### Title

Levels of interleukin (IL)-6 and IL-8 are elevated in serum and bronchoalveolar lavage fluid of haematological patients with invasive pulmonary aspergillosis




### Journal

Mycoses, 60(12)

### ISSN

0933-7407



### Authors

Heldt, Sven
Eigl, Susanne
Prattes, Juergen
et al.



### Publication Date

2017-12-01

### DOI

10.1111/myc.12679

Peer reviewed

Received: 04 June 2017 | Revised: 03 August 2017 | Accepted: 4 August 2017

DOI: 10.1111/myc.12679

ORIGINAL ARTICLE

WILEY mycoses Diagnosis, Therapy and Prophylaxis of Fungal Diseases

# Levels of interleukin (IL)-6 and IL-8 are elevated in serum and bronchoalveolar lavage fluid of haematological patients with invasive pulmonary aspergillosis

Sven Heldt[1,2] | Susanne Eigl[1] | Juergen Prattes[2,3] | Holger Flick[1] | Jasmin Rabensteiner[4] | Florian Prüller[4] | Tobias Niedrist[4] | Peter Neumeister[5] | Albert Wölfler[3,5] | Heimo Strohmaier[6] | Robert Krause[2,3] | Martin Hoenigl[1,2,3,7]

[1]Division of Pulmonology, Medical University of Graz, Graz, Austria

[2]Section of Infectious Diseases and Tropical Medicine, Medical University of Graz, Graz, Austria

[3]CBmed - Center for Biomarker Research in Medicine, Graz, Austria

[4]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria

[5]Division of Hematology, Medical University of Graz, Graz, Austria

[6]Center for Medical Research, Medical University of Graz, Graz, Austria

[7]Division of Infectious Diseases, Department of Medicine, University of California-San Diego, San Diego, USA

**Correspondence**
Martin Hoenigl, Section of Infectious Diseases and Tropical Medicine, Division of Pulmonology, Department of Internal Medicine, Medical University of Graz, Graz, Austria.
Email: martin.hoenigl@medunigraz.at

**Funding information**
This work was supported by funds of the Gilead Investigator Initiated Study IN-AT-131-1939, and the Oesterreichische Nationalbank (Anniversary Fund, project number 15346). This work has also partly been carried out with the K1 COMET Competence Center CBmed, which is funded by the Federal Ministry of Transport, Innovation and Technology (BMVIT); the Federal Ministry of Science, Research and Economy (BMWFW); Land Steiermark (Department 12, Business and Innovation); the Styrian Business Promotion Agency (SFG) and the Vienna Business Agency. The COMET program is executed by the FFG (The Austrian Research Promotion Agency, project number 844609). The funders had no role in the study design, data collection, analysis, interpretation, decision to publish, writing of the manuscript and decision to submit the manuscript for publication.

## Summary

*Aspergillus* spp. have been shown to induce T-helper cell (Th) 1 and Th17 subsets resulting in elevated levels of several cytokines. The objective of this study was to analyse a bundle of cytokines in serum and bronchoalveolar lavage fluid (BALF) in patients with and without invasive pulmonary aspergillosis (IPA). This nested case-control analysis included 10 patients with probable/proven IPA and 20 matched controls without evidence of IPA, out of a pool of prospectively enrolled (2014-2017) adult cases with underlying haematological malignancies and suspected pulmonary infection. Serum samples were collected within 24 hours of BALF sampling. All samples were stored at −70°C for retrospective determination of cytokines. IL-6 and IL-8 were significantly associated with IPA in both serum ($P = .011$ and $P = .028$) and BALF ($P = .006$ and $P = .012$, respectively), and a trend was observed for serum IL-10 ($P = .059$). In multivariate conditional logistic regression analysis, IL-10 remained a significant predictor of IPA in serum and IL-8 among BALF cytokines. In conclusion, levels of IL-6 and IL-8 were significantly associated with probable/proven IPA, and a similar trend was observed for serum IL-10. Future cohort studies should determine the diagnostic potential of these cytokines for IPA, and evaluate combinations with other IPA biomarkers/diagnostic tests.

**KEYWORDS**
*Aspergillus*, BAL, haematological malignancy, IFN-γ, IL-10, IL-17A, serum

Original data of this manuscript have been presented in part at ECCMID 2017, Vienna, Austria (poster presentation number 0989) 51st Annual Meeting DMYKg 2017, Muenster, Germany (oral presentation), and TIMM 2017, Belgrade, Serbia (poster presentation).

  

## 1 | INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is associated with high morbidity and mortality among patients with underlying haematological malignancies.[1,2] Due to the crude mortality of 80%-90% in the absence of adequate treatment, timely diagnosis and early start of antifungal therapy are key factors in the successful treatment of IPA.[3,4] The introduction of non-cultural diagnostic tests for IPA in blood and bronchoalveolar lavage fluid (BALF), including galactomannan antigen (GM) testing,[5,6] PCR,[7,8] the *Aspergillus*-specific lateral flow device test[9-12] and 1,3-β-D-glucan (BDG) testing,[13] was associated with a significant increase in the rate of IPA-diagnosed premortem (vs postmortem).[14] Despite these significant advancements, performance of these non-cultural diagnostic tests is varying, and the search for a reliable gold standard for diagnosis of IPA premortem continues.

Performance of currently available biomarkers 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.[15,16] However, diagnostic potential of these immunological markers for diagnosis of IPA in a clinical setting has not been evaluated yet. The objective of this nested case-control analysis of a prospective cohort study was to analyse a bundle of cytokines in serum and BALF in adult patients with underlying haematological malignancies with and without IPA.

## 2 | MATERIALS AND METHODS

This nested case-control study of prospectively collected data comprised paired routine serum and BALF samples obtained on the same day from 10 cases with IPA and 20 matched controls without IPA.

In total, 106 patients with haematological malignancies undergoing bronchoscopy were prospectively enrolled at the Medical University of Graz, Austria, between April 2014 and March 2017. Key inclusion criteria were (i) adult patients with (ii) underlying haematological malignancy (confirmed in all but one case who was admitted to the ICU and died within hours of admission and bronchoscopy, before the haematological malignancy could be confirmed) who were (iii) at risk for IPA according to the attending clinicians (eg, febrile neutropenia, induction chemotherapy for acute myeloid leukaemia, 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 March 2017 and signed informed consent where included in the cohort. GM was routinely performed in all serum and BALF samples and IPA 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).[17]

A total of 10 patients had probable or proven IPA and serum plus BALF samples available (n = 8 probable IA and n = 2 proven IA) and were included in this analysis. These 10 cases were matched with each 2 controls out of a pool of 56 patients not fulfilling IPA criteria enrolled into this cohort (ie, 34 patients with possible IPA and/or missing serum/BALF sample were excluded). Controls were individually matched to cases by factors determined previously to potentially influence outcome and cytokine levels: (i) presence and duration of neutropenia,[18] (ii) presence/absence of (a) allogeneic/autologous stem cell transplantation,[19] and (b) graft vs host disease (GVHD),[19,20] (iii) underlying diseases,[18,21] (iv) receipt of corticosteroids within a week before bronchoscopy[22] and (v) viral infection within 2 weeks before bronchoscopy (ie, detection of virus in blood or BALF using nucleic acid amplification testing).[21]

Serum samples of study participants were collected at the day of bronchoscopy and stored at −70°C for retrospective cytokine measurement. Cytokine concentrations of participants enrolled into this nested case-control study were determined by the Core Facility Imaging at the Center for Medical Research of the Medical University of Graz, Austria, between September 2016 and April 2017 with a ProcartaPlex® 11plex immunoassay (eBioscience, Vienna, Austria). Investigators measuring cytokine levels were blinded towards classification of cases into IPA 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.[15,23-36] The cytokines studied were: interleukin (IL)-4, IL-6, IL-8, IL-10, IL-15, IL-17A, IL-22, soluble IL-2 receptor (sIL-2r), tumours necrosis factor (TNF) α, interferon (IFN) γ and RANTES (chemokine ligand 5). Twenty-five microlitres of undiluted freshly thawed serum samples were processed in 96-well plates according to the manufacturer's instructions using magnetic beads. Standards for each cytokine were assayed in duplicates to generate standard curves using the reference concentrations as provided by the manufacturer. Data were obtained on a validated and calibrated Bio-Plex 200 system (Bio-Rad, Vienna, Austria) and analysed with Bio-Plex Manager 6.1 software (Bio-Rad, Vienna, Austria). The cytokine concentration was calculated from the standard curve using 5PL curve fitting. Cytokine levels below the standard range were extrapolated to give approximate values. Levels of cytokines are displayed in pg/mL.

Our study was conducted in accordance with the Declaration of Helsinki, 1996, Good Clinical Practice and applicable local regulatory requirements and law. The study protocol was approved by the local ethics committees, 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 23 (SPSS Inc., Chicago, IL, USA). Categorical data are displayed as proportions, continuous data as medians plus interquartile range (IQR) or means plus 95% confidence interval (95% CI) as appropriate. Comparisons between patient groups were performed using chi-squared test for proportions, the Mann-Whitney U test and Kruskal-Wallis test for nonparametric data. The *P* values were not corrected for multiple comparisons and are therefore only descriptive. Receiver operating characteristic (ROC) curve analyses were performed and area under the curve (AUC) values are presented including 95% CI, for cytokine levels using two approaches: (i) including extrapolated levels if below the standard range, and (ii)

levels below the standard range set to 0. Optimal cut-offs for discriminating patients with and without IPA were calculated by using the Youdens index. Utilising Cox survival analysis in SPSS, conditional logistic regression models were calculated for matched case-control pairs utilising these optimal cut-offs and adjusted for covariates used for matching, and hazard ratios (HR) including 95% CI were displayed. The sample size of 30 (with 10 cases and 20 controls) gave us 80% power ($\alpha = .05$) to detect a HR of 3.0 or above. Two-sided $P<.05$ was taken as cut-off for statistical significance.

## 3 | RESULTS

A total of 30 patients were included in the final analysis. Ten patients with proven (n = 2) or probable [n = 8; all 8 had a BALF GM ≥1 optical density index and 2/8 also had a positive serum GM result (≥0.5 optical density index) on the same day] IPA, and 20 patients classified as not having IPA according to EORTC/MSG 2008 criteria. Patients' characteristics are displayed in Table 1.

For all patients corresponding concurrent BALF and serum samples were collected within a time frame of <24 hours. Median, IQR, minimum and maximum cytokine levels in cases and controls are depicted in Table 2. Box plots for IL-6, IL-8, and IL-10 in serum are depicted in Figure 1, box plots for IL-6 and IL-8 in BALF are depicted in Figure 2.

AUCs for serum cytokines for differentiating between cases and controls with and without extrapolated levels are depicted in Table 3. When including extrapolated levels IL-6, IL-8, IL-10 and IL-17A were all significantly associated with IPA with AUCs between 0.73 and 0.81. After removing extrapolated levels below the standard range, however, only IL-6 and IL-8 were significantly associated with IPA and a trend was observed for IL-10 ($P = .059$). AUCs for BALF cytokines are also depicted in Table 3. Both IL-6 and IL-8 were significantly associated with IPA with AUCs of 0.810 and 0.785, respectively. ROC curves for IL-6, IL-8 and IL-10 in serum are displayed in Figure 3A, ROC curves for IL-6 and IL-8 in BALF are displayed in Figure 3B.

Cut-offs calculated for serum by using Youdens index as well as results of univariate conditional logistic regression analysis for predicting the event of IPA are depicted in Table 4. In multivariate conditional logistic regression analysis of serum cytokines, only IL-10 (cut-off 6.75 pg/mL; HR 10.568, 95% CI: 1.255-89.005; $P = .030$) remained a significant predictor of IPA, while IL-6, IL-8 as well as covariates used for matching were not significant. In multivariate conditional logistic regression analysis of BALF cytokines only IL-8 (cut-off 710 pg/mL;

**TABLE 1** Demographic data and underlying diseases of cases with probable/proven invasive pulmonary aspergillosis (IPA) and controls without evidence for IPA

| Demographic data, underlying diseases and other characteristics at the time of sampling | Probable/proven IPA (n = 10) | No evidence for IPA (n = 20) |
|---|---|---|
| Sex | | |
| Female | 5 (50%) | 14 (70%) |
| Male | 5 (50%) | 6 (30%) |
| Age, y | | |
| Range | 48-73 | 26-74 |
| Median | 54.5 | 60 |
| Underlying diseases | | |
| AML | 5 (50%) | 10 (50%) |
| NHL | 1 (10%) | 2 (10%) |
| MM | 1 (10%) | 2 (10%) |
| ALL | 2 (20%) | 3 (15%) |
| Others[a] | 1 (10%) | 3 (15%) |
| Other characteristics | | |
| Autologous SCT | 1 (10%) | 2 (10%) |
| Allogeneic SCT | 3 (30%) | 7 (35%) |
| GvHD | 2 (20%) | 5 (25%) |
| Systemic corticosteroid treatment within 14 d of sampling | 4 (40%) | 4 (20%) |
| Viral infection with immunomodulating viruses diagnosed within 14 d of sampling | 3 (30%) | 5 (25%) |
| Neutropenia (<500/μL) ≤10 d | 3 (30%) | 5 (25%) |
| Neutropenia >10 d (<500/μL) | 3 (30%) | 4 (20%) |

[a]Included cases of aplastic anaemia, chronic lymphatic leukaemia and active tuberculosis.
ALL, acute lymphocytic leukaemia; AML, acute myelogenous leukaemia; IPA, invasive pulmonary aspergillosis; MM, multiple myeloma; NHL, Non-Hodgkin lymphoma; SCT, stem cell transplantation; GvHD, graft vs host disease.

**TABLE 2** Median and interquartile range (IQR), as well as minimum (Min) and maximum (Max) of cytokine levels (pg/mL) in IPA cases (n = 10) vs matched controls without IPA (n = 20)

| | | Probable/proven invasive pulmonary aspergillosis | | | | No evidence for invasive pulmonary aspergillosis | | | |
|---|---|---|---|---|---|---|---|---|---|
| Material | Cytokine | Median | IQR | Min | Max | Median | IQR | Min | Max |
| Serum | IFNγ | 2.73 | 1.64-5.60 | 1.29 | 39.30 | 2.17 | 1.37-3.28 | 0.76 | 4.97 |
| | IL-10 | 7.66 | 0.41-37.46 | 0 | 358.7 | 0 | 0-1.94 | 0 | 220.2 |
| | IL-15 | 1.77 | 0-4.48 | 0 | 14.85 | 0 | 0-0 | 0 | 9.52 |
| | IL-17A | 0.39 | 0-1.58 | 0 | 3.65 | 0 | 0-0.2 | 0 | 0.65 |
| | sIL-2R | 66 654 | 22 210-104 229 | 11 325 | 158 267 | 44 086 | 32 786-66 060 | 9951 | 113 667 |
| | IL-22 | 0 | 0-120.4 | 0 | 813.7 | 0 | 0-0 | 0 | 140.0 |
| | IL-4 | 0.12 | 0-4.55 | 0 | 10.83 | 0 | 0-0.17 | 0 | 12.80 |
| | IL-6 | 292.5 | 16.98-1645 | 7.76 | 3049 | 9.90 | 3.68-36.64 | 0 | 3045 |
| | IL-8 | 225.3 | 11.76-938.4 | 1.98 | 2359 | 9.37 | 1.64-17.78 | 0.41 | 490.5 |
| | RANTES | 65.84 | 34.95-451.5 | 21.74 | 608.2 | 34.57 | 23.76-89.65 | 0 | 362.9 |
| | TNFα | 2.66 | 1.37-3.75 | 0.98 | 4.25 | 1.54 | 1.26-2.92 | 0.92 | 3.65 |
| BALF | IFNγ | 1.69 | 1.29-2.09 | 0.82 | 5.43 | 1.65 | 1.29-1.84 | 0.94 | 2.22 |
| | IL-10 | 0 | 0-1.50 | 0 | 15.96 | 0 | 0-0 | 0 | 2.98 |
| | IL-15 | 4.11 | 2.15-4.11 | 0 | 7.33 | 4.11 | 0-11.64 | 0 | 38.20 |
| | IL-17A | 0.80 | 0-4.32 | 0 | 5.04 | 0.12 | 0-1.23 | 0 | 8.58 |
| | sIL-2R | 253 | 139.5-533.9 | 0 | 1193 | 125.1 | 48.97-356.2 | 0 | 3689 |
| | IL-22 | 72.01 | 31.82-177.1 | 0 | 281.3 | 59.5 | 0-187.6 | 0 | 797.0 |
| | IL-4 | 1.95 | 1.35-4.05 | 0.87 | 11.25 | 4.05 | 2.33-5.78 | 1.02 | 7.67 |
| | IL-6 | 635.9 | 126.2-2267 | 56.30 | 2953 | 33.11 | 12.69-85.10 | 5.89 | 70 685 |
| | IL-8 | 1731 | 940.2-3916 | 263.7 | 5706 | 450.7 | 194.3-792.8 | 80.57 | 5673 |
| | RANTES | 8.74 | 3.40-31.38 | 2.62 | 109.8 | 6.92 | 2.61-16.79 | 0.88 | 34.14 |
| | TNFα | 2.14 | 1.49-3.49 | 0.92 | 6.19 | 1.47 | 0.96-2.53 | 0.62 | 17.32 |

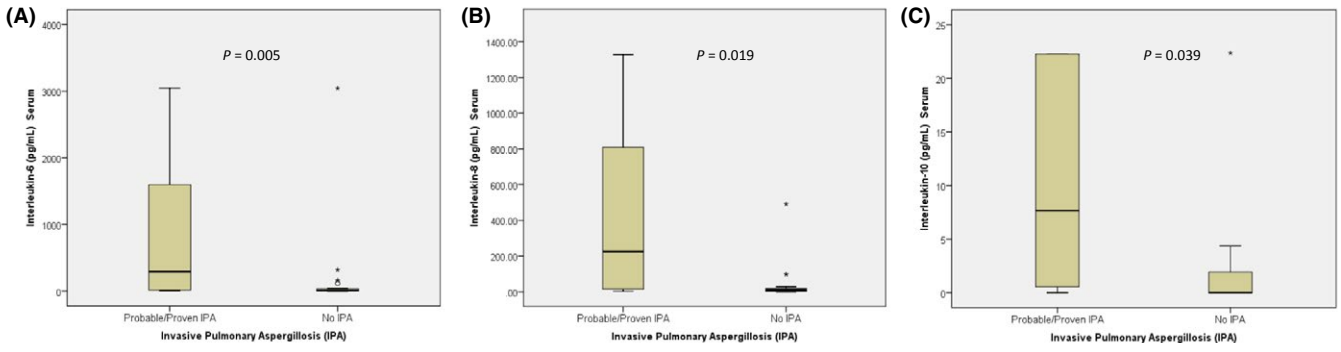


**FIGURE 1** Box plots of serum interleukin (IL) 6 (A), IL-8 (B) and IL-10 (C) serum levels in patients with probable/proven IPA vs matched controls without IPA. Extrapolated values below the standard range are included. *are outliers.

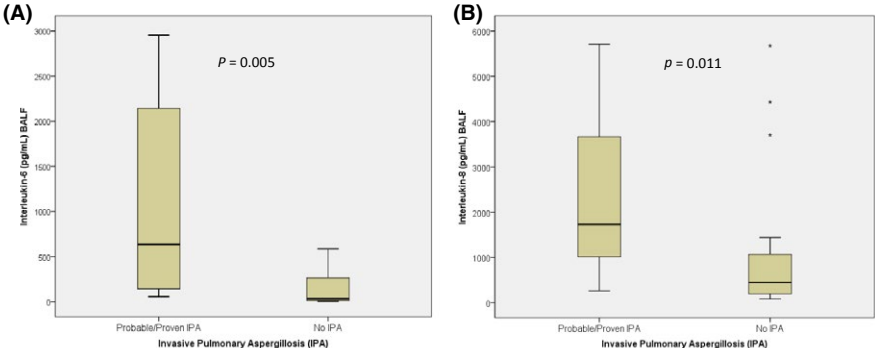


**FIGURE 2** Box plots of serum interleukin (IL) 6 (A) and IL-8 (B) bronchoalveolar fluid levels in patients with probable/proven IPA vs matched controls without IPA. Extrapolated values below the standard range are included. *are outliers.

**TABLE 3** Performance of cytokine levels in serum and bronchoalveolar fluid (BALF) for differentiating cases with probable/proven IPA (n = 10) from matched controls (n = 20)

| | Serum (including extrapolated values if below standard range) | | | Serum (excluding extrapolated values if below standard range) | | | BALF (including extrapolated values if below Standard range) | | | BALF (excluding extrapolated values if below standard range) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cytokine | AUC | 95% CI | *P* value | AUC | 95% CI | *P* value | AUC | 95% CI | *P* value | AUC | 95% CI | *P* value |
| IFNγ | 0.638 | 0.410-0.865 | .226 | 0.550 | 0.322-0.778 | .660 | 0.500 | 0.266-0.734 | 1.000 | 0.475 | 0.27256-0.694 | .826 |
| IL-10 | **0.735** | **0.535-0.935** | **.039** | 0.715 | 0.503-0.927 | .059 | 0.570 | 0.347-0.793 | .538 | 0.500 | 0.277-0.723 | 1.000 |
| IL-15 | 0.690 | 0.474-0.906 | .095 | 0.555 | 0.327-0.783 | .628 | 0.478 | 0.270-0.685 | .843 | 0.363 | 0.164-0.561 | .226 |
| IL-17A | **0.733** | **0.514-0.951** | **.041** | 0.600 | 0.370-0.830 | .379 | 0.593 | 0.359-0.826 | .416 | 0.610 | 0.384-0.836 | .333 |
| sIL-2R | 0.560 | 0.302-0.818 | .598 | 0.560 | 0.302-0.818 | .598 | 0.588 | 0.376-0.799 | .441 | 0.588 | 0.376-0.799 | .441 |
| IL-22 | 0.605 | 0.380-0.830 | .356 | 0.605 | 0.380-0.830 | .356 | 0.515 | 0300-0.730 | .895 | 0.515 | 0300-0.730 | .895 |
| IL-4 | 0.608 | 0.389-0.826 | .344 | 0.523 | 0.298-0.747 | .843 | **0.275** | **0.066-0.485** | **.048** | 0.528 | 0.301-0.754 | .809 |
| IL-6 | **0.810** | **0.650-0.970** | **.006** | **0.790** | **0.611-0.969** | **.011** | **0.810** | **0.655-0.965** | **.006** | **0.810** | **0.655-0.965** | **.006** |
| IL-8 | **0.765** | **0.578-0.952** | **.020** | **0.750** | **0.541-0.951** | **.028** | **0.785** | **0.617-0.953** | **.012** | **0.785** | **0.617-0.953** | **.012** |
| RANTES | 0.650 | 0.438-0.862 | .187 | 0.650 | 0.438-0.862 | .187 | 0.540 | 0.314-0.766 | .725 | 0.540 | 0.314-0.766 | .725 |
| TNFα | 0.658 | 0.438-0.877 | .166 | 0.500 | 0.277-0.723 | 1.000 | 0.655 | 0.453-0.857 | .173 | 0.500 | 0.277-0.723 | 1.000 |

Significant differences ($P < .05$) are in bold. AUC, area under the curve; CI, confidence interval

HR: 11.685, 95% CI: 1.423-95.915; $P = .022$) remained significant, while IL-6 as well as covariates used for matching were not significant.

Among patients with probable/proven IPA, 7 out of 10 had received mould active antifungals for more than 2 days before BALF and blood samples were obtained (median: 25 days, range: 8 to >360 days). Serum levels of IL-2R ($P = .033$) and IL-22 ($P = .017$) were significantly lower in those receiving antifungals >2 days, and trends towards lower levels were also observed for IL-8, IL-10 and IL-17A. In sub-analysis of only those three patients with probable/proven IPA who had received mould active antifungals for ≤2 days before sampling and their respective matched controls, serum IL-6, IL-8, IL-22 and RANTES were significantly higher in patients with IPA, while trends were also observed for IL-10 and IL-2R. In BALF, only IL-17A levels were significantly lower in those receiving antifungals >2 days versus those IPA cases without antifungals, with similar trends observed for IL-6 and IL-2R (all analyses after removal of extrapolated levels).

## 4 | DISCUSSION

In this nested case-control study, we investigated the diagnostic performance of a bundle of cytokines in serum and BALF for diagnosing IPA among patients with underlying haematological malignancies. We found that serum and BALF concentrations of IL-6 and IL-8 were significantly higher in patients with probable/proven IPA compared to those without evidence of IPA. We also found a trend towards elevated serum levels of IL-10, and in multivariate conditional logistic regression analysis serum IL-10 levels ≥6.75 pg/mL remained the sole predictor of probable/proven IPA.

Our main finding was that IL-6 and IL-8 levels in serum and BALF were significantly elevated in patients with probable/proven IPA vs controls with suspected pulmonary infection but no evidence of IPA. In median IL-6 and IL-8 levels were 20-30 times higher in serum and 4-20 times higher in BALF of those with probable/proven IPA vs those without evidence of IPA. Both cytokines are centrally involved in protective immunity against *Aspergillus* spp. In early stages of IPA, conidia are killed by local alveolar macrophages, and IL-8, also known as neutrophil chemotactic factor, is produced by macrophages and epithelial cells as an important chemoattractant for neutrophils.[15] Adaptive immunity develops when dendritic cells present fungal peptides to *Aspergillus*-specific $CD^{4+}$-naive T cells.[15] IL-6 plays an important role in T-cell recruitment and promotes differentiation of *Aspergillus*-specific $CD^{4+}$-naive T cells into Th2 and Th17 cells,[15,37] thereby influencing the Th1/Th2 balance which is known to be a critical factor determining the outcome of invasive fungal infections.[38]

The mechanism of IL-6 and IL-8 increase during IPA has been studied in a number of in vitro studies. In 1999, Borger and colleagues have reported an up-regulation of gene transcription by *Aspergillus fumigatus* proteases as cause of increased release of IL-6 and IL-8 by A549 pulmonary epithelial cells and primary epithelial cells.[39] More recent studies have shown that in vitro opsonisation of *A. fumigatus* conidia with H-ficolin,[40] L-ficolin [23] and M-ficolin,[41] which play essential roles in pathogen recognition and complement activation through

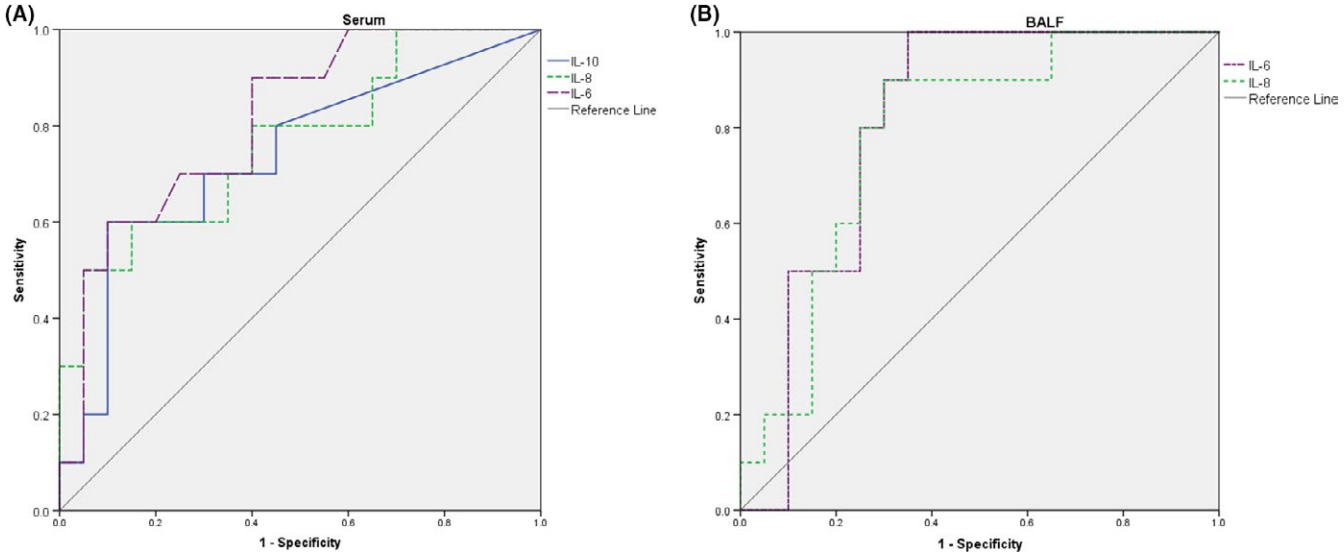


**FIGURE 3** Receiver operating characteristics (ROC) curve analysis of IL-6, IL-8 and IL-10 in serum and IL-6 and IL-8 in bronchoalveolar lavage fluid (BALF) for diagnosing probable/proven invasive pulmonary aspergillosis

**TABLE 4** Optimal cut-offs calculated using Youdens index for serum and BALF cytokines to differentiate cases with IPA from controls and results of conditional logistic regression analysis stratified by case-control triplets

| | | | | Conditional logistic regression (univariate) | | |
|---|---|---|---|---|---|---|
| **Cytokine** | **Cut-off (pg/mL)** | **Sensitivity** | **Specificity** | **Hazard ratios** | **95% confidence interval** | ***P* value** |
| *Serum* | | | | | | |
| IL-6 | >15 | 90 | 60 | 8.110 | −0.979-67.209 | .052 |
| | >200 | 60 | 90 | 10.568 | 1.255-89.005 | .030 |
| IL-8 | >88 | 60 | 85 | 76.775 | 0.044-135 210 | .255 |
| | >360 | 50 | 95 | 10.000 | 1.168-85.594 | .036 |
| IL-10 | >6.75 | 60 | 90 | 10.568 | 1.255-89.005 | .030 |
| *BALF* | | | | | | |
| IL-6 | >56 | 100 | 65 | 103.614 | 0.261-41 193.739 | .129 |
| IL-8 | 710 | 90 | 70 | 11.685 | 1.423-95.915 | .022 |

the lectin pathway, potentiate IL-8 secretion of A549 lung epithelial cells.[42] A similar mechanism has also been proposed for infections caused by *Aspergillus flavus*.[43] After in vitro stimulation with *A. fumigatus*, Kruppel-like Factor 4 has been shown to modulate IL-6 release in human dendritic cells.[37] Dectin-1-dependent IL-6 production regulates expression of iron chelators, haem and siderophore-binding proteins and hepcidin in infected mice and reduces systemic iron levels.[16,33,44] A pivotal role for IL-6 in protective immunity against *Aspergillus* has been reported in mice.[35] While IL-6 also plays a role in the transition from innate to acquired immunity during bacterial infection,[45] IL-6 may be predominantly elevated in IPA vs other infections of the lung, including *Pneumocystis carinii* pneumonia.[46]

We also found a trend towards increased serum levels of IL-10 in patients with probable/proven IPA and in serum IL-10 was the major predictor of IPA in multivariate conditional logistic regression analysis. IL-10 is an immunosuppressive cytokine and a central negative regulator of inflammatory responses, which has been attributed a largely detrimental role during fungal disease.[16,18,29,47] In a study by Potenza and colleagues, *Aspergillus*-specific T-cells producing non-protective IL-10 and protective IFN-γ were exclusively detected in haematologic malignancy patients with invasive aspergillosis and not in uninfected controls.[30] In contrast to findings of Potenza's study, we did not find an increase of IFN-γ levels in patients with IPA. We could also not verify findings by Ceesay and colleagues who reported that baseline IL-15, IL-2R, CCL2 and MIP-1α were significantly higher, while IL-4 was lower in patients with proven/probable invasive fungal infection compared to those with no evidence of fungal infection.[34] The latter study included a variety of fungal diseases, including yeast infections with very different immunological characteristics,[48] which may explain the difference to our nested case-control study which focused exclusively on IPA. IL-17A levels found in this study were very low in patients and controls, and once we excluded levels that were extrapolated below the standard range, not significantly different between cases and controls. This is in accordance with a previous study showing that *Aspergillus* is

a poor inducer of IL-17.[49] As an important limitation our nested case-matched control study design, which generally provides a better evidence level than a classic case-control study,[50] does not allow us to draw any conclusions regarding the added benefit of cytokine testing in addition to, for example, GM testing or PCR. Future larger cohort studies are needed to determine whether the diagnostic potential of IL-6, IL-8 and IL-10, without taking into account multiple covariates that may also result in higher cytokine levels, holds value for clinical routine. Also controls included in this study had suspected pulmonary infection for which they were undergoing routine bronchoscopy and microbiological workup of BALF samples. Other pulmonary infections may have caused increased levels of certain cytokines in controls explaining findings of this study that differ from previous studies which used uninfected controls. Cytokine levels may also vary according to underlying diseases and conditions for which our conditional analysis accounted for, however, larger studies are needed to evaluate whether cytokine levels can only be interpreted when taking into account these conditions. Finally, our study, although severely underpowered for subanalysis, showed some trends towards cytokines having less discriminatory power among those with ongoing mould-active antifungals. Future larger studies are also needed to evaluate this observation.

In conclusion, levels of IL-6 and IL-8 were significantly higher in patients with probable/proven IPA compared to controls without evidence of IPA. A trend was also observed for serum IL-10 levels. Future cohort studies should determine the diagnostic potential of these cytokines for IPA, and evaluate combinations of these cytokines with other IPA biomarkers/diagnostic tests, such as GM and PCR.

## ACKNOWLEDGMENTS

The authors acknowledge the support of Jennifer Ober and Sabrina Obersteiner in sample processing and testing.

## CONFLICTS OF INTEREST

J. Prattes received consulting fee from Gilead. A. Wölfler received speaker honoraria from Merck. M. Hoenigl received research grants from Gilead; served on the speakers' bureau of Gilead, Basilea and Merck. All other authors have no conflict of interest.

## ORCID

*Martin Hoenigl* http://orcid.org/0000-0002-1653-2824

## REFERENCES

1. Kontoyiannis DP, Marr KA, Park BJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the transplant-associated infection surveillance network (transnet) database. *Clin Infect Dis*. 2010;50:1091-1100.
2. Prattes J, Lackner M, Eigl S, et al. Diagnostic accuracy of the *Aspergillus*-specific bronchoalveolar lavage lateral-flow assay in haematological malignancy patients. *Mycoses*. 2015;58:461-469.
3. Greene RE, Schlamm HT, Oestmann JW, et al. Imaging findings in acute invasive pulmonary aspergillosis: clinical significance of the halo sign. *Clin Infect Dis*. 2007;44:373-379.
4. Lass-Florl C, Resch G, Nachbaur D, et al. The value of computed tomography-guided percutaneous lung biopsy for diagnosis of invasive fungal infection in immunocompromised patients. *Clin Infect Dis*. 2007;45:e101-e104.
5. Eigl S, Prattes J, Reinwald M, et al. Influence of mould-active antifungal treatment on the performance of the Aspergillus-specific bronchoalveolar lavage fluid lateral-flow device test. *Int J Antimicrob Agents*. 2015;46:401-405.
6. Hoenigl M, Salzer HJ, Raggam RB, et al. Impact of galactomannan testing on the prevalence of invasive aspergillosis in patients with hematological malignancies. *Med Mycol*. 2012;50:266-269.
7. Eigl S, Hoenigl M, Spiess B, et al. Galactomannan testing and Aspergillus PCR in same-day bronchoalveolar lavage and blood samples for diagnosis of invasive aspergillosis. *Med Mycol*. 2017;55:528-534.
8. Hoenigl M, Prattes J, Spiess B, et al. Performance of galactomannan, beta-d-glucan, Aspergillus lateral-flow device, conventional culture, and PCR tests with bronchoalveolar lavage fluid for diagnosis of invasive pulmonary aspergillosis. *J Clin Microbiol*. 2014;52:2039-2045.
9. Heldt S, Hoenigl M. Lateral flow assays for the diagnosis of invasive aspergillosis: current status. *Curr Fungal Infect Rep*. 2017;11:45-51.
10. Willinger B, Lackner M, Lass-Florl C, et al. Bronchoalveolar lavage lateral-flow device test for invasive pulmonary aspergillosis in solid organ transplant patients: a semiprospective multicenter study. *Transplantation*. 2014;98:898-902.
11. Prattes J, Flick H, Pruller F, et al. Novel tests for diagnosis of invasive aspergillosis in patients with underlying respiratory diseases. *Am J Respir Crit Care Med*. 2014;190:922-929.
12. Orasch T, Prattes J, Faserl K, et al. Bronchoalveolar lavage triacetylfusarinine C (TAFC) determination for diagnosis of invasive pulmonary aspergillosis in patients with hematological malignancies. *J Infect*. 2017. http://doi.org/10.1016/j.jinf.2017.05.014.
13. Reischies FM, Prattes J, Woelfler A, Eigl S, Hoenigl M. Diagnostic performance of 1,3-beta-d-glucan serum screening in patients receiving hematopoietic stem cell transplantation. *Transpl Infect Dis*. 2016;18:466-470.
14. Lewis RE, Cahyame-Zuniga L, Leventakos K, et al. Epidemiology and sites of involvement of invasive fungal infections in patients with haematological malignancies: a 20-year autopsy study. *Mycoses*. 2013;56:638-645.
15. Camargo JF, Husain S. Immune correlates of protection in human invasive aspergillosis. *Clin Infect Dis*. 2014;59:569-577.
16. Garcia-Vidal C, Viasus D, Carratala J. Pathogenesis of invasive fungal infections. *Curr Opin Infect Dis*. 2013;26:270-276.
17. De Pauw B, Walsh TJ, Donnelly JP, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis*. 2008;46:1813-1821.
18. Gresnigt MS, Joosten LA, Verschueren I, et al. Neutrophil-mediated inhibition of proinflammatory cytokine responses. *J Immunol*. 2012a;189:4806-4815.
19. Cunha C, Rodrigues F, Zelante T, Aversa F, Romani L, Carvalho A. Genetic susceptibility to aspergillosis in allogeneic stem-cell transplantation. *Med Mycol*. 2011;49(Suppl 1):S137-S143.
20. Tramsen L, Schmidt S, Roeger F, et al. Immunosuppressive compounds exhibit particular effects on functional properties of human anti-Aspergillus Th1 cells. *Infect Immun*. 2014;82:2649-2656.
21. Mayer-Barber KD, Yan B. Clash of the Cytokine Titans: counter-regulation of interleukin-1 and type I interferon-mediated inflammatory responses. *Cell Mol Immunol*. 2017;14:22-35.

22. Kyrmizi I, Gresnigt MS, Akoumianaki T, et al. Corticosteroids block autophagy protein recruitment in *Aspergillus fumigatus* phagosomes via targeting dectin-1/Syk kinase signaling. *J Immunol*. 2013;191:1287-1299.
23. Bidula S, Sexton DW, Abdolrasouli A, et al. The serum opsonin L-ficolin is detected in lungs of human transplant recipients following fungal infections and modulates inflammation and killing of *Aspergillus fumigatus*. *J Infect Dis*. 2015a;212:234-246.
24. Carvalho A, Cunha C, Bistoni F, Romani L. Immunotherapy of aspergillosis. *Clin Microbiol Infect*. 2012;18:120-125.
25. Chai LY, Vonk AG, Kullberg BJ, et al. Aspergillus fumigatus cell wall components differentially modulate host TLR2 and TLR4 responses. *Microbes Infect*. 2011;13:151-159.
26. Gessner MA, Werner JL, Lilly LM, et al. Dectin-1-dependent interleukin-22 contributes to early innate lung defense against *Aspergillus fumigatus*. *Infect Immun*. 2012;80:410-417.
27. Gresnigt MS, Netea MG, van de Veerdonk FL. Pattern recognition receptors and their role in invasive aspergillosis. *Ann N Y Acad Sci*. 2012b;1273:60-67.
28. Gresnigt MS, Rosler B, Jacobs CW, et al. The IL-36 receptor pathway regulates *Aspergillus fumigatus*-induced Th1 and Th17 responses. *Eur J Immunol*. 2013;43:416-426.
29. Loeffler J, Ok M, Morton OC, Mezger M, Einsele H. Genetic polymorphisms in the cytokine and chemokine system: their possible importance in allogeneic stem cell transplantation. *Curr Top Microbiol Immunol*. 2010;341:83-96.
30. Potenza L, Vallerini D, Barozzi P, et al. Characterization of specific immune responses to different Aspergillus antigens during the course of invasive Aspergillosis in hematologic patients. *PLoS ONE*. 2013;8:e74326.
31. Sun H, Xu XY, Shao HT, et al. Dectin-2 is predominately macrophage restricted and exhibits conspicuous expression during *Aspergillus fumigatus* invasion in human lung. *Cell Immunol*. 2013;284:60-67.
32. Werner JL, Gessner MA, Lilly LM, et al. Neutrophils produce interleukin 17A (IL-17A) in a dectin-1- and IL-23-dependent manner during invasive fungal infection. *Infect Immun*. 2011;79:3966-3977.
33. Camargo JF, Bhimji A, Kumar D, et al. Impaired T cell responsiveness to interleukin-6 in hematological patients with invasive aspergillosis. *PLoS ONE*. 2015;10:e0123171.
34. Ceesay MM, Kordasti S, Rufaie E, et al. Baseline cytokine profiling identifies novel risk factors for invasive fungal disease among haematology patients undergoing intensive chemotherapy or haematopoietic stem cell transplantation. *J Infect*. 2016;73:280-288.
35. Cenci E, Mencacci A, Casagrande A, Mosci P, Bistoni F, Romani L. Impaired antifungal effector activity but not inflammatory cell recruitment in interleukin-6-deficient mice with invasive pulmonary aspergillosis. *J Infect Dis*. 2001;184:610-617.
36. Lehrnbecher T, Kalkum M, Champer J, Tramsen L, Schmidt S, Klingebiel T. Immunotherapy in invasive fungal infection–focus on invasive aspergillosis. *Curr Pharm Des*. 2013;19:3689-3712.
37. Czakai K, Leonhardt I, Dix A, et al. Kruppel-like Factor 4 modulates interleukin-6 release in human dendritic cells after in vitro stimulation with *Aspergillus fumigatus* and *Candida albicans*. *Sci Rep*. 2016;6:27990.
38. Romani L. Immunity to fungal infections. *Nat Rev Immunol*. 2011;11:275-288.
39. Borger P, Koeter GH, Timmerman JA, Vellenga E, Tomee JF, Kauffman HF. Proteases from *Aspergillus fumigatus* induce interleukin (IL)-6 and IL-8 production in airway epithelial cell lines by transcriptional mechanisms. *J Infect Dis*. 1999;180:1267-1274.
40. Bidula S, Sexton DW, Yates M, et al. H-ficolin binds *Aspergillus fumigatus* leading to activation of the lectin complement pathway and modulation of lung epithelial immune responses. *Immunology*. 2015b;146:281-291.
41. Jensen K, Lund KP, Christensen KB, et al. M-ficolin is present in *Aspergillus fumigatus* infected lung and modulates epithelial cell immune responses elicited by fungal cell wall polysaccharides. *Virulence*. 2017. http://doi.org/10.1080/21505594.2016.1278337.
42. Houser J, Komarek J, Kostlanova N, et al. A soluble fucose-specific lectin from *Aspergillus fumigatus* conidia–structure, specificity and possible role in fungal pathogenicity. *PLoS ONE*. 2013;8:e83077.
43. Ghufran MS, Ghosh K, Kanade SR. A fucose specific lectin from *Aspergillus flavus* induced interleukin-8 expression is mediated by mitogen activated protein kinase p38. *Med Mycol*. 2017;55:323-333.
44. Leal Jr. SM, Roy S, Vareechon C, et al. Targeting iron acquisition blocks infection with the fungal pathogens *Aspergillus fumigatus* and *Fusarium oxysporum*. *PLoS Pathog*. 2013;9:e1003436.
45. Raggam RB, Wagner J, Pruller F, et al. Soluble urokinase plasminogen activator receptor predicts mortality in patients with systemic inflammatory response syndrome. *J Intern Med*. 2014;276:651-658.
46. Shen HP, Tang YM, Song H, Xu WQ, Yang SL, Xu XJ. Efficiency of interleukin 6 and interferon gamma in the differentiation of invasive pulmonary aspergillosis and pneumocystis pneumonia in pediatric oncology patients. *Int J Infect Dis*. 2016;48:73-77.
47. Cunha C, Goncalves SM, Duarte-Oliveira C, et al. IL-10 overexpression predisposes to invasive aspergillosis by suppressing antifungal immunity. *J Allergy Clin Immunol*. 2017. http://doi.org/10.1016/j.jaci.2017.02.034.
48. Krause R, Zollner-Schwetz I, Salzer HJ, et al. Elevated levels of interleukin 17A and kynurenine in candidemic patients, compared with levels in noncandidemic patients in the intensive care unit and those in healthy controls. *J Infect Dis*. 2015;211:445-451.
49. Chai LY, van de Veerdonk F, Marijnissen RJ, et al. Anti-Aspergillus human host defence relies on type 1 T helper (Th1), rather than type 17 T helper (Th17), cellular immunity. *Immunology*. 2010;130:46-54.
50. Ohneberg K, Wolkewitz M, Beyersmann J, et al. Analysis of clinical cohort data using nested case-control and case-cohort sampling designs. A powerful and economical tool. *Methods Inf Med*. 2015;54:505-514.

**How to cite this article:** Heldt S, Eigl S, Prattes J, et al. Levels of interleukin (IL)-6 and IL-8 are elevated in serum and bronchoalveolar lavage fluid of haematological patients with invasive pulmonary aspergillosis. *Mycoses*. 2017;60:818–825. https://doi.org/10.1111/myc.12679