis (IC). We evaluated different biomarkers for the diagnosis of IC in immunocompetent and immunocompromised patients at risk for developing invasive fungal diseases. The specificity of Candida albicans germ-tube antibodies (CAGTA) detection was high (89%−100%), but sensitivity did not exceed 61% even after raising the cut-off from 1/160 to 1/80. We developed enzyme-linked immunoassays detecting antibodies against C. albicans proteins (Als3-N, Hwp1-N, or Met6) that resulted more sensitive (66%−92%) but less specific than CAGTA assay. The combination of 1,3-beta-D-glucan (BDG) detection and CAGTA results provided the highest diagnostic usefulness in immunocompetent patients. However, in immunocompromised patients, anti−Met6 antibodies was the best biomarker, both, alone or in combination with BDG.

1. Introduction

Invasive candidiasis (IC) is a leading cause of mycosis-associated mortality in hospitals of developed countries with a high attributable mortality 40% to 50% despite treatment with antifungal agents (Edmond *et al.*, 1999, Gudlaugsson *et al.*, 2003). Population-based studies have reported incidence rates of 1.4 to 20 per 100,000 inhabitants (Quindós, 2014).

The incidence of IC in immunocompromised patients has increased as a consequence of factors such as long-term hospitalization, acquired immunodeficiency (HIV infection), treatment-induced immunodeficiency in patients receiving hematopoietic stem-cell or solid organ transplants, as well as those undergoing anticancer therapy (Kontoyiannis *et al.*, 2010, Pagano *et al.*, 2010, Pappas *et al.*, 2010). These patients can not eliminate efficiently this opportunistic and commensal fungus (Brown *et al.*, 2012). Furthermore, IC also extends the length of hospital stay and increases the cost of medical care (Quindós, 2014, Diekema *et al.*, 2012).

The diagnosis of IC is challenging because there are no specific clinical manifestations, and colonization and infection are difficult to distinguish. Conventional microbiological methods, such as the detection of the infecting fungus by histopathology and culture, usually lack both sensitivity, and specificity (Morrell *et al.*, 2005). Blood culture remains the "gold standard," delaying the possible therapeutic intervention and resulting in substantial morbidity and mortality (Lew, 1989). Because of these shortcomings, there is a great interest in developing reliable non−culture diagnostic tests, but none has found widespread clinical use yet.

A 12 to 24 hours delay in the introduction of the antifungal treatment can double the mortality of candidemia (Morrell *et al.*, 2005), so new early detection tests are needed to anticipate the specific antifungal treatment. Moreover, appropriate diagnostic techniques to exclude IC in patients at high risk, would allow de-escalation or discontinuation of antifungal agents, reducing healthcare costs, and the risk of inducing possible resistance to these drugs.

Abbreviations: Als-3-N, (amino terminal fraction of agglutinin like sequence protein); BDG, (1,3-beta-D-glucan); CAGTA, (Candida albicans germ tube antibodies); Hwp1-N, (amino terminal fraction of hyphal-wall protein 1); IC, (invasive candidiasis); IFD, (invasive fungal disease); Met6, (methionine synthase)

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Our group developed an indirect immunofluorescence assay to detect *Candida albicans* germ tube antibodies (CAGTA) in patients at risk of IC that allows distinguishing colonization from invasion (García-Ruiz *et al.*, 1997, Moragues *et al.*, 2004). Sáez-Rosón *et al.* (2014, 2015) identified agglutinin-like-sequence (Als3), hyphal-wall protein (Hwp1) and methionine synthase (Met6) among the main proteins recognized by CAGTA developed in a rabbit model of IC. Therefore, we aimed at evaluating the detection of specific antibodies against Als3, Hwp1, and Met6 by an enzyme linked immunosorbent assay (ELISA), as useful markers for the diagnosis of IC in immunocompetent, and immunocompromised patients with risk factors for developing an invasive fungal disease (IFD). Data were compared with the results of (Ardizzoni *et al.*, 2014, Brown *et al.*, 2012, Clancy *et al.*, 2008) 1,3-β-D-glucan (BDG) determination and CAGTA assay to assess the diagnostic utility of these serum biomarkers.

2. Patients and methods

2.1. Patients and methods

We studied 627 sera from 297 patients. Sera were collected prospectively at the Severo Ochoa University Hospital (Leganés-Madrid) and Cruces University Hospital (Barakaldo-Bizkaia), and stored at -20°C. Patients were classified into 3 groups according to clinical, and microbiological data. Group 1 included 126 patients (332 sera) with IC proven by a positive blood culture. Group 2 gathered 66 patients (164 sera) with other fungal infections, mainly *Aspergillus,* and *Pneumocystis* infections. Group 3 included 105 patients (131 sera) with no evidence of IFD. Patients were further classified as immunocompetent or immunocompromised. The sera collection is anonymized and registered (C.0005025) at the Instituto de Salud Carlos III, Spain.

2.2. Serum antibodies against germ tubes of C. albicans

The CAGTA titer was calculated as reported by Moragues *et al.* (2004) with some modifications. Briefly, 20 μL of 1/4 diluted serum in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, pH 7.2) was added to 80 μL of sorbent (heat-inactivated *C. albicans* blastospores 10^{10}/mL of PBS) to achieve a final 1/20 dilution of the sample. Twenty μL of 1/20 diluted serum and serial half dilutions in PBS were laid in each well of a slide containing fixed germinated *C. albicans* cells, and left to incubate for 30 minutes at 37° C in a humid chamber. The slides were washed with PBS for 5 minutes with gentle agitation, and left to dry in the air. Then, 10 μL of FITC-conjugated antihuman-IgG antibodies diluted 1/150 in PBS containing 0.05% Tween 20 and 0.05% Evans Blue was added and incubated once more for 30 minutes in the same conditions. The slides were washed again and left to dry. Coverslips were laid down with buffered glycerol (10% glycerol in PBS, pH 8.5−9) and slides were observed with a fluorescence microscope Eclipse 80i (Nikon Corporation, Tokio, Japan). The samples were interpreted as positive if green fluorescence was observed on the surface of the germ tubes and the blastoconidium section of the germinated cells stayed red. The CAGTA titer was established as the highest serum dilution that showed green fluorescence on the entire surface of germ tubes. Sample dilutions 1/160 or further are considered positive for IC.

2.3. Determination of serum antibodies against Als3-N, Hwp1-N and Met6 by enzyme immune assay (EIA)

The amino terminal fractions of *C. albicans* Als3 (Als3-N) and Hwp1 (Hwp1-N) proteins, whose immunogenic characters are located at the amino terminal end of their respective molecules (Hoyer *et al.*, 1998; Staab & Sundstrom, 1998), and Met6 were obtained as recombinant proteins following protocols reported previously (Laín *et al.*, 2007, Sáez-Rosón *et al.*, 2014). Microtiter plate wells (Costar, Cambridge, Massachusetts, USA) were loaded with 100 μL of purified recombinant antigen in 0.05 M sodium bicarbonate buffer (pH 9.6) (Als3-N or Hwp1-N 0.3 μg/mL, and Met6 1.25 μg/mL) and left overnight at 4°C; after removing the antigen solution, the wells were washed with PBS. The internal well surface was blocked with 200 μL of 1% bovine serum albumin in PBS (PBS-BSA) at 37°C for 1 hour, or overnight at 4°C. Microplates can be sealed and stored at -20°C after removing the blocking solution. Human sera were diluted 1/1000 in PBS and assayed in triplicate (100 μL/well). After 1 h of incubation at 37°C, the wells were washed 3 times with 200 μL/well of PBS-Tw (0.05% Tween 20 in PBS). Then, 100 μL of HRP-conjugated anti−human IgG (Sigma) diluted 1/1000 in PBS-Tw was added to each well and plates were incubated for 1 h at 37°C. The plates were washed as described above, and 100 μL of substrate solution (0.05 M citric acid, 0.1 M *di*-sodium hydrogen phosphate anhydrous, 0.05% of 30% hydrogen peroxide and 0.05% *o*-phenylenediamine (Sigma)) was added to each well. The plates were incubated in the dark for 30 minutes at room temperature. The reaction was stopped by adding 50 μL/well of 0.5 M sulfuric acid, and the absorbance was measured at 490 nm using a Microplate Autoreader (Bio-Tek Instruments, Montpelier, Vermont, USA). To normalize the results of different day assays, we run the same reference control serum in every plate; the absorbance data were divided by that of the reference serum, and results were reported as relative absorbance value.

2.4. Serum beta-D-glucan (BDG) determination

Serum BDG was estimated with the Fungitell kit (Associates of Cape Cod, Inc, E. Falmouth, MA, USA), following the instructions of the manufacturer. BDG concentration values <60pg/mL were considered negative, while values ≥80 pg/mL were interpreted as positive.

2.5. Statistics

Mean values of relative absorbance data of the 3 groups of patients were compared by ANOVA (GraphPad 6), and values of $P < 0.05$ were considered statistically significant. Receiver-operating-characteristic (ROC) curve analysis and area under the curve (AUC) values were used to establish the optimal cut-off value for each antibody (Youden index) and to evaluate the overall diagnostic performance of the tests. The sensitivity, specificity, positive, and negative predictive values and accuracy of assays were calculated as described by Kozinn et al (1978).

3. Results

3.1. Serum CAGTA, antibodies against Als3-N, Hwp1-N and Met6, and BDG determination

As shown in Fig. 1., regardless of their immune status, patients with proven IC (G1) achieved the highest CAGTA titers, and the geometric mean showed significant statistical differences with those of the G2 and G3 control groups ($P < 0.05$). The antibody response to Als3-N and Hwp-1-N displayed a similar behavior to CAGTA. It is noteworthy that, although immunocompromised patients showed lower antibody levels compared to immunocompetent patients, the G1 group mean values were always higher than the G2 and G3 control groups, and differences were statistically significant as well.

Regarding anti−Met6 antibodies, immunocompetent G1 patients showed a slightly higher mean value compared to patients with other fungal infections (G2), but this difference was not statistically significant. However, for immunocompromised patients, the differences in mean values of the 3 groups were statistically significant.

On the other hand, BDG concentration in the serum of patients with IFD (G1 and G2) did not show any significant differences

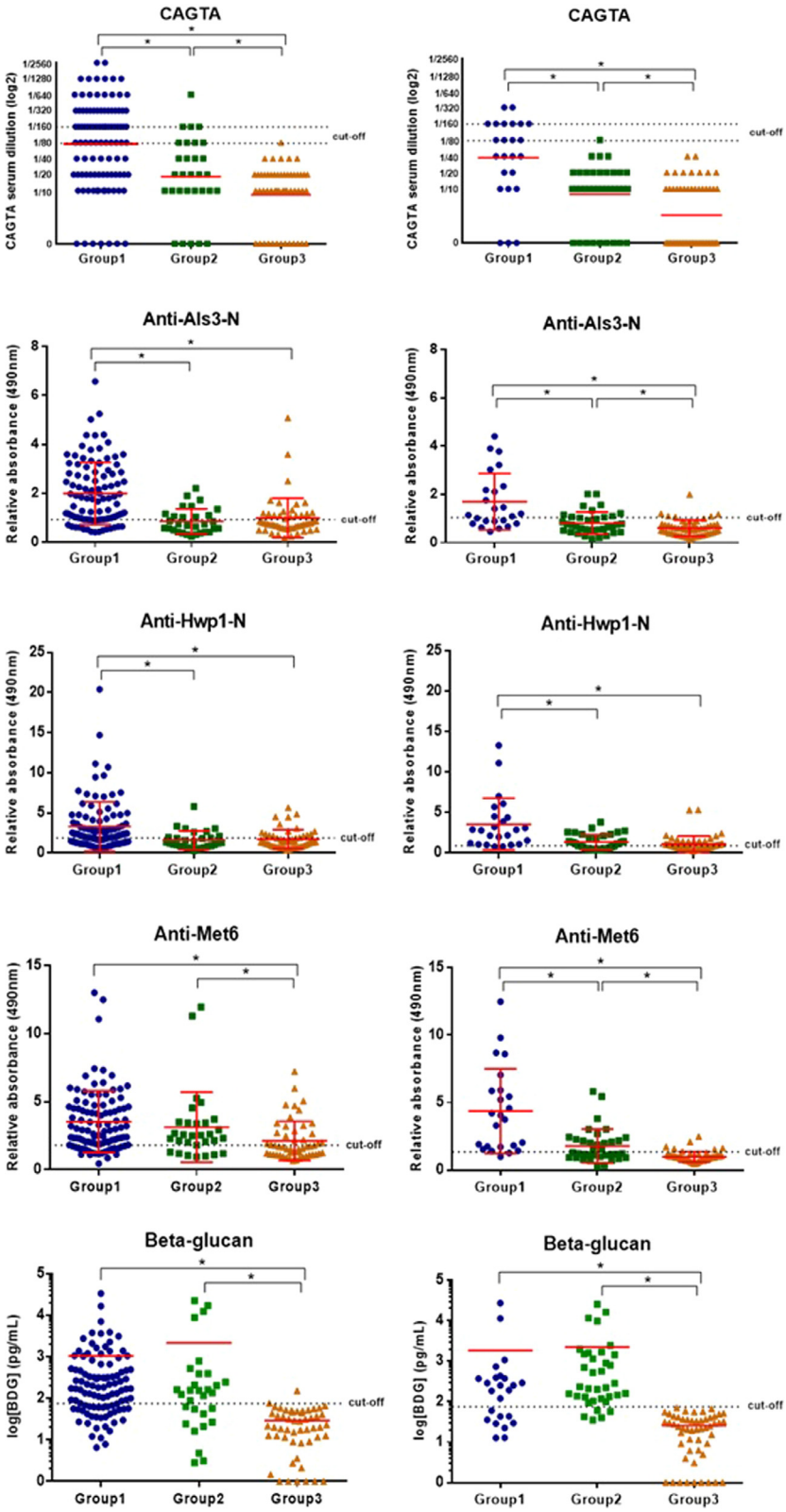

Fig. 1. Distribution of the results of assays for CAGTA and antibodies against Als3-N, Hwp1-N and Met6, and BDG determination in sera of immunocompetent (left column) and immunocompromised (right column) patients. Group 1: proven invasive candidiasis; Group 2: proven invasive fungal diseases different from candidiasis; Group 3: no evidence of fungal disease. Mean ± SD is represented with red lines; geometric mean is used for CAGTA titers (Color version of the figure is available online.).

between them, nor in relation to the immune status of the patients. This result was expected because BDG is a cell wall component of most fungi. Likewise, patients without evidence of fungal infection showed values below the published cut-off point (80 pg/mL).

3.2. ROC curves

The performance of the anti−Als-3, anti−Hwp1-N, and anti−Met6 antibodies tests for the diagnosis of IC was determined through the

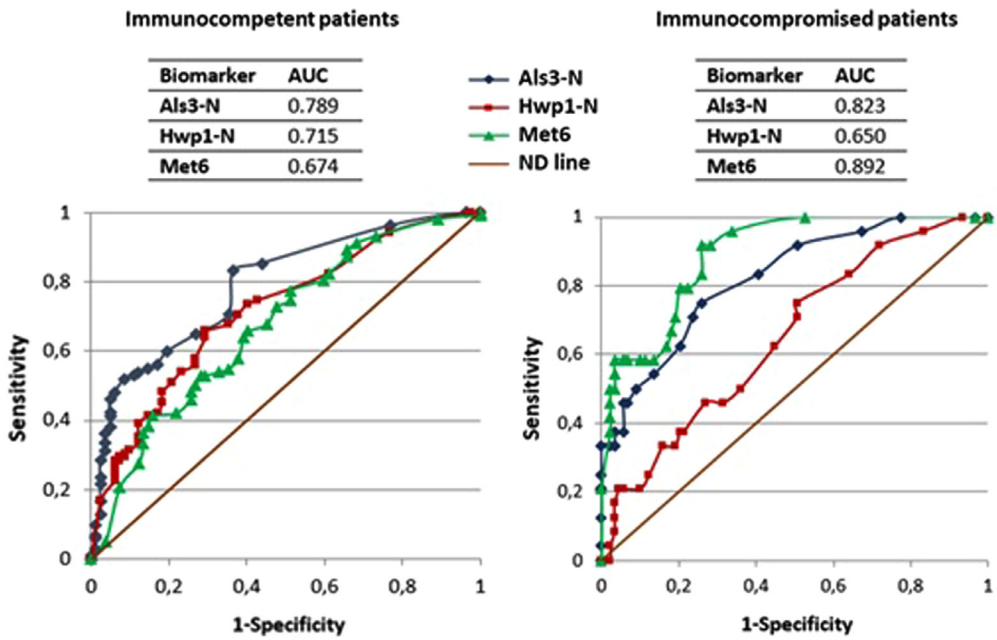

Fig. 2. Diagnosis of invasive candidiasis. ROC curves and AUC (area under the curve) values for the determination of specific antibodies against Als3-N, Hwp1-N, and Met6 in immunocompetent and immunocompromised patients. ND line = no discrimination line.

analysis of the ROC curves (Fig. 2), and the corresponding cut-off values were calculated independently for immunocompetent and immunocompromised patients.

The AUC value for the determination of anti–Als3-N antibodies in immunocompetent patients reached the highest value (0.789), corresponding to its discrimination ability, followed by the anti–Hwp1-N and anti–Met6 antibodies tests (Fig. 2, left panel). In contrast, in immunocompromised patients, the detection of anti–Met6 antibodies (AUC 0.892) reached the highest discriminating capacity, followed by anti–Als3-N, and anti–Hwp1-N antibodies tests (Fig. 2, right panel).

3.3. Diagnostic utility of biomarkers

The calculated parameters for the diagnosis of IC with the different biomarker assays are summarized in Table 1. For a 1/160 cut-off, CAGTA showed modest sensitivity, however specificity and PPV were high, even 100% in immunocompromised patients. Notably, when the cut-off was set to 1/80, sensitivity increased from 50% to 61% in immunocompetent patients, and from 33% to 50% in immunocompromised patients, at the cost of a slight decrease in specificity values, but a higher overall efficacy (accuracy 73% and 88%, respectively). Specific antibody tests outweighed the low sensitivity of CAGTA determination, most notably anti–Als3-N antibodies (83%), followed by anti–Met6 and anti–Hwp1-N antibodies among immunocompetent patients. However, in immunocompromised patients, the detection of anti–Met6 antibodies reached 92% sensitivity, with a high NPV (97%); in these patients, anti–Als3-N and anti–Hwp1-N antibodies tests sensitivity (75%), and NPV values (92% and 88%, respectively) were also remarkable.

BDG detection sensitivity for the diagnosis of IC was 62% to 68% (Table 1). Of note, in immunocompromised patients, PPV dropped to 33% while NPV reached 87%, with a discreet 65% accuracy.

3.4. Serum biomarkers and species of Candida and related genus

In further analysis, we tried to establish the sensitivity of biomarkers according to the yeast species causing invasive candidiasis in Group 1 patients (Table 2). The anti–Met6 antibodies test gave the best results for the 3 most represented species (*C. albicans* 84%, *Nakaseomyces glabrata* (formerly referred to as *Candida glabrata*) 88%, and *Candida parapsilosis* 100%), followed by anti–Als3-N antibodies (81%,

Table 1
Overall performance of CAGTA assay, detection of specific antibodies against Als3-N, Hwp1-N, and Met6, and BDG determination for the diagnosis of invasive candidiasis in immunocompetent and immunocompromised patients.

Biomarker	Patients' immunologic status	Sens[a] (%)	Spec[a] (%)	PPV[a] (%)	NPV[a] (%)	A[a] (%)
CAGTA-80[b]	competent	61	89	87	65	73
	compromised	50	98	86	88	88
CAGTA-160[b]	competent	50	95	93	60	70
	compromised	33	100	100	85	86
Anti–Als3-N antibodies	competent	83	63	74	75	74
	compromised	75	74	44	92	74
Anti–Hwp1-N antibodies	competent	66	71	74	62	68
	compromised	75	49	29	88	55
Anti–Met6 antibodies	competent	77	49	65	63	65
	compromised	92	74	49	97	78
BDG	competent	68	76	78	68	71
	compromised	62	65	33	87	65

[a] Sens: sensitivity; Spec: specificity; PPV: positive predictive value; NPV: negative predictive value; A: accuracy.
[b] CAGTA-80 and CAGTA-160 refer to the cut-off applied for calculations, 1/80 and 1/160 respectively

Table 2
Positive cases for each biomarker in relation to the yeast species responsible for infection in patients with proven candidiasis (Group 1).

		Antibodies anti–											
		Als3-N		Hwp1-N		Met6		BDG		CAGTA- 80[a]		CAGTA-160[a]	
Yeast Species	N	N	%	N	%	N	%	N	%	N	%	N	%
Candida albicans	57	46	81	39	68	48	84	44	77	36	63	35	61
Nakaseomyces glabrata[b]	26	21	81	17	65	23	88	14	54	20	77	14	54
Candida parapsilosis	17	15	88	12	71	17	100	10	59	11	65	6	35
Candida tropicalis	8	6	75	4	50	6	75	4	50	5	62	3	37
Pichia kudriavzevii[c]	4	3	75	2	50	4	100	2	50	1	25	0	0
Meyerozyma guilliermondii[d]	3	2	67	1	33	2	67	1	33	0	0	0	0
Clavispora lusitaniae[e]	2	2	100	2	100	2	100	2	100	1	50	1	50
Kluyveromyces marxianus	1	1	100	0	0	1	100	0	0	1	100	1	100
Candida spp.	16	13	81	11	69	15	94	10	62	5	31	4	25
Total	134												

[a] CAGTA-80 and CAGTA-160 refer to the cut-off applied for calculations, 1/80 and 1/160 respectively.
Yeast species formerly referred to as:
[b] Candida galbrata, [c]Candida krusei, [d]Candida guilliermondii, [e]Candida lusitaniae and [f]Candida kefyr.

81%, and 88% respectively). Although *Candida tropicalis*, and *Pichia kudriavzevii* and *Meyerozyma guilliermondii* (formerly referred to as *Candida krusei* and *Candida guilliermondii*, respectively) were less represented in this study, these 2 markers also provided the best sensitivity values for these 3 species.

3.5. Combination of test results

To improve the diagnostic indicators of IC obtained using the assays individually, we tested all possible combinations of the 5 assays. The best results were obtained for combinations of 2 biomarkers (Table 3); combinations of 3 or more biomarkers improved sensitivity but resulted in a dramatic decrease of specificity and accuracy of the diagnostic strategy (data not shown).

For immunocompetent patients, the combination of anti–Als3-N antibodies with anti–Met6 antibodies or BDG reached the highest sensitivity values (95% and 93%, respectively), followed by anti–Met6 antibodies + BDG (92%), with NPV ranging 83% to 85%. However, the best accuracy numbers corresponded to BDG + CAGTA (79 and 77%) followed by anti–Als3 antibodies + CAGTA (76%).

In contrast, in immunocompromised patients, the combination of results of anti–Met6 antibodies and CAGTA tests (92% sensitivity and 74% specificity), independently of the cut-off value applied for CAGTA rendered the best accuracy indexes (78%). In addition to 74% specificity, this combination reached a 97% NPV.

4. Discussion

Fungal culture and tissue histopathology from usually sterile sites remain the gold standard tests for IC diagnosis (Donnelly *et al.*, 2020, Pappas *et al.*, 2016, Pitarch *et al.*, 2018), but culture exhibits low sensitivity (approx. 50%) and it can take 24-48 hours to return a positive result. A delay of 12 to 24 hours in the introduction of antifungal treatment may double the attributed mortality (Garey *et al.*, 2006) so it is crucial to develop new diagnostic tests for the early detection of this fungal infection.

Candida mannan and anti–mannan detection techniques have been commercialized to support a diagnosis of IC. Reports for Platelia™ *Candida* Ag Plus (Bio-Rad) indicate sensitivity values of 85% and specificity of 95% (Sendid *et al.*, 2004), but one of its shortcomings is that it does not discriminate between colonization and invasion.

BDG is an interesting IFD biomarker detecting infections caused by *Candida, Aspergillus* or *Pneumocystis*. In this study, the sensitivity of BDG to detect an IFD (70%) was slightly superior to IC (62% to 68%), as expected for a panfungal marker, but specificity and PPV dropped dramatically in immunocompromised patients with IC. In these

Table 3
Diagnostic utility of combining tests for CAGTA, antibodies against Als3-N, Hwp1-N, and Met6, as well as BDG determination in immunocompetent and immunocompromised patients.

Immunocompetent patients						Immunocompromised patients				
Sens[a] (%)	Spec (%)[a]	PPV (%)[a]	NPV (%)[a]	A (%)[a]	Biomarkers	Sens (%)[a]	Spec (%)[a]	PPV(%)[a]	NPV (%)[a]	A (%)[a]
87	52	70	77	72	Als3-N + Hwp1	92	45	31	95	55
95	35	65	85	68	Als3-N + Met6	92	57	37	96	65
93	50	75	85	74	Als3-N + BDG	87	51	32	94	58
89	60	73	82	76	Als3-N + CAGTA-80[b]	83	73	45	94	75
87	62	74	80	76	Als3-N +CAGTA-160[b]	79	74	45	93	75
87	38	64	70	65	Hwp1-N + Met6	96	38	29	97	50
87	52	70	77	72	Hwp1-N + BDG	92	33	27	94	45
77	65	73	70	72	Hwp1-N +CAGTA-80[b]	92	48	32	96	58
75	70	76	69	72	Hwp1-N +CAGTA-160[b]	92	49	33	96	58
92	48	69	83	72	Met6 + BDG	96	55	37	98	64
86	51	69	75	71	Met6 + CAGTA-80[b]	92	74	49	97	78
83	54	69	72	70	Met6+CAGTA-160[b]	92	74	49	97	78
84	72	79	77	79	BDG + CAGTA-80[b]	75	65	37	91	67
78	74	79	73	77	BDG+CAGTA-160[b]	67	65	34	88	65

[a] Sens = sensitivity; Spec = specificity; PPV = positive predictive value; NPV = negative predictive value; A = accuracy.
[b] In this table "Als3-N" stands for anti–Als3-N antibodies, "Hwp1-N" for anti–Hwp1-N antibodies, and "Met6" for anti–Met6 antibodies; CAGTA-80 and CAGTA-160 refer to the cut-off applied for calculations, 1/80 and 1/160 respectively.

patients, our results showed a NPV of 87% for IC, in accordance with the current ESCMID guidelines recommending serial determination of BDG in adults at risk, especially to rule out this infection (Cuenca-Estrella *et al.*, 2012, Cornelius and Nguyen, 2014). The relatively low specificity of BDG for the diagnosis of IC could be compensated to some extent by combining results with other tests more specific.

CAGTA detection allows discriminating colonization from invasion (García-Ruiz *et al.*, 1997). Some authors question the usefulness of antibody detection in immunocompromised patients (Quindós *et al.*, 2004, Ellepola and Morrison, 2005), and we found that they have lower CAGTA titers than immunocompetent patients. Nevertheless, in both cohorts of patients, CAGTA titers of the proven IC groups were significantly higher than their respective control groups, including patients with other fungal infections. Importantly, CAGTA specificity was high (89%–100%) but, even raising the cut-off to 1/80, as recommended by Martínez-Jiménez *et al.* (2015), the sensitivity did not exceed 61% in immunocompetent patients, and these values agree with the recently reported data of Pini *et al.* (2019). Previous studies of our group claimed higher sensitivity values (García-Ruiz *et al.*, 1997, Moragues *et al.*, 2004), and Trovato *et al.* (2020) reported 91.3% sensitivity in long-stay ICU patients, emphasizing the value of serial monitoring to assess the kinetics of CAGTA for the clinical diagnosis of IC. Moreover, the occurrence of CAGTA has been associated with a lower mortality rate of ICU patients receiving antifungal treatment (Zaragoza *et al.*, 2009).

CAGTA determination offers an interesting support for clinical practice, but this technique is difficult to automate and requires trained personnel for microscopy register. Moreover, in IC patients, authors have identified specific antibodies against certain *C. albicans* proteins, which could be useful as serum biomarkers of infection (Laín *et al.*, 2008); some of these antigens are Met6, Hsp90, Pgk1, Eno1 (Pitarch *et al.*, 2006, 2007, 2011, 2014, 2016), Als3, or Hwp1 (Laín *et al.*, 2008, Naglik *et al.*, 2006, Ardizzoni *et al.*, 2014). Thus, we developed enzyme-linked immunosorbent assays, a commonly used technique in the clinical laboratory, to determine specific antibodies against recombinant *C. albicans* Als3-N, Hwp1-N, and Met6 proteins, and verified their usefulness for the diagnosis of IC.

Similar to CAGTA, mean antibody titers against recombinant proteins Als3-N, Hwp1-N, and Met6 were higher among immunocompetent patients than in immunocompromised patients, but in both cohorts, IC patients exhibited higher values than their respective control groups. ROC curves showed that anti–Als3-N antibodies determination had the highest discriminatory ability for the diagnosis of IC among immunocompetent patients, followed by anti–Hwp1-N. Conversely, in immunocompromised patients, the detection of antibodies against Met6 recorded the highest AUC value of the 3 proteins tested, followed by anti–Als3, and in both cases with higher discriminatory ability than for immunocompetent patients.

Detection of anti–Als3 antibodies in immunocompetent patients exhibited a sensitivity of 83%, much higher than reported 41.7% (Laín *et al.*, 2008) or 68.6% (Ardizzoni *et al.*, 2014). In immunocompromised patients, sensitivity was somewhat lower (75%) but with a high NPV (92%).

Regarding anti–Hwp1-N antibodies, in contrast to the high values of sensitivity 88.9%, and NPV 90.2% published by Lain *et al.* (2007), in our study the sensitivity reached only 75% in immunocompromised patients. On the contrary, Ardizzoni *et al.* (2014) were unable to assess any diagnostic capability for these antibodies. Although Hwp1 is a protein associated with *C. albicans* germ tube formation, other *Candida* species incapable of forming mycelia may contain proteins with regions homologous to Hwp1, and therefore induce cross-reacting antibodies in patients infected by other species different from *C. albicans* (Laín *et al.*, 2007).

In addition to *Candida*, other fungi express Met6 variants that can induce cross-reacting antibodies that would reduce the specificity of the assay. In this regard, in our study, immunocompetent patients with IC displayed higher anti–Met6 antibody titers than control patients did, although the difference from the other IFD group was not statistically significant. As a result, sensitivity was moderate (77%) with a rather low specificity (49%). Conversely, the detection of anti–Met6 antibodies in immunocompromised patients showed the best rates of the 3 tests developed (S 92%, E 74%, NPV 97%, A 78%). While sensitivity was superior to figures reported previously (60-65%), the specificity rate did not reach published values (82%–83%) (Pitarch *et al.*, 2007, Clancy *et al.*, 2008); the high specificity reported by these authors might be due to the absence of patients with other fungal diseases in their control groups.

From a clinical perspective, anti–Met6 antibodies detection in immunocompromised patients at risk of IC appears as a promising test since high sensitivity may guide the diagnosis of IC, and the negative predictive value of the test could serve as a support to rule out possible candidemia in this cohort. Consequently, anti–Met6 antibodies results can guide early antifungal treatment or de-escalation in the absence of specific antibodies. Moreover, (Pitarch, Nombela and Gil, 2011) found that high IgG antibody levels against *Candida* Met6, Hsp90, and Pgk1 was associated with good-prognosis and protective patterns in patients with IC, so anti–Met6 antibodies detection could be an interesting biomarker to predict patient clinical-outcome for individualized therapy of IC.

In addition to *C. albicans*, infections with *Nakaseomyces glabrata* or *C. parapsilosis* triggered measurable antibody responses with high frequency, especially against Als3-N (81% and 88% respectively) and Met6 (88 and 100%). Similarly, although less represented in patients of this study, *C. tropicalis, Pichia kudriavzevi* or *Meyerozyma guilliermondii* also elicited antibody responses to these 2 proteins. Moragues *et al.* (2004) also reported the presence of CAGTA in patients infected with non–*C. albicans* species. This phenomenon of cross-reactivity may be due to short sequence homologies of various proteins of different species of the genus *Candida* and related ones, as it has been described for *C. albicans* Hwp1 and other proteins of *C. parapsilosis, C. tropicalis, N. glabrata* or *Candida dubliniensis* (Laín *et al.*, 2007).

The detection of specific antibodies by EIA exceeded the sensitivity values of CAGTA in all cases, but at the cost of a significant loss in specificity; nevertheless, they maintained high NPV levels, especially in immunocompromised patients. In an attempt to exploit the advantages of the different assays, we analyzed the combinations of different test results, and found that combining 3 or more results increased the sensitivity for IC diagnosis; however, the specificity dropped dramatically, and therefore we focused on pairwise combinations. For immunocompetent patients, the best combinations of sensitivity (89-95%) and NPV (83%–85%) included anti–Als3 antibodies with CAGTA-80, anti–Met6 antibodies or BDG, as well as anti–Met6 antibodies with BDG, although the combination of BDG with CAGTA-80 showed the highest accuracy rate (79%). The combination of BDG with CAGTA has already been proposed to improve the specificity of BDG in IC cases (Pini *et al.*, 2019), and Martinez-Jiménez (2015) recommends raising the CAGTA cut-off point to 1/80. Conversely, in immunocompromised patients the best sensitivity (92%–96%) and NPV (96%–98%) results were obtained with combinations of anti–Met6 antibodies with anti–Als3 antibodies, BDG or anti–Hwp1 antibodies. Although the combination of anti–Met6 antibodies with CAGTA showed the highest accuracy rate (78%), the results did not improve the performance of anti–Met6 antibodies detection on its own.

5. Conclusions

In view of our results, we can conclude that EIA for detection of antibodies against *C. albicans* Als3-N, Hwp1-N, and Met6 proteins are more sensitive than CAGTA determination, but specificity is generally lower. However, in the case of immunocompromised patients, the specificity for anti–Als3-N and anti–Met6 antibodies remains 74%

while NPV goes up to 92% and 97% respectively. The combined results of BDG and CAGTA-80 offer the highest diagnostic efficacy in immunocompetent patients; however, for immunocompromised patients, anti−Met6 antibodies is the best biomarker for IC, both, alone or in combination with BDG. Laboratory surveillance of at-risk patients could result in an earlier initiation of antifungal therapy provided sensitive and specific diagnostic tests, and also cost effective, become available. De-escalation of established antifungal therapy may also rely on adequate surveillance of infection with biomarkers. Finally, evidence of immunologic response of patients against specific *Candida* antigens can help uncovering potential targets for vaccine design and immunotherapy against IC.

Authors' contributions

Ander Díez: Investigation, Formal analysis, Visualization, Writing - Original Draft. Giulia Carrano: Investigation. Marta Bregón-Villahoz: Investigation. María-Soledad Cuétara: Resources (selection of patients and samples). Juan Carlos García-Ruiz: Resources (selection of patients and samples). Iñigo Fernandez-de-Larrinoa: Supervision, Writing - Review & Editing. María-Dolores Moragues: Conceptualization, Methodology, Visualization, Project administration, Funding acquisition, Writing - Review & Editing.

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Declarations of competing interest

We declare that we have no competing interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2021.115509.

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