DEPARTMENT OF LABORATORY MEDICINE
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DIAGNOSTIC AND CLINICAL ASPECTS OF INVASIVE FUNGAL DISEASE AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Ola Blennow

Stockholm 2014

Cover picture: Thoracic CT showing a dense infiltrate with halo sign (top), and hyphae in a lung biopsy (bottom).

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Diagnostic and Clinical Aspects of Invasive Fungal Disease after Allogeneic Hematopoietic Stem Cell Transplantation

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ABSTRACT

Invasive fungal disease (IFD) is a major complication after allogeneic hematopoietic stem cell transplantation (HSCT). Effective prophylaxis has reduced the incidence of invasive candidiasis, but invasive mold infections (IMIs)—especially invasive aspergillosis (IA)—continue to be an important cause of non-relapse mortality. However, there are very few data regarding IFD and IMI after HSCT in the Nordic countries. The aim of this thesis work was to investigate epidemiological, diagnostic, and clinical aspects of IFD in HSCT recipients at Karolinska University Hospital, Huddinge.

In **paper I**, 99 patients who received reduced-intensity conditioning (RIC) were followed with weekly fungal PCR during the first 100 days after HSCT. Patients with a positive fungal PCR result were randomized to either treatment with liposomal amphotericin B or no treatment. We found that a single positive PCR test was not associated with IFD, irrespective of treatment. The cumulative incidence rate of proven or probable IA during the first year after transplantation was 9%, and significant risk factors in a multivariate model were grades II–IV acute-graft-versus host disease (aGVHD), cytomegalovirus- (CMV-) seronegative recipient with CMV-seropositive donor, and conditioning with alemtuzumab. In **paper II**, a possible influence of the intensity of the conditioning on pneumonia-related death was investigated. We found no significant differences in the cumulative incidence of pneumonia-related death between patients receiving myeloablative conditioning (MAC) and those receiving RIC: early death (< 100 days after HSCT) 2.8% vs. 2.1%, and overall death 8.2% vs. 10.5%. Etiology could be established in 40 of 60 patients (67%) who died from pneumonia, with proven or probable IMI in 19 patients (48% of patients with established etiology, 32% of all patients with pneumonia-related death). In the multivariate analyses, grades II–IV aGVHD, CMV infection, and treatment with mesenchymal stromal cells (MSCs) were factors associated with overall pneumonia-related death. In **paper III**, posaconazole tissue concentrations were examined in vivo. Tissue concentrations of posaconazole were analyzed in biopsies taken at autopsy of seven patients who received posaconazole prophylaxis, and they were compared with plasma concentrations in samples taken before death. Accumulation of posaconazole was found in heart, lung, kidney, and liver tissue, while concentrations in brain were approximately equal to the concentrations in plasma. The apparent tissue accumulation in vivo is in agreement with earlier in vitro findings and may explain the low incidence of breakthrough infections seen in prophylaxis trials despite low serum concentrations. In **paper IV**, incidence and risk factors for IMI were retrospectively investigated in 843 patients. The cumulative incidences of proven and probable IMI were 2.2% at day 100, 5.2% after 1 year, and 6.3% after 2 years. Factors significantly associated with a new IMI were older age (risk hazard 4.26 for 41–60 years of age and 9.0 for > 60 years of age, with 0–20 years as reference), grades II–IV aGVHD, treatment with MSCs, and transplantation with female donor to male recipient. In patients with grade II aGVHD, no IMIs were seen after onset of GVHD in 113 HSCTs performed in patients < 40 years of age, compared to 14 IMIs in 106 HSCTs (13.2%) in patients > 40 years of age ($p < 0.001$). Twelve of these 14 patients had signs of poor immune reconstitution

before onset of IMI. In patients with grade II aGVHD, few patients who are < 40 years of age appear to need mold-active prophylaxis, whereas in patients > 40 years of age prophylaxis is indicated if there are signs of poor immune reconstitution.

LIST OF SCIENTIFIC PAPERS

I. Blennow O, Remberger M, Klingspor L, Omazic B, Fransson K, Ljungman P, Mattsson J, Ringdén O. Randomized PCR-based therapy and risk factors for invasive fungal infection following reduced-intensity conditioning and hematopoietic SCT. *Bone Marrow Transplantation* 2010 Dec;45 (12):1710-8.

II. Forslow U, Blennow O, LeBlanc K, Ringden O, Gustafsson B, Mattsson J, Remberger M. Treatment with mesenchymal stromal cells is a risk factor for pneumonia-related death after allogeneic hematopoietic stem cell transplantation. *European Journal of Haematology* 2012 Sep;89 (3):220-7.

III. Blennow O, Eliasson E, Pettersson T, Pohanka A, Szakos A, El-Serafi I, Hassan M, Ringdén O, Mattsson J. Posaconazole concentrations in human tissues after allogeneic stem cell transplantation. *Antimicrobial Agents and Chemotherapy* 2014 Aug;58 (8):4941-3.

IV. Blennow O, Remberger S, Törlén J, Ringdén O, Ljungman P, Mattsson J. Incidence and risk factors for invasive mold infections after allogeneic stem cell transplantation. *Submitted manuscript*.

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

i. Blennow O, Mattsson J, Remberger M. Pre-engraftment blood stream infection is a risk factor for acute GVHD grades II-IV. *Bone Marrow Transplantation* 2013 Nov;48(12):1583-4.

ii. Uhlin M, Wikell H, Sundin M, Blennow O, Maeurer M, Ringden O, Winiarski J, Ljungman P, Remberger M, Mattsson J. 2014. Risk factors for Epstein-Barr virus-related post-transplant lymphoproliferative disease after allogeneic hematopoietic stem cell transplantation. *Haematologica* 2014 Feb;99(2):346-52.

iii. Blennow O, Ljungman P, Sparrelid E, Mattsson J, Remberger M. 2014. Incidence, risk factors, and outcome of bloodstream infections during the pre-engraftment phase in 521 allogeneic hematopoietic stem cell transplantations. *Transplant Infectious Diseases* 2014 Feb;16(1):106-14.

iv. Blennow O, Fjaertoft G, Winiarski J, Ljungman P, Mattsson J, Remberger M. 2014. Varicella-Zoster Reactivation after Allogeneic Stem Cell Transplantation without Routine Prophylaxis-The Incidence Remains High. *Biology of Blood and Marrow Transplantantation* 2014 Jun 7.

CONTENTS

LIST OF ABBREVIATIONS

aGVHD	acute graft-versus-host disease
A-Mn	anti-mannan antibodies
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BG	1,3-β-glucan
BG assay	assays detecting 1,3-β-glucan
CI	95% confidence interval
cGVHD	chronic graft-versus-host disease
CMV	cytomegalovirus
CNS	central nervous system
CT	computed tomography
CVC	central venous catheter
EAPCRI	European Aspergillus PCR Initiative
EBV	Epstein-Barr virus
GM	galactomannan
GM test	test detecting galactomannan
GVL	graft-versus-leukaemia
HCT-CI	hematopoietic cell transplantation specific comorbidity index
HLA	human leukocyte antigen
HR	hazard ratio
HSCT	In this thesis this abbreviation refers to allogeneic hematopoietic stem cell transplantation only
IA	invasive aspergillosis
IFD	invasive fungal disease
IMI	invasive mold infection
MIC	minimal inhibitory concentration
Mn	mannan
MSC	mesenchymal stromal cell
NK cell	natural killer cell
NPV	negative predictive value

PCP	pneumocystis pneumonia
PCR	polymerase chain reaction
PPV	positive predictive value
RIC	reduced-intensity conditioning regimen
RH	relative hazard
VZV	varicella-zoster virus

1 INTRODUCTION

In order to make it easier to read the text, allogeneic hematopoietic stem cell transplantation will be abbreviated to HSCT throughout the whole thesis. (This is not entirely correct since HSCT normally stands for all hematopoietic stem cell transplantation, i.e. both allogeneic and autologous.). Only proven and probable IFD are included in the studies mentioned in the text. (Definitions of IFD are explained in section 2.1).

1.1 HISTORY

The concept of allogeneic hematopoietic stem cell transplantation (HSCT) was born during the animal research performed in the wake of the first atomic bomb explosions. In 1949, Jacobsen found that shielding of the spleen with lead could save mice from otherwise lethal radiation (1). A short time later, it was shown that protection could also be conferred with an intravenous infusion of bone marrow (2). Investigations regarding the potential for autologous stem cell transplantation (i.e. from the same individual) and allogeneic stem cell transplantation (i.e. from another human being) in the treatment of leukemia were initiated, and the first transplantations in humans were performed during the latter half of the 1950s. One of the pioneers, E. D. Thomas, later received the Nobel Prize (1990) for his achievement in this field. Disappointingly, HSCT turned out to be unsuccessful—as reported in a review of 203 transplantations carried out between 1958 and 1968, with 125 graft failures, 49 cases of severe graft-versus-host disease (GVHD), and only 11 patients achieving long-term engraftment (3). It was concluded that the clinical application had been undertaken to soon and that more research in animals was needed. During the 1960s, important knowledge was obtained about high-dose conditioning regimens that reduced the risk of graft rejection, mechanisms and prophylaxis for GVHD, and human leukocyte antigen (HLA) typing (4). In the late 1960s, clinical studies in humans could be restarted, and the results have been increasingly successful ever since. Until the end of the 1990s, however, HSCT was not suitable for older patients due to the intensive conditioning. This led to the development of reduced-intensity conditioning (RIC) regimens (i.e. conditioning that allowed hematopoiesis to recover even without transplantation), with less toxicity and shorter duration of neutropenia, but with higher risk of relapse of the underlying disease. Different RIC protocols have been developed, and RIC is now the conditioning used in more than half of the patients in Europe and USA (5).

1.2 RATIONALE AND INDICATIONS

The rationale for performing HSCT differs between malignant and non-malignant diseases. In malignant diseases, the main reason is the graft-versus-leukemia (GVL) effect, i.e. that the new immune system will attack residual leukemia cells. In addition, HSCT permits more

intensive chemotherapy since toxicity to the bone marrow is usually the main limiting factor. The importance of the GVL effect in humans was first described by Weiden et al. in 1979. His group showed that in patients with grade II acute GVHD (aGVHD), relapse rate was 2.5 times less than in patients with no more than grade I aGVHD and than in patients transplanted from an identical twin (6). The same research group later found that chronic GVHD (cGVHD) also showed a clinically important GVL effect (7). The malignant indications are dominated by hematological diseases such as acute and chronic leukemia, myelodysplastic syndrome, lymphomas, and multiple myeloma.

In the non-malignant setting, HSCT is a replacement therapy in patients with congenital or acquired deficiencies of marrow function, the immune system, or storage functions. This includes a variety of diseases such as aplastic anemia, thalassaemia, severe combined immunodeficiency, and inborn errors of metabolism. Since the rationale for HSCT in these patients is in most cases to replace dysfunctional cells and not to treat malignancy, GVL—and thus GVHD—is not needed and should be avoided using intensive GVHD prophylaxis (8).

1.3 PROCEDURES

At least two important factors must be considered when deciding whether HSCT is indicated. First, treatment with HSCT must have a better outcome than the alternative treatment option(s). Second, the patient must be able to withstand the procedure so that the estimated non-relapse mortality due to HSCT will be low in relation to the outcome of the disease if an alternative treatment had been chosen. When the decision has been made, HLA typing of the recipient using DNA-based typing techniques is performed and the hunt for a suitable donor can begin (9). The donor should be as well HLA-matched as possible in order to minimize the risk of severe GVHD (8, 10). An HLA-matched sibling is preferred but only exists in approximately 30% of cases. The alternatives are to use a mismatched related donor (which includes haploidentical parents or children), to find a matched unrelated donor in the large registries that have been created for this purpose, or to use stem cells from umbilical cord blood (11). Hematopoietic stem cells are still poorly characterized, but are recognized by the expression of the marker CD34 on the cell surface. The cells can be harvested from bone marrow, through leukapheresis of peripheral blood after mobilization of stem cells with granulocyte colony stimulating factor, or from umbilical cord blood. The conditioning regimen is necessary to avoid rejection of the donor cells, and to kill malignant cells if the reason for HSCT is malignant disease. At our institution, in vivo T cell depletion with anti-thymocyte globulin is administered as part of the conditioning in patients with unrelated donors and patients with non-malignant disorders in order to reduce the risk of both graft failure and GVHD (12). Other T cell inhibiting drugs must also be administered before transplantation of the stem cells to dampen the reactivity of recipient T cells in order to avoid rejection of the graft and—after transplantation—to dampen donor T cells to avoid GVHD. The most common prophylaxis regime at our institution is cyclosporine combined with a

short course of methotrexate. Cyclosporine must be continued after the transplantation until immune tolerance has been achieved, i.e. the new T cells have learned to recognize the recipient tissues as being "self" and not to launch an attack. In the absence of treatment requiring GVHD, cyclosporine can usually be tapered over 3-6 months, but is usually given for more than one year in patients with non-malignant diseases.

The hematopoietic stem cells are infused via a central venous catheter (CVC) and they home to the marrow cavity quickly, directed by surface molecules (13). The duration of the period from cell infusion to engraftment, defined as absolute neutrophil count > 0.5×10^9/L in peripheral blood, differs depending on several factors such as the conditioning regimen, the source of stem cells, the number of CD34-positive cells transplanted, and the type of donor. Normally, patients receiving peripheral blood grafts have the shortest time to neutrophil recovery (approximately 14 days), followed by bone marrow grafts (approximately 18 days), while patients receiving umbilical cord grafts can have quite a long time to engraftment (approximately 30 days) (14, 15).

1.4 IMMUNE RECONSTITUTION

The cells that constitute innate immunity usually recover both in number and function during the first 1–2 months after transplantation, dramatically reducing the risk of bacterial and fungal infections (Figure 1) (16). The epithelial barriers are often disrupted by mucositis induced by the conditioning regimen, but recover quickly after engraftment. Neutrophil counts are rapidly normalized to pre-transplant levels, but important functions, such as chemotaxis and phagocytosis, are less efficient early on and take about two months to recover. If glucocorticoids are administered as treatment for GVHD, recovery is delayed (17). Monocyte counts are normalized by 1 month post-transplant. Macrophages are relatively resistant to conditioning therapies, so their number does not drop substantially, but they are gradually replaced with donor macrophages over several months (18). Most of the different dendritic cells reconstitute within 6 months, with the notable exception of follicular dendritic cells in the germinal centers of the lymph nodes and spleen (19, 20). These cells seem to recover more slowly and appear to be sparse even after 1 year. Since the follicular dendritic cells play an important role in the maturation of B cells in germinal lymph nodes, this may explain the slow reconstitution of memory B cells (19). Natural killer (NK) cell counts recover during the first weeks after transplantation. During 1–3 months after HSCT, the counts are supranormal and the NK cells kill target cells more efficiently, which may have a significant anti-leukemic effect (19).

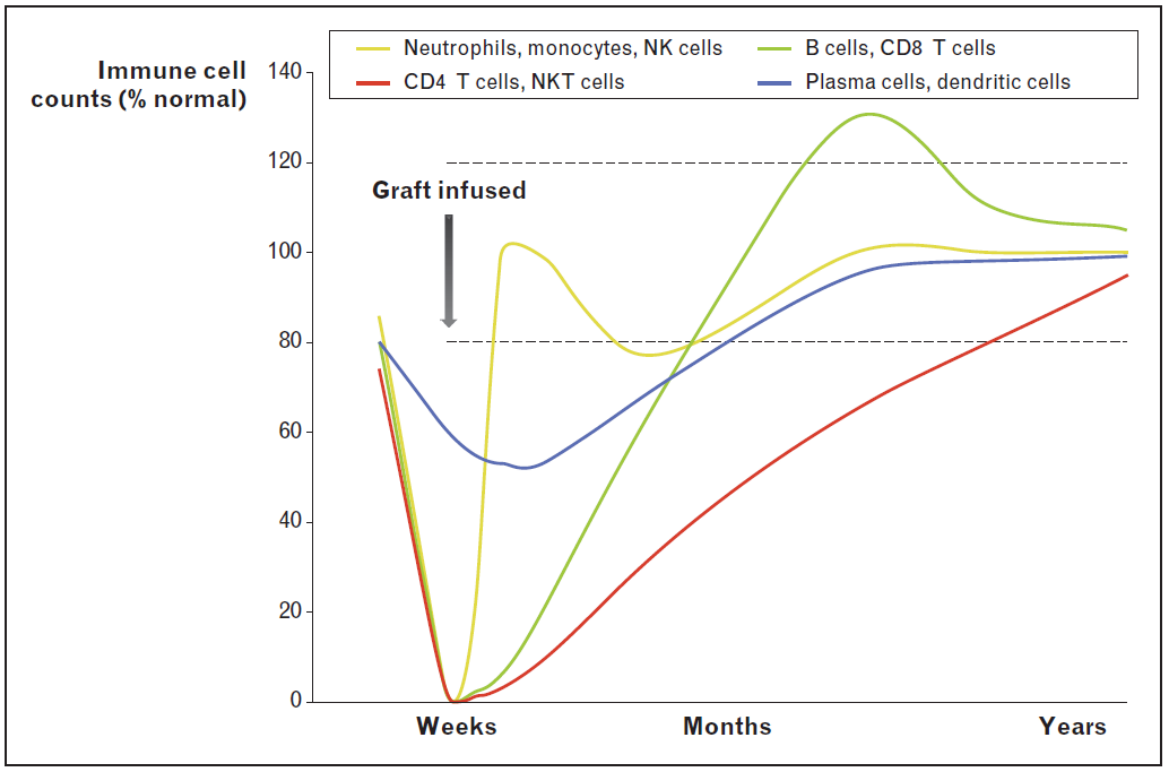


FIGURE 1. Approximate immune cell counts (expressed as percentage of normal counts) before and after myeloablative hematopoietic cell transplantation. Nadirs are higher and occur later after nonmyeloablative than myeloablative transplantation (not shown), as recipient cells persist after nonmyeloablative transplant for several weeks to months (in the presence of GVHD) or longer (in the absence of GVHD). The orange line represents innate immune cells [for example, neutrophils, monocytes and natural killer (NK) cells], the recovery of which is influenced by graft type (fastest with filgrastim-mobilized blood stem cells, intermediate with marrow and slowest with umbilical cord blood). Note the second nadir of these cells. The green line represents the recovery of CD8 T cells and B cells, the counts of which may transiently become supranormal. B-cell recovery is influenced by graft type (fastest after cord blood transplant) and is delayed by graft-versus-host disease (GVHD) and/or its treatment. The blue line represents the recovery of relatively radiotherapy/chemotherapy resistant cells such as plasma cells, tissue dendritic cells (for example, Langerhans cells) and, perhaps, tissue macrophages/microglia. The nadir of these cells may be lower in patients with acute GVHD because of graft-versus-host plasma cell/Langerhans cell effect. The red line represents $CD4^+$ T cells, the recovery of which is influenced primarily by the T-cell content of the graft and patient age (faster in children than adults). Adapted from [25].

Figure 1. *Approximate immune cells counts (expressed as percentage of normal count) before and after myeloablative hematopoietic stem cell transplantation. From Bosch et al. (16). Published with permission from Lippincott Williams & Wilkins.*

The reconstitution of B and T cells takes much longer. B cell reconstitution may take up to 2 years after HSCT, especially in the presence of cGVHD. B cells are undetectable for the first two months, after which they slowly rise and reach normal levels after 1–2 years. Early on, the vast majority of B cells are naïve cells, producing IgM rather than IgG or IgA—which is reminiscent of the situation in early childhood (21, 22). After transplantation, the serum isotype immunoglobulin levels are recovered in the same sequence as they develop in young children: IgM followed by IgG1/IgG3 followed by IgG2/IgG4/IgA (23). For efficient antibody responses, CD4+ T cells are needed, cells that are subnormal for up till 2 years after transplantation (24).

The speed and quality of immune reconstitution of T cells is of vital importance for the risk of developing viral and fungal infections after HSCT. CD8+ T cell counts reach normal levels approximately one year after transplantation, as compared to two years for CD4+ T cells, giving rise to the characteristic inverse CD4/CD8 ratio compared to healthy controls. CD8+ cells that are antigen-primed without "help" provided by CD4+ cells may develop normally but lack memory formation and proliferative capacity (25). After transplantation, T cells are regenerated by two pathways: (1) an early thymus-independent peripheral expansion of pre-existing T cells, either mature donor T cells from the graft or residual host T cells that survive the conditioning, and (2) a later thymus-dependent pathway whereby immature thymocytes are produced in the bone marrow and transported to the thymus, where they proliferate and are subsequently released as mature naïve T cells. Because the thymus-independent pathway cannot provide new T cells, the repertoire is limited early after HSCT. In order to have a functionally complete immune reconstitution, it is vital that new naïve T cells are generated, a process that takes from 6 months up to 2 years (26). Due to the slow T cell reconstitution and the dampened T cell activity induced by GVHD prophylaxis, all patients have a moderate risk of virus and mold infections. However, the risk is much higher in patients with factors that negatively affect the thymus function and hamper immune reconstitution—older age and GVHD being two of the most important (14, 27, 28).

1.5 GVHD AND IMMUNE RECONSTITUTION

The development of acute GVHD involves three important sequential steps or phases: (1) Activation of antigen presenting cells (APCs). Tissue damage caused by the conditioning regimen releases a storm of proinflammatory cytokines that promote activation of APCs. In addition, the conditioning causes injury to the gastrointestinal tract, allowing systemic translocation of inflammatory stimuli such as lipopolysacharides (LPS), which further enhance activation of host APCs. (2) Proliferation and differentiation of donor T cells in response to host APCs. (3) Destruction of target tissues by effector T cells. The resulting tissue damage leads to release of proinflammatory mediators, contributing to the cytokine storm that fuels aGVHD (29, 30).

There are several ways in which aGVHD affects immune reconstitution, including direct effects of the cytokine storm and bone marrow suppression caused by aGVHD-induced destruction of marrow stromal cells, and indirect side effects of the treatment given for aGVHD, usually high-dose glucocorticoids (31-33). Treatment with glucocorticoids delays reconstitution of neutrophil functions, such as chemotaxis and phagocytosis, and dampens the ability of functional neutrophils to respond to pathogens (17). A negative effect of aGVHD or its treatment has also been shown on the numbers of total and cytolytic NK cells and of dendritic cells (34). In addition, the total counts of both memory and naïve B cells are reduced, possibly due to aGVHD-induced destruction of bone marrow hematopoietic niches (31, 32, 34). Reconstitution of T cells requires a functional thymus with positive and negative selection, producing tolerant mature naïve T cells, a process that is severely impeded when

the thymus is attacked by aGVHD (26). The effect of aGVHD appears to be especially important in older patients, leading to a longstanding and sometimes irreversible negative effect on thymus function, even after successful treatment of the aGVHD (27).

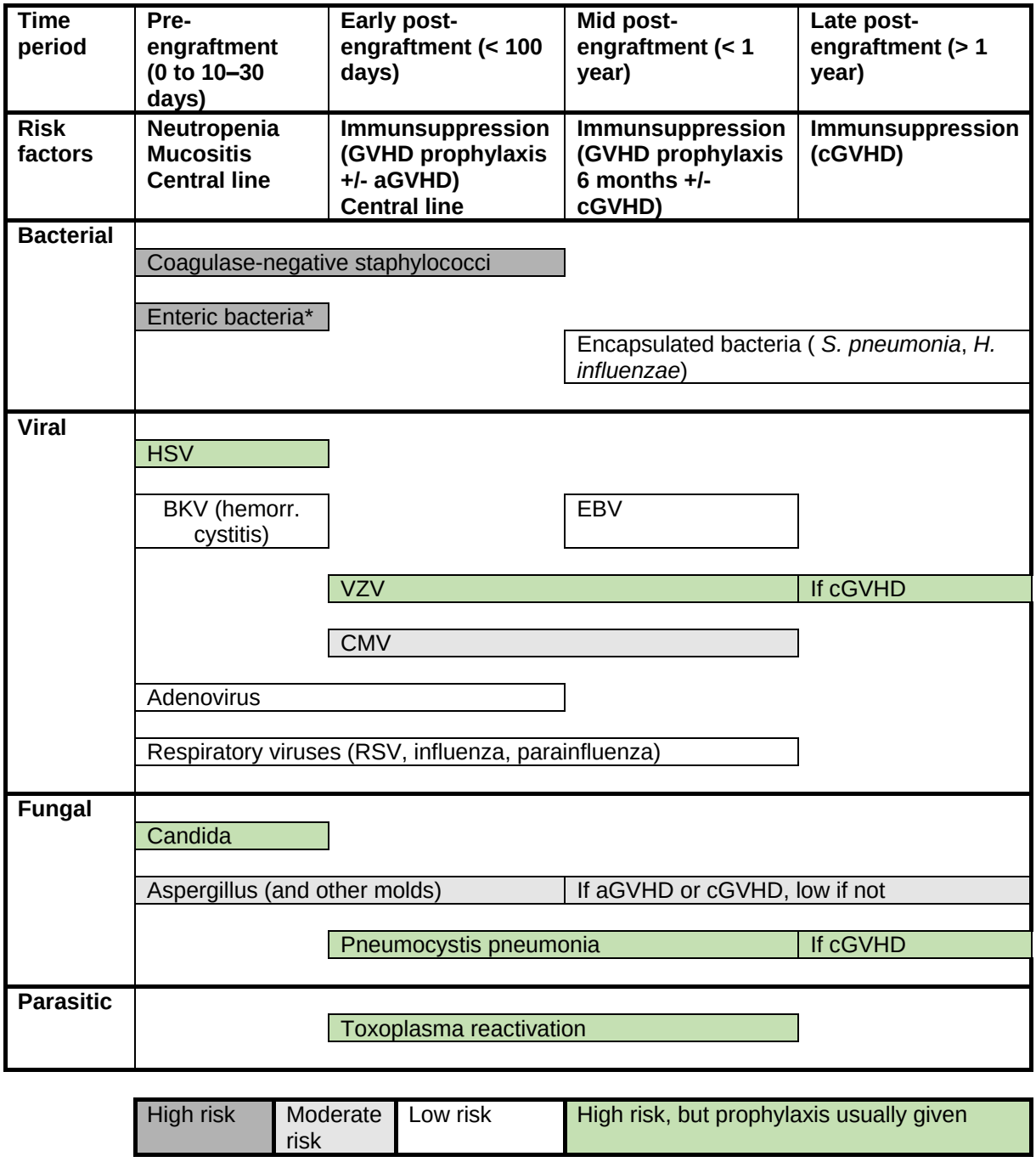
Chronic GVHD most often has a later onset and has features resembling autoimmune and other immunologic disorders such as scleroderma, Sjögren syndrome, primary biliary cirrhosis, and bronchioloitis obliterans (35). The diagnosis is based on diagnostic clinical signs, in combination with exclusion of other possible diagnoses, and is graded as mild, moderate, or severe (35). In contrast to acute GVHD, the pathophysiology of chronic GVHD remains poorly understood, but results in poor T cell reconstitution (30).

1.6 INFECTIONS AFTER HSCT

The spectrum and risk of infections differs with the amount of time that has passed after HSCT. These infections are usually divided into those that occur during the pre-engraftment phase, the early post-engraftment phase (< 100 days), the mid post-engraftment phase (< 1 year), and the late post-engraftment phase (> 1 year) (Figure 2). In the later phases, GVHD is the most important risk factor for infection, and in the absence of GVHD the risk is generally low after day 100.

1.6.1 Pre-engraftment phase

Due to neutropenia, mucositis, and indwelling CVCs, bacterial infections are common during the pre-engraftment phase (Figure 2). In a recent study investigating 521 HSCTs performed in our institution between 2001 and 2008, the incidence of at least one episode of bacteremia during the pre-engraftment phase was 20% (37). In accordance with other studies, the predominant etiologies were enteric gram-negative rods and gram-positive cocci, secondary to mucositis and bacterial translocation from the gastrointestinal tract, and coagulase-negative staphylococci originating from skin and CVC (37-40). Proven bacteremia is probably only the tip of the iceberg, as almost all HSCT patients have at least one episode of neutropenic fever—the majority of which are thought to be of bacterial origin. If not treated adequately, bacteremia in neutropenic patients carries a high mortality; in the event of fever, broad-spectrum antibiotics should always be administered immediately (after blood cultures have been performed) (41). The highest mortality has been reported for bloodstream infections with gram-negative rods, and prophylaxis with quinolones during neutropenia has been much debated. Several studies have shown a reduction in episodes of fever and bacteremia with prophylaxis, but no effect on overall survival. However, a Cochrane report from 2012 (including over 40 trials with more than 5,000 hematological patients) found both significantly lower all-cause mortality and infection-related mortality with quinolone prophylaxis (42). Another meta-analysis published earlier this year and including 1,453 stem cell recipients (407 of them allogeneic) found that bacterial prophylaxis reduced the number



*Gram-negative rods and gram-positive cocci.

Figure 2. *Timeline of infections after allogeneic stem cell transplantation. Adapted from Hammond (36).*

of febrile episodes and bacteremias, but not mortality (43). However, one weakness of the meta-analysis was that the survival data in patients receiving quinolone prophylaxis were mainly derived from studies including only autologous stem cell transplantations. At our institution, prophylaxis with ciprofloxacin during neutropenia has been used routinely since 1986. The incidence of gram-negative bacteremia was low after the introduction of prophylaxis (0.3%) but has increased significantly over the years, and was 4.0% in the period 2005–2008 (37).The risk of reactivation of herpes simplex virus (HSV) 1 and 2 is high during

the pre-engraftment period, and in most centers including ours, prophylaxis with acyclovir is given during this period. Respiratory viruses such as respiratory syncytial virus (RSV), influenza virus, parainfluenza virus, adenovirus, and metapneumovirus are important pathogens after HSCT, both during the pre-engraftment phase and later. Lower respiratory tract infections with RSV are serious complications with high mortality (44).

The risk of invasive candidiasis during neutropenia is considerable, at least after myeloablative conditioning, and prophylaxis is often given. The risk of early invasive aspergillosis (IA) (< 40 days after HSCT) has decreased during the past decade(s), with the majority of infections now occurring after engraftment, and in association with GVHD. However, this may be changing due to more patients with risk factors for early IA (such as previous IA and active leukemia) being transplanted (45). Fungal disease after HSCT will be discussed in more detail in Chapter 2.

1.6.2 Early post engraftment phase (< 100 days)

The risk of infections with enteric bacteria almost vanishes with the resolution of neutropenia, whereas the risk of bacteremia with coagulase-negative staphylococci is reduced but remains as long as the CVC is in place. Cytomegalovirus (CMV) reactivation is common even in the absence of aGVHD; Ljungman and colleagues found that 65% of patients had at least one CMV episode, occurring at a mean time of 26 days after HSCT (46). The cumulative incidence of CMV disease was 1.8% after 100 days and 6.3% after one year, with the most important risk factor being grades II–IV aGVHD (46). Reactivation of Epstein-Barr virus (EBV) leading to post-transplant lymphoproliferative disease (PTLD) is an important complication with high mortality. The incidence appears to have risen during the last 10 years and the cumulative incidence at our institution was found to be over 6% in 2011–2012, with a median time of onset of 71 days (47). Respiratory viruses, including adenovirus, continue to be a threat, especially in the presence of aGVHD grade II or more (44, 48). Reactivation of varicella-zoster virus (VZV) is common without routine prophylaxis (the cumulative 2-year incidence is 22.7% at our institution), and acyclovir should be prescribed to all seropositive patients for at least one year after HSCT, and longer if ongoing immunosuppression (49-51). Prophylaxis for pneumocystis pneumonia (PCP) is mandatory. Reactivation of toxoplasma is common in seropositive patients, and prophylaxis should be given. The risk of fungal disease is low in the absence of aGVHD.

1.6.3 Mid post engraftment phase (< 1 year)

The risk of infections after the first 100 days is coupled to development of chronic GVHD (cGVHD). In the absence of cGVHD, there is a modest risk of infections with encapsulated bacteria (*Streptococcus pneumonia, Haemophilus influenzae*). Prophylaxis for PCP should be given at least until GVHD prophylaxis has terminated, whereas VZV prophylaxis, as

mentioned above, should be prescribed for at least the first year after HSCT. All patients should be vaccinated against influenza.

In the setting of cGVHD, the risk of infections is determined by the severity of the cGVHD and the immunosuppressive treatment necessary, especially the glucocorticoid dose. Vaccination is very important for protection against encapsulated bacteria, and is started 3 months after HSCT (52). However, if severe cGVHD is present, prophylaxis with antibiotics may also be considered. Reactivation of CMV and EBV is not unusual, and continued monitoring may be considered in patients with severe cGVHD. Mold infections are a threat, and prophylaxis may be adequate in certain situations as discussed in section 5.4. The risk of PCP without prophylaxis is high.

1.6.4 Late post-engraftment phase (> 1 year)

The risk of infections during this late phase depends on the cGVHD status. In the absence of cGVHD, infections are usually no problem, even if there may still be a slight increased risk of VZV reactivation and of infections with encapsulated bacteria. If cGVHD is present, the risk is, as mentioned above, determined by the severity of the cGVHD and the immunosuppressive treatment necessary. Infections to consider are bacterial infections with encapsulated bacteria, influenza, CMV, PCP, VZV, and molds. Prophylaxis for PCP and VZV (and toxoplasma, if serpositive) should be administered for as long as immunosuppressive drugs to treat cGVHD are prescribed. Patients with severe cGVHD requiring glucocorticoids (prednisolone) $\geq$ 1 mg/kg have an increased risk of developing invasive mold infections (IMIs), and mold-active prophylaxis may be indicated. This will be discussed in more detail in Chapter 2 and in section 5.4.

2 INVASIVE FUNGAL DISEASE AFTER HSCT

2.1 DEFINITIONS OF INVASIVE FUNGAL DISEASE

The lack of a standard set of definitions for fungal infections used to be a major obstacle in clinical research, making it difficult to compare different clinical trials. As a result, defining criteria were published in 2002 by the European Organization for Research and Treatment of Cancer (EORTC)/National Institute of Allergy and Infectious Diseases Mycoses (MSG) Study Group (53). These criteria were revised in 2008, and are used as the "gold standard" in clinical research involving IFD (54). The infections are classified as proven, probable, or possible. Briefly, proven invasive mold infection (IMI) requires demonstration of fungal elements in tissues, accompanied by evidence of associated tissue damage (i.e. requires biopsy specimens). A proven invasive yeast infection requires recovery of a yeast isolate by culture from a sample obtained by a sterile procedure from a normally sterile site, usually blood. To reach the level of probable fungal infection, a combination of a susceptible host, clinical signs compatible with a fungal infection, and mycological evidence must be present (Table 1). Possible infection requires only a susceptible host and clinical signs compatible with a fungal infection, the most frequent being dense, well-circumscribed lesions visible on thoracic computed tomography (CT).

2.2 EPIDEMIOLOGY

2.2.1 Candida

Invasive candidiasis after HSCT was a major problem during the 1980s, with a reported incidence of over 10% and high attributable mortality (55-57). Unlike mold infections, candida infections are endogenously derived. Since neutropenic patients lack circulating cells with phagocytosis capacity, candida translocated from the gastrointestinal tract can spread to the bloodstream, giving rise to candidemia and sometimes to disseminated infection. Thus, the most important risk factor for invasive candidiasis after HSCT is neutropenia, and the risk is highest during the pre-engraftment phase. Other important risk factors during this period are mucositis, which enhances translocation from the gastrointestinal tract, and use of broad-spectrum antibiotics, which affects the normal bacterial flora of the gut and promotes colonization of candida (58). Also, having a CVC is a well-established risk factor for candidemia, something that most HSCT recipients have for at least three months after transplantation (58). The risk rapidly declines with the return of neutrophils and resolution of mucositis. The other period that is well known to have an increased risk is after onset of GVHD, especially if it involves the gastrointestinal tract, which is the case in more than half of all patients with aGVHD (59). The resulting mucosal ulcerations disrupt the mucosal barrier and facilitate translocation of candida, and the high doses of steroids needed for treatment of the GVHD have a negative effect on neutrophil chemotaxis and phagocytosis (60).

Table 1. *Criteria for probable invasive fungal disease except for endemic mycoses according to the revised EORTC/MSG criteria (53)*

Probable IFD: Requires the presence of a host factor, a clinical criterion, and a mycological criterion.

Possible IFD: Requires the presence of a host factor and a clinical criterion, but mycological criteria are absent.

Host factors

Recent history of neutropenia (< 0.5 × 10^9 neutrophils/L for > 10 days) temporally related to the onset of fungal disease.

Receipt of an allogeneic stem cell transplant.

Prolonged use of corticosteroids (excluding patients with allergic bronchopulmonary aspergillosis) at a mean minimum dose of 0.3 mg/kg of prednisone equivalent per day for 13 weeks.

Treatment with other recognized T cell immunosuppressants, such as cyclosporine, TNF-α blockers, specific monoclonal antibodies (such as alemtuzumab), or nucleoside analogues during the past 90 days.

Inherited severe immunodeficiency (such as chronic granulomatous disease or severe combined immunodeficiency).

Clinical criteria

Lower respiratory tract fungal disease: The presence of 1 of the following 3 signs on CT:

- Dense, well-circumscribed lesions(s) with or without a halo sign
- Air-crescent sign
- Cavity

Tracheobronchitis:

- Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis

Sinonasal infection: Imaging showing sinusitis plus at least 1 of the following 3 signs:

- Acute localized pain (including pain radiating to the eye)
- Nasal ulcer with black eschar
- Extension from the paranasal sinus across bony barriers, including into the orbit

CNS infection: 1 of the following 2 signs:

- Focal lesions on imaging
- Meningeal enhancement on MRI or CT

Disseminated candidiasis[3]*:* At least 1 of the following 2 entities after an episode of candidemia within the previous 2 weeks:

- Small, target-like abscesses (bull's-eye lesions) in liver or spleen
- Progressive retinal exudates on ophthalmologic examination

Mycological criteria

Direct test (cytology, direct microscopy, or culture)

Mold in sputum, bronchoalveolar lavage fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following:

- Presence of fungal elements indicating a mold
- Recovery by culture of a mold (e.g., *Aspergillus, Fusarium,* Zygomycetes, or *Scedosporium* species)

Indirect tests (detection of antigen or cell-wall constituents):

- Aspergillosis: galactomannan antigen detected in plasma, serum, bronchoalveolar lavage, or CSF
- Invasive fungal disease other than cryptococcosis or zygomycoses: 1,3-β-glucan detected in serum

Today, invasive candida infections after HSCT are a minor problem; large studies published during the last years have shown incidences under 2% (37, 45, 61). The main reason is probably the routine use of systemic antifungal prophylaxis during the pre-engraftment phase and during severe GVHD. Another important explanation is likely to be the increasing use of RIC and peripheral blood stem cells as stem cell source with less tissue damage, i.e. mucositis, and shorter duration of neutropenia.

2.2.2 Aspergillus

Invasive aspergillosis (IA) after HSCT remains a major complication with high mortality. As mentioned in section 1.6.1, the timing of IA changed during the 1990s, from early onset to late onset, often occurring in association with GVHD (62-64). However, this may be changing again as reported in a recent prospective surveillance study including 1,858 HSCTs performed in Italy in the period 2008–2010. The overall cumulative 12-month incidence of IFD in the study was 8.8%, 81% of which was due to IA. The majority of IA (57.3%) occurred early, which was thought to be due to more patients with risk factors for early infection (such as previous IA and active leukemia) being eligible for transplantation (45). A similar overall incidence of IFD was found in 6,666 HSCT recipients transplanted in the USA in 2001–2005, with 42% being due to IA (65). Numerous risk factors for development of IA after HSCT have been reported, including age > 40 years at transplantation, active leukemia at transplantation, cord blood as stem cell source, transplantation with an unrelated donor or mismatched related donor (as opposed to a matched related donor), delayed neutrophil engraftment, delayed lymphocyte engraftment, grades II–IV aGVHD, severe chronic GVHD, aGVHD before severe chronic GVHD, aGVHD in a patient transplanted with a donor other than a matched related one, and treatment with glucocorticoids (63, 66, 67). Most of these risk factors reflect poor T cell reconstitution, highlighting the importance of well-functioning T cells for avoidance of IMI. The fact that grades II–IV aGVHD is an important risk factor for IMI after HSCT has been well known for many years, but data showing the risk according to the level of aGVHD have been limited until recently. In the large study from Italy mentioned above, the 6-month cumulative incidence of IFD in patients with grade II aGVHD was 7.1% as compared to 12.3% in patients with grades III–IV (45). Furthermore, the risk of IFD in patients with grades II– IV aGVHD varied according to whether or not aGVHD was followed by cGVHD, and to the type of donor. The cumulative incidence of IFD in patients with aGVHD not followed by cGVHD was as low as 2.3% in patients with matched related donors, as compared to 10% in patients with other donors. In patients with aGVHD followed by cGVHD, the corresponding percentages were 10% and 25.3%.

The mortality from IA reported in larger studies has varied from 35% to over 70%, partly depending on differences in follow-up time, autopsy frequency, and whether overall or attributable mortality was reported (45, 64, 65, 68-70). Survival has increased during the last decade, at least partly due to increased use of voriconazole (64). Many risk factors for death have been reported and they include impairment in pulmonary function before

transplantation, neutropenia at diagnosis of IA, early IA, late IA, treatment with corticosteroids (prednisolone) at ≥ 2 mg/kg per day, mismatched donor, proven IA (as opposed to probable), and renal insufficiency (64, 69, 70).

2.2.3 Other molds

Invasive non-*Aspergillus* mold infections are uncommon, but the incidence is increasing and such infections are often fatal. The most common of these uncommon infections is mucormycosis, formerly known as zygomycosis. The reason for the change of name is a new classification of fungi together with the fact that the majority of zygomycosis infections are caused by members of the Mucorales, such as the genera *Rhizopus*, *Mucor*, *Lichtheimia*, *Cunninghamella*, and *Rhizomucor* (71, 72). The Mucorales can usually be found in decaying organic matter and infections are either caused by inhalation of spores or direct inoculation of wounds after penetrating trauma (73). Important risk factors after HSCT are neutropenia and receiving glucocorticoids as treatment of GVHD, whereas diabetes is the most important risk factor in non-HSCT patients (73). In a prospective surveillance study from the TRANSNET database including 16,200 stem cell transplantations performed in the USA in 2001–2006 (79% HSCT); 8% of all IFD was caused by mucormycosis, with a 12-month cumulative incidence of 0.29% (65, 72). In a study including 929 reported cases of mucormycosis, the mortality was around 40% for all patients but as high as 91% in HSCT recipients (74). In a recent study investigating risk factors for early death from pulmonary mucormycosis in hematology patients (39% HSCT recipients), the 4-week mortality was 37% for all patients but 75% in patients with high APACHE score, lymphocyte count under 0.1×10^9/L, and elevated lactate dehydrogenase (75).

Another uncommon but important mold infection is fusariosis, constituting 25% of the non-*Aspergillus* mold infections in the TRANSNET study (72). In a recent retrospective study of 233 cases diagnosed between 1985 and 2011, 92% of the patients had a hematological disease, 38% had undergone HSCT, and 75% were neutropenic at diagnosis (76). The 90-day survival probability improved significantly during the study period, from 22% in the period 1985–2000 to 43% in the period 2001–2011, and was highest for patients receiving voriconazole (60%). Variables associated with poor prognosis were receipt of glucocorticoids, neutropenia at the end of treatment, and treatment with amphotericin B deoxycholate (76).

2.3 ANTIFUNGAL AGENTS FOR SYSTEMIC USE

2.3.1 Amphotericin B derivates

Amphotericin B is the oldest antifungal drug still in regular use, with initial reports of antifungal activity published as early as 1956. Amphotericin B binds to ergosterole in the

fungal membrane, leading to alteration in permeability and leakage of cell components, and ultimately to cell death (Figure 3) (77). Only intravenous formulations can be used for systemic treatment since orally administered amphotericin B is not absorbed. The first drug available for systemic use, amphotericin B deoxycholate, had dose-limiting nephrotoxicity. As a result, during the second half of the 1980s, lipid formulations were developed that contained amphotericin B within liposomes—biodegradable vesicles that consist of an aqueous environment surrounded by phospholipid bilayers (78). These formulations have significantly less renal toxicity and have replaced amphotericin B deoxycholate in most transplant facilities, including our own. Amphotericin B formulations have a broad antifungal activity, including *Candida* spp. and the majority of molds involved in IFD after HSCT, such as *Aspergillus* spp. (with the exception of *A. terreus* which has higher minimal inhibitory concentrations (MICs)), Mucorales, and *Fusarium* spp. (79). Liposomal amphotericin B is the only lipid compound currently available in Sweden, and the usual dose used in the treatment of mold infections is 3–5 mg/kg per day.

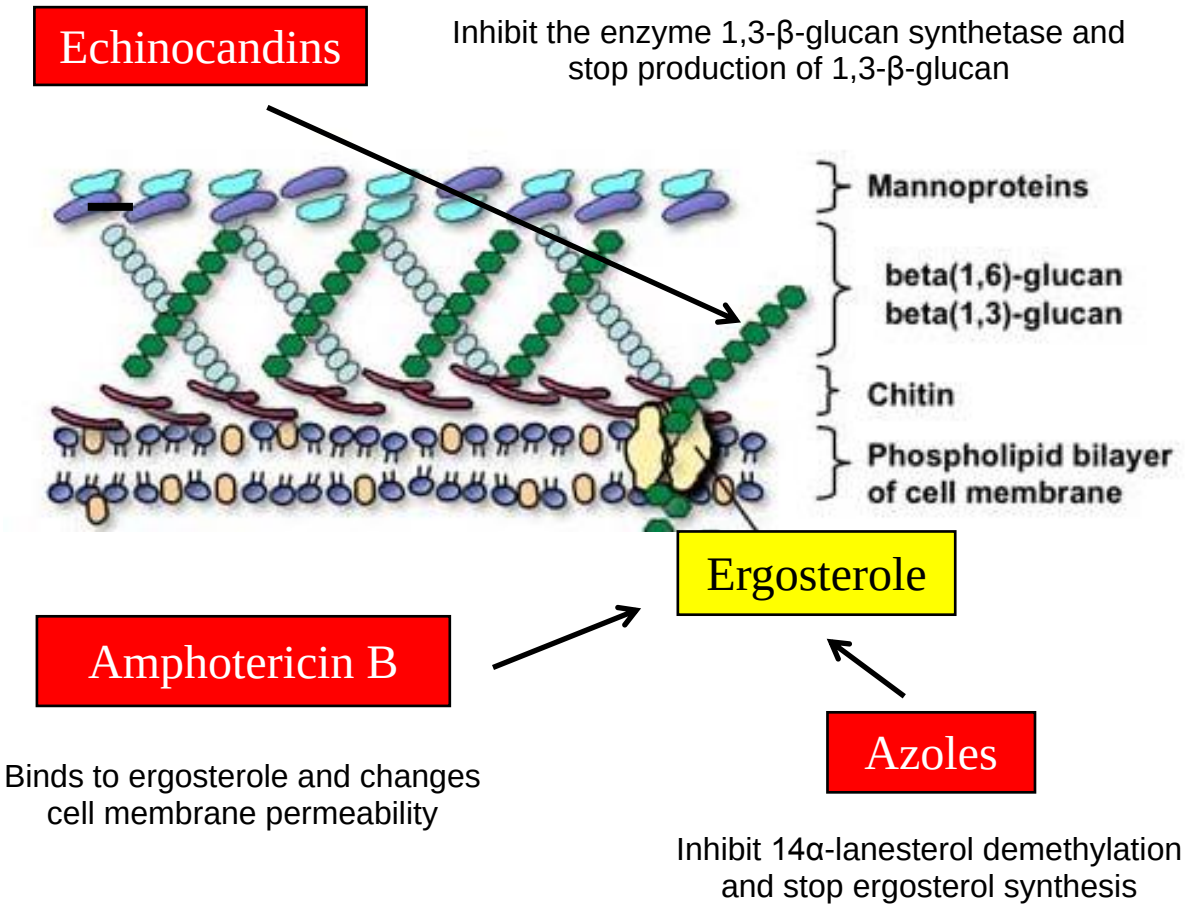


Figure 3. *Modes of action of antifungal drugs.*

2.3.2 Azoles

The first azoles in clinical use, the imidazoles, were available at the beginning of the 1970s. Miconazole was the first azole that could be used for systemic treatment, but it had to be administered intravenously and had many side effects such as phlebitis, fever, and chills (80). Ketoconazole, introduced in 1977, was the first azole that could be used orally, but its use was limited by variability in absorption, gastrointestinal intolerance, and liver toxicity (81). During the 1980s, a new group of azoles was synthesized, the triazoles, which all inhibit 14α-lanesterol de-methylation, a necessary step in ergosterol synthesis in the fungal cell membrane (Figure 3) (82). Fluconazole was developed during the latter half of the 1980s, and was shown to be a well-tolerated drug with good oral bioavailability and efficacy against *Candida* species with the exception of *C. krusei* and *C. glabrata* (83, 84). Itraconazole was introduced at approximately the same time as fluconazole, and was the first azole with anti-mold activity. Itraconazole has been a widely used drug, especially for prophylaxis, but variability in absorption, gastrointestinal tolerance, and hepatotoxicity have limited its usefulness. The next azole with anti-mold activity was voriconazole; it was approved in 2002 as the first of the second generation of triazoles. Voriconazole has good activity against *Candida* spp., *Aspergillus* spp, and *Fusarium* spp (85). Posaconazole was introduced in 2006 and has a broad antifungal activity covering *Candida* spp., *Aspergillus* spp., and some members of the Mucorales (85). There are several new azoles in clinical trials: isavuconazol, ravuconazole, and albaconazole.

2.3.3 Echinocandins

The echinocandins differ from the other antifungal agents in that they attack the cell wall and not the cell membrane. They inhibit the enzyme 1,3-β-glucan synthetase, which is necessary for production of 1,3-β-glucan (BG), a major component of the fungal cell wall which is fundamental for resistance to osmotic pressure (Figure 3). Generally, the echinocandins are well tolerated with few side effects, but they have the disadvantage of only being available as intravenous formulations. They are the first-hand choice when treating *Candida* infections (except *C. parapsilosis* and *C. guilliermondii*, which may have high MICs), and they are active against *Aspergillus* spp. but not against other molds (86-88).

At the moment, there are three registered echinocandins: caspofungin (the first to be approved in 2001), micafungin, and andulafungin. As all echinocandins have the same target, they have a very similar spectrum of activity. Caspofungin has the best documentation for use in patients with neutropenia, whereas anidulafungin appears to have the least hepatotoxicity, but these differences are of minor importance in clinical practice (86, 89, 90).

2.4 PROPHYLAXIS

2.4.1 Pre-engraftment until day 100

The antifungal drugs available for systemic treatment were very limited during the 1980s and consisted mainly of amphotericin B deoxycholate, with dose-limiting nephrotoxicity, and ketoconazole, with variable oral absorption and risk of severe liver toxicity (81). The situation changed with the introduction of fluconazole, a well-tolerated drug with good oral bioavailability and efficacy against most *Candida* species (83, 84). Since the risk of invasive candidiasis is highest during neutropenia, Goodman and colleagues performed a randomized trial comparing prophylaxis with fluconazole (400 mg once daily) with placebo during the pre-engraftment phase in 356 transplant patients (48% allogeneic) (57). The results showed a significantly lower incidence of invasive candidiasis in patients receiving fluconazole (2.8% vs. 15.8%) and lower candida-associated mortality (0.6% vs. 5.6%). In 1995, Slavin et al. reported data from a randomized study (with a long-term follow-up published in 2000) comparing prophylaxis with fluconazole (400 mg once daily) or placebo for 75 days in 300 patients undergoing HSCT (60, 91). Again, fluconazole recipients had a significantly lower overall incidence of invasive candidiasis (2.6% vs. 20.3%) and, importantly, also significantly better survival at 8-year follow-up (44.7% vs. 27.7%). Curiously, fluconazole prophylaxis had a prolonged protective effect: more patients in the placebo arm died of late (defined as > 110 days after HSCT) invasive candidiasis than those in the fluconazole arm (8 of 96 as opposed to 1 of 121). The explanation appeared to be that the incidence of severe GVHD involving the gut was significantly lower in fluconazole recipients, resulting in a lower risk of disseminated candida infection (60).

When an effective candida prophylaxis was used, it became evident that mold infections were major complications after HSCT, with high mortality. Subsequent prophylaxis trials therefore focused on comparing fluconazole with drugs that had activity against molds. Itraconazole prophylaxis for 180 days, or until 4 weeks after discontinuation of GVHD therapy, was compared with fluconazole, and was shown to provide significantly better protection against IMIs while patients were on treatment. However, there were no significant differences in IMI or invasive candidiasis at the end of the trial because of significantly more discontinuation of the study drug due to gastrointestinal intolerance in itraconazole recipients (92). Another, smaller study using prophylaxis for 100 days after HSCT also found itraconazole prophylaxis to be superior to fluconazole prophylaxis but, again, there were significantly more gastrointestinal side effects (93). It was concluded that even though itraconazole is more effective in preventing fungal infections, i.e. mold infections, hepatotoxicity and gastrointestinal intolerance limit its use.

Voriconazole, which has better gastrointestinal tolerance than itraconazole, was compared with fluconazole in a large prophylaxis study including 600 patients (61). The drugs were administered for 100 days after HSCT, or for 180 days in higher-risk patients (including aGVHD treated with prednisolone at > 1 mg/kg per day). Disappointingly, even though there was a trend of fewer IFDs in voriconazole recipients (7.3% vs. 11.2%), it did not reach

statistical significance. In addition, there was no significant difference in fungal-free survival. The authors stated that possible explanations included intensive monitoring with galactomannan (GM) tests and structured empiric antifungal therapy, enabling early treatment in patients with IFD and possibly reducing mortality in fluconazole recipients (61). A subgroup analyses showed that transplantation because of acute myeloid leukemia was a risk factor for IFD and that voriconazole was protective in this group. Thus, it appeared that the risk of mold infections in a mixed cohort of HSCT recipients might be too low for mold prophylaxis to be warranted, and that such prophylaxis should be reserved for higher-risk patients. This notion was indirectly supported by a study published in 2011 that compared voriconazole and itraconazole prophylaxis for 100 days after HSCT in 489 patients, which found incidences of IFD of 1.3% and 2.1%, respectively (94). A limitation in these trials was the reduced performance of the GM test in patients who received mold prophylaxis (95). This may have led to underestimation of IA, since the GM test is included in the EORTC/MSG definitions and—in most studies—is the most common mycological criterion fulfilled. With a false-negative GM test, many IAs will be classified as possible instead of probable, and thus be excluded from analysis (54).

Posaconazole prophylaxis during the pre-engraftment has not been properly evaluated, only two small studies involving 55 posaconazole recipients have been published (96, 97).

Micafungin was compared to fluconazole during neutropenia in a large study including 882 patients undergoing allogeneic transplantation (54%) or autologous transplantation (46%). The IFD incidence was low in both treatment arms (2.4% vs. 1.6%) and not significantly different (98). This was not unexpected, since patients undergoing autologous transplantation are not at risk of developing mold infections, so in reality the trial was underpowered.

The lipid formulations of amphotericin B have long half-lives, which may make them suitable for administration on alternate days in the prophylactic setting (99). The small studies that have been published so far have used either a high dose once a week, with reports of nephrotoxicity, or lower doses two or three times a week (97, 100). The results from a large placebo-controlled trial using 5 mg/kg liposomal amphotericin B twice weekly in patients with acute lymphatic leukemia are pending (NCT01259713), and may give a hint of whether this approach could also be useful after HSCT.

Inhalation of liposomal amphotericin B has—compared to fluconazole—been shown to significantly decrease invasive pulmonary aspergillosis in neutropenic non-HSCT patients (101). Aerosolized formulations of amphotericin B have also been tried after HSCT and appear to be safe, but the studies have been too small to be able to draw any conclusions regarding efficacy (102). It is important to remember that this prophylaxis only protects against lung aspergillosis, and that it must be combined with fluconazole or some other candida prophylaxis.

2.4.2 GVHD

In the setting of GVHD, prophylaxis with fluconazole was shown to be effective in preventing invasive candidiasis and candida-related death in the study by Marr and colleagues (60). Posaconazole has been shown to be as effective as fluconazole in prevention of all IFD in patients with grades II to IV aGVHD or chronic extensive GVHD, and superior in preventing IA (2.3% vs. 7.0%) at the end of the fixed 112-day treatment period (103). In the study by Wingard et al. comparing voriconazole with fluconazole, administration of the study drug was prolonged to 180 days after HSCT in high-risk patients, including patients treated with > 1 mg/kg prednisone per day on days 90–100. As mentioned above, there was a trend of fewer IFDs in voriconazole recipients (7.3% vs. 11.2%), but this did not reach statistical significance (61).

2.4.3 Secondary prophylaxis

With the introduction of RIC and less toxic antifungals (lipid formulation of amphotericin B, voriconazole and caspofungin), more patients with a history of IFD became eligible for HSCT. Retrospective studies strongly suggested a protective effect on relapse rates of IFD with secondary prophylaxis (104). A prospective study, conducted with voriconazole in 45 patients with a history of IFD (31 IA) prior to transplantation, confirmed the protective effect of secondary prophylaxis with only 3 patients developing an IFD the first year after HSCT (105). Recently, a prospective multicenter study from China reported a 1-year cumulative relapse rate of 25% in 130 patients with a history of IA before transplantation (106). In this study the antifungal used for treatment of IA was also used as secondary prophylaxis.

2.4.4 Other aspects of prophylaxis

Since the beginning of the 2000s, increasing azole resistance in clinical *Aspergillus* spp. isolates has been reported. The vast majority has been due to infection with an already resistant environmental isolate, resistance that is thought to be secondary to the use of agricultural fungicides (107). Acquired resistance still appears to be rare but has been reported in patients receiving long-term treatment because of chronic forms of aspergillosis (108). Development of resistance can also be due to selection of intrinsically resistant species, as reported in a prospective multicenter study of candidemia in France, in which recent exposure of fluconazole was associated with infection with an isolate with decreased susceptibility to fluconazole (109). Acquired azole resistance appears to be uncommon, but in a report from two tertiary cancer centers in the USA by Oxman et al., acquired rather than intrinsic resistance was thought to be the explanation in a third of cases with candidemia due to fluconazole-resistant isolates (110).

The rationale for recommending therapeutic drug monitoring when using voriconazole and posaconazole is the established dose-response effect combined with variable drug

concentrations when using standard dosages (111). The concept is uncontroversial for voriconazole because of its extremely variable pharmacokinetics. However, the recommendation when using posaconazole appears to more due to variable absorption of the oral solution than the pharmacokinetics, which is quite stable. With the introduction of a solid tablet formulation (already available in the USA, will be available during the fall in Sweden), the problem of variable absorption appears to be solved and drug monitoring during prophylaxis will be redundant (112).

2.4.5 Summary

Candida prophylaxis has been found to be beneficial both when administered during neutropenia and after engraftment up to day 75. Fluconazole prophylaxis is still a valid choice during the pre-engraftment phase unless there is a high local incidence of *C. glabrata* or *C. krusei*. The shorter duration of neutropenia after RIC probably reduces the risk of candidemia, but the data have not been altogether convincing, and administration of a safe, low-priced drug such as fluconazole seems reasonable. The effect on late-onset candida seen in the prolonged fluconazole prophylaxis trial by Slavin and Marr is probably less important today, since most patients with severe GVHD will receive mold-active prophylaxis. However, mold-active prophylaxis does not appear to be necessary as a routine prophylaxis after HSCT, due to the low incidence of early IMI, and should be reserved for high-risk patients such as those receiving corticosteroids because of GVHD. This will be explored in more detail section 5.4. Secondary prophylaxis is mandatory in patients with a history of IFD before transplantation. Although voriconazole has the best documented effect, it can also be an option to continue with the antifungal used for initial treatment.

2.5 DIAGNOSTICS

2.5.1 Candida

2.5.1.1 Candidemia and acute invasive candidiasis

The most common form of invasive candida infection in HSCT patients is candidemia. Usually the only symptom is fever, sometimes combined with signs of septic embolus to skin, lungs, or eyes. Undiagnosed candidemia during neutropenia can disseminate to the liver and spleen, resulting in hepato-splenic candidiasis (also known as chronic disseminated candidiasis), which usually cannot be diagnosed before recovery of neutrophils and the subsequent formation of multiple small abscesses.

Invasive candida infections can be quite difficult to diagnose. Blood culture is the gold standard, but the sensitivity has been reported to be only 50–75% (113). As a result, alternative tests based on the detection of fungal biomarkers and metabolites have been developed. Mannan (Mn) is a major component of the cell wall, and is one the main candida

antigens that circulate during infections (114). Initial observations showed an inverse relationship between circulating Mn and anti-mannan antibodies (A-Mn), which can both be measured with ELISA tests (Platelia® Ag Plus and Platelia® Ab Plus; Bio-Rad laboratories). A systematic review of studies evaluating Mn and A-Mn, published in 2010, found a sensitivity of 83% and a specificity of 86% when combining the two tests (115). Even though there was significant heterogeneity between the studies, it was concluded that a combined test was useful for diagnosis of invasive candidiasis (115, 116). A more recent case-control study including 56 patients with candidemia and 200 controls found a similar sensitivity—89%—but a specificity of only 63% when combining Mn and A-Mn (117). The reason for the poor specificity was the A-Mn test, which had a specificity of only 65% when used alone. However, using Mn test alone did not appear to be an option; even though the specificity was excellent at 97.5%, the sensitivity was only 59%. One explanation for this low sensitivity was that *C. parapsilosis* was not detected with the test (117).

Another test targets 1,3-β-glucan (BG), a major component in the cell wall of *Candida* spp, *Aspergillus* spp, *Pneumocystis jirovecii*, and many other fungi with the notable exceptions of the Mucorales and *Cryptococcus* spp. BG is released in blood and tissues during the course of an IFD, and is usually detected in plasma or serum. Detection in cerebrospinal fluid has also been shown to be useful, whereas the performance of BG assay in bronchoalveolar lavage fluid (BAL) is reported to be poor for fungal infections other than PCP (118, 119). There are four commercial tests available, three from Japan and one from the USA (Fungitell®; Beacon Diagnostics Laboratory, Cape Cod Inc.). They are all based on amoebocyte lysate from the horseshoe crab, but use different species. In the presence of BG, a coagulation cascade is initiated in the lysate, which leads to quantifiable transformation of a chromogenic substrate. Several prospective cohort studies have evaluated the performance of the BG assays, but differences in both populations and end-points make comparisons difficult. In addition, the cut-off values and number of positive tests required (one or two) have varied (116, 120-122). In a meta-analysis published in 2011, the performance of the BG assays was analyzed in patients with hematological malignancies (122). Six cohort studies including 1,771 adults with 215 IFDs were analyzed. Excellent specificity was found (98.9%)—but low sensitivity (49.6%)—when 2 consecutive positive tests were required. With an IFD prevalence of 10% (pre-test likelihood), the estimated positive and negative predictive values (PPV and NPV) were calculated to be 83.5% and 94.6%, respectively. With an IFD incidence of 2%, the PPV would decrease to 48% with a slight increase in NPV to 98.9%. Another large meta-analysis also including non-neutropenic patients, albeit with significant heterogeneity, found a pooled sensitivity of 76.8% and specificity of 85.3% in 2,979 patients with 594 proven or probable IFD (123). No difference in performance regarding *Candida* and *Aspergillus* infections was noted. Finally, another meta-analysis has shown BG assays to be of excellent value in diagnosing PCP, with a sensitivity of 94.8% (124).

The merits and usefulness of polymerase chain reaction (PCR) tests in blood specimens after HSCT will be discussed in section 5.1.

2.5.1.2 Chronic disseminated candidiasis

Reaching a diagnosis of chronic disseminated candidiasis can be difficult, since blood cultures are usually negative. BG assays and combined Mn and A-Mn testing is recommended. Imaging of liver and spleen should be performed, looking for focal lesions, and, if found, biopsied for microscopy, culture, and PCR. Biopsies are often microscopy-negative and culture-negative, possibly due to ongoing antifungal treatment or uneven spread of yeast in the lesions, in which case PCR has been shown to be of value (125, 126).

2.5.1.3 Summary

Due to the low incidence of invasive candida infections after HSCT when using candida-active prophylaxis, non-specific use of Mn and A-Mn, BG assays, and PCR tests is discouraged. In the event of prolonged neutropenic fever, the tests may be beneficiary but since the incidence of invasive candidiasis will also be low in this group, low PPV is a problem and may lead to a false-positive diagnosis. If signs of chronic disseminated infection are present on imaging of liver or spleen, low specificity will be less of a problem and BG and combined Mn and A-Mn testing in serum is recommended. In addition to the mandatory blood cultures, biopsies should be taken for microscopy, culture, and PCR.

2.5.2 Aspergillus

An invasive *Aspergillus* infection starts with inhalation of conidia, which germinate to form hyphae in the sinuses or lungs. After penetration of the epithelial barrier, it becomes an angioinvasive infection with risk of local bleeding and thrombosis. Occasionally the infection spreads hematogenously and becomes disseminated. The clinical picture varies according to the time period after HSCT. Early after HSCT, during the pre-engraftment phase, the first symptom of mold infections is usually fever, often followed by cough—sometimes with hemoptysis. Occasionally the first symptoms come from organs other than lungs and sinuses, such as the brain, due to unrecognized disseminated disease. In those cases, asymptomatic lung infiltrates are usually found on thoracic computed tomography (CT). *Aspergillus* infections after engraftment usually occur during treatment of GVHD, and, due to the presence of neutrophils, tend to have a slower course with low-grade fever and progressive respiratory symptoms.

The diagnostic tools of importance are culture and microscopy (of sputum, BAL, and biopsies), thoracic CT, GM test (serum and BAL), PCR test (blood specimens, BAL, and biopsies), and BG assays (serum).

2.5.2.1 Culture and microscopy

Microscopy is fast, easy to perform, and helpful to establish a diagnosis when positive (Figure 4). Since molds can be difficult to get to grow, microscopy should always be performed on biopsies, and it is also useful for sputum and BAL. The disadvantages of microscopy include variable sensitivity and not reaching a species identification (127).

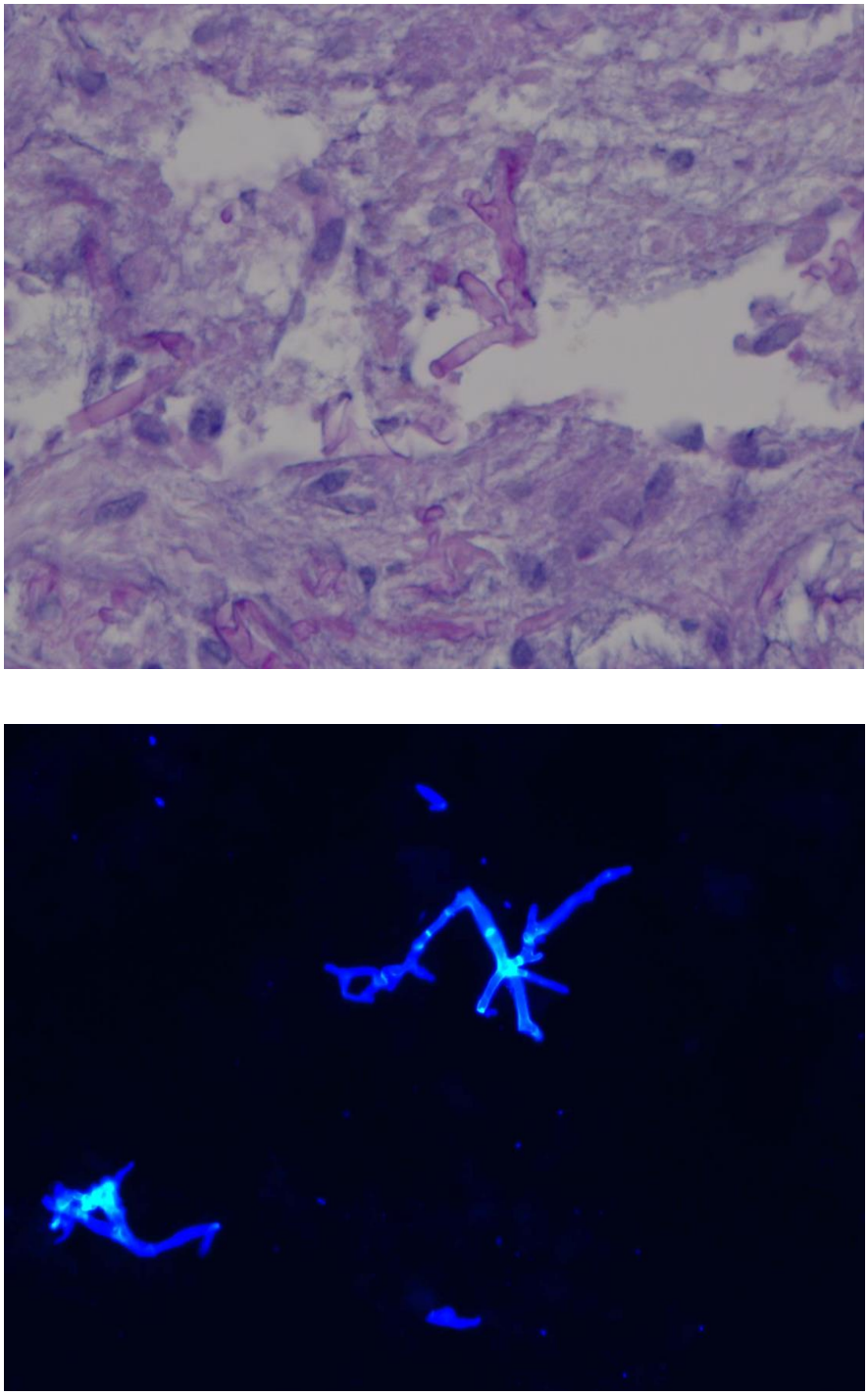


Figure 4a. Rhizopus microsporus *in a biopsy from sinus maxillaris, hematoxylin-eosin staining (top) and fluorescent staining (bottom).*

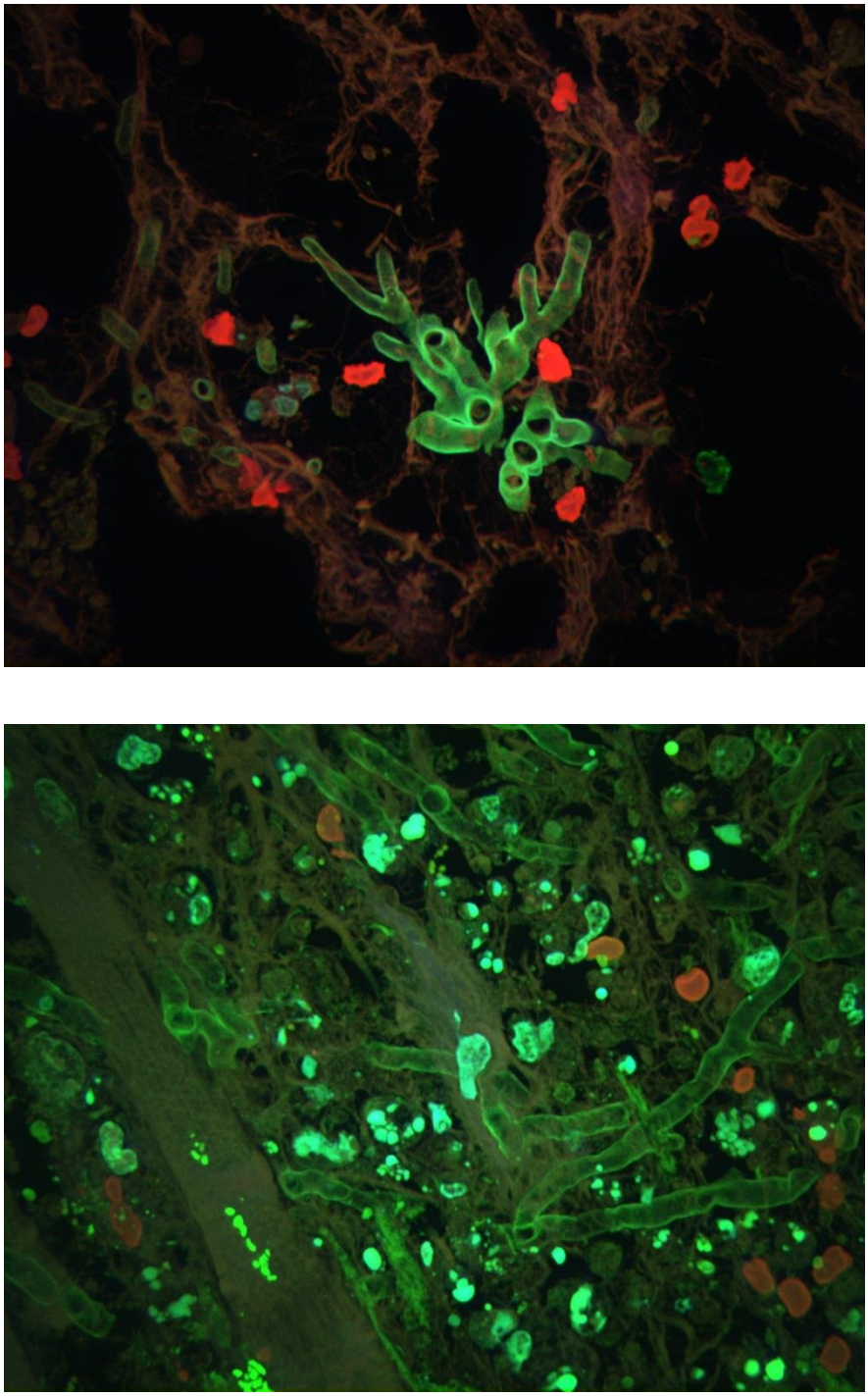


Figure 4b. *Biopsies from lung (top) and heart (bottom) taken at autopsy. Cultures showed growth of both* Aspergillus fumigatus *and* Fusarium spp. *The slides were stained by OmniFluorBrigth (OFB) stain mixture. The images were generated on a custom-built laser confocal microscope system at the Karolinska Imaging Core Facility (KIVIF) using the OFB Magic 5 computer program. Courtesy of Professor Laszlo Szekely, Department of Microbiology, Tumor and Cellbiology (MTC), Karolinska Institutet, Stockholm.*

Cultures from sputum, BAL, and biopsies remain important and allow both species identification and determination of MIC values, and they should always be performed. However, culture has low sensitivity (compared to galactomannan; for references, see *2.5.2.3 Galactomannan test*) and cannot discriminate between colonization and invasive infection.

2.5.2.2 CT

Thoracic CT is a very important diagnostic tool. Findings shown to be associated with IA are macronodules with or without halo sign, cavity (within a dense infiltrate), and air-crescent sign (54, 128). A macronodule is a dense, well-circumscribed lesion over 1 cm in diameter, with opacity that completely obscures the background (128). The halo sign has been defined as a macronodule surrounded by a perimeter of ground-glass opacity, the "halo" (Figure 5) (128). Histologically, this represents an infarction or necrosis surrounded by hemorrhaging. The air-crescent sign is made of a gas pocket between a lung sequestrum attributable to necrosis and a rim of viable lung (Figure 4) (128). Macronodules are often the first, early CT finding, followed by the halo sign, due to development of hemorrhaging around the perimeter, and, later on, by the air-crescent sign, as the necrosis gives rise to a lung sequester and the resulting cavity is filled by gas (Figure 5) (129). If the treatment is successful, regression of the air-crescent sign is gradually seen until only a scar is left (Figure 6). However, even with adequate treatment, the infiltrates will increase during the first week of treatment, will be stable during the second week, and will only then start receding (129).

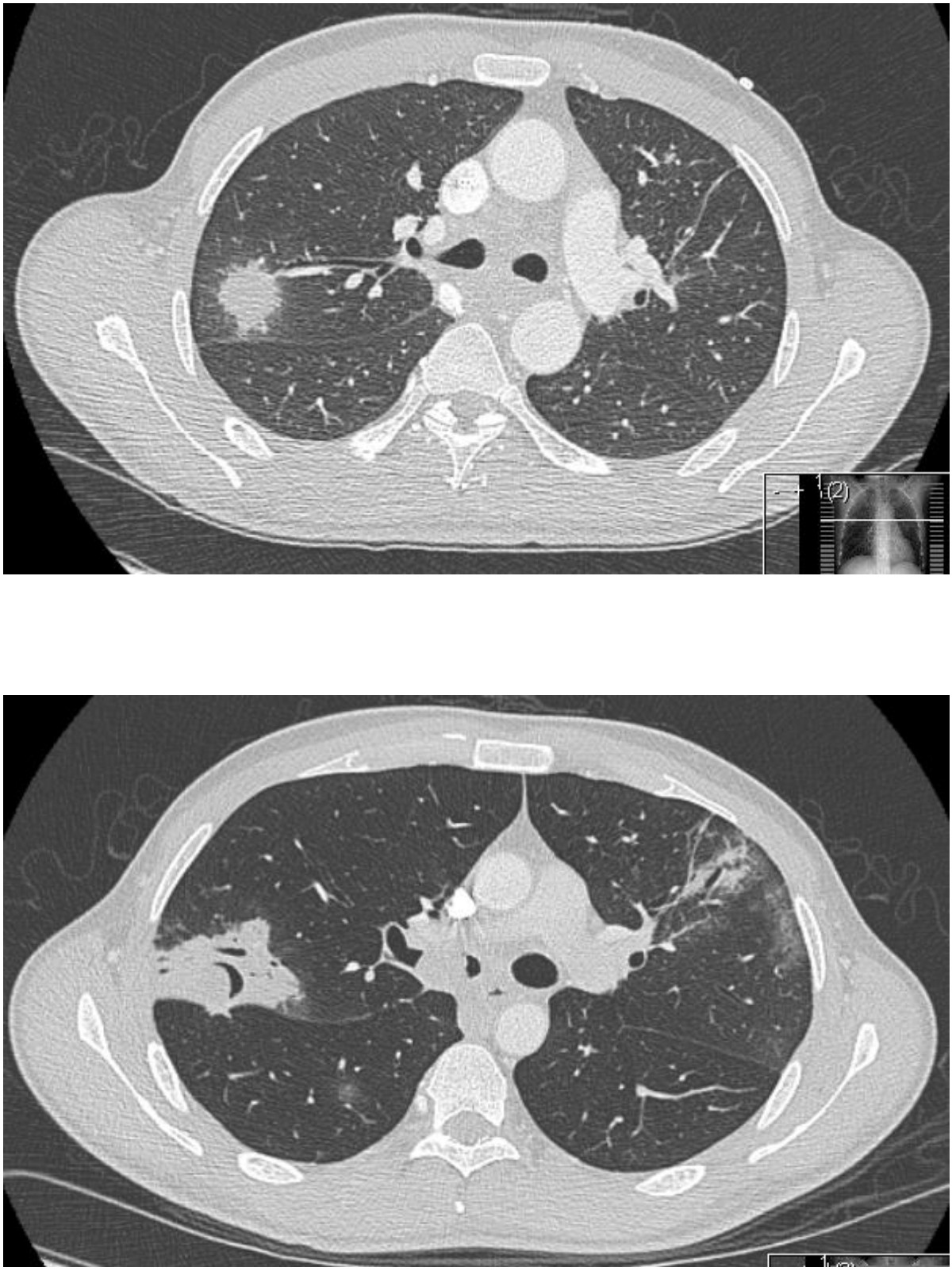


Figure 5. *Halo sign (top), followed by air crescent sign and new infiltrates in the left lung (bottom).*

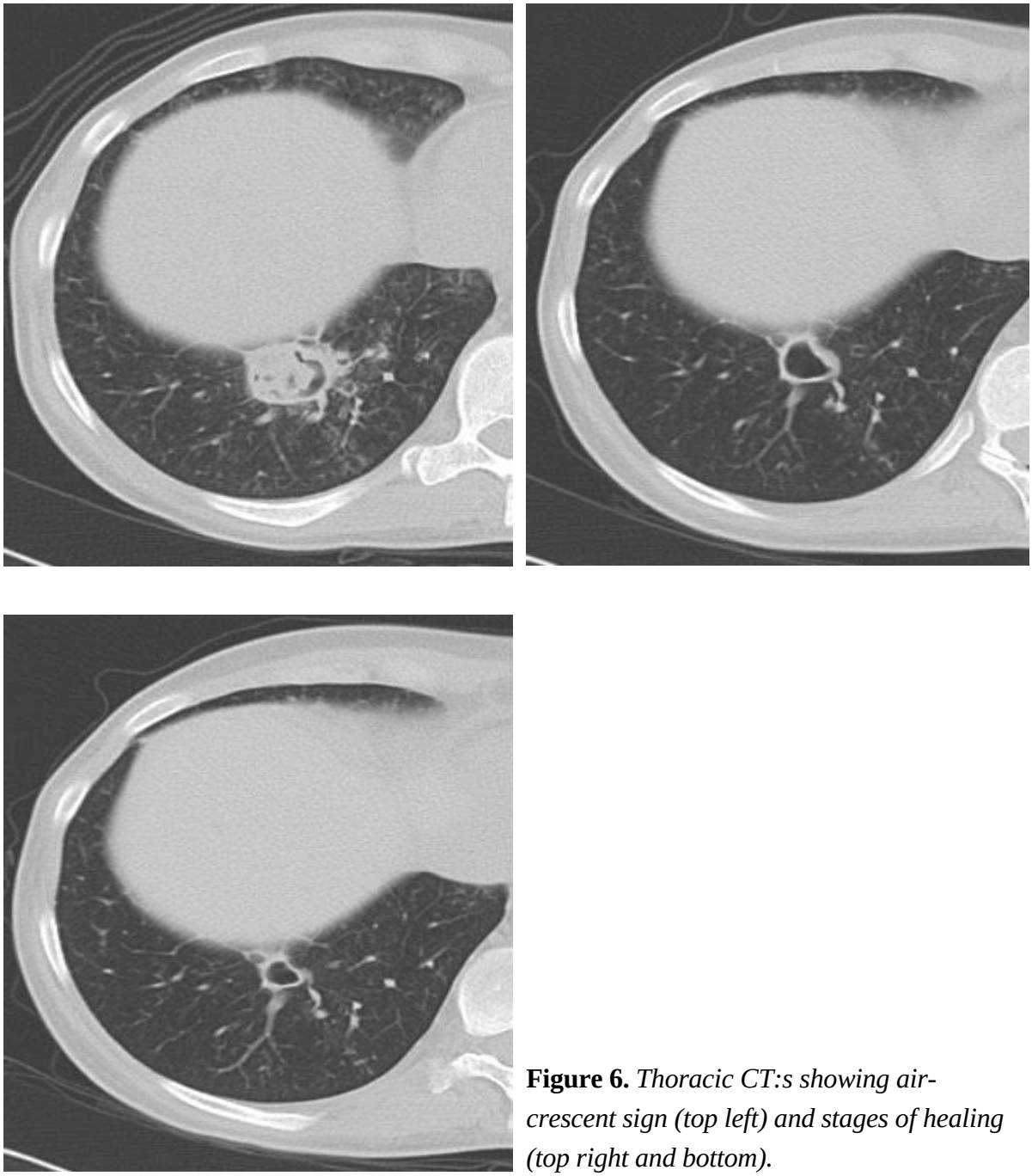


Figure 6. *Thoracic CT:s showing air-crescent sign (top left) and stages of healing (top right and bottom).*

2.5.2.3 Galactomannan (antigen) test

GM is a polysaccharide cell wall component of *Aspergillus* that is released by growing hyphae. Detection of GM in patients with IA was first described in 1979, initially in plasma, and later in BAL and CSF (130). A commercial enzyme immunoassay using monoclonal antibodies against GM became available in the late 1990s (Bio-Rad Laboratories). The performance of the test in plasma and BAL has been examined in a large number of studies, and several meta-analyses and guidelines reviewing the usefulness in clinical practice have been published (116, 131, 132). A problem that all of these studies have faced is that the GM test is incorporated into the criteria for probable IA (54). Thus, when evaluating the GM test,

the mycological criteria will have to be fulfilled primarily by a positive culture. As the potential benefit of the GM test is a higher sensitivity than culture, is has been difficult to establish which cases are true positives (i.e. probable IA) in the trials. Different approaches have been used, such as combining the criteria with clinical findings, or only including proven cases.

In a recent meta-analysis by Heng et al., the accuracy of GM in BAL was reviewed (131). This analysis included 16 studies with a total of 783 adult patients with hematological malignancies. Using an optical density cut-off value of 1.5, the pooled sensitivity and specificity for IA were as high as 92% and 98%, respectively. However, these results have been contradicted in a very recent study investigating 586 BALs performed in hematological patients because of respiratory symptoms and/or suspected IFD (133). IFD was classified as probable in 8.5% of the cases and as proven in 1.5%, according to the revised EORTC/MSG definitions (even though clinical judgment was stated to be the gold standard for IFD). The sensitivity, specificity, PPV, and NPV in this study were 50%, 73%, 16%, and 93%, respectively. An earlier meta-analysis investigating the performance of the GM test in plasma of hematological patients found the pooled sensitivity and specificity to be 58% and 95%, respectively (132). A more recent study by Koo et al. investigated GM in plasma from HSCT recipients and found a sensitivity of 64% and a specificity of 91% (134). Significant differences in the performance of the test have been reported, with better performance in neutropenic patients and worse performance in patients receiving active treatment or prophylaxis against molds (95, 135-138). This is not surprising, since *Aspergillus* is angioinvasive in the neutropenic setting, while in non-neutropenic patients the infection is often confined to the lung and less GM will be released into the blood. Active treatment or prophylaxis inhibits the growth of hyphae, leading to a lower level of GM in the blood and to reduced sensitivity of the test. One important reason for false-positive tests has been concomitant treatment with antibiotics, especially piperacillin/tazobactam (139). This is due to the presence of GM in batches of antibiotics, but the problem has been reported to be of less importance in recent years (140).

2.5.2.4 BG assays

The BG assays have mainly been tested for detection of candida infections. In addition, no large studies of BG assays have been performed in HSCT recipients, so currently there is no information about the usefulness of the test for diagnosis of IA in this population. However, more information will be available shortly, since the validity and usefulness of a BG assay (as well as a standardized PCR test) will be analyzed in an ongoing EORTC-initiated randomized trial comparing empirical and pre-emptive (diagnostic-driven) treatment in over 500 HSCT recipients (NCT01288378).

2.5.2.5 PCR

Aspergillus PCR has been tried on blood specimens, BAL, and biopsies. An important limitation is the lack of a standardized assay. A commercial PCR assay has been available for a couple of years, but in-house PCRs are performed at many institutions. Recently, a standardized *Aspergillus* PCR has been proposed by EAPCRI (the European Aspergillus PCR Initiative), a working group of ISHAM (the International Society for Human and Animal Mycology), and it is currently being evaluated in the EORTC trial mentioned above. The merits of current PCR assays in blood specimens after HSCT will be discussed in more detail in section 5.1.

The diagnostic performance of *Aspergillus* PCR in BAL has varied in the published literature. In a meta-analysis from 2011 including 17 studies with 1,191 patients defined as "immunocompromised or at-risk patients", the pooled sensitivities and specificities were as high as 91% (CI 79–96%) and 92% (CI 87–96%), respectively (141). Subgroup analysis showed that the performance of the PCR assay was influenced by the methodology, the primer design, and the methods of cell wall disruption and DNA extraction. In the meta-analysis by Heng investigating the diagnostic performance of GM in BAL from patients with hematological malignancies, the performance of *Aspergillus* PCR was evaluated in six of the studies included (131). The pooled sensitivities and specificities of PCR in these six studies were 57% (CI 31–80%) and 99% (CI 60–100%), respectively. The corresponding values for GM test in the six studies were 79% (CI 69–87) and 97% (CI 95–99%). When a positive result was defined by positivity of either GM test or *Aspergillus* PCR, the sensitivity increased but with a modest decrease in specificity: 84% (CI 79–88%) and 94% (CI 91–97%), respectively. Proposed possible reasons for the difference in results in the two meta-analyses were different patient groups (only hematological malignancies in the latter), the number of studies included, and potential differences in antifungal treatment. In a recent study including 116 patients with hematological malignancies, Heng reported that the sensitivity and specificity were 61% and 93%, respectively, for GM testing on BAL, and 78% and 79%, respectively, for PCR on BAL. Both had NPV between 85% and 90%. The authors concluded that the major use of the tests would be to rule out IA if results were negative.

In tissue samples (biopsies) from patients with proven IFD, PCR has been reported to as sensitive as microscopy, which is currently considered the gold standard (142). It has also been found to be superior to culture in establishing an etiological diagnosis in microscopy-positive biopsies (142-145). Furthermore, in a study of 165 microscopy-negative specimens (including both biopsies and BAL) from 162 patients, a broad-range PCR showed a sensitivity, specificity, PPV, and NPV of 57%, 97%, 80%, and 92%, respectively (142).

2.5.2.6 Summary

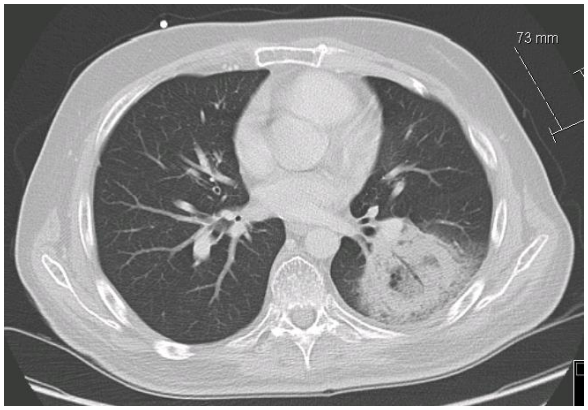
The primary diagnostic tools for diagnosis of IA are thoracic CT in combination with GM test in plasma. If infiltrates are found, BAL should be taken for analysis with culture,

microscopy, GM test, and perhaps PCR. The diagnostic performance of *Aspergillus* PCR in BAL appears to be similar to or a little worse than the performance of the GM test. Combining the two tests may be optimal for ruling out IA, but the cost-effectiveness of this strategy has not been evaluated. The usefulness of GM for surveillance will be discussed in section 2.6.2. Biopsies of suspected mold infections should always be pursued for analysis with microscopy, culture, and PCR. *Aspergillus* PCR assays in plasma or whole blood may be of value, but they will be discussed in more detail in section 5.1.

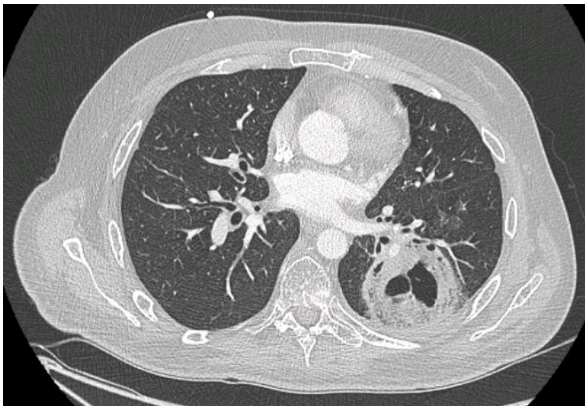
2.5.3 Other molds

Mucormycosis is characterized by development of tissue necrosis resulting from angioinvasion and subsequent thrombosis. The infection is often rapidly progressive and the mortality is high. The most common manifestations are pulmonary infection, rhinocerebral infection (both developed after inhalation of spores), cutaneous infection (resulting from direct inoculation with fungal spores into the skin), and disseminated infection (73). Symptoms of pulmonary infection are usually fever, cough, pleuritic thoracic pain, and sometimes hemoptysis, thus being indistinguishable from those of aspergillosis. As treatment of mucormycosis and aspergillosis differs, it is very important to reach an etiological diagnosis. Since there are no biomarkers for the diagnosis of mucormycosis, microscopy, culture, and PCR—of sputum, BAL, and biopsies—are the diagnostic tools available besides CT. Hyphae of the Mucorales have some features differentiating them from those of *Aspergillus*, such as irregular ribbon-like appearance, and that the angle of the branching includes wide-angle (90°) bifurcations (146). The CT findings are often the same as seen in invasive aspergillosis except for the reversed halo sign, a focal ground-glass attenuation surrounded by a ring of consolidation, which has been shown to be associated with a diagnosis of mucormycosis (Figure 7) (147).

Invasive *Fusarium* infections after HSCT often present as disseminated infection with metastatic skin lesions (148). Neutropenia and GVHD are important risk factors. The diagnosis is made through the identification of *Fusarium* spp. by culture, most often from blood and biopsies. No reliable biomarkers exist, but importantly, invasive fusariosis can give rise to a positive GM test, which may falsely be interpreted as being caused by IA (149).



Day 0



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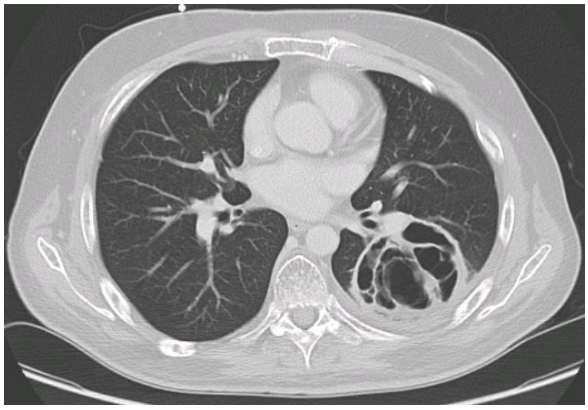

Day 15

Figure 7. *Thoracic CT showing reversed halo sign at the time of diagnosis (top left), after 11 days of treatment (top right) and after 15 days of treatment (bottom). Mold prophylaxis—posakonazole (200 mg three times daily)—was administered because of grade III aGVHD in a patient transplanted with a matched unrelated donor. Due to onset of fever and throat pain on day 111 after HSCT, a CT was performed, which showed an abscess in the thymus. Thoracic CT was performed as part of the diagnostic work-up and showed reversed (or inverse) halo sign. Fine-needle biopsy of the thymus was inconclusive and microscopy, culture, and PCR of BAL were negative. A new biopsy (using a thicker needle) of the thymus was performed, which showed hyphae, and Rhizopus microsporus was identified both by culture and PCR. Liposomal amphotericin B (5 mg/kg) was administered. New CTs after 11 days and 15 days of treatment showed an increasing cavity and suspected angioinvasion. To prevent major lung hemorrhage, operation with lobectomy (combined with removal of the abscess in the thymus) was performed. Peroperative cultures from the thymus showed growth of Rhizopus microsporus despite 20 days of treatment with liposomal amphotericin B.*

2.6 TREATMENT

2.6.1 Empirical treatment

IFD was shown as early as 1966 to be an important complication in neutropenic patients, and difficult to diagnose before death (150). As a consequence, physicians started to use amphotericin B deoxycholate as empirical fungal treatment in neutropenic patients with persistent fever. Eventually, two small underpowered randomized trials were published, both of which found fewer IFDs in persistent febrile neutropenic patients who received additional empirical treatment with amphotericin B than in patients who received antibiotics alone (151, 152). Other uncontrolled studies found similar results, and as a result empirical antifungal treatment became the standard of care (153). Several large randomized multicenter trials have compared different drugs for empirical antifungal treatment of neutropenic patients with persistent fever despite broad-spectrum antibiotics, but none have used placebo. The first large trial was published in 1999, and compared amphotericin B deoxycholate with liposomal amphotericin B in 687 patients (46% bone marrow recipients, but it was not stated how many were allogeneic). It found less breakthrough fungal infections, less infusion-related toxicity, and less nephrotoxicity with the lipid formulation (154). The next study, published in 2002, compared voriconazole and liposomal amphotericin B in 837 patients (18.5% HSCT). The overall success rate was a little higher in patients who received liposomal amphotericin B (26.0% vs. 30.6%), but voriconazole recipients had fewer breakthrough infections and fewer cases of nephrotoxicity (155). The echinocandins then came on the scene, and in 2004 a trial comparing caspofungin and liposomal amphotericin B in 1,095 febrile neutropenic patients was published. As in previous trials, the efficacy was similar but caspofungin was better tolerated with fewer infusion-related side effects and less nephrotoxicity (89).

There are at least two important reasons for the lack of difference in the trials other than the obvious one, i.e. that they had similar efficacy. Firstly, the primary outcome in all studies was a composite endpoint including several factors, such as resolution of fever, discontinuation of study drug, breakthrough infections, survival, and successful treatment of baseline fungal infection. Secondly, the vast majority of neutropenic patients with fever despite four days of treatment with broad-spectrum antibiotics do not have an IFD and do not need antifungal treatment.

2.6.2 Pre-emptive treatment

The knowledge that most neutropenic patients do not need empirical antifungal treatment led to a new approach: the pre-emptive or diagnostic-driven approach. This approach uses fungal surveillance, usually GM testing (with or without *Aspergillus* PCR) two to three times a week, during periods of high risk. If a test turns positive, a thoracic CT is performed, and if infiltrates are found, BAL is performed. In no infiltrate is seen, no fungal treatment is given and fungal surveillance is continued. If a test turns positive again, a new thoracic CT is performed, and so on. This approach has two theoretical advantages: a reduction in

unnecessary empirical fungal treatment in neutropenic patients with persistent fever despite broad-spectrum antibiotics, and an early detection of mold infections in patients with non-specific or discrete symptoms. The first group to test a pre-emptive approach with GM test in a prospective cohort was Maertens and colleagues in 2005 (156). They found a 78% decrease in the use of antifungals compared to when a standard empiric approach was used, with only one missed case of IFD (a case of mucormycosis). These findings led to a subsequent randomized trial by Cordonnier and colleagues, comparing a pre-emptive approach using the GM test with an empirical approach in 293 patients with hematological malignancies and an expected neutropenia of more than 10 days (157). The results showed reduced use of antifungals but a significantly higher incidence of IFD during induction therapy in the pre-emptive group. Since then, several small studies have shown good results using the pre-emptive approach, and in daily practice it is used in many centers in order to reduce side effects and avoid the cost of unnecessary antifungals (158). Some publications have reported successful incorporation of PCR in the pre-emptive approach (159, 160).

However, there are some potential caveats using a diagnostic-driven approach with GM testing. The pre-test likelihood of a true positive GM test is important: with an expected IA incidence of 10%, and a test sensitivity of 64% and specificity of 91%, as found by Koo et al. in HSCT patients, the PPV would be 44% (134). With an expected IA incidence of 5%, the PPV would be 27%. Thus, using a pre-emptive approach in a low-incidence population will give a lot of false-positive GM tests, resulting in unnecessary CTs and perhaps even unnecessary treatment. Another concern is that mold-active prophylaxis reduces the sensitivity of the GM test, and the two trials investigating the feasibility of the surveillance approach were performed in centers using fluconazole prophylaxis (156, 157). The problem with low incidence was nicely shown in a paper published by Duarte et al. in August this year, in which surveillance with GM tests was used in 262 high-risk episodes in hematological patients receiving posaconazole prophylaxis (138). Due to the low breakthrough rate of IA (1.9%), the PPV of the GM test was as low as 12%. False-positive GM tests were seen in 13.8% of all at-risk episodes. However, in patients with clinical suspicion of IA the PPV of GM tests was still high: 89.6% (138). The author concluded that the low pre-test likelihood of IA made the test unreliable for surveillance in asymptomatic patients. The pre-emptive approach may therefore be of value only in patients who are not receiving mold-active prophylaxis. Finally, GM is a test for aspergillosis and does not work for other mold infections such as mucormycosis and fusariosis, so there is a risk of delayed diagnosis of these infections if postponing thoracic CTs because of negative GM tests.

The role of PCR in the diagnostic-driven approach will be discussed in section 5.1.

2.6.3 Antifungal treatment at our institution

At our institution empiric treatment with liposomal amphotericin B for prolonged neutropenic fever was the predominant approach until the end of the 2000s, with the exception of the

patients included in paper I and during parts of the period 1998–2001 when the institution participated in a multicenter study of a PCR-based approach. Empirical treatment has now been replaced with an approach closer to the diagnostic-driven approach. Because of the low pre-engraftment incidence of IA found in paper I in this thesis, fungal surveillance with GM tests was judged not to be meaningful (62). Instead, an investigation is initiated when signs compatible with IMI occur—most often persistent neutropenic fever and/or cough. The investigation typically consists of a thoracic CT and GM testing in serum. If the CT shows infiltrates, a BAL is performed for culture, microscopy, and GM testing. If both GM test and CT are negative, no antifungals are administered. Interestingly, this approach resembles the one proposed in the recently published study by Duarte (138). However, if the patient deteriorates, empirical fungal treatment is usually initiated anyway, most often liposomal amphotericin B 3 mg/kg per day.

2.6.4 Candida

Several large randomized drug trials for treatment of candidemia have been performed, but the number of neutropenic patients included has been around 10% or less. First out was a comparison of fluconazole and amphotericin B deoxycholate in 1994, followed by caspofungin vs. amphotericin B deoxycholate in 2002, voriconazole vs. amphotericin B deoxycholate followed by fluconazole in 2005, and micafungin vs. caspofungin, anidulafungin vs. fluconazole, and micafungin vs. liposomal amphotericin B in 2007 (90, 161-165). All trials showed noninferiority regarding efficacy but, as expected, more nephrotoxicity and infusion-related side effects with the amphotericin B formulations. In the trial comparing anidulafungin with fluconazole, superiority of anidulafungin was noted but due to a possible center effect, the authors recommended that this result should be interpreted with caution (90). In addition, there was a trend of shorter time to first negative blood culture, possibly due to the fungicide effect of echinocandins on candida. In 2012, Andes and colleagues published a patient-level quantitative review including data from seven randomized trials for treatment of invasive candidiasis, and found that use of an echinocandin and removal of CVC were associated with improved survival (86).

Since the number of neutropenic patients included in the treatment studies was limited, the optimal treatment for patients with neutropenia and after HSCT has not been fully investigated. However, since echinocandins have been used successfully in neutropenic patients in trials investigating empirical antifungal therapy, they would appear to be the logical first-hand option for treatment of invasive candidiasis in these patients also. In addition, the shift towards invasive infections with non-albicans species makes resistance an issue with the azoles.

The duration of treatment for uncomplicated candidemia is 14 days after the first negative blood culture if neutropenia has resolved (166). Fundoscopy to rule out metastatic infection of the eye should always be performed, as the incidence in non-neutropenic patients is as high

as 16% in the setting of candidemia (167). The optimal treatment duration for hepato-splenic candidiasis remains to be decided, but a long duration (3 months or more) is usually needed.

Removal of CVCs is generally recommended in the non-neutropenic patients with candidemia (86). This principle has not been proven in neutropenic patients in whom the source of the candidemia is the GI-tract in the vast majority of cases. The recent recommendation of European Conference of Infections in Leukemia (ECIL) is, despite the lack of solid evidence, to remove the CVC when possible (www.Kobe.fr/ecil). If removal is not possible, treatment with an echinocandin or liposomal amphotericin B is recommended.

2.6.5 Invasive aspergillosis

The first randomized trial of importance for treatment of IA was published in 1998, and compared treatment with 1 mg/kg or 4 mg/kg of liposomal amphotericin B per day (168). The efficacy rates were similar in the two groups, but the study was small (87 patients) and today the majority of cases included would be classified as possible IA due to lack of mycological evidence. In 2002, Herbrecht and colleagues published a study comparing voriconazole and amphotericin B deoxycholate for treatment of IA (169). The study included 277 patients in the modified intention-to-treat population, 55% of whom had hematological malignancies, 45% had neutropenia at the time of diagnosis, and 24% were HSCT recipients. Treatment with voriconazole was found to be superior at follow-up (week 12), both regarding successful outcome of IA (53% vs. 32%) and overall survival (71% vs. 58%) (169). As expected, there was a big difference in the tolerability of the drugs: 80 of the patients receiving amphotericin B had to change therapy to other licensed antifungals due to side effects, as compared to 14 of the voriconazole recipients (170). However, even though side effects may have had some effect, difference in drug efficacy seemed to be of importance since differences in the mortality curves were evident as early as after 14 days of therapy. Voriconazole has been considered the first-hand option for treatment of aspergillosis ever since. Notably, the success rate for HSCT patients was lower than for other patients in both cohorts; it was 32% in voriconazole recipients. In 2007, the Ambiload trial was published, comparing treatment of IA with liposomal amphotericin B in 2 different regimens: 3 mg/kg per day vs. 10 mg/kg per day for 14 days followed by 3 mg/kg per day (171). The favorable response rate in patients receiving 3 mg/kg per day was 50% and overall survival at 12 weeks was 72%. The efficacy was similar in patients who received 10 mg/kg per day but with significantly more nephrotoxicity.

Even though it is difficult to compare two different trials, the voriconazole trial and the Ambiolad trial had very similar response rates (53% and 50%) and overall survival at 12 weeks (71% and 72%). More patients had hematological malignancies and neutropenia at baseline in the Ambiload trial, but there were more HSCT recipients in the voriconazole trial. Both trials accepted radiological findings of halo sign or air-crescent sign as fulfilling probable IA criteria in HSCT recipients and patients with recent neutropenia, even without

mycological evidence (modification of the EORTC/MSG criteria). However, in the Ambiload trial, 59% of all patients were included in this way, compared to 32% of the voriconazole recipients in the voriconazole trial. As this can be regarded as an early state of IA with a better prognosis, it may have worked in favor of liposomal amphotericin B (172). In addition, GM test was not available in the voriconazole study but was used in the Ambiload trial with only 17% of the patients having another mycological criterion fulfilled (169, 171). Again, this indicates early detection of IA and possibly a better prognosis. Other concerns with liposomal amphotericin B include the well-known nephrotoxicity when administered for long periods of time, requirement for intravenous administration, and a high price. Thus, voriconazole is the recommended drug for treatment of IA in patients not receiving prophylaxis with a mold-active azole. If voriconazole is not suitable because of intolerable side effects, drug interaction, or recent or ongoing prophylaxis with a mold-active azole, liposomal amphotericin B is the first-hand alternative.

Caspofungin has been the most used echinocandin for treatment of IA. In a non-comparative salvage study, the response rate was shown to be 45%, and in case series even higher response rates have been reported (173). In addition, is was well tolerated, and is an alternative in patients who do not tolerate voriconazole or liposomal amphotericin B. Data on the other echinocandins in the treatment of IA is limited.

In the near future, the results from one newly started trial and from two recently finished trials will probably change the picture. Posaconazole is only approved for salvage therapy of IA. However, with the recent approval of both an intravenous formulation and a tablet with good absorption, the time has come for a treatment trial comparing posaconazole with voriconazole. Such a trial has just started recruiting patients, and is planned to be completed in 2017. A recently completed trial compared isavuconazole with voriconazole for the treatment of IA, and according to preliminary data presented at ECCMID earlier this year, isavuconazole reached non-inferiority. The paper has not been published yet but according to a press release from Astellas, the all-cause mortality at day 42 in the intention-to-treat population (n = 516) was 18.6% in the isavuconazole treatment group and 20.2% in the voricanozole treatment group. In addition, patients receiving isavuconazole experienced significantly fewer study drug-related adverse events. Thus, it looks like isavuconazole will get a first-hand recommendation beside voriconazole.

An important recent trial compared combination therapy with voriconazole and anidulafungin vs. voriconazole alone for IA. The preliminary data have been presented at ECCMID 2012 by Marr and colleagues, who reported a trend of better overall survival at week 6 with combination treatment, but this did not reach statistical significance (174). In a subgroup analysis of patients with probable IA diagnosed with GM test only (218 of 277 patients in the modified intention-to-treat population), the difference was statistically significant. One interpretation of this finding would be that in patients with IA diagnosed early, combination therapy is superior, whereas in patients diagnosed later in the course of the infection, the prognosis is poor regardless of the kind of therapy. The full paper has not been published, but

will probably have a huge impact on the long-lasting discussion about the merits of combination treatment, something that many physicians have used in desperate situations even though evidence has been lacking.

Favorable outcomes with granulocyte transfusions in neutropenic patients with fungal infections have been reported in case series, but there have only been a few randomized studies, difficult to interpret (175, 176). The latest randomized study investigated granulocyte transfusion in neutropenic patients with fever and pulmonary infiltrates, or tissue infection, or IFD, and was published in 2008. Unfortunately, due to methodological difficulties and a large proportion of rapidly recovering patients in both treatment arms, no conclusions could be made except that the treatment was safe (177). However, recently a randomized trial, designed to include 114 patients with IFD or severe bacterial infection, has been completed and will hopefully provide some answers regarding the clinical use in neutropenic patients with IFD (ClinicalTrials.gov, NCT00627393).

The first randomized trial showing successful therapy of IA with T cell therapy was published in 2005 by Perruccio and colleagues (178). Donor derived T cell clones specific for *Aspergillus fumigatus* were administered to patients with IA, which resulted in markedly reduced serum levels of GM and only 1/10 dying in the therapy group vs. 6/13 in the control group. However, the generation of the specific T cells was laborious and time-consuming, taking 4-6 week for cell expansion, thus limiting the clinical potential. Since then new methods have shortened the time to 14 days, making adoptive T cell therapy a future option for treatment of IA (179). Very recently another approach was published, creating genetically modified T cells using a gene-transfer system to enforce expression of a chimeric antigen receptor that recapitulates the specificity of Dectin-1, a fungal pattern-recognition receptor (180). The cells were shown to inhibit hyphal growth both in vitro and in vivo, and may be an important treatment option in the future.

2.6.6 Other molds

The recommended first-line treatment for mucormycosis is liposomal amphotericin B (5 mg/kg per day), combined with surgical debridement if possible (146). Some of the Mucorales are sensitive to posaconazole, which may either be used as follow-up treatment after a good response to liposomal amphotericin B, or as salvage therapy if liposomal amphotericin B cannot be used or has failed (146). A recently published, small retrospective study found a possible benefit of combined liposomal amphotericin B and posaconazole (181). Granulocyte colony stimulating factor is highly recommended in the case of neutropenia, and treatment with glucocorticoids should be stopped if possible. Time to diagnosis and treatment is of the essence since these infections often have very rapid progress.

There are no data regarding the optimal treatment for fusariosis after HSCT. In a retrospective study of 233 cases, the 90-day survival probability during 2001–2011 was 60% with voriconazole and 53% with liposomal amphotericin B (76).

3 AIMS

3.1 GENERAL AIMS

To improve our knowledge of the incidence and risk factors for IFD and IMI after HSCT in Sweden. To try to identify which patients will benefit the most from mold-active prophylaxis.

3.2 SPECIFIC AIMS

Paper I — To determine whether fungal PCR-guided early antifungal treatment reduces the incidence and mortality of proven and probable IFD after RIC HSCT. To determine whether PCR contributes to reaching a diagnosis of probable or proven IA after RIC HSCT. To investigate risk factors for IA after RIC HSCT.

Paper II — To determine whether pneumonia-associated early death (before day 100 after HSCT) is reduced with RIC relative to MAC. To investigate etiologies and risk factors for pneumonia-associated death after HSCT.

Paper III — To determine the relationship between tissue concentrations and plasma concentrations in patients receiving posaconazole prophylaxis.

Paper IV — To determine the incidence and risk factors for IMI in a large cohort of HSCT recipients performed in a center with a high autopsy frequency.

4 MATERIAL AND METHODS

4.1 SUBJECTS

4.1.1 Paper I

This study investigated the effect of randomizing patients with a positive fungal PCR test to antifungal treatment or no treatment. All patients undergoing HSCT after RIC at the Karolinska University Hospital, Huddinge, between April 2002 and November 2006 were eligible for inclusion, the only exclusion criterion being hypersensitivity to liposomal amphotericin B (the patient characteristics are presented in Table 2). In total, 99 patients were included and followed by fungal PCR once a week for the first 100 days after transplantation. Patients with a positive PCR result were randomized to antifungal treatment with liposomal amphotericin B or to no treatment. Liposomal amphotericin B was given at a dose of 3 mg/kg per day for patients with positive PCR result for *Aspergillus*. After 3 days, the dose was reduced to 1 mg/kg per day if the patient was in a stable clinical condition. Patients with a positive *Candida* PCR received 1 mg/kg per day. Treatment was continued until there was one negative PCR test in patients without fever and neutropenia, and two consecutive negative PCR tests in patients with fever and/or neutropenia. Depending on the patient's clinical status, the treatment could be prolonged according to the judgment of the treating physician. Patients with fever above 38.5°C for 3 days despite treatment with antibiotics, and with unknown etiology, were considered eligible for antifungal treatment regardless of the PCR results.

4.1.2 Paper II

In this study, the incidence of and risk factors for early and overall death due to pneumonia after HSCT were investigated. All patients transplanted at Karolinska University Hospital, Huddinge, between January 2000 and December 2009 were screened (n = 691; the patient characteristics are presented in Table 2). All the patients who died during the study period were identified (n = 273), and their clinical data were investigated. Patients in whom pneumonia could possibly have contributed to death (n = 155) were included for further analysis regarding causes of death and pneumonia etiology.

4.1.3 Paper III

In this study, tissue concentrations of posaconazole were analyzed and compared to serum concentrations. Seven HSCT recipients who had received posaconazole at the time of death, or had received posaconazole shortly before the time of death, were included. Multiple biopsies from brain, heart, lung, liver, and kidney were taken during routine autopsy and stored at -80°C until analysis. One to three blood samples per patient were collected during the posaconazole treatment period and stored at -80°C until analysis.

Table 2. *Patient characteristics in papers I, II, and IV*

	Paper I 99 RIC 2002-2006		**Paper II Cons. pat. 2000–2009**		**Paper IV Cons. pat. 2001–2012**
	No IA n = 90	**IA n = 9**	**RIC n = 336**	**MAC n = 355**	**All n = 843**
Median age(range)	54 (11-69)	59 (48-65)	51 (0-71)	24 (0-61)	38 (<1-71)
Sex (M/F)	59/31	5/4	191/45	222/133	508/335
Malignancy	85	8	274	317	705 (84%)
Non-malignancy	5	1	62	38	138 (16%)
Disease stage (early/late)[#]			137/140 (41/59%)	179/175 (50/50%)	413/389 (49/51%)
Donor					
MRD	37 (41%)	4 (44%)	135 (40%)	128 (36%)	292 (35%)
MUD	45 (50%)	5 (56%)	167 (50%)	158 (45%)	429 (51%)
MM	8 (9%)	0	34 (10%)	69 (19%)	122 (14%)
TNC dose ($\times 10^8$/kg)	11.1 (0.2-33)	10.8 (8.4-16.1)	10.1 (0.15-86.3)	7.7 (0.14-63.8%)	8.9 (0.1-86.3)
CD34 dose ($\times 10^6$/kg)	7.8 (0.1-25.1)	8.3 (5.2-18.5)	6.9 (0.04-80)	6.8 (0.03-66)	6.9 (0.01-80.0)
Previous SCT (Auto/Allo)	17	1	79 (59/20) (24%)	22 (10/12) (6%)	132 (16%)
MAC	0	0	0	355	396 (47%)
RIC	90	9	336	0	447 (53%)
GVHD prophylaxis					
-Cya/tacrolimus + MTX/MMF	81	8	258	283	624
-Tacrolimus + Sirolimus			41	26	104
-Other	9*	1*	37	46	115
ATG	63 (70%)	0	260 (77%)	237 (67%)	577 (69%)
Alemtuzumab	13 (14%)	3 (33%)			46 (5%)
Female to Male	59/31	5/4	58 (17%)	71 (20%)	150 (18%)
BM/PBSC/CB	12/76/2	1/8/0	68/248/20 (20/74/6%)	118/203/34 (33/57/10%)	233/548/62 (28/65/7%)
GVHD					
aGVHD I–IV	40				539 (64%)
aGVHD II–IV	18				358 (42%)
cGVHD (Y/N)	25/	1/			224/600 (27%)
BSI before engraftment					169 (20%)
MSCs			26 (8%)	27 (8%	106 (13%)

RIC: reduced-intensity conditioning; IA: invasive aspergillosis; MAC: myeloablative conditioning; Disease stage: Early: first complete remission (CR1)/first chronic phase (CP1) or non-malignancy, Late: later stages; MRD: matched related donor; MUD: matched unrelated donor (at least HLA-A, -B, and -DR matched); MM: mismatched donor; TNC: total nucleated cell; SCT: stem cell transplantation; Cy: cyclophosphamide; Bu: busulphan; fTBI: fractionated total body irradiation; Flu: fludarabine; Treo: treosulphan; Cya: cyclosporine;

Mtx: methotrexate; MMF: mycophenolate mofetil; ATG: anti-thymocyte globulin; BM: bone marrow; PBSC: peripheral blood stem cell; CB: cord blood; aGVHD: acute graft-versus-host disease; cGVHD: chronic graft-versus-host disease; BSI: bloodstream infection; MSCs: mesenchymal stromal cells.

[#]*Disease stage not applicable for HSCTs performed because of solid tumor.*

**Other than Cya/tacrolimus + MTX/MMF.*

4.1.4 Paper IV

This retrospective study investigated the incidence and risk factors for IFD after HSCT. All patients transplanted at Karolinska University Hospital, Huddinge, between January 2001 and September 2012 were included (n = 843; the patient characteristics are presented in Table 2). Data regarding the HSCT procedure were collected prospectively and entered into a database for research purposes. Data regarding IFD were collected retrospectively. Patients with relapse of underlying disease or diagnosis of a secondary malignancy were censored at the date of diagnosis.

4.2 METHODS

4.2.1 IFD definitions (Papers I and IV)

In paper I, IFD was classified as probable or proven according to the EORTC/MSG criteria from 2002, and in paper IV it was classified according to the revised criteria from 2008 (52, 53). In the revised definitions, the "major" and "minor" clinical criteria were changed in favor of more characteristic and objective evidence, in most cases findings from medical imaging, in order to exclude dubious cases (53) (Table 1). Regarding the revised mycological criteria, BG assays in serum were included, the requirement for two positive GM tests in serum was reduced to one, and findings of candida in urine cultures were excluded. PCR was not included because of lack of a standard assay that had been clinically validated (53).

4.2.2 Fungal PCR (Paper I)

Blood samples were incubated with hypotonic red cell lysis buffer. Following lysis of the erythrocytes, the samples were centrifuged and the pellets were transferred to tubes containing glass beads and vortexed thoroughly for three minutes. Then DNA was extracted using a fully automated laboratory robot, the MagNA Pure LC instrument (Roche). The primers and hybridization probes for *Candida* spp. were developed by Klingspor et al. (182); the *Aspergillus* probe was that described by Loeffler et al. (183). The LightCycler system

(Roche) was used for PCR amplification and detection with a cut-off value at 40 cycles. Each PCR analysis included a negative control consisting of hypotonic red cell lysis buffer, an extraction control, and a positive control containing fungal DNA. The following precautions were taken to avoid contamination. Before analysis was performed, cabinets, pipettes, racks, and DNA extraction robots were wiped down with DNAZap® (Ambion). The different steps were carried out in separate laboratories that were independently equipped. Both the manual steps and the robot extraction of DNA were performed in special rooms with positive pressure and hepa-filtered incoming air. Filter tips were used on the pipettes, DNA-grade water was used, and one negative control was added for every 5 clinical samples.

4.2.3 Definitions of pneumonia and pneumonia-associated death (Paper II)

A diagnosis of pneumonia was established if there were respiratory symptoms such as cough, dyspnoea, and an increased amount of mucus, together with new infiltrates on thoracic X-ray and/or CT compatible with infectious pneumonia. In most cases, these were combined with positive results from histology or cultures derived from autopsy, sputum culture, or BAL culture. In addition, exclusion of the idiopathic pulmonary syndrome was required (184). Early pneumonia was defined as pneumonia occurring within 100 days after HSCT. The cause of death was defined as the most important immediate cause, or the last event that had occurred before the chain of events leading to death.

4.2.4 Determination of posaconazole concentration in serum and tissues (Paper III)

The concentration of posaconazole in plasma was determined using a routine liquid chromatography-mass spectrometry- (LC-MS-) based method (described below) at the Department of Clinical Pharmacology, Karolinska University Hospital, Stockholm. When analyzing tissue concentrations, calibration curves were based on posaconazole (Schering-Plough) and internal standard- (UK-115794; Janssen) spiked homogenates of C57BL/6N mouse brain, heart, lung, liver, and kidney, respectively. Sample preparation was done by sonication of tissue in sodium chloride (0.9% w/v), followed by protein precipitation with two volumes of acetonitrile. The supernatant was injected into an LC-MS-system consisting of an Agilent 1100 MSD and an Agilent 1100 LC (Agilent) with a C18-column (Phenomenez Kinetex). The performance of the method was monitored with quality control samples at two levels. Chromatography was performed using a gradient based on aqueous 25 mM formic acid as solvent A and 100% acetonitrile as solvent B. Mass spectrometry was operated in selective ion monitoring using electrospray ionization in positive mode at the following ions: m/z 351 for posaconazole and m/z 348 for internal standard. The calibration curve was linear over a concentration range of 500–10,000 ng/mL for all organs. The quantification analysis was based on the ratio of peak height analyte to peak height internal standard and equal weighting by linear regression analysis. Quantifications were done using Agilent LC/MSD

ChemStation software rev. B.04.02.SP. When comparing the individual plasma concentration with tissue concentrations, the density of plasma was approximated to 1.0 g/mL.

4.2.5 Statistical methods

Incidences of IFD and IMI were estimated using a non-parametric estimator of cumulative incidence curves taking competing events (death without IFD or IMI) into consideration. Univariate and multivariate risk factor analyses for IMI were performed using Cox regression models (to estimate risk hazards (RHs)). Factors with $p < 0.10$ in the univariate analysis were introduced into a backwards-elimination multivariate analysis.

The incidence of death from pneumonia was estimated using an estimator of cumulative incidence curves taking competing events (death unrelated to pneumonia) into consideration. Predictive analyses for death from pneumonia were based on the proportional hazards model for sub-distribution of competing risks. Univariate and multivariate analyses were then performed using Gray's test and the proportional sub-distribution hazard regression model developed by Fine and Gray (185). All tests were two-sided. The type-I error rate was fixed at 0.05 for factors potentially associated with time-to-event outcomes. Factors with $p < 0.10$ in the univariate analysis were introduced into a backwards-elimination multivariate analysis.

Categorical parameters were compared using the Chi-square test and continuous variables were compared using the Mann-Whitney test. Analysis was performed using the cmprsk software package (developed by Gray, June 2001), Splus 6.2 software (Insightful, Seattle, WA, USA) and Statistica software (StatSoft, Tulsa, OK, USA).

5 RESULTS AND DISCUSSION

5.1 PCR SCREENING AND PRE-EMPTIVE TREATMENT (PAPER I)

When this study was initiated in 2001, no biomarkers for the diagnosis of IFD had been firmly established. The first reports on the performance of the GM test and *Aspergillus* PCR in blood specimens in patients at risk of fungal disease were published during the latter part of the 1990s (186). Hebart et al. showed in 2000 that prospective weekly monitoring with a pan-fungal PCR in high-risk neutropenic patients had a sensitivity of 100% and a specificity of 73% for diagnosis of proven or probable IFD (187). Other studies reported that a positive PCR result preceded symptoms and clinical diagnosis of IFD (188, 189). These findings raised the question of whether PCR-guided early antifungal treatment could reduce the incidence and mortality of IFI after HSCT, in an analogous way to the successful development of pre-emptive treatment of CMV (190).

In order to try to answer this question, patients with a positive fungal PCR result within 100 days of RIC HSCT were randomized to receive either treatment with liposomal amphotericin B or no treatment. Unfortunately, several factors prevented a conclusion being made. First, no appropriate power calculation was performed, which led to inclusion of an insufficient number of patients. Secondly, several patients with a single positive PCR result were not randomized because the treating physician did not want to subject well-feeling, asymptomatic outpatients to hospitalization in order to receive an intravenous drug. Thirdly, the shift towards later onset of IA after HSCT, especially after RIC, was not established knowledge when the study was planned. In addition, fungal prophylaxis administered during the pre-engraftment period was changed from non-absorbable amphotericin B to fluconazole during the study period, thus reducing the risk of invasive candidiasis.

During the first 100 days after HSCT, 41 of the 99 patients had at least one positive PCR result (*Aspergillus,* n = 18; *Candida,* n = 29; both, n = 6), but only 21 patients (51% of those who were positive) were randomized. No treatment was given to the 20 patients who were not randomized; instead, PCR was repeated with a negative result in all patients. Thus, initiating treatment on the sole basis of one positive PCR would have led to considerable overtreatment. A similar result was found in a much larger multicenter study by Hebart et al., performed between 1998 and 2001 (191). Even though our institution was one of the participating centers, the results were not published until 2009 and therefore were not known at the time of planning or performance of our study. In the study by Hebart, 403 patients were randomized to receive either PCR-directed therapy combined with empirical therapy or empirical therapy only, during the first 100 days after HSCT. As expected, more patients in the PCR group than in the empirical group received antifungal treatment: 57.1% vs. 36.7%. However, the increased antifungal treatment did not result in lower total incidences of proven and probable IFD (8.2% in both groups) or IA (4.1% in the PCR group vs. 2.4% in the empirical group) at day 100 (191).

Earlier this year, a meta-analysis was published investigating the diagnostic performance of *Aspergillus PCR* on blood specimens in high-risk hematological patients, including 25 studies with a total of 2,595 patients (192). The pooled diagnostic performance showed a sensitivity of 84% (CI 75–91%) and a specificity of 76% (CI 65–84%), which in low-risk patients (≤ 5% pre-test probability), such as the patients included in our study and in the Hebart study, would give a PPV of only 16%, but an excellent NPV of 99%. Requirement of at least two positive PCR test results increased the specificity to 95% but decreased the sensitivity to 64% (192). In low-risk patients (≤ 5% pre-test probability), this would increase the PPV to 46% with a slight decrease in NPV, 98%.

Obviously, antifungal treatment cannot be initiated on the sole basis of a test with an estimated PPV of only 16% (or 46%). Instead, *Aspergillus* PCR has been incorporated into a diagnostic-driven pathway in hematological patients at risk of IFD. Barnes and colleagues recently reported results using a neutropenic care pathway with GM and PCR testing twice a week in a cohort of 549 high-risk hematological patients (22% HSCT recipients) (159). They found that when both tests were used together, the sensitivity was 98% and the NPV was 99.6%, permitting a diagnosis of IA to be excluded and obviating the need for empirical treatment. Multiple positive PCR and GM test results enabled accurate diagnosis of IA, with a specificity of 95%. In addition, biomarkers preceded clinical signs in 85% of proven and probable invasive disease. Last year, Morrissey and colleagues published a randomized controlled trial comparing surveillance of hospitalized patients with GM and PCR testing twice weekly (once weekly for outpatients), with an empirical approach based on culture and histology (160). Of the 240 patients included, 80% had undergone HSCT. Significantly less patients in the surveillance group than in the empirical group received antifungal treatment: 15% vs. 32% ($p = 0.002$). There were no differences in overall mortality or IA-related mortality (160).

However, it is important to acknowledge that a major problem in the interpretation of this and other PCR studies is the lack of standardization of *Aspergillus* PCR. For instance, in the above-mentioned meta-analysis, 20 studies used whole blood and 6 used serum. Of the 20 studies using whole blood, eight used bead beating as recommended by EAPCRI. Sixteen studies used an 18S primer and seven used another ribosomal primer. Ten studies had one deviation or less from the recommendation of EAPCRI, while 16 had more than one deviation (192). In addition, four different DNA extraction methods were used. Interestingly, if only including studies that used a PCR method with no more than one deviation from the recommendation by EAPCRI, and two positive tests were required, the specificity was as high as 98% with a sensitivity of 67% (192). This indicates that the poor performance of fungal PCR that has been found in some studies may have been due to inferior PCR assays with poor methodology, and not problems linked to the whole concept of PCR (192). In order to overcome this problem, a standardized PCR method approved by the EACPRI is currently being tested in a large randomized study by the EORTC (NCT01288378), which will hopefully establish the role of PCR in diagnostic-driven pathways after HSCT.

5.2 TREATMENT WITH MESENCHYMAL STROMAL CELLS IS A RISK FACTOR FOR PNEUMONIA-ASSOCIATED DEATH AND IMI (PAPERS II AND IV)

The cumulative incidence of pneumonia-related death was 9.3% (CI 6.6–12.0) and risk factors in a multivariate regression analysis were grades II–IV aGVHD (HR = 2.61, CI 1.54–4.34, $p < 0.001$), CMV infection (defined as > 1,000 copies/mL of whole blood; HR = 2.27, CI 1.31–3.93, $p = 0.004$), and having received mesenchymal stromal cells (MSCs) (HR = 3.16, CI 1.75–5.69, $p < 0.001$). IMI was the most common etiology identified and was found in 32% of all patients (19 of 60) with pneumonia-related death, and in 48% of patients with established etiology (19 of 40). MSCs had been administered to 14 of the 60 patients with pneumonia-related death and eight (57%) of these died from a mold infection.

The association between MSCs and pneumonia-related death was not unexpected since treatment with MSCs appears to a risk factor for a variety of infections. We have previously reported that treatment with MSCs is a significant risk factor for EBV-driven PTLD, reactivation of VZV, and—in patients with severe GVHD—IFD (47, 49, 193). Since IMI was the most common etiology to pneumonia-related death in the study, the association between treatment with MSCs and pneumonia-related death may at least in part be explained by an association between treatment with MSCs and IMI. This is supported by the results in paper IV, in which treatment with MSCs was a significant risk factor for development of a new IMI after 797 HSCTs (relative hazard (RH) = 3.90, CI 2.02–7.55, $p < 0.001$). Since a major indication for treatment with MSCs is steroid-resistant GVHD, a serious condition with high risk of IMI, the multivariate analysis was recalculated after omitting patients treated because of grades III–IV aGVHD. The association was still significant (RH = 3.2, CI 1.3–7.9, $p = 0.01$), indicating that it is the powerful immunosuppressive properties of MSCs that constitute the risk of development of IMI and not (only) the underlying immune suppression caused by GVHD. MSCs, which were described for the first time in 1941 in bone marrow, are pluripotent cells that have the capacity to differentiate into mesenchymal lineages including bone, cartilage, and fat (194, 195). Major research in this field has been performed during the last two decades, especially in the transplant setting after Le Blanc and colleagues reported successful treatment of GVHD with haplo-identical MSCs in 2004 (196). Still not fully characterized, the minimal criteria for defining MSCs are adherence to plastic when maintained in standard culture, specific surface antigen expression, and multipotent differentiation potential (197). The exact mechanisms responsible for the increased risk of IMI remain to be defined, but the anti-inflammatory effect of MSCs is probably important. In an experimental sepsis model in mice, treatment with MSCs showed powerful anti-inflammatory abilities through downregulation of inflammation-related genes, preventing sepsis-induced acute lung injury and organ dysfunction (198). Similar effects have been shown in other animal studies, and MSCs have been proposed for treatment of acute respiratory syndrome. In vitro studies have shown that MSCs have a strong immunosuppressive effect on alloreactive T cells (199). Our group has shown inferior thymus T cell output, measured by TREC analysis, in patients receiving MSCs as engraftment

support in cord blood transplantation (200). Another, more specific mechanism for the increased risk of IMI may be MSC-induced indoleamine 2,3-dioxygenase (IDO). IDO blocks tryptophan metabolism along the kynurenine pathway, leading to suppression of T cell responses, an effect that has been shown to facilitate lethal pulmonary aspergillosis in a mouse model (201, 202).

Due to the findings in this study, we believe that mold prophylaxis should be strongly considered in HSCT recipients treated with MSCs from a third party donor, and that patients should be carefully monitored for signs of respiratory symptoms.

5.3 TISSUE CONCENTRATIONS OF POSACONAZOLE (PAPER III)

Posaconazole concentrations in biopsies, taken at autopsy of seven patients receiving posaconazole prophylaxis because of GVHD, were analyzed and compared to plasma concentrations in samples collected before death. The results showed that posaconazole concentrations in all the tissues examined, except brain, were higher than in plasma, indicating accumulation (Table 3, 4). As the time elapsed from last administered dose of posaconazole to death was quite long for several patients in the study (a median of 58 hours), the actual accumulation might be considerably higher in patients taking posaconazole regularly. Unfortunately, no conclusions could be drawn regarding the exact relationship between plasma and tissue concentrations because of (1) variability in posaconazole dosage, and (2) variability in time elapsed between doses administered and determination of plasma concentrations. However, the same pattern of accumulation in tissues was seen in all patients irrespective of dosage.

These findings are in agreement with a recent case report in which accumulation was seen in lung, liver, spleen, and kidney tissues of a renal transplant patient treated with posaconazole (203). They are also in accordance with the finding that posaconazole accumulates in alveolar macrophages and white blood cells (204-206). Furthermore, accumulation has been shown in an in vitro model where posaconazole was found to accumulate in pulmonary epithelial cell membranes, especially in the endoplasmic reticulum, and to persist in the membranes for at least 48 hours after removal of extracellular posaconazole (207, 208). When the epithelial cells came in contact with *Aspergillus fumigatus* conidia, posaconazole was rapidly transferred. Thus, the membrane appeared to function like a reservoir, releasing posaconazole when the cell came in contact with a target for the drug. The authors suggested that the accumulation of posaconazole in cell membranes is an important mechanism in prophylaxis and is a possible explanation for the prolonged antifungal effect (more than 48 hours after removal of extracellular posaconazole) seen in the experiments (207, 208). Our observation of accumulation of posaconazole in tissues in vivo is in accordance this hypothesis, and it may be an important explanation for the low breakthrough rates of IFD seen in clinical trials despite relatively low serum levels of posaconazole (Cavg $\leq$ 0.49 µg/mL in 50% of patients in the neutropenia prophylaxis trial, and MIC for *Aspergillus* spp typically 0.5 µg/mL) (207,

209-212). This finding also raises questions about the clinical use of therapeutic drug monitoring during prophylaxis.

Posaconazole concentrations in brain tissue were low compared to other tissues in all patients. One reason for this might be that none of the patients had an infection in the central nervous system (CNS). The posaconazole concentrations in cerebrospinal fluid presented in case reports have ranged from undetectable levels in patients with no CNS infection to twice the plasma levels in patients with brain abscesses and ventriculitis (213-215). The duration of treatment for CNS infections is long, and one can speculate that the inflammation will probably diminish during therapy, leading to lower susceptibility of the blood-brain barrier. If this is true, the results from this study would suggest that there may be a potential risk of sub-therapeutic concentrations in the CNS during long-term treatment with posaconazole. Thus, it may be pertinent to strive for high plasma concentrations in these patients.

The major limitations of the study were the variability in posaconazole dosage and in the time elapsed between administration of doses and determination of plasma concentrations. Furthermore, since posaconazole concentrations were analyzed in homogenized tissue, differences in extracellular and intracellular distribution could not be investigated. Minor breakdown of posaconazole during the interval between death and autopsy and between autopsy and tissue analysis cannot be ruled out, so the original tissue concentrations may have been higher.

Table 3. *Posaconazole dosages and plasma concentrations in paper III*

Pat	Time from initiation of treat-ment to death (days)	Dose (mg)	Plasma concentration determination closest to death					Interval bet-ween last dose and death (hours)
			Time between dose and sampling (hours)	Steady state reached?	Result (ng/ mL)	Time from sampling to death (days)	Doses administered between sampling and death	
1	5	200 mg, 2 doses 5 days before death, 1 dose 3 days before death	21	No	30	2	None	81
2	4	400 mg, 2 doses 4 days before death, 200 mg, 2 doses 1 day before death	57	No	10	1	200 mg, 2 doses	16
3	31	200 mg q12h, every second day	33	Yes	40	0	None	65
4	42	200 mg q12h, every second day	9	Yes	70	0	None	24
5	196	200 mg q12h, every second day	34	Yes	50	4	200 mg, 2 doses	79
6	19	200 mg q12h, every second day	33	Yes	330	3	200 mg, 1 dose	58
7	22	200 mg q8h, 200 mg q12h the last 2 days before death	15	Yes	390	0	None	19

q12h, every 12 hours; q8h, every 8 hours.

Table 4. *Tissue concentrations of posaconazole in paper III*

Pat	Serum concentration (ng/mL)*	Tissue concentration (ng/g)				
		Brain	Kidney	Liver	Heart	Lung
1	30	160	480	620	310	140
2	10	80	320	660	180	200
3	40	40	280	260	ND	110
4	70	ND	510	1,000	510	550
5	50	60	330	500	260	670
6	330	260	4,600	7,460	1,790	4,530
7	390	320	1,550	2,290	1,730	890

**Reproduced from Table 3.*

ND, not determined.

5.4 INCIDENCE AND RISK FACTORS FOR A NEW IMI AFTER HSCT AND IMPLICATIONS FOR PRIMARY MOLD PROPHYLAXIS (PAPERS I AND IV)

In paper I, the cumulative incidence rates of proven or probable IFD, IA, and invasive candidiasis during the first year after transplantation were 12%, 9%, and 2%, respectively. Significant risk factors in a multivariate model were grades II–IV aGVHD (RH = 13.1, CI 2.20–77.5, p = 0.005), CMV-seronegative recipient with CMV-positive donor (RH = 6.72, CI 1.43–31.7, p = 0.016), and conditioning with alemtuzumab (RH = 7.87, CI 1.77–34.9, p = 0.007).

The incidence of mold infections had been thought to be low in Sweden due to the cold climate and a 1-year cumulative IA incidence as high as 9% was therefore somewhat surprising. However, since relatively few patients were included in the study (n = 99), and all had received RIC, the results could not be generalized. Consequently, we performed a large retrospective cohort study including 843 consecutive HSCT recipients transplanted between January 2001 and September 2012 (paper IV). Apart from investigating the incidences of IFD and IMI, the aim was to identify risk factors for a new IMI, trying to identify which patient groups would benefit from primary mold prophylaxis. After Ullmann et al. found that primary posaconazol prophylaxis prevented IA in patients with grades II–IV aGVHD or cGVHD, posaconazole prophylaxis has been recommended to all patients with aGVHD grades II–IV in most guidelines (103, 216-218). However, in the study the grade of aGVHD appeared to be important for the risk of developing IFD in patients receiving fluconazole, with a total incidence of 8% in patients with grade II aGVHD and 19% in patients with grade III aGVHD (103). In addition, in the large prospective study from Italy, the risk of IMI in patients with aGVHD varied quite considerably, and the cumulative incidence of IFD in patients with aGVHD that was not followed by cGVHD was as low as 2.3% in patients with

matched related donors (45). Thus, there may be a risk of overtreatment by prescribing posaconazole to all patients with grade II GVHD, resulting in increased risk of side effects, resistance, and drug interactions—as well as considerable costs.

We found that the total cumulative incidence of IFD was 8.4%, and that the cumulative incidence of IMI was 2.2% at day 100, 5.2% after 1 year, and 6.3% after 2 years. As mentioned above, the main goal of the study was to try to identify patients who would benefit from administration of primary mold prophylaxis. Therefore all patients receiving secondary mold prophylaxis because of IFD before HSCT (n = 46), were excluded from the risk factor analysis. Factors significantly associated with a new IMI in a multivariate analysis were higher age (RH = 4.26 for 41–60 years of age and 9.0 for > 60 years of age, with 0–20 years as reference), grades II–IV aGVHD, treatment with MSCs, and transplantation with female donor to male recipient (Table 5). HCT-specific comorbidity index (HCT-CI) was available for 507 patients, all of whom were over 20 years of age (219). Multivariate analysis in these patients revealed that a comorbidity score of > 5 (a high value only reached by 4.1% of all patients) was associated with IMI (RH = 2.8, CI 1.1–6.8, p = 0.03).

In patients with grade II aGVHD, a new IMI was diagnosed after 14 of 219 HSCTs (6.4%) performed in 212 patients. All 14 IMIs occurred in patients > 40 years of age (14 of 106 (13.2%) vs. 0 of 113 (0%), p < 0.001). Additional indications of poor immune reconstitution beside aGVHD were seen in 12 of 14 patients with IMI (Table 6). IMI was judged to be the main cause or a contributory cause of death in 8 patients, constituting 32% of non-relapse mortality (NRM) and 16% of all deaths in patients > 40 years of age with grade II aGVHD.

A unique feature of our study was the high autopsy frequency, 68% in patients over 18 years of age who did not die from relapse or rejection, making the IMI incidence quite robust. Mold infections are notoriously difficult to diagnose before death, a notion supported here by the detection of 15 previously unknown IMIs in 75 autopsies.

The main finding of the study was the strong influence of age on the risk of a new IMI after HSCT. The increase in risk found in patients above 60 years of age (1-year cumulative incidence 16.4% in patients > 60 years of age, as compared to 7.1% in patients aged 41–60, 2.1% in patients aged 21–40, and 1% in patients aged 1–20) is important, since HSCT is increasingly being performed in elderly patients. The impact of age was especially evident in patients with grade II GVHD. No IMI was diagnosed after onset of GVHD in 113 transplantations performed in patients ≤ 40 years of age, as compared to 14 of 106 (13.2%) in patients > 40 years of age. Low autopsy frequency in younger patients did not appear to explain this finding: only six patients who died of other causes than relapse did not have an autopsy performed; nor did administration of mold-active prophylaxis (administered after 4% of the HSCTs), empirical treatment (> 3 weeks of treatment administered after 4% of HSCTs), transplantation with matched related donor (used in a minority of the HSCTs: 34%), or low initial corticosteroid dosage when treating aGVHD (typical starting dose during the study period: 2 mg/kg prednisolone per day) (220). One important explanation is probably that younger patients generally have little comorbidity and experience few complications

Table 5. *Risk factor analysis for development of a new invasive mold infection after HSCT (excluding 46 HSCTs with secondary prophylaxis because of IFD prior to transplantation)*

	No IMI	IMI	Univariate	Multivariate (RH, 95% CI, p)
No. of HSCTs	757	40	797	776*
Age, years				
0–20				Reference
21–40				0.90, 0.22-3.77, 0.89
41–60				4.26, 1.60-11.3, <0.01
> 60				9.01, 3.11-26.1, <0.001
Sex (M/F)	460/297	27/13	0.56	
Malignacy	626	34	0.91	
Non-malignancy	131	6		
Disease stage (Early/Late)#	375/347	15/19	0.47	
Donor:				
MRD	275 (36%)	11 (28%)	0.29	
MUD	379 (50%)	24 (60%)	0.22	
MM	103 (14%)	5 (13%)	0.99	
Donor age	32 (0-72)	40 (0-67)	0.03	
TNC dose	8.6 (0.1-86.2)	10.1 (0.2-28.3)	0.33	
CD34 dose	6.8 (0.1-80)	8.7 (0.2-28)	0.10	
Previous SCT	120 (16%)	8 (20%)	0.63	
MAC/RIC	366/391	12/28	0.035	
TBI-based	222 (29%)	11 (28%)	0.94	
Chemo-based	534 (71%)	29 (73%)		
ATG	519 (69%)	29 (73%)	0.73	
Alemtuzumab	37 (5%)	6 (15%)	0.016	
Female to Male	132 (17%)	12 (30%)	0.07	2.16, 1.09-4.27, 0.02
BM/PBSC/CB	221/480/56	5/31/4	0.03	
GVHD				
aGVHD I-IV	475 (63%)	35 (88%)	0.003	
aGVHD II-IV	308 (41%)	31 (78%)	<0.001	4.28, 2.00-9.17, <0.001
cGVHD (Y/N)	197/481 (26%)	14/22 (35%)	0.28	
BSI	154 (20%)	10 (25%)	0.61	
MSCs-all indications	79 (10%)*	15 (38%)*	<0.001*	3.90, 2.02-7.55, <0.001
PMCs	21 (3%)	1 (3%)	0.70	

Disease stage: Early: first complete remission (CR1)/first chronic phase (CP1) or non-malignancy, Late: later stages; MRD: matched related donor; MUD: matched unrelated donor; MM: mismatched donor; TNC: total nucleated cell; SCT: stem cell transplantation; MAC: myeloablative conditioning; RIC: reduced-intensity conditioning; TBI: total body irradiation; ATG: anti-thymocyte globulin; BM: bone marrow; PBSCs: peripheral blood stem cells, CB: cord blood; aGVHD: acute graft-versus-host disease; cGVHD: chronic graft-versus-host disease, BSI: bloodstream infection, MSCs: mesenchymal stromal cells; PMCs: placenta-derived mesenchymal cells.

**21 HSCTs excluded in univariate analysis of MSCs and in all multivariate analyses because of a randomized trial with MSCs or placebo; code not broken.*

#Disease stage not applicable for HSCTs because of solid tumor.

after HSCT if they respond well to treatment with glucocorticoids and do not develop grades III–IV aGVHD or severe cGVHD. This leads to a low net immune suppression and a low risk of IMI. The situation for older patients with grade II aGVHD is quite different, however. Advanced age and aGVHD have been shown to decrease thymic function and the de novo production of naïve T-cells, which play an important part of the immune reconstitution after HSCT (26). In addition, aGVHD has been shown to have a longer impact on thymic function in patients who are > 25 years of age, indicating that a severely impaired T-cell reconstitution can be expected in older patients with aGVHD (27). Older patients also have more comorbidities than younger patients, resulting in higher rates of complications. This might increase the net immune suppression and increase the risk of IMI, a notion supported by the finding that an HCT-CI score of > 5 (a high value usually reached only in older patients) was a significant risk factor in the multivariate analysis. Another factor of importance is that older patients more often have older donors (mainly because their siblings are about the same age but also because older unrelated donors are accepted for older patients), which have been shown to be associated with low counts of naïve CD4 T cells on days 180–365 after HSCT (221).

Even so, the risk of IMI appears to vary also in older patients with grade II GVHD, reflecting the heterogeneity of GVHD. In patients > 40 years of age, the risk was low in the absence of other signs of poor immune reconstitution. It therefore appears that in patients with grade II aGVHD, primary mold prophylaxis can in most cases be safely withheld in those who are < 40 years of age, as well as in patients > 40 years of age with a good response to glucocorticoids and no other signs of poor immune reconstitution. These conclusions are in line with those presented in a recently published guideline regarding primary antifungal prophylaxis after HSCT (66). In patients > 60 years of age, the threshold for administration of primary mold prophylaxis should be low, and should probably include all patients with GVHD grade II (or greater) and cGVHD, as well as patients with signs of poor immune reconstitution such as recurrent CMV infection.

The most important limitation of this study was its retrospective nature. In addition, the autopsy frequency was significantly lower in patients < 18 years of age—25% as compared to 68% for patients over 18 years of age—which may have led to an underestimation of the incidence of IMI in children and adolescents, thus overestimating the importance of older age as a risk factor for IMI in the whole cohort. However, since the cumulative incidences of IMI in patients aged 1–20 and 21-40 was comparable (1% and 2.1%, respectively) it seems unlikely that many cases were missed. The strengths of the study includes a high rate of

autopsies performed in patients over 18 years of age dying from causes other than relapse or disease progression, and long follow-up time.

Table 6. *IMI and grade II aGVHD in paper IV*

	0−40 years	**> 40 years**
Number of HSCTs*	113	106
Donor		
-MRD	41 (36%)	34 (32%)
-Other	73 (64%)	74 (68%)
Mold prophylaxis		
-Number (%)	5 (4%)	16 (15%)
-Median duration (range)	32 (11−329)	55 (7−650)
Empirical antifungal treatment[#]		
-Number (%)	14 (12%)	18 (17%)
-Median duration (range)	10 (1−622)	14 (2−125)
Dead		
-Total	34 (30%)	53 (50%)
-Relapse, progression	22 (19%)	28 (26%)
-Non-relapse mortality	12 (11%)	25 (24%)
Autopsy frequency in non-relapse, non-malignancy deaths	6 (50%)	21 (84%)
Chronic extensive GVHD	1 (1%)	6 (6%)
Invasive mold infection		
-Number (%)	0 (0%)[¤]	14 (13.2%)[¤]
Donor		
-MRD (% of all MRDs)		7 (21%)
-Other donor		7 (10%)
Possible contributory factors/signs of poor immune reconstitution:		
-Reactivation of EBV/PTLD		4
-Multiple CMV reactivations		4
-Varicella reactivation, severe		1
- > 100 mg prednisolone per day for > 6 weeks		1
-Late-onset neutropenia		1
-Chronic extensive GVHD		1
-No factor identified		2

GVHD: graft-versus-host disease; HSCT: allogeneic stem cell transplantation; MRD: matched related donor; EBV: Epstein-Barr virus; PTLD: post-transplant lymphoproliferative disease; CMV: cytomegalovirus.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The major conclusions in this thesis are as follows:

(1) Invasive mold infections are major complications after HSCT in Sweden, despite the cold climate;

(2) Older age is an important risk factor for development of a new invasive mold infection;

(3) Treatment with mesenchymal stromal cells from a third party is a risk factor both for death from pneumonia and development of a new invasive mold infection;

(4) Surveillance with fungal PCR of blood specimens after HSCT cannot alone guide pre-emptive antifungal treatment;

(5) When given as prophylaxis, posaconazole accumulates in tissues relative to plasma concentrations, with the exception of brain.

If IA (which by far constitutes the majority of all IMIs) is such a dangerous complication, why not give mold-active prophylaxis to all HSCT patients? Indeed, if the prophylactic drugs available had been like acyclovir for VZV reactivation—i.e. cheap, well-tolerated, and (almost) without resistance problems—this might have been an acceptable solution. However, this is not the case. Posaconazole costs more than 100 euros per day and has considerable gastrointestinal side effects, and disturbing reports of increasing azole resistance are being published from several countries.

So how should we reduce the morbidity and mortality of IA and IMI after HSCT?

As has been discussed in this thesis, there are at least three possible strategies: pre-emptive treatment using fungal surveillance, administration of primary mold-active prophylaxis to all patients, or administration of primary mold-active prophylaxis to groups of patients with a high risk, i.e. targeted prophylaxis. A surveillance-based strategy is only suitable if the incidence of IA is high, since the pre-test likelihood is fundamental for the performance of surveillance tests. Primary mold-active prophylaxis also requires a high incidence in order to be effective, otherwise the number of patients needed to treat to avoid one IA or IMI will be too high. Thus, in the high-risk setting, either surveillance or primary mold-active prophylaxis to all patients can be used, but if prophylaxis is chosen, no surveillance should be performed due to the low pre-test likelihood. Should PCR be included in a surveillance approach? As shown in paper I, PCR cannot guide therapy on its own, but the performance of the test in studies following EAPCRI guidelines is encouraging. However, until the results from the ongoing EORTC study have been published, the test is probably best used on biopsies—and together with the GM test in BAL to rule out IA.

In a setting with a low incidence of IMI, such as ours, the PPV of a surveillance approach will be too low, and the number needed to treat with prophylaxis to avoid one IA or IMI will be too high. Thus, targeted mold prophylaxis, dividing patients into groups based on the risk of developing IA or IMI, is probably the best option. In paper IV, patients with a new IMI could be found either in patients with grades III and IV aGVHD or severe cGVHD, or in patients > 40 years of age with grade II aGVHD and signs of poor immune reconstitution. The guidelines at our institution regarding primary mold-active prophylaxis in patients with no more than grade II aGVHD have been changed accordingly, with no prophylaxis being administered to patients < 40 years of age or to patients > 40 years of age with a good response to glucocorticoids (tapered to 20 mg prednisolone per day within 3–4 weeks). In patients > 40 years of age with a poor or slow response to glucocorticoids or with other signs of poor immune reconstitution (such as multiple CMV reactivations or EBV reactivation), prophylaxis is prescribed. Finally, prophylaxis is administered to all patients > 60 years of age with grade II GVHD.

However, even if targeted prophylaxis based on clinical data will hopefully both reduce the use of mold-active prophylaxis and the incidence of IA and IMI, there will still be patients receiving unnecessary prophylaxis and patients needing prophylaxis but not getting it. To avoid this, reliable, objective, tools for measurement of net immune suppression in an individual are needed. HSCT has changed considerably over the years, moving from myeloablative conditioning to all patients via reduced protocols to more and more tailored conditioning based on factors such as patient age, diagnosis, underlying conditions, donor, type of stem cells, and graft manipulation. After adding post-transplantation factors that affect immune reconstitution, such as GVHD and infections, it is obvious that the net immune suppression will differ significantly between patients. And yet, when estimating the risk of IA and IMI, we rely on clinical factors such as age, donor, and GVHD. Recently, a paper reported an association between genetic PTX3 deficiency and IA after HSCT (222). Reports of polymorphisms being associated with IA have been published before, but often the results have not been consistent in repeated studies. Genetic markers for vulnerability might very well be useful in the future, and eventually be incorporated into clinical guidelines, but they will probably not solve the issue of measuring net immune suppression. In fact, the complexity of the immune system makes it questionable whether such tests will be possible to develop. However, an article published earlier this year by Shekhar and Brodin, may have been the beginning of the future (223). In this paper, the author described the development of a computational tool that can handle the raw data retrieved from mass cytometry, automatically stratifying cells in phenotypic sub-populations based on the distribution of a large number of different proteins (223). This enables a much more refined identification of immune cell sub-populations and the way they interact. In the future, it may be possible to measure the immune status of the patients using this technique and identifying patients with risk of specific infections, such as IA. These patients can then receive prophylaxis, either old fashioned style with a mold-active drug, or with the genetically modified T cells described in an very interesting work from MD Anderson, in which a gene-transfer system was used to

enforce expression of a chimeric antigen receptor with fungal pattern-recognition properties (180). T cell therapies have several possible advantages, such as better efficacy, no drug interactions, and no problem with drug resistance. Another possible strategy in the future may be vaccination, however, until now research within this field have been mainly unsuccessful.

A test measuring immune suppression would also be important in helping to decide when prophylaxis can be stopped, something we do not know today. The fixed treatment duration of posaconazole prophylaxis in the GVHD trial was 112 days (103). No large prophylaxis trial in GVHD patients has been performed since, so there are no data regarding the optimal duration of prophylaxis. Even if we keep refining our guidelines and keep trying to find out exactly which patients are at risk of IMI and would therefore benefit from mold-active prophylaxis, we still do not know for how long we should give it. With the price running at 100 euros per day and an increasing azole resistance in several countries, this is an issue that must be addressed in future trials. Until more data become available, our approach will be to stop prophylaxis when the glucocorticoid dose is under 20 mg/kg prednisolone per day, but with closely scheduled follow-up.

During the more foreseeable future, we aim to build a prospective database for IFD after HSCT in Scandinavia, covering the majority of transplantation centers. Since both the patient population and the transplant procedures are fairly homogeneous in the whole of Scandinavia, we hope to be able to compare the outcome of our recently changed prophylaxis guidelines with the outcomes of the prophylaxis approaches used in the other centers of similar size.

7 SAMMANFATTNING PÅ SVENSKA

Infektioner är mycket vanliga efter allogen stamcellstransplantation (med stamceller från en annan människa), tidigare kallad benmärgstransplantation. Det har flera orsaker. Ofta är orsaken till en transplantation blodcancer, vilket innebär att mycket cellgiftsbehandling redan getts innan det är dags för transplantationen. I samband med själva transplantationen måste också kraftig cellgiftsbehandling ges, både för att döda eventuellt kvarvarande cancerceller och för att slå ner det gamla immunförsvaret, så att de nya stamcellerna inte stöts bort. Dessa hittar sedan snabbt till benmärgen och börjar tillverka nya röda och vita blodkroppar. Men det tar tid, oftast någonstans mellan 14 och 21 dagar, innan de nya vita blodkropparna är tillräckligt många för att kunna bekämpa infektioner. De transplanterade cellerna behöver successivt vänja sig vid den nya omgivningen för att inte uppfatta den som farlig och attackera, vilket kallas transplantat-mot-värdsjukdom (förkortas GVHD på engelska). Under tillvänjningsperioden, som brukar vara cirka sex månader, måste man ge mediciner som dämpar det nya immunförsvaret, vilket gör att patienterna blir infektionskänsliga. Om man ändå drabbas av GVHD måste ännu mer dämpande behandling ges, och infektionsrisken ökar då ytterligare.

En av de allvarligaste infektionerna är svampinfektioner, som har hög dödlighet. Det finns data publicerade från andra länder om dessa svampinfektioner, men mindre är känt om förekomsten i Sverige (och Norden). Målet med denna avhandling var därför att undersöka förekomst och riskfaktorer för svampinfektioner, speciellt mögelsvampsinfektioner, hos patienter som genomgått allogen stamcellstransplantation på Karolinska Universitetssjukhuset, Huddinge.

I det **första arbetet** undersöktes hur användbart det är att med PCR-test regelbundet leta efter svamp-DNA i blodprov. Tanken var att med detta känsliga test skulle svampinfektioner kunna upptäckas i ett tidigt skede och behandling ges innan infektionen blivit allvarlig. Vi fann att ett ensamt positivt PCR-test inom de 100 första dagarna efter transplantation, vilket 41 av 99 patienter hade, inte talade för att det faktiskt fanns en svampinfektion och att man inte kunde förlita sig enbart på detta prov för att sätta in svampbehandling. Vidare fann vi att 9 % av alla patienter hade en mögelsvampinfektion inom det första året efter transplantationen, och att en viktig riskfaktor för att få en sådan infektion var GVHD.

I det **andra arbetet** undersöktes hur vanligt det är med lunginflammation som leder till döden och om mängden cellgifter som ges vid transplantationen påverkar denna risk. Vi fann att 2,5 % av alla transplanterade patienter dog av lunginflammation inom de första 100 dagarna, och 9,4 % inom det första året. Cellgiftsbehandlingens intensitet hade ingen signifikant påverkan på risken att dö i lunginflammation. I två tredjedelar av fallen (67 %) kunde man hitta vad som orsakat lunginflammationen, och av dessa var mögelsvampsinfektion vanligast (48 % av de fall där orsak kunde fastställas, 32 % av alla lunginflammationer med dödlig utgång).

I det **tredje arbetet** undersökte vi vävnadskoncentrationer av ett svampläkemedel, posakonazol. De sju patienter som ingick i studien fick alla förebyggande svampbehandling

med posakonazol på grund av svår GVHD fram till dess att de dog. Vid obduktionerna (som görs rutinmässigt på alla patienter som inte dör av återfall i sin grundsjukdom) sparades små vävnadsbitar som senare analyserades med bestämning av mängden posakonazol. Dessa mängder jämfördes med mängden av posakonazol i blodprov tagna medan patienterna levde. Vi fann att posakonazol ansamlades i vävnader från hjärta, lunga, njure och lever, men inte från hjärna. Slutsatsen blev att då mängden av posakonazol i blod ofta är relativt låg, kan ansamlingen i vävnader vara en viktig förklaring till att posakonazol klarar av att skydda mot svampinfektioner.

I det **fjärde arbetet** undersökte vi förekomsten av, och riskfaktorer för, mögelsvampsinfektion. Denna studie innefattade 843 patienter som transplanterades mellan 2000 och 2012. Vi fann att frekvensen av mögelsvampsinfektioner var 2,2 % efter 100 dagar, 5,2 % efter 1 år och 6,3 % efter 2 år. Viktiga riskfaktorer för en ny mögelsvampsinfektion var 1) ålder vid transplantation (ökande risk från 40 års ålder och uppåt med högst risk över 60 års ålder), 2) GVHD och 3) behandling med mesenkymala stamceller (celler med starkt immunsupprimerade egenskaper som ibland ges som behandling mot GVHD). För patienter med måttligt svår GVHD var åldern avgörande för risken att drabbas av en mögelsvampsinfektion. Ingen av de under 40 år fick en sådan infektion mot 13 % av dem över 40 år. Förebyggande mögelsvampmedicin finns (förstahandsmedel är posakonazol som ingick i det tredje arbetet), men den är mycket dyr och har en del biverkningar, varför det inte är genomförbart att ge den till alla patienter. Det viktiga med fynden i det fjärde arbetet är att vi nu bättre kan identifiera vilka patienter som har hög risk för att få en, oftast dödlig, mögelsvampsinfektion. Därmed kan vi ge förebyggande svampbehandling med mer precision.

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