The Journal of Infectious Diseases

MAJOR ARTICLE

Treatment Monitoring and Outcome Prediction in Invasive Aspergillosis Using Immunologic Markers

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Background. Invasive aspergillosis (IA) is a leading cause of mortality despite antifungal therapy. Current biomarkers for treatment monitoring and outcome prediction are limited, with host biomarkers showing potential in other fungal diseases but not in IA yet.

Methods. In this ECMM multicenter study, we prospectively evaluated a cohort of 51 patients with probable or proven IA with underlying hematological malignancies. Serial serum samples, collected over a 3-week period, were analyzed using the Olink Target 96 Inflammation panel. Differential expression analyses and survival analysis for outcome prediction were performed.

Results. Early after treatment, all differentially expressed proteins were upregulated in patients showing clinical improvement compared with those with worsening disease, with the exception of CCL23. Multiple proteins significantly correlated with the need to switch from primary to salvage antifungal therapy. For late treatment response, matrix metalloproteinase-10 (MMP-10) emerged as predictor, with consistently lower expression during clinical improvement during Weeks 2 and 3. Moreover, low-serum concentrations of CXCL6 at Week 2 and MMP-10 at Week 3 were associated with higher survival probability.

Conclusions. The dynamics of immune markers across disease states may help predicting responses to antifungal treatment. Future studies should establish their usefulness in clinical practice.

This multicenter study evaluated circulating immune biomarkers in patients with invasive aspergillosis to monitor treatment and predict outcome. Several proteins were associated with treatment response and survival, demonstrating potential clinical value, although further validation is required.

Keywords. biomarkers; proteomics; invasive fungal infections; aspergillosis; treatment monitoring.

Invasive mold infections are life-threatening diseases that frequently occur in immunocompromised individuals [1]. The most common of these fungal diseases, invasive aspergillosis (IA), has high mortality rates that vary amongst patient groups and can reach ~90% [1, 2]. Heterogeneous clinical presentations, diverse comorbidities, and limitations of diagnostic efficacy often result in delayed or missed identification of IA and, consequently, worse patient outcomes [3]. Further

Received 17 October 2025; accepted 26 February 2026; published online 4 March 2026

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The Journal of Infectious Diseases®

https://doi.org/10.1093/infdis/jiag140

exacerbating the need for high-quality diagnostics and disease monitoring is the increasing incidence and elevated mortality associated with isolates resistant to antifungal therapy [4]. This threat to public health recently led the World Health Organization to highlight *Aspergillus fumigatus* as a critical priority pathogen and calling for the development of accurate diagnostics, particularly for high-risk groups such as cancer patients [5].

Patients with hematological malignancy (HM) have a high risk of developing IA, even though many centers have adopted antifungal prophylaxis regimens for this group due to very high mortality rates of ∼50% [6]. However, postmortem analyses indicate that many cases remain undetected [3]. This diagnostic failure is caused by insufficient sensitivity, limited availability, and long turnaround times of current diagnostic [7]. While positive histopathology is the gold-standard for diagnosis, biopsies often cannot be safely performed in this patient population. Moreover, sensitivity of fungal culture from tissue or respiratory samples remains low [8]. The sensitivity and specificity of mycological tests, including fungal markers such as *Aspergillus* galactomannan (GM) and β-1,3-d-glucan (BDG), and molecular methods, including quantitative polymerase chain reaction (qPCR), vary significantly depending on the sample type, underlying disease, immunological status of patients, and prior exposure to antifungal prophylaxis and treatment [9].

Inconsistencies in the reliability of existing diagnostic approaches not only reduce survival rates due to delayed initiation of appropriate antifungal therapy but also constrain their utility for early prognosis prediction and treatment monitoring [10]. Optimizing early-response monitoring is essential for improving outcomes in IA, as delays in detecting refractory disease and appropriate adjustment to salvage therapy can result in a nearly 2-fold increase in mortality rates, longer hospital stays, and increased costs [11]. Therefore, dynamic assessment and prediction of treatment failure, requiring drug-switching to salvage therapy, could optimize antifungal usage to improve patient outcomes.

Recently, signaling proteins and receptors involved in host immune response during IA have been explored as potential immunological biomarkers of inflammation [12]. Observations of raised concentrations of several cytokines, such as interleukin-8 (IL-8), and immune regulators, for example, matrix metalloproteinase-10 (MMP-10), in the serum of HM patients with IA have led to immune markers being proposed as valuable for early diagnostics when combined with mycological tests [13–15]. Exploratory results indicate that host status biomarkers also have the potential to monitor and predict early antifungal drug response in this group [16]. However, to facilitate timely therapeutic adjustments and improve patient outcomes, further investigation is required to establish the role of inflammation indicators for dynamic disease monitoring in IA. To address this, we employed an unbiased approach to explore the distinguishing potential of immunologic serum biomarkers to predict antifungal drug response in IA patients with HM. Specifically, our goals were to identify immunological biomarkers that predict early and late treatment responses and overall survival probabilities. Moreover, the necessity of switching from primary to salvage antifungal therapy in cases of inadequate response to treatment, as well as the presence of severe neutropenia at the time of diagnosis were tested for potential correlation with the circulating concentrations of inflammatory proteins.

METHODS

Study Design and Sample Collection

In this multicenter study, we prospectively evaluated a cohort of 51 patients with underlying HM that were diagnosed with probable/proven IA between 2020 and 2024 according to revised standard criteria from the European Organization for Research and Treatment of Cancer/Mycology Study Group [17]. Sampling was conducted according to Good Epidemiological Practice requirements from European legislation and approved by the Ethics Committee for Research in Life and Health Sciences of the University of Minho, Braga, Portugal (CEICVS 028/2020). Each participating hospital was required to obtain local institutional review board confirmation or approval as deemed necessary by local regulations or authorities.

Serial blood samples were collected from each patient, starting on the day of diagnosis (day with the first positive mycological result)—timepoint 1 (T1), and on day 3 and/or 4, followed by twice per week for the 2 subsequent weeks (Figure 1). In clinical practice, it was not possible to collect blood at each of the intended timepoints. Therefore, in the final analysis, samples from days 3 and 4 were combined into a single timepoint (hereon considered T4). When samples from both days were available for the same patient, only the sample from day 4 was retained to avoid repeated measures and potential overestimation of the results. For samples collected during Weeks 2 and 3 (hereon W2 and W3), a similar approach was applied to maximize the sample size per timepoint. In this case, when 2 samples were available for the week, the earliest sample was selected for analysis, while later samples were excluded.

Serum samples were stored at −80°C at each site and sent in batch to the Radboud University Medical Centre, Nijmegen, The Netherlands in 2024. The clinical state of each patient—improvement, stable, or worsening—was assessed by clinicians 7 and 30 days after the start of antifungal treatment, following the Segal et al [18] clinical trial response criteria for IA and taking into account clinical, radiological, and mycological responses. Prophylaxis with a mold-active azole, in accordance with ESCMID and ECIL guideline recommendations for aspergillosis [19, 20], was used consistently across all centers. In patients who did not receive mold-active prophylaxis, serum GM screening was performed 2 to 3 times per week. For all patients, including those receiving mold-active

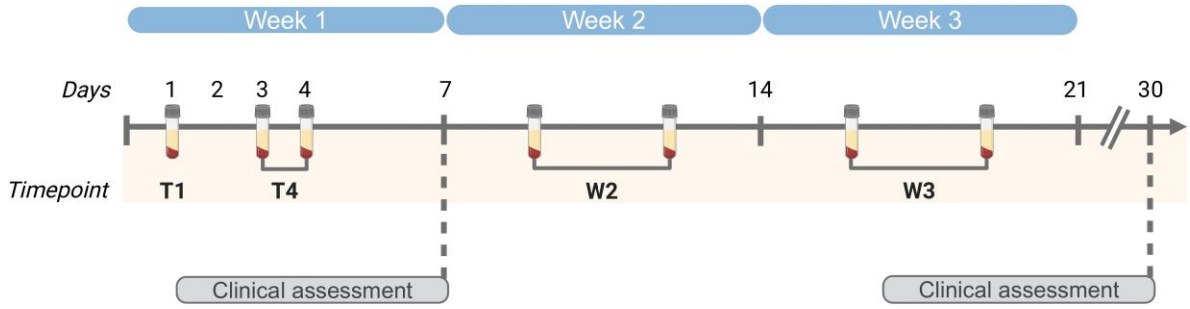

Figure 1. Sampling diagram. Blood samples were sequentially collected at day 1 (T1) and day 3 and/or day 4 (T4) after treatment initiation and then twice per week during weeks 2 and 3 (W2 and W3). The clinical state of each patient (improvement, stable, or worsening) was assessed on days 7 and 30.

prophylaxis, serum GM and bronchoalveolar lavage (BAL) GM testing, along with other fungal diagnostic procedures, were conducted whenever there was clinical suspicion of IA.

Analyses of Inflammation-Related Biomarkers in IA

Serum samples were analyzed using the Olink Target 96 Inflammation panel, which is a high-throughput proteomic immunoassay that measures 92 inflammation-related proteins based on a highly sensitive and specific proximity extension assay. In this assay, target proteins are recognized by double antibodies and coupled with specific cDNA barcodes, which are subsequently amplified and quantified using a high-throughput microfluidic real-time PCR instrument, BiomarkHD (Fluidigm, South San Francisco, CA). The final assay readout is presented in log2-normalized protein expression values (NPX). Proteins detected in less than 75% of the samples and samples that deviated more than 0.3 NPX from the plate median were excluded from the dataset. Moreover, protein concentrations under the detection threshold were replaced with the protein's lower limit of detection (LOD).

Statistical Analysis

Dataset heterogeneity was assessed for the variables age, sex, underlying disease, severe neutropenia at the time of diagnosis, and the antifungal treatment used as a primary therapy, using a permutational multivariate ANOVA (PERMANOVA). Severe neutropenia was defined as an absolute neutrophil count < 500/μL. Global expression profiles were visualized using heatmaps of z-scored median protein expression over time, for clinical state (improvement, stable, or worsening) at day 7 and 30 after initiation of treatment. Differential expression analyses were performed to identify potential biomarkers for early and late treatment responses (7 and 30 days after treatment initiation, respectively) by comparing improvement and worsening cases at each timepoint (T1, T4, W2, and W3), using linear regression models with the *limma* method [21]. False discovery rate (FDR) *P*-value correction was applied using the Benjamini–Hochberg method; however, due to a lack of significant results using FDR, nominal *P*-values were used to define significance. A fold change threshold of 0.5 was used.

For early treatment response prediction, the kinetics of significantly differentially expressed proteins were also analyzed across T1 and T4, comparing improvement and worsening cases assessed at day 7. Differences in mean expression levels between groups at each timepoint were tested using the Wilcoxon test. We followed the approach of selecting more than one consecutive timepoint because it provides the flexibility necessary for clinical practice applications of potential biomarkers. Pearson correlations between switching from primary antifungal to salvage therapy (defined as any change from the initial antifungal agent to a different drug) within the first 20 days of treatment and NPX at T1 were estimated (n = 17 patients who switched antifungal drug within the first 20 days), as well as correlation between protein expression at T1 and severe neutropenia at the time of diagnosis of the fungal infection (n = 23 patients).

For predicting outcome, we performed a survival analysis with Kaplan Meier curves for the 2 proteins with the highest significance in differential expression at Week 2 and Week 3 until last follow-up, which was censored at 6 weeks. The 6-week survival threshold was chosen in accordance with other clinical trials in patients with IA (eg [22]).

All clinical data were retrieved from an electronic case report form hosted on www.clinicalsurveys.net (EFS Summer 2021, TIVIAN GmbH, Cologne, Germany). Detailed statistical methods are available in the supplementary material. All analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria), and statistically significant differences were defined at a *P*-value < .05.

RESULTS

Study Cohort

A total of 51 patients with IA were enrolled over the 4-year study period, from the following participating centers: University of Cologne, Germany (n = 18); Innsbruck Medical

University, Austria (n = 10); UZ Leuven, Belgium (n = 10); IFIGEN study group, including Hospital de Santa Maria in Lisbon and the Portuguese Institute of Oncology—Porto, Portugal (n = 8); Hospital Universitari de Bellvitge, Barcelona, Spain (n = 2); University of Genova, Italy (n = 2); University Hospital of Rennes, France (n = 1), and Medical University of Graz, Austria (n = 1). Samples from 6 patients were excluded due to issues in storage and transport. An additional subset of 4 patients was excluded due to insufficient clinical information for data analysis. After quality control of the readouts from the Olink assay, a total of 81 out of 92 proteins and 39 out of 41 patients met the criteria for inclusion in the analyses, ie proteins detected in <75% of the samples and samples that deviated more than 0.3 NPX from the plate median were excluded.

From the final cohort of 39 patients with probable/proven IA, 16 had detection of either *Aspergillus* spp. (n = 15) via culture or PCR, while the others had positive GM results in BAL and/or serum. The 30-day mortality was 12.8% (5/39), with 4 of the 5 deaths attributable to IA, all preceded by disease progression or worsening. An additional 4 patients died between day 30 and day 80, all of whom also experienced worsening IA prior to death. The attributable mortality was therefore 10% at day 30% and 20% at day 90. Demographic and clinical characteristics of the study cohort are displayed in Table 1 and in the Supplementary Material 2.

Protein Expression Across the Samples

Examining principal component analysis (PCA) representations of all samples throughout the study revealed no evident clustering of protein expression for any demographic variable (Supplementary Figure 1), and no significant differences were found in the protein expression (Supplementary Table 1). Two sample outliers were identified in the PCAs with the raw data and were subsequently removed from the dataset. Visualization of z-scored NPX using heatmaps demonstrated considerable differences in treatment responses (Figure 2*A* and 2*B*), showing a striking contrast between improving and worsening cases across timepoints. Additionally, it is noteworthy the high variability in protein expression throughout Weeks 2 and 3 in the worsening cases compared with other clinical states (Figure 2*A* and 2*B*). The expression kinetics for all proteins relative to patients' clinical state at days 7 and 30 can be found in the Supplementary Figures 2 and 3.

Day 7 Response to Treatment and Switching to Antifungal Salvage Therapy

To predict early treatment response, we performed differential expression analysis on the timepoints T1 and T4 relative to the clinical state at day 7. A total of 7 inflammation-related proteins were significantly differentially expressed in, at least, one of the early-response timepoints when comparing the improvement with the worsening cases (Figure 3*A*). All proteins were upregulated in patients who clinically improved, except for CCL23, which was the only downregulated protein (Figure 3*A*). When analyzing the pattern of differentially expressed proteins across the initial timepoints (T1 and T4) in relation to the clinical state at day 7, Flt3L, MCP-1, and TRAIL showed consistently increased expression in improvement cases across early treatment timepoints (Figure 3*B*). However, none of the proteins were consistently significantly up- or downregulated in both T1 and T4.

The correlation analysis of protein expression at T1 and the necessity to switch from antifungal primary therapy to salvage therapy within the first 20 days of treatment revealed several significant correlations. IL-10RA, IL-20RA, NRTN, and Flt3L showed the strongest negative correlations with primary treatment failure and the necessity to switch to antifungal salvage therapy (Figure 3*C*). Additionally, patients with severe neutropenia showed significant positive correlations with the expression of MCP-4, TWEAK, and IL5 (Supplementary Material, Figure 4).

Day 30 Response to Treatment and Survival Analysis

For predicting Day 30 treatment response, we performed protein differential expression analysis relative to the clinical state at day 30 for timepoints W2 and W3. A total of 14 significantly differentially expressed proteins were found at W2, and 9 proteins were significantly differentially expressed at W3, when comparing the improvement with the worsening cases (Figure 4*A* and 4*B*, left side). MMP-10, MCP-3, IL-6, and CCL23 concentrations were consistently downregulated at Weeks 2 and 3 in the improving cases. Survival analysis on the proteins with the highest significance on Week 2 (MMP-10 and CXCL6) and on Week 3 (MMP-10 and MCP-3) showed a significantly higher survival probability in patients with low concentrations of CXCL6 at W2 (Figure 4*A*, right side). No significant differences were found at W3, although there was a trend for improved survival probability in patients with low-expression levels of MMP-10 and MCP-3.

DISCUSSION

In this multicenter study, we identified several inflammatory proteins that were differentially expressed when comparing patients showing improvement and those experiencing disease progression throughout antifungal treatment. We have found that circulating concentrations during the first week of treatment are highly variable among proteins, with Flt3L identified as upregulated in improving patients, as well as significantly negatively correlated with the need for switch to antifungal salvage therapy. Notably, CXCL6 and MMP-10 emerged as potential predictors of late treatment outcome and overall survival.

Despite advances in antifungal therapies for IA [23], treatment monitoring and early identification of patients who do or do not respond to antifungal treatment remains a critical

Table 1. Demographic and Clinical Characteristics of the Analyzed Cohort

		Clinical Assessment at Day 7[a]				Clinical Assessment at Day 30[a]			
		Impr.	Stable	Wors.	Unkn.	Impr.	Stable	Wors.	Unkn.
Sex	Female	5 (50.0)	14 (73.7)	5 (71.4)	1 (33.3)	8 (53.3)	9 (69.2)	4 (80.0)	4 (66.7)
	Male	5 (50.0)	5 (26.3)	2 (28.6)	2 (66.7)	7 (46.7)	4 (30.8)	1 (20.0)	2 (33.3)
Age range	18–29	4 (40.0)	…	1 (14.3)	1 (33.3)	4 (26.6)	1 (7.7)	…	1 (16.7)
	30–49	2 (20.0)	2 (10.5)	1 (14.3)	1 (33.3)	2 (13.3)	2 (15.4)	1 (20.0)	1 (16.7)
	50–69	2 (20.0)	14 (73.7)	2 (28.6)	…	5 (33.3)	7 (53.8)	4 (80.0)	2 (33.3)
	70–89	2 (20.0)	3 (15.8)	3 (42.9)	1 (33.3)	4 (26.7)	3 (23.1)	…	2 (33.3)
Underlying disease	Acute leukemia	5 (50.0)	12 (63.2)	4 (57.1)	2 (66.7)	8 (53.3)	9 (69.2)	3 (60.0)	3 (50.0)
	Aplastic anemia	1 (10.0)	…	…	…	1 (6.6)	…	…	…
	Blackfan-Diamond anemia	1 (10.0)	…	…	…	1 (6.6)	…	…	…
	Chronic lymphocytic leukemia	1 (10.0)	…	…	…	1 (6.6)	…	…	…
	Myelodysplastic syndrome	1 (10.0)	1 (5.3)	3 (42.9)	…	1 (6.6)	2 (15.4)	…	1 (16.7)
	T-cell prolymphocytic leukemia	1 (10.0)	…	…	…	3 (20)	1 (7.7)	1 (20.0)	…
	Lymphoma	…	4 (21.1)	…	…	…	1 (7.7)	…	…
	Multiple myeloma	…	1 (5.3)	…	…	…	…	…	1 (16.7)
	Chronic myelomonocytic leukemia	…	…	…	1 (33.3)	…	…	1 (20.0)	…
	Unknown	…	1 (5.3)	…	…	…	…	…	1 (16.7)
Neutropenia at diagnosis	No	5 (50.0)	6 (31.6)	2 (28.6)	1 (33.3)	5 (33.3)	5 (38.5)	2 (40.0)	2 (33.3)
	Yes	4 (40.0)	3 (15.8)	2 (28.6)	…	5 (33.3)	3 (23.1)	…	1 (16.7)
	Unknown	1 (10.0)	10 (52.6)	3 (42.9)	2 (66.7)	5 (33.3)	5 (38.5)	3 (60.0)	3 (50.0)
Neutropenia within 30 d of diagnosis	No	5 (50.0)	8 (42.1)	1 (14.3)	…	5 (33.3)	4 (30.8)	4 (80.0)	1 (16.7)
	Yes	5 (50.0)	10 (52.6)	6 (85.7)	3 (100)	9 (60.0)	9 (69.2)	1 (20.0)	5 (83.3)
	Unknown	…	1 (5.3)	…	…	1 (6.7)	…	…	…
Antifungal treatment	Isavuconazole	1 (10.0)	3 (15.8)	…	1 (33.3)	1 (6.67)	3 (23.1)	…	…
	Posaconazole	1 (10.0)	3 (15.8)	2 (28.6)	…	1 (6.67)	1 (7.7)	1 (20.0)	1 (16.7)
	Voriconazole	8 (80.0)	6 (31.6)	4 (57.1)	…	2 (13.3)	2 (15.4)	…	…
	Amphotericin B liposomal	…	3 (15.8)	…	1 (33.3)	3 (20.0)	4 (30.8)	…	…
	Fluconazole	…	…	…	1 (33.3)	…	1 (7.7)	…	…
	Micafungin	…	4 (21.0)	…	…	8 (53.3)	2 (15.4)	4 (80.0)	4 (66.7)
	Caspofungin	…	…	1 (14.3)	…	…	…	…	1 (16.7)

[a]Data are given as absolute numbers, with percentages per group in parentheses. Neutropenia was defined as an absolute neutrophil count < 500/μL. Abbreviations: *Impr.,* improvement; *wors.,* worsening, *unkn.,* unknown.

challenge. This is exacerbated by increasing antifungal resistance and the emergence of rare molds that are often intrinsically resistant to antifungal agents used in IA [24, 25]. Inflammatory biomarkers have emerged as potential tools for predicting the response to treatment [13, 26]. In our study, we found contrasting patterns of biomarker concentrations between early and late stages of treatment. Our analysis revealed that most significantly expressed proteins were upregulated in improvement cases at the initial stages of treatment (T1 and T4), while most significantly expressed proteins at later stages (Weeks 2 and 3) were downregulated in improving patients. This temporal pattern suggests that upregulation of inflammatory proteins is fundamental at the initial phase of treatment for disease resolution during the activation of antifungal immunity but may be detrimental if maintained at higher levels during later stages of the disease. Moreover, the upregulation of cytokines and chemokines shown in improving patients during the first days might indicate an effective immune response in controlling the initial hyperinflammatory state and facilitating a shift toward tissue repair [27]. Nonetheless, the hypothesis that lower cytokine levels in worsening patients at early stages could reflect underlying cytopenia cannot be ruled out.

The identification of 7 differentially expressed inflammation-related proteins during early treatment highlights the rapid mobilization of immune responses following antifungal therapy initiation. We have found that protein expression during the first week of treatment is highly variable among proteins, with Flt3L identified as upregulated in improving patients and significantly negatively correlated with the need to switch from primary to salvage antifungal therapy. Flt3L (FMS-like tyrosine kinase 3 ligand) plays a crucial role in dendritic cell (DC) development and function [28]. Research in IA models has shown the important role of DCs as regulators of the inflammatory response by shaping T helper responses [29]. In our study, sustained levels of Flt3L in improving patients at early treatment stages may suggest that effective antifungal response may depend on Flt3L-mediated development and function of DCs and other immune cells that drive pathogen recognition and immune coordination. Furthermore, decreased Flt3L levels at the time of diagnosis correlated with an increased need to progress from primary to salvage antifungal

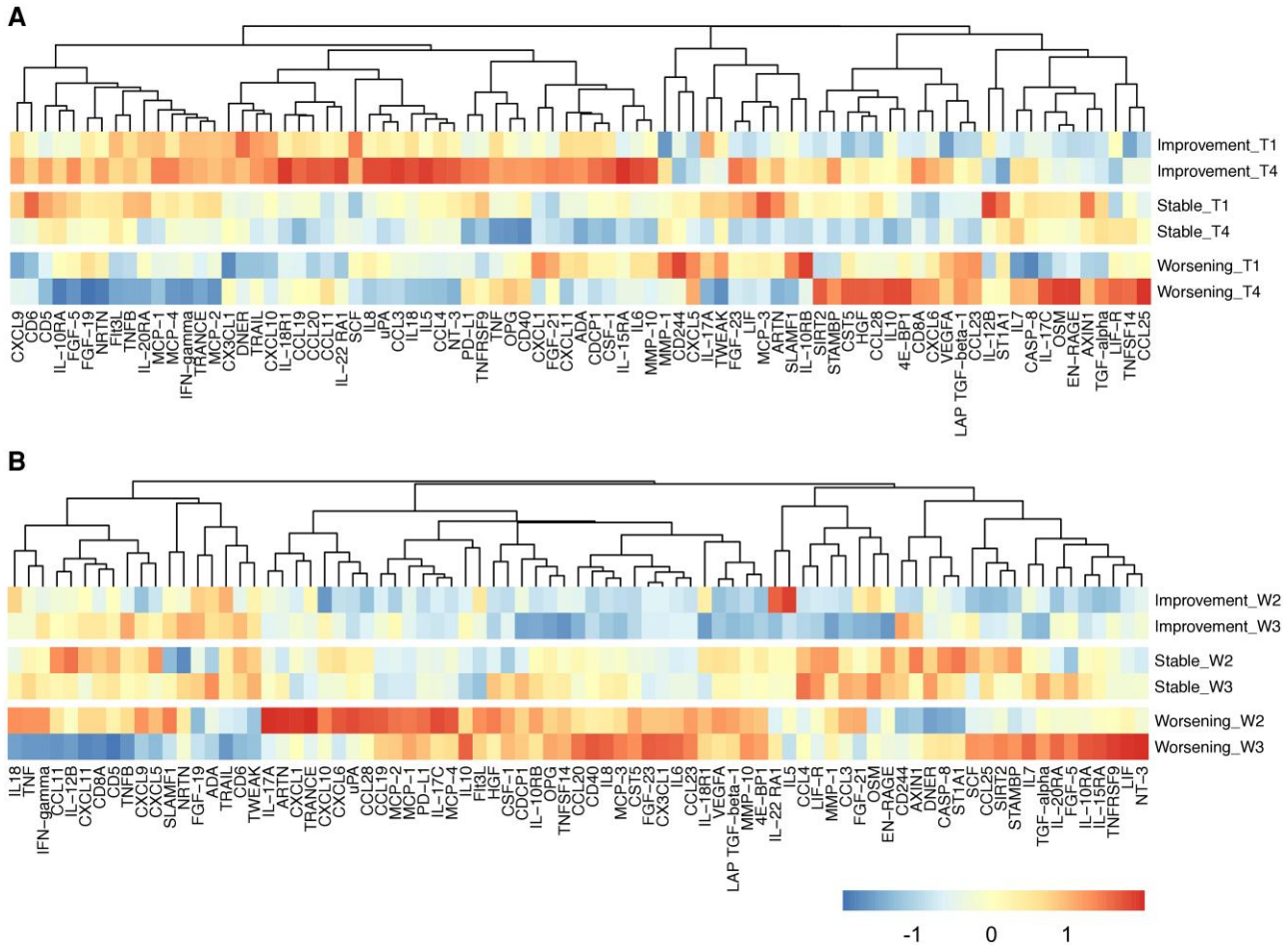

Figure 2. Heatmaps of the global protein expression profile over time. *A*, Protein expression relative to the clinical state (improvement, stable, or worsening) at day 7, for timepoints T1 and T4; (*B*) Protein expression relative to the clinical state at day 30, for timepoints W2 and W3.

therapy. Although Flt3L has been reported to be downregulated in cases of IA compared with noninfected controls [13], our results also suggest that Flt3L may act as a marker of immune function capable of identifying IA patients who could benefit from increased surveillance or more intensive treatment.

In patients with severe neutropenia at the time of diagnosis, a positive correlation with the expression of MCP-4 (monocyte chemotactic protein 4) and IL-5, an eosinophil-related cytokine, was found. Although not directly involved in neutrophil recruitment, the high circulating concentration of these proteins in patients with severe neutropenia suggests a compensatory immune response where the failure of neutrophil-mediated antifungal defenses triggers alternative inflammatory pathways involving eosinophil and monocyte recruitment [30]. Indeed, research in mice suggested a role for eosinophils in antifungal defense against *A. fumigatus* [31].

In the later stages of treatment, the significant downregulation of MMP-10 was consistently observed in patients with clinical improvement at day 30, as well as a trend of higher survival probability associated with low circulating concentrations of MMP-10 at Week 3. Matrix metalloproteinase-10 is involved in the breakdown of extracellular matrix components and also plays a significant role in the pathogenesis of several infectious diseases, including lung diseases [32]. Indeed, high concentrations of MMPs have been associated with the progression of several pulmonary infections and disease [33], and MMP-10 has been proposed as a potential biomarker in idiopathic pulmonary fibrosis (IPF) [34]. Similarly, high levels of CXCL6 (C-X-C motif chemokine ligand 6), which is a proinflammatory chemokine that plays a key role in neutrophil recruitment and activation, have been found in bronchoalveolar lavage fluid from IPF patients and in bleomycin-instilled mice [35]. In our study, low expression of CXCL6 at later treatment stages was associated with an improved clinical state and higher survival rates, suggesting that these patients successfully resolved the acute inflammation stage. On the other hand, patients with increased expression of CXCL6 at Weeks 2 and 3 may have persistent fungal burden, inadequate treatment response, or dysregulated immune responses that continue driving inflammation, leading to poorer outcomes.

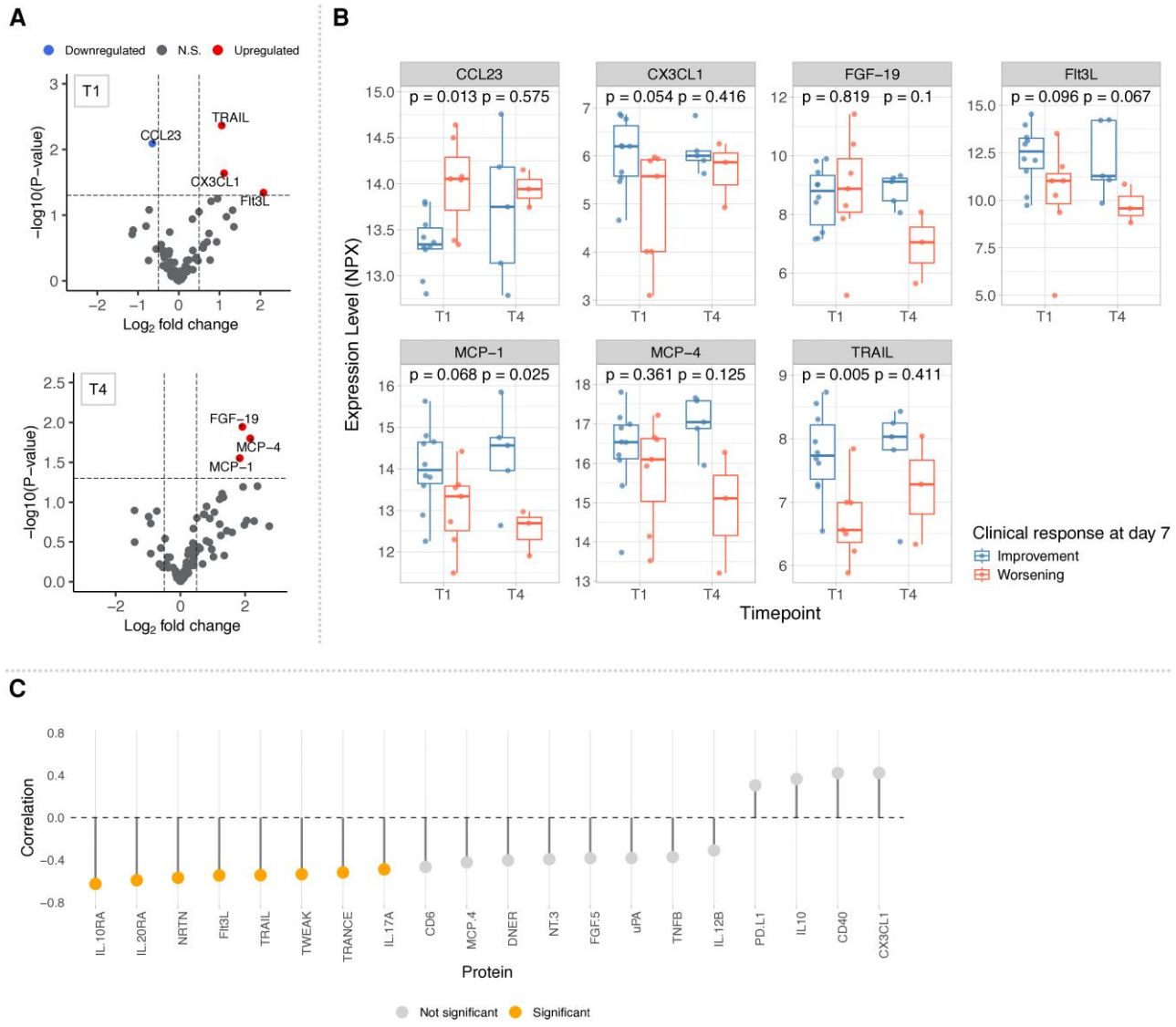

Figure 3. Inflammatory markers involved in the early response to antifungal treatment. *A*, Differentially expressed proteins at T1 (top) and T4 (bottom) between improving versus worsening cases at day 7. Significant upregulated proteins are shown in red, downregulated in blue, and nonsignificant in gray (N.S.); *B*) Mean expression levels of the differentially expressed proteins across timepoints T1 and T4. Improvement cases are shown in blue and worsening cases are shown in red. *C*, Correlation of inflammatory protein expression at T1 with switching from primary therapy to antifungal salvage therapy within 20 days of treatment (n = 24 patients; from which 6 patients switched to salvage therapy based on progression of IA under primary treatment). Plots show Pearson correlation of proteins with an absolute correlation value higher than 0.30. Significant proteins are shown in colored dots.

The identification of potential protein biomarkers such as Flt3L, MMP-10, and CXCL6 presents significant potential for predicting treatment outcomes in IA. The correlation between protein expression at the time of diagnosis and antifungal switching suggests that biomarker-guided therapy modification could optimize treatment outcomes while minimizing unnecessary treatment changes, although a larger sample size and the combination of immunological and clinical parameters would be necessary to identify the most robust monitoring algorithm. Furthermore, the prognostic value of late-timepoint protein measurements provides opportunities for risk stratification and long-term management of the disease. Finally, the integration of classical inflammatory markers with clinically actionable measurements, such as C-reactive protein levels [36] should be considered in future studies.

The major limitation of this study is the small sample size, which, together with the multiple comparisons performed, resulted in limited statistical power and reduced our ability to detect significant differences and draw robust conclusions. Also, enrollment into the study was delayed due to the COVID-19 pandemic, resulting in an extended enrollment period of nearly 4 years to reach the targeted number of 51 patients with IA.

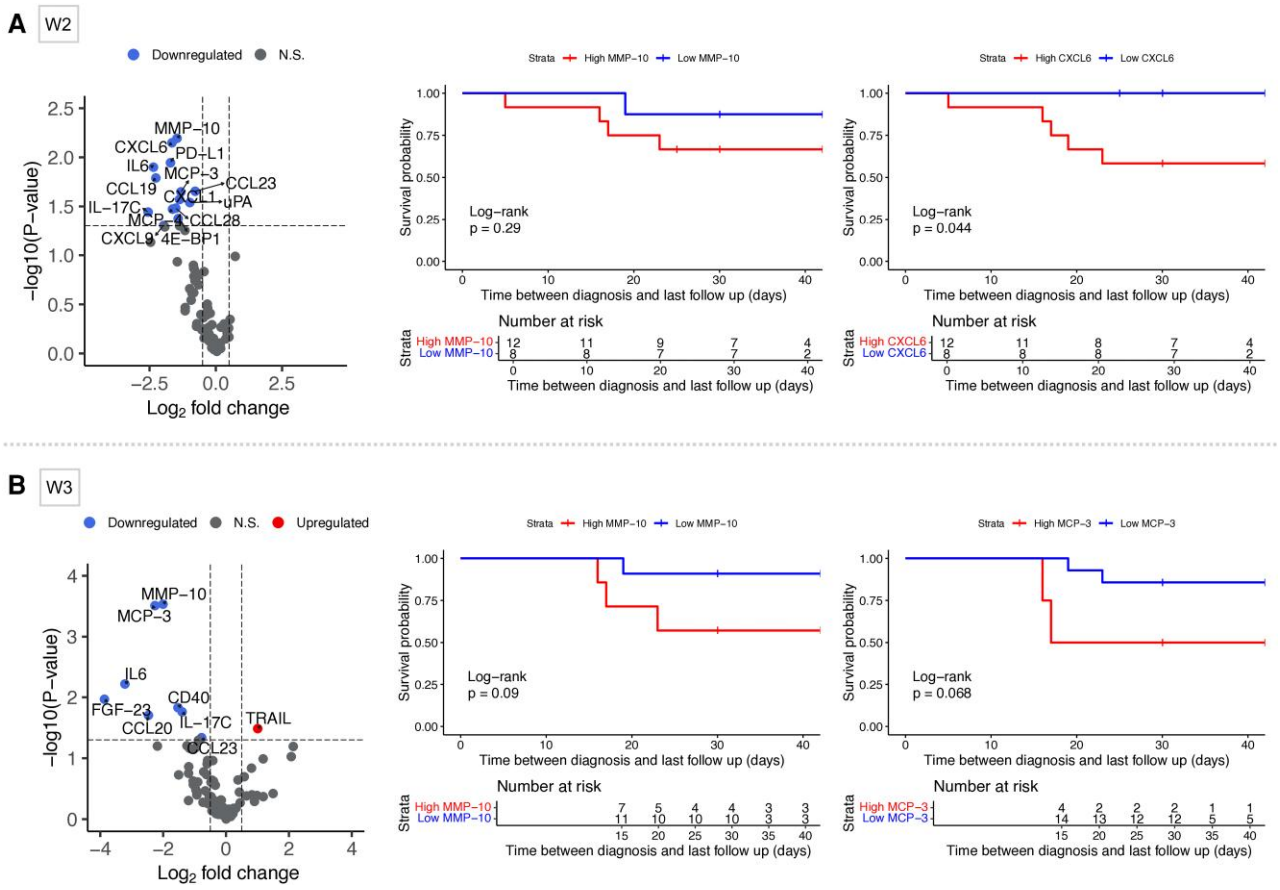

Figure 4. Inflammatory markers involved in the response to antifungal treatment at day 30. *A*, Differentially expressed proteins between improving versus worsening cases at W2 (left side) and Kaplan–Meier curves for the proteins with the highest significance at W2, MMP-10 and CXCL6 (right side). *B*, Differentially expressed proteins between improving versus worsening cases at W3 (left side) and Kaplan–Meier curves for the proteins with the highest significance at W3, MMP-10, and MCP-3 (right side). For volcano plots, significantly upregulated proteins are shown in red, downregulated in blue, and nonsignificant in gray (N.S.); for survival plots, high expression levels are represented in red, low-expression levels in blue, and the + symbol indicates censored observations.

In conclusion, our study suggests that circulating concentrations of certain protein biomarkers, particularly Flt3L, MMP-10, and CXCL6 may be promising for treatment monitoring and outcome prediction in patients with IA. The temporal nature of protein expression changes observed in this study highlights the importance of longitudinal monitoring in IA, suggesting that biomarker treatment monitoring strategies at early treatment stages should incorporate temporal dynamics rather than relying on single-timepoint measurements, ideally combining both fungal- (eg, serum GM in neutropenic patients with positive results at baseline) and host-derived markers. Moreover, focusing exclusively on neutropenic patients with positive serum GM at time of diagnosis, and incorporating serial assessments of GM alongside immunological profiling could clarify whether late responses have true predictive value or represent surrogate markers of treatment response. Future studies that validate these biomarkers in larger cohorts of patients, including larger subcohorts, eg of patients with switch to salvage therapy due to fungal disease progression, will be critical to establishing their utility in clinical practice, enabling more precise, personalized treatment monitoring strategies for IA, which would be especially important in immunocompromised patients.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Author Contributions. Conceived study: A. C. and M. H. Designed study and experiments: M. G. N, C. C., A. C., and M. H. Performed measurements: A. S. and V. K. Collected

clinical samples and data: R. S., R. A., C. L. -F., M. M., S. S., C. G.-V., J.-P. G., D. R. G., J. P., J. G., P. M.-G., M. B., J. M., P. K. Analyzed and interpreted data: A. P., J. S., S. B., C. C., A. C., M. H. Supervision: A. C. and M.H. Acquired funding: A. C. and M. H. Wrote the initial draft: A. P. and J. S. Edited the final version: all the authors.

Data availability statement. All data are incorporated into the article and its online Supplementary material.

Financial support. The study was supported by an Investigator Initiated Research Grant from Gilead Sciences (Project: Immunologic Markers for Treatment Monitoring and Diagnosis in Invasive Mold Infection; IN-EU-131-5720). A. P., J. S., C. C., and A. C. were supported by the Fundação para a Ciência e a Tecnologia (FCT) (UID/06304/2023, LA/P/0050/2020, 2022.06674.PTDC, and PMTargets/0003/2024), the "la Caixa" Foundation and FCT under the agreement LCF/PR/HR24/52440014 (FUNPATH), the "la Caixa" Foundation under the agreement LCF/PR/HR22/52420003 (MICROFUN), and the EU Horizon 2020 research and innovation program under the agreement no 847507 (HDM-FUN).

Potential conflicts of interest. M. G. N. is a scientific founder of TTxD, Biotrip, and Lemba. M. H. received research funding from Gilead, Astellas, MSD, Mundipharma, Melinta, Shionogi, GSK, Pulmocide, Euroimmune, Scynexis, F2G and Pfizer. AC reports speaker honoraria from Gilead. J. M. reports grants or contracts from F2G, Gilead, MSD, Mundipharma, Pfizer; Consulting fees from AbbVie, Servier, Basilea, Takeda, Daiichi Sankyo, Cidara, Asahi Kasei Pharma Corporation, Gilead, Elion Therapeutics, Novo Nordisk, MedPace, Menarini, Mundipharma, Pfizer, Scynexis, Shionogi; Speaker and lecture honoraria from Takeda, AbbVie, Gilead, Medscape/WebMD, MSD, Mundipharma, Pfizer, Shionogi; Payment for expert testimony from Cidara, Mundipharma, F2G. P. K. reports grants or contracts from German Federal Ministry of Research and Education (BMBF) B-FAST (Bundesweites Forschungsnetz Angewandte Surveillance und Testung) and NAPKON (Nationales Pandemie Kohorten Netz, German National Pandemic Cohort Network) of the Network University Medicine (NUM), the State of North Rhine-Westphalia and the Dr. Heinz Lux-Stiftung; Consulting fees from Ambu GmbH, Gilead Sciences, infill healthcare communication GmbH, Mundipharma Research Limited, Noxxon N.V. and Pfizer Pharma; Honoraria for lectures from Akademie für Infektionsmedizin e.V., Ambu GmbH, Astellas Pharma, BioRad Laboratories Inc., Datamed GmbH, European Confederation of Medical Mycology, Gilead Sciences, GPR Academy Ruesselsheim, HELIOS Kliniken GmbH, Jazz Pharmaceuticals Germany GmbH, Lahn-Dill-Kliniken GmbH, medupdate GmbH, MedMedia GmbH, MSD Sharp & Dohme GmbH, Pfizer Pharma GmbH, Sanofi-Aventis Deutschland GmbH, Scilink Comunicación Científica SC, streamedup! GmbH, University Hospital and LMU Munich and VITIS GmbH; Participation on an Advisory Board from Ambu GmbH, Gilead Sciences, Mundipharma Research Limited and Pfizer Pharma; A filed patent at the German Patent and Trade Mark Office (DE 10 2021 113 007.7); Other nonfinancial interests from Elsevier, Wiley and Taylor & Francis online outside the submitted work. J. P. -G. received honoraria for talks in congresses from AstraZeneca, Pfizer and Shionogi. R. S. reports grants from the German Center for Infection Research and the Ministry of Culture and Science of the State of North Rhine-Westphalia, lecture and speaker honoraria from Akademie für Infektionsmedizin e.V., Ärztliche Akademie für medizinische Fort- und Weiterbildung in Nordrhein, Forum für medizinische Fortbildung GmbH, Infektio Saar Netz, Hikma, Mundipharma and Pfizer. DRG reports investigator-initiated grants from Pfizer, Shionogi, Tillotts Pharma, Menarini, Advanz Pharma, bioMérieux, and Gilead Italia, travel support from Pfizer and bioMérieux, and speaker and/or advisor fees from Pfizer, bioMérieux, Menarini, Advanz Pharma, and MSD. MB reports research grants and/or personal fees for advisor/consultant and/or speaker/chairman from BioMérieux, Cidara, Gilead, Menarini, MSD, Pfizer, and Shionogi. C. L. -F. reports grants from F2G, Gilead, Mundipharma; Consulting and speakers fees from Basilea, Gilead, Mundipharma, Pfizer, Shionogi. Other authors have no conflicts of interest to declare.

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