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PREVENTION AND DIAGNOSTICS OF INVASIVE FUNGAL INFECTIONS in acute leukaemia and allogeneic stem cell transplantation

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Academic Dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles which are referred to in the text by citing the appropriate Roman numerals:

I. Nihtinen A, Anttila V-J, Richardson M, Meri T, Volin L, Ruutu T. The utility of intensified environmental surveillance for pathogenic moulds in a stem cell transplantation ward during construction activity to monitor the efficacy of HEPA filtration. Bone Marrow Transplant 2007;40:457-460.

II. Nihtinen A, Anttila V-J, Elonen E, Juvonen E, Volin L, Ruutu T. Effect of fluconazole prophylaxis on the incidence of invasive candida infections and bacteraemias in patients with acute leukaemia. Eur J Haematol 2008;80:391-396.

III. Nihtinen A, Anttila V-J, Richardson M, Ruutu T, Juvonen E, Meri T, Volin L. Invasive *Aspergillus* infections in allo-SCT recipients: environmental sampling, nasal and oral colonization and galactomannan testing. Bone Marrow Transplant 2010;45:333-338.

IV. Nihtinen A, Anttila V-J, Richardson M, Ruutu T, Juvonen E, Meri T, Volin L. Factors influencing the performance level of *Candida* mannan antigen testing in allogeneic stem cell transplant recipients not receiving fluconazole prophylaxis. Transpl Infect Dis 2011;13:266-272.

V. Nihtinen A, Anttila V-J, Ruutu T, Juvonen E, Volin L. Low incidence of invasive aspergillosis in allogeneic stem cell transplant recipients receiving amphotericin B inhalation prophylaxis. Transpl Infect Dis 2011, July 12, epub ahead of print.

ABBREVIATIONS

Ab	antibody
Ag	antigen
aGvHD	acute graft-versus-host disease
ALL	acute lymphoblastic leukaemia
AmB	amphotericin B
AML	acute myeloid leukaemia
ATG	antithymocyte globulin
BAL	bronchoalveolar lavage
BMT	bone marrow transplantation
BU	busulfan
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
cGvHD	chronic graft-versus-host disease
CI	confidence interval
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CNS	central nervous system
CSF	cerebrospinal fluid
CY	cyclophosphamide
D-AmB	amphotericin B deoxycholate
DNA	deoxyribonucleic acid
ECIL	European Conference of Infections in Leukaemia
ELISA	enzyme-linked immunosorbent assay
EORTC/MSG	European Organization for Research and Treatment of Cancer/ Invasive Fungal Infections Cooperative Group/ National Institute of Allergy and Infectious Diseases Mycoses Study Group
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
GM	galactomannan
GvHD	graft-versus-host disease
Gy	Gray
HEPA	High-Efficiency Particulate Air
HLA	human leukocyte antigen
HRCT	high-resolution computerized tomography
HSC	hepatosplenic candidiasis

HUCH	Helsinki University Central Hospital
IA	invasive aspergillosis
IC	invasive candidiasis
ICU	intensive care unit
IDSA	Infectious Diseases Society of America
IFI	invasive fungal infection
IPA	invasive pulmonary aspergillosis
I.V.	intravenous
LAF	laminar air flow
L-AmB	lipid formulations of amphotericin B
LAT	latex agglutination technique
MDS	myelodysplastic syndrome
MF	myelofibrosis
MM	multiple myeloma
MP	methylprednisolone
MRI	magnetic resonance imaging
MTX	methotrexate
NHL	non-Hodgkin lymphoma
NPV	negative predictive value
ODI	optic density index
OR	odds ratio
OS	overall survival
PCR	polymerase chain reaction
PPV	positive predictive value
RIC	reduced intensity conditioning
SAA	severe aplastic anaemia
SCT	stem cell transplantation
TBI	total body irradiation

ABSTRACT

Invasive fungal infections (IFIs) constitute a potentially lethal complication in haematological patients, particularly in patients with acute leukaemia and in allogeneic stem cell transplant (SCT) recipients. Most of these infections are caused by *Aspergillus* or *Candida* species (spp.). The key issues in improving the prognosis of patients with these infections are prevention, prompt diagnostics and early start of antifungal therapy.

The series of studies in the present thesis were performed to investigate the prevention and serological diagnostic methods of invasive aspergillosis (IA) and invasive candidiasis (IC) in patients receiving chemotherapy for acute leukaemia and in allogeneic SCT recipients.

Two studies assessed the role of environmental exposure to *Aspergillus* spores and the colonization of nasal cavities with *Aspergillus* spp. as risk factors for IA. In Study I, these factors were investigated in a prospective way during a period of heavy construction activity in the immediate vicinity of the SCT ward. Such periods can cause outbreaks of IA. The air quality remained good, and the patients did not have nasal colonization with *Aspergillus* spp. No cases of IA were detected in the 55 patients treated in the ward during the construction period. In the other study (Study III), environmental sampling was performed under normal conditions over a 2.5-year period. *Aspergillus* spp. were detected in 6.1% of the environmental samples. This study had 102 allogeneic SCT recipients, three of whom (2.9%) had *Aspergillus* spp. in their nasal samples. Two patients (2%) had IA. The feasibility of *Aspergillus* galactomannan antigen (Ag) testing from serum as a diagnostic marker of IA was also assessed. Of the 2071 serum samples, 12 (0.6%) yielded positive results in nine patients (8.8%). The serum samples yielded positive results in one of the two patients with IA. The sensitivity and specificity of the Ag test was 50% and 92%, respectively.

The role of oral colonization with *Candida* spp. and *Candida* mannan Ag testing of serum samples was also investigated in the group of 102 allogeneic SCT recipients (Study IV). Of the 657 oral samples, 92 (14%) yielded positive results in 38 (37%) patients. Of the 2071 serum samples, 98 (4.7%) yielded positive and 78 (3.8%) borderline positive results. One patient (1%) had IC. In this patient, six out of nine serum samples yielded positive and one sample borderline positive results. False positive or borderline positive *Candida* Ag test results were detected in 75 patients (73.5%) and in 169 (8.1%) serum samples. False results were associated with the use of acyclovir and valacyclovir.

Two retrospective studies evaluated the role of antifungal prophylaxis. In Study II, the role of fluconazole prophylaxis was assessed in 1089 adult patients with acute leukaemia by comparing the incidence of IC in 847 patients not receiving prophylaxis (years 1978-1999, Period 1) to 242

patients receiving fluconazole prophylaxis (years 2000-2004, Period 2). The incidence of IC was 8.7% and 1.6%, respectively ($P < 0.001$). A larger proportion of patients in Period 2 compared to Period 1 had bacteraemias, 65% vs. 52%, respectively ($P < 0.001$).

In Study V, the efficacy and tolerability of Amphotericin B (AmB) inhalation prophylaxis were analysed in allogeneic SCT recipients. Antifungal prophylaxis was not given to 257 patients transplanted in 1996-2000 (Period I), whereas in the 354 patients transplanted in 2001-2005 (Period II) AmB inhalation prophylaxis was started in cases of acute graft-versus-host disease (aGvHD) requiring therapy with high-dose methylprednisolone (MP). IA was detected in 17 (6.6%) vs. 9 (2.5%) of the patients in Period I and Period II ($P = 0.007$), respectively. Breakthrough IA was detected in only one the 111 patients (1%) who used the prophylaxis in Period II. The inhalations were well tolerated.

In conclusion, the environmental surveillance of the SCT ward showed constantly low numbers of fungal spores indicating well functioning air filtration. Colonization of the nasal cavities with *Aspergillus* spp. was rare and IA was detected in 2% of the allogeneic SCT recipients. IC occurred in only 1% of the patients despite the fact that oral colonization with *Candida* spp. was detected 38% of the patients. In a population of patients with such a low incidence of IA and IC, the galactomannan and mannan Ag tests were not helpful in predicting the risk of invasive fungal infections. Fluconazole prophylaxis was effective in reducing the incidence of IC in patients with acute leukaemia. AmB inhalations were similarly effective as prophylaxis of IA in allogeneic SCT recipients with aGvHD.

INTRODUCTION

Invasive fungal infections are a major cause of death in immunocompromised hosts. The majority of IFIs are caused by *Aspergillus* and *Candida* spp. The groups of haematological patients at highest risk of IFIs are patients with acute leukaemia and allogeneic SCT recipients. In patients with IC the mortality rate is 20-50% (156,169,184,255); in patients with IA it may be even higher, 70-90% (93,174,250,266).

The profile of IFIs in allogeneic SCT recipients has changed in the past decades. The incidence of IA rose from 5-6% to 10-12% from the 1980´s to the 1990´s and has thereafter stabilized (19,70,107,158,240,278). The incidence of IC has fallen, probably due to the widespread use of fluconazole prophylaxis (79,156,184,199,256).

The poor prognosis of patients with IFIs is associated with delays in the diagnosis of these infections. The yield of blood cultures is low, and radiological findings can be unspecific even in disseminated infections. Obtaining a histological sample to confirm the diagnosis is not often possible due to the fragile condition of the patients. Different serological methods to detect fungal antigens from patient samples have thus been developed to help with the earlier diagnosis of IFI.

Colonization of the mucous membranes is the first step in the pathogenesis of IFI. With *Candida* infections the colonization occurs in the gastrointestinal tract. *Aspergillus* spores, in turn, enter the body from the air to the lungs. High numbers of spores can be released into the air during construction activity. This enhances the risk of IA. Several studies have reported outbreaks of IA in immunocompromised patients after construction activity in nearby areas of hospitals (17,239,262). Colonization with *Candida* and *Aspergillus* spp. can never be totally avoided. Patients with risk factors for IFI, such as prolonged neutropenia or graft-versus-host disease (GvHD) after allogeneic SCT may therefore benefit from antifungal prophylaxis.

Minimizing the risk of colonization, giving antifungal prophylaxis to high-risk patients, early diagnosis, and a prompt start of antifungal therapy are the key issues in the prevention of IFIs as well as in improving the prognosis of patients with these infections.

The studies in this thesis were performed in adult patients with acute leukaemia and in allogeneic SCT recipients with the focus on risk factors, prevention, and serological diagnostics of IA and IC in these patient groups.

REVIEW OF THE LITERATURE

Invasive fungal infections enhance morbidity, mortality, costs, and hospital days in immunocompromised hosts. The subgroups of haematological patients at a particularly high risk for IFI are patients receiving chemotherapy for acute leukaemia and SCT recipients. The majority of IFIs are caused by *Aspergillus* and *Candida* spp. Table 1 shows the incidence of *Aspergillus* and *Candida* infections and the mortality rates in leukaemia patients and in SCT recipients.

As Table 1 shows, autologous SCT recipients have a low risk of IFI. In patients with acute leukaemia, the risk of IFI is higher after induction chemotherapy than after consolidation therapies (141,200,231). IFI-related mortality rate is high. The prognosis is especially dismal in allogeneic SCT recipients with IA. Early diagnosis and start of therapy improve the prognosis of IFIs (4,68,75,99,180). The fundamental dilemma regarding IFIs has been the inability to diagnose these infections early (75,99,180). Due to more accurate serological and radiological diagnostic methods, a better understanding of the risk factors for these infections, and new antifungal agents, the prognosis of IFIs has somewhat improved (88,184,187,200). However, the mortality still often exceeds 50% (199,266). Table 2 shows the risk factors for IFIs in patients with acute leukaemia and in allogeneic SCT recipients.

Some of the risk factors listed in Table 2 deserve special attention. First of all, the degree and duration of neutropenia correlate with the risk of IFI (90,171,223,278). Gerson et al. estimated that the risk of IA rises 1% by each day during the second and third week of neutropenia (76). From the fourth week on, the risk rises 4.3% each day.

Second, colonization is the first step towards IFI. In *Candida* infections, the colonization is mostly endogenous. *Candida* spp. are a part of the normal flora of the skin, mouth, and intestinal tract. Mucositis often occurs after intensive chemotherapy opening the route for IC. The overgrowth of *Candida* spp. is enhanced by the use of broad-spectrum antimicrobial agents in neutropenic patients (53). Cross-infections i.e. exogenous acquisition has also been reported, especially with *Candida parapsilosis* (139,267). In *Aspergillus* infections the route of infection is exogenous. Ingress of fungal conidia from the air into the lungs leads to colonization of the airways and, possibly, to IA.

Third, in allogeneic SCT recipients the risk of IFI remains significant even after neutrophil recovery. The underlying disease, the conditioning, and the medication used as GvHD prophylaxis disturb both cell-mediated and humoral immunity. The immunity is even further disturbed if therapy against GvHD is required. GvHD can enhance the risk of IFI from two- to seven-fold (74,157,164).

Table 1. Incidence of IA or IC and mortality rates of haematological patients with these infections.

IA		Number of patients	Incidence %	Mortality of patients with IA %	Reference
Patients with acute leukaemia					
		54	16.7	78	(242)
		256	2.3	NR	(288)
		675	7.1	27	(188)
		1625 (AML)	8	64	(51)
		1000 (ALL)	6.3	56	(51)
		283	1	NR	(192)
		231	2.6	NR	(90)
Autologous SCT recipients					
Year of Tx:	1978-1991	114	0	0	(166)
	1993-1996	354	0.8	33	(186)
	1994-1999	2115	1.1	42	(51)
	1990-2001	1188	0.8	29	(108)
	2001-2002	2588	0.5	54	(179)
	2000-2008	62	8	20	(214)
Allogeneic SCT recipients					
Year of Tx:	1986-1990	322	5.6	78	(240)
	1987-1993	2008	7.1	93	(278)
	1989 -1993	142	11	93	(106)
	1993-1998	1682	10	80	(157)
	1994-1999	1175	12.8	71	(51)
	2001-2002	2033	2.9 - sibling donor; 2.3 - MUD; 3.9	76.3	(179)
	1999-2003	1249	6.3	77	(199)
	2000-2005	157	12.9	25	(19)

IC		Number of patients	Incidence %	Mortality of patients with IC %	Reference
Patients with acute leukaemia					
		54	18.5	NR	(242)
		283	4.2	NR	(192)
		231	4.3	NR	(90)
		70	11-21	NR	(56)
		138	11.6	NR	(36)
		442	6.3	17.8	(221)
		95	8.9	NR	(248)
Autologous SCT recipients					
Year of Tx:	1990-2001	1188	0.6	50	(108)
	1999-2003	1979	0.8	43.8	(199)
	2000-2008	62	2	NR	(214)
Allogeneic SCT recipients					
	1980-1986	1506	11.4	39 if candidaemia 90 if disseminated candidiasis	(80)
Year of Tx:	1989-1993	142	3	25	(106)
	1993-1996	92	4.3	0	(186)
	1994-1997	585	4.6	20	(156)
	1997-1998	94	8.5	NR	(22)
	1996-2000	395	3	8.3	(164)‡
	1999-2003	1249	1.2	57.1	(199)

‡ all patients transplanted with peripheral blood stem cell grafts from sibling donors

Abbreviations: NR; not reported, AML; acute myeloid leukaemia, ALL; acute lymphoblastic leukaemia, SCT; stem cell transplantation, Tx; transplantation, MUD; matched unrelated donor.

Table 2. Risk factors for IFI in haematological patients.

Risk factor	Risk for IA	Risk for IC	Reference
Degree and duration of neutropenia	X	X	(90,133,157,223,236,252,278)
Colonization of airways or gut	X	X	(36,56,98,117,123,156,162,198,242,278)
Therapy with broad-spectrum antibiotics	X	X	(53,198,223,252)
Age of the patient	X	X	(158,236,252,278)
Active disease/relapse	X	X	(42,90,174,223)
Previous IFI	X		(118,194,227)
Iron overload	X		(74,127)
Genetic susceptibility	X		(122,172,235,249,296)
Unrelated donor	X		(74,158,278)
Mismatched donor	X		(74,158)
T cell depletion of the graft	X		(157,285)
RIC	X		(87,126)
GvHD	X		(74,157,164,174,252,278)
Therapy with corticosteroids	X		(74,82,164,174,193,278)
Lymphopenia	X		(74,157,174)
Cytomegalovirus disease	X	X	(74,82,156,157,174)
No air filtration	X		(7,278)
Construction work in the vicinity of SCT ward	X		(188,278)
Central venous catheters		X	(2,117,198,255)

Abbreviations: RIC; reduced intensity conditioning, GvHD; graft-versus host disease, SCT; stem cell transplantation

Clinical features of invasive aspergillosis

The site of *Aspergillus* infections is in the lungs in about 90% of the cases (23,107,199). Ingress of fungal conidia from the air leading to colonization of the airways is the usual route of infection. Antimicrobial-resistant fever, cough, and pleuritic chest pain are typical symptoms of invasive pulmonary aspergillosis (IPA). In patients with haematological malignancies 5-20% of IA cases disseminate to other organs, including the central nervous system (CNS) (23,68,174,199). Some studies have even reported CNS involvement in 40-55% of the patients (107,147,240). IA occurs in the form of sinusitis in 5-10% of the patients (199,200). *Aspergillus fumigatus* is the most frequently isolated spp., followed by *A. flavus*, *A. niger, and A. terreus* (23,107,164,199).

Aspergillus in the environment

Decaying vegetation is the primary source of *Aspergillus* spp. which are present everywhere in the environment; in the air, in soil, and in water. *Aspergillus* conidia are 2.5-3 µm in diameter. The spores can remain airborne for long periods of time due to their small size. They move with a velocity of 0.5-1 m/hour (204). After having landed on any surface, the spores can become airborne repeatedly. The spores enter buildings through air intakes, doors, and windows (251). The amount of spores in the air and on surfaces can be investigated with several techniques, such as air sampling, gravity air sedimentation plates, and contact plates (181).

Colonization of the airways

When inhaled, *Aspergillus* conidia are able to enter the human bronchioles due to their small size. In most healthy people fungal colonization of the airways does not cause any clinical problems. In immunocompromised hosts such as solid organ transplant recipients, allogeneic SCT recipients, and patients receiving chemotherapy for acute leukaemia colonization of the airways can lead to IA of the lungs or the paranasal sinuses. The quantity of fungal conidia of the alveolar space sufficient to cause IA in these patients is unknown. The amount is probably quite small, equal to air concentration of one colony-forming unit(CFU)/m^3 of spores or less (18,220,252).

Air filtration and quantity of fungal spores

The number of conidia in the air falls with air filtration. Therefore, immunocompromised patients may benefit from air filtration. Some early studies reported a fall in the incidence of IA even with course air filtration techniques (229,230). However, with these techniques significant amounts of fungal conidia remain in the air (102). More refined techniques, i.e. High-Efficiency Particulate Air (HEPA) filtration and laminar air flow (LAF), are more effective in clearing the air from fungal conidia. HEPA filtration clears the air of particles ≥ 0.3 µm in diameter with 99.97% efficacy, making at least 12 air exchanges per hour. LAF, a costly technique, adds a component of circulating the filtrated air in parallel flowing planes and 400 air exchanges per hour.

In the study by Leenders et al. the concentration of all types of spores was 400 CFU/m^3 in the outside air, 32 CFU/m^3 inside the hospital building, 7 CFU/m^3 inside the haematology ward, and <2 CFU/m^3 in the HEPA-filtered rooms (138). Another study showed a fall in the number of *Aspergillus* conidia from 15 CFU/m^3 inside the hospital during renovation work to 0.18 CFU/m^3 of the HEPA-filtered rooms (196).

Placing high-risk patients in rooms with HEPA±LAF filtration has reduced the incidence of IA (27,252). In a survey conducted by the International Bone Marrow Transplantation Registry, the

use of HEPA filtration was even connected to lower transplant-related mortality by day 100 post-SCT. In this study, HEPA filtration also correlated with a reduction in the incidence of IA in patients with unrelated donors (205). Another study not only reported a fall of spore counts from 1.7 ± 0.2 CFU/m^3 in the ward corridor to 0.008 ± 0.003 CFU/m^3 in the HEPA-filtered rooms but also a reduction in the incidence of IA in immunocompromised patients after initiation of HEPA filtration (195). Barnes et al. reported similar results in a pediatric bone marrow transplantation (BMT) unit with LAF (25). In contrary to these two studies, Hospenthal et al. reported that the number of conidia in the ward air did not correlate with the incidence of IA (102). This study, however, concerned oncology patients and wards with course air filtration, not HEPA or LAF. The current CDC/IDSA (Centers for Disease Control and Prevention/Infectious Diseases Society of America) guidelines recommend placing SCT recipients in rooms with HEPA filtration and positive pressure compared with the ward corridor (41). LAF is considered optional in these guidelines.

The protective environment of HEPA-filtered patient rooms applies only to periods of hospitalization. After being discharged from hospital the patients are unavoidably subjected to *Aspergillus* conidia. This fact is supported by the finding that the timing of IA in allogeneic SCT recipients is bimodal. Currently, only 20-30% of these infections occur within the first 30-40 days after transplantation when the patients are usually hospitalized (74,128,158). The majority of IA cases, however, are detected 90-140 days after the transplantation (164,250).

Construction work and risk of invasive aspergillosis

The number of fungal conidia in the air rises during construction or renovation activity. Construction work inside or adjacent to the hospital puts therefore the HEPA filtration system under maximal strain and can cause outbreaks of IA in immunocompromised patients. These outbreaks can occur due to dysfunction of the HEPA filtration or contamination of the air ventilation channels with *Aspergillus* conidia (131,144,183). Insufficient protective measures during construction activity can also lead to outbreaks of IA (12,17,132). A review article analysed 53 studies reporting outbreaks of IA (277). Construction activity was the probable cause in half of the outbreaks. Of the 458 patients in these 53 studies, 65.3% had haematological malignancies. In this subset of patients the mortality rate was the highest, 57.6%. These data emphasize the importance of well-executed protective measures for high-risk patients. The CDC/IDSA guidelines recommend protective measures for SCT recipients during construction activity (84). These measures include building protective barriers around the construction site and creating negative air pressure inside it, closing the air intakes near the construction, specific routes of entry and exit for construction use only, and thorough wet-cleaning of the construction area and its vicinity when the work is finished.

The awareness of periods of construction activity as a risk factor for IA is growing. Several studies reporting the results of prospective environmental surveillance during such periods have been published in the past decades. Table 3 summarizes some of these studies.

Table 3. Prospective environmental surveillance studies.

Techniques used in the environmental sampling	Duration of surveillance	Result	Reference
Air sampling for spore counts	75 minutes after demolition	Number of spores rose > 1.5 log outside, < 1 log in the hallway of BMT unit.	(257)
Air sampling for spore counts Nasal cultures	1 year	ICU spore counts mostly stable; three bursts. 6.4% of nasal swabs positive. No cases of IA.	(78)
Particle measurements Air sampling for spore counts	7.5 months	Particle & spore counts of BMT unit significantly lower than at the construction site.	(197)
Air sampling for spore counts Surface cultures by swabs	2 years	Spore counts of HEPA rooms rose from 4 to 24.7 CFU/m^3 (mean). LAF needed to keep the rooms clean. Fewer SCTs performed during construction. Incidence of IA stable.	(50)
Air sampling for spore counts	6 years	More positive air samples after construction inside the hospital, CFU's remained stable. No outbreaks of IA.	(216)
Air sampling for spore counts	4.5 months	Spore counts stable in 4 hospital floors, median 0 CFU/m^3. No outbreaks of IA.	(46)
Surface cultures by gravity air setting plates	6 months	8% of BMT unit samples positive, mean 0 CFU/m^3. Incidence of IA stable.	(129)
Air sampling for spore counts	6 months	Damages caused by rain water during construction detected early enough to prevent outbreaks.	(182)
Air sampling for spore counts	1 year	Concentration of viable fungi lower in BMT unit vs. outdoors; no difference in the concentration of *Aspergillus* spp. 3 peaks in the spore counts.	(52)
Air sampling for spore counts Surface cultures by swabs	7 years	Intensified protective measures after outbreak in haematology wards. Proportion of positive samples stable. Incidence of IA fell.	(29)
Particle measurements Air sampling for spore counts	1 year	Particle & spore counts rose after demolition at the construction site. Extensive protective measures. Incidence of IA stable in oncology & BMT patients.	(91)

Abbreviations: BMT; bone marrow transplantation, ICU; intensive care unit, HEPA; High-Efficiency Particulate Air, CFU; colony corming unit, LAF; laminar air flow, SCT; stem cell transplantation.

Aspergillus in water and food

In addition to air, *Aspergillus* spp. are present in water. Community water reservoirs contain moulds, but their quantity seems to vary according to the primary origin of the water supply. Surface water is more contaminated with *Aspergillus* spp. than ground water (282). Some studies have detected *Aspergillus* spp. in tap water, taps and showerheads of patient rooms, and in the bathroom air after showering (11,281). One group reported a patient with lymphoma and IA, in whom the *Aspergillus* genotype was identical to that found in the patient-room water (9). The same authors noticed that thorough cleaning of the bathroom before showering decreases the number of spores in the air (10). However, the correlation between the number of spores in water and IA is unclear. No outbreaks connected to water have been described. Also, other studies have reported absence of *Aspergillus* spp. in both community water and tap water of hospital wards (85,203).

Since *Aspergillus* spp. also live in the soil, food is a potential source of infection for immunocompromised hosts. Tea, pepper, skin of fruits, and freeze-dried soups, for instance, contain *Aspergillus* spp. (34,54). This must be taken into consideration during the preparation of food and the handling of food products. Tobacco also contains *Aspergillus* spp., as shown by Verweij et al. (275). Singh et al. reported a correlation between cigarette smoking and risk of IA in liver transplant recipients (254). Smoking may be a risk factor for IA also in other immunocompromised patients.

Due to the ubiquitous nature of *Aspergillus* spores, separating nosocomial IA from community-acquired cases is difficult. No consensus exists over the criteria for nosocomial IA. Patterson et al. have suggested that this should be done by looking at the timing of the infection (206). According to this group, IA should be considered nosocomial if it is detected more than seven days after hospital admission or less than 14 days after the patient was last discharged from the hospital.

Diagnostics of invasive aspergillosis

Diagnosing IA is challenging. The clinical symptoms and the findings on the chest radiograph are unspecific. Histopathological demonstration of fungi in tissue specimens or fungal growth in culture are the only ways to confirm the diagnosis of IA.

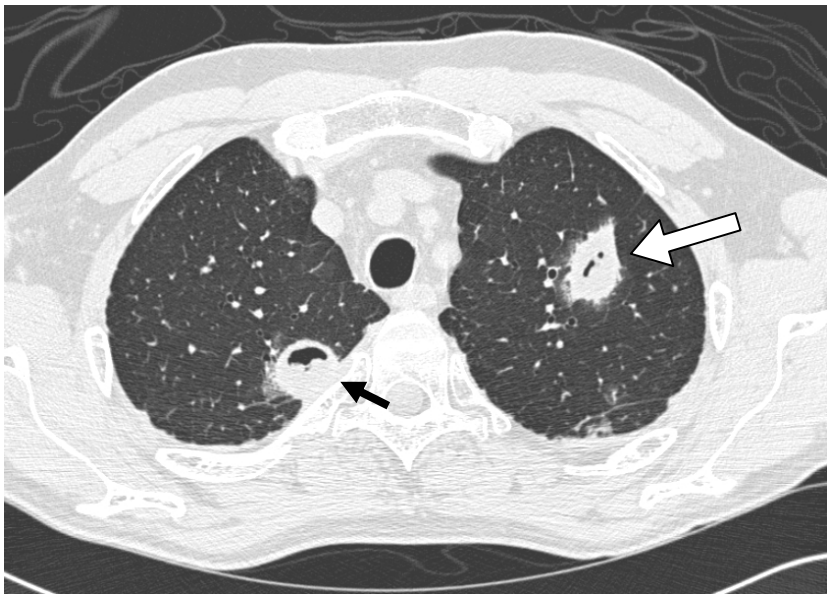
Due to the fragile condition of most high-risk patients, obtaining a histological sample by biopsy is not often possible. Blood cultures remain negative in over 90% of the patients even in disseminated IA (113). Obtaining representative sputum samples from these severly ill patients is not often possible either and, even if samples are collected, the sensitivity of the cultures is

approximately only 30% (68). The positive predictive value (PPV), though, is 50-82% and even higher in neutropenic patients (101,208,295).

Bronchoscopy with bronchoalveolar lavage (BAL) is a less invasive technique than biopsy for obtaining samples from the bronchial tree. As with sputum samples, the sensitivity of BAL cytological samples and fungal cultures in SCT recipients is modest; 30-64% (140,218). However, the specificity and negative predictive value (NPV) are over 90% (147).

In high-resolution computerized tomography (HRCT), findings indicative of IPA are multiple nodules more than 1 cm in diameter, the so-called halo sign (a nodular consolidation with a surrounding ground-glass opacity), and, less frequently, cavitation or air crescent sign (31,71,81). Figure 1 shows IPA findings. These findings are not specific for IA, as they can be present in other types of pulmonary infections, such as *Pseudomonas*, *Mycobacterium, Nocardia* or viral infections (137). If the localisation of suspected fungal lesions and the condition of the patient allow it, computerized tomography-guided needle biopsy should be obtained. At best, the sensitivity and PPV of a biopsy are high; 70-80% and 100%, respectively (39,189).

Figure 1. HRCT of a 51-year old male non-Hodgkin lymphoma patient with proven IA. Air crescent (black arrow) and halo sign (white arrow). Courtesy of Docent Anneli Piilonen M.D., Radiology Department, Helsinki University Central Hospital (HUCH).



Antibody (Ab) production is not a reliable method to confirm the diagnosis of IA in patients with haematological malignancies (294). In allogeneic SCT recipients the production of antibodies is hampered for months or even years (143). Recent diagnostic efforts in these patients have thus been focused on detecting circulating fungal antigens by serological methods rather than measuring the host response.

The optimal serological test should be rapid, sensitive, specific, and repeatable. Two *Aspergillus* Ag tests are currently commercially available. The targets of these tests are structural components of the fungal cell; galactomannan (GM) and 1,3-β-D-glucan. In addition to these tests, the polymerase chain reaction-technique (PCR) can be used to detect fungal deoxyribonucleic acid (DNA) from biological samples.

Galactomannan antigen test

GM is a polysaccaride part of the cell wall of *Aspergillus* and *Penicillium* spp. The GM molecule consists of the non-immunogenic mannan core part and the immunoreactive galactofuranoside side chain.

The first GM Ag test was performed with latex agglutination technique (LAT). The test sensitivity was 23-50%, and the test yielded positive results late, usually simultaneously with the clinical or radiological findings or even after them (13,100,111,115,153).

To improve sensitivity, the GM sandwich enzyme-linked immunosorbent assay (GM ELISA) test was developed (258). The threshold of detection of this test is 1 ng/ml of GM compared with the 15 ng/ml of LAT. The ELISA test uses a rat monoclonal antibody (Ab) both to detect and capture the 1→5-β-galactofuranoside side chains of the GM molecule. The result is expressed as optic density index (ODI) which is the ratio between the optical density of the patient sample and the control sample. A commercial kit is available for this test (Platelia *Aspergillus*, Bio-Rad, Hercules, CA, USA).

Two early studies reported GM ELISA test sensitivity of 82.5-90% in neutropenic patients and SCT recipients (260,273). The ELISA test yielded positive results before the LAT test in both studies with a maximum of five days in one study and a median of 27 days in the other. Since then several other groups have reported the results of the GM ELISA test in neutropenic patients and in allogeneic SCT recipients. Tables 4 and 5 summarize the results of the largest prospective studies in this field.

The cut-off levels, serum sampling frequencies, and the antifungal prophylaxis of the patients in these studies have been different (Tables 4 and 5). The best performance level is achieved when

Table 4. Studies of the GM ELISA test in patients with haematological malignancies, serum sampling during neutropenia only.

Number of - patients - samples	Frequency of sampling	Cut-off ODI	Antifungal prophylaxis	Patients with IA, % (proven&probable)	sens. %	spes. %	PPV %	NPV %	Other observations	Ref.
135 (34 allog. SCT) 507	once a week	1.5 1.0	none	11.8	69 100	96 100	 64	 100	Ag test positive before other signs of IA in 12.5% of patients wit IA	(265)
104 (39 allog. SCT) 1642	twice a week until engraftment/IFI	different cut-offs tested	fluconazole or itraconazole	27.9	96.5	98.6	98.6	98.4	best performance, cut-off 0.5 in two consecutive samples	(148)
203 (239 episodes, 74 allog. SCT) 4884	twice a week	different cut-offs tested	fluconazole or itraconazole	18.7	92.1	97.5	87.5	98.5	best performance, cut-off 0.5 in two consecutive samples	(150)
200 (28 allog. SCT) NA	twice a week	0.5	none	11.5 8.5	100 100	97.2 93.8	82.1 60.7	100 100	when Ag test included in diagnostic criteria vs. not included	(207)

Abbreviations: ODI; optic density index, IA; invasive aspergillosis, sens; sensitivity, spes; specificity, PPV; positive predictive value, NPV; negative predictive value, Ref; reference, allog. SCT; allogeneic stem cell transplantation, Ag; antigen, GvHD; graft-versus-host disease, i.v; intravenous, AmB; amphotericinB

Table 5. Studies of the GM ELISA test in patients with haematological malignancies, serum sampling beyond the neutropenic phase.

Number of - patients - samples	Frequency of sampling	Cut-off ODI	Antifungal prophylaxis	Patients with IA, % (proven& probable)	sens. %	spes. %	PPV %	NPV %	Other observations	Ref.
215 2161	1st month: weekly, then monthly	1.0	fluconazole + ketoconazole	18.6	82.5	81	54	95		(260)
186 (40 allog. SCT) 2172	twice a week when hospitalized, then weekly until 6 months in allogeneic SCT only	1.0	itraconazole	17.7	92.6	95.4	92.6	95.4	test performance based on post mortem confirmation of IA. Ag test positive 6 days (median) before clinical symptoms of IA in 66% of patients with IA	(145)
797 (450 adult allog. SCT) 6209	twice a week during neutropenia or GvHD, then monthly	1.5 in two consecutive samples	not reported	6.6	88.6	97.5			result of the adult allogeneic SCT recipients	(261)
100 2695	twice a week when hospitalized, then weekly until stop of GvHD/immunosuppression	1.0 in two consecutive samples	itraconazole or i.v. AmB + AmB inhalations	18	94.4	98.8	94.4	98.8	test performance based on post mortem confirmation of IA. Ag test positive 14 days (median) before confirmation of IA in 88.8% of patients with IA	(147)
74 832	1st episode: twice a week, then weekly until stop of immunosuppression	1.5	fluconazole for all itraconazole if GvHD	8.1	75	100	100	97	Ag test positive before chest radiograph in 25% of patients wit IA	(232)
121 1523	twice a week when hospitalized, as outpatient: clinician´s decision	0.5	not reported	10	50	94	46	94	Ag test positive before other signs of IA in 33% of patients	(70)

Abbreviations: ODI; optic density index, IA; invasive aspergillosis, sens; sensitivity, spes; specificity, PPV; positive predictive value, NPV; negative predictive value, Ref; reference, allog. SCT; allogeneic stem cell transplantation, Ag; antigen, GvHD; graft-versus-host disease, i.v.; intravenous, AmB; amphotericin B

two consecutive results with a cut-off ODI of 0.5 are used as the criterion of test positivity and the serum samples are obtained at least twice a week (148,150). At best the test can yield positive results several days, even weeks before clinical symptoms or abnormalities in the chest radiograph (145,146,261). Combining prospective GM ELISA surveillance with early HRCT scanning confirms or rules out IA with better accuracy than either method alone (37,96,149). Interestingly, the Ag level of the GM ELISA test seems to correlate with the clinical outcome of patients with IA (146,151,173,292).

The GM ELISA test has some limitations. First, the test performs best in neutropenic patients (47). In non-neutropenic patients, such as allogeneic SCT recipients after engraftment, intensive care unit (ICU) patients, and solid organ transplant recipients the sensitivity is only 25-56% (35,69,70,103,160,167). Animal models indicate that the fungal infection is less angioinvasive in non-neutropenic than in neutropenic individuals, thus releasing less Ag into the bloodstream (24). Also, some investigators have reported that the test does not yield positive results early enough to help to establish earlier diagnosis of IA (70,232).

Second, the performance level of the GM ELISA test is hampered by the use of antifungal agents (47,160,161). Marr et al. showed that the test sensitivity was significantly lower in patients who had received itraconazole, voriconazole or AmB within two weeks prior to the serum sampling than in those who had not (160).

Third, false positive results occur in one fifth of the patients with the GM ELISA test (19,228,260). Therapy with piperacillin-tazobactam, amoxicillin±clavulanate, and some gluconate-containing intravenous (i.v.) solutions can cause false positive results because of residuals of GM from moulds used in the production process of these preparations (3,21,165,217). GvHD of the gut may cause leakage of GM into the bloodstream, also leading to false positive Ag test results (19).

The GM ELISA test can be performed in samples other than serum, such as BAL, urine or cerebrospinal fluid (CSF) (237,259,274). In the BAL fluid of neutropenic patients the test sensitivity is 100% if the sample is taken before the initiation of antifungals (26,207). In solid organ transplant recipients and ICU patients the test performs better in BAL fluid, bronchial aspirates or sputum than in serum (44,104,253). When analysed from BAL samples, however, the test does not necessarily help to establish early diagnosis of IA, since BAL is usually performed after abnormalities are detected on HRCT.

1,3-β-D-glucan test

Beeta-D-glucan is a cell wall component of various fungi such as *Candida*, *Aspergillus*, *Fusarium*, and *Pneumocystis* spp. The Ag can be detected by using a calorimetric assay. Four test kits are currently commercially available for the analysis. In neutropenic patients with acute leukaemia the test has yielded sensitivity, specificity, PPV, and NPV of 60-100%, 65-90%, 43-74%, and 91-100%, respectively (192,248). False positive results may occur during bacteraemias, overgrowth of *Candida* spp. in the gastrointestinal tract after antimicrobial therapy during mucositis, haemodialysis, and therapy with i.v. immunoglobulins (105,119,212,248). High serum concentrations of bilirubin or triglycerides lead to false negative results (212). The PPV of the test may be better if two consecutive positive results are used as the criterion for the test positivity (192,248). The feasibility of this test in allogeneic SCT recipients is unknown. Due to the panfungal nature of the test, a histological or culture sample is required to specify the causative fungus.

Polymerase chain reaction

Studies of *Aspergillus* PCR in blood samples of patients with haematological malignancies have shown sensitivity, specificity, PPV, and NPV of 75-100%, 65-100%, 22-100%, and 100%, respectively (89,92,112,134,284). At best, the PCR test has yielded positive results 14 days before the confirmation of IA diagnosis (89). The somewhat suboptimal sensitivity is probably connected to the short duration of fungal DNAemia in blood. Colonization of the airways and contamination of the samples can lead to false positive test results and, thus, to low PPV. Several investigators have concluded that a single positive result should not be interpreted as a positive result (89,134). Overall, comparing the results of different studies is hampered by the use of in-house primers. Lack of inter-laboratory validation and standardization of methods means that PCR is currently not considered a standard method in the diagnostics of IA.

The diagnosis of IA is usually confirmed after a chain of events. Symptoms such as cough or fever unresponsive to broad-spectrum antimicrobials, abnormalities on chest radiograph or a positive serum GM Ag test may cause a suspicion of IA. Further investigations such as HRCT serve as a guide for obtaining samples (needle biopsy or BAL) for microscopy, culture or GM Ag testing.

Clinical features of invasive candidiasis

Bodey et al. suggested classifying IC as candidaemia or disseminated candidiasis (32). In this classification candidaemia is defined as the isolation of *Candida* spp. in at least one blood culture without signs of deep organ involvement. Fever not responding to broad-spectrum antimicrobials is often the only clinical finding. Acute disseminated candidiasis is characterised by fungemia and fungal dissemination to more than one deep organ during the neutropenic period. Skin lesions are present in 10% and endophtalmitis in 5-50% of cases, but the infection can involve any internal organ. Chronic disseminated candidiasis is characterized by fever unresponsive to bacterial antibiotics and persisting after recovery from neutropenia. Signs of liver function abnormalities, especially elevated alkaline phosphatase, can be detected. Abdominal pain and hepato- or splenomegaly or both are also part of this syndrome. The term hepatosplenic candidiasis (HSC) is often used instead of chronic disseminated candidiasis. In both forms of candidiasis, *Candida* spp. should be found in histological or culture samples from deep organs or tissues to confirm the diagnosis.

Diagnostics of invasive candidiasis

Blood cultures yield positive results in about half of the cases of disseminated candidiasis and the incubation time of the the cultures is long (28). Ophtalmological examination can reveal endophtalmitis. In HSC, MRI reveals round hyperintense lesions less than 1 cm in diameter in liver and/or spleen. At best, the sensitivity and specificity of MRI are 100% and 96%, respectively, thus making it a superior technique compared with ultrasound or CT scanning (14,244).

Non-culture methods to diagnose *Candida* infections focus on identifying cell wall components (mannan, 1,3-β-D-glucan), cytoplasmic antigens (enolase), metabolites (arabinitol), and fungal DNA. The methods regarding 1,3-β-D-glucan and DNA identification have been discussed previously in this review (page 23).

Enolase

Enolase is a sytoplasmic Ag of *Candida* spp. In the prospective study by Walsh et al., the sensitivity and specificity of enolase detection by immunoassay in 24 patients with IC were 54% and 96%, respectively (279). The test sensitivity rose to 71% if two consecutive positive results were considered. The test performed better in patients with disseminated candidiasis than in patients with candidaemia. Two other studies of enolase measurements reported sensitivity of 65-71.8% and specificity of 97.1-100%, respectively (86,175). None of these studies reported the timing of enolase positivity with respect to the first positive blood cultures. Besides the

unsatisfactory sensitivity of enolase testing, it is unclear whether the assay can help in yielding earlier diagnosis of IC.

Arabinitol

Arabinitol is a metabolite of *Candida albicans*. Arabinitol can be detected from serum or urine. The level of this metabolite rises in cases of renal insufficiency, since it is cleared by glomerular filtration. Falsely high results can be avoided by calculating the arabinitol/creatinine ratio (DA/Cr) or by measuring the ratio of fungal/non-fungal (D/L) arabinitol. Walsh et al. studied the DA/Cr ratio with an enzymatic assay in more than 3000 serum samples from 274 patients with cancer (280). The DA/Cr ratio was elevated in 74% of the patients with candidaemia and in 40% of those with disseminated candidiasis. In the study of Arendrup et al., 74 out of 93 patients had haematological malignancies (16). The sensitivity, specificity, PPV, and NPV of D/L-arabinitol ratio measurement with gas chromatography were 41.7%, 86.4%, 76.9%, and 57.6%, respectively. The test was more informative as a marker of IC in neutropenic than in non-neutropenic patients (16). Use of arabinitol as a serological marker of IC is hampered by the fact that the level of tissue invasion affects its concentration. Frequent (daily) testing might therefore be necessary (280). Also, some non-*albicans* spp. such as *C. krusei* do not secrete arabinitol (73,280). Chromatography is a time-consuming technique and usually allows only a small number of samples to be processed per day, whereas the enzymatic assay is more rapid.

Mannan antigen and antibody test

Mannan is a polysaccharide part of the cell wall of *Candida* spp. with various mannose residues in different *Candida* spp. Mannan is highly immunogenic and can thus stimulate Ab production. As with *Aspergilllus* GM, the earliest *Candida* mannan Ag tests were performed with LAT. The specificity of the test was good, 97-100%, but the sensitivity was only 52-60% (94,125,263). Similarly to the GM Ag story, a 15-times more sensitive sandwich ELISA test for *Candida* mannan (*Candida* Ag test) was developed and is currently commercially available (Platelia, Bio-Rad, Hercules, CA, USA). The test uses a monoclonal rat Ab both to detect and to capture the β-1-5 oligomannosides of *Candida albicans*. The threshold of detection is 0.25 ng/ml. Due to the immunogenic nature of mannan, a sandwich ELISA Ab test was added to the test panel.

The studies of the *Candida* Ag and Ab test have been performed with fairly small numbers of patients, heterogenous patient populations, various sampling frequencies, and different cut-off levels for both tests. The studies with only or mainly neutropenic haematological patients have reported sensitivity and specificity of 31-100% and 49-100% for the Ag test, and 52-100% and 38-100% for the Ab test, respectively (16,61,215,226,247,272,283). Using the Ag and Ab test together has improved the performance level of the ELISA test. However, some studies indicate that

colonization with *Candida* might cause false positive results more often in the Ab than the Ag test (209,245). It should also be noted that the primary targets of the ELISA tests are the mannose residues of *C. albicans* and thus both the Ag and Ab test are less sensitive in non-*albicans* infections, particularly in those caused by *C. parapsilosis* or *C. krusei* (246).

In patients with a haematological malignancy the Ag test tends to yield positive results earlier than the Ab test (16,61). Interestingly, either one or both of the tests seem to work particularly well in cases of HSC yielding positive results 10-14 days before radiological abnormalities or an otherwise confirmed diagnosis (61,215). Of the aforementioned studies, those by Rimek et al. and Verduyn-Lunel et al. had SCT recipients, but the serum samples were obtained only during neutropenia (226,272). No previous data therefore exists of the performance of the ELISA Ag or Ab test in allogeneic SCT recipients during the post-transplantation months when Ab production in usually almost non-existent (143). Currently, ECIL (European Conference of Infections in Leukaemia) concludes that *Candida* mannan Ag and Ab test may offer diagnostic help in patients with IC and recommends using both the Ag and Ab test rather than either test alone (154).

Diagnostic criteria of invasive aspergillosis and invasive candidiasis

In 2002, the EORTC/MSG (Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer and Mycoses Study Group of the National Institute of Allergy and Infectious Diseases) consensus group published the definitions for IFIs in order to unify the criteria for these infections in clinical work and in research (20). The definitions were updated in 2008 and they are currently the gold standard of diagnostics (55). The definitions classify IFIs as proven, probable, and possible cases. The diagnostic criteria contain host factors, such as neutropenia and prolonged use of corticosteroids, clinical criteria, and mycological criteria. Tables 6 and 7 show the criteria in more detail.

Table 6. EORTC/MSG criteria for proven IA or IC. Adapted from de Pauw et al. (55).

Analysis, specimen
Microscopy of sterile material
Histopathologic/cytopathologic examination or direct microscopy of biopsy or needle aspirate from a normally sterile site showing hyphae/yeast-like forms/yeast cells & associated tissue damage
Culture of sterile material
Positive culture of a specimen obtained by a sterile procedure from a normally sterile site with clinical or radiological evidence of infection. Does not include BAL/urine/cranial sinus cavity specimen
Culture, blood
Positive culture and clinical signs of infection. Does not include *Aspergillus* spp.

Abbreviation: BAL; bronchoalveolar lavage

Table 7. EORTC/MSG criteria for probable IA or IC. Adapted from de Pauw et al. (55).

Probable IA or IC requires a host factor, a clinical criterion, and a mycological criterion. Cases meeting only host factor and clinical criteria are classified as possible.

Host factors
Recent history of neutropenia (ANC < 0.5 x 10^9/l) for > 10 days
Allogeneic SCT recipient
Prolonged use of corticosteroids
Treatment with other immunosuppressants, such as CsA, TNFα-blockers etc.
Inherited severe immunodeficiency
Clinical criteria
Lower respiratory tract fungal disease; presence of at least one of the following on CT:
Dense, well-circumscribed lesions ± a halo sign
Air-crescent sign
Cavity
Tracheobronchitis in bronchoscopy
Sinonasal infection; sinusitis on imaging and at least one of the following:
Acute pain of sinonasal area
Nasal ulcer
Extension from the paranasal sinuses to the bone structures
Central nervous system infection; one of the following:
Focal lesions on imaging
Meningeal enhancement on CT or MRI
Disseminated candidiasis; at least one of the following within two weeks of candidaemia:
Small, target-like lesions in liver or spleen
Progressive retinal exudates
Mycological criteria
Direct test = cytology/microscopy/culture
Mould in sputum, BAL fluid, bronchial brush or sinus aspirate
Indirect tests
GM Ag detected in plasma, serum, BAL fluid or CSF
β-D-glucan detected in serum

Abbreviations: ANC; absolute neutrophil count, SCT; stem cell transplantation, CsA; cyclosporine A, TNFα; tumour necrosis factor-alfa, CT; computerized tomography, MRI; magnetic resonance imaging, BAL; bronchoalveolar lavage, GM; galactomannan, Ag; antigen, CSF; cerebrospinal fluid

Antifungal prophylaxis

Systemic antifungal prophylaxis should be considered for high-risk haematological patients mostly in two circumstances; during neutropenia and the early months after allogeneic SCT, especially if GvHD is present. The decision of starting antifungal prophylaxis should be based on individual risk assessment of the patient and on knowledge of local epidemiology.

Prolonged neutropenia is one of the most important risk factors for IFIs. Neutropenic patients are usually hospitalized. The combination of protective environment i.e. HEPA filtration and systemic antifungal prophylaxis may prevent colonization and thereby infections by *Aspergillus* and *Candida* spp.

After being discharged from hospital the patients are unavoidably subjected to *Aspergillus* conidia. In patients with leukaemia the risk of IA is small after recovery from neutropenia. In allogeneic SCT recipients the risk remains significant. GvHD further enhances the risk of IA (74,106,174). The very-high-risk allogeneic SCT recipients may therefore benefit from systemic antifungal prophylaxis with anti-mould activity. The optimal prophylactic drug should be easy to administer, efficacious, well tolerated, and have few interactions with other drugs.

Polyens

The use of amphotericin B deoxycholate (D-AmB) is limited by severe adverse effects, especially cumulative nephrotoxicity (33). With lower doses toxicity might be avoided, though at the expense of efficacy (124,291). The lipid formulations of AmB are less toxic but costly. Also, a randomised prospective study comparing liposomal AmB to placebo as prophylaxis in SCT recipients failed to show any difference in efficacy (120). Another disadvantage of all polyens is that they can not be used for systemic prophylaxis in oral forms.

Triazoles

Three randomised placebo-controlled studies have evaluated the efficacy of fluconazole prophylaxis in patients with acute leukaemia and in autologous SCT recipients. The study by Winston et al. failed to show benefit of fluconazole prophylaxis regarding the incidence of IFIs or overall survival (OS) (288). The study by Rotstein et al. reported significantly fewer IFIs in patients on fluconazole prophylaxis (231). Fluconazole even reduced IFI-associated mortality in this study. It should be noted, however, that in this study oesophagitis and urinary tract infections were also classified as IFIs. In the third study by Laverdiere et al. the incidence of proven or probable IFIs also fell from 24.4% to 6.7% in the fluconazole group ($P < 0.001$) (135). This study did not report survival figures. Overall, the efficacy of fluconazole prophylaxis in patients with acute leukaemia is considered controversial, and better fungal-free survival was reported in only one study (231).

Contrary to leukaemia patients, fluconazole did show significant efficacy against IFIs in allogeneic SCT recipients in the studies by Goodman et al. and Slavin et al. (79,256). The difference between these two randomised, placebo-controlled studies was the duration of prophylaxis, 22 vs. 64 days (median), respectively. The study by Slavin et al. even showed a survival benefit in patients who received fluconazole. Marr et al. updated the results of the study by Slavin et al. (155). At a median of eight years after the randomisation, the beneficial effect of fluconazole prophylaxis was still evident. Based on these studies fluconazole prophylaxis is widely used in allogeneic SCT recipients, whereas the practises in patients with acute leukaemia vary.

With the generalised use of fluconazole prophylaxis in both high-risk haematological and non-haematological patients, some studies have reported an overall rise in the proportion of non-*albicans Candida* infections (1,2). The retrospective studies by Wingard et al. and Alangaden et al. reported the same trend in leukaemia patients and SCT recipients (6,286). Also, in the prospective study by Ellis et al., colonization with *C. glabrata* was more common in patients with fluconazole prophylaxis than with placebo (60). However, five randomised, placebo-controlled trials with over 1200 leukaemia patients and SCT recipients have not indicated a rise in IFIs caused by *C. glabrata* or *C. krusei* associated with fluconazole prophylaxis (79,121,231,256,288).

Fluconazole has no effect against *Aspergillus* spp., whereas several other triazoles have. Itraconazole is the oldest of these drugs. Of the three randomised placebo-controlled studies in neutropenic patients, one reported a decline in the incidence of IFIs with itraconazole, whereas two did not (116,168,191). In a German multicentre study neutropenic patients were randomised to receive either itraconazole or fluconazole prophylaxis (77). No difference was observed in the incidence of IFIs or IAs, IFI-related mortality or OS between the two groups. The overall incidence of IFIs in this study was quite low, 2%. Two randomised studies have compared the efficacy of itraconazole to fluconazole in allogeneic SCT recipients (159,289). The study by Winston et al. reported a reduction in the incidence of IFIs but not of IAs with itraconazole (289). The second study, by Marr et al., showed a reduction of invasive mould infection incidence from 12% to 5% with itraconazole (P = 0.03) (159). Neither study reported improved fungal-free survival or OS with itraconazole. In addition to questions concerning its efficacy, itraconazole has limited tolerability, poor bioavailability in oral forms, and clinically important interactions with other drugs (38,77,133,159,289). These qualities limit the use of itraconazole.

Of the newer triazoles, voriconazole was equal to fluconazole prophylaxis in allogeneic SCT recipients in a large, multicentre study with 600 patients (287). The incidence of proven, probable or possible IFIs was 7.3% with voriconazole and 11.2% with fluconazole, respectively. No difference was detected in OS either. In leukaemia patients, the voriconazole study by Vehreschild

et al. was interrupted early with only 25 patients enrolled when the results of the posaconazole study were analysed (271). The posaconazole study was conducted in 602 patients with acute leukaemia. This study showed a reduction in the incidence of IFIs and IFI-related mortality and a better OS with posaconazole (48). Simultaneously with the leukaemia study, a prospective study of posaconazole vs. fluconazole in allogeneic SCT recipients was published (264). In this study the prophylaxis was targeted on patients with grade 2-4 aGvHD or extensive cGvHD. The duration of prophylaxis was designed to be 112 days. The incidence of IA was 2.3% in the posaconazole group and 7% in the fluconazole group (P = 0.006). Fewer breakthrough infectios occurred in the posaconazole group than in the fluconazole group. The difference was particularly clear regarding breakthrough *Aspergillus* infections which were detected in 1% of the patients in the posaconazole group versus 5.9% in the fluconazole group (P = 0.001). No difference was detected in OS, though.

Echinocandins

With regard to echinocandins, a Japanese retrospective analysis reported good efficacy of micafungin prophylaxis in patients with acute leukaemia (97). The incidence of IFIs fell from 12.3% to 1.5% in this study. Micafungin was prospectively compared to fluconazole over the period of neutropenia in SCT recipients in the study by van Burik et al. (269). The prophylaxis was successful 80% vs. 73.5% of the patients in the micafungin and fluconazole groups, respectively (P = 0.003). Breakthrough IFIs were detected in about 2% of the patients in both groups. No survival benefit was detected. Half of the patients in this study received autologous SCT. Chou et al. reported their experience of caspofungin prophylaxis in allogeneic SCT recipients (43). In this retrospective analysis, breakthrough IFIs were detected in 7.3% of the patients. This is a higher proportion than in the micafungin study by van Burik et al. (269). In should be remembered, though, that the risk profile of the patients in these two studies was different, since the study by Chou et al. had mostly allogeneic SCT recipients (43).

Based on the aforementioned and several other studies, various groups of experts have given their recommendations for the use of primary antifungal prophylaxis in patients with acute leukaemia and in allogeneic SCT recipients. Table 8 summarizes some of these guidelines. None of the guidelines recommend antifungal prophylaxis for autologous SCT recipients.

Table 8. Summary of recommendations by IDSA, ECIL, ESCMID (European Society of Clinical Microbiology and Infectious Diseases), and the German Society of Haematology and Oncology for primary antifungal prophylaxis in haematological patients. Only A1 level recommendations are included in the table.

Guideline (Reference)	Recommended for patients with acute leukaemia receiving induction chemotherapy	Recommended for allogeneic SCT recipients
IDSA (72)	fluconazole, itraconazole, voriconazole, posaconazole, micafungin or caspofungin (also recommended for salvage chemotherapy)	fluconazole, itraconazole, voriconazole, posaconazole, micafungin or caspofungin until day +75 or stop of immunosuppression
ECIL (152)	posaconazole	fluconazole or voriconazole during neutropenia (provisional recommendation for voriconazole) posaconazole if GvHD (provisional recommendation for voriconazole)
ESCMID* (64)	none	early neutropenia: - morbidity reduction: fluconazole, voriconazole or micafungin - survival advantage: fluconazole between engraftment and day +100: - morbidity reduction: fluconazole or voriconazole - survival advantage: fluconazole moderate to severe GvHD: - morbidity reduction: fluconazole or posaconazole - survival advantage: none
German Society of Haematology and Oncology (49)	posaconazole	fluconazole prior to GvHD posaconazole after onset of severe GvHD

*Gives recommendations for *Candida* prophylaxis only

Abbreviations: SCT; stem cell transplantation, GvHD; graft-versus-host disease

Alternative routes of antifungal prophylaxis

Since 90% of IA infections affect the lungs, giving antifungal prophylaxis in the form of inhalations seems a tempting approach. This would enable targeted prophylaxis directly to the site of intended action with potentially fewer adverse effects or drug interactions than with systemic antifungal prophylaxis.

Amphotericin B nasal sprays

The earliest studies of alternative routes for antifungal prophylaxis were focused on AmB nasal sprays. Meunier-Carpentier et al. reported significant reduction in the incidence of IA in neutropenic patients who received AmB nasal sprays in a randomised study (170). Thereafter, Jeffery et al. retrospectively analysed the efficacy of these sprays in a group of 130 patients, including 21 allogeneic SCT recipients (109). The use of prophylaxis did not reduce nasal colonization with *Aspergillus* spp. but the incidence of proven cases of IA fell. However, updated analyses from the same centre eight years later revealed that the fall in the incidence of IA was related to the initiation of HEPA filtration rather than to the prophylactic AmB nasal sprays (290). Similarly, disappointing results were reported by Jorgensen et al. in a group of 15 leukaemia patients who did not benefit from the nasal sprays (110). The lack of efficacy of AmB nasal sprays may be due to the fact that they do not reach the lower airways which *Aspergillus* spores are able to enter.

Amphotericin B inhalations

The studies of the pharmacokinetics of AmB inhalations indicate good efficacy and low toxicity. Beyer et al. performed dynamic scintigraphy on healthy volunteers after one inhalation of Tecnetium-labeled AmB (30). The scintigraphies showed that 3.5-4% of the total drug activity was evenly dispersed in the lungs and significant activity still remained 14 hours after the inhalation. In the same study, serum concentrations of AmB were measured in autologous SCT recipients after AmB inhalation. AmB was detected in the serum of 11% of the patients with a minimal, non-toxic concentration. Monforte et al. measured AmB concentrations of BAL fluid of lung transplantation recipients after D-AmB inhalation of 6 mg (176). The concentration of BAL fluid was above the minimal inhibitatory concentration of AmB for *Aspergillus* at four hours after the inhalation and remained sufficient in the distal airways even at 24 hours.

Several studies have reported that AmB inhalations are effective as prophylaxis in lung transplant recipients (142,202,219). According to the survey by Dummer et al., the majority of U.S. centres give AmB inhalation prophylaxis to lung transplant recipients (59). D-AmB might cause adverse effects such as cough, wheezing, nausea, and vomiting more often than the lipid formulations (L-AmB) of the drug (57). Also, studies of drug concentration in BAL fluid indicate that L-AmB might have a longer effect than D-AmB, thus allowing less frequent dosing (176,177).

Two prospective, randomised studies have evaluated the issue of AmB inhalation prophylaxis in haematological patients (224,243). The results of these studies were contradictory regarding the efficacy of the prophylaxis in neutropenic patients. In the study by Schwartz et al. the incidence of IA was 4% with the D-AmB inhalation prophylaxis and 7% without it and the prophylaxis was deemed ineffective (243). Rijnders et al., in turn, reported a significant fall of IA incidence from 14% to 4% in patients receiving liposomal AmB inhalation prophylaxis compared to placebo (224).

Two additional studies reported good efficacy and tolerability of the conventional and lipid complex forms of the drug in SCT recipients (8,95). Recently, an Italian group reported good results with the combination of fluconazole and D-AmB inhalations as prophylaxis in allogeneic SCT recipients (178). ECIL currently gives a provisional B1 recommendation for aerosolized liposomal AmB in combination with fluconazole to neutropenic patients (152). D-AmB inhalations are not recommended.

AIMS OF THE STUDY

The purpose of the present study was to improve the prevention and diagnostics of IFIs in allogeneic SCT recipients and in patients with acute leukaemia in order to improve the prognosis of the patients.

The specific aims were:

- To evaluate the degree of environmental exposure to moulds and colonization of the patient rooms and patients with moulds under normal conditions and during heavy construction activity in the vicinity of the SCT ward.
- To determine whether colonization of the upper airways with *Aspergillus* spp. or the degree of oral colonization by *Candida* spp. predicts IA and IC.
- To study the feasibility of two antigen tests, *Aspergillus* galactomannan and *Candida* mannan, from serum as a diagnostic marker of IA and IC in allogeneic SCT recipients.
- To analyse the effect of fluconazole prophylaxis on the incidence of IC infections and bacteraemias in patients with acute leukaemia.
- To assess the impact of D-AmB inhalation prophylaxis on the incidence of IA and the tolerability of the inhalations in allogeneic SCT recipients.

MATERIALS AND METHODS

Patients

Allogeneic SCT recipients

All adult allogeneic SCT recipients transplanted in HUCH (Helsinki University Central Hospital) between January 1, 1996 and December 31, 2005 were included in the study regarding AmB inhalation prophylaxis (Study V). Patients transplanted between January 1, 2001 and December 31, 2002 were eligible for Studies III and IV unless they received reduced intensity conditioning (RIC) that does not lead to severe neutropenia.

Conditioning regimens. The most common conditioning was cyclophosphamide (CY) and total body irradiation (TBI). CY 60mg/kg was given once daily i.v. on days 1 & 2 and TBI of 12 Grays (Gy) in six fractions on days 3-7 (lungs 10 Gy). For some patients, i.v. busulfan (BU) 3.2 mg/kg daily in divided doses for four days was combined with CY. A third type of conditioning was a combination of treosulfan 10-14 g/m^2 daily on days 1-3 and i.v. fludarabine 30 mg/m^2 daily on days 1-5 (40). Patients with aplastic anaemia were treated with CY 50 mg/kg on four consecutive days. Antithymocyte globulin (ATG) on three consecutive days was added to the CYTBI, BUCY, and CY conditioning regimens in patients receiving their graft from an unrelated donor. ATG was administered with four different dosing regimens during the ten-year period. In addition to the treosulfan-based conditioning of 10-12g/m^2, RIC was given with fludarabine 30mg/m^2 on days 1-3 plus TBI of two Gy or CY 1g/m^2 on days 1-2 plus fludarabine 25mg/m^2 on days 1-5.

GvHD prophylaxis and treatment. Cyclosporine A and methotrexate (MTX) served as GvHD prophylaxis. A short course of MTX was used from June 1997 on. MP was used from day +8 or +14 to day +110 with a maximum dose of 1 mg/kg for all patients until June 1999 and thereafter for patients with sibling donors only (234). In cases of aGvHD, MP was given as the first-line therapy with a minimum starting dose of 2mg/kg and a maximum dose of 10mg/kg daily.

Infection prophylaxis and treatment. All patients were placed in the HEPA-filtered private rooms from the beginning of the conditioning and all received cotrimoxazole (if sibling donor) or ciprofloxacin (if unrelated donor) prophylaxis until engraftment. Acyclovir served as antiviral prophylaxis from day -4 until day +35. During neutropenia, broad-spectrum antimicrobials were administered for fever higher than 38°C. Ceftriaxone and tobramycin served as the first-line therapy. Empirical antifungal therapy with i.v. AmB was initiated if neutropenic fever persisted for five days during therapy with broad-spectrum antimicrobials. As topical therapy to prevent oral yeast infections, the patients used miconazole gel 2,5 ml four times a day for three months. Systemic antifungal prophylaxis was not used routinely. Since the beginning of 2001, D-AmB

inhalations were prescribed as antifungal prophylaxis to all patients treated with high-dose MP (10 mg/kg) for aGvHD. The patients started the prophylaxis at the beginning of the high-dose MP therapy and continued it for two to three months according to the decision of the attending physician. For the inhalation 25 mg of D-AmB for i.v. infusion was dissolved in 5 ml of sterile water. The drug was then inhaled with a nebulizer over 10-15 minutes once a day. The patients took one or two doses of salbutamol (0.1 mg/dose) prior to the AmB inhalation to prevent bronchial obstruction.

Patients with acute leukaemia

All adult patients treated with chemotherapy for acute leukaemia in HUCH 1978-2004 were included (Study II).

Infection prophylaxis and treatment. No systemic antifungal prophylaxis was used in 1978-1999. From the beginning of the year 2000 on, all patients received fluconazole prophylaxis with a dose of 400 mg daily over each period of neutropenia. The prophylaxis was started when the neutrophil count fell below 1.0 x 10^9/l and continued until the count was above 0.2 x 10^9/l and possible mucosal damage was cured or until the start of empirical antifungal therapy. During neutropenia, broad-spectrum antimicrobials and empirical antifungal therapy with i.v. AmB was initiated according to the the same principles as in allogeneic SCT recipients.

Methods

Environmental surveillance

During the time of the study the adult SCT ward of HUCH was situated on the ground floor of the 15-storey hospital complex. The ward had 13 HEPA-filtered single patient rooms (Studies I,III,IV,V).

The continuous environmental surveillance was performed in the ward between May 2000 and October 2002 at one to three week intervals by settled dust analyses using plastic cups that were left at five locations inside the SCT ward (Study III). The locations were two patients rooms, the bathroom of one patient room, the vestibule between the double doors of the entry of one patient room, and the drug dispensary.

Heavy construction work was performed on land immediately adjacent to the SCT ward between October and December 2005. A barrier was built around the construction area and the ventilation intake ducts. A five-step prophylactic environmental surveillance system was designed to prevent an outbreak of IA (Study I). First, the pressure of the ventilation channels was checked daily. Second, particle counts for particles more than 0.3 µm in diameter were measured in all the patient rooms five times a week using a Particle Scan Pro® portable counter (IQ Air, Incen AG, Goldach,

Switzerland). For comparison, the particle count of the outside air at the hospital main entrance was measured on six occasions. Third, air sampling for fungal spores was performed with a single-stage Surface Air Sampler (SAS 100, pbi International, Milan, Italy) (Study I). One thousand litres of air was impacted onto a malt agar plate (Envirocheck Rodac H + S, Merck, Darmstadt, Germany), which was then inoculated for a maximum of 14 days. The air sampling was performed weekly (with the exception of one week) in three patient rooms, at the construction area and at the hospital main entrance. Fourth, surface samples from three patient rooms were obtained once a week using malt agar contact plates.

Colonization of nasal and oral cavities

Swab samples were obtained from both nostrils and the dorsum of the tongue of the patients once a week whenever hospitalized during the first post-transplant year (Studies III and IV) and on three randomly selected dates during the surveillance period (Study I). All samples were cultured using standard techniques for the isolation and speciation of fungi (65).

Monitoring of serum markers

For the analyses of the serum markers 1-2 ml of blood was obtained in a prospective way once a week until 12 weeks after transplantation and thereafter 1-2 times a month (Studies III and IV). The samples were stored at -20ºC. The analyses were performed according to the manufacturer's instructions. Briefly, after heat treatment and centrifugation, the serum samples were placed in the wells of the microtitration plates coated with the monoclonal Ab. Next, the Ab-containing conjugate was added to the wells. After incubation and washing, a chromogen solution was added. After a second incubation period, the reaction was stopped with a acid-containing solution. The ODIs were then read using a plate reader. With the GM ELISA test (Platelia *Aspergillus*, Bio-Rad, Hercules, CA, USA), ODI of ≥ 0.5 was used as the criterion of test positivity (Study III). Concentrations of 0.25-0.5 ng/ml were considered borderline and concentrations above 0.5 ng/ml were deemed positive with the Platelia *Candida* Ag test (Platelia *Candida*, Bio-Rad, Hercules, CA, USA) (Study IV). The first serum samples were obtained prior to the start of the conditioning regimen, and the sampling and follow-up of clinical data was continued until one year after the transplantation, death or relapse.

Definitions

Cases of IA and IC were defined according to the EORTC/MSG criteria (Tables 6 and 7, pages 27-28). Only proven and probable infections were included. The cases of IA or IC occurring after the progression or relapse of the underlying disease after SCT were censored from the final analyses (Studies III,IV, and V). The *Candida* infections were classified according to the definitions by Bodey et al. as candidaemia and disseminated candidiasis (32).

Data collection

Data regarding invasive *Aspergillus* and *Candida* infections was collected from the patient charts for all studies.

The HUCH Diagnosis Registry provided a list of adult patients who had been diagnosed with acute leukaemia between January 1, 1978 and December 31, 2004. The records of the Microbiology Laboratory of HUCH of blood cultures positive for yeasts or bacteria from the same time period were also reviewed (Study II).

In allogeneic SCT recipients data of the underlying disease, disease status, transplant-related factors, duration of neutropenia, presence of aGvHD or chronic GvHD (cGvHD), the number and causative agents of bacteraemias, duration of any antimicrobial, antiviral or antifungal therapy, and use of parenteral nutrition were reviewed (Studies III,IV, and V). Data of the adverse effects of the D-AmB inhalations was collected (Study V). The study end-points were IA or IC by one year after the transplantation, relapse or death (Studies III and IV) and IA or death (Studies I and V).

STATISTICAL ANALYSES

The comparison of categorical variables was made by using Fisher's exact test or chi-square test and by Student's t-test or Mann-Whitney-U test for continuous variables. The difference in numbers of CFU in different locations of environmental sampling was assessed by Kruskal-Wallis test (Study III). Absence or presence of aGvHD or cGvHD, positive blood cultures, use of any antimicrobial, antiviral or antifungal drugs, and use of parenteral nutrition were used as variables to find out correlations with the Ag test results (Study IV). Antimicrobial drugs were analysed by groups (cephalosporins, carbapenems, tetracyclines, aminoglycosides, macrolides) when applicable. All variables were tested with the Mantel-Haenszel analysis and a logistic regression (Study IV). The median Ag concentrations of patient groups were compared with the Mann-Whitney test (Study IV). The cumulative incidence of IA and the OS of the patients were estimated by the Kaplan-Meier method (Study V). The statistical analyses were performed with SPSS, versions 13.0, 16.0, and 17.0 for Windows (SPSS Inc, Chicago, Illinois, USA). *P* values of less than 0.05 were considered statistically significant. All *P* values are two-sided.

RESULTS

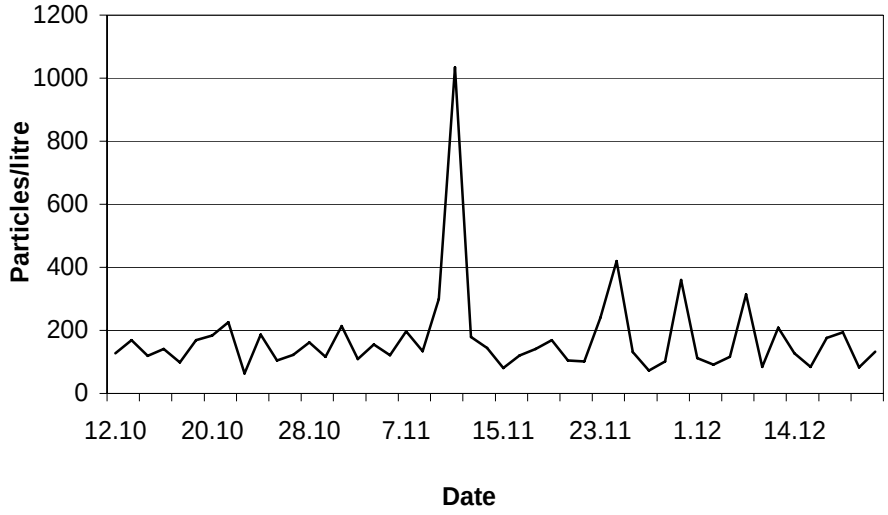
Environmetal surveillance (Studies I and III)

Air filtration, particularly by laminar air flow (LAF) or HEPA filters, has been shown to reduce the level of fungal contamination in the air and the incidence of IA in allogeneic SCT recipients. Construction work inside or adjacent to the hospital can cause IA outbreaks particularly if the ventilation system is faulty or if the protective measures around the construction area are not sufficient. The objectives of the environmental sampling of the present studies were to determine whether continuous environmental surveillance of the SCT ward can predict the risk of IA and prevent an outbreak of IA after construction work in the immediate vicinity of the SCT ward.

Over the 2.5-year surveillance period, 245 settled dust samples were obtained (Study III). Of the 20 positive samples (8.8%), all yielded single pathogens with a median of 1 CFU/m^3 (range 1-7). *A. fumigatus* was the most common pathogen (14 samples). A small cluster of nine positive samples was detected during a five-week period three months after the environmental surveillance was initiated. The remaining 11 positive surveillance samples were detected at random instances throughout the surveillance period. No statistically significant differences were detected in the proportion of the positive samples or the median numbers of CFUs in the five locations examined.

During the construction period (Study I), the pressure in the ventilation channels remained stable. The median particle count of the patient rooms was between 63 and 420 particles/l except one peak of 1034 particles/l (Fig. 2). This peak was suspected to have been caused by heavy drilling during in-hospital renovation work four floors above the SCT ward. The particle counts of the outside air at the hospital main entrance ranged from 110 806 to 292 624 particles/l (mean 173 659 particles/l). Of the 33 the patient room air samples, 31 yielded negative results. The two positive samples yielded non-pathogenic environmental fungi, but the other sample was also positive for *A. niger* with 1 CFU/m^3. In contrast, all samples from the construction area and the hospital main entrance yielded positive results, with 2-21 (median 9) and 1-31 (median 7) CFU/m^3, respectively The most common spp. were *Penicillium* (13 samples), *Rhizopus* (5 samples), and *A. fumigatus* (4 samples). Of the 33 patient room surface samples, 23 yielded negative results and seven yielded non-pathogenic, environmental fungi. Of the three *Aspergillus*-positive samples, *A.fumigatus* was detected in two consecutive samples from one patient room (1 CFU/m^3 each) during the second and the third week of the surveillance period. The samples from this room were thereafter negative. The third sample yielded *A. versicolor* in another patient room.

Figure 2. Particle counts in the 13 patient rooms, median count of each day.



Colonization

Colonization of the airways or the gastrointestinal tract with fungi are known risk factors for IFIs. The present studies assessed whether fungal cultures from the nasal and oral cavities of the patients can predict IA or IC.

Nasal colonization with Aspergillus spp. (Studies I and III)

Swab samples from nasal cavities of the 102 patients were obtained 657 times (median number six samples per patient, range 1-23) (Study III). *Aspergillus* spp. were detected in seven nasal samples from three patients. *A. fumigatus* was detected in two patients. Neither of these patients was diagnosed with IA. The remaining four samples with *A. niger* were obtained during the last two weeks of life from a patient with probable IA. In addition to these *Aspergillus*-positive samples, five other patients had nasal colonization with other fungi.

During the construction period, swab samples were obtained from 24 patients (Study I). All the 70 nose samples yielded negative results.

Oral colonization with Candida spp. (Study IV)

Of the 657 oral samples, a total of 91 (13.8%) samples in 38 (37.2%) patients yielded *Candida* spp. and *A. fumigatus* was detected in one sample (Study IV). *C. albicans* was the most common spp. found in the oral samples. It was detected in 82 samples (89.1% of the positive samples), *C.*

glabrata in seven (7.6%), and *C. parapsilosis* in two (2.2%) samples, respectively. The number of positive samples per patient ranged from one to 13. More than one *Candida* spp. was detected in seven patients over the time of the follow-up. The first sample, obtained at a median of one day after arriving to the ward, yielded positive results in 12 patients. In seven out of 39 (18%) patients, the oral colonization resolved without systemic antifungal therapy. Of the 63 patients whose oral samples remained negative throughout the time of follow-up, 20 (32%) never received any systemic antifungals.

Invasive *Aspergillus* and *Candida* infections (Studies I, III, and IV)

During the construction period, 55 patients were treated on the ward (Study I). Allogeneic SCT was performed on 15 patients, autologous SCT on seven patients, and 11 patients were treated with high-dose MP for aGvHD. With a follow up time of 2-247 (median 214) days from the beginning of the construction work, no new cases of IA were detected.

During the study period of 2001-2002, 138 patients received allogeneic SCT (Studies III and IV). A total of 36 patients were excluded due to RIC or patient refusal; thus, 102 patients were included. The median age of the patients was 44 years (range 18-60) Three patients were re-transplanted due to graft rejection. The diagnoses were AML (30 patients), CML (25), ALL (20), MDS (11), CLL (5), MF (4), MM (2), NHL (2), SAA (2), and Diamond-Blackfan anaemia (1). Table 9 shows the transplant-related factors in these patients.

Two patients (2%) had IA and one (1%) had IC. A 56 year-old female patient with AML had aGvHD on day +23 after SCT from a human leukocyte antigen (HLA)-identical sibling donor. Chest radiograph abnormalities were detected on day +100. IPA was confirmed by a fine needle biopsy of the lung tissue on day +109 (*A. fumigatus).* The last two of the 19 serum samples obtained from this patient yielded positive results with ODI's of 4.5 (day +106) and 3.2 (day+113). All nasal and oral samples obtained from this patient yielded negative results. The patient had been on D-AmB inhalation prophylaxis for 76 days at the time of IPA diagnosis. The inhalations were replaced by i.v. liposomal AmB. The patient died 23 days after the diagnosis of IA.

Another patient had probable IA. This patient with CML in the second chronic phase and mismatched unrelated donor was transplanted twice with a 97-day interval due to graft rejection. On day +152 after the first transplantation, the nasal swabs yielded *A. niger*. HRCT revealed changes indicative of IPA and *A. niger* was cultured from BAL fluid on day +154. All 22 serum samples yielded negative results for GM, including the last sample from day +159. The patient succumbed without engraftment on day +162 after the first transplantation despite therapy with i.v. liposomal AmB. Post mortem examination was not allowed.

Table 9. The main transplant-related characteristics of the patients in Studies III and IV.

Characteristics		Total patients n = 102
Conditioning [1]		
CYTBI		93
BUCY		4
Treosulfan-fludarabine		4
CY		2
Other		2
Donor type		
HLA-identical sibling		60
unrelated		41
syngenic		1
Graft source [1]		
Bone marrow		52
Peripheral blood		47
Both		3
Duration of neutropenia, days, med. (range)[2]		16 (0-137)
aGvHD	grade I-IV	36
	grade II-IV	15
cGvHD	limited	21
	extensive	11
OS 1 year		75

1) Three patients received a second transplantation from the same donor due to rejection
2) Absolute neutrophil count ≤ 0.5. x 10^9/l

Abbreviations: CY; cyclophosphamide, TBI; total body irradiation, BU; busulfan, HLA; human leukocyte antigen, aGvHD; acute graft-versus-host disease; cGvHD; chronic graft-versus-host disease, OS; overall survival

IC occurred in a 46-year-old male patient with ALL in 3rd remission. He received two transplantations with a 34-day interval because of engraftment failure. The patient had severe complications after the transplantations. These complications included polyomavirus-related cystitis leading to severe haematuria, renal insufficiency requiring haemodialysis, aspiration pneumonia, and convulsions. Empirical D-AmB 1 mg/kg was administered from day +14 to day +33 and fluconazole 400 mg daily from day +34 to day +61. On day +61 the patient went into septic shock caused by fluconazole-sensitive *C. albicans* which was treated with liposomal AmB. Eight days later the blood cultures still remained positive for *C. albicans*. The patient died of multiorgan failure on day +75. Autopsy confirmed the diagnosis of disseminated candidiasis. Two oral samples obtained on days -9 and -2 yielded *C. albicans*. The third positive sample, from day +40, yielded *C. glabrata*. Of the nine serum samples from this patient, six samples yielded positive, two negative, and one sample a borderline result. The first positive sample was obtained 49 days before first the blood culture showing *C. albicans*. Of the remaining six serum samples thereafter, five yielded positive and one borderline positive results.

Antigen test results (Studies III and IV)

Diagnosis of IFI is challenging because of the difficulty in obtaining appropriate histological samples. Blood cultures often yield negative results. Early diagnosis and start of antifungal therapy improve the prognosis of patients with IFIs. Different serological tests have been developed over the years for the early diagnostics of IFI. The objectives of the current studies were to determine whether cases of IA and IC can be detected early by galactomannan and mannan Ag screening in allogeneic SCT recipients.

A total of 2071 blood samples were obtained for the antigen test analyses. The median number of samples was 22 per patient (range 4-38).

GM ELISA

With the GM ELISA test, 12 samples (0.6%), obtained from nine patients yielded positive results (Study III). The test was positive in two consecutive samples only in the patient with proven IA. The remaining ten positive samples were obtained from eight patients without IA. Of the 2059 negative GM ELISA samples, 22 (1.1%) were obtained from the patient with a probable IA. In the per-patient analysis with two cases of IA, the sensitivity, specificity, PPV, and NPV of the GM ELISA test were 50%, 92%, 11%, and 99%, respectively.

Candida Ag

With the *Candida* Ag test, 98 (4.7%) samples obtained from 55 patients yielded positive and 78 (3.8%) samples from 56 patients borderline positive results (Study IV). All samples yielded negative results in 26 patients. The median number of positive samples was one per patient (range 1-6). Two consecutive samples yielded positive results in five patients. Three consecutive positive samples were only seen in the patient with IC. In this patient, six samples yielded positive, one a borderline result, and two samples negative results. Thus, 92 of the 98 positive and 77 of the 78 borderline test results were considered false results. The median Ag concentrations were significantly higher in the true positive samples than in the false positive samples, 1,60 ng/ml (range 0.96-2.15 ng/ml) vs. 0.62 ng/ml (range 0.52-2.8 ng/ml), respectively ($P < 0.001$).

In the multivariate analysis, two factors correlated with the false positive and borderline positive *Candida* Ag test results. These factors were the use of valacyclovir (Mantel-Haenszel analysis**,** $P = 0.0347$) and the use of acyclovir (logistic regression model, OR 1.676; 95% CI 1.2066-2.328, $P = 0.0021$) at the time of serum sampling. Acyclovir served as antiviral prophylaxis for the first 35 days after the transplantation in all patients, but 23 patients also received this drug later during the first year. Valacyclovir was given to 50 (49%) patients. After day +35, either or both of these antivirals were given in 84 episodes. The indication for antiviral therapy was mucositis in 26 (30.9%), herpes

simplex infection of the skin or genital area in 23 (27.4%), herpes zoster in 18 (21.4%), antiviral prophylaxis in 14 (16.7%), and encephalitis in three (3.6%) of the episodes.

The proportion of false positive or borderline positive samples before or after transplantation were analysed by dividing the serum samples into four groups: samples obtained before transplantation (105 samples, Group I, baseline situation), within the first month (349 samples, Group II), within 31-100 days (788 samples, Group III), and within 101-365 days (820 samples, Group IV) after the transplantation. As Table 10 shows, the proportion of false results did not change.

Table 10. Timing of false positive or borderline positive *Candida* antigen test results in relation to stem cell transplantation.

Timing of samples	**No. of samples***	**No. of positive & borderline positive samples (%)**	**No. of negative samples (%)**	**Positive & borderline vs. negative results; *P***
Before SCT (I)	105	13 (12.4)	92 (87.6)	
0-30 days after SCT (II)	349	37 (10.6)	312 (89.4)	I vs. II; 0.610
31-100 days after SCT (III)	788	58 (7.3)	730 (92.7)	I vs. III; 0.065
101-365 days after SCT (IV)	820	61 (7.4)	759 (92.6)	I vs. IV; 0.088

* 9 samples taken from the patient with IC excluded.

Abbreviation: SCT; stem cell transplantation

Antifungal prophylaxis

Colonization of the gut with *Candida* spp. and of the airways with *Aspergillus* spp. during chemotherapy-induced neutropenia and during the early months after allogeneic SCT, especially if GvHD is present, enhance the risk of IFIs. In such periods, the patients may therefore benefit from systemic antifungal prophylaxis. In the present study fluconazole prophylaxis was investigated in patients with acute leukaemia and AmB inhalation prophylaxis in allogeneic SCT recipients with aGvHD.

Fluconazole prophylaxis in patients with acute leukaemia (Study II)

During the period of 1978-2004, 1089 adult patients received chemotherapy for acute leukaemia at HUCH; 847 in 1978-1999 (Period 1, no fluconazole prophylaxis) and 242 between the years 2000 and 2004 (Period 2, fluconazole prophylaxis). The median age of the patients was 53 years (range 16-88 years); 795 (73%) of the patients had AML and 294 (27%) had ALL.

In Periods 1 and 2, IC was detected in 74 (8.7%) and 4 (1.6%) of the patients, respectively ($P < 0.001$) (Table 11). The difference was mainly based on the reduction in the incidence of disseminated candidiasis, which was detected in 53 (6.2%) and 1 (0.4%) of the patients, respectively ($P = 0.001$). Changes in the intensity of the chemotherapy courses during the 27-year period may have influenced the results; thus, the results of the patients treated with non-intensive chemotherapy in the late 1970's and early 1980's were eliminated from the analysis. The results of the remaining 440 AML patients treated in 1984-1999 and of the 149 patients with ALL treated in 1987-1999 (Period 1b) were compared with the results of Period 2. The intensity of the chemotherapy courses in these two periods was very similar. In Period 1b, 16 patients (2.7%) had candidaemia and 44 (7.5%) had disseminated candidiasis. The difference in the incidence of candidiasis between Period 1b and 2 reached statistical significance ($P < 0.001$), whereas the incidence of candidaemias did not ($P = 0.305$).

The proportion of patients with at least one bacteraemia was higher in Period 2 than in Period 1 or 1b ($P < 0.001$ and $P = 0.005$). The numbers and types of bacteraemias are listed in Table 12**.**

Table 11. The numbers on invasive *Candida* infections in patients with acute leukaemia.

	Period 1 1978-1999 no prophylaxis n = 847	***P***	**Period 2 2000-2004 fluconazole prophylaxis n = 242**	***P***	**Period 1b 1984-1999(AML) 1987-1999(ALL) no prophylaxis n=589**
Candidaemia	**21** (2.5%)	0.325	**3** (1.2%)	0.305	**16** (2.7%)
- *C.albicans*	7 (0.8%)		1 (0.4%)		
- non-*albicans* spp.	9 (1.1%)[1)]		2 (0.8%)[2)]		
- unspes. yeast	5 (0.6%)		0		
Disseminated candidiasis	**53** (6.2%)	0.001	**1** (0.4%)	<0.001	**44** (7.5%)
Total	**74** (8.7%)	< 0.001	**4** (1.6%)	<0.001	**60** (10.2%)

[1)] non-*albicans* spp. = C*. krusei* (5), C. *tropicalis* (3) and C. *glabrata* (1).

[2)] non-*albicans* spp. = C*. krusei* (2)

Table 12. Bacteraemias in patients with acute leukaemia.

	Period 1 1978-1999 no prophylaxis n = 847	***P***	**Period 2 2000-2004 fluconazole prophylaxis n = 242**	***P***	**Period 1b 1984-1999(AML) 1987-1999(ALL) no prophylaxis n=589**
Number of patients with ≥ 1 bacteraemia	440 (52%)	<0.001	157 (65%)	0.005	319 (54%)
Number of bacteraemias	990		272		842
- gram-positive	494 (49.9%)	0.05	154 (56.6%)	0.026	411 (48.8%)
- gram-negative	367 (37.1%)	0.007	77 (28.3%)	0.008	313 (37.2%)
- mixed	129 (13%)	0.382	41 (15.1%)	0.664	118 (14%)

AmB inhalation prophylaxis in allogeneic stem cell transplant recipients (Study V)

Over the study period, 611 patients received allogeneic SCT; 257 patients in 1996-2000 (Period I, no inhalation prophylaxis) and 354 patients in 2001-2005 (Period II, inhalation prophylaxis). Double allogeneic transplantation was performed on 11 patients; the total number of transplantations was thus 622.

There were some differences in the baseline characteristics of the patients in Period I vs. Period II; the median age; 44 years vs. 47 years ($P = 0.005$), the proportion of patients with CML (29.2% vs. 13.8%, $P < 0.001$), with MM (8.9% vs. 15.5%, $P = 0.019$) or with advanced disease (45.5% vs. 59.9%, $P < 0.001$), respectively. A larger proportion of transplantations were performed with

peripheral blood stem cell graft and RIC in Period II than in Period I, 61.8% vs. 24% and 23.4% vs. 11%, respectively ($P < 0.001$ for both).

The incidence of aGvHD and cGvHD was similar in the two periods. Grade 2-4 aGvHD and extensive cGvHD were detected more often in Period I than in Period II, 21% vs.15.8% (P = 0.009) and 25.3% vs.19.8% (P = 0.006), respectively. Acute GvHD occurred on day +34 in Period I vs. day +26 in Period II (P = 0.014).

The OS was 42.4% and 59% ($P = 0.001$) with a median follow-up of 3.5 years (range 4 days – 13 years) and 4.6 years (range 10 days – 9.5 years) in Period I and Period II, respectively.

IA was detected in 17 (6.6%) vs. 9 (2.5%) of the patients in Period I and Period II (P = 0.007, logrank test). Table 13 summarizes the data of the observed cases of IA.

Empirical fluconazole and i.v. AmB were administered to 163 (63%) vs. 164 (46.3%) ($P < 0.001$), and 49 (19%) vs. 43 (12%) ($P = 0.018$) of the patients in Period I and Period II, respectively. In addition to these drugs, four patients in both periods received other antifungals. These antifungals included caspofungin (three patients), itraconazole (three patients), posaconazole (one patient), and flucytosine (one patient).

During Period II, 111 patients with aGvHD used the AmB inhalation prophylaxis for a median time of 84 days (range 1-297 days). None of these patients discontinued the prophylaxis due to adverse effects. Breakthrough IA occurred during the prophylaxis in only one patient (1%). IA was detected in five additional patients at a median of 148 days (range 56-987 days) after finishing the prophylactic inhalations.

Table 13. Cases of invasive aspergillosis.

	Period I 1996-2000 n = 257 (%)	**Period II 2001-2005 n = 354 (%)**	***P***
IA	17 (6.6)	9 (2.5)	0.007
proven	13	6	
probable	4	3	
Timing of IA			
≤40 days after SCT	4 (23.5)	1 (11)	0.628
>40 days after SCT	13 (76.5)	8 (89)	
Median time from SCT to IA, days (range)	95 (16 days-5.5 years)	155 (21 days-2.9 years)	0.225
Localisation of IA			
pulmonary	10 (59)	7 (78)	0.418
disseminated	7 (41)	2 (22)	
Antifungal therapy for IA			
AmB	13	2	
caspofungin	2	3	
itraconazole	2	-	
flucytosine (combined to others)	2	-	
voriconazole	-	5	
Survived after IA	1 (5.9)	3 (33)	0.104
Median survival from diagnosis of IA, days (range)	69 (0 days-8.6 years)	53 (8 days-5.6 years)	0.048

Abbreviations: IA; invasive aspergillosis, SCT; stem cell transplantation, AmB; amphotericin B

DISCUSSION

The series of studies in the present thesis had their focus on the prevention and early diagnostics of IFIs in high-risk haematological patients. Prevention and early diagnostics are the key issues in reducing the significant mortality of patients with IFIs. Colonization of the respiratory tract with *Aspergillus* spores from the air is a risk factor for IA. With *Candida* infections, the colonization occurs via the gastrointestinal tract, particularly in neutropenic patients with mucositis and therapy with broad-spectrum antimicrobial agents. Colonization can never be totally avoided and high-risk patients may therefore benefit from antifungal prophylaxis. Making the diagnosis of IFI and starting the antifungal therapy early correlate with the outcome of the patient. Serological tests from serum samples may help with earlier diagnosis.

Environmental surveillance of the stem cell transplantation ward

During the 2.5-year environmental surveillance period of the SCT ward, the settled dust analyses yielded positive results in 8.2% of the samples with a constantly low amount of fungi (median 1 CFU/m^3) and no seasonal variation indicating that the HEPA filtration system was working well (Study III). *Aspergillus* spores were detected in 15 (6%) of the samples.

Continuous environmental surveillance could detect disturbances in the air filtration system. The role of continuous environmental sampling as a tool for assessing the risk of IA, however, is not well established. In the study by Falvey et al. the ten-year monthly air sampling revealed two bursts of *Aspergillus* conidia (66). In the BMT ward, one third of the samples yielded *Aspergillus* spp. with a mean of 1 CFU/m^3. The authors concluded that routine air sampling does not predict or prevent nosocomial infections. Two other studies reported the results of extensive environmental surveillance (67,136). In these studies, a great majority of air or surface samples of HEPA-filtered patient rooms yielded negative results for *Aspergillus* spp. indicating good infection control measures. No prediction of possible outbreaks could be made. Rupp et al. reported that 16.7% of air samples from the SCT ward corridor were positive for *Aspergillus* spp. during a seven-year period (233). No correlation was detected between air samples with *Aspergillus* growth and cases of IA. Overall, continuous environmental sampling may not be helpful in predicting the risk of IA.

Large amounts of *Aspergillus* spores are released into the air during construction or renovation activity. Such activity near or inside the SCT ward can cause outbreaks of IA in SCT recipients through contaminated HEPA filters, ventilation ducts, staircases or even vacuum cleaners (12,131,132,239). The environmental surveillance of the present study during heavy construction work did not show increased amounts of fungi in the air or colonization of the patient rooms or patients (Study I). The protective measures were thus effective in preventing an outbreak of IA.

With the increased interest in of the role of environmental exposure to moulds, several prospective studies have been conducted during construction activities. In the study by Overberger et al. the protective measures kept the spore counts of the BMT area under 3 CFU/m^3 vs. 355 CFU/m^3 of the construction area during an in-hospital renovation (197). Krüger at al. took 1043 samples with gravity air setting plates during a six-month period. Of the samples from the HEPA-filtered rooms, 8% yielded positive results with low CFUs vs. 39% of the samples from the ward corridor. The incidence of IA in allogeneic SCT recipients did not change (129). Another German study described well-planned protective measures during the demolition of an old hospital building (91). The particle and spore counts rose significantly in the hospital area. The incidence of IA, however, did not rise in the oncology or BMT patients. Similarly to the present study, all of the three aforementioned studies reported extensive protective measures with good results and no aspergillosis outbreaks. Environmental surveillance during periods of construction or renovation seems feasible.

No recommendations exist of any preferred technique when performing environmental surveillance. The combination of techniques used during the high-risk period of construction in the present study was designed to cover the different phases of *Aspergillus* aerobiology. Particle measurements are a quantitative method and air sampling a qualitative technique to detect airborne particles. Surface sampling can detect particles that have already landed and are no longer airborne. Colonization of the upper airways of the patients could be the first sign of an outbreak.

Particle measurements are mostly used in occupational settings. This technique is not a standardized method in the setting of infection control. However, we found particle measurements easy to perform, quick and cheap, and feel that they are a useful quantitative part of environmental surveillance.

In the present study preparations were made early at the beginning of the construction period for procedures in case signs of fungal contamination occurred. Table 14 shows these procedures.

Table 14. Procedures in case of signs of fungal contamination.

Observation	Procedures
Elevated air pressure of ventilation channels	Change the HEPA filters
High particle counts in patient rooms	Check the HEPA filters Search for other sources; window frames etc.
Air or surface samples repeatedly positive for moulds	Remove the patients from the ward Cleaning of the ward
Nasal/oral samples positive for moulds = colonization	Alarming; pre-emptive therapy
New cases of IA	Failure of system; antifungal therapy

Nasal colonization with *Aspergillus* species

In the present study colonization of the nasal cavities of the patients by *Aspergillus* spp. was rare (Study III). Only three patients (3%) had positive nasal swabs. Two patients, colonized with *A. fumigatus*, did not have IA. The third colonized patient who had *A. niger* in her nasal cavity, developed signs of a probable IA two days after the first positive samples were obtained. The use of AmB inhalation prophylaxis (39 patients) may have affected the low incidence of nasal colonization.

Aisner et al. reported that 8.8% of the neutropenic patients had nasal colonization with *Aspergillus* spp. compared with 3% in the present study (5). Colonization was more common in the study by Richardson et al., where *A. fumigatus* was detected in 24% the of patients (222). A correlation between colonization and the risk of IA was not observed. That study, however, included various types of haematological patients, who were not all treated in HEPA-filtered rooms.

Colonization of the airways is a risk factor for IA. Regarding samples that measure colonization, the sensitivity of sputum and BAL samples is 30% and 30-64%, respectively (68,140,218). Nasal samples are easier to obtain than sputum or BAL fluid. Very few studies have, however, assessed how well nasal colonization predicts IA in haematological patients. Aisner et al. reported that nasal colonization by *A. flavus* is a clear risk factor for IA and colonization by *A. fumigatus* a possible risk factor (5). Newman et al. also found that nasal colonization by *A. flavus* often led to IA (185). Nucci et al. conducted a prospective study in neutropenic patients with haematological malignancies (190). Nasal swabs yielded *Aspergillus* spp. in 18% of the treatment episodes. The overall PPV and NPV of the nasal cultures were 6.4% and 100%, respectively. The PPV was slightly better, 20%, if neutropenia lasted more than six days.

Oral colonization with *Candida* species

Oral colonization with *Candida* occurred in 37% of the patients of the present study (Study III). In some previous studies oral colonization has been detected in 23-44% of patients during systemic antifungal prophylaxis (62,156). Lack of routine fluconazole prophylaxis in the present study did not enhance oral colonization. Colonization by non-albicans spp. was rare, since *C. glabrata* was present only in 7.6% of the positive oral samples and *C. krusei* was absent. The proportion of these two *Candida* spp. was lower than in a study by Marr et al., where *C. glabrata* and *C. krusei* were detected in over 50% of the patients during fluconazole prophylaxis (156).

The risk of IC after colonization varies between different *Candida* spp. IC occurs in less than a third of patients colonized with *C. albicans*, whereas *C. tropicalis* colonization leads to IC in 80-100% of the cases (162,210,238). The fact that all oral samples remained negative in almost two thirds of the patients in the current study and colonization by non-albicans spp. was rare agrees with the observed low incidence of IC.

Candida spp. are a part of the normal flora of the mucous membranes of the human body. In fact, 30% of the population are colonized with *Candida* at any given moment (270). IC occurs when the host defensive mechanisms fail to control the yeasts. In immunocompromised hosts, the degree of colonization can be assessed by samples from the nasopharynx, urine, anal region, stool or vagina. Previous studies indicate that repeated colonization of a single body site or simultaneous colonization of several sites with *Candida* spp. enhance the risk of IC (162,163,263). Martino et al. stated that the combination of cultures from the oropharynx and rectal swabs or stool samples are adequate to evaluate colonization (163). The current study provides some information about the degree of oral *Candida* colonization only. The oral samples were taken during periods of hospitalization, not throughout the first year. Oral samples alone, however, may be enough to asses the risk of IC. The study by Marr et al. in allogeneic SCT recipients used only mouthwashings to assess *Candida* colonization within 75 days after transplantation. Patients with oral colonization had a three-fold risk of IC (156). Also, in the study by Zollner-Schwetz et al. oral *Candida* colonization correlated with intestinal colonization in SCT recipients (297).

Invasive *Aspergillus* and *Candida* infections

The incidence of IA in the present study was 2% (Study III). The observed incidence is much lower than in a previous study from our centre which included patients transplanted in 1989-1995 (107). In that study, 11% of the patients had IA. Other previous studies reported a rise in the incidence of IA from 5-6% in the 1980's to 10-12% in the 1990's (158,278). The finding of the present study is therefore somewhat surprising, although some other recent studies have reported that the

incidence of IA may be falling (179,199). This may be connected to the general development in the field of SCT, such as more accurate HLA tissue typing techniques, increased use of peripheral blood grafts, and wider options in therapy against GvHD. The fact that 38% of the patients in the present study received AmB inhalation prophylaxis may also have played a role in the low incidence of IA.

Despite the fact that fluconazole prophylaxis was not routinely used, IC was detected only in one patient (1%) (Study IV). Based on randomised, placebo-controlled studies from the 1990's, fluconazole prophylaxis is widely recommended for allogeneic SCT recipients (79,256). The finding of a low incidence of IC in the present study may be a refletion of an overall low incidence of *Candida* infections in Finland, as indicated by national epidemiological data of *Candida* bloodstream infections (213). The present finding emphasizes the fact that local epidemiology should always be considered before applying prophylaxis recommendations from general guidelines into clinical practise.

Antigen tests

Galactomannan ELISA

The GM ELISA test was positive in one of the two patients with IA in the present study (Study III). The positive GM ELISA result did not precede other clinical signs of infection in this patient. False positive GM ELISA test results were detected in 7.8% of the patients and in 0.5% of the samples. The false positive results were not connected to the use piperacillin-tazobactam, amoxicillin or amoxicillin-clavulanate which are the best known causes for false positive results. Other studies have reported false positive results in 18-19% of patients (19,260).

It is recommended that the GM ELISA test should be performed at least twice a week. In the current study, the test was performed once a week for the first 12 weeks and thereafter 1-2 times a month until one year. This may have affected the performance of the test. The present objective was, however, to target the testing more on the most typical time point of IA which is 3-4 months after the transplantation (164,250). In a Japanese study, the median time of IA was even later, 204 days after transplantation (19). The timing of IA creates challenges for the use of serological methods.

Studies regarding the performance of the GM ELISA test in patients with haematological malignancies have, at best, reported sensitivity, specificity, PPV, and NPV of 94-96%, 98%, 94-98%, and 98%, respectively (147,148). The test can yield positive results several days, even weeks before any other signs of IA are detected (147,148,160). These studies have used different cut-off levels and sampling frequencies, which makes it difficult to compare the results. A meta-analysis that included 18 studies of patients with haematological malignancies and six studies with

BMT recipients only, reported an overall test sensitivity and specificity of 65% in BMT recipients (211). The prevalence of IA also affected the test performance; with a prevalence of 5%, the PPV of the GM ELISA test was 31% and the NPV was 98% (211). The performance status of the GM ELISA test was undoubtedly affected by the low incidence of IA in the present study.

Candida mannan

In the present study, false positive Ag test results were detected in 53% of the patients and in 4.4 % of the serum samples with a cut-off level of 0.5 ng/ml (Study IV). A single positive result, therefore, does not seem to predict IC. In the only patient with IC, however, the *Candida* Ag test yielded a positive result 49 days prior to candidaemia.

The use of acyclovir and valacyclovir correlated with false positive results. This could be connected to mucositis caused by the conditioning, herpes virus infections or GvHD. Mucositis could have caused leakage of mannan into the bloodstream. The fact that the proportion of false positive or borderline positive results did not change during the different time points after the transplantation compared with the pre-transplantation results makes mucositis an unlikely explanation. Also, mucositis was the indication for the use of acyclovir and/or valacyclovir in less than one third of the treatment episodes.

Some previous studies have been focused on the *Candida* Ag test in patients with haematological malignancies. Rimek et al. investigated 469 serum samples obtained from 62 neutropenic patients (226). The sensitivity and specificity of the Ag test was 67% and 49%, respectively. The test was positive in all three patients with a proven IC. SCT was performed on 34 patients, although the authors did not report the type of SCT. The study by Ellis et al. included 86 patients with haematological malignancies (61). The Ag test had a sensitivity of 82% and a specificity of 68% at day 10 of fever of unknown origin. In a retrospective study of 53 patients by Prella et al., the Ag test yielded sensitivity, specificity, PPV, and NPV of 29-31%, 92-96%, 80-89%, and 53-57%, respectively, depending on the cut-off level (215). False positive Ag test results were detected in only 4% of the patients compared with 53% in the present study.

Due to the highly immunogenic qualities of mannan, some investigators have recommended the combining of the Ag test with the Ab ELISA test in order to improve sensitivity (245,246). In patients with a haematological malignancy, however, the Ab test tends to yield positive results later than the Ag test which has yielded positive results several days before the blood cultures in some patients with candidaemia (61,215,225,247,293). Also, the study by Arendrup et al. reported that the Ab levels were higher in non-haematological vs. haematological patients, which may reflect the immune status of different patient groups (16). Due to the slow immune reconstitution in allogeneic SCT recipients it was chosen to use only the Ag test in the present study (143).

In the present study serum sampling was done once a week. Since mannan is removed from the bloodstream within less than 12 hours, the serum samples have been obtained once or twice a week or even daily in other studies (61,114,215,226,272). Even with the once-a-week sampling false positive or borderline positive results were recorded in three quarters of the patients in the present study indicating that single postivie results occur often.

The false positive *Candida* Ag test results of the present study may have, in fact, been true positive results connected to yeast infections that were not detected, since these infections were either cured by antifungal therapy or the patients died. The median durations of fluconazole and i.v. AmB therapies were six and five days, respectively. It seems unlikely for an IC to be cured with such short term therapy. A Post mortem examination was performed on ten of the 27 patients who died, and no new cases of IFI were detected.

Antifungal prophylaxis

Fluconazole prophylaxis in patients with acute leukaemia

In the present study fluconazole prophylaxis reduced the incidence of disseminated candidiasis in patients with acute leukaemia significantly but had no effect on candidaemias (Study II). The incidence of candidaemia, however, was quite low even without fluconazole (2.5%). The fall in the incidence of IC might also be connected to other factors such as chemotherapy intensity, duration of neutropenia, and severity of mucositis. Due to the retrospective nature of the current study, the role of these factors could not be assessed.

Disseminated candidiasis virtually disappeared during fluconazole prophylaxis. In a previous study from HUCH, HCS was detected in 6.8% of adult leukaemia patients treated in 1980-1993 (15). This incidence is similar to that reported by Chen et al (42). The post-mortem-based study by van Burik et al. evaluated the incidence of HSC in SCT recipients (268). Fluconazole prophylaxis reduced the incidence of HSC from 14% to 1.2%. Sallah et al. analysed the incidence and risk factors of HSC in patients with acute leukaemia (236). HSC was detected in 5.4% of the patients. Younger age, duration of neutropenia for more than 15 days, and use of fluoroquinolone prophylaxis were recognised as risk factors. The authors stated that patients with these risk factors should be put on antifungal prophylaxis.

The role of fluconazole prophylaxis in patient groups other than allogeneic SCT recipients is controversial. In the randomised study by Winston et al., fluconazole did not reduce the incidence of IFIs or mortality in a cohort of 256 patients with acute leukaemia (288). In a Canadian study, however, the incidence of proven and probable IFIs fell from 24% to 6% with fluconazole prophylaxis in a cohort of 274 patients (231). IFI-related mortality also fell with fluconazole in that

study. It should be noted, however, that oesophagitis and urinary tract infections were classified as proven IC and 37.2% of the patients received autologous SCT. Autologous SCT is generally not considered to cause a high risk for IFI. The third randomised, placebo-controlled study of Laverdiere et al. also reported a significant decline in the incidence of IFIs with fluconazole prophylaxis (135). Again, a large proportion of patients (44%) were autologous SCT recipients. Currently, ECIL does not recommend fluconazole prophylaxis for patients with acute leukaemia, since evidence for its use is considered insufficient, whereas posaconazole is recommended (152).

Fluconazole prophylaxis may lead to more infections caused by non-*albicans* spp. This phenomenon was not observed in the present study (Study II). The overall incidence of IC during prophylaxis was only 1.6%. In three randomised studies of fluconazole vs. placebo with nearly 600 leukaemia patients, infections caused by *C. krusei* or *C. glabrata* did not rise with fluconazole prophylaxis (121,231,288). In a retrospective study of 465 haematological patients, however, Wingard et al. reported an incidence of 8.3% vs. 1.2% of *C. krusei* and 2.4% vs. 0.9% of *C. glabrata* infections in patients receiving fluconazole vs. patients with other types of prophylaxis, respectively (286). This phenomenon was not detected in two other retrospective studies in cancer patients or haematological patients (98,130). Interestingly, five of the 21 cases (23.8%) of candidaemia in Period 1 of the present study were caused *C. krusei* and one (4.7%) by *C. glabrata*. These yeasts were thus more common in the pre-fluconazole era in the current study than in some previous studies (6,60).

In the present study the proportion of patients with bacteraemias and the proportion of gram-positive bacteraemias rose during fluconazole prophylaxis. Kern et al. also reported that the leukaemia patients with fluconazole prophylaxis had more gram-positive bacteraemias than those without the prophylaxis; 33% vs. 13%, respectively (121). Schaffner et al., in turn, showed a trend towards more gram-negative bacteraemias in patients with fluconazole prophylaxis in a cohort of 154 patients (241). Viscoli et al. reviewed four trials with more than 3000 patients with various types of antifungal prophylaxis (276). Absorbable antifungals, i.e. azoles, enhanced the risk of bacteraemias. Similar findings concerning bacteraemias were also reported with itraconazole prophylaxis by Menichetti et al. (168). Why triazole prophylaxis would cause more bacteraemias remains unclear. In a study with ketoconazole prophylaxis it was speculated that drug interactions between the antifungal drug and the chemotherapeutic agents might enhance the cytotoxic effect of chemotherapy (201). In the present study, drug interactions are an unlikely explanation, since fluconazole prophylaxis was started after the chemotherapy, at the onset of neutropenia. Again, the intensity of chemotherapy may play a role in the incidence of bacteraemias. In the subanalysis of patient groups with similar chemotherapy intensity, however, the difference in the proportion of bacteraemias was still evident.

Amphotericin B inhalation prophylaxis in allogeneic stem cell transplant recipients

In the present study, the incidence of IA in allogeneic SCT recipients fell from 6.6% to 2.5% after the initiation of D-AmB inhalation prophylaxis (Study V). The D-AmB inhalations were well tolerated. One breakthrough IA was detected. The retrospective nature of the present study creates some limitations. The role of general advances in the field of SCT during the ten-year period such as more accurate HLA-tissue typing techniques, more options in the therapy of GvHD, and better supportive care must have played a role in the incidence of IA and the better OS in Period II. In adult patients transplanted in 1989-1995 in HUCH IA occurred in 11% of the patients (107). The incidence of IA started to fall gradually from 1996 on, but the lowest incidence was detected during AmB inhalation prophylaxis.

GvHD enhances the risk of IA (157,174). This is reflected in the timing of IA; the majority of these infections occur 90-140 days after the transplantation (141,164,250). Instead of anti-mould prophylaxis for all allogeneic SCT recipients, targeted prophylaxis to patients with GvHD seems more logical. The randomised study by Ullman et al. had allogeneic SCT recipients with grade 2-4 acute or extensive cGvHD (264). IA was detected in 2.3% vs. 7% of patients with posaconazole vs. fluconazole prophylaxis, respectively. Breakthrough IA occurred in 1% of the patients during posaconazole prophylaxis. In the present study the AmB inhalation prophylaxis was also targeted, but only to patients treated with a high dose of MP due to aGvHD. The incidence of breakthrough IA was similar to that of the study by Ullman et al. (264).

The fall of IA incidence in the present study was not connected with the use of new antifungals as only four patients received posaconazole or caspofungin. With regard to the old antifungals, fewer patients received systemic fluconazole or AmB in Period II compared with Period I. The role of the inhalation prophylaxis is further supported by the fact that despite an earlier occurrence of aGvHD in Period II, IA tended to occur later.

Giving antifungal prophylaxis in the form of inhalation seems logical, since it would target the prophylaxis directly to the site of colonization and infection. The adverse effects and drug interactions connected to systemic antifungals might be avoided. Two prospective, randomized studies have evaluated the efficacy of AmB inhalation prophylaxis in neutropenic patients with hematological malignancies with contradictory results. In the study by Schwartz et al. D-AmB inhalations were deemed ineffective, since the incidence of IA was 4% with the prophylaxis and 7% without it (243). That study had no allogeneic SCT recipients. Rijnders et al. reported a significant fall of IA incidence from 14% to 4% in patients receiving liposomal AmB inhalation prophylaxis instead of placebo (224). In that study, 11% of the patients received allogeneic SCT.

Two observational studies assessed the efficacy of AmB inhalation prophylaxis in allogeneic SCT recipients. The study by Hertenstein et al. included 303 patients (271 allogeneic SCT recipients) who received D-AmB inhalations over the neutropenic period (95). With a follow-up of 120 days IA was detected in 2% of the allogeneic SCT recipients. Breakthrough IA was detected in two patients (0.7%) during the prophylaxis. In the study by Alexander et al. 40 allogeneic SCT recipients received lipid complex AmB inhalation and fluconazole prophylaxis for 13 weeks (8). No IA cases were observed. In the present study, the median duration of the AmB inhalation prophylaxis was similar, 12 weeks, and breakthrough IA occurred in 1% of the patients indicating good efficacy of the prophylaxis.

The potential adverse effects of AmB inhalations include couch, bad taste, dyspnea, bronchial obstruction, nausea, and vomiting. These have led to the discontinuation of prophylaxis in 7-22% of haematological patients in previous studies, whereas some studies have reported no significant adverse effects (45,58,63,83,95,178,243). L-AmB inhalations may cause fewer adverse effects than D-amB as reported by Drew et al. in lung transplant recipients (57). In the present study, the D-AmB inhalations were well tolerated, since no discontinuations occurred. The use of a pre-inhalation bronchodilator might play a role in this.

Due to the low number of patients with IA in Period II the risk factors for IA could not be analysed or compared. A post-mortem examination was performed on 41.3% of the patients who died, which may lead to underestimation of the true incidence of IA. The incidence of IA, however, reached its minimum after the initiation of the AmB inhalation prophylaxis. Only one breakthrough IA was detected. The need for a prospective, randomized study of the efficacy of the AmB inhalation prophylaxis in patients with GvHD is obvious. We have found this prophylaxis effective and well tolerated and continue to use it in our centre.

SUMMARY AND CONCLUSIONS

Prevention and early diagnostics of IFIs were the focus of the studies in this thesis. Colonization of the respiratory tract with *Aspergillus* spp. and of the gastrointestinal tract with *Candida* spp. are risk factors for IFIs. Colonization was studied by assessing the air quality of the SCT ward and by performing fungal cultures of the nasal and oral cavities of allogeneic SCT recipients. *Aspergillus* and *Candida* antigen testing was performed from serum samples of allogeneic SCT recipients to see if the diagnosis of IFI could be made earlier. Since colonization can never be totally avoided, high-risk patients may benefit from antifungal prophylaxis. This aspect was studied with fluconazole in patients wit acute leukaemia and with AmB inhalations in allogeneic SCT recipients with aGvHD.

Routine environmental surveillance of the SCT ward with settled dust analysis did not show elevated levels of *Aspergillus* spores or seasonal variation. The environmental surveillance during heavy construction work also showed that HEPA filters were successful in keeping the patient rooms almost clear of fungal spores throughout the construction period. Colonization of the patient rooms or patients was not detected. No *Aspergillus* infections were seen after the construction period. An environmental surveillance, such as in the present study, can be recommended in a SCT ward during construction or renovation activity.

Colonization of the nasal cavities of allogeneic SCT recipients with *Aspergillus* spp. was rare, and IA was detected in 2% of the patients. With the constantly low spore count of air samples and low incidence of nasal colonization it is difficult to estimate the correlation between these factors and the risk of IA. The GM ELISA test yielded positive results in one of the two patients with IA, but only after radiological signs of the infection. The test was thus not helpful for the earlier diagnosis of IA, and routine use of this test does not seem useful in a population of patients with a low incidence of IA. However, earlier prospective studies have shown that the GM ELISA test is a valuable additional diagnostic tool in patient populations with a higher incidence of IA, especially when combined to other methods such as HCRT and BAL.

Oral colonization with *Candida* spp. occurred in 37% of allogeneic SCT recipients, but the incidence of IC was only 1%. With a cut-off of 0.5 ng/ml, single positive *Candida* mannan Ag test results were detected in over 50% of the patients without clinical signs of yeast infection. In the only patient with IC, however, the test was positive seven weeks before the diagnosis of IC was confirmed. Routine use of the *Candida* mannan Ag test is not useful in a population of patients with such a low incidence of IC.

Fluconazole prophylaxis was effective in reducing the incidence of IC in patients with acute leukaemia without signs of rise in non-*albicans* infections. Bacteraemias, however, increased. We have found fluconazole highly effective and continue to use it in patients with acute leukaemia receiving chemotherapy.

The incidence of IA fell from 6.6% to 2.5% in allogeneic SCT recipients after the initiation of the AmB inhalation prophylaxis for patients with aGvHD and high-dose MP therapy. Breakthrough IA occurred in 1% of the patients during the prophylaxis. The inhalations were effective and well tolerated and we continue to use this prophylaxis in our centre.

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