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Combined real-time PCR and galactomannan surveillance improves diagnosis of invasive aspergillosis in high risk patients with haematological malignancies

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Summary

Invasive aspergillosis (IA) is a leading complication of intensive treatment for haematological malignancies. Earlier diagnosis should facilitate effective antifungal therapy and prevent progression to invasive disease, which is often lethal. Polymerase chain reaction (PCR) assays, targeting the 28S and ITS ribosomal gene regions respectively, were evaluated for early detection of *Aspergillus* DNA and for diagnostic utility in patients receiving treatment in two busy haematopoietic stem cell transplant centres. Patients undergoing intensive chemotherapy, autologous or allogeneic transplant were eligible for inclusion in the study. EDTA blood and serum samples for circulating *Aspergillus* biomarkers, including galactomannan (GM), were collected twice-weekly on a prospective basis from all study patients who were categorized according to international consensus criteria for defining invasive fungal disease (IFD). Of 278 patients recruited there were 44 probable IA cases and only one proven case. Moderate sensitivity and specificity, poor positive predictive value (50–80%), but good negative predictive value (>80–90%) were common to both PCR assays. Overall biomarker performance could be improved by combining positive results of either PCR assay with GM taken within a 12-d period. The addition of PCR to GM monitoring in high-risk patients with haematological malignancies provides greater diagnostic accuracy in invasive aspergillosis.

Keywords: *Aspergillus fumigatus*, PCR, galactomannan, invasive aspergillosis, diagnosis.

Invasive aspergillosis (IA) has become the leading cause of invasive fungal disease (IFD) following intensive remission-induction chemotherapy, or haematopoietic stem cell transplantation for acute leukaemia and lymphoproliferative disorders (Lortholary *et al*, 2011). *Aspergillus fumigatus* is the species most commonly isolated from cases of IA (Kontoyiannis *et al*, 2010; Pagano *et al*, 2010). A major factor in determining the poor outcome of this disease is delay in detection of *Aspergillus* infection and diagnosis of disease, and consequent sub-optimal therapy (Ostrosky-Zeichner, 2012).

A combined clinical, radiological and laboratory approach to diagnosis of IFD is standard practice for haematologists treating patients with haematological malignancy (HM; Cuenca-Estrella *et al*, 2011). Detection of circulating fungal antigens, galactomannan (GM) or ß-D-glucan, using commercially available kits has become accepted practice although these assays have problems with variable performance (Cuenca-Estrella *et al*, 2011; Ostrosky-Zeichner, 2012). Over the past decade there has been a concerted effort to improve polymerase chain reaction (PCR)-based methodologies for the diagnosis of IA (Hope *et al*, 2005; White *et al*, 2006a; Mengoli *et al*, 2009). However, it has not been incorporated into the revised definitions for IFD of the European Organization for Research and Treatment of Cancer/National Institutes of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG; de Pauw *et al*, 2008), and even though there is increasing standardization, the role of PCR in the management of IA is still not established (Klingspor & Loeffler, 2009; Cuenca-Estrella *et al*, 2011).

The need for diagnostic accuracy is a strong motive for the continued development and standardization of PCR assays for early detection of *Aspergillus* infection before

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disease becomes established. Integrating new non-culture diagnostic assays into patient management depends on having reproducible results from validated assays that have been tested with sufficient power. In this prospective study we evaluated the performance of two real-time PCR assays in two busy stem cell transplant units over a 2-year period. The aim was to establish if PCR used alone or in combination with GM could improve *Aspergillus* biomarker performance compared with the current standard of GM detection when applied to a high-risk patient cohort.

Patients and methods

Ethics statement

Prior approval for the study was obtained from the St James's Hospital (SJH) Research Ethics committee and the Ethical Committee of the University Hospital of Wuerzburg (UKW).

Patient populations

Patients undergoing remission-induction chemotherapy for acute leukaemia, lymphoma, or myeloma, autologous or allogeneic bone marrow or stem cell transplant (allo-SCT), were eligible for inclusion. Over the course of the study 146 patients were recruited from SJH and 132 from UKW.

In the SJH unit all patients in the study were nursed in single rooms, each with a lobby, positive pressure ventilation and air input through HEPA filters. Patients with acute leukaemia and those undergoing allo-SCT received antifungal prophylaxis with three times weekly intravenous Ambisome (1 mg/kg) or caspofungin (70 mg) throughout the neutropenic period, or longer in allo-SCT patients receiving immunosuppression for graft-versus-host disease. In patients with antibiotic unresponsive febrile neutropenia or with clinical or radiological signs suggestive of fungal infection, mould active antifungal therapy, different from the prophylactic agent, was given according to individual physicians' discretion as was the decision regarding referral of the patient for computerized tomography (CT) scan and/or bronchoscopy and lavage.

In the UKW unit, patients undergoing chemotherapy for acute leukaemia were nursed on open wards whereas allo-SCT patients were nursed in dedicated single rooms with positive pressure and HEPA filtration of incoming air. Antifungal prophylaxis for chemotherapy patients was with fluconazole 400 mg daily for at least the duration of neutropenia and in allo-SCT patients for up to day 30 post-transplant. A chest CT scan was considered for patients presenting with fever for more than 3 d or in the event of pulmonary signs or symptoms. Bronchoscopy and lavage was considered in cases of pulmonary infiltrate with unknown aetiology. Mould active intravenous antifungal therapy was started in cases with persistent fever, with/without clinical signs suggestive of IFD.

Diagnosis of invasive fungal disease

The EORTC/MSG definitions were used for categorization of patients with IFD including IA (de Pauw *et al*, 2008). All CT scans were reported by local radiologists as part of clinical management and, in an attempt to classify patients as accurately as possible according to the EORTC/MSG criteria, we recruited an independent expert to review the CT images who was blinded as to the patient treatment group, and other clinical and mycological information.

Sample collection, storage and GM testing

Patient blood samples were collected twice weekly; in UKW the EDTA blood samples were logged and processed prospectively while, in SJH, they were frozen at −80°C and processed in retrospective batches. DNA extracts were stored at −20°C until they were processed by the second PCR assay. Clotted blood samples were routinely processed in each centre for serum GM detection using the Platelia sandwich enzyme-linked immunosorbent assay (ELISA; BioRad, Marnes-La-Coquette, France) according to the manufacturer's instructions. An index of 0·5 or greater was taken as positive.

DNA extraction

DNA was extracted using an adaptation of a previously described protocol (Springer *et al*, 2011). Blood samples which had been stored at −80°C (SJH) were thawed and equilibrated to room temperature. Samples from both centres were subjected to two cycles of red cell lysis, a white cell lysis step (SJH only), bead beating (710–1180 μm acid washed glass beads, Sigma [SJH]; MagnaLyser Green Beads, Roche Diagnostics Ltd, West Sussex, UK [UKW]) and final purification using the High Pure PCR Template Preparation Kit (Roche Diagnostics Ltd). Negative and positive extraction controls consisted of 3 ml healthy volunteer blood in an EDTA collection tube with 50 conidia added for the positive control. The eluate volumes of 65 μl (SJH), and 100 μl (UKW) were processed immediately for ITS qPCR in UKW or stored at −20°C until processed by the other assays in both centres.

PCR assays

All primers and probes used in this study are listed in Table SI.

28S nested qPCR

This *Aspergillus*-specific nested PCR assay was performed according to a previously described protocol (White *et al*, 2006b). Second round amplification was carried out using Taqman Gene Expression Mastermix (Applied Biosystems, Warrington, UK) on an ABI 7000 (SJH) or StepOnePlus (UKW) real time PCR machine (Applied Biosystems).

ITS single round qPCR

The ITS qPCR assay targeting the ITS1/5.8S ribosomal operon was a previously described *Aspergillus*-specific assay (Springer *et al*, 2011). Briefly, a 20 μl reaction mix containing *Aspergillus*-specific primers, hybridization probes, Light Cycler FastStart DNA Master HybProbe (Roche Diagnostics Ltd.), 6·25% dimethyl sulfoxide, and 10 μl of DNA extracts was analysed on a Light Cycler machine (model 1.5, Roche Diagnostics Ltd.). The amplification protocol was: 95°C for 9 min, 50 cycles of 95°C for 10 s, 54°C for 30 s, and 72°C for 25 s followed by melting curve analysis.

In SJH the assay was modified as follows: 20 μl reactions contained 0·25 μmol/l primers Asp fum F and Fungi5.8_R, 0·375 μmol/l hydrolysis probe ITS-PF, 10 μl Taqman Gene Expression Mastermix (Applied Biosystems) and 7 μl template DNA. Amplification was on an ABI 7500 real time PCR machine (Applied Biosystems) with the following conditions; 95°C for 10 min, then 45 cycles of 95°C for 15 s and 60°C for 30 s. The shortened extension and annealing step meant that only *A. fumigatus* could be detected in the SJH assay, however *A. fumigatus* comprised >95% of isolates from previously documented cases in that centre.

Internal control PCR

The internal control (IC) was an assay for detection of the meningococcal *ctra* gene (Corless *et al*, 2001). The IC plasmid (provided by P. L. White, Public Health Wales, Cardiff, UK) was added, 5 μl of 10 pg/μl solution, to the DNA extracts. Inhibition was indicated by a significant increase in Cq of >1 cycle, compared to extraction negative and positive controls.

Optimization of the PCR assays

Analytical sensitivity of the qPCR assays was tested by spiking 3 ml whole-blood EDTA samples obtained from healthy volunteers with *A. fumigatus* conidia at concentrations of 100, 50, 20, 10, 0 conidia/ml (Morton *et al*, 2010). Triplicate samples at each concentration were extracted as described above and both PCR assays were performed on the eluates. Potential contamination in the batches of blood collection tubes was determined by filling ten EDTA tubes each with 3 ml healthy volunteer blood and 10 with PBS buffer, followed by DNA extraction and ITS-qPCR.

Data analysis

For the analysis of assay performance, patients categorized as having either proven or probable IA were classed as a true positive and those diagnosed as unclassified were true negative (de Pauw *et al*, 2008). Individuals categorized as possible IA were not included in the analysis. As a single positive GM assay result of $\geq 0{\cdot}5$ was part of the mycological criteria for defining a case of probable IA the performance of the GM assay was only assessed where there were consecutive positive results of $\geq 0{\cdot}5$ or two positive results within a 12-d period during which four samples were processed for each assay. The performance of each PCR assay was assessed for individual or consecutive positives, or two or more positive results within the 12-d period. Receiver operator curves (ROC) were generated using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). Diagnostic statistics including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), diagnostic odds ratios (DOR) with confidence intervals, and diagnostic accuracy were calculated by previously described methods (Armitage & Berry, 1994).

Results

Categorization of patients by EORTC/MSG criteria

The numbers of cases of proven, probable, and possible IA in SJH were: one, 14, and 11 respectively with 120 unclassified cases, an overall incidence of proven/probable IA of 10%. The corresponding numbers for UKW were: none, 30, and 19 and 83 respectively, incidence 22·7%. The majority of probable cases in both centres were allo-SCT recipients (Table I). UKW had a more heterogeneous spread of probable cases with 63% in allo-SCT patients, compared to 93% for SJH, and 37% vs. 7% for leukaemia chemotherapy patients (*P* value 0·0384). Three other IFD were documented in SJH: two *C. parapsilosis* candidaemias, one an allo-SCT patient who subsequently had probable IA, and a lymphoma chemotherapy patient, unclassified for IA; the third case was *C. albicans* candidaemia in a Hodgkin lymphoma patient also unclassified for IA. In UKW there was one case of *C. krusei* septic arthritis, with probable IA; two candidaemias, one with *C. tropicalis*, and one with *Candida* sp., both in the unclassified IA group, and two cases of zygomycosis both in possible IA cases.

Performance of PCR assays

A limit of detection of six conidia per ml of volunteer blood was determined for both PCR assays (Cq for ITS was 35–37, second round of 28S assay had a Cq of 6) which was in accordance with previously published detection limits (White *et al*, 2011). No *Aspergillus* DNA was detected in any of the blood collection tubes or reagents. We did not apply a detection cut-off.

The ITS qPCR in SJH and the 28S nested assay in UKW yielded positive results with similar frequencies (8·5% and 8·1%); there was a lower percentage of positive assays using the 28S PCR in SJH while the ITS qPCR in UKW gave unexpectedly low numbers of positive results (Table II). Compared to the GM assay the PCR assays yielded more single positive sample results among the unclassified cases (SJH, ITS *P*: 0·0016, 28S *P*: 0·0244; UKW: 28S *P*: 0·0001), with the

Table I. Incidence of Invasive Aspergillosis according to patient groups treated in each centre.

	Number of patients		Number of probable/ proven cases (% of treatment group)		Number of possible cases (% of treatment group)		Number of unclassified cases (% of treatment group)	
Treatment groups	SJH	UKW	SJH	UKW	SJH	UKW	SJH	UKW
Allogeneic stem cell transplant								
Matched unrelated donor	26	33	6 (23)*	8 (24)	2 (8)	4 (12)	18 (69)	21 (64)
Sibling donor	44	39	8 (18)	9 (23)	4 (9)	10 (26)	32 (73)	20 (51)
Mismatch donor	–	4	–	2 (50)	–	0	–	2 (50)
Acute Leukaemia/Chemotherapy								
Acute myeloid leukaemia	27	42	1 (4)	9 (21)	2 (7)	4 (10)	24 (89)	29 (69)
Acute lymphoblastic leukaemia	5	12	–	2 (16)	1 (20)	1 (8)	4 (80)	9 (76)
Lymphoma/myeloma	44	2	–	–	2 (5)	0	42 (95)	2 (100)
Total	146	132	15	30	11	19	120	83

*Includes one case of proven aspergillosis. SJH: St James's Hospital; UKW: University Clinic Wuerzburg.

Table II. Summary of characteristics and number of diagnostic assays performed.

	28S qPCR assay		ITS qPCR assay		GM ELISA	
	SJH	UKW	SJH	UKW	SJH	UKW
Sample volume	3 ml blood		3 ml blood		–	
Template volume	5 µl		7 µl	10 µl	300 µl	
Proportion of DNA eluate per PCR assay	8%	5%	11%	10%	–	
Total samples	1828	2017	1828	2087	1839	1992
Positive (%)	5·6	8·1	8·5	2·2	7·5	7·8

qPCR, quatitative polymerase chain reaction; GM ELISA, galactomannan enzyme-linked immunosorbent assay; SJH, St James's Hospital; UKW, University Clinic Wuerzburg.

exception of the ITS qPCR in UKW, (*P*: 0·0663; Table III). Cases of probable IA had a higher proportion of positive results than the unclassified cases; this difference increased when assays were interpreted in terms of confirmed positive results (Table IV). This was supported by the results of ROC curve analysis (Table SII). The GM results also reflected this trend because the median number of samples for probable cases was 4 (range 0–17), possible 0 (range 0) and unclassified 0 (range 0–14).

The detection of true positive (proven/probable) cases was optimal when only a single positive assay result was assessed; however this led to high numbers of false positive results (Table IV). Confirmation by a second positive assay result reduced false positives at the expense of sensitivity, this was also observed when data from only the allo-SCT patients was analysed (Table SIII). The highest DOR values were for the combination of ITS+GM in each centre; confirmed ITS in UKW was not considered due to its poor sensitivity even though the lack of false positive assays gave it the highest DOR value (Fig. 1). Overall, the diagnostic parameters and DOR values for the different assays indicated that a combination of positive PCR/GM results would provide the best diagnostic accuracy (Table IV, Fig. 1). However, when data from the two centres were merged the combined PCR/GM performance was comparable to a confirmed GM positive result (Table SIV).

Timing of assay positives relative to CT scan

In the probable/proven IA cases we investigated the temporal relationship between a first positive assay result, whether GM, PCR, or a combination of these, and the date of the first CT scan that met the criteria for a diagnosis of IFD. The few cases where a CT scan had predated sample collection for the PCR assays were excluded from this analysis. The 28S and ITS assays were positive prior to or at the time of a positive CT scan in 55% and 48% of the probable IA cases respectively; 67% of cases had a positive GM result before the positive CT scan; 56% of the cases had both a GM and PCR positive prior to the CT scan; 15% did not have any PCR positive assay results (Fig. 2). In 26 cases (81% overall; 82% in TCD and 81% in UKW) the patients had at least one non-culture test (qPCR or GM) positive result prior to the first positive CT scan.

Discussion

Invasive aspergillosis continues to be a devastating complication of intensive chemotherapy or SCT for leukaemia. In a recent single-centre study of 269 patients receiving chemotherapy for acute myeloid leukaemia the incidence of probable/proven IA was 18% with an attributable mortality of 10% and excess cost per patient of €15 000 (Slobbe *et al*, 2008). In a large US multicentre surveillance study of IFD following SCT the cumulative incidence was highest for IA (Kontoyiannis *et al*, 2010). The prominence of IA among leukaemia cases with IFD diagnosed at autopsy suggests that the diagnosis is often delayed or missed (Maertens *et al*, 2005; Chamilos *et al*, 2006).

Table III. Prevalence of positive assay results in patients categorized as proven/probable, possible or unclassified IA.

	No. of patients		No. (%) of proven/probable cases		No. (%) of possible cases		No. (%) of unclassified cases	
Assay	SJH*	UKW†	SJH	UKW	SJH	UKW	SJH	UKW
Single positive result								
GM	43	53	15 (100)	28 (97)	0	0	28 (23)	25 (30)
ITS	69	32	12 (80)	16 (55)	5 (45)	2 (10)	52 (43)	14 (17)
28S	64	83	13 (87)	20 (69)	6 (54)	10 (52)	45 (37)	53 (64)
Confirmed positive result								
GM	19	23	9 (60)	14 (48)	0	0	10 (8)	9 (11)
ITS	25	7	7 (47)	7 (24)	3 (27)	0	15 (12)	0
28S	9	35	0	10 (34)	0	3 (16)	9 (7)	22 (26)
GM + ITS	21	15	11 (73)	12 (41)	0	0	10 (8)	3 (4)
GM + 28S	14	28	8 (53)	14 (48)	0	0	6 (5)	14 (16)

GM, galactomannan.
Confirmed positive assay:consecutive positive assays or second positive within 12 d.
*SJH–146 patients (15 Proven/Probable; 11 Possible; 120 Unclassified).
†UKW–132 patients (30 Proven/Probable; 19 Possible; 83 Unclassified).

Table IV. Diagnostic parameters for each assay depending on use of single positive or confirmed positive criteria for defining a case of probable or proven IA.

	Sensitivity		Specificity		Positive predictive value		Negative predictive value		Diagnostic test accuracy	
Assay	SJH	UKW	SJH	UKW	SJH	UKW	SJH	UKW	SJH	UKW
Single positive										
ITS	0·8	0·55	0·57	0·84	0·19	0·53	0·96	0·85	0·6	0·76
28S	0·87	0·69	0·63	0·36	0·22	0·27	0·97	0·78	0·65	0·45
Confirmed positive										
GM	0·6	0·48	0·92	0·89	0·47	0·61	0·95	0·84	0·88	0·79
ITS	0·47	0·24	0·88	1·0	0·32	1·0	0·93	0·79	0·83	0·81
28S	0	0·34	0·93	0·74	0	0·31	0·88	0·77	0·82	0·64
GM + ITS	0·73	0·41	0·91	0·96	0·50	0·8	0·96	0·83	0·89	0·82
GM + 28S	0·53	0·48	0·95	0·84	0·57	0·50	0·94	0·83	0·90	0·75

Patients classified as possible IA were excluded from the analysis.
SJH, St James's Hospital; UKW, University Hospital Wuerzburg; GM, galactomannan.

In our two-centre study the incidence of IA was 20% (SJH) and 25% (UKW) respectively in allo-SCT patients, compared with 3% (SJH) and 20% (UKW) respectively in acute leukaemia patients undergoing chemotherapy. The fact that the UKW chemotherapy patients were treated on an open ward without receiving mould active antifungal prophylaxis may have relevance to the higher incidence of IA in that cohort.

Antifungal strategies for the prevention or early therapy of *Aspergillus* infection have increasingly employed diagnostic biomarkers (Rogers *et al*, 2011). The potential value of circulating GM detection for predicting development of IA has been recognized for over two decades (Rogers *et al*, 1990). Although meta-analyses have shown that the commercially available GM assay has reasonable reliability there continue to be concerns about false positive results due to cross-reacting unrelated fungi, or piperacillin-tazobactam co-administration, and false negative results from using the 0·5 index cut-off (Pfeiffer *et al*, 2006). Currently the EORTC/MSG definitions, which are recommended only for research studies, permit a single positive GM result that is temporally related to a positive CT scan to be categorized as probable IA (de Pauw *et al*, 2008). However, in a recent prospective clinical trial that evaluated pre-emptive therapy, prompted by positive GM (using a cut-off of 1·5) together with specified clinical criteria, there were more fungal infections in the pre-emptive arm compared to those receiving standard empirical therapy (Cordonnier *et al*, 2009).

The primary aim of our study was to determine if PCR assays targeting fungal DNA extracted from whole blood could increase the diagnostic accuracy for detection of *Aspergillus* disease in high-risk HM patient populations. The methodological approaches used in each centre were predetermined by local factors including availability of PCR

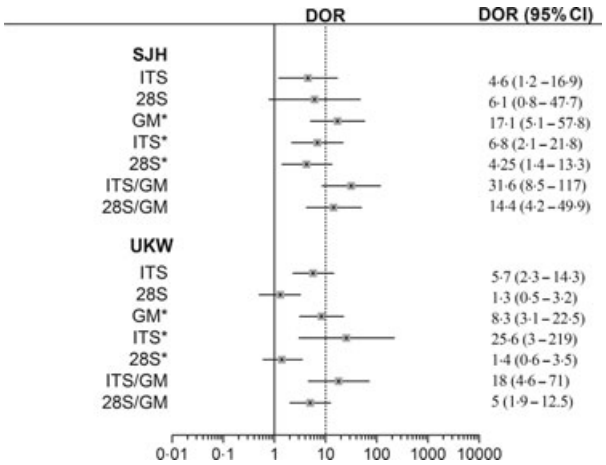

Fig 1. Plot showing the Diagnostic Odds Ratios (DOR) for each assay in each centre. Values for galactomannan (GM) assays are also shown. *Confirmed positive. In the case of St James's Hospital (SJH) GM, University Clinic Wuerzburg (UKW) ITS* and SJH 28S* there were zero values for false positive or true positive respectively; requiring substitution of 1 to enable representation of a DOR on the chart. The data are plotted on a Log_{10} scale and horizontal bars indicate the 95% confidence interval.

equipment. Recently, the European *Aspergillus* PCR Initiative (EAPCRI) has shown that the PCR platform does not significantly affect assay performance (White *et al*, 2010a).

We selected the nested PCR assay as 'gold standard' based on the encouraging results of an earlier study in a similar high-risk population (White *et al*, 2006b). However, as nested-PCR assays have an inherent risk of contamination by airborne spores and carry-over of amplicons we chose for comparison a single-round, probe-based PCR assay with the ITS gene region as target (Springer *et al*, 2011).

Guidelines published after our study had started recommend that lysis of white cells (WCL) in blood samples for *Aspergillus* DNA extraction is critical (White *et al*, 2010a,b). However, this was not performed in UKW because earlier data (unpublished) indicated that omitting WCL did not substantially influence the lower detection limit of the PCR assay. However the poor performance of the ITS assay in UKW would appear to be explained by this exclusion. The explanation could be that omitting WCL leads to insufficient release of intra-leucocyte *Aspergillus* DNA as well as to insufficient digestion of proteins present in human blood. Additionally, the omission of WCL leads to high concentrations of human DNA that can potentially inhibit the PCR assay. This did not however substantially influence the sensitivity of the 28S PCR. Interestingly, both assays utilize different probe formats, the 28S PCR was performed by using hydrolysis probes, whereas in the ITS qPCR assay in UKW the LightCycler FRET system was used.

It is striking that the PPV of the *Aspergillus* PCR assays was relatively low, as has been reported in comparable studies (White *et al*, 2006b), especially when compared with those from PCR assays that detect viral pathogens (Hebart *et al*, 1996). Cytomegalovirus or parvovirus B19 infections result in extremely high template numbers being present in blood, often out of the linear range of the respective PCR

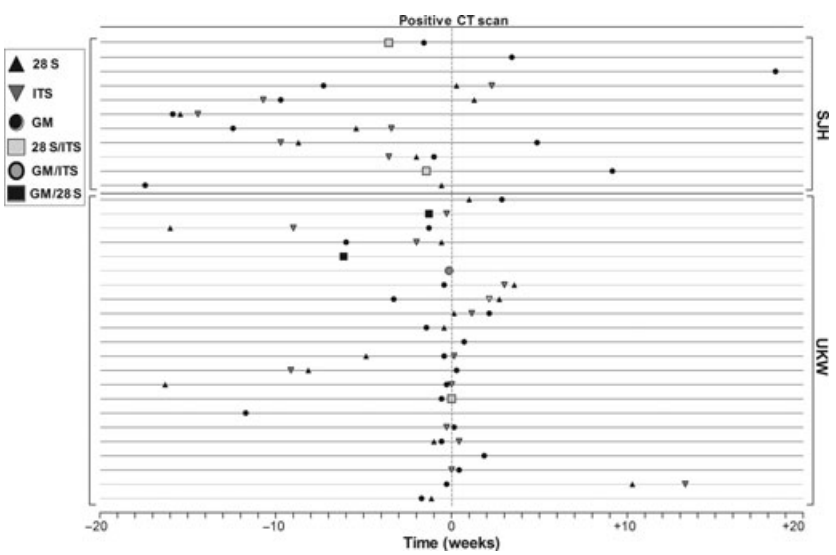

Fig 2. Relationship between the time point of first biomarker positive assay and of first positive computerized tomography (CT) scan according to European Organization for Research and Treatment of Cancer/National Institutes of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria (de Pauw *et al*, 2008). A small number of patients had a CT scan shortly prior to the start of PCR study and these were excluded from the analysis. The data for both centres indicate that 81% of patients had a single positive or confirmed assay result prior to identification of probable invasive aspergillosis. In each assay, 55% of 28S, 48% of ITS and 67% of GM first positive results preceded a diagnosis of probable IA. The median number of days prior to positive CT for each positive assay in both centres was 4 d for GM, 9 d for 28S and 1·5 d for ITS. The dark grey horizontal lines indicate that the individual had undergone Allo-SCT. SJH: St James's Hospital; UKW: University Hospital Wuerzburg.

assays (Von Muller *et al*, 2002). In *Aspergillus* infection only low copy numbers in blood are expected, often at the detection limit of the assay, even in patients with proven or probable IA (Klingspor & Loeffler, 2009). In order to reduce such problems, EAPCRI and others recommend performing identical multiplicities (duplicates, ideally triplicates) from each DNA patient sample. A challenge for the development and evaluation of diagnostic assays for IA is use of the current EORTC/MSG criteria for classification, given that cases with strong laboratory evidence of *Aspergillus* infection will be falsely categorized as unclassified if they don't have clinical criteria such as a positive CT scan.

The specificity of the biomarker assays was significantly improved by analysing confirmed positive results. This was largely due to a reduction in positives in the unclassified patient group. While this was at the expense of test sensitivity, the overall diagnostic accuracy of either combined PCR/GM or confirmed GM was close to 90% in SJH and 80% in UKW. The attraction of combining GM with PCR is that they target distinct biological markers: an immunodominant cell wall antigen and DNA, respectively.

Other studies have evaluated the utility of detection of GM or *Aspergillus* DNA by PCR in bronchoalveolar lavage samples. A recent systematic review (Avni *et al*, 2012) found that combining the results of these two tests gave optimal sensitivity with no loss of specificity for diagnosis of IA.

A single biomarker was positive in over 80% of the probable/proven IA cases prior to the first CT scan that established the diagnostic category, and while this decreased to 67% if a combined positive PCR/GM result was used, the greater diagnostic accuracy achieved with confirmed positive assay results suggests that this might provide greater confidence in identifying patients who are likely to progress to invasive disease.

As far as we are aware, this is the largest prospective study to date that has evaluated PCR for diagnosis of IA. Our finding of an improved performance with combined results of blood biomarkers for detection of *Aspergillus* infection and documentation of IA cases should inform clinical practice and may help to improve patient care by more reliable documentation of invasive aspergillosis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. PCR primers and probes used in this study.

Table SII. Area under curve for ROC curves full data generated in PAWS 18 (SPSS Inc.).

Table SIII. Performance for each assay in Allo-SCT patients in each centre depending on use of single positive or confirmed positive test criteria for defining a positive case.

Table SIV. Performance for each assay for both centres combined depending on use of single positive or confirmed positive test criteria for defining a positive case.

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