Triacetylfusarinine C: a urine biomarker for diagnosis of invasive aspergillosis

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Abstract

**Objectives:** Early diagnosis of invasive aspergillosis (IA) remains challenging, with available diagnostics being limited by inadequate sensitivities and specificities. Triacetylfusarinine C, a fungal siderophore that has been shown to accumulate in urine in animal models, is a potential new biomarker for diagnosis of IA.

**Methods:** We developed a method allowing absolute and matrix-independent mass spectrometric quantification of TAFC. Urine TAFC, normalized to creatinine, was determined in 44 samples.
from 24 patients with underlying hematologic malignancies and probable, possible or no IA according to current EORTC/MSG criteria and compared to other established biomarkers measured in urine and same-day blood samples.

**Results:** TAFC/creatinine sensitivity, specificity, positive and negative likelihood ratio for probable versus no IA (cut-off ≥3) were 0.86, 0.88, 6.86, 0.16 per patient.

**Conclusion:** For the first time, we provide proof for the occurrence of TAFC in human urine. TAFC/creatinine index determination in urine showed promising results for diagnosis of IA offering the advantages of non-invasive sampling. Sensitivity and specificity were similar as reported for GM determination in serum and bronchoalveolar lavage, the gold standard mycological criterion for IA diagnosis.

**Keywords**
aspergillosis; biomarker; diagnosis; siderophore; urine

**Introduction**

Invasive aspergillosis (IA) is the most common airborne fungal infection mainly affecting individuals with a suppressed immune system including those with prolonged neutropenia, and hematopoietic stem cell transplantation (1). IA is caused by infection with different *Aspergillus* species, with *A. fumigatus* accounting for ~90% of all cases (1). IA has become one of the leading causes of death in immunocompromised patients, with 6-week mortality rates of around 30% (2).

A major reason for the high mortality of IA is delay of diagnosis. Due to the absence of a single “gold standard” test for diagnosis of IA, different assays are employed combined with clinical, radiological, serological, molecular biological, mycological and histological methods. Diagnostic approaches for IA include fungal culture from bronchoalveolar lavage fluid (BALF) and biopsies, immunodetection of the cell wall component galactomannan (GM) in serum and BALF, immunodetection of the cell wall component 1,3-β-D-glucan (BDG) in serum, detection of an *Aspergillus*-specific cell wall protein via a lateral flow device, and detection of *Aspergillus*-specific DNA via PCR in blood and BALF (3–7). The most commonly used non-culture based approach for IA diagnosis is GM detection. Depending on the patient cohort, the sensitivity and specificity of the GM testing varies between 48–77% and 81–100% for serum (4, 5) and 79% and 70% for urine samples (6). Notably, GM measurement in urine is still an investigational method.

The suboptimal diagnosis is also reflected by the fact that antimold prophylaxis is now widely used in hematological malignancy patients at highest risk for IA (8). While antimold prophylaxis is successful in reducing the burden of IA in those patients by about 70%, cases of breakthrough IA do occur (9–11). Diagnosis of these breakthrough cases is particularly challenging because sensitivities of available diagnostic tests decrease even further in the presence of antimold prophylaxis or treatment (12–16). Hence, there is an urgent need for improved diagnostic markers for IA (17–19).
IA usually originates from inhalation of fungal conidia, subsequent germination and tissue- and angioinvasive growth in case of insufficient local immune response. To obtain iron for growth and to overcome iron restriction by the host, *Aspergillus* species secrete low-molecular mass iron chelators, termed siderophores (20). Genetic inactivation of siderophore production renders *A. fumigatus* avirulent in a mouse model for IA emphasizing the in vivo production of siderophores, which is further supported by transcriptional upregulation of the siderophore system during murine infection (21–25). Uptake of siderophore-iron chelates is mediated by specific siderophore transporters exclusively found in fungi. The major secreted siderophore of *A. fumigatus* is triacetylfusarinine C (TAFC). Importantly, TAFC is produced only by actively growing cells and is not present in conidia.

Imaging studies showed that TAFC chelating the radionuclide $^{68}$Gallium (instead of iron) is accepted by siderophore transporters and can be employed for in vivo imaging of IA in mouse and rat models (26, 27). $^{68}$Gallium-TAFC, after being injected intravenously, circulates in the blood stream and is selectively accumulated by fungal cells, which can be monitored via Positron Emission Tomography (PET). These studies also demonstrated that TAFC, which is a small (905,323 g/mol) and uncharged molecule, has a short half-life in blood and is rapidly excreted by the kidneys in intact form: within 45 minutes, about 90% of injected TAFC was found in kidneys and bladder. In agreement, TAFC was recently detected in urine in a rat model for IA employing mass spectrometric methods (28). In humans, TAFC has previously been detected in BALF (29), and in serum samples from patients with IA using ultra performance liquid chromatography tandem mass spectrometry (30).

The objective of this study was to evaluate the potential of TAFC as a urine biomarker for human IA.

**Materials and Methods**

**Samples and Reagents**

The analyzed urine samples included four samples of a probable IA patient collected in 2015 at the Division of Hematology, University Hospital of Wuerzburg, Germany, 44 samples of midstream urine from 24 hematological malignancy patients collected between 2012 and 2015 at the Division of Hematology, Medical University of Graz, Austria, and 15 samples from healthy volunteers collected in 2015. Samples were stored at −70°C until analysis. Analysis of clinical specimens was carried out in a blinded fashion.

The study adhered to the Declaration of Helsinki, 2013, Good Clinical Practice, and was approved by the local ethics committees, Medical University Graz, Austria (EC-number 23–343 ex 10/11), and University Hospital Wuerzburg, Germany (#233/14).

**Production of TAFC and $^{13}$C-labelled TAFC**

For the generation of $^{13}$C-isotope-labelled TAFC, *A. fumigatus* ($10^6$ spores/mL) was grown for 24 h at 37°C in *Aspergillus* minimal medium (31) shake culture containing 20 mM NH$_4$NO$_3$ and 1% w/v $^{13}$C-labelled D-glucose (U-13C6, 99%, Eurisotop) as sole nitrogen and carbon sources, respectively. For preparation of $^{12}$C-TAFC, standard D-glucose (Roth, Karlsruhe, Germany) was used. Addition of iron to the growth medium was omitted to
generate iron starvation and consequently induce the production of siderophores. Subsequently, siderophores in the culture supernatant were saturated with iron by adding FeCl$_3$ to a final concentration of 1 mM. TAFC was purified by reversed-phase HPLC as described previously (32).

**Extraction of TAFC from urine**

300 μL of urine was spiked with 10 ng $^{13}$C-TAFC in a volume of 10 μL for absolute quantification. Moreover, 10 μL of a 10 mM FeSO$_4$ solution was added to iron-saturate potential iron-free siderophores. The resulting samples were pre-extracted with 300 μL chloroform/diethylether (1:1, Roth, Karlsruhe, Germany) and centrifuged at 20,000 relative centrifugal force (rcf) at 4 °C for 3 min. The organic phase was discarded in order to reduce compounds responsible for ion suppression effects. Subsequently, samples were extracted three times with 300 μL chloroform, centrifuged as described above. The solvent of the pooled organic phases was evaporated at 60 °C in a thermo-shaker. Dry samples were stored at −20 °C until measurement. For analysis, TAFC was resolubilized in 10 μL of 0.1% (v/v) formic acid (Sigma-Aldrich, Vienna, Austria).

**Capillary electrophoresis (CE)-electrospray ionization (ESI)-mass spectrometry (MS)**

For CE-ESI-MS analysis of TAFC, a CESI 8000 CE-ESI system (Sciex, Brea, CA), equipped with a bare fused-silica capillary (total length: 90 cm, i.d.: 30 μm, o.d.: 150 μm, Sciex, Brea, CA), was coupled to a Thermo Scientific Q Exactive HF hybrid quadrupole Orbitrap mass spectrometer (Bremen, Germany). A terminal porous segment of the bare fused-silica capillary inside the sprayer interface acted as nanospray emitter; a secondary capillary filled with conductive liquid enabled the electric contact. The background electrolyte (BGE) used for CE was 0.1% (v/v) formic acid.

Prior to each analysis, the conductive liquid capillary was rinsed with BGE for 1 minutes at 100 psi; the separation capillary was rinsed with acetone for 2 minutes at 100 psi followed by BGE for 2.5 minutes at 100 psi. Samples were injected for 30 sec at 5 psi, which corresponds to a sample volume of 25 nL. The analysis was performed at +20 kV for 10 minutes with a pressure of 10 psi at the capillary inlet to transport the analyte towards the MS.

Mass spectra were acquired in positive ion mode applying a data-independent automatic switch between survey scan (from m/z 400 – 1000), SIM scan (from m/z 900 – 1000) and MS/MS acquisition. Full scan MS spectra were acquired with a resolution of R = 120,000 in profile mode. Automatic gain control target was set to 3e6 and maximum ionization time was 50 ms. For data-independent MS/MS acquisition ions at m/z = 906.33 were isolated and fragmented using higher-energy collisional dissociation (HCD) applying a normalized collision energy of 28.0. Isolation window was set to m/z =3.0.

**MS data analysis, TAFC quantification, transition analysis, determination of limit of quantification (LOQ)**

For data analysis, the four ion adducts [$+H^+$, $+NH_4^+$, $+Na^+$, and $+K^+$] of $^{12}$C TAFC and $^{13}$C TAFC internal standard were extracted from SIM scans using Qual Browser software (part
of Thermo Xcalibur 3.0.63). Mass tolerance for ion extraction was set to 10 ppm. Peak areas were integrated using Qual Browser software and total intensities of $^{12}$C TAFC and the $^{13}$C TAFC internal standard were calculated, respectively. The intensities of all four TAFC ions were summed up with $^{12}$C TAFC being normalized to the spiked 10 ng $^{13}$C TAFC using the formula yielding the endogenous $^{12}$C TAFC concentration in ng/mL:

$\left(\frac{\text{TAFC in extracted sample}}{\text{TAFC in extracted sample}}\right) \times \frac{\text{[ng]/300 [μl]}}{1000}$.

Transitions specific for the most intense fragments of $^{12}$C TAFC+H$^+$ (m/z=622.19, 888.32 and 906.33) were extracted from MS/MS scans (performed at m/z = 906.33) at a mass tolerance of 10 ppm. These transitions were used for identity verification only.

The limit of quantification (LOQ) was determined by repetitive measurement (11-times) of a TAFC standard concentration of 1 ng/mL yielding ion signal intensities for TAFC+H$^+$ of 36,000 – 65,300 with a medium of 45,500 ± 8,800. For LOQ calculation, this most abundant TAFC ion was used. The lowest measured ion signal intensity of 36,000 was taken to define the LOQ. As an ion signal intensity of even 10,000 still allows reliable quantification (Gaussion distributed signal), the technical LOQ was set to 0.28 ng/mL.

### Analysis of GM, (1→3)-β-D-glucan (BDG) and creatinine

At the Medical University of Graz, Austria, urine samples were prospectively tested for GM (Platelia Aspergillus Ag ELISA; Bio-Rad Laboratories, Munich, Germany) and creatinine levels. Urine GM levels were then normalized to the urine creatinine content, by dividing GM absolute urine levels by creatinine urine levels and multiplication with the factor 100. Following previous recommendations, a GM/creatinine index of >0.25 was defined as positive (6). Blood samples were collected simultaneously (i.e., on the same day) with urine samples and tested routinely for GM and BDG levels (Fungitell assay; Associates of Cape Cod, Inc, East Falmouth, USA) as described previously (14, 33). Samples were stored at −70°C and shipped in 2016 on dry ice to the Innsbruck Medical University, for retrospective TAFC determination. In part these samples have been published before (6, 34, 35).

### Classification of IA and Statistical Analysis

IA was classified according to the revised EORTC/MSG criteria with one modification: exclusion of BDG as mycological criterion (36). Following the criteria, PCR results and urine biomarker results were not used for classification of IA. Investigators in Innsbruck were blinded towards IPA classification of the samples.

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. Sensitivity, specificity, negative likelihood ratio (NPV), and positive likelihood ratio (PPV) were calculated, and displayed including 95%CI. Receiver operating characteristic (ROC) curve analyses were performed and area under the curve (AUC) values are presented including 95%CI, for differentiating probable IA versus possible or no IA using two approaches: per patient and per sample. ROC curves were compared using the method by Hanley and
McNeil (37). The optimal cut-off for the TAFC/creatinine index was determined using Youdens index. Two-sided P<0.05 was taken as cut-off for statistical significance.

Results

Establishment of TAFC determination by Capillary electrophoresis (CE)-electrospray ionization (ESI)-mass spectrometry (MS)

The first goal was establishing a mass spectrometry (MS)-based method for determination of TAFC. Using liquid chromatography (LC)-MS, we observed TAFC absorptions and carry-over effects negatively affecting the quantification. Therefore, we switched from LC-MS to CE-ESI-MS, which did not show these negative effects. TAFC exhibited four ion adducts [+H⁺ at m/z=906.33, +NH₄⁺ at 923.36, +Na⁺ at 928.32, +K⁺ at 944.29] with the [+H⁺] adduct being the most abundant TAFC ion. For quantification, signal intensities of all four ions were summed up. TAFC determination displayed linearity over the range of 0.1 to 10,000 ng/mL with a correlation coefficient of 0.9987 (Figure 1A). The limit of detection (LOD) for TAFC was 0.1 ng/mL. The technical CE-ESI-MS limit of quantification (LOQ) for TAFC was 0.28 ng/mL (see Material & Methods). The chloroform extraction procedure used for prepurification and desalting results in a 30-fold enrichment of TAFC before CE-ESI-MS quantification, which significantly decreases the LOQ for TAFC levels in urine samples of this analytical method, theoretically down to 0.01 ng/mL.

In some patient samples, spiking with TAFC revealed ion suppression effects, which cause an underestimation of the TAFC concentration. To enable matrix-independent absolute quantification of TAFC, we generated ¹³C isotope-labelled TAFC from A. fumigatus cultures grown with ¹³C isotope-labelled glucose as sole carbon source. With respect to extraction and chromatography, ¹³C TAFC displays the same features as ¹²C TAFC, but it is distinguishable due to its higher molecular mass. Like native ¹²C TAFC, ¹³C TAFC exhibited four ion adducts [+H⁺ at m/z=945.46, +NH₄⁺ at 962.49, +Na⁺ at 967.45, +K⁺ at 983.42]. For absolute matrix-independent TAFC quantification, patient samples were spiked with 10 ng ¹³C TAFC as internal standard and the endogenous ¹²C TAFC concentration was calculated by normalization to the internal ¹³C TAFC standard. Figure 1B and C show exemplary CE-ESI-MS analyses of a patient sample displaying the patient ¹²C TAFC and spiked ¹³C TAFC ions used for quantification.

A limitation of testing biomarkers in urine specimens is the variation of urine concentrations. To account for differences in urine concentration, urine TAFC levels were normalized to the urine creatinine (crea) levels, which reflect the urine concentration, using the formula (TAFC concentration) [ng/mL] / (creatinine) [mg/dL] x 100, yielding the TAFC/crea index. Such a normalization was found to be instrumental for using GM as a urinary IA biomarker (6).

Noteworthy, TAFC is a very stable molecule: TAFC stability in water is not affected by storage for five years at −20°C or cooking for 1 hour; moreover, stability of TAFC spiked into urine was not affected by a 7-times cycle consisting of heating to 60°C for 30 minutes followed by storage at −20°C for 23.5 hours (data not shown).
**TAFC in urine samples from healthy volunteers and a patient with IA**

CE-ESI-MS analysis of urine specimens from nine healthy individuals revealed TAFC concentrations of 0.10 to 0.29 ng/mL and a TAFC/crea index of median 0.1 [interquartile range (IQR) 0.1–0.3; range 0.1–0.5] indicating background TAFC levels (Figure 2).

Next, we analyzed four serial urine specimens of an acute myeloid leukemia patient (AML) diagnosed with probable IA (Figure 3A). This patient received allogeneic stem cell transplantation and empirical fluconazole therapy followed by posaconazole treatment upon diagnosis with probable IA. Urine samples contained TAFC concentrations of 28.2 to 146.8 ng/mL and TAFC/crea indexes of 6.9 to 444.1 being 36- to 2,476-fold higher than the background level in healthy individuals. Remarkably, the TAFC/crea indexes were elevated not only after probable IA diagnosis (i.e., both positive serum GM and serum *Aspergillus* PCR), but already were elevated 15 days before serum GM and PCR became positive.

**Validation of urine TAFC as a diagnostic marker for IA**

Samples from 24 hematological malignancy patients with and without IPA (Table 1) were retrospectively selected for measurement of TAFC in urine. Seven patients had probable IA, one had possible IA and 16 had no IA. Twenty-one samples were tested from patients with evidence for probable IA at the day of sampling (ongoing mold active antifungal prophylaxis or treatment in all 21 samples; 19/21 samples collected during absolute neutropenia), two samples from patients with evidence for possible IA (both ongoing mold-active antifungal prophylaxis and absolute neutropenia) and 21 samples from patients without evidence for IA (12 of those during ongoing mold-active antifungal prophylaxis, 10 during absolute neutropenia).

**Per sample analysis and cutoff determination:** TAFC/crea index was significantly lower in samples from no or possible IA compared to samples from probable IA (median 1.4, IQR 0.5–2.6 versus median 7.6, IQR 3.8–12.8; p<0.001) but significantly higher in samples from no or possible IA versus healthy volunteers (p=0.003; Figure 2). AUC for differentiating probable versus possible or no IA in the per-sample analysis was 0.883 (95%CI 0.773 – 0.993) for TAFC/crea index, which was significantly higher (p=0.04) than the AUC for GM/crea index (0.683; 95%CI 0.515 – 0.851). ROC curves are displayed in Figure 4. AUCs of TAFC/crea index were comparable to serum BDG (AUC 0.880; 95%CI 0.772 – 0.988) and serum GM (AUC 0.910; 95%CI 0.822 –0.998), despite the fact that the latter was used for probable IA classification and performance for serum GM is therefore likely an overestimation. Using Youdens index, a TAFC/crea index ≥3 was found to be optimal for differentiating between probable IA and no IA. TAFC/crea sensitivity, specificity, positive and negative likelihood ratio for probable versus possible/no IA samples (cut-off ≥3) were 0.81 (95%CI 0.58 – 0.95), 0.90 (95%CI 0.68 – 0.99), 8.1 (95%CI 2.1 – 30.6), 0.21 (95%CI 0.09 – 0.52). Performance was similar to serum GM, while urine GM/crea index (sensitivity 0.52, specificity 0.95) and serum BDG (sensitivity 0.47, specificity 1.00) were less sensitive (Table 2).

**Per patient analysis:** Heat map presentations for biomarker results in serial samples of four patients with probable IA are displayed in Figure 3B-E. Figure 3B shows a patient with...
probable IA, deteriorating despite treatment, displaying continuous positivity of both serum GM and TAFC/crea index. Figure 3C patient displays positivity of all four biomarkers tested at the day of IA diagnosis without follow up samples. Figure 3D shows a patient with positivity of both urine GM and TAFC/crea index in all samples but serum GM positivity only at the day of diagnosis. Figure 3E illustrates a patient displaying high positivity of serum GM, serum BDG and TAFC/crea index at the day of diagnosis with subsequent rapid decrease of all three biomarkers under successful therapy reflected by the clinical improvement of the patient. Per patient analysis for differentiating cases with probable IA versus no IA yielded an AUC for TAFC/crea index of 0.835 (95%CI 0.585 – 1.000), and for GM/crea index of 0.830 (95%CI 0.579 – 1.000). Sensitivity, specificity, positive and negative likelihood ratio for patients with probable versus no IA (cut-off ≥2) were 0.86 (95%CI 0.49 – 0.97), 0.88 (95%CI 0.64 – 0.97), 6.86 (95%CI 1.81 – 25.96), 0.16 (95%CI 0.03 – 1.01) for TAFC/crea index. Performance of other biomarkers in urine and serum is displayed in Table 2 (except for serum GM, which was used as mycological criterion for defining probable IA and therefore not evaluable).

Discussion

Invasive aspergillosis progresses rapidly and remains difficult to diagnose - especially at early stages the clinical and radiological presentation of IA is non-specific and can be atypical or insidious (16, 38). Mycological diagnosis also is challenging, particularly since cultures of lower respiratory secretions have a low diagnostic yield and sensitivity (39) and specimens used for fungal biomarkers (e.g., GM) and PCR testing usually require invasive sampling (blood or BALF). Moreover, performance of these diagnostic tests may be limited in patients without neutropenia and those receiving antifungal prophylaxis or treatment (12, 14, 15). Therefore, improvement of IA diagnostics is needed.

TAFC is the major siderophore of *A. fumigatus* and *Aspergillus nidulans*, while *Aspergillus terreus* and *Aspergillus niger* produce other siderophore types (40). Consequently, TAFC is not a biomarker for all *Aspergillus* spp infections. Nevertheless, *A. fumigatus* is the major cause of IA and *A. nidulans* is a common pathogen in patients with chronic granulomatous disease (41). Among plant pathogens, *Fusarium graminearum* was found to produce TAFC, while *Alternaria brassicicola*, *Cochliobolus* spp. were found to produce other siderophore types (42). Importantly, TAFC is produced neither by Mucorales nor by bacteria. These differences in siderophore production might be instrumental for species-specific diagnostics. We established a new method for detecting the *Aspergillus* secreted siderophore TAFC by CE-ESI-MS in urine including extraction for enrichment, spiking with $^{13}$C isotope-labelled TAFC allowing highly sensitive and matrix-independent absolute quantification, and normalization to creatinine for normalization to urine concentration. $^{13}$C TAFC spiking was found to be crucial for detection of ion suppression effects, which negatively affect TAFC determination.

Previously published LC-MS/MS-mediated TAFC determination in blood reported a LOD of ≥1 ng/mL and a LOQ of 5 ng/mL (30). TAFC was also detected in urine and blood samples of rats with reported LODs and LOQs of 0.28 and 0.85 ng/mL for serum and 0.02 and 0.05 ng/mL for urine, respectively (28). However, these methods do not allow absolute
matrix-independent TAFC determination. The technical LOQ of the CE-ESI-MS method reported here is 0.28 ng/mL, which increases for clinical samples due to the applied enrichment to ≥0.01 ng/mL, which is the most sensitive method reported so far.

When validating the method clinically, TAFC/crea index determination in urine samples showed a promising performance for diagnosis of IA, with sensitivities and specificities comparable to those reported for GM in serum and BALF (12, 14, 43), the current gold standard for IA diagnosis, and superior to urine GM testing. In addition, our findings indicate that the TAFC/crea index declines rapidly in the presence of successful antifungal treatment, suggesting that the index may be useful also for treatment stratification and outcome prediction. Future studies with larger sample sizes are needed to confirm these findings.

While this is the first study that evaluated urine TAFC levels for diagnosis of IA in humans, two recent studies reported elevated levels of TAFC in BALF and serum from patients who developed IA versus controls. One study revealed that sensitivity of BALF GM can be increased from 53% to 73% (1.0 ODI GM cut-off), and from 73% to 87% (0.5 ODI GM cut-off), when combined with BALF TAFC (29). In another recent study, serum TAFC levels were elevated in patients with IA versus controls (30). Compared to these results, diagnostic performance of TAFC levels in urine seems to be superior, which may be explained by TAFC accumulation in the bladder as shown in recent animal models (26, 27).

Interestingly, the TAFC/crea index, although significantly lower than in those with probable IA, was found to be higher in patients with hematological malignancies at risk but without evidence of IA when compared to healthy controls. Explanations may include increased translocation of food-derived TAFC from the gastrointestinal tract due to increased intestinal permeability, or subclinical infections that are controlled by antifungal prophylaxis or the immune system.

Our study is subject to a number of limitations, including the low sample size of clinical samples and prospective cohort studies are needed to validate the method, before it can be established in clinical routine. Furthermore, CE-ESI-MS is not typically found in routine diagnostic laboratories. However, the promising results of this study are highly encouraging to establish alternative detection methods, e.g. via immunoassays.

In conclusion, TAFC/crea index determination showed good discriminatory power for differentiating IA versus no IA in urine samples from patients with hematologic malignancies, and was superior to urine GM testing. This novel biomarker may be the first to allow for reliable diagnosis of IA in urine samples, which would offer several advantages in clinical routine such as easy repetition and non-invasive sampling.

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collection, analysis, interpretation, decision to publish, in the writing of the manuscript, and in the decision to submit the manuscript for publication.

**Abbreviations:**

| Abbreviation | Definition |
|--------------|------------|
| Amb          | liposomal amphotericin B |
| BALF         | bronchoalveolar lavage fluid |
| BGE          | background electrolyte |
| Casp         | empirical therapy with caspofungin |
| CE           | capillary electrophoresis |
| crea         | creatinine |
| EORTC/MSG    | European Organization for Research and Treatment of Cancer/Mycoses Study Group |
| ESI          | electrospray ionization |
| Flu          | fluconazole |
| GM           | galactomannan |
| IA           | invasive aspergillosis |
| ICU          | submission to intensive care unit |
| IQR          | interquartile range |
| LC           | liquid chromatography |
| ms           | mass spectrometry |
| ODI          | optical density index |
| pIA          | day of diagnosis "probable invasive aspergillosis" |
| Pos          | posaconazole |
| TAFC         | triacetylfusarinine C |
| Tx,          | day of transplantation |
| Vor iv       | voriconazole intravenously |

**References**

1. Denning DW. 1998 Invasive aspergillosis. Clin Infect Dis 26:781–803; quiz 804–5. [PubMed: 9564455]
2. Garcia-Vidal C, Peghin M, Cervera C, Gudiol C, Ruiz-Camps I, Moreno A, Royo-Cebrecos C, Rosello E, de la Bellaca J, Ayats J, Carratala J. 2015 Causes of death in a contemporary cohort of patients with invasive aspergillosis. PLoS One 10:e0120370. [PubMed: 25803853]
3. Thornton CR. 2014 Breaking the mould - novel diagnostic and therapeutic strategies for invasive pulmonary aspergillosis in the immune deficient patient. Expert Rev Clin Immunol 10:771–80. [PubMed: 24689528]

4. Acosta J, Catalan M, del Palacio-Perez-Medel A, Lora D, Montejo JC, Cuetara MS, Moragues MD, Ponton J, del Palacio A. 2011 A prospective comparison of galactomannan in bronchoalveolar lavage fluid for the diagnosis of pulmonary invasive aspergillosis in medical patients under intensive care: comparison with the diagnostic performance of galactomannan and of (1→3)-beta-d-glucan chromogenic assay in serum samples. Clin Microbiol Infect 17:1053–60. [PubMed: 20825441]

5. Racil Z, Kocmanova I, Toskova M, Buresova L, Weinbergerova B, Lengerova M, Rolencova M, Winterova J, Hrncirova K, Volfpa C, Skrickova J, Mayer J. 2011 Galactomannan detection in bronchoalveolar lavage fluid for the diagnosis of invasive aspergillosis in patients with hematological diseases-the role of factors affecting assay performance. Int J Infect Dis 15:e874–81. [PubMed: 22040924]

6. Reischies FM, Raggam RB, Prattes J, Krause R, Eigl S, List A, Quehenberger F, Strenger V, Wolff A, Hoenigl M. 2016 Urine Galactomannan-to-Creatinine Ratio for Detection of Invasive Aspergillosis in Patients with Hematological Malignancies. J Clin Microbiol 54:771–4. [PubMed: 2669701]

7. Koo S, Bryar JM, Page JH, Baden LR, Marty FM. 2009 Diagnostic performance of the (1→3)-beta-D-glucan assay for invasive fungal disease. Clin Infect Dis 49:1650–9. [PubMed: 19863452]

8. Patterson TF, Thompson GR., 3rd, Denning DW, Fishman JA, Hadley S, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Nguyen MH, Segal BH, Steinbach WJ, Stevens DA, Walsh TJ, Wingard JR, Young JA, Bennett JE. 2016 Executive Summary: Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America. Clin Infect Dis 63:433–42. [PubMed: 27481947]

9. Duarte RF, Sanchez-Ortega I, Cuesta I, Arnan M, Patino B, Fernandez de Sevilla A, Gudiol C, Ayats J, Cuenca-Estrella M. 2014 Serum galactomannan-based early detection of invasive aspergillosis in hematology patients receiving effective antimold prophylaxis. Clin Infect Dis 59:1696–702. [PubMed: 25165088]

10. Cornely OA, Maertens J, Winston DJ, Perfect J, Ullmann AJ, Walsh TJ, Helfgott D, Holowiecki J, Stockelberg D, Goh YT, Petrini M, Hardalo C, Suresh R, Angulo-Gonzalez D. 2007 Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. N Engl J Med 356:348–59. [PubMed: 17251531]

11. Hoenigl M, Raggam RB, Salzer HJ, Valentín T, Valentín A, Zollner-Schwetz I, Strohmeier AT, Seeber K, Wolff A, Sill H, Krause R. 2012 Posaconazole plasma concentrations and invasive mould infections in patients with haematological malignancies. Int J Antimicrob Agents 39:510–3. [PubMed: 22481947]

12. Eigl S, Prates J, Reischies F, Spiess B, Neumeister P, Zollner-Schwetz I, Raggam RB, Flick H, Buchheidt D, Krause R, Hoenigl M. 2015 Influence of mould-active antifungal treatment on the performance of the Aspergillus-specific bronchoalveolar lavage fluid lateral-flow device test. Int J Antimicrob Agents 46:401–5. [PubMed: 26187363]

13. Prattles J, Lackner M, Eigl S, Reischies F, Raggam RB, Koidl C, Flick H, Wurm R, Palfner M, Wolff A, Neumeister P, Thornton CR, Krause R, Lass-Florl C, Hoenigl M. 2015 Diagnostic accuracy of the Aspergillus-specific bronchoalveolar lavage lateral-flow assay in haematological malignancy patients. Mycoses 58:461–9. [PubMed: 26103209]

14. Eigl S, Hoenigl M, Spiess B, Heldt S, Prattles J, Neumeister P, Wolff A, Rabenstein J, Prueller F, Krause R, Reinwald M, Flick H, Buchheidt D, Boch T. 2017 Galactomannan testing and Aspergillus PCR in same-day bronchoalveolar lavage and blood samples for diagnosis of invasive aspergillosis. Med Mycol 55:528–534. [PubMed: 27744310]

15. Springer J, Lackner M, Nachbaur D, Girschikofsky M, Risslegger B, Mutschlechner W, Fritz J, Heinz WJ, Einsele H, Ullmann AJ, Loffler J, Lass-Florl C. 2016 Prospective multicentre PCR-based Aspergillus DNA screening in high-risk patients with and without primary antifungal mould prophylaxis. Clin Microbiol Infect 22:80–86. [PubMed: 26400571]

16. Heldt S, Prattles J, Eigl S, Spiess B, Flick H, Rabenstein J, Johnson G, Prueller F, Wolff A, Niedrist T, Boch T, Neumeister P, Strohmeier H, Krause R, Buchheidt D, Hoenigl M. 2018 Diagnosis of Invasive Aspergillosis in Hematological Malignancy Patients: Performance of
Cytokines, Asp LFD, and Aspergillus PCR in Same Day Blood and Bronchoalveolar Lavage Samples. J Infect doi:10.1016/j.jinf.2018.05.001.

17. Cornely OA, Lass-Florl C, Lagrou K, Arsic-Arsenijevic V, Hoenigl M. 2017 Improving outcome of fungal diseases - Guiding experts and patients towards excellence. Mycoses 60:420–425. [PubMed: 28497502]

18. Hoenigl M, Eigl S, Heldt S, Duettmann W, Thornton C, Prattes J. 2018 Clinical evaluation of the newly formatted lateral-flow device for invasive pulmonary aspergillosis. Mycoses 61:40–43. [PubMed: 28922489]

19. Heldt S, Eigl S, Prattes J, Flick H, Rabensteiner J, Pruller F, Niedrist T, Neumeister P, Woffler A, Strohmaier H, Krause R, Hoenigl M. 2017 Levels of interleukin (IL)-6 and IL-8 are elevated in serum and bronchoalveolar lavage fluid of haematological patients with invasive pulmonary aspergillosis. Mycoses 60:818–825. [PubMed: 28877383]

20. Haas H 2014 Fungal siderophore metabolism with a focus on Aspergillus fumigatus. Nat Prod Rep 31:1266–76. [PubMed: 25140791]

21. Schreitl M, Bignell E, Kragl C, Joechli C, Rogers T, Arst HN, Jr., Haynes K, Haas H 2004 Siderophore biosynthesis but not reductive iron assimilation is essential for Aspergillus fumigatus virulence. J Exp Med 200:1213–9. [PubMed: 15504822]

22. Hissen AH, Wan AN, Warwas ML, Pinto LJ, Moore MM. 2005 The Aspergillus fumigatus siderophore biosynthetic gene sidA, encoding L-ornithine N5-oxygenase, is required for virulence. Infect Immun 73:5493–503. [PubMed: 16113265]

23. Schreitl M, Bignell E, Kragl C, Sabiha Y, Loss O, Eisendle M, Wallner A, Arst HN, Haynes K, Haas H. 2007 Distinct Roles for Intra- and Extracellular Siderophores during Aspergillus fumigatus infection. PLoS Pathog 3:e128.

24. Yasmin S, Alcazar-Fuoli L, Grundlinger M, Puempel T, Cairns T, Blatzer M, Lopez JF, Grimalt JO, Bignell E, Haas H. 2012 Mevalonate governs interdependency of ergosterol and siderophore biosyntheses in the fungal pathogen Aspergillus fumigatus. Proc Natl Acad Sci U S A 109:E497–504. [PubMed: 22106303]

25. McDonagh A, Fedorova ND, Crabtree J, Yu Y, Kim S, Chen D, Loss O, Cairns T, Goldman G, Armstrong-James D, Haynes K, Haas H, Schreitl M, May G, Nierman WC, Bignell E. 2008 Subtelomere directed gene expression during initiation of invasive aspergillosis. PLoS Pathog 4:e1000154. [PubMed: 18787699]

26. Petrik M, Haas H, Laverman P, Schreitl M, Franssen GM, Blatzer M, Decristoforo C. 2014 68Ga-triacetylfusarinine C and 68Ga-ferrioxamine E for Aspergillus infection imaging: uptake specificity in various microorganisms. Mol Imaging Biol 16:102–8. [PubMed: 23818006]

27. Petrik M, Vlckova A, Novy Z, Urbanek L, Haas H, Decristoforo C. 2015 Selected (6)(8)Ga-siderophores versus (6)(8)Ga-colloid and (6)(8)Ga-citrate: biodistribution and small animal imaging in mice. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 159:60–6. [PubMed: 25363728]

28. Luptakova D, Pluhacek T, Petrik M, Novak J, Palyzova A, Sokolova L, Skriba A, Sediva B, Lemr K, Havlicek V. 2017 Non-invasive and invasive diagnoses of aspergillosis in a rat model by mass spectrometry. Sci Rep 7:16523. [PubMed: 29184111]

29. Orasch T, Prattes J, Faserl K, Eigl S, Duettmann W, Lindner H, Haas H, Hoenigl M. 2017 Bronchoalveolar lavage triacetylfusarinine C (TAFC) determination for diagnosis of invasive pulmonary aspergillosis in patients with hematological malignancies. J Infect 75:370–373. [PubMed: 28576596]

30. Carroll CS, Amankwa LN, Pinto LJ, Fuller JD, Moore MM. 2016 Detection of a Serum Siderophore by LC-MS/MS as a Potential Biomarker of Invasive Aspergillosis. PLoS One 11:e0151260. [PubMed: 26974544]

31. Pontecorvo G, Roper JA, Hemmons LM, Macdonald KD, Button AW. 1953 The genetics of Aspergillus nidulans. Adv Genet 5:141–238. [PubMed: 13040135]

32. Gsaller F, Eisendle M, Lechner BE, Schreitl M, Lindner H, Muller D, Geley S, Haas H. 2012 The interplay between vacuolar and siderophore-mediated iron storage in Aspergillus fumigatus. Metallomics 4:1262–70. [PubMed: 23151814]
33. Pruller F, Wagner J, Raggam RB, Hoenigl M, Kessler HH, Truschnig-Wilders M, Krause R. 2014 Automation of serum (1→3)-beta-D-glucan testing allows reliable and rapid discrimination of patients with and without candidemia. Med Mycol 52:455–61. [PubMed: 24906361]

34. Duettmann W, Koidl C, Troppan K, Seeger K, Buzina W, Wolfler A, Wagner J, Krause R, Hoenigl M. 2014 Serum and urine galactomannan testing for screening in patients with hematological malignancies. Med Mycol 52:647–52. [PubMed: 24939321]

35. Raggam RB, Fischbach LM, Prattes J, Duettmann W, Eigl S, Reischies F, Wolfler A, Rabenstein J, Prueller F, Krause R, Hoenigl M. 2015 Detection of (1→3)-beta-D-glucan in same-day urine and serum samples obtained from patients with haematological malignancies. Mycoses 58:394–8. [PubMed: 25959065]

36. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Munoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE. 2008 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 46:1813–21. [PubMed: 18462102]

37. Hanley JA, McNeil BJ. 1982 The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 143:29–36. [PubMed: 7063747]

38. Nucci M, Nouer SA, Grazziutti M, Kumar NS, Barlogie B, Anaissie E. 2010 Probable invasive aspergillosis without prespecified radiologic findings: proposal for inclusion of a new category of aspergillosis and implications for studying novel therapies. Clin Infect Dis 51:1273–80. [PubMed: 21034199]

39. Maertens JA, Raad, II, Marr KA, Patterson TF, Kontoyiannis DP, Cornely OA, Bow EJ, Rahav G, Neofytos D, Aoun M, Baddley JW, Giladi M, Heinz WJ, Herbrecht R, Hope W, Karthaus M, Lee DG, Lortholary O, Morrison VA, Oren I, Selleslag D, Shoham S, Thompson GR, 3rd, Lee M, Maher RM, Schmitt-Hoffmann AH, Zeiher B, Ullmann AJ. 2016 Isavuconazole versus voriconazole for primary treatment of invasive mould disease caused by Aspergillus and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial. Lancet 387:760–9. [PubMed: 26684607]

40. Haas H, Zadra I, Stöffler G, Angermayr K. 1999 The Aspergillus nidulans GATA factor SREA is involved in regulation of siderophore biosynthesis and control of iron uptake. J Biol Chem 274:4613–9. [PubMed: 9988696]

41. Falcone EL, Holland SM. 2012 Invasive fungal infection in chronic granulomatous disease: insights into pathogenesis and management. Curr Opin Infect Dis 25:65869.

42. Oide S, Moeder W, Haas H, Krasnoff S, Gibson D, Yoshioka K, Turgeon BG. 2006 NPS6, encoding a non-ribosomal peptide synthetase involved in siderophoremated iron metabolism, is a conserved virulence determinant of plant pathogenic ascomycetes. Plant Cell 18:2836–53. [PubMed: 17056706]

43. Cordonnier C, Botterel F, Ben Amor R, Pautas C, Maury S, Kuentz M, Hicher Y, Bastuji-Garin S, Bretagne S. 2009 Correlation between galactomannan antigen levels in serum and neutrophil counts in haematological patients with invasive aspergillosis. Clin Microbiol Infect 15:81–6.
### Highlights

- Diagnosis of invasive aspergillosis (IA) is unsatisfying with current methods.
- New method for quantification of an *Aspergillus* siderophore (TAFC) was established.
- TAFC determination in urine yielded promising results for diagnosing IA.
- Detection in urine offers the advantage of non-invasive sampling.
Figure 1.
Overview of TAFC analysis by CE-MS. (A) Calibration curve for quantification of TAFC by CE-MS. The linear regression equation is $y = 27400 x^{1.09}$. (B) Full-scan ESI mass spectra of an equimolar mixture of TAFC (red) and $^{13}$C TAFC standard (blue). Adduct ions for ammonium, sodium and potassium were observed. (C) Extracted ion electropherograms of TAFC+H$^+$, $^{13}$C TAFC+H$^+$ and accompanying adduct ions when analyzing an equimolar mixture of standards. Mass tolerance for ion extraction was set to 10 ppm.
Comparison of TAFC/creatinine (TAFC/crea) indexes of healthy individuals (median 0.1, interquartile range (IQR) 0.1–0.3), patients at risk but without invasive aspergillosis (IA) or possible IA (median 1.4, IQR 0.5–2.6) and patients with probable IA (median 7.6, IQR 3.8–12.8). Statistical difference: **, p=0.003; ***, p<0.001. Each dot represents a patient sample and the columns display the median value. Classification of patients was based on updated EORTC/MSG criteria (36).
Figure 3.
Representative heat map presentation of biomarkers in patients with probable IA. Positive samples are highlighted in red. (A) Patient with IA from Wuerzburg, where TAFC/crea resulted positive 15 days before the first positive serum GM level and serum PCR test result (no urine GM measured). (B) Patient B with IA who died 12 days after initiation of liposomal amphotericin B (Amb) therapy. No drop in TAFC levels could be observed. (C) Patient C diagnosed with IA without follow up samples. (D) Patient D with probable IA improving under treatment. (E) Patient E developed IA under empirical caspofungin therapy (Casp), was switched to i.v. voriconazole (Vor) and improved clinically. TAFC levels decreased rapidly after voriconazole was initiated. The color code of the heat map starting with the cut-off values for positivity of each test is represented in the bottom right.

Abbreviations: BDG, 1,3-beta-d-glucan (pg/mL); crea, creatinine; Flu, fluconazole; GM, galactomannan (measured as ODI = optical density index); ICU, submission to intensive care unit; pIA, day of diagnosis “probable invasive aspergillosis”; Pos, posaconazole; TAFC, triacetlyfusarinine C; Tx, day of transplantation.
Figure 4.
Receiver operating characteristics (ROC) curve analysis for TAFCC/crea index (black) and GM/crea index (grey) for differentiating probable invasive aspergillosis (IA) from no evidence for IA or possible IA. Notably, both tests were performed with the same samples, but at different points of time.
Table 1
Demographic data and underlying diseases of the study population

| Overall cohort | Probable/Possible IA | No IA |
|----------------|----------------------|-------|
| Patients (N)   | 24                   | 8 (7/1)| 16   |
| Samples (N)    | 44                   | 23    | 21   |
| Age median, yrs (range) | 58 (19 – 75) | 64 (49 – 75) | 57 (19 – 74) |
| Female         | 10                   | 3     | 7    |

Underlying diseases

- Acute myeloid leukemia: 8/1/7
- Acute lymphoid leukemia: 4/2/2
- Lymphoma: 3/1/2
- Multiple myeloma: 2/1/1
- Myelodysplasia: 1/1/-
- Chronic lymphoid leukemia: 1/-/1
- Others: 5/2/3
- Absolute Neutropenia (<500 neutrophils per mL): 15/7/8
- Allogeneic Stem Cell Transplantation: 13/3/10
Table 2.
Per patient and per sample performance of urine TAFC/crea and urine GM/crea indexes as well as serum BDG (all biomarker/sample combinations displayed were not utilized for classifying IA). Displayed are sensitivity, specificity, positive likelihood ratio (PLR), and negative likelihood ratio (NLR) including 95% confidence intervals.

|                  | Sensitivity     | Specificity   | PLR      | NLR     |
|------------------|-----------------|---------------|----------|---------|
| **Per sample**   |                 |               |          |         |
| Urine TAFC/crea  | 0.81 (0.58 – 0.95) | 0.90 (0.68 – 0.99) | 8.10 (2.1 – 30.6) | 0.21 (0.09 – 0.52) |
| Urine GM/crea    | 0.52 (0.30 – 0.74) | 0.95 (0.75 – 1.00) | 10.48 (1.8 – 73.9) | 0.50 (0.32 – 0.79) |
| Serum BDG, pg/mL | 0.47 (0.26 – 0.70) | 1.00 (0.83 – 1.00) | perfect  | 0.52 (0.35 – 0.79) |
| **Per patient**  |                 |               |          |         |
| Urine TAFC/crea  | 0.86 (0.49 – 0.97) | 0.88 (0.64 – 0.97) | 6.9 (1.8 – 26) | 0.16 (0.03 – 1.01) |
| Urine GM/crea    | 0.71 (0.29 – 0.96) | 0.94 (0.70 – 1.00) | 11.43 (1.6 – 80.7) | 0.30 (0.09 – 0.99) |
| Serum BDG, pg/mL | 0.71 (0.29 – 0.96) | 1.00 (0.79 – 1.00) | perfect  | 0.29 (0.09 – 0.92) |

Abbreviations: BDG, beta-D-glucan; Crea, creatinine; GM, galactomannan; TAFC, triacetylfusarine C.