Diagnostic Value of Microbial Cell-free DNA Sequencing for Suspected Invasive Fungal Infections: A Retrospective Multicenter Cohort Study

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Background. An early diagnosis and treatment of invasive fungal disease (IFD) is associated with improved outcome, but the moderate sensitivity of noninvasive diagnostic tests makes this challenging. Invasive diagnostic procedures such as bronchoalveolar lavage (BAL) have a higher yield but are not without risk. The detection and sequencing of microbial cell-free DNA (mcfDNA) may facilitate a noninvasive diagnosis.

Materials. In a prospective observational study, we collected plasma in the 120 hours preceding or following a BAL in patients with hematological malignancies suspected for a pulmonary IFD. The EORTC/MSGERC2020 criteria were used for IFD classification. Sequencing was performed by Karius (Redwood City, CA) using their Karius Test (KT) on plasma and a "research use only test" on BAL fluid if available. Cases with a probable/proven IFD were identified based on standard diagnostic tests on serum and BAL (microscopy, polymerase chain reaction, galactomannan, culture) and used to calculate the sensitivity, specificity, and additional diagnostic value of the KT.

Results. Of 106 patients enrolled, 39 (37%) had a proven/probable invasive aspergillosis, 7 (7%) a non-*Aspergillus* IFD, and 4 (4%) a mixed IFD. The KT detected fungal mcfDNA in 29 (28%) patients. Compared with usual diagnostic tests, the sensitivity and specificity were 44.0% (95% confidence interval [CI], 31.2–57.7) and 96.6% (95% CI, 88.5%–99.1%). Sensitivity of the KT was higher in non-*Aspergillus* IFD (*Mucorales*:2/3, *Pneumocystis jirovecii*: 3/5). On BAL, the sensitivity was 72.2% (95% CI, 62.1–96.3), and specificity 83.3% (95% CI, 49.1–87.5).

Conclusions. Sequencing of mcfDNA may facilitate a noninvasive diagnosis of IFD in particular non-*Aspergillus* IFD. However, on plasma and similar to currently available diagnostics, it cannot be used as a "rule-out" test.

Keywords. hematological malignancy; invasive fungal disease; invasive fungal infection; microbial cell-free DNA; next generation sequencing.

Invasive fungal diseases (IFDs) pose a significant threat to immunocompromised patients, particularly those with hematological malignancies or after allogeneic stem cell transplantation [1–3]. An early and accurate diagnosis of invasive fungal infections (IFIs) allows for prompt initiation of antifungal therapy, ultimately reducing IFD-related morbidity and mortality [4, 5]. Currently, routine IFD diagnosis is based on conventional microscopy, fungal culture, biomarkers such as galactomannan (GM) and beta-D-glucan, and to a lesser extent also polymerase chain reaction (PCR)–based DNA detection methods. They are part of the 2020 EORTC/MSGERC consensus definitions that are frequently used to classify IFIs [6]. Unfortunately, the existing diagnostic armamentarium is far from perfect. Conventional microscopy and fungal culture have a low sensitivity, and in addition, fungal culture typically takes days but occasionally much longer to grow [4]. Although GM shows improved performance compared with fungal culture, its efficacy is influenced by the patient population and the material used. The use of serum GM is especially useful in patients with neutropenia but is less validated in other populations. In addition, validation studies showed that the sensitivity of GM in bronchoalveolar lavage (BAL) fluid is higher than in serum at a minor cost of specificity [7]. Similarly, beta-D-glucan displays reasonable sensitivity but lacks specificity because it is produced by many fungi, including

Candida, and bacteria [8]. PCR-based tests were recently introduced in the EORTC/MSGERC consensus definitions but cannot distinguish airway colonization from invasive disease [9, 10]. Semi-invasive or invasive sampling methods, such as bronchoscopy or lung biopsy, respectively, have a higher diagnostic yield but are time-consuming and not always possible [11, 12]. It is clear that novel noninvasive diagnostics are needed that facilitate the timely detection of fungal pathogens.

Cell-free DNA (cfDNA) is freely circulating in body fluids including blood. Over the past 2 decades, cfDNA sequencing has been shown to enable noninvasive diagnosis of fetal abnormalities and characterization/monitoring of cancer [13]. More notably, cfDNA originating from microbes (microbial cell-free DNA [mcfDNA]) and extracted from blood plasma has been successfully sequenced as well [14].

Karius Inc., a clinical diagnostics company located in California, has developed an mcfDNA test (The Karius test [KT]) capable of detecting DNA of more than 1000 pathogens including bacteria, DNA viruses, and fungi from 1 mL of plasma [15]. A benefit of this technique is detecting DNA from multiple species simultaneously.

In this multicenter study, we compared the performance of the KT on plasma to standard IFD diagnostics in a cohort of patients with an underlying hematological malignancy in whom an IFD was suspected. Additionally, we extended our investigation to include the sequencing of mcfDNA on BAL fluid in a subgroup of patients to explore the broader potential of the KT in diagnosing IFIs in various bodily fluids.

METHODS

Study Design and Population

This was a multicenter study conducted at 3 tertiary care centers in the Netherlands and Belgium: Erasmus University Medical Center Rotterdam, University Hospital Ghent, and AZ Sint-Jan Bruges. The study involved patients with an underlying hematological malignancy and a clinical and radiological suspicion of an IFD. Written consent was obtained from all patients who planned to undergo or had already undergone a BAL in the previous 5 days and who were included in the ARPOS study (NL62004.078.17). Plasma samples were collected and together with any available leftover BAL fluid were stored at −80 °C for research on new IFD diagnostic methods. The inclusion of patients and the sample collection was therefore prospective, but the KT was performed at a later time. All patients included in the ARPOS study for whom material was available at that time were included in this study.

Diagnostic Criteria

The classification of IFD was based on the 2020 EORTC/MSGERC consensus definitions. All patients met the host factor criterion. Additionally, the radiological criterion for IFD diagnosis was determined based on the chest computed tomography scan closest to the BAL sampling. BAL was performed as part of routine clinical care. More details about the diagnostic tests that were done as part of routine care on serum and/or BAL are described in Supplementary Methods 1.

Microbial Cell-free DNA Sequencing (KT)

BAL fluid and plasma samples were sent under frozen conditions to Karius (Redwood City, CA) for mcfDNA sequencing using a certified courier (World Courier). Because the plasma and BAL samples had been collected in the context of the ARPOS study, testing was not performed in real time. For plasma, the KT details and performance have been previously described [15]. Briefly, following mcfDNA extraction, enriched library preparation, sequencing, discarding of human sequences, and alignment of the remaining sequences to a curated database of reference genomic sequences, test results were generated using the Karius version 3.13 analytic pipeline.

KT reports consisted of all microorganisms deemed to be significantly higher than real-time background control specimens [15]. These microorganisms were quantified in molecules per microliter as an equivalent to the number of DNA fragments per microliter. For BAL fluid, Karius used a research use only pipeline (RUO-KT) using the same process as in plasma cfDNA sequencing aside from a 1:10 predilution, and with a distinct Clinically Reportable Reference list that includes 981 bacteria, fungi, DNA viruses, and parasites that have the potential to cause pneumonia. The relative abundance of each reported potential pathogen was reported and calculated by dividing the number of pathogen DNA reads by median background flora DNA reads. A preliminary reference range for each pathogen was also provided and defined as the 97.5 percentile relative abundance detected in BALs from a cohort of 66 patients clinically adjudicated negative for a probable cause of pneumonia [16]. Finally, "status" is reported as an indicator of relative abundance compared with the reference range for each pathogen. An example of a report of the test on plasma and on BAL from the same patient is available in Supplementary Methods 2 and 3.

Data Collection and Analysis

Clinical data were extracted from electronic health records, including patient characteristics such as age, sex, underlying disease and treatment, neutrophil count, and antifungal treatment. Mycological evidence was collected as part of routine clinical care (BAL microscopy, culture, and galactomannan); *Aspergillus* PCR and serum galactomannan were also performed centrally at Erasmus University Medical Center Rotterdam. Results from the KT/RUO-KT were classified as positive, negative, or failed. Because the ARPOS study focuses on invasive fungal disease, the potential clinical impact of bacterial, viral, or parasitic DNA that was detected by the KT/RUO-KT was not evaluated in this study. Also, fungal species that were detected but not typically associated with invasive pulmonary disease

Table 1. Baseline Characteristics

	Total (N = 106)	Proven/probable Invasive Aspergillosis (N = 35)	Proven/probable Non-Aspergillus IFD (N = 7)	Proven/probable IA + non-Aspergillus IFD (N = 4)	Possible IFD (N = 48)	No IFD (N = 12)
Age (y)—median (IQR)	64 (54–70)	65 (56–69)	70 (54–72)	65 (16–68)	62 (51–70)	65 (56–75)
Male—no. (%)	72 (68%)	25 (71%)	6 (86%)	2 (50%)	33 (70%)	9 (75%)
Underlying disease						
AML—no. (%)	51 (48%)	17 (49%)	4 (57%)	1 (25%)	22 (47%)	2 (17%)
MDS—no. (%)	11 (10%)	1 (3%)	…	3 (75%)	4 (9%)	1 (8%)
ALL—no. (%)	8 (7%)	2 (5%)	…	…	2 (4%)	1 (8%)
MM—no. (%)	6 (6%)	1 (3%)	…	…	1 (2%)	3 (25%)
CLL—no. (%)	5 (5%)	…	…	…	…	2 (17%)
DLBCL—no. (%)	4 (4%)	3 (8%)	…	…	3 (6%)	1 (8%)
Other—no. (%)	21 (20%)	11 (31%)	3 (43%)	…	16 (33%)	2 (17%)
HSCT						
Allogeneic HSCT—no. (%)	22 (24%)	10 (29%)	2 (29%)	2 (50%)	9 (19%)	2 (17%)
Autologous HSCT—no. (%)	6 (6%)	3 (9%)	…	…	1 (2%)	2 (17%)
Time between HSCT and plasma sampling (mo)—median (IQR)	8 (1–31)	3 (1–27)	14 (14–14)	16 (14–)	11 (0–30)	52 (0–)
Neutropenia[a] (<500/mL)—no. (%)	52 (49%)	19 (54%)	2 (29%)	4 (100%)	26 (54%)	1 (8%)
Diagnosis (standard of care tests)[b]—no. (%)						
No fungal disease[c]	60 (57%)	…	…	…	…	…
Probable IA	35 (34%)	…	…	…	…	…
Probable PJP	3 (3%)	…	…	…	…	…
Probable IM	4 (4%)	…	…	…	…	…
Mixed probable IA and PJP	2 (2%)	…	…	…	…	…
Mixed probable IA and IM	2 (2%)	…	…	…	…	…

Abbreviations: ALL, acute lymphatic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphatic leukemia; DLBCL, diffuse large B-cell lymphoma; IA, invasive aspergillosis; IM, invasive mucormycosis; HSCT, hematopoietic stem cell transplantation; L-AmB, liposomal Amphotericin B; MDS, myelodysplastic syndrome; MM, multiple myeloma; PJP, *Pneumocystis*-related pneumonia.

[a]Around time of plasma sampling (± 48 h).

[b]Diagnosis is based on modified EORTC/MSGERC criteria. Diagnosis of IM could also be based on a single positive *Mucorales* PCR on bronchoalveolar fluid and/or plasma that is not implemented in the consensus criteria; 1 patient had a positive *Mucorales* culture.

[c]Patients with a possible IA were also accounted for the group "No fungal disease": No IFD (N = 12), possible IA (N = 48).

(eg, *Candida* species, *Penicillium* species) were not considered as cases of IFD in this study.

Categorical variables were reported as frequencies, continuous variables were reported as median and quartiles. Results from the KT/RUO-KT were compared with the 2020 EORTC/MSGERC consensus definitions, proven and probable IFD were used as cases, and possible and no IFD were used as controls.

To assess the performance of the KT/RUO-KT, the sensitivity, specificity, positive and negative predictive value, and positive and negative likelihood ratio were calculated along with 95% confidence intervals (CIs). Subgroup analyses were conducted based on the presence of neutropenia, treatment with mold-active antifungals, and patients who recently underwent an allogeneic stem cell transplantation. Finally, the performance of the KT on plasma was calculated separately for the 3 most frequent IFDs: *Aspergillus*, *Mucorales*, and *Pneumocystis jirovecii*.

RESULTS

Plasma samples, diagnostic test results, and clinical data were collected from 109 patients between June 2018 and May 2022. After excluding 3 patients because of the long (>5 days) interval between BAL and plasma sampling, 106 patients were included in the analysis. Sufficient BAL fluid remained to perform the RUO-KT on 34 patients.

Baseline Characteristics

Of the 106 patients, 72 (68%) were male and the median age was 64 (interquartile range: 54–70) years. Fifty-one patients had underlying acute myeloid leukemia and 11 had underlying myelodysplastic syndrome. Twenty-eight (30%) had received a hematopoietic stem cell transplantation. At the time of plasma sampling, the neutrophil count was <500/mL in 52 (49%). Antifungal prophylaxis or therapy was given to 98 of the 106 (92%) patients but in only 25 (24%) was the agent was active against molds. More details are shown in Table 1.

Standard of Care Diagnosis

Thirty-nine (37%) patients had a probable IA. A probable mucormycosis was found in 6 (6%) and a probable *P jirovecii*–related pneumonia (PCP) in 5 (5%) patients. Additionally, a second IFD was diagnosed in 4 (4%) patients with a probable

invasive aspergillosis (IA; 2 also had a probable mucormycosis and 2 a probable *Pneumocystis*-related pneumonia [PJP]).

From the 39 patients with a probable IA, BAL culture was positive in 7 (18%) and BAL GM in 27 (69%). Serum GM was ≥1.0 in only 7 (18%) and ≥0.5 in 14 (36%) patients with a probable IA.

The *Aspergillus* PCR was positive on BAL from 37/106 (35%) patients, of whom 24 (23%) fulfilled the "PCR positive in duplicate" criterion as required in the EORTC/MSGERC consensus definitions. In 8 patients, the diagnosis of probable IA was solely based on a positive *Aspergillus* PCR on BAL fluid. So, overall, the PCR and/or GM test on BAL was the only positive mycological test in the majority (25/39) of IA cases.

In the 6 patients with a probable mucormycosis, BAL culture and *Mucorales* PCR on plasma was positive in 1 (16%); the other patients were diagnosed based on a positive PCR test in plasma (N = 1) or BAL fluid (N = 2) or both (N = 2).

In all 5 patients with a probable PJP, diagnosis was based on a positive *P jirovecii* PCR on BAL. More details on mycological evidence for IFD diagnosis can be found in Supplementary Data 4 (Supplementary Table 1).

In 60 patients, diagnosis of a probable/proven IFD could not be established based on the EORTC/MSGERC consensus definitions. In 10 of these patients, the *Aspergillus* PCR was positive; however, the duplicate positive criterion was not met and spare BAL fluid was unavailable so we were unable to repeat the PCR. Finally, 1 patient with a positive *Mucorales* PCR on BAL was not classified as probable IFD because the patient survived without any antifungal treatment and the cycle threshold value at which the PCR became positive was 40.

Results of the KT

Plasma. The KT was successful in 105/106 (99%) of the plasma samples, meaning that human and/or microbial DNA could be sequenced. Overall, the test was positive for any bacterial, viral, or fungal DNA in 79 (75%) patients. Fungal DNA was detected and successfully sequenced in 29 (27%) of them. In 3 patients, 2 different species were found (1: *Aspergillus flavus/oryzae* and *Rhizomucor* pusillus, 2: *Aspergillus fumigatus* and *A oryzae*, 3: *Penicillium nalgiovense* and *Debaryomyces hansenii*). Fungal DNA from species known to cause invasive pulmonary disease were found in 24 patients. *Aspergillus* DNA in 17 and *Mucorales* and *Pneumocystis* DNA in 4 patients each (in 1 patient both *Aspergillus* and *Mucorales* DNA was detected). We refer to Tables 2 and 3 for details on all the detected fungal species, including fungi not typically associated with pulmonary disease.

Bronchoalveolar Lavage. The RUO-KT was performed on BAL fluid from 34 patients and was successful for 33. It was positive in 31 (94%): in 17 (51%) fungal DNA was detected whereas in 14 of these the detected fungus was known to cause invasive

Table 2. Identification of Fungal Species by Next-generation Sequencing of Microbial Cell-free DNA (Karius Test on Plasma and Research-only-use Test on BAL Fluid)

Fungal Pathogens	Plasma[b] (N = 32)	BAL[c] (N = 20)
Aspergillus species	18 (56%)	12 (60%)
– A fumigatus	– 15 (47%)	– 11 (55%)
– A oryzae	– 2 (6%)	– 0
– A flavus/oryzae	– 1 (3%)	– 1 (5%)
Candida species[a]	4 (13%)	5 (25%)
– C albicans	– 2 (6%)	– 3 (15%)
– C glabrata	– 1 (3%)	– 1 (5%)
– C tropicalis	– 0	– 1 (5%)
– D hansenii (C famata)	– 1 (3%)	– 0
Pneumocystis jirovecii	4 (13%)	2 (10%)
Mucorales species	4 (13%)	1 (5%)
– Rhizomucor pusillus	– 2 (6%)	– 0
– Rhizopus microsporus	– 2 (6%)	– 1 (5%)
Penicillium nalgiovense[a]	2 (6%)	0

[a]In general, these species are no causative pathogens for invasive pulmonary disease and are therefore not defined as cases of invasive fungal disease in this study.

[b]In total, 32 fungal pathogens were found across 106 patients in plasma, in 3 of these patients, 2 fungal pathogens were found.

[c]In total, 20 fungal pathogens were found across 106 patients in BAL fluid. In 1 patient, 2 fungal pathogens were found.

pulmonary disease (Table 2). *Aspergillus* DNA was found in 12 patients. In 1 of these 12, *P jirovecii* DNA was also found, whereas in 2 of these 12 *Candida* species were found as well. Finally, *Mucorales* and *Pneumocystis* DNA was detected in 1 patient each. Results of DNA from fungi not associated with pulmonary disease that was detected by the RUO-KT can be found in Tables 2 and 3.

Comparison of mcfDNA Sequencing on Plasma and BAL. Refer to Table 3 for an overview of all patients in whom fungal DNA was detected and sequenced either in BAL and/or plasma. In 3 of the 14 patients in whom the RUO-KT detected DNA from pathogenic fungi in BAL fluid, the same DNA was detected on plasma (all *A fumigatus*). In 4 patients, there was a partial agreement; in 3 of them fungal DNA that was detected in plasma (*Aspergillus* in 3, *P jirovecii* in 1) was also detected in BAL but additional fungi were detected in BAL as well (*Aspergillus* in 1, *Candida* in 2). In 1 patient, both *Aspergillus* and *Mucorales* species were found in plasma but only *Aspergillus* was found in BAL. In 7 patients, no agreement was found between the RUO-KT in BAL compared with the KT in plasma. In 5 of these patients, *A fumigatus* was detected in BAL fluid, but not in plasma. In 1 patient, *P jirovecii* was found in BAL, but not in plasma, and in 1 patient BAL fluid showed *Rhizomucor microsporus* in contrast to plasma, which only showed *A oryzae*. In 69 patients, no fungal species were detected with mcfDNA sequencing.

Performance of mcfDNA Sequencing. To compare the performance of the KT on plasma and the RUO-KT on BAL with standard of care diagnostics typically available in a tertiary

Table 3. Overview of Patients in Which Sequencing of Microbial Cell-free DNA (Karius Test) Showed a Fungal Species + Associated Standard Diagnostics

	Karius test[a] Plasma	RUO-Karius Test[a] BAL	Serum GM	BAL GM	BAL Culture	BAL Aspergillus PCR[b]	BAL Mucor PCR	BAL Pneumocystis PCR[c]	Plasma Mucor PCR[c]	BAL Microscopy	Radiology	
1	A fum	A fum	0	NP	–[e]	–	NP	NP	NP	–	Noduli + halo	Possible IA
2	A fum	A fum	0.1	1.52	A fum	++	NP	NP	NP	–	Noduli + halo	Probable IA
3	A fum	A fum	0.1	0.1	–	++	NP	NP	NP	–	Noduli + halo	Probable IA
4	A flavus/oryzae R pusillus	A flavus/oryzae	0.6	4.7	A fum	++	–	NP	+ (35.91)	–	Nodulary consolidation + wedge-shaped consolidation	Mixed probable IA and IM
5	A fum	A fum C albicans	0.2	2.22	–	–	NP	NP	NP	NP	Nodule + halo	Probable IA
6	A fum	A fum C glabrata	0.5	6.07	–	++	NP	NP	NP	–	Noduli + halo	Probable IA
7	P jirovecii	A fum P jirovecii	0.31	0.09	–	++	NP	+ (29.2)	NP	–	Wedge-shaped consolidation	Mixed probable IA and PJP
8	A fum	A fum	0	0.12	–	++	NP	NP	NP	NP	Cavity	Probable IA
9		. fum	0.1	0.79	–	++	NP	NP	NP	NP	Noduli + halo	Probable IA
10	P nalgiovense	A fum	0.1	0.19	–	+	NP	NP	NP	–	Noduli + halo	Possible IA
11		A fum	0.1	2	A fum	NP	NP	NP	NP	+	Noduli + halo	Probable IA
12		A fum	0	2.6	–	++	NP	NP	NP	–	Noduli + halo	Probable IA
13	P jirovecii	P jirovecii	0.1	0.25	–	+	NP	+	NP	–	Centrilobular ground-glass opacities	Probable PJP
14	A oryzae	R microsporus	0.1	0.22	–	–	+ (33.5)	NP	–	–	Nodule + halo	Probable Mucormycosis (PCR)
15	C albicans	C albicans	0.1	0.06	–	–	NP	NP	NP	–	Bilateral ground glass	No mycological evidence
16	C albicans	C albicans	0.1	0.2	–	–	NP	NP	NP	Yeast	Bilateral patchy consolidations	No mycological evidence
17	C tropicalis	C tropicalis	0.12	0.1	–	–	NP	NP	NP	NP	Lobar consolidation	No mycological evidence
18	NP	R pusillus	1	4.07	–	++	+ (45)	NP	+ (32)	–	Noduli + halo	Mixed probable IA and IM
19	NP	A fum	0.2	3.08	A fum	++	NP	NP	NP	–	Nodule without halo	Probable IA
20	NP	A fum	0.1	0.46	–	++	NP	NP	NP	–	Nodule + air-crescent	Probable IA
21	NP	C glabrata	1.6	6.5	–	++	NP	NP	NP	–	Nodulary consolidation + halo	Probable IA
22	NP	A fum	0.1	0.6	–	++	NP	NP	NP	–	Noduli + halo + tree-in-bud	Probable IA
23	NP	A fum	12.2	7.2	–	++	NP	NP	NP	–	Consolidation + halo	Probable IA
24	NP	A fum	0.1	1	–	++	NP	NP	NP	–	Noduli + halo	Probable IA
25	NP	C albicans	0	0	–	–	NP	NP	NP	NP	Noduli + halo + tree-in-bud	Possible IA
26	NP	P jirovecii	0	1.3	–	–	NP	+ (31.2)	NP	Yeast	Bilateral peribronchovascular ground glass noduli	Mixed probable IA and PJP
27	NP	A fum	1.5	0.7	–	–	NP	+ (42.4)	NP	–	Nodulary consolidation + halo + tree-in-bud	Probable IA
28	NP	P jirovecii	0.1	0.8	–	Species +	NP	+ (30)	NP	–	Wedge-shaped consolidation + bilateral ground-glass opacities	Probable PJP
29	NP	R microsporus	0	0.08	Mucor species	NP	NP	NP	+ (36.4)	+	Reverse halo	Probable Mucormycosis (culture)
30	NP	A fum A oryzae	0.1	3.26	–	++	NP	NP	NP	–	Noduli + halo	Probable IA
31[d]	NP	P jirovecii	0	0.07	–	–	NP	+	NP	–	Bilateral ground-glass opacities	No IFD found

Table 3. Continued

	Karius test[a] Plasma	RUO-Karius Test[a] BAL	Serum GM	BAL GM	BAL Culture	BAL Aspergillus PCR[b]	BAL Mucor PCR	BAL Pneumocystis PCR[c]	Plasma Mucor PCR[c]	BAL Microscopy	Radiology
32	A fum	NP	4.2	6.8	–	NP	NP	NP	NP	-	Noduli + halo	Probable IA
33	R microsporus	NP	0.1	0.3	–	–	+ (36.59)	NP	+ (38.5)	–	Wedge-shaped consolidation + ground glass	Probable Mucormycosis (PCR)
34	A fum	NP	0.2	8	A fum	++	NP	NP	NP	+	Wedge-shaped consolidation + halo	Probable IA
35	C albicans	NP	0.1	0.1	–	NP	NP	NP	NP	–	Noduli + halo	Possible IA
36	D hansenii P nalgiovense	NP	0	0.3	–	–	NP	NP	NP	–	Noduli + halo + wedge-shaped consolidation	Possible IA
37	A fum	NP	0.8	2.9	–	NP	NP	NP	NP	–	Noduli + halo + wedge-shaped consolidation	Probable IA

Abbreviations: −, the mycological test being performed but the result being negative; A fum, Aspergillus fumigatus; IA, invasive aspergillosis; IFD, invasive fungal disease; IM, invasive mucormycosis; NP, not performed; PJP, Pneumocystis jirovecii pneumonia.

[a]In patients 1 to 3, the Karius test on plasma shows a full agreement with the RUO-Karius test on BAL fluid, in patients 4 to 7, there is a partial agreement. In patients 8 to 14, there is no agreement between the Karius test on BAL and plasma. In patients 15 to 37, the Karius test was not performed on BAL fluid.

[b]Aspergillus PCR was performed in duplicate and this was deemed as positive mycological evidence according to the 2020 EORTC/MSGERC consensus definitions. This is depicted in the table with "++". If only 1 PCR test was positive, this is depicted as a single "+" symbol.

[c]A positive Mucorales PCR was deemed as sufficient mycological evidence for the diagnosis of an invasive mucormycosis in combination with clinical and radiological signs.

[d]In patient 31, Pneumocystis PCR was positive; however, there was a very low fungal load. There was no radiological suspicion for a PJP. The patient made a full recovery under broad spectrum antibiotic therapy. No treatment for PJP was initiated.

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care center, we considered all probable/proven IFD cases according to the 2020 EORTC/MSGERC consensus definitions as true positives, whereas those classified as possible or no IFD were retained as IFD-negative cases (Table 4). Four patients fulfilled the definition for 2 different fungal causes of infection; in total, 109 events were therefore registered for performance analysis instead of 105. According to this analysis, the KT on plasma had a sensitivity of 44.0% (95% CI, 31.2–57.7) and a specificity of 96.6% (95% CI, 88.5–99.1). The sensitivity of the RUO-KT on BAL fluid was 72.2% (95% CI, 49.1–87.5) but the specificity was lower at 88.2% (95% CI, 65.7–96.7).

From the 60 patients without a diagnosis of a probable IFD based on the EORTC/MSGERC definitions, the KT found a possible cause for infection in 3 patients on plasma (Figure 1A). The KT therefore had a possible additional diagnostic value of 2.9% (3/105) in the overall population and 5% (3/60) in the nonprobable subgroup. For the 33 patients in whom the RUO-KT was also performed on bronchoalveolar fluid, the test had a possible additive diagnostic value of 6.1% (2/33) (Figure 1B).

Subgroup analysis showed that in patients who had undergone an allogeneic stem cell transplantation, the sensitivity of the KT was 50.0%; it was 44.8% in patients with neutropenia. For the 14 patients with a serum galactomannan of $\geq$0.5 OD, the sensitivity and specificity was 53.3% and 100%. Importantly, the sensitivity of the KT was not impacted by the use of mold-active prophylaxis (sensitivity 7/14, 50%; specificity 13/13, 100%). Finally, the sensitivity of the KT on plasma was higher in patients with a Mucorales or P jirovecii infection compared with those with an IA (4/6 [66.7%], 3/5 [60.0%] versus 15/39 [38.5%], respectively), whereas the specificity was high across fungal species, ranging between 97% and 100%.

DISCUSSION

Diagnosing an IFD is challenging and often not possible without an invasive diagnostic procedure. In this study, we explored the potential of mcfDNA sequencing as a novel noninvasive diagnostic tool in 106 patients with underlying hematological malignancies with a suspected IFD. We compared the performance of the KT on plasma or BAL to a combination of diagnostic tests typically used in these patients. When performed on plasma, the overall sensitivity and specificity was 44.0% and 96.6%. In contrast to the impact that mold-active antifungal therapy can have on the yield of fungal cultures and serum galactomannan testing, this did not impact the yield of the KT [17]. Although the specificity was consistently high across all species and patient subgroups, the sensitivity varied substantially across fungal species. A low sensitivity (38.5%) was observed in those with an IA, whereas only 4 of the 11 cases of a non-Aspergillus IFD were missed (sensitivity 64%). This is in line with previous studies. In a cohort of hematopoietic stem cell recipients with a

Table 4. Diagnostic Performance of Next-generation Sequencing of Microbial Cell-free DNA (Karius Test on Plasma and Research-only-use Test on BAL Fluid) Compared With the Modified EORTC/MSGERC Definitions

General Performance of the Karius Test	N[a]	Sensitivity (%)	95% CI	Specificity (%)	95% CI	PPV (%)	95% CI	NPV (%)	95%CI	LR+	95% CI	LR−	95% CI
PLASMA	109	44.0	31.2–57.7	96.6	88.5–99.1	78.6	60.5–89.8	70.3	59.7–79.2	13.0	3.2–52.5	0.6	.45–.74
BAL	35	72.2	49.1–87.5	88.2	65.7–96.7	81.3	57.0–93.4	79.0	56.7–91.5	6.1	1.6–23.3	0.3	.15–.68
PLASMA + BAL (all patients)	110	56.9	43.4–69.5	94.9	86.1–98.3	82.9	67.3–91.9	74.7	63.8–83.1	11.2	3.6–34.6	0.45	.33–63
PLASMA + BAL (patients with both tests)	36	73.7	51.2–88.2	88.2	65.7–96.7	82.4	70.2–98.8	79.0	56.7–91.5	6.3	1.7–23.7	0.30	.14–65
Performance of the Karius test[b] per IFD	**N**	**Sensitivity (%)**	**95% CI**	**Specificity (%)**	**95% CI**	**PPV (%)**	**95% CI**	**NPV (%)**	**95% CI**	**LR+**	**95% CI**	**LR−**	**95% CI**
Probable IA	105	38.5	24.9–54.1	97	89.6–99.2	88.2	65.7–96.7	72.7	62.6–80.9	12.9	3–52.6	0.6	.49–82
Probable PJP	105	60.0	23.1–88.2	99.0	94.6–99.8	75	30.1–95.4	98.0	93.1–99.5	60	7.5–479	0.4	.14–18
Probable IM	105	66.7	30–90.3	100	96.3–1	100	51–100	98	93.1–99.5	-	-	0.3	.1–1
Performance of the Karius test[b] in subgroups	**N**	**Sensitivity (%)**	**95% CI**	**Specificity (%)**	**95% CI**	**PPV (%)**	**95% CI**	**NPV (%)**	**95%CI**	**LR+**	**95% CI**	**LR−**	**95% CI**
Neutropenia	56	44.8	28.4–62.5	96.3	81.7–99.3	72.2	49.1–87.5	68.4	52.5–80.9	12.1	1.7–86.4	0.6	.41–8
AMT	27	50.0	26.8–73.2	100	77.2–100	77.8	45.3–93.7	72.2	49.1–87.5	-	-	0.5	.3–84
No AMT	75	40.6	25.5–57.7	95.4	84.5–98.7	76.5	52.7–90.4	70.7	58.0–80.8	8.7	2.1–36.1	0.6	.46–84
aSCT	27	50.0	28.0–72.0	100	74.1–100	80.0	49–94.3	64.7	411.3–82.7	-	-	0.5	.3–82

Abbreviations: AMT, anti-mold therapy; aSCT, allogeneic stem cell transplantation; IA, invasive aspergillosis; IM, invasive mucormycosis; NPV, negative predictive value; PJP, *P jiroveci*–related pneumonia; PPV, positive predictive value.

[a]The Karius test was successful in 105 patients on plasma and in 33 patients on BAL fluid. If multiple pathogens were found with the standard diagnostic tests or Karius test, patients were counted more than once; therefore, the values in the table are sometimes >105.

[b]Only the results from the Karius test on plasma are accounted for in this table.
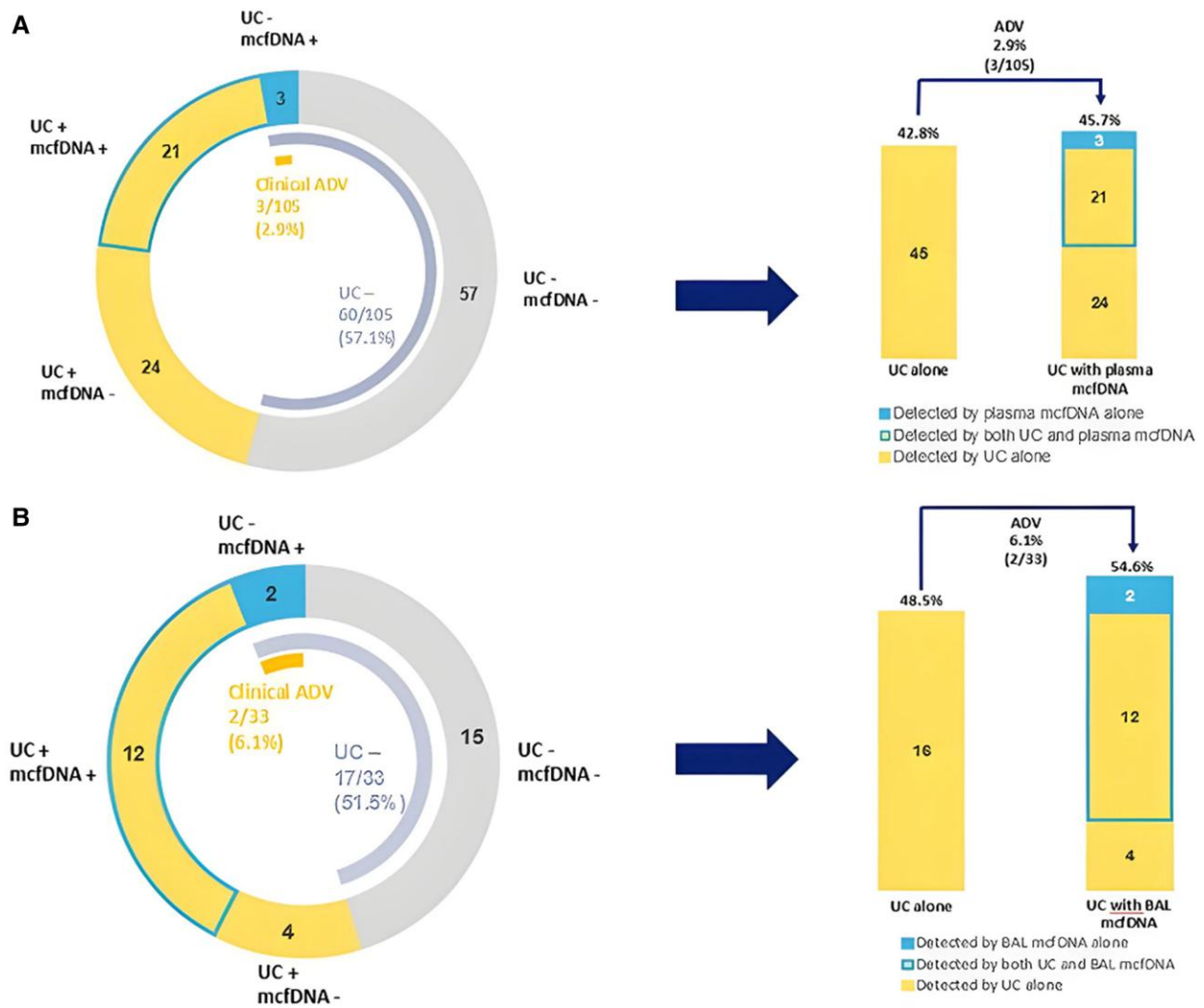
Figure 1. Additional diagnostic value (ADV) of the Karius Test on plasma and research-only-use test on BAL fluid compared to the EORTC/MSGERC definitions. *A*: Diagnostic value of the Karius test (mcfDNA) on plasma compared with the modified EORTC/MSGERC consensus definitions (Mucorales PCR added to the definitions for IM). NGS on plasma was successful in 105 patients. In 60 patients, EORTC/MSGERC criteria were not fulfilled for an invasive fungal infection disease (IFID). In 3 (2.9%) of these patients, the KT (mcfDNA test) showed a fungal pathogen with potential to cause an IFIIFD. This was categorized as a possible ADV. Importantly, 3 patients had a co-infection next to an IA; however, only the co-infection could be confirmed by the KT test (*Pneumocystis* in 2 patients, *Mucorales* in 1 patient). Additionally, 1 patient had a probable IM, but the KT only showed *Aspergillus* in plasma. These patients was also categorized in the "UC + mcfDNA+" group. *B*: Diagnostic value of the research-use-only Karius test (mcfDNA) on bronchoalveolar fluid (BALf) compared with the modified EORTC/MSGERC consensus definitions (*Mucorales* PCR added to the definitions for IM). NGS on BALf was successful in 33 patients. In 17 patients, EORTC/MSGERC criteria were not fulfilled for an invasive fungal infection disease (IFDI). In 2 (6.1%) of these patients, the KT (mcfDNA test) showed a fungal pathogen with potential to cause an invasive fungal infection disease (IFID). This was categorized as a possible ADV. Importantly, 1 patient was suspected for an *Aspergillus* and *Mucorales* co-infection based on the modified EORTC/MSGERC definitions. The KT test could only confirm the *Aspergillus* species. Abbreviations: ADV, additional diagnostic value; KT, Karius test; mcfDNA, microbial cell-free DNA; UC, usual care.

suspected IFD, Hill and colleagues reported an overall sensitivity of 51%, which is comparable to the 50% sensitivity we observed in this subgroup. Similar to our findings, they observed a higher sensitivity in non-*Aspergillus* mold infections (79%) than for IA (31%) [18]. Hong et al. also reported that in all 5 proven cases of non-*Aspergillus* mold infections, the KT was positive [14]. More recently, Hoenigl et al. showed a high sensitivity of the KT in patients with COVID-associated pulmonary aspergillosis [19]. In our study, we found that the KT on plasma potentially had an additional diagnostic value in only 3 (2.9%) patients compared with the already available diagnostic armamentarium based on culture, GM, and PCR. This increased to 6.1% when the RUO-KT was performed on BAL fluid. In a recent study by Bergin et al., plasma mcfDNA testing resulted in a higher additional diagnostic value of 12.1% (95% CI, 7.7–18.0). Because they also took the bacterial and viral DNA sequencing results into account, this higher additive diagnostic value is not surprising [16]. Additional diagnostic value will be higher in centers unable to perform PCR-based testing for *Mucorales*, *Aspergillus*, and *P jirovecii* DNA. Another strategy related to mcfDNA sequencing

might be optimizing a cfDNA PCR on serum or plasma instead of sequencing. Currently, a plasma cfDNA fungal panel has also been created by the group of Banaei and colleagues that was able to identify *Aspergilli*, *Mucorales*, *Pneumocystis*, and other species. When a large plasma volume (>2 mL) was used for DNA extraction, sensitivity and specificity were 69.6% (95% CI, 57.9–79.2) and 99.5% (95% CI, 99.0–99.8), respectively. This panel additionally showed a high clinical impact [20] cfDNA PCRs targeting a single species had an even better performance, but at the cost of losing the ability to identify multiple species using 1 test [21, 22].

In this study, and for the first time, the diagnostic performance of the RUO-KT test was evaluated on BAL fluid as well. As expected, the sensitivity was substantially higher on BAL compared with plasma (72.2% vs 44.0%) and fungal DNA detected in plasma was only missed in BAL fluid in 2 patients. Therefore, in cases where conventional diagnostic tests have failed and BAL fluid sampling is pursued, performing the RUO-KT on BAL fluid could prove beneficial. The disadvantage of using BAL fluid is that DNA from commensal flora will often be detected and clinical adjudication will be essential to distinguish invasive pathogens from commensal flora. This may also explain the lower specificity we observed for the RUO-KT on BAL (88.2%) compared with plasma (96.6%). Furthermore, the abundance of commensal DNA could occasionally mask the presence of DNA from invasive pathogens [15].

Our study has limitations. First, the patients included in this study had been enrolled in an ongoing study with a case report form limited to the diagnosis and treatment of IFD. We were therefore unable to look at any additional diagnostic value for bacterial, viral, or parasitic pulmonary infections [16]. Despite being 1 of the largest published studies on the value of the KT in immunocompromised patients with a suspected IFD, the number of patients with an invasive pulmonary aspergillosis (n = 39) still remained relatively small and invasive mucormycosis or PJP was diagnosed in less than a handful of patients. This precludes definite conclusions on the latter pathogens. Second, the decision of the treating physician to test for the presence of non-*Aspergillus* species (eg, using a *Mucorales* or *Pneumocystis* PCR) was not standardized but was typically made in a patient with negative IA diagnostics. We can therefore not exclude that 1 or more invasive mucormycosis or PJP infections were missed.

From a practical point of view, the actual turnaround time of the KT will depend on the transportation time to and the location of the KT site as well as the time between sample arrival and the generated report. Currently, there is only 1 test site in the United States [15, 18]. As with any other fungal diagnostic test, measuring the actual clinical impact of the KT on the outcome of patients with a suspected IFD is not possible based on observational data. Ideally, this should be investigated in future prospective randomized clinical trials but it is unlikely that such studies will be performed in the (near) future. The

moderate sensitivity of the KT on plasma in patients with an IA means that it cannot be used as a rule-out test for IA. However, the better sensitivity in non-*Aspergillus* mold infections suggests that in patients in which the readily available *Aspergillus* diagnostic tests turn out negative, it may be a sensible next step to use mcfDNA sequencing to look for the presence of DNA from other fungal pathogens as well as fungal mimickers like *Nocardia* or *Legionella*.

Future research is necessary to explore other patient populations in whom plasma mcfDNA sequencing may proof useful. These may be patients with a suspicion of deep-seated infections but negative cultures (eg, endocarditis, spondylodiscitis, suspected mycotic aortic aneurysms) [23]. In addition, a decrease in the DNA load over time may serve as a biomarker of treatment response as suggested in a small case series [24]. Finally, future studies should look into the utility of mcfDNA testing in other bodily fluids (eg, joint aspirate, cerebrospinal fluid).

In conclusion, detecting and sequencing fungal mcfDNA in plasma or BAL fluid had a moderate sensitivity but high specificity, with a better performance for non-*Aspergillus* IFD compared with IA. Further studies should assess its potential clinical impact in diverse clinical scenarios.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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