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Journal

Mycoses, 60(7)

ISSN

0933-7407

Authors

Cornely, Oliver A
Lass-Flörl, Cornelia
Lagrou, Katrien
et al.

Publication Date


2017-07-01

DOI

10.1111/myc.12628

Peer reviewed

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 Eigl¹ | Juergen Prattes^{2,3} | Holger Flick¹ |
Jasmin Rabensteiner⁴ | Florian Prüller⁴ | Tobias Niedrist⁴ | Peter Neumeister⁵ |
Albert Wölfler^{3,5} | Heimo Strohmaier⁶ | Robert Krause^{2,3} | Martin Hoenigl^{1,2,3,7} 

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

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

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

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

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

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

⁷Division 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

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 test⁹⁻¹² 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 were 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 bronchoscopy²² 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;

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 ($< 500/\mu\text{L}$) ≤ 10 d	3 (30%)	5 (25%)
Neutropenia > 10 d ($< 500/\mu\text{L}$)	3 (30%)	4 (20%)

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

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)

Material	Cytokine	Probable/proven invasive pulmonary aspergillosis				No evidence for invasive pulmonary aspergillosis			
		Median	IQR	Min	Max	Median	IQR	Min	Max
Serum	IFN γ	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
	TNF α	2.66	1.37-3.75	0.98	4.25	1.54	1.26-2.92	0.92	3.65
BALF	IFN γ	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
	TNF α	2.14	1.49-3.49	0.92	6.19	1.47	0.96-2.53	0.62	17.32

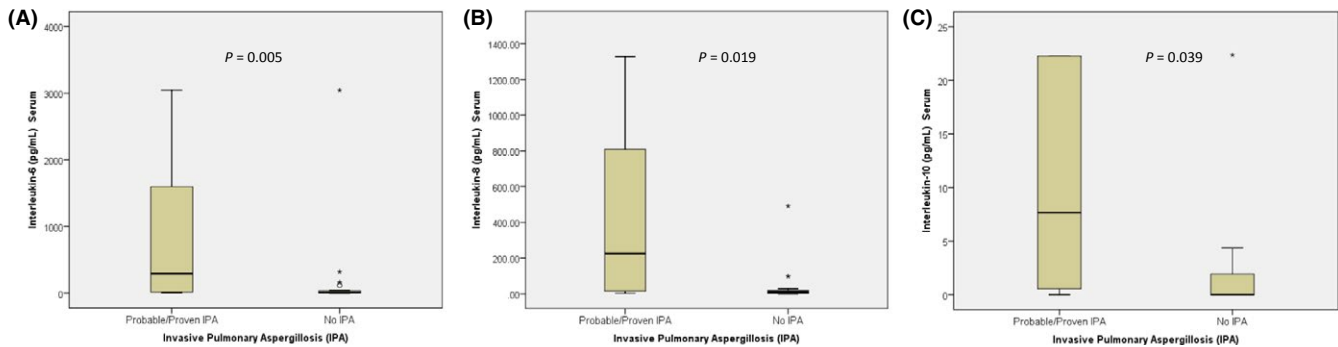


FIGURE 1 Box plots of serum interleukin (IL) 6 (A), IL-8 (B) and IL-10 (C) serum levels in patients with probable/proven IPA vs matched controls without IPA. Extrapolated values below the standard range are included. *are outliers.

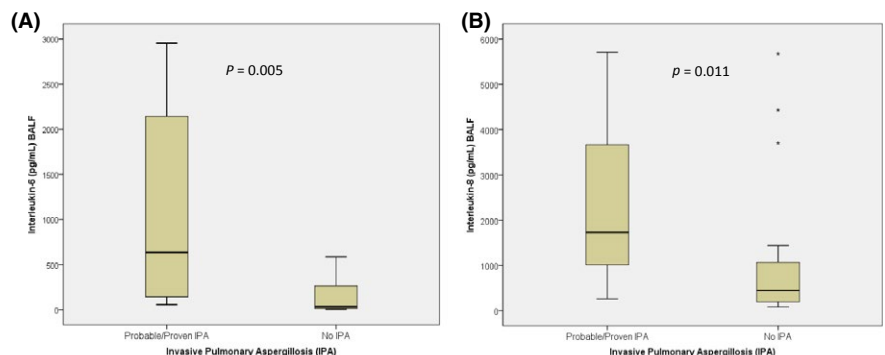


FIGURE 2 Box plots of serum interleukin (IL) 6 (A) and IL-8 (B) bronchoalveolar fluid levels in patients with probable/proven IPA vs matched controls without IPA. Extrapolated values below the standard range are included. *are outliers.

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)

Cytokine	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)		
	AUC	95% CI	P value	AUC	95% CI	P value	AUC	95% CI	P value	AUC	95% CI	P value
IFN γ	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	0.300-0.730	.895	0.515	0.300-0.730	.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
TNF α	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 CD⁴⁺-naive T cells.¹⁵ IL-6 plays an important role in T-cell recruitment and promotes differentiation of *Aspergillus*-specific CD⁴⁺-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-ficolin²³ and M-ficolin,⁴¹ which play essential roles in pathogen recognition and complement activation through

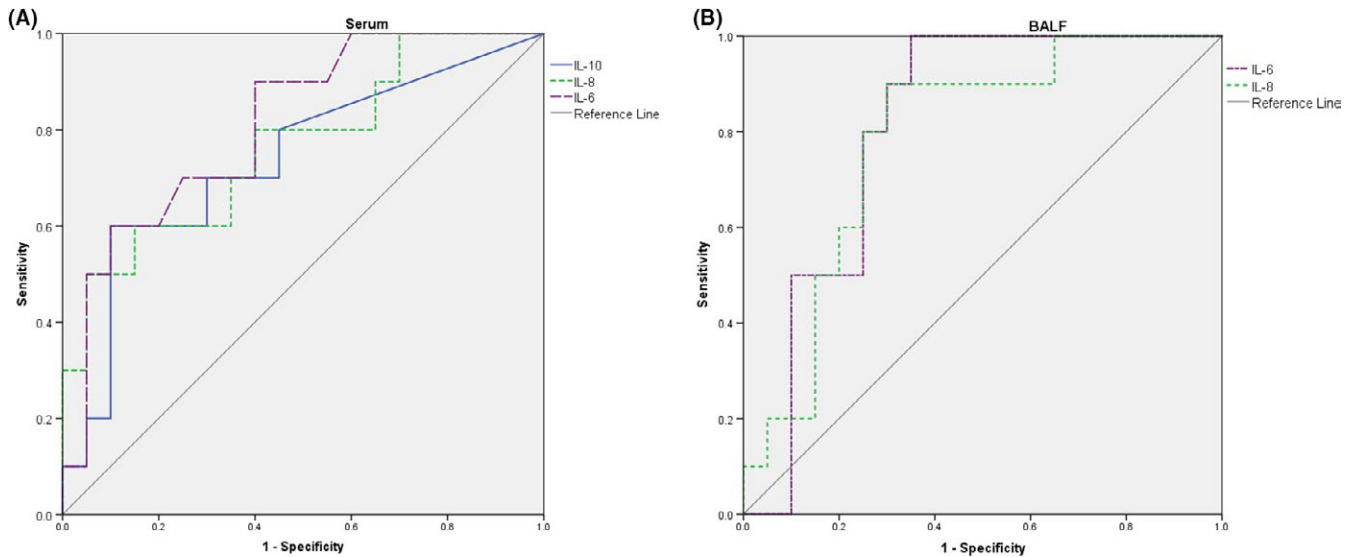


FIGURE 3 Receiver operating characteristics (ROC) curve analysis of IL-6, IL-8 and IL-10 in serum and IL-6 and IL-8 in bronchoalveolar lavage fluid (BALF) for diagnosing probable/proven invasive pulmonary aspergillosis

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

Cytokine	Cut-off (pg/mL)	Sensitivity	Specificity	Conditional logistic regression (univariate)		
				Hazard ratios	95% confidence interval	P value
<i>Serum</i>						
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
<i>BALF</i>						
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 hematologic 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-1 α 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 sub-analysis, 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>

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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>