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Diagnosis of invasive aspergillosis in hematological malignancy patients: Performance of cytokines, *Asp* LFD, and *Aspergillus* PCR in same day blood and bronchoalveolar lavage samples☆

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SUMMARY

Background: *Aspergillus* spp. induce elevated levels of several cytokines. It remains unknown whether these cytokines hold value for clinical routine and enhance diagnostic performances of established and novel biomarkers/tests for invasive aspergillosis (IA).
Methods: This cohort study included 106 prospectively enrolled (2014–2017) adult cases with underlying hematological malignancies and suspected pulmonary infection undergoing bronchoscopy. Serum samples were collected within 24 hours of bronchoalveolar lavage fluid (BALF) sampling. Both, serum and BALF samples were used to evaluate diagnostic performances of the *Aspergillus*-specific lateral-flow device test (LFD), *Aspergillus* PCR, β-D-glucan, and cytokines that have shown significant associations with IA before.
Results: Among 106 cases, 11 had probable IA, and 32 possible IA; 80% received mold-active antifungals at the time of sampling. Diagnostic tests and biomarkers showed better performance in BALF versus blood, with the exception of serum interleukin (IL)-8 which was the most reliable blood biomarker. Combinations of serum IL-8 with either BALF LFD (sensitivity 100%, specificity 94%) or BALF PCR (sensitivity 91%, specificity 97%) showed promise for differentiating probable IA from no IA.
Conclusions: High serum IL-8 levels were highly specific, and when combined with either the BALF *Aspergillus*-specific LFD, or BALF *Aspergillus* PCR also highly sensitive for diagnosis of IA.

Introduction

Invasive mold infections (IMI) including invasive aspergillosis (IA) are associated with high morbidity and mortality among patients with underlying hematological malignancies.[1,2] Mycological diagnosis is challenging, with cultures of bronchoalveolar lavage fluid (BALF) having a low sensitivity.[3,4] Consequently, fungal biomarkers such as galactomannan [GM], beta-D-glucan [BDG] and also molecular diagnostic tests (polymerase chain reaction [PCR]) of blood or BALF, have emerged and are now widely used for diagnosing IA.[4,5] However, performance of these biomarkers and tests has been shown to be far from perfect, particularly among patients receiving mold-active prophylaxis or treatment, which has been shown to reduce sensitivity of diagnostic tests for IA.[5–10] Despite continuing advances in the diagnostic arsenal, with emerging new diagnostic tests such as the *Aspergillus*-specific lateral-flow device

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test [LFD], and new biomarkers[11] on the horizon, IA remains difficult to diagnose.

Performance of currently available biomarkers and tests for early diagnosis of IA and IMI may be enhanced by combination with sensitive and specific immunological markers. In fact, *Aspergillus* spp. have been shown to induce T-helper cell (Th) 1 and Th17 subsets resulting in elevated levels of several cytokines,[12,13] and recent studies have indicated – after adjusting for multiple covariates also associated with higher cytokine levels – that particularly Interleukin (IL)-8 and IL-6 may show promise as diagnostic markers.[14,15] It remains however unknown whether these cytokines hold value for clinical routine. Also the added benefit of cytokine testing in addition to, for example, GM testing or PCR has not been evaluated yet.

The objective of this prospective cohort study was to determine the diagnostic potential of IL-8, IL-6 and other cytokines, as well as established and emerging tests for IA and IMI in patients with underlying hematological malignancies in a setting that uses mold-active prophylaxis.

Materials and methods

This prospective cohort study comprised paired routine serum and BALF samples obtained on the same day from cases with underlying hematological malignancies who underwent routine bronchoscopy due to suspicion of pulmonary infections.

Study cohort

In total 122 cases undergoing bronchoscopy were prospectively enrolled at the Medical University of Graz, Austria, between April 2014 and July 2017. Key inclusion criteria were i.) adult patients with ii.) underlying hematological malignancy who were iii.) at risk for IPA according to the attending clinicians (e.g. febrile neutropenia, induction chemotherapy for acute myeloid leukemia, allogeneic stem cell transplantation) and had iv.) a BALF sample obtained in clinical routine due to suspicion of infection. All patients who met inclusion criteria between April 2014 and July 2017 and signed an informed consent were included in the cohort. After signing the informed consent serum and whole blood samples were collected at the day of bronchoscopy. A total of 16 cases had to be excluded due to the following reasons: 1.) same day blood samples (i.e. collected within 24-h) were not available ($n = 14$); 2.) BALF volume after routine testings' were insufficient for further diagnostic work up within the study protocol ($n = 1$); 3.) hematological malignancy was suspected but not confirmed because of mortality within days of admission ($n = 1$). After exclusion of these 16 cases, 106 patients remained in the final analysis.

Biomarker testing

Conventional culture as well as BALF and serum GM concentrations (Platelia EIA; Bio-Rad Laboratories, Vienna, Austria) were prospectively determined in clinical routine at the Medical University of Graz. Given that the vast majority of patients received mold-active antifungals at the time of bronchoscopy cut-offs of 0.5 GM optical density index (ODI) where used for serum and BALF, following previous evidence that the 0.5 ODI cutoff is preferable in patients on mold-active antifungals.[16]

BDG testing was performed in part prospectively and in part retrospectively at the Medical University of Graz, using the commercial available Fungitell® assay (Cape Cod Diagnostics, Falmouth, MA) with an adopted protocol suitable for use on a routine BCS XP® coagulation analyzer, as described previously.[17] BDG testing was only performed in serum samples, as BALF BDG testing yields very low specificity, which is explained by the fact that non-pathogenic *Candida* colonization in the lung is leading to high BDG values.[18–20] For serum BDG we used the recommended cut-off of ≥ 80 pg/mL to define positivity.

All blood and BALF samples were initially stored at 4 °C. Aliquots of 2 mL were shipped overnight to the Department of Hematology and Oncology, University Hospital of Mannheim, Heidelberg University where a nested *Aspergillus* PCR assay was performed prospectively in all study samples according to the protocol modified by Skladny et al. [21,22] PCR assays were performed according to the protocol for blood and BALF samples.[21] Extraction of DNA was processed according to the protocol of Sambrook et al.[22]. As an internal control, a 138-bp PCR fragment encoded by the human glucose-6-phosphate dehydrogenase gene was amplified in each clinical sample.

Remaining blood and BALF isolates were stored at −70 °C. Cytokine concentrations were determined in serum and BALF samples at the Center for Medical Research of the Medical University of Graz, Austria, between 09/2016 and 10/2017 with a personalized ProcartaPlex® 11plex immunoassay (eBioscience, Vienna, Austria) as described before.[14] Investigators measuring cytokine levels were blinded towards classification of cases into IA and IMI categories and all other clinical and demographic information. The 11 cytokines in the immunoassay were selected based on published literature in human and animal models showing an increase or decrease of these cytokines in blood and/or BALF of cases with IPA.[14,15,23–28] The cytokines measured were: IL-4, IL-6, IL-8, IL-10, IL-15, IL-17A, IL-22, soluble IL-2 receptor (sIL-2r), tumors necrosis factor (TNF) α, interferon (IFN) γ, and RANTES ("regulated on activation, normal T cell expressed and secreted", synonym: chemokine ligand 5). For inclusion into the main analyses we selected only those cytokines that have shown significant associations with IA in the previously conducted nested case-control analysis matched for multiple covariates, including neutrophil status, immunosuppressant and concomitant viral and bacterial infections.[14]

Testing with the new *Asp* LFD (OLM Diagnostics, Newcastle-upon-Tyne, United Kingdom) was performed in the Microbiology Laboratory at the Medical University of Graz in 12/2017. Stored non-hemorrhagic BALF samples where thawed, vortexed, centrifuged at 14,000 G and immediately tested by applying 70 µL of BALF supernatant to the test, as described previously,[19,29] with results read 10 and 15 min later. Following the manufacturer's instructions, hemorrhagic BALF (after being vortexed and centrifuged) and serum samples were mixed 1:2 with the accompanying buffer, vortexed, heated and centrifuged at 14,000 G. 70 µL supernatant was applied to the LFD and results were read after 15 min and also after 45 min.

IA classification and Statistical analysis

IA was graded in accordance with the revised criteria by the European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group (EORTC) and the Mycoses Study Group of the National Institute of Allergy and Infectious Disease (MSG),[30] with the exclusion of BDG as a mycological criterion. IMI was graded according to the same criteria but with the inclusion of serum BDG as mycological criterion (i.e., included only in the absence of Pneumocystis or Candida infection).

Our study was conducted in accordance with the Declaration of Helsinki, 2013, Good Clinical Practice. The study protocol was approved by the local ethics committee, Medical University Graz, Austria (EC-numbers 25–221 and 23–343), and registered at ClinicalTrials.Gov (Identifier: NCT02058316 and NCT01576653). Statistical analysis was performed using SPSS, version 24 (SPSS Inc., Chicago, IL, USA). For continuous data, including cytokine levels,

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