Prospective multicentre PCR-based *Aspergillus* DNA screening in high-risk patients with and without primary antifungal mould prophylaxis

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Abstract

Invasive aspergillosis (IA) is associated with significant morbidity and mortality, and, among other factors, this is due to a delay in diagnosis performed with conventional techniques. A prospective, multicentre study was conducted to evaluate the efficacy of *Aspergillus* DNA screening in the early diagnosis of IA. Patients undergoing haematopoietic stem cell transplantation or chemotherapy for acute leukaemia were enrolled for biomarker screening. Three centres applied the same protocol for in-house PCR, which was compliant with the European *Aspergillus* PCR Initiative recommendations, to guarantee the highest diagnostic standards. Two thousand one hundred and twenty-eight sera from 213 patients were investigated and stratified according to the revised European Organization for the Research and Treatment of Cancer/Mycoses Study Group criteria for invasive fungal disease. The incidence rates of probable and possible IA were 18% and 38%, respectively. The sensitivity, specificity and positive predictive value (PPV) of PCR were superior in antifungal drug-naive patients, being 71.4%, 92.3%, and 62.5%, respectively. The last of these key performance indicators (PPV) was moderate in patients receiving primary prophylaxis, at 5.4%. Negative predictive values for both strategies applied were 100% with and 98.3% without antifungal mould prophylaxis. PCR has the potential to play a decisive role in the diagnosis and management of *Aspergillus* infections in centres not applying primary antifungal mould prophylaxis.

Keywords: Antifungal mould prophylaxis, *Aspergillus*, diagnosis, DNA screening, PCR
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Introduction

Invasive aspergillosis (IA) is a common opportunistic fungal infection, and usually occurs in immunocompromised patients. Early diagnosis is critical for a favourable outcome, but is difficult to achieve with conventional methods. Hence, a range of molecular and serological techniques have been investigated. The detection of galactomannan (GM) via Platelia *Aspergillus* enzyme immunoassay (EIA) has been incorporated into the diagnostic criteria for IA, but its specificity and sensitivity vary from 40% to 100%, and depend on the population tested [1,2]. Measurement of blood (1,3)-β-D-glucan may be useful as a preliminary screening tool, but this antigen is a pan-fungal marker. The sensitivities and specificities in patients with haematological malignancies and IA ranged from 55% to 95% and from 77% to 96%, respectively [3–5]. Recently, the *Aspergillus* Lateral Flow Device was shown to be as accurate as the standard serological

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marker GM [6]. The detection of *Aspergillus* DNA lacks technical standardization, and a relatively poor understanding of DNA release prevents its broad application. Various PCR assays have been developed, and the sensitivities and specificities range from 43% to 100% and from 64% to 100%, respectively [7–14]. Not a single nucleic acid-based molecular test has yet provided enough accuracy to be incorporated into expert guidelines [15,16]. This multicentre study applied prospective biomarker-based screening in patients at risk for IA, using an identical protocol for PCR, which was compliant with the European *Aspergillus* PCR Initiative (EAPCRI) [17] recommendations to guarantee the highest diagnostic standards and to enhance PCR performance for the diagnosis of IA.

Materials and methods

Patient samples

Over a period of 2 years, twice-weekly serum samples were taken from allogeneic haematopoietic stem cell transplant recipients and patients receiving myelosuppressive chemotherapy during hospitalization and admitted to the Department for Internal Medicine II, University Hospital Würzburg (UKW), Germany, Internal Medicine V, University Hospital Innsbruck, and Internal Medicine, Hospital Elisabethinen, Linz (HEL), Austria. Clinical signs and symptoms of invasive fungal disease (IFD) and microbiological data were recorded for each individual patient according to revised European Organization for the Research and Treatment of Cancer (EORTC)/Mycoses Study Group (MSG) criteria [18]. Sera were analysed with an in-house PCR and the GM test (Platelia *Aspergillus* EIA; BioRad, Vienna/Austria) as a control at UKW, HEL, and the Division of Hygiene and Medical Microbiology, Medical University of Innsbruck (MUI). Whereas patients at the MUI and HEL received micafungin and/or posaconazole and/or voriconazole as primary antifungal prophylaxis, patients hospitalized at UKW did not did not receive mould-active prophylaxis. If an IFD was suspected, an intense diagnostic work-up was started, covering the performance of various microbiological cultures and high-resolution computed tomography scans of the chest. Bronchoscopy, biopsy sampling and antifungal treatment were performed in accordance with institutional protocols. The study was approved by the local ethics committees of the MUI (Ethikommission der Medizinischen Universität Innsbruck, UN4529). UKW and HEL tested their samples as part of routine diagnostic care.

PCR assay

DNA extraction was performed according to EAPCRI recommendations for processing serum [17]. One millilitre of serum was extracted by use of the QIAamp UltraSens virus kit (Qiagen, Hilden, Germany), with the following modification: (a) no carrier RNA was used (addition of cRNA caused non-specific amplification); (b) lysate centrifugation was adjusted to 3000 ***g***; and (c) the elution volume was adjusted to 35 µL. The elution buffer was incubated on the column for 2 min, before tubes were spun for 2 min. In each DNA extraction run, one negative control (human serum) and one *Bacillus*-positive serum (spiked with 10 000 plasmid copies of *Bacillus* DNA) were included as a quality control. Sera from HEL were shock frozen at −80°C and sent on dry ice to the MUI for PCR testing. DNA extraction and detection procedures were validated by testing different EAPCRI panels, and showed comparable performance in detecting the thresholds set by the EAPCRI [18]. An *Aspergillus*-specific real-time PCR assay targeting the internal transcribed spacer 1 (ITS1)-5.8S rRNA gene region was used to detect fungal DNA, and was performed in duplicate. Briefly, 21-µL reaction mixtures contained 0.3 µM primer Asp fum_F degen, 0.3 µM primer Fungi 5.8_R, 0.15 µM hydrolysis probe ITS-PF [19], 10 µL of SsoFast Probe Supermix (BioRad), and 10 µL of template DNA. In a second reaction, *Bacillus* DNA was detected. This internal control was tested independently of the *Aspergillus* target (monoplex), but within the same PCR run. The reaction mixtures (21 µL in total) contained 0.12 µM primer 16S S (5′-ggTCTT gAC ATC CTC TgA cAA tCC TA-3′), 0.12 µM primer 16S A (5′-AAC TgA ATg CTg gCA ACT AAg ATC A-3′), 0.07 µM hydrolysis probe 16S TM (5′-JOE-AgA gTg ACA ggT ggT gCA Tgg TTg TC-BHQ1-3′), 10 µL of SsoFast Probe Supermix (BioRad), and 10 µL of template DNA.

Amplification was carried out at UKW and the MUI with a StepOnePlus machine (Applied Biosystems, Vienna/Austria) and CFX96 Touch (BioRad), respectively, with the following steps: 95°C for 2 min, and 60 cycles of 95°C for 5 s, 54°C for 15 s (detection step), and 72°C for 1 s. Negative and positive PCR controls were included in each run, and were analysed in duplicate (*Aspergillus*). With the use of ten-fold dilutions of plasmid DNA from 10^6 to 100 copies, PCR efficiency was 110% for *Aspergillus fumigatus*, 111% for *Aspergillus terreus*, and 122% for *Aspergillus flavus*. With the use of ten-fold dilutions of genomic DNA from 10^6 to 100 copies, PCR efficiency was 100% for *A. fumigatus*, 98% for *A. terreus*, and 99% for *A. flavus*. The limit of detection was below two copies for all tested *Aspergillus* species. No cross-reactivity with other fungi or human genomic DNA was observed, and species identification was implemented as previously published [8]. DNA extracts of *A. fumigatus* (CBS 457.75), *A. terreus* (CBS 601.65) and *A. flavus* (CBS 100927) were used as quality controls.

All centres performed quantification on the day of sampling for GM with the Platelia *Aspergillus* GM ELISA (Bio-Rad), according to the manufacturer's instructions, with a cut-off optical density of 0.5.

Patients with proven or probable IA according to EORTC/MSG criteria [18] were classified as true positives, and those without any IFD present were classified as true negatives.

Statistics

For sample size calculation, we applied the value of 0.95 as an estimation of test sensitivity [19]. To ensure that the lower 95% CI was >0.75, the number of cases was calculated to be 34; an estimated 20% incidence of IA resulted in a sample size of 170 patients. All patients' characteristics and underlying diseases were analysed descriptively and according to the Standards for Reporting of Diagnostic Accuracy Initiative [20]. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), including 95% CIs, were calculated. Statistical analysis was performed separately for the two subgroups, i.e. prophylaxis vs. non-prophylaxis. Performance measures were compared between the two groups by the use of Fisher's exact test. Within groups, biomarker positivity was compared by use of the McNemar χ^2 test [21]. A significance level of $\alpha = 0.05$ (two-tailed) was applied. Statistical analyses were conducted in R version 3.1.1.

Results

Patient details and incidence of IA

The entire set of samples comprised 2259 sera collected from 235 patients at high risk for IFI. From this cohort, patients with fewer than three specimens ($n = 12$) and samples showing any failure/inhibition of the internal control or of the entire PCR reaction ($n = 56$) were excluded. Thus, our population evaluated herein consisted of 213 patients with 2128 sera investigated; this gave a mean of 10.0 sera (range, 3–30) per patient. Detailed demographic patient information is given in Table 1. Eighteen and 38 cases of probable and possible IA, and 151 with no IFD, were identified according to revised EORTC/MSG criteria [17]. Eight cases of proven IFD were documented; proof of IFD at the MUI showed that the frequency of rare fungal pathogens was increasing, whereas IA was dominant at UKW. The incidence rates of proven, probable and possible IFD/IA were 41.1% for UKW, 20.5% for the MUI, and 5.0% for HEL (Table 1). In the planning of the trial, we expected the incidence of patients with proven and probable IA to be approximately 25%. This turned out to be an overestimation, as, in reality, it was approximately 20%. The lack of IA cases led to wider CIs for the performance measures, but did not bias the endpoint results (Tables 2–4).

Biomarker positivity according to the EORTC/MSG classification

The performance of biomarker screening is shown in Table 3. At UKW, the centre without application of primary antifungal mould prophylaxis, GM screening yielded 14 cases of probable IA, and in-house PCR missed one case (92.9%). However, when two positive PCR results were gathered per patient, ten of 14 cases were detected (71.4%). Among patients suffering from possible IA, only two of 36 showed multiple positive PCR results. PCR screening failed to be of clinical value in detecting IA

TABLE 1. Characteristics of patients enrolled in the multicentre *Aspergillus* biomarker study

Underlying diseases (patients)	Study centres			
	UKW	HEL	MUI	Total
AML (*n*)	42	24	33	99
ALL (*n*)	7	5	6	18
CLL (*n*)	6	0	0	6
MDS (*n*)	20	3	3	26
Lymphoma (*n*)	17	3	1	21
MM (*n*)	37	0	1	38
Solid tumours (*n*)	0	5	0	5
HSCT (*n*)	119	40	44	203
Male/female ratio (*n*)	82/47	22/18	28/16	132/81
Female mean age in years (range)	53 (23–79)	54.1 (25–74)	53.9 (22–80)	53.5 (22–80)
Male mean age in years (range)	53.6 (20–77)	49.8 (19–76)	54.2 (20–73)	52.7 (19–77)
Fungal infections				
Possible IA (*n*)	36[a]	1	1	38
Probable IA (*n*)	14	1	3	18
Proven IFD (*n*)	3[b]	0	5[c]	8
No IFD (*n*)	78	38	35	151
Total incidence of IA (%)	10.9	2.5	6.8	20.2
Total incidence of IFD (%)	41.1	5.0	20.5	30.1
Incidence of proven and probable IFD only (%)	13.2	2.5	18.2	12.2
Total patients (*n*)	129	40	44	213

ALL, acute lymphatic leukaemia; AML, acute myeloid leukaemia; CLL, chronic lymphatic leukaemia; HEL, Hospital of Elisabethinen, Linz; HSCT, allogenic haematopoietic stem cell transplantation; IA, invasive aspergillosis; IFD, invasive fungal disease; MDS, myelodysplastic syndrome; MM, multiple myeloma; MUI, Innsbruck Medical University; UKW, University Hospital Würzburg.
[a]Two patients suffering from possible IA and simultaneously from proven invasive *Candida* infections.
[b]IFD = invasive *Candida* species infection.
[c]IFD = *Malassezia restricta*, *Hormographiella aspergillata*, *Aureobasidium* species, *Mucor* species, one unknown fungal species (hyphal positivity in lung biopsy).

TABLE 2. Serum biomarker positivity according to European Organization for the Research and Treatment of Cancer (EORTC)/Mycoses Study Group (MSG) criteria for the University Hospital Würzburg study centre (without antifungal mould prophylaxis)

Biomarker	EORTC/MSG classification	
	% of patients with probable IA ($n = 14$)	% of patients with possible IA ($n = 36$)
≥1 positive GM[a]	100.0	0.0
≥1 positive PCR[b]	92.9	41.7
≥2 positive PCRs[b]	71.4	5.6

GM, galactomannan; IA, invasive aspergillosis.
[a]Cut-off value ≥0.5.
[b]Detailed information for in-house PCR assay is given in Materials and methods.

in patients receiving primary antifungal mould prophylaxis (Table 3).

Diagnostic parameters

The data gained from probable IA cases demonstrate that a biomarker screening approach is meaningful for patients who are not receiving primary antifungal mould prophylaxis (Table 2). The PPV for double PCR positivity was 62.5%. A major finding of this study is the fact that PCR resulted in a high ratio of false positivity in patients receiving antifungal mould prophylaxis. PCR was positive in 45.5% of patients with no IFD/IA according to EORTC/MSG criteria. In contrast, UKW showed low levels of false-positive PCR results (7.7%) (Table 4). However, in general, a high NPV was found for both strategies applied, both without (98.3%) and with (100%) antifungal prophylaxis.

Discussion

In our study, the incidence rates of proven and probable IA were 2.5% (HEL), 6.8% (MUI) and 10.9% (UKW) in allogeneic haematopoietic stem cell transplant patients and acute leukaemia patients receiving chemotherapy. In contrast, the percentages of rare fungal pathogens such as Mucorales were 0% for UKW, and 2.5% for HEL, and 11.4% for the MUI. HEL and the MUI applied antifungal mould prophylaxis, whereas UKW did not. *Aspergillus* PCR screening resulted in a high rate of false positivity in centres with prophylaxis as compared with UKW. PCR revealed positive results in 45.5% of patients categorized as not suffering from IA at the MUI, and in 42.1% at HEL (Table 4). The findings obtained are of great importance, and underline the limited value of PCR screening in patients receiving antifungal mould prophylaxis. Only 6.4% false-positive results were obtained at UKW (Table 4). Overall, only 2.4% of patients from the MUI and HEL were categorized as suffering from possible IA, in contrast to 27.9% of patients from UKW.

The primary aim of our study was to determine whether PCR targeting fungal DNA extracted from serum could increase the diagnostic accuracy for detection of *IA* in high-risk patients. On the basis of the encouraging results of an earlier study, we selected a PCR assay [22] in which the use of serum is expected to produce fewer false-negative results, owing to the smaller amount of host DNA [23,24]. We focused on unifying all technical procedures to ensure in-house *Aspergillus* PCR reliability in the appointed study centres. It is striking that the PPV of the in-house *Aspergillus* PCR assays was relatively low (5.4%) in patients receiving antifungal mould prophylaxis, whereas it was sufficient (62.5%) in patients without antifungal prophylaxis (Table 3). The reasons for these differences are not clear, but may arise from multiple sources. A major reason for the inconsistency between the results reported by studies is the definition of PCR positivity. Investigators used either one or two positive PCR results per patient tested [25]; a recent meta-analysis of 16 studies evaluating PCR assays for *Aspergillus* in blood found that two positive PCR results had the same sensitivity as, but superior specificity to, a single positive result [26]. Nevertheless, our data show the same trend for prophylaxis vs. non-prophylaxis in centres using either one or two positive samples; it is clear that a single positive PCR result is of limited value. Another reason for false-positive PCR results may be contamination from blood collection tubes; Harrison *et al.* [27] found that 18% of collection tubes were contaminated with *Aspergillus* DNA. So far, participating centres have used tubes not carrying *Aspergillus* DNA [22,28].

To obtain reliable PCR results, the highest-quality standards were applied in this survey. DNA processing was compliant with EAPCRI recommendations [17], and internal quality control monitoring was ensured by performing regular sample exchanges of both centres and training of technicians. In each PCR run, negative controls (pretested human serum from healthy donors and PCR-grade water), controls spiked with *Bacillus* DNA (extraction control), positive controls with low copy numbers of *Aspergillus* DNA and inhibition controls were included. The external quality control monitoring consisted of participating EAPCRI and Quality Control for Molecular Diagnostics-initiated round-robin tests. It is therefore unlikely that single positive PCR results simply reflect laboratory contamination rather than true *Aspergillus* DNAemia.

The reason for the high rate of false-positive PCR results at centres applying antifungal mould prophylaxis is not yet known. It could have been caused by various factors, but we speculate that antifungal treatment influenced PCR testing. On the other hand, it is possible that prophylactic drugs act on fungal cells, thus providing a source of fungal DNA. Hence, in an ongoing study, we will clarify whether the drugs that we applied influence PCR testing. In any case, additional diagnostic procedures are necessary in false-positive cases.

IA disappeared in centres with prophylaxis, but other rare fungal representatives, such as Mucorales causing breakthrough infections, are increasing in frequency. We are aware of the possibility of selecting rare fungal pathogens when antifungal prophylaxis is applied, but, on the other hand, we decreased fungal incidence with this regimen [29]. A closer look at UKW shows that the proportion of possible IA cases is 27.9%; this category was only 2.4% for centres applying antifungal prophylaxis. If only one-third of these UKW patients would, in reality, shift to proven or probable cases (e.g. through intensive diagnostic work-ups), the incidence would increase adequately.

TABLE 3. Serum biomarker performance in patients suffering from probable and proven invasive aspergillosis (IA)

Evaluation of biomarkers	Biomarker performance in patients with probable IA										p-values[a] for centres without prophylaxis vs. centres with prophylaxis				
	Centre without primary antifungal prophylaxis (UKW)					Centres with primary antifungal prophylaxis (MUI and HEL)									
	1 × GM+[b]	1 × PCR+[c]	2 × PCR+[d]	GM/1 × PCR	GM/2 × PCR	1 × GM+[e,f]	1 × PCR+[c]	2 × PCR+[d]	GM/1 × PCR[g]	GM/2 × PCR[h]	1 × GM+	1 × PCR+	2 × PCR+	GM/1 × PCR	GM/2 × PCR
True positives (*n*)	14	13	10	13	10	4	4	2	4	2	—	—	—	—	—
True negatives (*n*)	73	57	72	75	76	53	20	38	55	60	—	—	—	—	—
False positives (*n*)	5	21	6	3	2	20	53	35	18	13	—	—	—	—	—
False negatives (*n*)	0	1	4	1	4	0	0	2	0	2	—	—	—	—	—
Sensitivity, % (95% CI)	100.0 (76.8–100.0)	92.9 (66.1–99.8)	71.4 (41.9–91.6)	92.9 (66.1–99.8)	71.4 (41.9–91.6)	100.0 (39.8–100.0)	100.0 (39.8–100.0)	50.0 (6.8–93.2)	100.0 (39.8–100.0)	50.0 (6.8–93.2)	1.000	1.000	0.569	1.000	0.569
Specificity, % (95% CI)	93.6 (85.7–97.9)	73.1 (61.8–82.5)	92.3 (84.0–97.1)	96.2 (89.2–99.2)	97.4 (91.0–99.7)	72.6 (60.9–82.4)	27.4 (17.6–39.1)	52.1 (40.0–63.9)	75.3 (63.9–84.7)	82.2 (71.5–90.2)	0.001	<0.001	<0.001	<0.001	0.002
PPV, % (95% CI)	73.7 (48.8–90.9)	38.2 (22.2–56.4)	62.5 (35.4–84.8)	81.3 (54.4–96.0)	83.3 (51.6–97.9)	16.7 (4.7–37.4)	7.0 (1.9–17.0)	5.4 (0.7–18.2)	18.2 (5.2–40.3)	13.3 (1.7–40.5)	<0.001	<0.001	<0.001	<0.001	<0.001
NPV, % (95% CI)	100.0 (95.1–100.0)	98.3 (90.8–100.0)	94.7 (87.1–98.5)	98.7 (92.9–100.0)	95.0 (87.7–98.6)	100.0 (93.3–100.0)	100.0 (83.2–100.0)	95.0 (83.1–99.4)	100.0 (93.5–100.0)	96.8 (88.8–99.6)	1.000	1.000	1.000	1.000	0.696
DOR	2045.0	34.5	29.4	—	—	425.0	161.0	1.1	—	—	—	—	—	—	—

The mathematical formulas were: TP/(TP + FN) for sensitivity; TN/(FP + TN) for specificity; TP/(TP + FP) for PPV; TN/(FN + TN) for NPV; and (TP × TN + 0.5)/(FP × FN + 0.5) for DOR.
DOR, diagnostic OR; FN, false negative; FP, false positive; GM, galactomannan; HEL, Hospital of Elisabethinen, Linz; MUI, Medical University of Innsbruck; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive; UKW, University Hospital of Würzburg.
[a]Fisher's exact test.
[b]Probable patients with one positive GM test from serum (definition of IA according to EORTS/MSG criteria).
[c]Probable patients with one positive serum PCR test; detailed information on the design of the in-house PCR assay is given in Naterials and methods.
[d]Probable patients with a minimum of two positive serum PCR tests.
[e]Probable patients with one positive GM test from serum or bronchoalveolar lavage fluid (definition of IA according to European Organization for the Research and Treatment of Cancer (EORTS)/Mycoses Study Group (MSG) criteria).
[f]Random retesting of positive samples revealed positive results in 99%.
[g]Probable patients with one positive GM test from serum or bronchoalveolar lavage fluid (definition of IA according to EORTS/MSG criteria) plus one positive serum PCR test; detailed information on the design of the in-house PCR assay is given in Materials and methods.
[h]Probable patients with one positive GM test from serum or bronchoalveolar lavage fluid (definition of IA according to EORTS/MSG criteria) plus two positive serum PCR tests; detailed information on the design of the in-house PCR assay is given in Materials and methods.

TABLE 4. Centre-specific serum biomarker positivity in patients without invasive fungal disease (IFD)/invasive aspergillosis according to the European Organization for the Research and Treatment of Cancer (EORTC)/Mycoses Study Group (MSG) classification

	% of patients with no IFD according to the EORTC/MSG classification		
Biomarker	**UKW (n = 78)**	**HEL (n = 38)**	**MUI (n = 35)**
≥1 positive PCR[a]	26.9	73.7	59.1
≥2 positive PCRs[a]	7.7	42.1	45.5

HEL, Hospital of Elisabethinen, Linz; MUI, Medical University of Innsbruck; UKW, University Hospital of Würzburg.
[a]Detailed information on the in-house PCR assay is given in Materials and methods.

Recently, colleagues showed that a combined monitoring strategy based on serum GM and *Aspergillus* DNA was associated with earlier and improved diagnosis in high-risk haematological patients not receiving anti-mould prophylaxis [30].

Our study suffers from several limitations, as the number of patients with proven IA was limited, and patients were receiving different antifungal regimens, because our study was a real-life study. Another potential drawback of the approach used in our trial is the fact that the biomarker-based strategy does not detect non-*Aspergillus* mould infections. The statistical analyses of performance measures of GM vs. PCR suffer from the limitation that GM is part of the EORTC/MSG criteria for discriminating between probable or possible IA; therefore, by definition, all patients with probable IA are positive for GM, and all patients with possible IA are negative for GM. This incorporation bias leads to an optimistic estimation of performance measures for GM, and might also negatively influence performance measures of PCR; this fact could not be overcome in the current study, owing to the limited number of proven IA cases.

Taking all of the above-mentioned factors into consideration, it becomes evident that PCR has the potential to play a decisive role in the diagnosis and management of *Aspergillus* infections in centres not applying prophylaxis. PCR screening is of limited value in patients receiving antifungal mould prophylaxis. In this setting, any test should be used in a diagnostic way to confirm the aetiology of any breakthrough infections.

Transparency declaration

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Author contributions

J. Springer and M. Lackner optimized and developed the new PCR assay. B. Risslegger and W. Mutschlechner performed data monitoring. J. Fritz performed statistical analysis and data analysis. D. Nachbaur, H. Einsele, W. J. Heinz and A. J. Ullmann contributed to the collection and final analysis of data. J. Löffler and C. Lass-Flörl designed the study and provided regulatory advice. C. Lass-Flörl drafted the manuscript. All authors contributed to data interpretation and revision of the final manuscript.

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