

Prospective clinical evaluation of lower cut-offs for galactomannan detection in adult neutropenic cancer patients and haematological stem cell transplant recipients

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Received 9 April 2004; accepted after
publication 28 June 2004

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The incidence of angio-invasive aspergillosis (IA) has increased over the past decade, both in transplant recipients and in patients with haematological disorders (Verweij & Denning, 1997; Wald *et al*, 1997; Chandrasekar *et al*, 2000; Singh, 2001; Marr *et al*, 2002a). *Aspergillus* has now become the leading cause of infectious mortality in many haematology units and haematopoietic stem cell transplant (HSCT) centres in the US and Europe (Groll *et al*, 1996; Marr *et al*, 2002b; Martino *et al*, 2002). In addition, the introduction of new modalities for HSCT, such as the use of non-myeloablative conditioning regimes and the use of novel immunosuppressive agents, will probably further contribute to the increasing incidence of fungal infections worldwide (Fukuda *et al*, 2003; Hagen *et al*, 2003). Unfortunately, the overall mortality rate of IA remains high (Ribaud *et al*, 1999; Patterson *et al*, 2000; Lin *et al*, 2001). Although the recent availability of voriconazole as a new agent for the primary treatment of IA represents an important therapeutic advance, approximately 50% of all patients will still

Summary

The recent advent of an improved commercial serum enzyme-linked immunosorbent assay (ELISA) for the detection of circulating galactomannan (GM), a major constituent of *Aspergillus* cell walls, has contributed to the diagnosis of invasive aspergillosis (IA) in many haematology and transplant centres. However, the optimal threshold for positivity remains a matter of debate. We prospectively evaluated the impact of lowering the cut-off in 124 neutropenic episodes with a high pretest probability for IA. Two new cut-off points, lower than previously accepted, are proposed: (a) a 'static' cut-off at 0.8 and (b) a 'dynamic' cut-off at 0.5. A single assay with an optical density (OD) index ≥ 0.8 warrants the initiation of anti-*Aspergillus* therapy. A further lowering of the 'static' threshold seems not clinically feasible given the drop in positive predictive value (PPV). However, the demonstration of at least two sequential sera with an OD ≥ 0.5 ('dynamic' threshold) increased the specificity and the PPV to 98.6% and the efficiency to 98%. Applying both cut-offs to a subgroup of 21 'possible' fungal infections further identified and upgraded six cases of IA. However, the clinical benefit of lower cut-offs (particularly for earlier diagnosis) depends upon the kinetics of antigenaemia and the intensity of serum sampling.

Keywords: invasive aspergillosis, serology, galactomannan, cut-off value, haematological malignancy, early diagnosis.

be unresponsive to this intervention (Herbrecht *et al*, 2002a). In addition, for patients with profound persistent neutropenia or for those with uncontrolled graft-versus-host disease, IA often progresses irrespective of the type of antifungal therapy (Denning, 1996; Herbrecht *et al*, 2002b).

Early treatment may improve the outcome, especially in neutropenic patients (Aisner *et al*, 1977; von Eiff *et al*, 1995). However, early diagnosis of IA remains problematic, largely because of the lack of specific clinicoradiological signs and symptoms [with the possible exception of the 'halo-sign' on high-resolution computed tomography (CT) scanning (Caillot *et al*, 1997)] and the low sensitivity of conventional diagnostic tests, such as culture and microscopy of lower respiratory tract specimens (Denning, 2000). In addition, histopathological examination of infected tissue – the diagnostic gold standard – is, in many instances, not feasible given the often-critical condition of these patients and the underlying coagulation abnormalities. The advent of an improved commercial serum

enzyme-linked immunosorbent assay (ELISA) for the detection of circulating galactomannan (GM), the major constituent of *Aspergillus* cell walls, has facilitated the diagnosis of IA in many European centres (Stynen *et al*, 1992, 1995). Previous studies have shown that serial screening for GM in the high-risk population can help to establish an earlier diagnosis of the disease, even before the onset of clinical symptoms or radiological signs (Verweij *et al*, 1995; Sulahian *et al*, 1996; Maertens *et al*, 1999, 2001, 2002; Sulahian *et al*, 2001).

In recent years, it has become clear that the performance of the assay can be affected by many variables, including the selection of the cut-off to define positivity, as well as the number of positive samples required to define a true positive result. In Europe, many studies have employed the index value of 1.5 (as recommended by the manufacturer) or 1.0, although lower cut-offs have been suggested (Herbrecht *et al*, 2002b). Recently, this assay (Platelia[®] *Aspergillus*; Bio-Rad Laboratories, Marnes-La-Coquette, France) was cleared by the Food and Drug Administration (FDA) for diagnostic use in the US, accepting an index value of 0.5 or higher as positive (Wheat, 2003). While lowering the cut-off will result in an earlier diagnosis, it also increases the risk of misclassification as a result of false-positive assays. The aim of this study was to further evaluate the feasibility of using lower cut-offs in neutropenic patients with a high pretest probability for IA.

Patients and methods

Study population and design

From July 2001 to December 2002, we prospectively measured serum GM levels in a consecutive series of adult (>16 years of age) haemato-oncological patients who were considered to be at risk for developing IA. Eligible patients were receiving intensive chemotherapy for acute myeloid leukaemia or myelodysplastic syndrome with an expected neutropenia ($<0.5 \times 10^9/l$) for at least 14 d or were undergoing myeloablative allogeneic HSCT. Patients with acute lymphoblastic leukaemia receiving high dose steroids as part of their remission-induction chemotherapy were also eligible.

All patients were hospitalized in single reverse-isolation rooms in a unit equipped with high-efficiency particulate air filters until neutrophil recovery ($>0.5 \times 10^9/l$). All patients received antifungal prophylaxis with itraconazole capsules 400 mg/d (serum levels were not measured) or fluconazole 400 mg p.o./i.v. in case of intolerance or inability to take oral itraconazole.

Serum samples were taken at least twice weekly, starting at the beginning of cytoreductive therapy. Sampling was stopped at the end of neutropenia or, in case of fungal infection, until the end of hospitalization or death. Sera were stored at -20°C and analysed twice weekly by the same technicians who were unaware of the clinical status of the patient. Clinicians were not blinded from the laboratory results but indices <1.0 were reported as 'negative'. Indices ≥ 1.0 were reported numerically.

During hospitalization, patients were surveyed for the development of fever and sinopulmonary signs and symptoms. Physical examinations were carried out daily. Conventional chest X-rays were performed at admission and once to twice weekly during hospitalization (or more frequently during periods of fever). Blood and sputum cultures were performed as clinically indicated. We performed weekly surveillance cultures for bacterial and fungal growth from stool, urine samples and oral washes. In case of clinical suspicion of invasive fungal disease [neutropenic fever not responding to at least 5 d of broad-spectrum antibacterial therapy, development of a new infiltrate on chest X-ray, CT scan abnormality compatible with invasive fungal disease, positive culture of mould or microscopy from a respiratory tract sample], a diagnostic work-up was initiated, including high-resolution pulmonary CT scan followed by bronchoalveolar lavage, if feasible. Lavage samples were submitted for bacterial, fungal, mycobacterial and *Legionella* cultures, direct immunofluorescent staining or polymerase chain reaction (PCR) for *Pneumocystis jiroveci*, acid-fast staining and viral cultures. No endobronchial biopsies were taken in cytopenic patients. Other diagnostic procedures were performed on clinical indication. Empirical antibacterial therapy consisted of cefepime or meropenem.

Antigen detection

The sandwich-ELISA assay (Stynen *et al*, 1995) was modified to a semi-automated protocol. Briefly, 300 μl of test serum was mixed with 100 μl of 4% EDTA treatment solution and heated at 100°C for 3 min to dissociate immune complexes and to precipitate serum proteins that could interfere with the test. After centrifugation at $10\,000 \times g$ for 10 min, 50 μl of the supernatant was pipetted into a microtitration plate. The wells of the microtitration plate were coated with the monoclonal anti-GM antibody EB-A2. The rest of the procedure was not performed manually as described by the manufacturer but automatically with a (BEP III, Dade Behring, Marburg, Germany). The BEP III is a semi-automatic analyser used to process ELISA tests in microtitre plates. It was programmed to perform the processing of the sample and the OD measurement as described by the manufacturer. Briefly, 50 μl of a reaction mixture containing peroxidase-labelled anti-GM monoclonal antibody EB-A2 was added to the wells. After a 90-min incubation, the plates were washed extensively before adding 100 μl of a substrate-chromogen solution containing tetramethylbenzidine. Then the plates were incubated for another 30 min in darkness at room temperature, followed by the addition of 100 μl 1.5 N sulphuric acid to stop the reaction. The OD was read at 450/620 nm. All reagents were purchased from Bio-Rad. Positive and negative controls were included in each run, as well as cut-off controls for conversion of the measured absorbances into indices. The OD index was calculated by dividing the OD of the clinical sample by the OD of the control sample obtained in the same run. As recommended by other studies, an index of ≥ 1.0 was

considered positive while an index <1.0 was reported as negative. A result was considered as a true positive when at least two consecutive samples for that patient were positive.

Case definition and classification

Treatment episodes were classified as proven IA, probable IA, or possible invasive fungal infection, based on the 2002 standardized Invasive Fungal Infections Group of the European Organization for the Research and Treatment of Cancer/Mycoses Study Group (EORTC-IFIG/MSG) case definitions (Ascioglu *et al*, 2002), with the necessary modification that GM levels were not included in the microbiological criteria. Episodes without evidence of IA were assumed to be true negative episodes and formed the control group.

Statistical analysis

As a patient could be included in the study on more than one occasion (patients diagnosed with IA were no longer eligible for subsequent inclusion until complete remission of the infection), analysis was performed according to per treatment episode. Samples from episodes with proven or probable IA were utilized to estimate sensitivity; sera from the control group were used for specificity. Positive predictive value (PPV), negative predictive value and efficiency (true positive + true negative/total number of tests) were calculated from the combination of both groups. Although the true status of the disease remained inconclusive for episodes of possible fungal infection, subsequent analyses were performed considering these possible episodes as either proven/probable or control episodes.

Results

Treatment episodes

Overall, 124 treatment episodes from 104 patients were analysed. The median age of the patients was 49 years (range 16–79 years). Demographic characteristics, number of antigen assays per episode and underlying disorders summarized according to the probability of IA, are shown in Table I.

Antigen testing

A total of 1642 sera were collected (mean 13.2 samples/episode). Eight hundred and sixty-six consecutive sera were analysed from 74 episodes (mean 11.7 sera/episode) without clinical or radiological signs of IA. The distribution of serum OD index values is depicted in Fig 1. All sera were negative when using a cut-off index of 1.5 or 1.0 (specificity of 100%). When lowering the positivity threshold to 0.5, 848 sera (97.9%) were negative; 18 sera (2.1%) were positive from 11 different treatment episodes, including six allogeneic HSCT procedures. However, the positivity was confirmed by

consecutive positive samples (0.5, 0.6, 0.7, respectively) in only one episode. This patient had previously been diagnosed with proven *Aspergillus* tracheobronchitis during consolidation chemotherapy for acute leukaemia; she subsequently underwent a sibling allogeneic HSCT without clinicoradiological evidence of relapse of fungal disease. The remaining episodes with positive samples (≥ 0.5) presented with either fluctuant antigenaemia or with an isolated positive value. Using the ≥ 0.5 cut-off, the specificity on a per episode basis was 85.1% for a single positive sample (63/74 episodes) and 98.6% for two consecutive positive samples (73/74 episodes).

A total of 507 consecutive sera were investigated from 29 episodes with proven ($n = 16$) or probable ($n = 13$) IA. Circulating antigen was detected in 24 (82.7%) and 27 (93.1%) episodes when using a single positive sample with a cut-off index of 1.5 and 1.0, respectively. Requiring at least two consecutive positive sera reduced the sensitivity to 62% (18/29) and 79.3% (23/29) when using a cut-off of 1.5 and 1.0, respectively. Using the cut-off point of 0.5, the sensitivity of the assay was 96.5% (28/29) both for a single positive sample and for two consecutive sera. The only patient with constant negative assays died of rapidly progressive veno-occlusive disease in the early post-transplant period; however, at autopsy, he was found to have a necrotic pulmonary lesion with fungal hyphae compatible with *Aspergillus* (yet the culture was negative). Typically, a progressive and constant increase in antigenaemia or decrease following successful therapy was found without abrupt fluctuations (Fig 2).

The sensitivity, specificity, PPV and negative predictive value and efficacy of the assay was re-evaluated at different cut-off points, ranging from 0.3 to 1.5 (Table II). Despite a superior sensitivity and slightly increased negative predictive value (NPV) at the 0.5 or 0.6 cut-off when compared with the higher indices of 1.5 (e.g. +13.7% increased sensitivity) and 1.0, the specificity and PPV of the lower cut-offs was markedly reduced, resulting in a poorer clinical efficiency. Considering a single serum with an OD index equal to or greater than a given cut-off as truly positive (static cut-off point), it seems clinically feasible to lower the cut-off index to 0.8 in an attempt to make an earlier diagnosis. Lower thresholds would result in a too high number of false-positive samples.

However, the presence of two or more consecutive sera with an index ≥ 0.5 (dynamic cut-off point) increased the specificity and the PPV of the assay to 98.6% and the clinical efficiency to 98%. Differences in the performance of the assay related to the underlying disease or treatment (allogeneic transplant *versus* no transplant; acute lymphoblastic leukaemia *versus* myeloid malignancy) were not observed (data not shown).

We also identified 21 episodes of possible fungal infection. Two hundred and sixty-nine consecutive sera were analysed in this subgroup (mean 12.8/episode). Sera 1 and 2, respectively, were positive when using a cut-off of 1.5 or 1.0; however, positivity was not confirmed by a subsequent sample. On a per episode basis (and considering these episodes as true episodes of IA together with the proven and probable cases), this

Table I. Characteristics of treatment episodes and serum samples.

	Proven IA (n = 16)	Probable IA (n = 13)	Possible IFI (n = 21)	No IA (n = 74)	Total (n = 124)
No. of patients*	16	13	20	59	104
Age (years)					
Mean	51	57.5	46.7	41.9	46.7
Median	54	58	49	42	49
Range	16–79	32–79	20–68	17–70	16–79
Sex (male/female)	10/6	8/5	16/5	42/32	76/48
Underlying disorder (n)	16	13	21	74	124
AML	5	6	10	21	42
ALL	3	1	5	6	15
MDS	0	2	2	8	12
Relapsed AL	1	3	2	10	16
Allo-HSCT	7	1	2	29	39
Duration neutropenia					
Mean (d)	14.5	19	22.2	18.1	19.1
Median (d)	18	20	23	18	18
Range†	0–64	0–50	0–50	6–45	0–64
Samples per episode (n)					
Mean‡	16	16.4	12.2	11.7	13.2
Median‡	16	15.5	11	9	10
Range	7–38	4–32	6–28	5–41	4–41

IA, invasive aspergillosis; IFI, invasive fungal infections; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; MDS, myelodysplastic syndrome; AL, acute leukaemia; allo-HSCT, allogeneic haematopoietic stem cell transplantation.

*Patients could be screened during multiple consecutive treatment episodes; therefore the total of the four individual columns adds up to 108 instead of 104.

†Includes three patients with ALL (one proven, one probable and one possible case, respectively) who did not develop neutropenia ($<0.5 \times 10^9/l$) during remission–induction therapy with vincristine, daunorubicin, asparaginase and high-dose corticosteroids (prednisone $60 \text{ mg/m}^2 \times 28 \text{ d}$).

‡The larger number of ELISAs performed in proven and probable cases is because of the prolonged hospitalization of patients for treatment of IA.

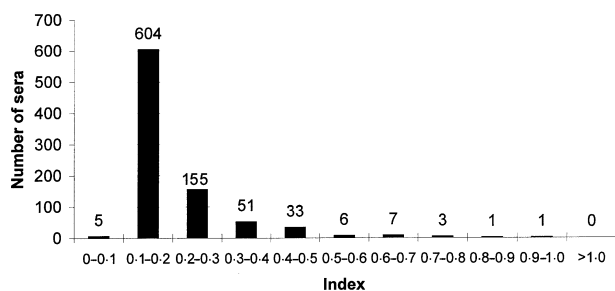


Fig 1. Distribution of 866 serum index values from 74 treatment episodes without invasive aspergillosis. Eighteen samples were positive when using the ≥ 0.5 cut-off.

reduced the sensitivity to 50% and 58% for a single positive sample with a cut-off of 1.5 or 1.0, respectively, and to 36% and 46% for two consecutive samples. The specificity of 100% remained unchanged when considering these 21 episodes as true negative episodes (together with negative control group) and requiring at least two consecutive positive samples ≥ 1.5 or ≥ 1.0 , respectively. When applying the above mentioned static and dynamic cut-offs for positivity to these 21 episodes, 15 episodes were constantly negative for both criteria; six episodes fulfilled the criterion of having at least two consecutive samples

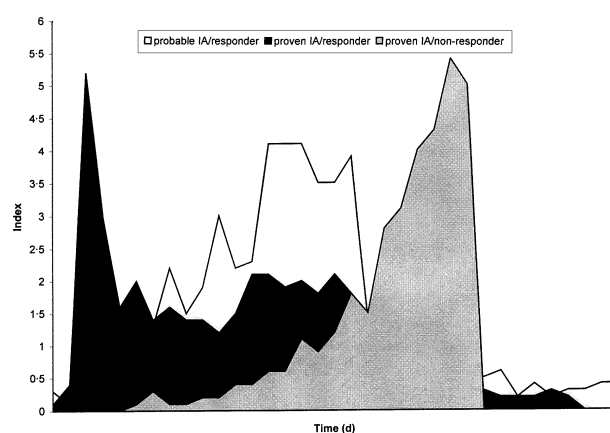


Fig 2. Time course of antigenaemia in three selected patients. Two patients, both survivors, cleared GM while receiving antifungal therapy. The third patient is representative of a larger group of 16 patients with rising antigen titres despite adequate therapy; they all died of or with IA.

with an OD index of ≥ 0.5 (28.5%). Two of these latter episodes were also positive when using the static cut-off of ≥ 0.8 . The implementation of these thresholds enabled us to further upgrade these six possible episodes to probable invasive aspergillosis. A high resolution CT scan demonstrated the

Table II. Impact of modified cut-off values on ELISA performance in adult patients*.

OD index cut-off:	Static									Dynamic 2 × ≥0.5
	1.5	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	
Sensitivity (%)	82.7	93.1	96.5	96.5	96.5	96.5	96.5	96.5	100	96.5
Specificity (%)	100	100	98.6	97.3	93.2	86.4	85.1	71.6	60.8	98.6
PPV (%)	100	100	96.5	93.3	84.8	73.7	71.8	57.1	50	98.6
NPV (%)	93.7	97.4	98.6	98.6	98.6	98.5	98.4	98.1	100	98.4
Efficacy (%)	95.1	98	98	97	94.2	89.3	88.3	78.6	71.8	98

*Excluding possible cases of fungal infection.

PPV, positive predictive value; NPV, negative predictive value.

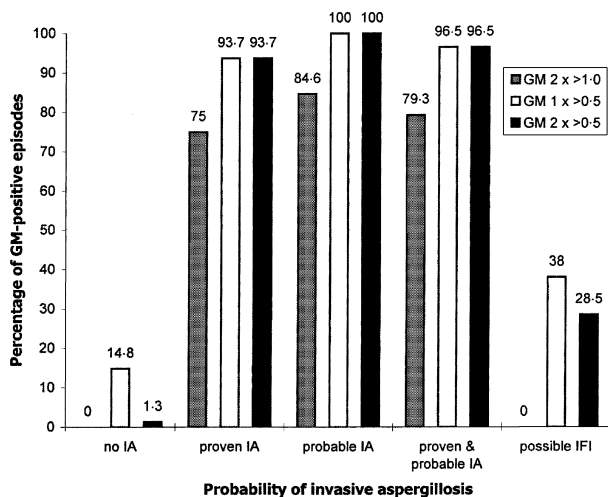


Fig 3. Galactomannan-positive episodes in patient groups, defined according to the EORTC/MSG probability of invasive aspergillosis. Three different cut-offs for positivity are shown: two consecutive sera with OD ≥ 1.0, one serum sample with OD ≥ 0.5, and two consecutive sera with OD ≥ 0.5.

presence of a halo sign and the formation of an air crescent sign in two of these cases as well as non-specific pulmonary abnormalities in the remainder. All six patients responded favourably to empiric antifungal therapy. In addition, proven IA was documented during a subsequent course of chemotherapy in two of these patients. Figure 3 shows the distribution of patient episodes according to EORTC/MSG criteria and the GM results in the different groups using different thresholds for positivity.

Chronological relationships between the first positive immunoassays and clinical diagnosis and start of antifungal treatment are summarized in Table III and Fig 4. Using a conventional cut-off of 1.5, only 12 (41.3%) episodes were identified to have positive ELISA results before or at the start of antifungal therapy (median 2 d; range 0–24 d). In contrast, with the new reduced cut-offs (dynamic or static), the first positive immunoassay preceded or coincided with the initiation of antifungal therapy in 21 (72.4%) and 20 (68.9%) episodes, respectively (median 5 d; range 0–24 d). Interestingly, in the

latter group, patients who were seronegative at the start of antifungal therapy but who became positive thereafter were initially treated empirically; the OD index at the start of empirical therapy was low and ranged from 0.3 to 0.9. Subsequent seropositivity was a sign of breakthrough infection or of refractoriness to therapy. As shown in Table III, time to positivity was not affected by the underlying patient population or the site of infection.

Discussion

In recent years, there has been some progress in the early diagnosis of IA in haemato-oncological patients, although largely because of the use of CT scanning (Caillot *et al*, 1997; Denning, 2000). More recently, GM, an exo-antigen released from *Aspergillus* hyphae while invading host tissue, can be detected in serum and other body fluids by an ELISA-based immunocapture assay (Stynen *et al*, 1995). In contrast to PCR techniques for the detection of fungal DNA, the detection of soluble *Aspergillus* antigen has been standardized because of the availability of a commercial kit. Over the past 5 years, several studies in Europe have shown that serial screening for GM in the high-risk neutropenic population can help to establish a diagnosis of IA (Verweij *et al*, 1995; Sulahian *et al*, 1996; Maertens *et al*, 1999, 2001; Sulahian *et al*, 2001), sometimes before the onset of clinical symptoms or radiological signs (Maertens *et al*, 2002). Although the sensitivity of the assay in prospective evaluations varied between approximately 50% and 90%, this wide range can be partly explained by critical differences in the study population, in the case definitions, in the frequency of sampling, and in the requirement for demonstration of persistent positivity for classification as a true positive (Wheat, 2003).

Using different cut-off levels for defining a positive sample may also contribute to variations in the performance of the assay. The manufacturer recommends a cut-off point of ≥1.5, whereas an index between 1.0 and 1.5 is to be considered indeterminate. In their analysis of reproducibility of the test, Verweij *et al* (1998) suggested that the threshold should be reduced for negative samples, from 1.0 to 0.8, and for positive samples, from 1.5 to 1.0. Using the criterion of two consecutive

Table III. Diagnosis of proven and probable IA: patient characteristics and documentation.

Episode no.	Patient characteristics				Underlying condition	Outcome	Host factors	Clinical evidence	Site of infection	Time elapsed between clinical diagnosis and positive ELISA (d)*			OD index at start of antifungal therapy (and trigger for initiating therapy)
	Age (years)	Sex	IA	Sex						2 × ≥0.5	1 × ≥0.8	1 × ≥1.5	
1	62	F	P	P	ALL	Dead	N, F	Halo sign	P (bil)	-2	-2	0	2.6 (CD + GM)
2	63	F	P	P	AML	Dead	N, F	New infiltrate, pleuritic pain	P (uni)	-6	-6	-1	1.9 (CD + GM)
3	31	M	P	P	Allo	Dead	G, S, A	Halo sign	Dis	Indeterminate†			1.8 (GM)
4	70	F	P	P	AML	Dead	N, F	New infiltrate, <i>A. fumigatus</i> BAL	P (uni)	+3	+6	NA	0.4 (E)
5	68	M	P	P	ALL	Dead	N, S	New infiltrates, hemoptysis	P (bil)	-24	-24	-24	3.1 (CD)
6	64	M	P	P	AML	Dead	N, F	Halo sign, <i>A. fumigatus</i> sputum	P (bil) oesophagus	-6	-5	-2	3.7 (CD + GM)
7	21	F	P	P	Allo	Dead	N, F, G, S, A	Hemiparesis, <i>A. fumigatus</i> (brain biopsy)	Dis	-4	0	NA	1.2 (CD)
8	56	M	P	P	Allo	Dead	N, F	None	P (uni)	NA	NA	NA	NA
9	79	M	P	P	AML	Dead	N, F	Halo sign, <i>A. fumigatus</i> BAL	Dis	-7	-7	-3	2.6 (CD + GM)
10	17	M	P	P	AML	Dead	N, F	New infiltrate, <i>A. ustus</i> BAL	P (bil)	-6	-6	-3	2.8 (CD + GM)
11	45	M	P	P	ReAL	Dead	N, F	Periorbital swelling, Halo sign	P (bil)	-7	-5	0	5.3 (CD + GM)
12	47	M	P	P	ALL	Dead	N, F	Seizure, <i>A. fumigatus</i> (brain biopsy)	brain	+14	+15	+16	0.3 (E)
13	54	F	P	P	Allo	Dead	N, F	Halo sign	Dis	Indeterminate†			NA
14	49	F	P	P	Allo	Dead	N, G, S, A	New infiltrate, <i>A. fumigatus</i> BAL	Dis	-3	-1	+3	0.9 (E)
15	50	M	P	P	Allo	Dead	N, F	Halo sign	P (uni)	+15	+15	+17	0.4 (E)
16	69	M	P	P	Allo	Dead	N, F	New infiltrates, <i>A. fumigatus</i> BAL	P (bil)	-5	-5	0	5.6 (CD)
17	63	M	PP	PP	MDS	Alive	N, F	Halo sign, <i>A. fumigatus</i> sputum	P (uni)	-3	-2	+4	0.9 (E)
18	54	M	PP	PP	ALL	Dead	F, S	New infiltrate, <i>A. fumigatus</i> BAL	P (bil)	-3	+1	+8	0.9 (Cult)
19	68	M	PP	PP	AML	Alive	N, F	Halo sign, <i>A. fumigatus</i> BAL	P (bil)	-7	-7	-4	5.2 (GM)
20	66	M	PP	PP	MDS	Alive	F, S	Halo sign, <i>A. fumigatus</i> BAL	P (uni)	-1	-1	NA	0.9 (CD)
21	65	F	PP	PP	AML	Dead	N, F	New infiltrate, <i>A. fumigatus</i> BAL	P (bil)	+5	+5	+7	0.4 (E)
22	51	M	PP	PP	Allo	Alive	N, F	Halo sign, <i>A. fumigatus</i> BAL	P (bil)	-3	-3	NA	1.1 (CD + cult)
23	62	F	PP	PP	ReAL	Dead	N, F	Halo sign, <i>A. terreus</i> BAL	P (bil)	-7	-6	+1	NA
24	52	F	PP	PP	AML	Alive	N, F	Halo sign, <i>A. fumigatus</i> BAL	P (bil)	-8	-8	0	3.2 (CD + GM)
25	32	M	PP	PP	ReAL	Dead	N, F	Nodular infiltrate, <i>A. fumigatus</i> BAL	P (bil)	0	+1	+4	0.5 (E)

Table III. Contd.

Episode no.	Patient characteristics			Outcome	Host factors	Clinical evidence	Site of infection	Time elapsed between clinical diagnosis and positive ELISA (d)*			OD index at start of antifungal therapy (and trigger for initiating therapy)
	Age (years)	Sex	Underlying condition					2 × ≥0.5	1 × ≥0.8	1 × ≥1.5	
26	79	M	AML	Dead	N, F	Halo sign, <i>A. fumigatus</i> sputum	P (bil)	-11	-9	+5	3.1 (CD + cult)
27	54	F	ReAL	Alive	N, F	Halo sign, Microscopy BAL	P (uni)	0	+2	+4	1.3 (CD)
28	49	M	AML	Alive	N, F	Halo sign, Microscopy BAL	P (bil)	+4	+8	+10	0.3 (E)
29	74	F	AML	Dead	N, F	Halo sign, <i>A. fumigatus</i> BAL	P (bil)	-5	-5	-2	3.5 (CD)

F, female; M, male; P, proven; PP, probable; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; Allo, allogeneic hematopoietic stem cell transplantation; ReAL, relapsed acute leukaemia; MDS, myelodysplastic syndrome; N, neutropenia; F, persistent fever; G, graft-versus-host disease; S, use of corticosteroids; A, anti-thymocyte globulin; BAL, bronchoalveolar lavage; P (uni), unilateral pulmonary involvement; P (bil), bilateral pulmonary involvement; Dis, disseminated fungal disease; CD, clinical diagnosis; GM, galactomannan ELISA; E, empirical initiation of antifungal therapy; cult, positive culture result; NA, not applicable.

*Values are numbers of days elapsed between clinical suspicion of IA and positive ELISA results according to the different cut-offs. Negative numbers indicate the number of days ELISA results were positive before clinical suspicion.

†Indeterminate, clinical diagnosis could not be made because of confounding factors, such as concomitant viral or parasitic pulmonary infections and/or bronchiolitis obliterans with organizing pneumonia.

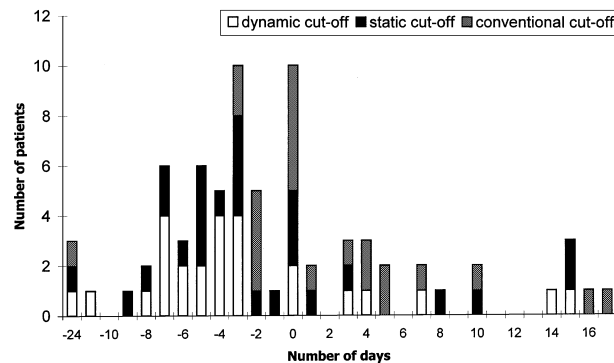


Fig 4. Number of days from a positive ELISA result to the onset of treatment (day 0), using a threshold OD index of 2 × ≥0.5 (dynamic cut-off), 1 × ≥0.8 (static cut-off), and 1 × ≥1.5 (conventional cut-off) in the 26 patients with proven/probable IA who received antifungal therapy. A positive ELISA preceded or coincided with the start of antifungal therapy in 21, 20 and 12 patients, using a dynamic, static or conventional cut-off respectively. Those patients who were not seropositive before the start of antifungal therapy when using the lower thresholds were all receiving empirical broad-spectrum antifungals. Subsequent seropositivity in these latter patients was often a first sign of breakthrough or therapy-refractory aspergillosis.

sera with an OD index ≥1.0, we previously obtained a sensitivity and specificity of 92.6% and 95.4%, respectively (Maertens *et al*, 1999). In addition, others have recommended that the cut-off value should be lowered to 0.7, at least in adult non-allogeneic HSCT patients (Herbrecht *et al*, 2002b).

More recently, the performance of the sandwich ELISA has also been evaluated in cancer patients and bone marrow transplant recipients in the US and found to be both sensitive (81%) and specific (89%). In May 2003, the assay was cleared by the FDA as an aid in the diagnosis of IA in cancer patients, accepting an index cut-off for positivity of ≥0.5 (Wheat, 2003). Clearly, lowering the cut-off might result in an earlier diagnosis – given the fact that the level of antigenaemia corresponds with fungal burden and that patients with proven IA normally display a progressive increase in antigenaemia over time – but may be counterbalanced by a rise in false-positive results. In our study, 11 of 74 episodes (14.9%) from the control patient population had an index value of ≥0.5. The resulting specificity of 85.1% for a single positive result was significantly lower compared with previously reported specificities using higher cut-off values (Verweij *et al*, 1995; Sulahian *et al*, 1996, 2001; Maertens *et al*, 1999, 2001); a similar drop in specificity (from 99.4% to 88.7%) has also been reported when the cut-off was reduced from 1.5 to 0.6 (Herbrecht *et al*, 2002b). In addition, accepting a single positive assay of ≥0.5 as a diagnostic criterion also had a markedly negative impact on PPV and clinical efficiency when compared with a cut-off value of 0.8, 0.9 or 1.0. However, sensitivity remained fairly stable between 0.4 and 0.9.

Nevertheless, this study corroborates the use of index cut-offs in adult haematology patients that are lower than previously accepted: (a) a ‘static’ cut-off at 0.8 and (b) a

'dynamic' cut-off at 0.5. As depicted in Table II, a single assay with an OD index of ≥ 0.8 in the appropriate patient population warrants the initiation of anti-*Aspergillus* therapy, even if the diagnosis is not later confirmed. Besides, the low rate of false positivity does outweigh the high mortality of established disease, particularly because better-tolerated new anti-*Aspergillus* agents have become available. A further lowering of the 'static' threshold does not seem to be clinically feasible because of a drop in the PPV. However, given the gradual rise in antigenaemia in proven and probable cases of IA – as opposed to the stable or fluctuating false-positive results – a 'dynamic' threshold at 0.5 proved indeed to be clinically feasible. The demonstration of at least two sequential sera with OD ≥ 0.5 increased the specificity and PPV of the assay to 98.6% and the clinical efficiency to 98%. So, the performance of the ELISA using either or both criteria is virtually identical and should enable an earlier diagnosis.

We also identified 21 episodes of possible fungal disease. However, a proportion of these possible cases, as defined by the EORTC/MSG criteria, are likely to have non-fungal infections or have non-infectious abnormalities. In practice, many of these patients will receive empirical antifungal therapy. When applying the newly proposed cut-offs to this subgroup, six cases of possible fungal disease were identified and upgraded to probable IA. Some of these patients demonstrated supportive radiological abnormalities, while others developed fatal proven IA during the subsequent course of cytoreductive therapy. All six cases demonstrated a protracted increase in GM level (similar to proven and probable cases), followed by a gradual decrease while receiving empirical antifungal therapy. Although it remains impossible to estimate precisely the proportion of IA cases in this subgroup in the absence of histopathological evaluation, six of 21 cases corresponds with a previously reported 28% incidence in an autopsy-controlled study (Maertens *et al*, 2001).

However, the clinical benefit of lowering the cut-off value is not primarily the capture of an otherwise unrecognized cases (e.g. during empirical therapy) but rather the earlier detection of cases that would not otherwise meet the criteria for anti-*Aspergillus* treatment until some time later. How much later remains precisely the nub of the problem. For instance, in this series, antifungal treatment could have started earlier in approximately 70% of the cases when applying the lower cut-offs. However, the potential beneficial impact of lower cut-off values depends upon the kinetics of GM release and the frequency of sampling. For instance, a lower threshold will have little impact on earlier diagnosis in patients with a rapid increase in antigenaemia and/or with infrequent sampling. Given this complexity, future studies should address the kinetics of GM release as well as the optimal frequency of sampling. Finally, ELISA performance – especially sensitivity – may differ in centres with other clinical practices. As shown also in this study, the early implementation of empirical antifungal therapy seems to suppress or delay the expression of GM antigenaemia in neutropenic patients until a higher fungal

burden has been achieved (Mennink-Kersten *et al*, 2004). In addition, the intravenous administration of piperacillin–tazobactam – an antibiotic combination not used in our series – has been shown to be associated with false-positive ELISA results (Sulhian *et al*, 2003; Adam *et al*, 2004; Viscoli *et al*, 2004). In view of the widespread empirical use of this drug combination in febrile neutropenic patients, this observation is a matter of concern because it may lead to inappropriate invasive investigations and overtreatment with toxic and/or expensive drugs.

In conclusion, lower than previously accepted cut-off levels for GM detection by the Platelia® sandwich-ELISA method can be used in adult neutropenic oncohaematological patients with a highly expected prevalence of invasive aspergillosis. The benefit, however, will probably depend upon the kinetics of antigenaemia, the intensity of sampling, and the further investigation of other confounding factors.

Acknowledgements

The authors are indebted to Nicole Pieters, Katrijn Overloop, and Miet Van Vlerken for performing *Aspergillus* serology testing.

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