Langenbeck's Archives of Surgery (2019) 404:309–325
https://doi.org/10.1007/s00423-019-01769-y

ORIGINAL ARTICLE

New approaches for the detection of invasive fungal diseases in patients following liver transplantation—results of an observational clinical pilot study

Sebastian O. Decker[1] · Albert Krüger[1] · Henryk Wilk[1] · Silke Grumaz[2] · Yevhen Vainshtein[2] · Felix C. F. Schmitt[1] · Florian Uhle[1] · Thomas Bruckner[3] · Stefan Zimmermann[4] · Arianeb Mehrabi[5] · Markus Mieth[5] · Karl Heinz Weiss[6] · Markus A. Weigand[1] · Stefan Hofer[7] · Kai Sohn[2] · Thorsten Brenner[1]

Received: 7 August 2018 / Accepted: 21 February 2019 / Published online: 5 March 2019

Abstract

Purpose Despite antifungal prophylaxis following liver transplantation (LTX), patients are at risk for the development of subsequent opportunistic infections, such as an invasive fungal disease (IFD). However, culture-based diagnostic procedures are associated with relevant weaknesses.

Methods Culture and next-generation sequencing (NGS)-based fungal findings as well as corresponding plasma levels of ß-D-glucan (BDG), galactomannan (GM), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin (IL)-2, -4, -6, -10, -17A and mid-regional proadrenomedullin (MR-proADM) were evaluated in 93 patients at 6 consecutive time points within 28 days following LTX.

Results A NGS-based diagnostic approach was shown to be suitable for the early identification of fungal pathogens in patients following LTX. Moreover, MR-proADM and IL-17A in plasma proved suitable for the identification of patients with an IFD.

Conclusion Plasma measurements of MR-proADM and IL-17A as well as a NGS-based diagnostic approach were shown to be attractive methodologies to attenuate the weaknesses of routinely used culture-based diagnostic procedures for the determination of an IFD in patients following LTX. However, an additional confirmation within a larger multicenter trial needs to be recommended.

Trial registration German Clinical Trials Register: DRKS00005480.

Keywords *Candida* spp. · *Aspergillus* spp. · Next-generation sequencing · Interleukin-17A · ß-D-glucan · Mid-regional proadrenomedullin

Thorsten Brenner and Kai Sohn share senior authorship.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00423-019-01769-y) contains supplementary material, which is available to authorized users.

✉ Kai Sohn

✉ Thorsten Brenner
thorsten.brenner@med.uni-heidelberg.de

[1] Department of Anesthesiology, Heidelberg University Hospital, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany

[2] Fraunhofer IGB, Nobelstraße 12, 70569 Stuttgart, Germany

[3] Institute of Medical Biometry and Informatics, University of Heidelberg, Im Neuenheimer Feld 130, 69120 Heidelberg, Germany

[4] Department of Infectious Diseases, Medical Microbiology and Hygiene, Heidelberg University Hospital, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany

[5] Department of General, Visceral and Transplant Surgery, Heidelberg University Hospital, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany

[6] Department of Internal Medicine IV, Heidelberg University Hospital, Im Neunheimer Feld 410, 69120 Heidelberg, Germany

[7] Department of Anesthesiology, Westpfalzklinikum, Hellmut-Hartert-Straße 1, 67655 Kaiserslautern, Germany

Introduction

Liver transplantation (LTX) is a routinely used therapeutic option for patients with end-stage liver diseases (ESLDs). Due to improvements in medical care within the last years, the outcome of patients following LTX has steadily improved. These efforts are mainly due to the use of better immunosuppressive drugs [1]. However, the improved efficacy of newly developed immunosuppressive drugs is associated with an increased risk for invasive fungal diseases (IFDs) [2, 3]. Patients suffering from an IFD are hallmarked by a significantly increased mortality [3, 4]. In this context, *Candida albicans*, *Candida glabrata*, and *Aspergillus fumigatus* are of most relevance [5, 6]. The attributed mortality for IFDs ranges from 10 to 67% in the case of *Candida* spp. [7–10] and up to 80% in the case of invasive aspergilloses (IA) [11, 12]. However, routinely used culture-based diagnostic procedures are associated with relevant weaknesses, since blood cultures often remain negative despite the presence of an IFD [13, 14]. Hence, in patients with persistent leucocytosis or fever despite broad-spectrum antimicrobial therapy, the presence of an IFD needs to be taken into account. Moreover, a high fungal burden at non-sterile body sites or in drainages may support the diagnosis of an IFD most frequently caused by *Candida* spp. [15]. The diagnosis of an IFD caused by molds (e.g., *Aspergillus* spp.) is even more difficult, since the EORTC/MSG definitions were developed for hematological patients and presentation of IA or other IFD might be different in other populations [16, 17]. These diagnostic procedures are time-intensive and may result in a delay until an antifungal therapy is initiated [16, 18, 19]. The value of polymerase chain reaction (PCR)-based diagnostic procedures remains unclear [18]. Moreover, the value of biomarkers, such as plasmatic ß-D-glucan (BDG) and galactomannan (GM), in bronchoalveolar lavage fluid (BALF) or plasma for the diagnosis of an IFD is limited due to high rates of false-positive test results [20–22]. The poor performance of these biomarkers can be explained by patients' characteristics, such as the use of ß-lactam antibiotics, renal replacement therapy, transfusions, and gastrointestinal translocations [20]. In order to prevent opportunistic IFDs, an antifungal prophylaxis is applied in most cases [23, 24]. Unfortunately, these drugs are not effective in all cases and are associated with relevant side effects [25, 26]. Moreover, interleukins 10 and 17A, as well as mid-regional proadrenomedullin (MR-proADM) have recently been shown to be of potential value for the diagnosis of an IFD in patients suffering from septic shock [27]. However, the diagnostic value in patients following LTX has not been investigated yet.

The aims of this study were, therefore, (1) to assess the cumulative incidence of an IFD as well as to determine its influence on patients' outcome, (2) to compare characteristics of patients with and without an IFD, (3) to evaluate the additional benefit of a next-generation sequencing (NGS)-based diagnostic approach, and (4) to assess the diagnostic value of ß-D-glucan (BDG), galactomannan (GM), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukins (IL)-2, -4, -6, -10, -17A and mid-regional proadrenomedullin (MR-proADM) in patients following LTX.

Materials and methods

Study design

The observational clinical study was approved by the local ethics committee (Ethics Committee of the Medical Faculty of Heidelberg, Trial Code No. S-098/2013/ German Clinical Trials Register DRKS00005480) and conducted in the surgical intensive care unit of the Heidelberg University Hospital, Germany, between February 2014 and March 2016. All study patients gave written informed consent. In total, 93 patients suffering from an ESLD undergoing orthotopic LTX were enrolled in this study, representing the only inclusion criterion. Missing written consent and age below 18 years were defined as exclusion criteria. The treatment of patients was based on the Heidelberg Manual for LTX [28]. Plasma samples for NGS-based diagnostics and the different biomarker measurements were collected directly after LTX (T0) and 1 day (T1), 2 days (T2), 7 days (T3), 14 days (T4), 21 days (T5), and 28 days (T6) thereafter. Additionally, blood cultures were taken at T0, T3, T4, and T6. Relevant baseline data (demographic data, primary site of infection), clinical data (disease severity scores, such as Simplified Acute Physiology (SAPS II) score, Sequential Organ Failure Assessment (SOFA) score, and Acute Physiology Health Evaluation (APACHE II) score, surgical procedures, antifungal therapy, outcome parameters) and routine infection parameters (leukocytes, C-reactive protein (CRP), procalcitonin (PCT), body temperature) were also collected. The primary endpoint of this study was to assess the cumulative incidence of an IFD in patients following LTX by the use of a multifaceted approach. Apart from culture-based microbiological procedures, the additional benefit of a next-generation sequencing (NGS)-based diagnostic approach, as well as the diagnostic value of ß-D-glucan (BDG), galactomannan (GM), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukins (IL)-2, -4, -6, -10, -17A, and mid-regional proadrenomedullin (MR-proADM) for the diagnosis of an IFD in patients following LTX was assessed. The influence of an IFD following LTX on patients' outcome was defined as a secondary endpoint.

Moreover, characteristics of patients with and without an IFD following LTX were compared.

Group definitions

Candida spp. in the respiratory tract or in fluids from drainages were classified as colonization. Positive results in blood cultures, intraoperative swabs, and *Aspergillus* spp. in deep respiratory tract specimens with accompanying pulmonary infiltrates characteristic of an IA were classified as infection.

Detailed information about immunoassays, flow cytometry analyses, and microbiological are presented in the Supplementary Digital Content (SDC) S1 and described elsewhere [27].

NGS-based diagnostics

Plasma preparation and nucleic acid isolation

Plasma was prepared from blood samples by centrifugation for 10 min at 292 ×*g* and 4 °C, snapped frozen, and stored at − 80 °C until further processing. Nucleic acids were isolated from thawed plasma after a centrifugation step of 5 min at 16,000 ×*g* at 4 °C with the QIAsymphony sample preparation (SP) instrument (Qiagen) and the QIAsymphony DSP Circulating DNA Kit following the manufacturer's protocol. To ensure preparation from equal volumes of 1000 μl, 1200 μl starting volume of plasma was loaded per sample. The following were variations from the protocol: if plasma volumes were below 1000 μl after centrifugation, the respective samples were excluded from the analysis. Nucleic acids were eluted in 60 μl of molecular biology-grade water (5 Prime, Germany). Contamination controls were prepared following the same procedure, starting from 1200 μl of molecular biology-grade water (5 Prime, Germany). The cfDNA was quantified with the Qubit dsDNA HS Assay Kit (Life Technologies), and quality was assessed with the HS NGS Fragment Analysis Kit and the Fragment Analyzer instrument (AATI).

Preparation of NGS libraries and sequencing

Library preparation and sequencing were carried out as previously described [29] from 1 ng cfDNA using the Nextera XT library preparation kit (Illumina), with a Biomek FXP liquid handling robot (Beckman Coulter). Sequencing of the libraries was performed on a HiSeq2500 (Illumina), resulting in 20 million 100-bp single end reads, on average, per sample.

Bioinformatics

Bioinformatics processing, classification, and SIQ score calculation were carried out as described previously [29]. Seven control samples of liver transplant patients directly following liver transplantation and 1 day later were available to serve as the control population. Species hits were excluded according to the following criteria: (a) taxonomy in RefSeq database does not show a clear species assignment; (b) hit is a phage; (c) bacterial/viral hits with less than ten or fungal hits with less than two normalized counts; (d) p value above 0.05; (e) hits belong to known contaminant genus according to Salter and colleagues [30], except SIQ score above 500 and species previously described as a pathogen (labeled with contaminant tag a); (f) species counts in more than 50% of all samples detected according to the complete investigated cohort, except SIQ score above 500 and species previously described as a pathogen (labeled with contaminant tag b); (g) all hits from *Ralstonia pickettii*, because they show a SIQ score of 500 or above in more than 25% of the samples according to the complete investigated cohort.

Statistical analyses

The resulting data were entered into an electronic database (Excel 2010; Microsoft Corp., Redmond, USA) and evaluated using SPSS software (Version 24.0; SPSS, Inc., Chicago, USA). Figures were drawn using GraphPad Prism (GraphPad Software, La Jolla, USA). Categorical data were summarized using absolute and relative frequencies. Quantitative data were summarized using median with quartiles. The Kolmogorov–Smirnov test was applied to check for normal distribution. Due to non-normally distributed data, non-parametric methods for evaluation were used (Chi-square test for categorical data, Mann–Whitney U test for continuous data). Appropriate cut-off values for the detection of an IFD were calculated using ROC analyses. A p value less than 0.05 was considered statistically significant. Concerning symbols and higher orders of significance, the following were utilized: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

Results

Patients' characteristics

In total, 93 patients were included in this study. Patients' characteristics are presented in Table 1, whereas detailed information about the perioperative course of the patients is presented in Table 2.

Table 1 Patients' characteristics

Parameter	Unit	All patients ($n = 93$)	With fungal isolates ($n = 23$)	Without fungal isolates ($n = 70$)	p for patients without fungal isolates vs. with fungal isolates
Male		58 (62.0%)	11 (47.8%)	47 (67.0%)	0.080
Age	(years)	52 (42–58)	52 (40–59)	52 (44–58)	0.883
BMI	(kg/m^2)	25.5 (23.0–29.9)	25.7 (23.4–29.9)	25.5 (23.0–29.8)	0.718
MELD score		18.0 (11.0–28.0)	32.0 (20.0–37.5)	16. (10.–21.3)	0.001**
Causes of liver cirrhosis					
Alcohol		27 (29.0%)	7 (30.4%)	20 (28.6%)	0.530
Hepatitis B		6 (6.5%)	2 (8.7%)	4 (5.7%)	0.463
Hepatitis C		10 (10.8%)	1 (4.3%)	9 (12.9%)	0.234
HCC		25 (26.9%)	7 (30.4%)	18 (25.7%)	0.424
PSC		16 (17.2%)	4 (17.4%)	12 (17.1%)	0.601
PBC		5 (5.4%)	2 (8.7%)	3 (4.3%)	0.361
NASH		7 (7.5%)	0 (0.0%)	7 (10.0%)	0.127
Others		20 (21.5%)	5 (21.7%)	15 (21.4%)	0.592
Need for catecholamines before LTX		3 (3.2%)	3 (13.0%)	0 (0.0%)	0.014*
NYHA 0-I		90 (96.8%)	22 (95.7%)	68 (97.1%)	0.578
Diabetes mellitus		18 (19.4%)	3 (13.0%)	15 (21.4%)	0.290
Arterial hypertension		28 (30.1%)	5 (21.7%)	28 (28.6%)	0.133
Coronary heart disease		10 (10.8%)	2 (8.7%)	8 (11.4%)	0.926
Chronic obstructive lung disease		7 (7.5%)	2 (8.7%)	5 (7.1%)	0.590
Smoker		21 (22.6%)	6 (26.1%)	15 (21.4%)	0.830
Renal insufficiency		20 (21.5%)	6 (26.1%)	14 (20.0%)	0.376
Pre-existing ARF		10 (10.8%)	5 (21.7%)	5 (7.1%)	0.91
Pre-existing thrombosis		18 (19.3%)	8 (34.8%)	10 (14.3%)	0.092
Neurological disorder		43 (46.2%)	13 (56.2%)	30 (42.9%)	0.327
High-urgency		32 (34.4%)	5 (21.7%)	27 (38.6%)	0.109
Re-LTX		16 (17.2%)	7 (30.4%)	9 (12.9%)	0.057
Immunosuppressive medication					
Corticosteroids		93 (100%)	23 (100%)	70 (100%)	–
Mycophenolat mofetil		92 (98.6%)	23 (100%)	69 (96.6%)	0.731
Ciclosporin		39 (41.9%)	10 (43.5%)	29 (41.4%)	0.495
Tacrolimus		54 (58.1%)	13 (56.5%)	41 (58.6%)	0.495

Data are presented either as number (with the corresponding percentage value) or as median (with accompanying quartiles (Q1–Q3)).

BMI, body mass index; MELD, model of end-stage liver disease; HCC, hepatocellular carcinoma; PSC, primary sclerosing cholangitis; PBC, primary biliary cirrhosis; NASH, non-alcoholic fatty liver disease; NYHA, New York Heart Association; ARF, acute renal failure; LTX, liver transplantation

Concerning symbolism and higher orders of significance: $p < 0.05$: *, $p < 0.01$**

Fungal pathogens and infection sites

As assessed by culture-based diagnostic procedures, 23 patients (24.8%) revealed fungal isolates in different specimens within the 28-day observation period, whereas 70 patients (75.2%) were found to be negative for fungal isolates (Fig. 1). Fungal isolates were found in single and multiple locations in 20 (87.0%) and 3 (13.0%) patients, respectively, and were located at the following sites: respiratory tract ($n = 10$; 43.5%), abdominal site ($n = 10$; 43.5%), blood culture ($n = 2$; 8.7%), and one single case of a disseminated *Rhizopus microsporus* infection ($n = 1$; 4.3%) (double-naming feasible; Fig. 1).

Preoperative bilirubin levels as well as corresponding MELD scores were significantly increased in patients with fungal isolates in comparison to those patients without any

Table 2 Details of the ICU and hospital stay

Parameter	Unit	All patients ($n = 93$)	With fungal isolates ($n = 23$)	Without fungal isolates ($n = 70$)	p for patients without fungal isolates vs. with fungal isolates
APACHE II^{+}		27.0 (17.0–32.0)	29.0 (21.5–34.5)	25.0 (17.0–31.0)	0.072
SOFA^{+}		13.0 (7.0–15.0)	15.0 (10.0–17.5)	11.5 (5.0–15.0)	0.013*
SAPS^{+}		52.0 (30.0–69.0)	61.0 (44.0–78.0)	43.5 (25.8–68.5)	0.012*
Time of mechanical Ventilation	(days)	1 (1–4)	2 (1–13)	1 (1–3)	0.002**
Tracheostomy		11 (11.8%)	8 (34.8%)	3 (4.3%)	0.000***
Hospital stay before LTX	(days)	1.0 (1.0–7.0)	7.0 (1.0–21.5)	1.0 (1.0–1.0)	0.002**
ICU stay	(days)	13.0 (8.0–24.)	22.0 (12.5–40.5)	11.5 (8.0–20.0)	0.021*
Hospital stay	(days)	34.0 (25.0–52.0)	45.0 (27.0–65.0)	32.0 (24.3–50.0)	0.138
90-day survival		73 (78.5%)	13 (56.5%)	60 (85.7%)	0.005**
28-day survival		86 (92.8%)	20 (87.0%)	66 (94.3%)	0.232
ALF after LTX		14 (16.1%)	7 (30.4%)	9 (11.4%)	0.057
ARF after LTX		31 (33.3%)	12 (52.2%)	19 (27.1%)	0.027*
Dialysis					
Directly after LTX		6 (6.5%)	4 (17.0%)	2 (2.9%)	0.030*
In time course		27 (20.0%)	14 (60.1%)	13 (18.6%)	0.000***
BDA		22 (23.7%)	6 (26.1%)	16 (22.9%)	0.565
Duration of surgery	(min)	347 (289–405)	375 (340–405)	332 (285–409)	0.049*
Intraoperative blood loss	(L)	3 (1.5–4.4)	3 (1.6–7.6)	2.6 (1.4–4.0)	0.040*
Rejection		20 (21.5%)	6 (26.1%)	14 (20.0%)	0.364
Administration of					
PRBC	(n.o.p.)	67 (72.0%)	21 (91.3%)	46 (65.7%)	0.028*
FFP	(n.o.p.)	75 (80.6%)	22 (95.7%)	53 (75.7%)	0.008**
Platelets	(n.o.p.)	66 (70.9%)	18 (78.3%)	48 (68.6%)	0.013*
Perforation of the intestine or stomach		4 (4.3%)	3 (13.0%)	1 (2.5%)	0.045*
BDA-insufficiency (patients with BDA; $n = 22$)		5 (22.7%)	3 (50.0%)	2 (12.5%)	0.095
Stenosis of the bile duct		9 (9.7%)	3 (13.0%)	6 (15.0%)	0.391
Leakage of the bile duct		10 (10.8%)	2 (8.7%)	8 (11.4%)	0.138
Need for surgical intervention		44 (47.3%)	18 (78.3%)	26 (37.1%)	0.001*
Vascular complications		13 (14.0%)	3 (13.0%)	10 (14.3%)	0.594
Need for endoscopic diagnostics		13 (14.0%)	5 (21.7%)	8 (11.4%)	0.184

Data are presented either as number (with the corresponding percentage value) or as median (with accompanying quartiles (Q1–Q3)).

APACHE II score, Acute Physiology And Chronic Health Evaluation score; SOFA, Sequential Organ Failure Assessment score; SAPS, Simplified Acute Physiology score; ICU, intensive care unit; LTX, liver transplantation; ARF, acute renal failure; ALF, acute liver failure; BDA, biliodigestive anastomosis; n.o.p., number of patients; PRBC, packed red blood cells; FFP, fresh frozen plasma, $^{+}$ calculated at the first day after LTX

Concerning symbolism and higher orders of significance: $p < 0.05$: *, $p < 0.01$**, $p < 0.001$***

fungal findings (bilirubin: median 9.4 mg/dl (Q1 1.7 mg/dl–Q3 28.4 mg/dl) vs. 2.95 mg/dl (1.2–8. 5 mg/dl) → $p = 0.014$*/MELD score: 32 (20.0–37.5) vs. 16 (10.0–21.3) → $p = 0.001$*) (Table 1). Moreover, in patients with fugal isolates, allogenic blood products were administered more frequently during LTX in comparison to patients without fungal isolates (fresh frozen plasma (FFP): 22 patients (95.7%) vs. 53 patients (75.7%) → $p = 0.008$*/packed red blood cells (PRBC): 21 (91.3%) vs. 46 (65.7%) → $p = 0.028$*, platelets: 18 (78.3%) vs. 48 (68.6%) → $p = 0.013$*) (Table 2). Apart from that, patients with fungal isolates were shown to be more seriously ill following LTX, proven by a prolonged need for mechanical ventilation, a higher incidence of acute renal failures (ARF), a longer ICU stay, as well as significantly increased disease severity scores and a reduced survival at 90 days (Table 2).

Based on the group definitions, patients with fungal isolates ($n = 23$) were defined to be fungally colonized in 13 cases, whereas an IFD was present in 10 patients. A detailed description of the identified fungal pathogens is given in Table 3,

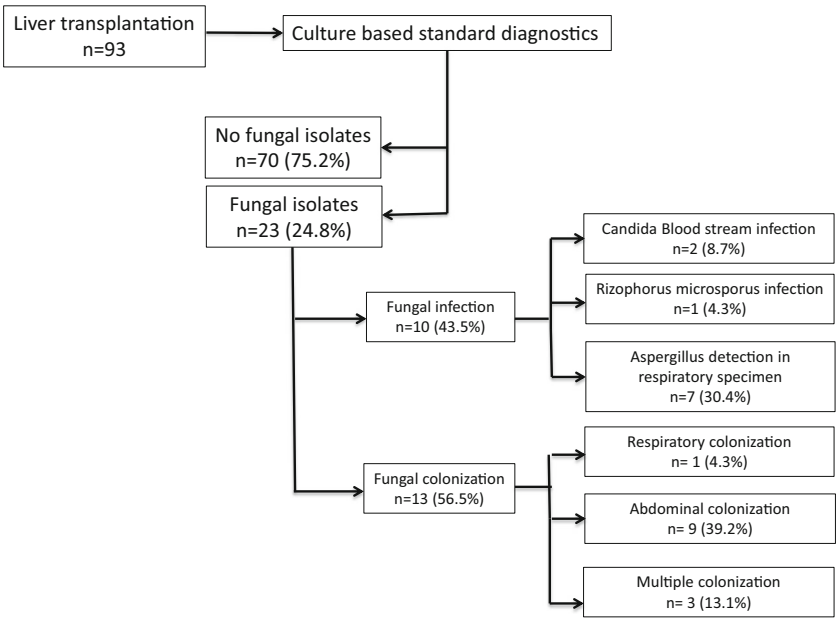

Fig. 1 Identification of fungal pathogens in patients following LTX ($n = 93$)

whereas patients' characteristics as well as a summary of the antifungal treatment concept are given in Table 4 and SDC S2 (including Table T1). Patients suffering from an IFD were shown to have a significantly increased need for mechanical ventilation, suffered from an ARF more frequently, and revealed a significantly decreased 90-day survival (Table 5). Conversely, both subgroups did not differ significantly with respect to the need for allogenic blood products (Table 5).

NGS-based microbiological diagnostics

Analogous to the previously described NGS methodology [29], we calculated SIQ scores, including modified fungal SIQ scores for each time point and compared it to the clinical microbiology data within the observation period of 28 days.

Within this manuscript, we would like to present detailed data of two exemplary cases, describing the suitability of a NGS-based diagnostic approach for the detection of an IFD in two different scenarios:

(1) Confirmation of candidemia by fungal SIQ score. Patient L89 suffered from candidemia, as confirmed by a positive blood culture at T0. Fungal SIQ score revealed *C. albicans* at days 1, 2, 7, and 14 after LTX, confirming candidemia up to 14 days after LTX. A detailed description of the patient's course is given in Fig. 2a and SDC 4.
(2) Detection of an IFD by fungal SIQ score even in patients which have so far been classified as colonized based on culture-based diagnostic procedures. Due to restrictions, including the limited sensitivity of culture-based

Table 3 Detailed fungal findings

	Invasive fungal disease ($n = 10$)			Colonization ($n = 13$)				
	Fungemia	Invasive aspergillosis	Disseminated	Tracheal secretion	Abdominal wound swab	Abdominal drainage	Bile fluid	Urine
Candida albicans	2	0	0	2	3	2	2	2
Candida glabrata	0	0	0	1	0	2	1	1
Candida spp.	0	0	0	0	0	1	0	3
Candida norvegensis	0	0	0	0	0	1	0	0
Candida dublinensis	0	0	0	0	0	1	0	0
Aspergillus spp.	0	7	0	0	0	0	0	0
Rhizopus microsporus	0	0	1	0	0	0	0	0

Data are presented as numbers. Double-naming feasible

Table 4 Characteristics of patients with an IFD or with a fungal colonization

Parameter	Unit	Fungal infection ($n = 10$)	Fungal colonization ($n = 13$)	p for patients fungal infections vs. patients with colonization
Male gender		2 (20.0%)	9 (69.2%)	0.026*
Age	(years)	48.0 (34.5–57.5)	54.0 (45.0–60.0)	0.410
BMI	(kg/m^2)	25.6 (23.9–31.0)	27.3(23.0–29.1)	0.648
MELD score		28.5 (21.5–37.3)	32.0 (14.0–37.0)	0.738
Causes of liver cirrhosis				
Alcohol		3 (30.0%)	3 (23.1%)	0.663
Hepatitis B		1 (10.0%)	1 (7.7%)	0.692
Hepatitis C		0 (0.0%)	1 (7.7%)	0.565
HCC		2 (20.0%)	5 (38.5%)	0.313
PSC		1 (10.0%)	3 (23.1%)	0.404
PBC		2 (20.0%)	0 (0.0%)	0.178
NASH		0 (0.0%)	0 (0.0%)	
Others		1 (10.0%)	4 (30.8%)	0.251
Need for catecholamines before LTX		2 (20.0%)	1 (7.7%)	0.398
NYHA 0-I		1 (10.0%)	2 (15.4%)	0.602
Diabetes mellitus		1 (10.0%)	2 (15.4%)	0.602
Arterial hypertension		2 (20.0%)	3 (23.1%)	0.314
Coronary heart disease		1 (10.0%)	1 (7.7%)	0.712
Chronic obstructive lung disease		1 (10.0%)	1 (7.7%)	0.692
Smoker		3 (30.0%)	3 (23.1%)	0.441
Renal insufficiency		2 (20.0%)	4 (30.8%)	0.463
Pre-existing ARF		2 (20.0%)	3 (23.1%)	0.663
Pre-existing thrombosis		5 (50.0%)	8 (61.5%)	0.057
Neurological disorder		7 (70.0%)	6 (46.2%)	0.112
High-urgency		2 (20.0%)	3 (23.1%)	0.633
Re-LTX		3 (30.0%)	4 (30.8%)	0.663
Immunosuppressive medication				
Corticosteroids		10 (100%)	13 (100%)	–
Mycophenolat mofetil		10 (100%)	13 (100%)	–
Ciclosporin		4 (40.0%)	6 (46.2%)	0.552
Tacrolimus		6 (60.0%)	7 (53.8%)	0.552

Data are presented either as number (with the corresponding percentage value) or as median (with accompanying quartiles (Q1–Q3)

BMI, body mass index; MELD, model of end-stage liver disease; HCC, hepatocellular carcinoma; PSC, primary sclerosing cholangitis; PBC, primary biliary cirrhosis; NASH, non-alcoholic fatty liver disease; NYHA, New York Heart Association; ARF, acute renal failure; LTX, liver transplantation

Concerning symbolism and higher orders of significance: $p < 0.05$: *

diagnostics, some patients might be falsely classified as colonized despite the presence of an IFD. Accordingly, a more sensitive and reliable diagnostic procedure would be of value in order to minimize false-negative test results. Patient L74 (Fig. 2b and SDC 4) was classified as being colonized (as assessed by culture-based diagnostics in drainage fluid at 21 days after LTX), although calculation of the SIQ score clearly revealed signs for an IFD caused by an infection with *C. albicans* already at day 0 and day 1 after LTX. Knowledge of these NGS-based results might have led to a change in antifungal therapy, which might have been of relevance for the presented patient. The patient further developed an infection with *Pseudomonas aeruginosa* and *Klebsiella*

Table 5 Details of the ICU and hospital stay for patients with fungal isolates

Parameter	Unit	Fungal infection ($n = 10$)	Fungal colonization ($n = 13$)	p for patients fungal infections vs. patients with colonization
APACHE II^{+}		30.5 (26.5–34.8)	29.0 (21.0–34.0)	0.563
SOFA^{+}		16.0 (13.5–18.8)	15.0 (10.0–16.0)	0.648
SAPS^{+}		64.0 (56.5–78.5)	60.0 (40.0–74.0)	0.257
Time of mechanical ventilation	(days)	14.0 (3.0–19.0)	1.5 (1.0–2.8)	0.012*
Tracheostomy		6 (60.0%)	2 (15.4%)	0.037*
Hospital stay before LTX	(days)	4 (1–13)	10 (1–31)	0.410
ICU-stay	(days)	31.5 (14.3–46.3)	21.0 (12.0–25.0)	0.376
Hospital stay	(days)	47.0 (34.0–51.0)	43.0 (26.5–71.3)	0.917
90-day survival		2 (20.0%)	11 (84.6%)	0.003**
28-days survival		7 (70.0%)	13 (100%)	0.068
ALF after LTX		5 (60.0%)	2 (7.7%)	0.092
ARF after LTX		9 (90.0%)	3 (23.1%)	0.002**
Dialysis				
Directly after LTX		1 (10.0%)	3 (23.1%)	0.712
In time course		5 (50.0%)	4 (30.8%)	0.306
BDA		2 (20.0%)	3 (23.1%)	0.633
Duration of surgery	(min)	382.5 (375.0–403.8)	360.0 (325.0–420.0)	0.563
Intraoperative blood loss	(L)	2.5 (2.0–6.3)	3.3 (1.5–7.6)	0.771
Administration of				
PRBC	(n.o.p.)	10 (100%)	11 (84.6%)	0.308
FFP	(n.o.p.)	10 (100%)	12 (92.3%)	0.565
Platelets	(n.o.p.)	7 (70.0%)	11 (84.6%)	0.367
Rejection		2 (20.0%)	4 (30.8%)	0.463
Perforation of the intestine or stomach		2 (20.0%)	1 (7.7%)	0.398
BDA-insufficiency (in patients with BDA)		1 of 2 (50.0%)	2 of 3 (66.7%)	0.602
Stenosis of the bile duct		1 (10.0%)	2 (15.4%)	0.602
Leakage of the bile duct		1 (10.0%)	1 (7.7%)	0.355
Need for surgical intervention		9 (90.0%)	9 (69.2%)	0.251
Vascular complications		3 (30.0%)	0 (0.0%)	0.068
Need for endoscopic diagnostics		4 (40.0%)	1 (7.7%)	0.089

Data are presented either as number (with the corresponding percentage value) or as median (with accompanying quartiles (Q1–Q3)

APACHE II score, Acute Physiology And Chronic Health Evaluation score; SOFA, Sequential Organ Failure Assessment score; SAPS, Simplified Acute Physiology Score; ICU, intensive care unit; LTX, liver transplantation; ARF, acute renal failure; ALF, acute liver failure; BDA, biliodigestive anastomosis; n.o.p., number of patients; PRBC, packed red blood cells; FFP, fresh frozen plasma, $^{+}$ calculated at the first day after LTX

Concerning symbolism and higher orders of significance: $p < 0.05$: *, $p < 0.01$: **

pneumoniae, as confirmed by cultivation of drainage fluids, blood culture as well as SIQ scores.

(1,3)-ß-D-glucan

Measurements of BDG did not differ significantly between patients with an invasive fungal disease (IFD) and those with a fungal colonization. Moreover, most of the BDG measurements remained below the critical cut-off value of < 80 pg/ml in all subgroups of patients, so that plasma levels of BDG proved unsuitable for early identification of an IFD following LTX (Fig. 3).

Galactomannan

Plasmatic GM revealed positive test results in 12 patients without any culture-based fungal findings. In these 12 cases, increased plasma concentrations of GM were most

probably attributable to the underlying antibiotic therapy (e.g., piperacillin/tazobactam), which is well known to be associated with increased GM concentrations [31, 32]. Moreover, plasmatic GM was shown to be increased above the plasmatic cut-off > 0.5 only in 3 out of 7 patients with an IA. Conversely, concentrations of GM in BALF were increased beyond the cut-off of > 1.0 in all seven cases with proven IA (data not shown).

Inflammation and infection marker levels

Plasma levels of acute phase proteins (e.g., C-reactive protein (CRP) or procalcitonin (PCT)), leukocytes as well as general inflammation marker levels (such as IL-2, TNF-α) in patients without any fungal isolates, suffering from a fungal colonization or an IFD, are presented in SDC S3.

IL-17A was shown to be significantly increased in patients suffering from an IFD following LTX in comparison to (1) patients without any fungal findings at T1 and T2 and (2) patients with a fungal colonization at T1 (Fig. 4a). Therefore, IL-17A was found to be a suitable marker for early identification of patients at high risk for the development of an IFD as assessed by receiver-operating characteristic (ROC) analyses (ROC area under the curve (AUC) for patients with an IFD vs. non-infected patients (i.e., patients without any fungal isolates + colonized patients) e.g., at T1: AUC = 0.792; cut-off 16.41 pg/ml → Sens. 1.000; Spec. 0.530; at T2: AUC = 0.742; cut-off 19.71 pg/ml → Sens. 0.900; Spec. 0.630) (Fig. 4b). No significant differences could be observed with regard to the causing pathogen (*Aspergillus* spp. vs. *Candida* spp.) within the group of patients suffering from an IFD.

The same holds true for plasma levels of MR-proADM, which were shown to be significantly increased in infected patients in comparison to both colonized patients as well as those patients without any fungal findings at several time points within the 28-day observation period (Fig. 5a). Accordingly, MR-proADM was shown to be a suitable tool for the identification of patients with an IFD as assessed by ROC analyses (ROC AUC for patients with an IFD vs. non-infected patients, e.g., at T0: AUC = 0.776; cut-off 2.84 nmol/l → Sens. 0.889; Spec. 0.544; at T3: AUC = 0.895; cut-off 4.90 nmol/l → Sens. 0.889; Spec. 0.860; at T4: 0.893; cut-off 6.29 nmol/l → Sens. 0.889; Spec. 0.930) (Fig. 5b).

Discussion

Within this clinical investigation, a NGS-based diagnostic approach and plasma measurements of MR-proADM and/or IL-17A were shown to add value for a comprehensive, reliable, and fast diagnosis of an IFD in patients following LTX.

Incidence, risk factors, and outcome relevance of IFDs following LTX—an everyday problem in transplantation centers

Due to the intake of immunosuppressive drugs and the associated reduced immunocompetence [33, 34], patients following LTX are at high risk of colonization or fungal infections with *C. albicans*, *C. glabrata*, and *Aspergillus* spp. [5, 35]. In case of fungal findings, patients were shown to be more seriously ill and, therefore, hallmarked by a reduced long-term survival [36, 37], often combined with a reduced kidney function [38–40]. Moreover, transfusion of allogenic blood products is closely linked to the risk of developing an IFD [35], which can also be supported by the presented investigation. The need for re-transplantation, presence of a respiratory failure, preoperative intake of steroids, insulin-dependent hyperglycemia during ICU stay, hepatic artery thrombosis, and hepatic vein complications represent further risk factors for the development of an IFD following LTX [39–42], of which none were shown to be relevant within the presented investigation.

The status quo of fungal diagnostics in patients following LTX

Culture-based diagnostics are associated with relevant weaknesses [43]. Positive blood cultures can only be obtained in 60–80% of patients despite an underlying IFD due to rapid clearance from the bloodstream, especially in cases of gastrointestinal translocations [13, 14]. Accordingly, molecular approaches might be able to overcome the aforementioned limitations. However, PCR methods were examined during the last years but only revealed a limited power to discriminate between contaminations, colonization, and IFD [18, 44].

Looking for ways out of this quandary—NGS-based diagnostics of fungal infections in patients following LTX

NGS, in contrast to PCR-based technologies, is an open platform approach which detects bacterial, fungal, and viral pathogens by a quantitative and unbiased method and allows for the discrimination of signal reads from noise (caused by contaminant or commensal species) via SIQ score calculation. A comparable NGS-based diagnostic approach for early diagnostics of bacteremia has recently been published by our workgroup and others [27, 29, 45, 46] and could show this benefit for fungal diagnostics in septic patients [47]. By using this newly designed "fungal SIQ-score" in plasma samples of patients following LTX within the presented investigation, we were able to (1) confirm culture-based diagnostics of candidemia and (2) detect IFDs even in those patients, which have, so far, been classified as colonized

a

L89

	Candida albicans	*Enterococcus spec.*	*Candida albicans*	*Candida albicans*	*Enterococcus spec.*	*Candida albicans*	*Enterococcus spec.*	*CoNS*	*Candida albicans*	*Enterococcus spec.*	*Klebsiella pneumoniae*	*Enterococcus faecium*	*Bacteroides vulgatus*	*Parabacteroides distasonis*	*Escherichia coli*
NGS	-	-	+	+	-	+	-	-	+	-	-	+	+	+	+
BC	+	-	n.d.	n.d.	n.d.	-	-	-	-	-	+	+	-	-	-
Tracheal secretion	-	+	n.d.	n.d.	n.d.	-	+	+	-	+*	-	n.d.	n.d.	n.d.	n.d.
BAL	n.d.	n.d.	n.d.	-	+	n.d.	n.d	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Time point	T0		T1	T2		T3			T4			T5			

* positive results were obtained from specimens taken within 48h of the respective time point

b

L74

	Candida albicans	*Candida albicans*	*Micrococcus luteus*	*Dinoroseobacter shibae*	*Ochrobactrum anthropi*			*E. faecium*	*Candida albicans*	*Klebsiella pneumoniae*	*Enterococcus faecalis*	*Pseudomonas spec./aeruginosa*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Torque Teno Virus*
NGS	+	+	+	+	+	-	-	-	+	-	-	+	+	+	+	+
BC	-	n.d.	n.d.	n.d.	n.d.	n.d.	-	+	-	+	-	-	-	-	-	-
Drainage fluid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+*	+	+	+*	+	n.d.	n.d.	n.d.	n.d.
Time point	T0	T1				T2	T3	T4	T5				T6			

* positive results were obtained from specimens taken within 48h of the respective time point

based on culture-based diagnostic procedures. Moreover, the NGS-based approach would have revealed signs for an IFD already prior to the availability of the first culture-based results of fungal pathogens, thus providing the opportunity to initiate a targeted life-saving antifungal therapy in infected patients much earlier than previously possible. Even our approach seems to be more sensitive and specific than the state-of-the-art methods. However, due to the small number of patients within our collective, further studies will be necessary.

◀ **Fig. 2** Time course fungal SIQ analyses and comparison to conventional clinical microbiology data for patients following LTX. The respective species identified for patients L89 (**a**) and L74 (**b**) are shown at the top of the graph. All results are grouped by time points T0 to T6. Species identified by fungal SIQ score analyses (NGS) are highlighted by green-colored boxes, while species identified by BC are highlighted with red-colored boxes. A match to species identified from other specimens by cultivation is highlighted by gray-colored boxes. n.d. indicates that the respective analysis was not performed at the corresponding time point, while a minus (−) or a plus (+) symbol indicates a negative or positive result, respectively. An asterisk highlights those positive results that were obtained from a sample not directly at the indicated time point but within 48 h of it. (**a**) L89—a 63-year-old man was listed as a candidate for LTX due to ethyl toxic liver cirrhosis and was allocated for first transplantation in 09/2015. Five months following the first transplantation, the patient suffered from ischemic graft failure; so, re-transplantation was performed in 01/2016. The MELD score prior to re-transplantation was calculated to be 20. Following the re-transplantation procedure, immunosuppression was maintained by the combined use of corticosteroids, mycophenolat mofetil as well as tacrolimus. Meropenem (MEM), linezolid (LZD), and caspofungin (CFG) were given in terms of a perioperative anti-infective prophylaxis. Immediately following the re-transplantation, *Candida albicans* could be detected in blood cultures; so, a targeted treatment regime with caspofungin (CFG) was maintained. Moreover, hygiene smears showed rectal colonization with vancomycin-resistant *Enterococcus faecium*. Besides, at day 1, after re-transplantation, the patient was in need for a surgical revision due to an intra-abdominal hematoma. Three days later, the bile duct and the intestine were connected in terms of a biliodigestive anastomosis. At day 14 following the re-transplantation, *Klebsiella pneumonia* could be detected in blood cultures; so, a targeted treatment with ceftazidime was initiated. Due to severe worsening of the patient's condition, tigecycline was also administered to the patient in terms of a calculated antibiosis. Additionally, piperacillin/tazobactam was added instead of ceftazidime, since the patient's conditions were not changing. Moreover, the patient showed an ischemic colon; so, a subtotal colectomy was performed 20 days after the re-transplantation. At day 21, blood cultures revealed *Enterococcus faecium* (VSE). Unfortunately, the patient died at day 22 following the re-transplantation. BC, blood culture; BAL, bronchoalveolar lavage; CoNS, coagulase-negative staphylococci; NGS, species identified by fungal SIQ score analyses via next-generation sequencing. (**b**) L74—a 60-year-old man was listed as a candidate for LTX due to ethyl toxic liver cirrhosis. The MELD score prior to transplantation was calculated to be 10. Following the transplantation procedure, immunosuppression was maintained by the combined use of corticosteroids, mycophenolat mofetil as well as tacrolimus. Cefuroxime and fluconazole were given in terms of a perioperative anti-infective prophylaxis. Due to suspected pneumonia, an empiric treatment regime with moxifloxacin was initiated at day 3 after LTX. At day 14 following LTX, *Enterococcus faecium* (VSE) was found in blood cultures. Moreover, *Candida* spp. were found in abdominal drainages at 21 days after the transplantation procedure, which was interpreted as fungal colonization. At day 45 following LTX, the patient suffered from a biliary leakage due to a necrotic bile duct; so, the bile duct was resected and a percutaneous bile drainage was brought in place. BC, blood culture; NGS, species identified by fungal SIQ score analyses via next-generation sequencing

Biomarkers for the diagnosis of IFDs in patients following LTX

The use of non-invasive diagnostic tools, such as BDG or GM, aims to attenuate the above-mentioned inefficiencies of the routinely used diagnostic approaches for the detection of IFDs, although these biomarkers are also far from perfect. The specificity of BDG in LTX is known to be low [20]. Among others, the presence of a bacterial infection and/or the need for an antibiotic treatment were accused to be associated with false-positive BDG levels in patients not affected by an IFD [48, 49]. However, recent evidence suggests that the influence of some of these factors was somehow overestimated [50–53]. Nevertheless, within the presented investigation, plasma levels of BDG definitely failed to be of diagnostic value for the identification of an IFD in patients following LTX. Accordingly, the use of BDG plasma levels in terms of a screening tool cannot be recommended. IA is especially common in neutropenic patients or following hematopoietic stem cell transplantation [54], but it also represents an opportunistic infection in patients following LTX [5]. GM represents a heat-stable polysaccharide present in the fungal wall of most *Aspergillus* spp. [55]. Although GM works fine in neutropenic patients, its diagnostic value is much lower in patients following LTX with a high rate of false-positive results [20–22]. It is, therefore, clear as to why neither BDG nor GM is currently recommended for use as diagnostic tools in the identification of IFDs in patients following LTX by the Infectious Disease Society of America (IDSA) [18].

Immune monitoring for the detection of IFDs following LTX

The immunocompetent body is steadily confronted with a myriad of microorganisms at its interfaces between the internal and external environment. Especially, *Candida* spp. frequently colonize mucosal surfaces but without inducing any infection. This only works because our body has adapted to this condition by establishing physical barriers (e.g., mucosal and skin) as well as stationing immune cells at these delicate interfaces. In patients following LTX, this sensitive balance is disturbed due to the intake of immunosuppressive drugs in order to avoid a rejection of the graft [33, 34].

Several biomarkers are involved in the fighting cascade against fungal infections. Accordingly, IL-17A was shown to be suitable for the detection of an IFD in patients suffering from sepsis [27, 56]. The same seems to hold true for LTX patients. Analogous to septic patients suffering from an IFD, patients undergoing LTX also revealed significantly increased levels of IL-17A in comparison to patients with a fungal colonization, as well as patients without any fungal findings. However, two things have to be kept in mind with regard to these findings: (1) IL-17A is primarily associated with the defense against *Candida* spp., whereas *Aspergillus* spp. are known to be

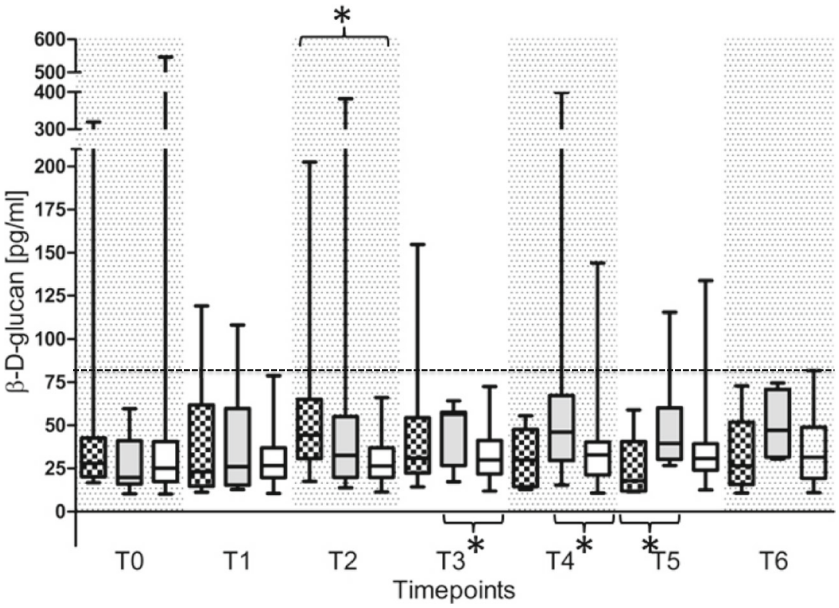

Fig. 3 Plasma concentrations of ß-D-glucan (BDG) in patients following LTX. Plasma concentrations of BDG were measured in patients following LTX with an IFD (black squared box), a fungal colonization (gray box), or without any fungal findings (white box). Plasma samples were collected immediately following LTX (T0), and 1 day (T1), 2 days (T2), 7 days (T3), 14 days (T4), 21 days (T5), and 28 days (T6) afterwards. Data in box plots are given as median, 25th percentile, 75th percentile with the 10th as well as 90th percentile at the end of the whiskers. Concerning symbolism and higher orders of significance: $p < 0.05$: *. The dotted line at 80 pg/ml indicates the critical cut-off value of the Glucatell®-Kit (Pyroquant Diagnostik GmbH)

a less strong inductor [57]. Accordingly, high plasma levels of IL-17A in patients suffering from IA might be, at least in part, caused by an accompanying fungal colonization with *Candida* spp. (2) IL-17A is also known to serve as a prognostic parameter for acute rejections following transplantation [58]. Moreover, IL-17-producing T helper 17 cells (Th17) have recently been described to be of importance in the pathogenesis of psoriasis [59] and play a crucial role in the host defense against a variety of pathogens, including bacteria and viruses [60]. However, since plasma levels of IL-17A differed significantly between the three subgroups (patients with an IFD vs. colonization vs. without any fungal pathogens) at several time points, whereas the rate of rejections, cases of psoriasis or the number of non-fungal infections (caused by bacteria or viruses) did not, the observed differences of IL-17A plasma levels seem to be most likely attributable to the presence of fungal pathogens, rather than the occurrence of the aforementioned other influencing factors. In summary, plasma levels of IL-17A might, therefore, be a promising biomarker for the anticipation of an IFD in patients following LTX. MR-proADM is known to be of diagnostic value for the discrimination of critically ill patients with all-cause sepsis (independent of the underlying microbial pathogen) from those without an infectious stimulus [61]. Moreover, ADM appears to play a critical role in the host defense against systemic infections, especially fungal infections [27]. Accordingly, MR-proADM was proven to be suitable for the identification of patients suffering from an IFD following LTX within the presented investigation. However, increased levels of MR-proADM are also known to serve as an indicator for severe complications following LTX [62], especially for those of vascular origin [63]. Moreover, renal impairment is known to be associated with increased levels of MR-proADM following liver transplantation [64]. Therefore, patients' renal function and other influencing factors, such as the presence of a vascular problem of the graft, have to be taken into account.

Limitations

Although the results of our observational, prospective, clinical investigation are congruent, the following limitations need to be addressed in connection with the presented manuscript. The clinical investigation was performed in terms of an observational single-center study and is, therefore, characterized by a small number of participating patients, representing a highly selective cohort of patients with the need for an orthotopic LTX due to an underlying ESLD, in which statistical errors may not be precluded. Since the presented work was realized in terms of a pilot study, no α-adjustment was performed, and obtained knowledge is based on descriptive data. Accordingly, an additional confirmation within a larger multicenter trial needs to be recommended.

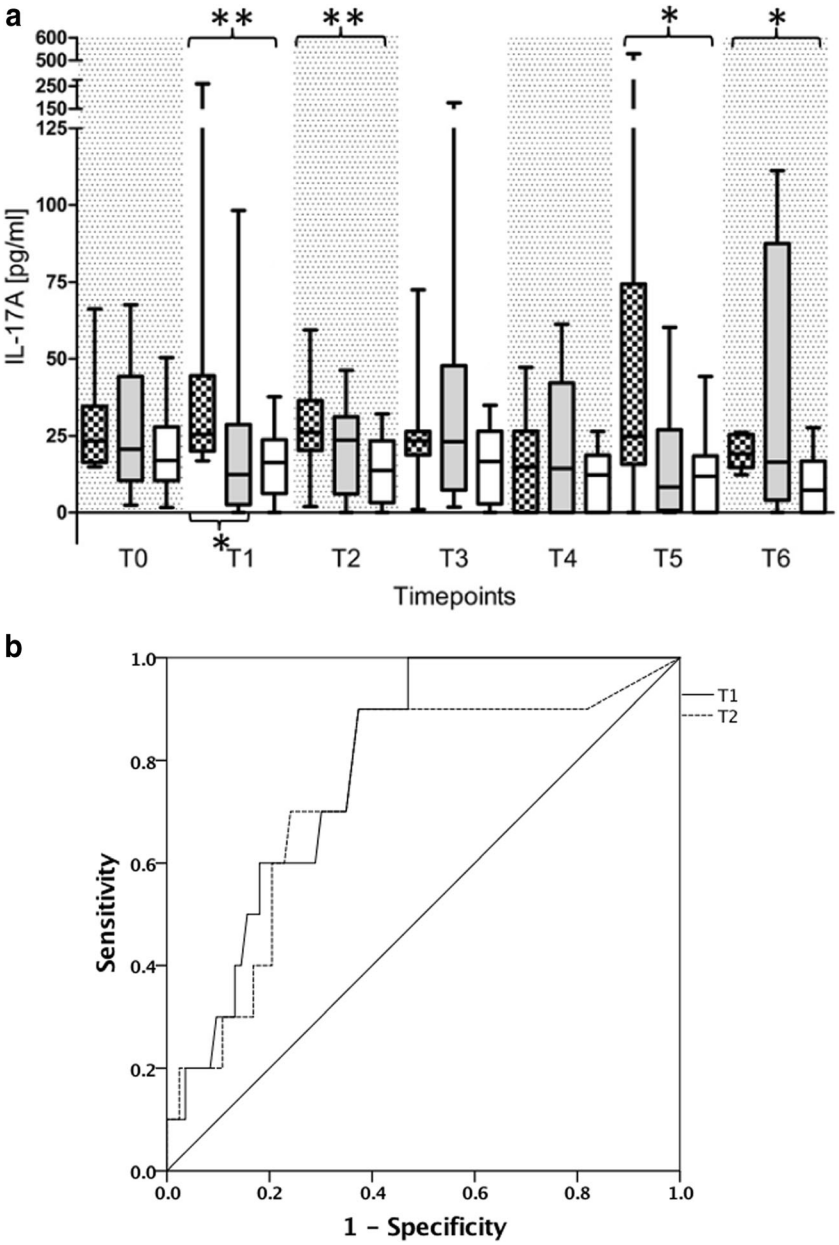

Fig. 4 Plasma concentrations of interleukin (IL)-17A in patients following LTX. (**a**) Plasma concentrations of IL-17A were measured in patients after LTX with a fungal infection (black squared box), a fungal colonization (gray box), or without any fungal findings (white box). Plasma samples were collected directly after LTX (T0), and 1 day (T1), 2 days (T2), 7 days (T3), 14 days (T4), 21 days (T5), and 28 days (T6) afterwards. Data in box plots are given as median, 25th percentile, 75th percentile with the 10th as well as 90th percentile at the end of the whiskers. Concerning symbolism and higher orders of significance: $p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***. (**b**) Receiver-operating characteristic (ROC) analysis with IL-17A in all participating patients 1 day after LTX (T1) and 2 days (T2) afterwards with regard to the prediction of a fungal infection up to day 28. Patients suffering from a fungal infection represented the target group, whereas both patients with a fungal colonization as well as patients without any fungal isolates served as controls for this ROC analysis

Moreover, due to weaknesses of culture-based diagnostics (including BC), representing the gold standard for the identification of patients suffering from an IFD, a relevant number of IFD episodes may also be missed also within the presented investigation. However, this BC-related problem is well-known but has not been satisfactorily solved yet, representing the main justification for the presented study here [13, 14].

Conclusions

Fungal pathogens are quite common in patients following LTX and are associated with an increased morbidity, necessitating a comprehensive, reliable, and fast diagnosis. In this respect, a NGS-based diagnostic approach might be a suitable tool for the detection of fungal pathogens in plasma samples of patients following LTX. Moreover, the fungal SIQ score

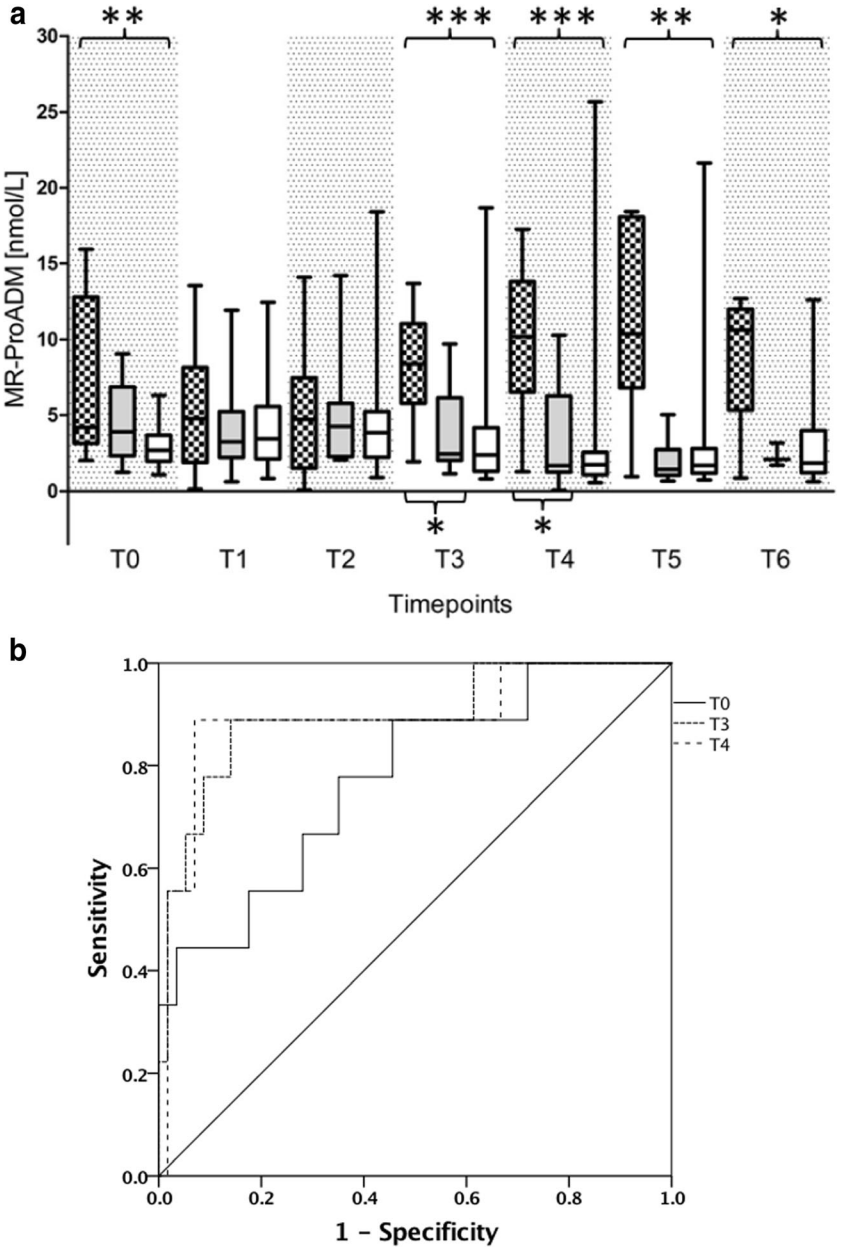

Fig. 5 Plasma concentrations of mid-regional proadrenomedullin (MR-proADM) in patients following LTX. (**a**) Plasma concentrations of MR-proADM were measured in patients following LTX with a fungal infection (black squared box), a fungal colonization (gray box), or without any fungal findings (white box). Plasma samples were collected directly after LTX (T0), and 1 day (T1), 2 days (T2), 7 days (T3), 14 days (T4), 21 days (T5), and 28 days (T6) afterwards. Data in box plots are given as median, 25th percentile, 75th percentile with the 10th as well as 90th percentile at the end of the whiskers. Concerning symbolism and higher orders of significance: $p < 0.05$: *, $p < 0.01$: **. (**b**) Receiver-operating characteristic (ROC) analysis with MR-proADM in all participating patients directly after LTX (T0), and 7 days (T3), as well as 14 days (T4) afterwards with regard to the prediction of a fungal infection up to day 28. Patients suffering from a fungal infection represented the target group, whereas both patients with a fungal colonization as well as patients without any fungal isolates served as controls for this ROC analysis

allowed for the identification of an IFD even in those patients, which have so far been classified as colonized (based on culture-based diagnostic procedures). A NGS-based diagnostic platform, therefore, might represent a feasible approach for the identification of IFDs, even in those cases where classic microbiological or molecular diagnostic approaches fail. The identification of patients with an IFD can be further facilitated by plasma measurements of MR-proADM as well as IL-17A. Accordingly, the implementation of MR-proADM and IL-17A measurements in routine diagnostics following LTX should be taken into account for the detection of an IFD. Conversely, plasma levels of BDG did not significantly differ among patients without any fungal pathogens, colonized as well as infected patients.

Acknowledgements We would like to thank Ute Krauser and Karolina Glanz for their excellent technical assistance.

Authors' contributions SOD conceived the study, participated in its design and coordination, and helped in drafting the article. Furthermore, he performed data acquisition, carried out the measurements in the laboratory, and prepared the tables and figures. AK and HW performed data acquisition and were involved in critical revision of the article. FU, FCFS, AM, MM, KHW, MAW, and SH participated in the design of the study and were involved in revising the article. TBru participated in the design of the study and performed the statistical analysis. SZ performed all microbiological analyses and was involved in critical revision of the article. SG, YV, and KS were responsible for NGS-based diagnostics and revised the article critically. TBre conceived the study, participated in its design and coordination, and drafted the article. All authors read and approved the final manuscript.

Funding The study was conducted with the financial support of the Department of Anesthesiology (Heidelberg University Hospital, Germany) and Fraunhofer IGB (Stuttgart, Germany). Furthermore, this study received financial grants from the B. Braun Foundation, Melsungen, as well as the Heidelberg Foundation of Surgery, and was supported by the European Union grant Fungitect. MR-proADM measurements were carried out by BRAHMS GmbH.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Trunecka P (2013) Immunosuppression after liver transplant, now and in future. Vnitr Lek 59(8):671–677
2. Reed A, Herndon JB, Ersoz N, Fujikawa T, Schain D, Lipori P, Hemming A, Li Q, Shenkman E, Vogel B (2007) Effect of prophylaxis on fungal infection and costs for high-risk liver transplant recipients. Liver Transpl 13(12):1743–1750. https://doi.org/10.1002/lt.21331
3. Singh N (2003) Fungal infections in the recipients of solid organ transplantation. Infect Dis Clin N Am 17(1):113–134 **viii**
4. Neofytos D, Fishman JA, Horn D, Anaissie E, Chang CH, Olyaei A, Pfaller M, Steinbach WJ, Webster KM, Marr KA (2010) Epidemiology and outcome of invasive fungal infections in solid organ transplant recipients. Transpl Infect Dis 12(3):220–229. https://doi.org/10.1111/j.1399-3062.2010.00492.x
5. Pappas PG, Alexander BD, Andes DR, Hadley S, Kauffman CA, Freifeld A, Anaissie EJ, Brumble LM, Herwaldt L, Ito J, Kontoyiannis DP, Lyon GM, Marr KA, Morrison VA, Park BJ, Patterson TF, Perl TM, Oster RA, Schuster MG, Walker R, Walsh TJ, Wannemuehler KA, Chiller TM (2010) Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin Infect Dis 50(8):1101–1111. https://doi.org/10.1086/651262
6. Andes DR, Safdar N, Baddley JW, Alexander B, Brumble L, Freifeld A, Hadley S, Herwaldt L, Kauffman C, Lyon GM, Morrison V, Patterson T, Perl T, Walker R, Hess T, Chiller T, Pappas PG, Investigators T (2016) The epidemiology and outcomes of invasive Candida infections among organ transplant recipients in the United States: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Transpl Infect Dis 18(6):921–931. https://doi.org/10.1111/tid.12613
7. Shah T, Lai WK, Gow P, Leeming J, Mutimer D (2005) Low-dose amphotericin for prevention of serious fungal infection following liver transplantation. Transpl Infect Dis 7(3–4):126–132. https://doi.org/10.1111/j.1399-3062.2005.00108.x
8. Husain S, Tollemar J, Dominguez EA, Baumgarten K, Humar A, Paterson DL, Wagener MM, Kusne S, Singh N (2003) Changes in the spectrum and risk factors for invasive candidiasis in liver transplant recipients: prospective, multicenter, case-controlled study. Transplantation 75(12):2023–2029. https://doi.org/10.1097/01.TP.0000065178.93741.72
9. Eschenauer GA, Kwak EJ, Humar A, Potoski BA, Clarke LG, Shields RK, Abdel-Massih R, Silveira FP, Vergidis P, Clancy CJ, Nguyen MH (2015) Targeted versus universal antifungal prophylaxis among liver transplant recipients. Am J Transplant 15(1):180–189. https://doi.org/10.1111/ajt.12993
10. Fortun J, Martin-Davila P, Moreno S, Barcena R, de Vicente E, Honrubia A, Garcia M, Nuno J, Candela A, Uriarte M, Pintado V (2003) Prevention of invasive fungal infections in liver transplant recipients: the role of prophylaxis with lipid formulations of amphotericin B in high-risk patients. J Antimicrob Chemother 52(5):813–819. https://doi.org/10.1093/jac/dkg450
11. Nagao M, Fujimoto Y, Yamamoto M, Matsumura Y, Kaido T, Takakura S, Uemoto S, Ichiyama S (2016) Epidemiology of invasive fungal infections after liver transplantation and the risk factors of late-onset invasive aspergillosis. J Infect Chemother 22(2):84–89. https://doi.org/10.1016/j.jiac.2015.11.005
12. Barchiesi F, Mazzocato S, Mazzanti S, Gesuita R, Skrami E, Fiorentini A, Singh N (2015) Invasive aspergillosis in liver transplant recipients: epidemiology, clinical characteristics, treatment, and outcomes in 116 cases. Liver Transpl 21(2):204–212. https://doi.org/10.1002/lt.24032
13. Silveira FP, Kusne S, Practice ASTIDCo (2013) Candida infections in solid organ transplantation. Am J Transplant 13(Suppl 4):220–227. https://doi.org/10.1111/ajt.12114
14. Clancy CJ, Nguyen MH (2013) Finding the "missing 50%" of invasive candidiasis: how nonculture diagnostics will improve understanding of disease spectrum and transform patient care. Clin Infect Dis 56(9):1284–1292. https://doi.org/10.1093/cid/cit006
15. Vazquez JA, Miceli MH, Alangaden G (2013) Invasive fungal infections in transplant recipients. Ther Adv Infect Dis 1(3):85–105. https://doi.org/10.1177/2049936113491936
16. 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, European Organization for R, Treatment of Cancer/Invasive Fungal Infections Cooperative G, National Institute of A,

Infectious Diseases Mycoses Study Group Consensus G (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(12):1813–1821. https://doi.org/10.1086/588660

17. Bassetti M, Scudeller L, Giacobbe DR, Lamoth F, Righi E, Zuccaro V, Grecchi C, Rebuffi C, Akova M, Alastruey-Izquierdo A, Arikan-Akdagli S, Azoulay E, Blot SI, Cornely OA, Lass-Florl C, Koehler P, Cuenca-Estrella M, de Lange DW, De Rosa FG, De Waele JJ, Dimopoulos G, Garnacho-Montero J, Hoenigl M, Kanj SS, Maertens J, Martin-Loeches I, Munoz P, Kullberg BJ, Agvald-Ohman C, Poulakou G, Rello J, Sanguinetti M, Taccone FS, Timsit JF, Torres A, Vazquez JA, Calandra T, from the Study Group for Infections in Critically Ill P, the Fungal Infection Study Group of the European Society of Clinical M, Infectious D, European Society of Intensive Care M, European Confederation of Medical M, Mycoses Study Group E, Research C (2018, 2019) Developing definitions for invasive fungal diseases in critically ill adult patients in intensive care units. Protocol of the FUNgal infections definitions in ICU patients (FUNDICU) project. Mycoses. https://doi.org/10.1111/myc.12869
18. 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) Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis 63(4): e1–e60. https://doi.org/10.1093/cid/ciw326
19. Singh N, Husain S, Practice ASTIDCo (2013) Aspergillosis in solid organ transplantation. Am J Transplant 13(Suppl 4):228–241. https://doi.org/10.1111/ajt.12115
20. Singh N, Winston DJ, Limaye AP, Pelletier S, Safdar N, Morris MI, Meneses K, Busuttil RW, Wagener MM, Wheat LJ (2015) Performance characteristics of galactomannan and beta-d-glucan in high-risk liver transplant recipients. Transplantation 99(12): 2543–2550. https://doi.org/10.1097/TP.0000000000000763
21. Leeflang MM, Debets-Ossenkopp YJ, Wang J, Visser CE, Scholten RJ, Hooft L, Bijlmer HA, Reitsma JB, Zhang M, Bossuyt PM, Vandenbroucke-Grauls CM (2015) Galactomannan detection for invasive aspergillosis in immunocompromised patients. Cochrane Database Syst Rev 12:CD007394. https://doi.org/10.1002/14651858.CD007394.pub2
22. Pfeiffer CD, Fine JP, Safdar N (2006) Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. Clin Infect Dis 42(10):1417–1427. https://doi.org/10.1086/503427
23. Ljungman P (2001) Prophylaxis against herpesvirus infections in transplant recipients. Drugs 61(2):187–196
24. Slifkin M, Doron S, Snydman DR (2004) Viral prophylaxis in organ transplant patients. Drugs 64(24):2763–2792
25. Giannella M, Bartoletti M, Morelli M, Cristini F, Tedeschi S, Campoli C, Tumietto F, Bertuzzo V, Ercolani G, Faenza S, Pinna AD, Lewis RE, Viale P (2016) Antifungal prophylaxis in liver transplant recipients: one size does not fit all. Transpl Infect Dis 18(4):538–544. https://doi.org/10.1111/tid.12560
26. Fortun J, Muriel A, Martin-Davila P, Montejo M, Len O, Torre-Cisneros J, Carratala J, Munoz P, Farinas C, Moreno A, Fresco G, Goikoetxea J, Gavalda J, Pozo JC, Bodro M, Vena A, Casafont F, Cervera C, Silva JT, Aguado JM, Grupo de Estudio de Infeccion en Pacientes Trasplantados-Grupo de Estudio de Micologia M, Red Espanola de Investigacion en Patologia I (2016) Caspofungin versus fluconazole as prophylaxis of invasive fungal infection in high-risk liver transplantation recipients: a propensity score analysis. Liver Transpl 22(4):427–435. https://doi.org/10.1002/lt.24391
27. Decker SO, Sigl A, Grumaz C, Stevens P, Vainshtein Y, Zimmermann S, Weigand MA, Hofer S, Sohn K, Brenner T (2017) Immune-response patterns and next generation sequencing diagnostics for the detection of mycoses in patients with septic shock—results of a combined clinical and experimental investigation. Int J Mol Sci 18(8). https://doi.org/10.3390/ijms18081796
28. Hospital HU (2006) Heidelberger Manual der Lebertransplantation. (2.Auflage)
29. Grumaz S, Stevens P, Grumaz C, Decker SO, Weigand MA, Hofer S, Brenner T, von Haeseler A, Sohn K (2016) Next-generation sequencing diagnostics of bacteremia in septic patients. Genome Med 8(1):73. https://doi.org/10.1186/s13073-016-0326-8
30. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P, Parkhill J, Loman NJ, Walker AW (2014) Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol 12:87. https://doi.org/10.1186/s12915-014-0087-z
31. Boonsarngsuk V, Niyompattama A, Teosirimongkol C, Sriwanichrak K (2010) False-positive serum and bronchoalveolar lavage Aspergillus galactomannan assays caused by different antibiotics. Scand J Infect Dis 42(6–7):461–468. https://doi.org/10.3109/00365541003602064
32. Metan G (2013) The interaction between piperacillin-tazobactam and Aspergillus galactomannan antigenemia assay: is the story over? Infection 41(1):293–294. https://doi.org/10.1007/s15010-012-0327-5
33. Morris PJ (2004) Transplantation—a medical miracle of the 20th century. N Engl J Med 351(26):2678–2680. https://doi.org/10.1056/NEJMp048256
34. Sayegh MH, Carpenter CB (2004) Transplantation 50 years later—progress, challenges, and promises. N Engl J Med 351(26):2761–2766. https://doi.org/10.1056/NEJMon043418
35. Li C, Wen TF, Mi K, Wang C, Yan LN, Li B (2012) Analysis of infections in the first 3-month after living donor liver transplantation. World J Gastroenterol 18(16):1975–1980. https://doi.org/10.3748/wjg.v18.i16.1975
36. Kaltenborn A, Hartmann C, Salinas R, Ramackers W, Kleine M, Vondran FW, Barthold M, Lehner F, Klempnauer J, Schrem H (2015) Risk factors for short- and long-term mortality in liver transplant recipients with MELD score >/= 30. Ann Transplant 20:59–69. https://doi.org/10.12659/AOT.892322
37. Sganga G, Bianco G, Frongillo F, Lirosi MC, Nure E, Agnes S (2014) Fungal infections after liver transplantation: incidence and outcome. Transplant Proc 46(7):2314–2318. https://doi.org/10.1016/j.transproceed.2014.07.056
38. Smoter P, Nyckowski P, Grat M, Patkowski W, Zieniewicz K, Wronka K, Hinderer B, Morawski M (2014) Risk factors of acute renal failure after orthotopic liver transplantation: single-center experience. Transplant Proc 46(8):2786–2789. https://doi.org/10.1016/j.transproceed.2014.09.044
39. Kawagishi N, Satoh K, Enomoto Y, Akamatsu Y, Sekiguchi S, Fujimori K, Satomi S (2006) Risk factors and impact of beta-D glucan on invasive fungal infection for the living donor liver transplant recipients. Tohoku J Exp Med 209(3):207–215
40. Fortun J, Martin-Davila P, Moreno S, De Vicente E, Nuno J, Candelas A, Barcena R, Garcia M (2002) Risk factors for invasive aspergillosis in liver transplant recipients. Liver Transpl 8(11): 1065–1070. https://doi.org/10.1053/jlts.2002.36239
41. Gladdy RA, Richardson SE, Davies HD, Superina RA (1999) Candida infection in pediatric liver transplant recipients. Liver Transpl Surg 5(1):16–24
42. Nieto-Rodriguez JA, Kusne S, Manez R, Irish W, Linden P, Magnone M, Wing EJ, Fung JJ, Starzl TE (1996) Factors associated with the development of candidemia and candidemia-related death among liver transplant recipients. Ann Surg 223(1):70–76

43. Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, Meersseman W, Akova M, Arendrup MC, Arikan-Akdagli S, Bille J, Castagnola E, Cuenca-Estrella M, Donnelly JP, Groll AH, Herbrecht R, Hope WW, Jensen HE, Lass-Florl C, Petrikkos G, Richardson MD, Roilides E, Verweij PE, Viscoli C, Ullmann AJ, Group EFIS (2012) ESCMID* guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients. Clin Microbiol Infect 18(Suppl 7):19–37. https://doi.org/10.1111/1469-0691.12039
44. Avni T, Leibovici L, Paul M (2011) PCR diagnosis of invasive candidiasis: systematic review and meta-analysis. J Clin Microbiol 49(2):665–670. https://doi.org/10.1128/JCM.01602-10
45. Cummings LA, Kurosawa K, Hoogestraat DR, SenGupta DJ, Candra F, Doyle M, Thielges S, Land TA, Rosenthal CA, Hoffman NG, Salipante SJ, Cookson BT (2016) Clinical next generation sequencing outperforms standard microbiological culture for characterizing polymicrobial samples. Clin Chem 62(11): 1465–1473. https://doi.org/10.1373/clinchem.2016.258806
46. Long Y, Zhang Y, Gong Y, Sun R, Su L, Lin X, Shen A, Zhou J, Caiji Z, Wang X, Li D, Wu H, Tan H (2016) Diagnosis of sepsis with cell-free DNA by next-generation sequencing technology in ICU patients. Arch Med Res 47(5):365–371. https://doi.org/10.1016/j.arcmed.2016.08.004
47. Grumaz C, Kirstahler P, Sohn K (2017) The molecular blueprint of a fungus by next-generation sequencing (NGS). Methods Mol Biol 1508:361–383. https://doi.org/10.1007/978-1-4939-6515-1_21
48. Digby J, Kalbfleisch J, Glenn A, Larsen A, Browder W, Williams D (2003) Serum glucan levels are not specific for presence of fungal infections in intensive care unit patients. Clin Diagn Lab Immunol 10(5):882–885
49. Marty FM, Lowry CM, Lempitski SJ, Kubiak DW, Finkelman MA, Baden LR (2006) Reactivity of (1–>3)-beta-d-glucan assay with commonly used intravenous antimicrobials. Antimicrob Agents Chemother 50(10):3450–3453. https://doi.org/10.1128/AAC.00658-06
50. Prattes J, Schneditz D, Pruller F, Jaindl E, Sauseng N, Hoenigl M, Schilcher G, Krause R (2017) 1,3-ss-d-Glucan testing is highly specific in patients undergoing dialysis treatment. J Inf Secur 74(1):72–80. https://doi.org/10.1016/j.jinf.2016.09.005
51. Metan G, Koc AN, Kaynar LG, Atalay A, Ozturk A, Eser B, Cetin M (2013) What is the role of the (1–>3)-beta-D-glucan assay in the screening of patients undergoing autologous haematopoietic stem-cell transplantation? Mycoses 56(1):34–38. https://doi.org/10.1111/j.1439-0507.2012.02195.x
52. Mikulska M, Furfaro E, Del Bono V, Raiola AM, Ratto S, Bacigalupo A, Viscoli C (2012) Piperacillin/tazobactam (Tazocin) seems to be no longer responsible for false-positive results of the galactomannan assay. J Antimicrob Chemother 67(7):1746–1748. https://doi.org/10.1093/jac/dks111
53. Furfaro E, Mikulska M, Del Bono V, Guolo F, Minetto P, Gobbi M, Ghiso A, Bacigalupo A, Viscoli C (2014) Bloodstream infections are an improbable cause of positive serum (1,3)-beta-D-glucan in hematology patients. Clin Vaccine Immunol 21(9):1357–1359. https://doi.org/10.1128/CVI.00214-14
54. Denning DW (1998) Invasive aspergillosis. Clin Infect Dis 26(4): 781–803 quiz 804-785
55. Zedek DC, Miller MB (2006) Use of galactomannan enzyme immunoassay for diagnosis of invasive aspergillosis in a tertiary-care center over a 12-month period. J Clin Microbiol 44(4):1601. https://doi.org/10.1128/JCM.44.4.1601.2006
56. Krause R, Zollner-Schwetz I, Salzer HJ, Valentin T, Rabensteiner J, Pruller F, Raggam R, Meinitzer A, Prattes J, Rinner B, Strohmaier H, Quehenberger F, Strunk D, Heidrich K, Buzina W, Hoenigl M (2015) Elevated levels of interleukin 17A and kynurenine in candidemic patients, compared with levels in noncandidemic patients in the intensive care unit and those in healthy controls. J Infect Dis 211(3):445–451. https://doi.org/10.1093/infdis/jiu468
57. Cheng SC, van de Veerdonk F, Smeekens S, Joosten LA, van der Meer JW, Kullberg BJ, Netea MG (2010) Candida albicans dampens host defense by downregulating IL-17 production. J Immunol 185(4):2450–2457. https://doi.org/10.4049/jimmunol.1000756
58. Kim N, Yoon YI, Yoo HJ, Tak E, Ahn CS, Song GW, Lee SG, Hwang S (2016) Combined detection of serum IL-10, IL-17, and CXCL10 predicts acute rejection following adult liver transplantation. Mol Cell 39(8):639–644. https://doi.org/10.14348/molcells.2016.0130
59. Hawkes JE, Yan BY, Chan TC, Krueger JG (2018) Discovery of the IL-23/IL-17 signaling pathway and the treatment of psoriasis. J Immunol 201(6):1605–1613. https://doi.org/10.4049/jimmunol.1800013
60. Li Y, Wei C, Xu H, Jia J, Wei Z, Guo R, Jia Y, Wu Y, Li Y, Qi X, Li Z, Gao X (2018) The immunoregulation of Th17 in host against intracellular bacterial infection. Mediat Inflamm 2018(6587296):1–13. https://doi.org/10.1155/2018/6587296
61. Angeletti S, Battistoni F, Fioravanti M, Bernardini S, Dicuonzo G (2013) Procalcitonin and mid-regional pro-adrenomedullin test combination in sepsis diagnosis. Clin Chem Lab Med 51(5): 1059–1067. https://doi.org/10.1515/cclm-2012-0595
62. Miguel D, Prieto B, Alvarez FV (2013) Biological variation and prognosis usefulness of new biomarkers in liver transplantation. Clin Chem Lab Med 51(6):1241–1249. https://doi.org/10.1515/cclm-2012-0713
63. Fabrega E, Crespo J, Casafont F, de La Pena J, Garcia-Unzueta MT, Amado JA, Pons-Romero F (1999) Adrenomedullin in liver transplantation and its relationship with vascular complications. Liver Transpl Surg 5(6):485–490
64. Geny B, Ellero B, Charloux A, Brandenberger G, Doutreleau S, Piquard F (2003) Circulating adrenomedullin is increased in relation with increased creatinine and atrial natriuretic peptide in liver-transplant recipients. Regul Pept 114(1):61–66