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Commentary

Triacetylfusarinine C: A urine biomarker for diagnosis of invasive aspergillosis

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SUMMARY

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.

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

Abbreviations: 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.

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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% and 77% and 81% and 100% for serum[4,5], while a sensitivity of 79% and a specificity of 70% has been reported 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 min, 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-labeled TAFC

For the generation of ^{13}C-isotope-labeled 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_4NO_3 and 1% w/v ^{13}C-labeled 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 min at 100 psi; the separation capillary was rinsed with acetone for 2 min at 100 psi followed by BGE for 2.5 min at 100 psi. Samples were injected for 30 s at 5 psi, which corresponds to a sample volume of 25 nL. The analysis was performed at +20 kV for 10 min 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 to 1000), SIM scan (from m/z 900 to 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:

(^{12}C TAFC in extracted sample) / (^{13}C TAFC in extracted sample) x 10 [ng] / 300 [µl] x 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 $45{,}500 \pm 8800$. 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 (Gaussian-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 $m/z = 906.33$, $+NH_4^+$ 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 (Fig. 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 ^{13}C isotope-labelled TAFC from *A. fumigatus* cultures grown with ^{13}C isotope-labeled glucose as sole carbon source. With respect to extraction and chromatography, ^{13}C TAFC displays the same features as ^{12}C TAFC, but it is distinguishable due to its higher molecular mass. Like native ^{12}C TAFC, ^{13}C TAFC exhibited four ion adducts [$+H^+$ at $m/z = 945.46$, $+NH_4^+$ at 962.49, $+Na^+$ at 967.45, $+K^+$ at 983.42]. For absolute matrix-independent TAFC quantification, patient samples were spiked with 10 ng ^{13}C TAFC as internal standard and the endogenous ^{12}C TAFC concentration was calculated by normalization to the internal ^{13}C TAFC standard. Fig. 1B and C shows exemplary CE–ESI–MS analyses of a patient sample displaying the patient ^{12}C TAFC and spiked ^{13}C 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 h; moreover, stability of TAFC spiked into urine was not affected by a 7-times cycle consisting of heating to 60 °C for 30 min followed by storage at −20 °C for 23.5 h (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 (Fig. 2).

Next, we analyzed four serial urine specimens of an acute myeloid leukemia patient (AML) diagnosed with probable IA (Fig. 3A). This patient received allogeneic stem cell transplantation and empirical fluconazole therapy followed by posaconazole treatment upon diagnosis with probable IA. Urine samples contained

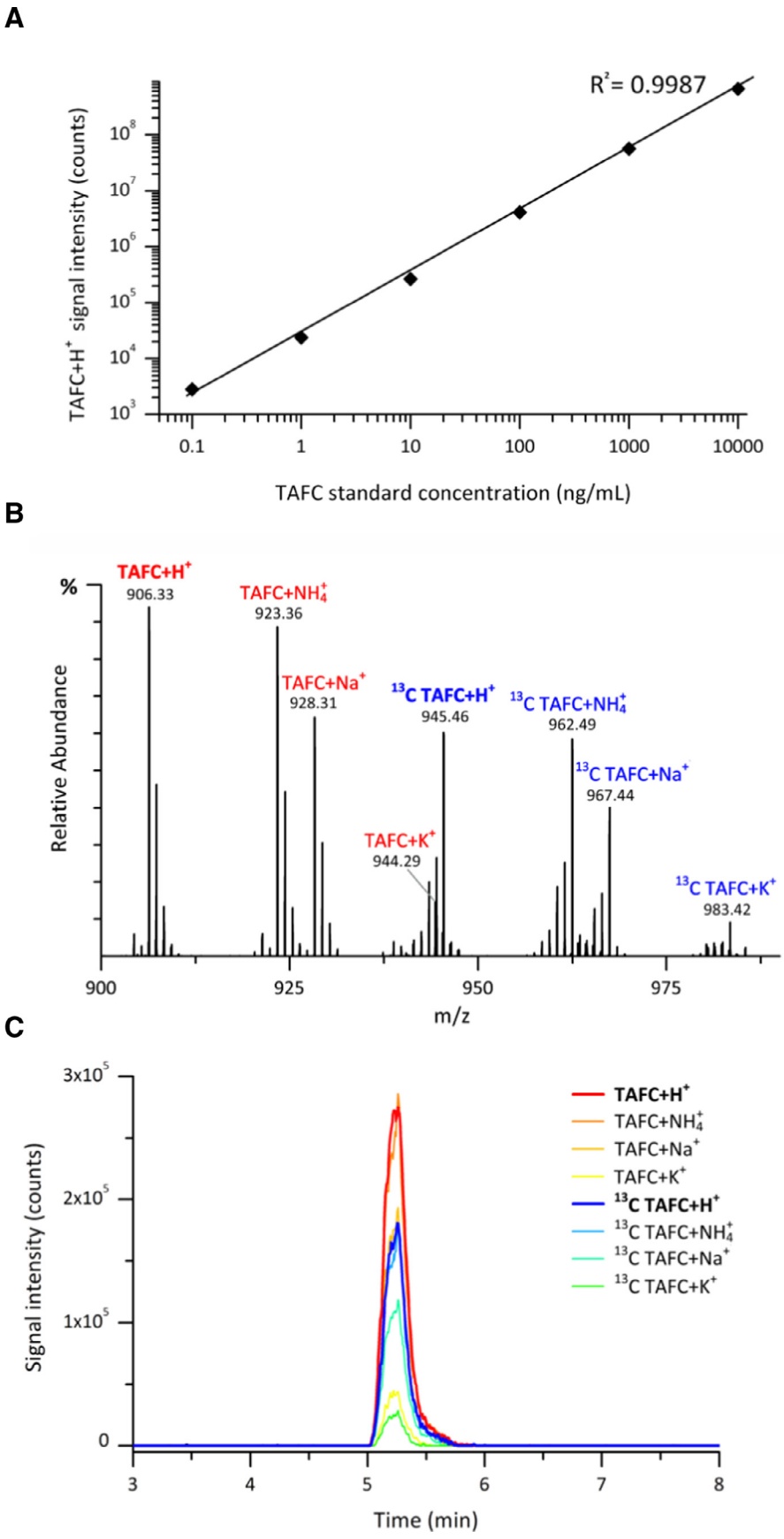

Fig. 1. Overview of TAFC analysis by CE–MS. **(A)** Calibration curve for quantification of TAFC by CE–MS. The linear regression equation is $y = 27{,}400\ x^{1.09}$. **(B)** Full-scan ESI mass spectra of an equimolar mixture of TAFC (red) and ^{13}C TAFC standard (blue). Adduct ions for ammonium, sodium 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.

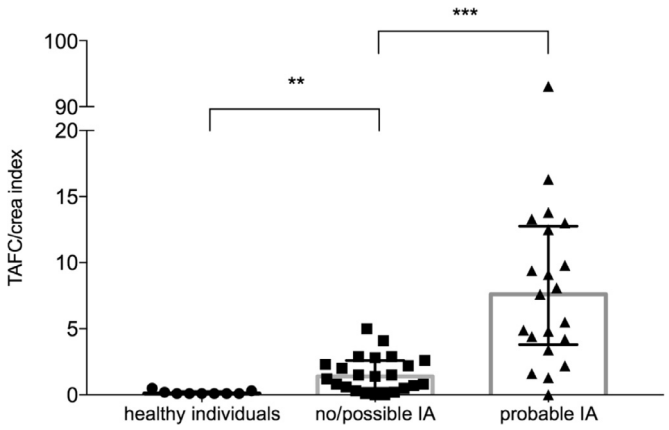

Fig. 2. Comparison of TAFC/creatinine (TAFC/crea) indexes of healthy individuals (median 0.1, interquartile range (IQR) 0.1-0.3), patients at risk but without invasive aspergillosis (IA) or possible IA (median 1.4, IQR 0.5-2.6) and patients with probable IA (median 7.6, IQR 3.8-12.8). Statistical difference: **, $p = 0.003$; ***, $p < 0.001$. Each dot represents a patient sample and the columns display the median value. Classification of patients was based on updated EORTC/MSG criteria (36).

TAFC concentrations of 28.2 to 146.8 ng/mL and TAFC/crea indexes of 6.9 to 444.1 being 36- to 2476-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 ($p = 0.003$; Fig. 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 Fig. 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 Fig. 3B–E. Fig. 3B shows a patient with probable IA, deteriorating despite treatment, displaying continuous positivity of both serum GM and TAFC/crea index. Fig. 3C patient displays positivity of all four biomarkers tested at the day of IA diagnosis without follow up samples. Fig. 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. Fig. 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 ≥ 3) were 0.86 (95%CI 0.49–0.97), 0.88 (95%CI 0.64–0.97), 6.86

A

TAFC urine		28.2		48.2									146.8			9.6			
TAFC/crea		133.3		6.9									444.1			43.6			
GM serum	0.06		0.08		0.07			0.09		0.52		>5.0		>5.0	>5.0		>5.0		
PCR serum (Cq)	nn		nn		nn			nn		43.81		33.75		37.37	41.20		42.74		
Diagnosis/clinics						Tx					pIA							ICU	death
Antifung. therapy							Flu	Flu	Flu	Flu	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	
Day	1	7	12	13	15	16	17	19	21	22	25	26	27	29	33	34	36	39	65

B

TAFC urine	4,99	6,13	1,47	8,20	
TAFC/crea	9,1	13,3	16,3	13,0	
GM serum	0,78	0,73	0,80	0,73	
GM/crea urine	0,19	0,29	1,44	0,13	
BDG serum	79	37	33	186	
Diagnosis/clinics	pIPA				death
Antifung. therapy	Amb	Amb	Amb	Amb	Amb
Day	1	6	8	11	12

C

TAFC urine		0,15	0,93
TAFC/crea		0,5	5,5
GM serum		0,40	1,14
GM/crea urine		0,22	0,52
BDG serum		47	287
Diagnosis/clinics			pIPA
Antfung. therapy	CaPr	CaPr	Vor iv
Day	1	7	9

D

TAFC urine		0,92	0,73	1,37	0,76
TAFC/crea		4,2	8,1	9,8	4,8
GM serum		0,52	0,24	0,26	0,36
GM/crea urine		3,77	0,92	0,89	3,42
BDG serum		51	40	24	15
Diagnosis/clinics		pIPA			
Antifung. therapy	CaPr	Amb	Amb	Amb	Amb
Day		1	5	6	7

E

TAFC urine		80.3	1.48	0.86
TAFC/crea		93.1	12.5	1.3
GM serum		0.99	0.59	0.10
GM/crea urine		0.23	0.65	0.10
BDG serum		2133	109	86
Diagnosis/clinics		pIPA	Clin improv	
Antifung. therapy	CaPr	Vor iv	Vor iv	Vor iv
Day	1	8	13	16

TAFC/crea	>3	>15
GM serum	>0.50	>1
GM/crea urine	>0.25	>0.75
BDG serum	>80.00	>150

Fig. 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 iv) 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.

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

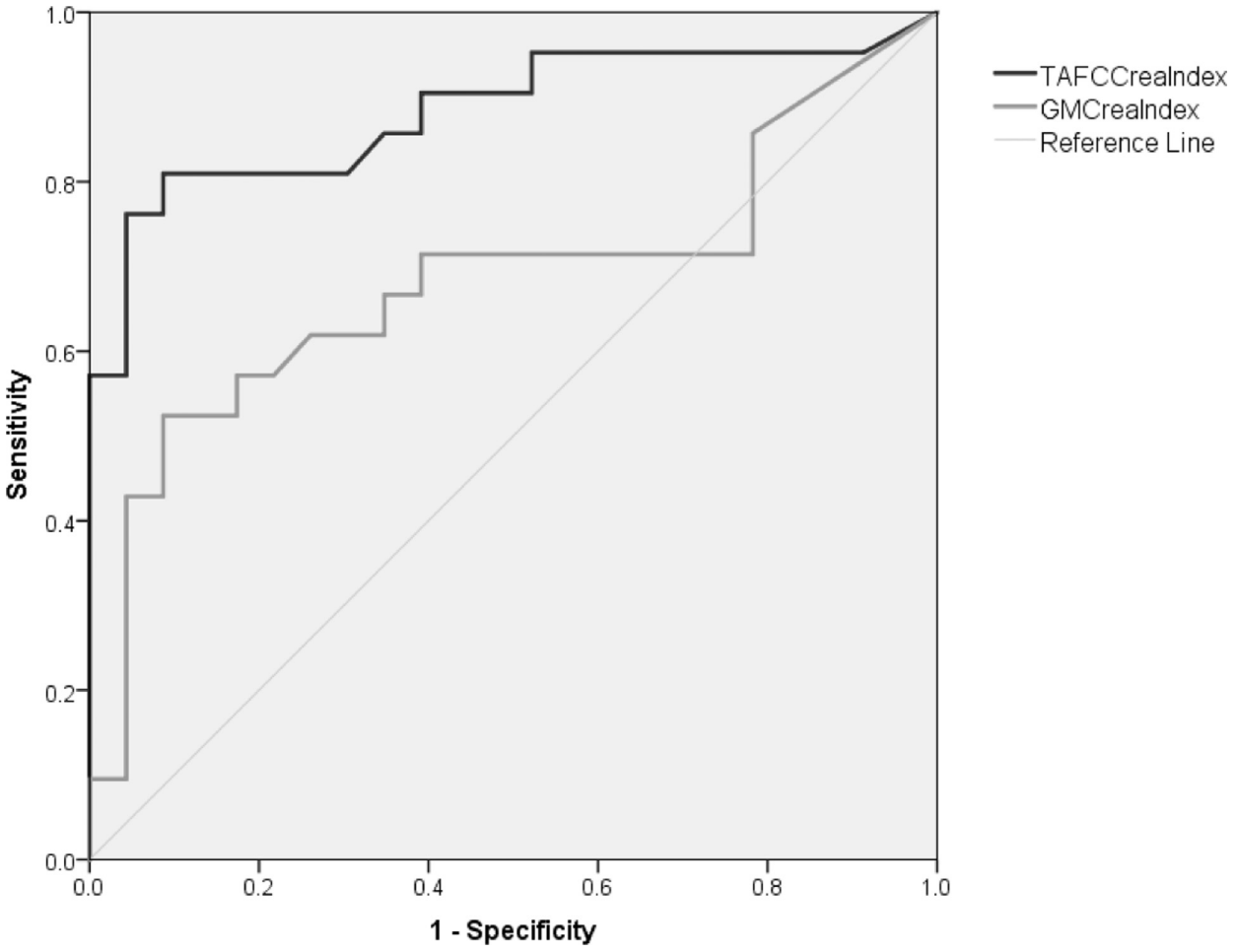

Fig. 4. Receiver operating characteristics (ROC) curve analysis for TAFC/crea index (black) and GM/crea index (grey) for differentiating probable invasive aspergillosis (IA) from no evidence for IA or possible IA. Notably, both tests were performed with the same samples, but at different points of time.

(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 antimold 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

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)

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

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-labeled 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 $\geq$ 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 $\geq$ 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 antimold 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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Conflicts of interest

H. Haas received research grant from Vical. M. Hoenigl received research grants from Gilead; and served on the speakers' bureau of Gilead, Basilea and Merck. J. Prattes received travel grant from Pfizer. All other authors declare no conflict of interest.

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