Diplomarbeit

Antifungal agents and Invasive fungal infections (IFI´s) in hematological malignancies: the AIHM study

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Graz, im Juni 2012 *Anna-Teresa Strohmeier*

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Zusammenfassung

Ziel

Invasive Mykosen bei Patienten mit maligner hämatoonkologischer Grunderkrankung stellen noch immer ein großes Problem dar und sind mit Morbidität, Mortalität und Kosten für das Gesundheitssystem verbunden. Um Klarheit und Einheitlichkeit im Umgang mit invasiven Mykosen zu gewährleisten, entwickelte die European Organisation for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) Richtlinien, welche diese Infektionen definieren. Anhand der Grundlage dieser EORTC/MSG Kriterien werden die Patienten hinsichtlich der invasiven Mykose in „bewiesen, wahrscheinlich und möglich“ eingeteilt. Dabei spielt der Galaktomannan Test, eine Methode zum Nachweis von Aspergillus Antigen, eine wichtige Rolle.

Unsere Studie hat sich mit der Inzidenz von opportunistischen invasiven Mykosen bei Patienten mit maligner hämatoonkoogischer Grunderkrankung, assoziierten Wirtsfaktoren, verschriebener antifungaler Therapie und mit der Nutzung von Galaktomannan als wichtiges und empfindliches Instrument bei deren frühen Diagnose beschäftigt.

Methoden

In diese prospektive Studie der hämatologischen Abteilung der Medizinischen Universität Graz wurden 729 Patienten, von denen 129 eine antifungale Therapie erhielten, eingeschlossen. Die Studiendauer betrug sieben Monate (April 2010 bis Oktober 2010). Die Daten der 129 Patienten mit antifungaler Therapie wurden zweimal wöchentlich erhoben und umfassten die jeweilige Grunderkrankung, die antifungale Therapie und deren Dosierung sowie Dauer und Grund dieser Behandlung. Im Falle einer invasiven Mykose wurden die Patienten nach den EORTC/MSG Kategorien in „bewiesen, wahrscheinlich und möglich“ eingeteilt und hinsichtlich ihrer Wirtsfaktoren, Pilzspezies und Therapieerfolg nach zwölf Wochen untersucht.

Resultate

Innerhalb der Studiendauer wurden 200 antifungale Therapien verabreicht. Der am häufigsten verwendete Wirkstoff war Posaconazol, welches in 81/129 Patienten verabreicht wurde, gefolgt von Caspofungin bei 65/129 (50%), Itraconazol in 19/129 (5%) und Voriconazole in 18/129 (14%) Patienten. Das liposomale Amphotericin B wurde 9/129 Patienten verschrieben, während Fluconazole sogar nur 7/129 Patienten verabreicht wurde. 74/129 Patienten (57%) wurden prophylaktisch, 57/129 (44%) empirisch, 39/129 (30%) präemptiv und 8/129 Patienten gezielt behandelt. 13/28 Patienten litten an einer wahrscheinlichen und 6/28 Patienten an einer bewiesenen invasiven Mykose. 24/28 Patienten mit invasiver Mykose entwickelten eine invasive Infektion mit Schimmelpilzen, von denen neun mögliche, 13 wahrscheinliche und zwei bewiesene Infektionen waren. In 94/128 Patienten mit antifungaler Therapie wurde eine Testung mittels Aspergillus-Antigen durchgeführt. In 10 von diesen 94 Patienten gab es positive Test-Resultate, während neun von diesen zehn Patienten die Kriterien einer wahrscheinlichen invasiven Aspergillose erfüllten, erfüllte ein Patient die Kriterien einer gesicherten Infektion mit dem Schimmelpilz *Geosmithia Argillacea*.

Zusammenfassung

Verglichen mit einer früheren Studie an derselben Institution konnte, aufgrund des Galaktomannan-Antigen Tests, eine signifikant höhere Anzahl von wahrscheinlichen oder gesicherten invasiven Schimmelpilzinfektionen nachgewiesen werden. Dieses spezielle Diagnostikmittel ermöglicht eine exaktere Klassifikation gemäß der EORTC/MSG Kriterien von wahrscheinlichen invasiven Aspergillosen.

Abstract

Aim

Invasive fungal infections in patients with hematological malignancies continue to be a major problem resulting in morbidity, mortality and health care related costs. To provide clarification and uniformity in the diagnosis of invasive fungal infections, the EORTC/MSG developed guidelines to define these infections. On the basis of the EORTC/MSG criteria, patients were classified into “proven, probable and possible” IFI. In this context, Galactomannan-Testing (GM), a method for the evidence of Aspergillus antigen, plays an important role. Our study dealt with the incidence of opportunistic invasive fungal infections in patients with hematological malignancies, associated host factors, prescribed antifungal therapy and the use of Galactomannan as an important and sensitive tool for the early diagnosis.

Method

In this prospective study of the Division of Hematology at the Medical University of Graz, 729 patients were included of whom 129 patients received an antifungal therapy. The duration of study was a total of eight months, from April 2010 until October 2010. Data of the 129 patients with antifungal therapy were collected twice a week and contained underlying diseases, antifungal therapy and dosage, as well as duration and reason for treatment. In the case of IFI, patients were classified into the EORTC/MSG categories of “proven, probable and possible” and accurately examined concerning the host factors, fungal species and the outcome after twelve weeks.

Results

Throughout this study, 200 courses of antifungal agents were administered. The most common antifungal agent was posaconazole in 81/129 cases (63%), followed by caspofungin in 65/129 (50%) cases, itraconazole in 19/129 (15%) and voriconazole in 18/129 (14%) patients. Liposomal amphotericin B was used in 9/129 (7%) patients, whilst fluconazole was only used in 7/129 (5%) patients. 74/129 (57%) patients were treated prophylactically, 57/129 (44%) empirically, 39/129 (30%) preemptively and 8/129 patients (6%) were treated directly.

9/28 were possible, 13/28 were probable and 6/28 patients with antifungal therapy were proven IFI. 24/28 patients with IFI had an IMI of whom 9/24 were possible, 13/24 were probable and 2/24 patients were proven IMI.
In 94/128 patients with antifungal therapy, serum GM-testing was conducted. In 10/94 patients, GM testing was positive. Nine of these ten cases were probable IA, whilst one of the cases was proven IMI with the mould *Geosmithia argillacea.*

Conclusion

Compared to a previous study at the same institution, it was possible to establish, because of the GM testing, a significantly higher number of probable and proven IFI. This special diagnostic tool enabled a more exact classification of probable IFI according to the EORTC/MSG criteria.

Inhaltsverzeichnis

Abbreviation

ALL = acute lymphatic leukemia

Allo = allogeneous

AML = acute myeloid leukemia

Auto = autologous

BAL= broncho alveolar lavage

BG = 1-3-ß-D-Glucan

BSC = bronchoscopy

CML = chronic myeloid leukemia

CLL = chronic lymphoid leukemia

CRF = case report form

CSF = cerebrospinal fluid

CT = computed tomography

EORTC = European Organisation for Research and Treatment of Cancer

GM = galactomannan

GvHD = Graft-versus-Host-Disease

HEPA = high efficiency particulate airfilter

HRCT = high resolution computed tomography

HSC = hepatosplenic candidiasis

HSCT = human stem cell transplantation

IA = invasive aspergillosis

IC = invasive candidiasis

IFI = invasive fungal infection

IMI = invasive mould infection

MDS = myelosysplastic syndrome

MEDOCS = Medical and nursing documentation network of Styria

MM = multiple myeloma

MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group

NCA = non candida albicans

NHL = non Hodgkin Lymphoma

Tables and Figures

1 Introduction

1.1 Mycology

Fungi are eukaryotes with a cell-membrane including the genom, being higher developed than bacteria. Fungi are a heterogeneous group, yet all characterized by cell-walls including chitin, glucan and mannan but lacking chlorophyll. These characteristics show the difference between fungi, animals and plants. Animal cells have no cell walls, while plant cells are sourrounded by cell walls mainly made of cellulose. Fungi are able to reproduce themselves in a sexual and asexual way. Sexual forms are named „Fungi Perfecti“, asexual forms are called „Fungi imperfecti“. Most fungi are environmental pathogens, with 150 species being able to colonize and harm the human being. Most of the pathogenic fungi have a minimal virulence and need ideal conditions to act as opportunists and cause local or systemic mycoses (1, 2).

1.1.1 General structure of fungi

Fungi contain a big genom with thousands of genes which are distributed on several chromosomes. The set of chromosomes varies between the species. *Aspergillus*, for example, owns a haploid set of chromosomes while *Candida albicans* owns a diploid one (1).

The fungal genom is similiar to the human genom. The cell membrane divides the genetic material from the cytoplasm. The fungal cytoplasm includes mitochondria, ribosomes, golgi apparatus and peroxisomes. The difference between the fungal-like cytoplasm and the human-like cytoplasm is ergosterol as opposed to cholesterol as a component. The fungal cell has a complex cell wall which consists of glucan and chitin. Proteins, mannans, mannoproteins and melanin are also found in the cell wall (1). The fungal cell is protected by the cell wall from osmotic pressure changes and environmental burden. The cell wall allows the fungal cell to interact with its surroundings and represents a highly dynamic structure, which maintains its plasticity during growth, cell division and spore germination (3, 4).

1.1.2 Morphology

Different morphological forms are found in fungi. The basic elements of filamentous fungi are hyphae which show a ramified and tubular structure of 2-10 µm. The mycelium is a plexus of hyphae. Substratmycelium (nutrition) penetrates into the nutritive substance, while the airmycelium develops above the nutritive substance. The fungal-thallus is the entirety of the mycelium and is also known as a fungal colony. Yeast is a basic element of the unicellular fungi and is also named Blastospore, which is round to oval with a diameter of 3-10µm. Pseudohypha are several, elongated yeast cells which are linked to each other in a chain and similar to real hyphae. Some of the fungi are able, depending on environmental issues, to occur in yeast or in mycelium-forms, defined as dimorphism. Dimorphic fungi occur in the parasitic status (pathogenic germs) as yeasts, in saprophytic status (on artificial nutrient medium) as mycelium (5).

1.1.3 Biological characteristics and performances of pathogenic fungi

Pathogenic fungi have adherence-ability to the cells of the macroorganism and show invasive growth in the host organism. Yeasts are able to resorb through the wall of the intestine into the hematic-lymphatic system. Fungi are resistant to digestive enzymes, phagocytosis and to antibiotics, using dead body cells from the host as a nutri-substrat. Fungi also show a thermal tolerance and are independent of atmospheric oxygen (5).

Because of their specific surface structure, fungi act as antigens. The major components of the cell wall are peptidoglucomannans with antigenic properties. The most important antigens are mannan complexes which are solute and heat stable. The cell content are somatic and zytoplasmatic antigens from proteins, gylcoproteins and galactomannans. Metabolic antigens are complex metabolic products with high protein content released by fungi. The humoral immune system as well as the cellular response are stimulated by the fungal antigens. This is followed by the immediate and the delayed reaction type. Consecutively antibodies are produced. The T-cell activity is very important to protect the organism from mycoses. The fungal antigenicity is species-specific (6).

Fungi are able to sensitize man and cause allergic reactions. Dermatophytes and yeasts are able to cause fungal id reactions on the body surface while mucous membrane lesions (rhinitis allergica, bronchial asthma) are caused through contact with airborne spores. Due to their small size (2-10µm), spores are able to reach the lung. The mucous membrane of the nose and the throat build a barrier to spores. The allergenic potency of the mycelium is much lower than of spores. The real allergens are protein complexes (6).

Fungal cells have to produce enzymes to decompose organic substances. Yeasts have a broad spectrum of enzymes. These enzymes are important to distinguish between the fungal strains (6). Another biological characteristic of fungi is toxin production. Mycotoxins are mould-metabolits of the secondary metabolism. These metabolits are low-molar substances and do not cause an immune response. They are induced into the organism through the air or spoiled food and are able to cause acute or chronical toxic effects (1). Mycotoxics are thermoduric with nephro, neuro, mutagenic, teratogenic and carcinogenic effects. The most important medical mycotoxin is ethyl alcohol, which is produced by yeasts (6).

1.1.4 Some important mycotoxins in medicine

Aflatoxins have carcinogenic effects (primary liver cancer). Ochratoxins cause kidney and liver damage. Ergot alkaloids have a wide variety because of their different receptors; ergotamine triggers contraction of smooth muscles and causes abortions. Ergotamine also causes the increase of blood pressure because of peripheral vasoconstriction (5).

1.1.5 Clinical classifications of mycoses (5)

Infections due to fungi occur more frequently than allergies or mycotoxicoses.
The clinical classification of mycoses is:

- Primary mycoses: Coccidoidomycosis, Histoplasmosis, Blastomycosis
- Opportunistic mycoses: deep and superficial yeast.mycoses, aspergillosis, cryptococcosis, mucormycosis, phaeohyphomycosis, hyalohyphomycosis, penicillosis, pneumocystiosis
- Subcutaneous mycoses: Sporotrichosis, chromomycosis, maduarmycosis
- Cutaneous mycoses: dermatomycosis, pityriasis vesicolor, tinea nigra piedra

1.2 Opportunistic mycoses

Opportunistic mycoses are caused by

- Yeasts
- Moulds

Both can harm the skin, mucous membrane and inner organs. A further prerequisite for opportunistic mycoses is a pronounced weakness of immune response (5).

1.2.1 Yeasts

Yeasts are mono-cellular eukaryotes in oval shape. Especially *Candida species* play a major role in Medicine.

There are more than 200 candida species. The most important opportunistic pathogen is ***Candida albicans***. Other important candida species are ***Candida tropicalis, Candida glabrata and Candida krusei (1).***

While in healthy subjects, *Candida* just acts as a colonizer in the oral, vaginal or gastrointestinal flora without pathological relevance, it may be causative of life threatening infection in the immunocompromised (1).

1.2.2 *Candida albicans*

C. albicans is a facultative, pathogenic yeast and part of the *Ascomycota*. *C. albicans* causes dermal and mucous membrane mycoses, catheter-related infections and sepsis. It is the most ubiquitous pathogen causing mycoses. The cell wall contains mannans, glucan, mannoproteins, chitin and proteins. Chitin and glucan do not have antigenic properties. Mannans are immunogen. Mannoproteins of the cell wall convey the cell's adherence to epithels and endothels. *Candida spp.* produce different extracellular proteinases and lipases. Acid aspartate-proteinases destroy immunoglobulins and complement, decompose mucus as a barrier and release nitrogen through elimination of the host's proteins. The main reservoir of *C.albicans* are animals and humans. The pathogen occurs as a saprophyt on intestinal and urogenital mucous membrane (7).

Most *Candida spp*., mainly *C. albicans*, act as facultative pathogenic germs. In a host with optimal milieu conditions, opportunists are able to spread, either superficially or invasively, into different organs. Yeasts use specific virulence factors and display adhesive surface structures (mannoproteins). Proteinase molecules and other molecules can promote the attachment of yeast cells on epithelial cells. The epithelial barrier is cleaved by lytic enzymes, proteinases and phospholipases. Normal defensive reactions of the body, for instance the complement reaction, are inhibited. New phenotypes are generated to distract the immune system. Polymorphous granulocytes and T- lymphocytes are of major importance in preventing yeast infection. T-lymphocytes secrete cytokines and activate macrophages. The humoral immunity plays only a minor role (1). Polymorphous granulocytes are able to phagocyte and kill pseudo-mycelium and blastospores. Neutropenic patients are predetermined to get a candidosis. Malfunctions of T-lymphocytes dispose the development of mucocutaneous candidose (thrush, candida-oesophagitis). T-lymphocytes play a major role in the immunity of the mucous-membrane (7).

1.2.3 Factors of the host facilitating *Candida* infection

The reduction or removal of the physiological bacterial flora due to antibiotics, high pH-value in the vagina or surplus of estrogen by oral contraceptives and gravidity facilitate the infection with *Candida spp*. Suppression of the specific/unspecific defense by leukemia or iatrogenic processes (transplantations, radiotherapy, cytostatics) also facilitate infections with *Candida spp*. Diabetes mellitus, hyperglycemia and diabetic ketoacidosis reduce the defensive function of phagocytes followed by dissemination (1).

1.2.4 Virulence properties of *Candida albicans* colonization

Virulence properties of *C.albicans* are short recreation times, resistance to environmental variations and adherence to epithelium and endothelium. *C.albicans* is able to invade by secretion of proteinases and phospholipases and to establish appropriate morphological structures. The persistence is enhanced by phenotypic switching and antigenic mimicry (1).

1.2.5 Other *Candida species*

Candida glabrata

In the past*, C.glabrata* was considered to be nonpathogenic and rarely caused infections in men. Due to the use of immunosuppressants and broad-spectrum antibiotics mucosal and systemic infections caused by *C.glabrata* have shown a significant increase. After *C.albicans, C.glabrata* is the most common reason for candidiasis (8). *C. glabrata* is an asexual form and belongs to the group Fungi Imperfecti. Above 37°C, *C. glabrata* is the only *Candida spp.* which does not build pseudohyphae. *C.glabrata* acts as a commensal and is found in the mucosa of healthy asymptomatic individuals. *C. albicans* and *C. glabrata* show significant differences and interactions in the host (9).

C. glabrata is mainly found in the thrush of AIDS patients under fluconazole therapy. Over the last decades, however, it has been increasingly identified as cause of infection in ICU patients. The main reason for that development is the increasing use of fluconazole prophylaxis in the ICU. In contrast to *C.albicans*, *C.glabrata* is mostly resistant to fluconazole. Positive blood cultures under poly-chemotherapy are no rarity. A massive fungal increase on the skin and mucous membrane is followed by dissemination (1).

Candida parapsilosis

Candida parapsilosis was first isolated in Puerto Rico from the feces of a patient who suffered from diarrhea. In 1940*, C.parapsilosis* was the causative pathogen of a mortal case in an intravenous drug user who died of an endocarditis.

Over the last decade, the incidence of infections with *C.parapsilosis* showed a dramatic increase. *C.parapsilosis* is the second most frequently isolated *Candida spp*. from blood cultures. The cells are oval to round or show a cylindrical form. *C. parapsilosis* can occur in a yeast phase or in a pseudohyphal form. True hyphae were not built by *C. parapsilosis* (10).

C. parapsilosis has also been isolated in non-human sources and is therefore not an obligate human pathogen. *C. parapsilosis* acts as a commensal in humans. In the immune-compromised host, *C.parapsilosis* represents an enormous danger for infection. The prolonged use of central venous catheters as well as prior antibiotic

therapy, parenteral nutrition, prior surgery, prior immunosuppressive therapy, malignancy, transplant receipt, neutropenia are also hazard factors for infections with *C.parapsilosis* (10).

Candida krusei

C.krusei is a *Candida spp.* which shows a lower virulence than *C.albicans*, for instance. Studies showed an increase in the infection with *C.krusei* because of the prophylactic use of antifungal agents. The use of posaconzole for prophylaxis may be accountable for the growth of *C.krusei*. In hospital, it is very important to control the presence of *C.krusei* and other resistant pathogens because a bad response to treatment leads to a longer hospital stay (11).

1.2.6 Other yeast species

Cryptococcus neoformans

C. neoformans is an opportunistic pathogen and mainly found in patients with AIDS with a diameter of 3-5 µm (1, 5). *C. neoformans* is a pathogenic yeast and part of the *Basidiomycota. C. neoformans* is surrounded by a capsule. In the capsule, there are different polysaccharides (mannose, xylosyl/beta-glucoronyl). The main part of the capsule is glucoronxylomannan. The cell wall contains beta-glucan and chitin. The difference to *C. albicans* is the small proportion of 1-3 Beta-D-Glucan in the cell wall (7). *C. neoformans* is found in the ground, in types of grasses and grains. The dispersal through birds is important for the human being. Mainly pigeon droppings are potential sources of infection (1).

C. neoformans is the only *Cryptococcus spp.* that may cause infection in humans. The pathogen is able to escape the host's immune defense because of the polysaccharide capsule and the cell wall's melanin. The capsule prevents from phagocytosis and melanin protects the fungal against oxidation by macrophages. The capsule's antigens stimulate the humoral and cellular immune system (7). Cryptococcosis is an aerogenic infection and is caused by inhalation of contaminated dust. The site of predilection is the lung followed by sub-clinical symptoms. In people who are immune-compromised (patients with AIDS, Corticosteroid therapy, Mb. Hodgkin), the pathogen is able to invade other organs

with the focus on the CNS (1, 5). The fungal persists in the brain tissue without causing an acute inflammatory reaction, namely meningoencephalitis (1).
Within the first two years of/after infection, a relapse can occur. Regular check-ups of the liquor, urine and sputum are important. Immune-compromised patients may even receive a lifetime prophylaxis of fluconazole (7).

1.2.7 Moulds

Moulds are ubiquitous and live as saprophytes on dead organic material. As pathogens they are able to cause opportunistic infections and allergies (1).

Aspergillus

Aspergillus spp. are saprophytes and the most common fungi in the human environment. *Aspergillus* is found in nuts, grain and in the soil of potted plants. A high concentration of *Aspergillus spp*. is found on compost piles. There are more than 200 *Aspergillus species*. Only a few *Aspergillus spp*. have a clinical relevance. The most important species is *Aspergillus fumigatus. Aspergillus niger, terreus, nidulans or versicolo*r are much rarer (1). *Aspergillus spp*. are part of higher fungals. Their characteristic sign is a filamentous, partitioned hypha with a diameter of 3-4 µm. The cell wall contains mannans and glucans (1, 5, 7).

Extracellular products of *Aspergillus spp*. are proteases, catalases, phospholipases and ribonuclease. All these products are important pathogenic factors. SAPs are able to cause thrombosis and hemorrhagic ischemia by converting coagulation factor X. PksP produced by *Aspergillus fumigatus* is a species-specific pigment and protects the fungus from reactive oxygen-radicals. Gliotoxin, also produced by *Aspergillus fumigatus*, is cytotoxic and inhibits the cellular immunity (7).

Aspergillus spp. are ubiquitous and our skin and mucous membrane are in permanent touch with the spores. A good working immune system takes care of eliminating these spores. The fungus reaches the organism via the respiratory tract. Because of their small size (2-4µm) spores can easily reach the alveoli and are eliminated by macrophages. In the immune-compromised host, moulds can cause infections of the lung or disseminate into other organs (1).

Predisposing factors for infections are damage to lung issues (TBC), cellular and humoral disorders and a reduced number and function of neutrophil granulocytes. Different *Aspergillus spp.* act as opportunists and acquire a parasitic life form. *Aspergillus fumigatus*, for instance, adheres to the host's cells, colonizes and spreads into the tissue. In the immune-compromised host, *Aspergillus fumigatus* shows an intravascular growth followed by activation of the coagulation system. The first step in immune defense against *Aspergillus* infections is the elimination of conidia by alveolar macrophages. If conidia are not eliminated completely, they are able to germinate and build hyphae. As a consequence, complement is activated and attracts macrophages and granulocytes**.** Both of them are able to phagocyte hyphae but no conidia. Opsonization with phagocytosis strengthening is followed by inflammatory reaction (1, 7).

Aspergillus flavus

A. flavus produces aflatoxins B1 and B2 and is an important opportunist in the immunocompromised host and the most common trigger for allergical aspergillosis. *A. flavus* is part of the section *Flavi* and is heterothallic. In patients with hematological malignancies *A. flavus* acts as an opportunistic human pathogen for invasive fungal infections and is detectable in paranasal sinus and otitis externa as well as in the oral mucosa and subcutaneous issue. *A. flavus* is the second most important *Aspergillus spp*., which is responsible for the systemic infections of immune-compromised patients (7, 12).

Aspergillus niger

A. niger, a filamentous ascomycete fungus, is also accused of acting as an opportunist in humans. *A. niger* is known for its production of citric acid and its important role in the global carbon cycle (13). Most commonly found in patients with otitis externa and patients with severe immunodeficiency, it leads to invasive fungal infections or aspergillum (4, 14).

Aspergillus terreus

A. terreus is, apart from the other *Aspergillus spp*, an uncommon but important human pathogen, which causes IFI and is often resistant to antifungal therapy with amphotericin B**.** *A. terreus* causes, in 10% of all infections with *Aspergillus spp*.,

allergic and different organ manifestations in lung, eyes, skin, tissue, articulations and disseminated infections in the immune-compromised host (7, 15). Infections with *A.terreus* range from 3%-12.5%. *A.terreus* was identified being more firm to amphotericin B than *A. fumigatus, A. flavus and A. niger* (16).

1.2.8 Other mould species

Penicillium spp.

Penicillium spp. are ubiquitous and exist in plants and in the ground. They are needed for composition of dead plant material. *Penicillium spp*. are characterized by a brush-shaped structure and show a similar morphology to *Aspergillus spp*. The most famous metabolic product of certain *Penicillium spp.* is Penicillin. In human medicine, *Penicillium spp*. act as mycotoxins, allergens and in very rare cases as pathogens causing infections. Mycotoxins are consumed with decayed food and are able to cause toxic symptoms. Through inhalation of spores, allergies are caused. These spores decay and cytoplasmatic proteins with allergenic effects are released. Allergenic reactions can occur as rhinitis, bronchitis and alveolitis. Compared to *Aspergillus*, *Penicillium* does not have the ability for invasive growth. Only *Penicillium marneffei* has small potency for invasive growth in immune-compromised patients. In Thailand, this is a disease associated with AIDS and intravenous antifungal therapy may be indicated (1, 7).

Fusarium spp.

Fusarium spp. are a heterogenous group of moulds. Their presence is ubiquitous in mud and they play a major role in food production. Only in exceptional cases, *Fusarium* causes infections in humans. *Fusarium* produces mycotoxins which are neurotoxic, teratogenic and immunotoxic. Predisposing factors for infections are injured skin, inoculation with contaminated products and iatrogen (invasive diagnostic and therapy). The clinical manifestations of *Fusarium spp.* are keratitis, endophtalmitis, nail mycoses and disseminated infections with fever and multiple skin lesions in the immune-compromised host (1, 7).

Zygomycetes

Zygomycetes are filamentous fungi with an unsepted mycelium. Their presence is ubiquitous containing 600 different species and just a few of the *Zygomyctes* are

human-pathogens. A very important human pathogen is *Absidia corymbifera*. *Zygomycetes* are lentogenic and act only as opportunists in predisposed patients. In patients with immune suppression or metabolic diseases, *Zygomycetes* are inhaled and colonize the respiratory mucous membrane. They are able to invade into the vascular system and cause thromboses. Clinical manifestations of an infection with *Zygomycetes* are rhinocerebral mycoses, pulmonary mycoses and gastrointestinal mycoses (1, 17).

1.3 The immune-compromised host

Hematological malignancies (leukemia, lymphoma) are the most common reason for immunodeficiency in hematological patients. Cellular or humoral immune-deficiency is caused by iatrogenic measures (chemotherapy) or by the disease itself and followed by an increased susceptibility to infections (18).

Fungal infections are an important cause of morbidity and mortality in hematological patients. On the one hand, bacterial infections are more common than fungal infections, but on the other hand, fungal infections cause a higher morbidity and mortality. The diagnosis of invasive fungal infections is very difficult and the effect of antifungal therapy is different in the hematological, immuno-compromised host. An important factor of invasive fungal infections in immune-compromised patients is neutropenia. Not every patient with hematological malignancy has the same potential for getting an infection. The disease's risk correlates with the gravity and duration of neutropenia (19).

1.3.1 Neutropenia

Neutropenia is defined as a decrease of neutrophil granulocytes < 1.830/µl (20). Epidemiology, development, treatment and prognosis of infections in neutropenic patients are different to non-neutropenic ones (21).

There are three risk levels of neutropenia causing opportunistic fungal infections (19):

Low-risk: Neutropenia < 500/µl for < 5 days; standard-therapy in lymphomas with high malignancy; solid tumors

Standard-risk: Neutropenia < 500/µl for 5-10 days; Salvage-therapy in lymphomas with high malignancy; autologous bone marrow transplantation (auto-SCT)

High-risk: Neutropenia < 500/µl for > 10 days; acute myeloid leukemia (AML), allogenous bone marrow transplantation (allo-SCT), high-dose therapy with glucocorticoids (> 20mg/d as long- term medication), inherited immune deficiencies

1.3.2 Infections occurring with neutropenia in hematologic patients

The most common reason for neutropenia (< 500/µl) is a paraneoplastic chemotherapy. At the same time, an impairment of the intestinal mucous barrier exists. Neutropenia with granulocytes < 500/µl may predisposes fungal and opportunistic bacterial infection (21). The susceptibility for infections in hematologic patients is caused by the lack of circulating neutrophil granulocytes. This lack is an intentional effect of causing a remission. Neutropenia may take several days to weeks. Prolonged neutropenic phases are observed after bone marrow transplantations. Hematological patients with neutropenia > 10 days are predisposed to fungal infections. Due to the modern antimicrobial agents, patients survive the early neutropenic phase and fungal infections increase (22, 23).

1.4 Opportunistic invasive fungal infections

Opportunistic invasive fungal infections play an important role in hematological and immuno-compromised patients. Antineoplastic therapy, immune-suppressive agents and broad-spectrum antibiotics are responsible for the increase in invasive fungal infections (24).

The microbiological diagnosis of IFIs succeeds only in some of the cases. Yeasts physically colonize the immunocompromised host. Therefore, detection of fungi by

culture is not necessarily a reason for therapy. It is important to differ between colonization and invasion. An exact and definitive diagnosis can only be made on the basis of a biopsy procedure (microscopic detection of hyphae in tissue) or positivity of blood cultures (22).

1.4.1 Host factors for invasive fungal infections (24)

- Neutropenia < 500 neutrophils/mm3 for > 10 days
- Allogeneic HSCT
- Prolonged use of corticosteroids >21 days
- Treatment with T-cell suppressants, cyclosporine, Tnf-alpha blockers, monoclonal antibodies
- Congenital severe immunodeficiency

For the therapy of hemoblastoma (AML, NHL, CML), human stem-cell transplantation is applied (HSCT). HSCT is a transplantation of multi-potent, hematopoetic stem cells. There is an autologous form and an allogeneic form. Autologous refers to the transplantation of the patient's stem cells which are produced naturally in the body and were stored in a freezer. Allogeneic means stem-cell transplantation with the healthy cells of a donator (cells of relatives, siblings or other donators). GVHD is the reaction of the patient's immune system to allogeneic stem-cell transplantation. This reaction runs from T-lymphocytes of the recipient, directed against the donator's tissue. Despite of the HLA identity, the patient's immune system detects the donor cells as a foreign tissue. There is an acute form (< 100 days after transplantation) and a chronic form (>100 days after transplantation). The most common forms are GVHD of the skin and of the gut. Graft-versus-Host-Disease (GvHD) may occur in recipients of allo SCT. GVHD itself is a predisposing factor for development of IFI. Patients with HSCT, GVHD and AML for instance, are generally at high risk for IFI and these patients were treated prophylactically. Prophylactic treatment doesn't need an evidence of infection or clinical signs. GVHD consists of high dose corticosteroids which are an important host factor for IFI (18).

1.4.2 Candidiasis

Mucocutanous forms of *C.albicans* infections are skin and nail infections, deeper dermatitis, vulvovaginitis, balanitis, thrush, oesophagitis and gastrointestinal infections (1). Candidiasis starts with colonization or oral mucocutaneous candidosis. In immune-compromised patients, especially in neutropenic ones, candidosis leads to a septic process or to disseminated organ infections. These organ infections generally occur to lungs, liver and kidney. Apart from organ-manifestations even skin infections can occur (26). Skin lesions are observed in neutropenic patients and are a late symptom of disseminated infections (19). Systemic forms (isolated or disseminated) are cystitis, pyelitis, renal abscess, pericarditis, peritonitis, meningitis, uveitis. In the immunocompromised host especially hepatic and splenic infections increased while the incidence of these complications showed a decrease. *Candida* arthritis and osteomyelitis were once rare and show now an increase in their occurrence. Also the appearance of *Candida* endocartitis increased significantly. Fungemia and sepsis are also systemic forms of the clinical manifestation of *C.albicans* (1, 25).

The mortality rate in patients with invasive *Candida* infections is estimated at about 40%, disseminated candidiasis in particular accounts for a 25% mortality rate (26, 27). There is a wide spectrum of clinical signs in patients with candidemia and disseminated candidiasis, ranging from fever alone up to organ specific manifestations and sepsis. Clinically, it is very difficult to distinguish between acute candidemia and bacterial sepis or shock (28).

Hepatosplenic candidiasis (HSC) belongs to the forms of chronic systemic candidiasis. HSC represents a complication during neutropenia in the immuno-compromised host. The term chronic systemic candidiasis is chosen because of other manifestations in the human body. It is reported that HSC has increased significantly in the last two decades. One reason for that increase may be improved diagnostic imaging. In one study patients who suffered from HSC have a hematological malignancy, febrile neutropenia and were treated with cytotoxic chemotherapy and anti-microbiological agents. Symptoms are antibiotic-resistant fever, abdominal pain and the infiltration of neutrophiles granulocytes in the liver

and spleen. Lab-markers show an increase in leukocytes and serum alkaline phosphatase. Hepatic transaminases usually remain the same (28).

Candida is physiologically found on the skin, in genitourinary and gastrointestinal tracts (13). The most popular species is *Candida albicans*. Others, such as *Candida glabrata* are also frequently isolated and part of the group "*Non albicans Canida*" (NAC). Treatment failure and reduced susceptibility to antifungal agents are responsible for the increase of NAC (27).

In the years 1992-94, a prospective surveillance of 30 clinical centers was done. There was a significant difference between hematological patients with neoplasm and patients with solid tumors concerning distribution of pathogens. The most common identified pathogen was *C. albicans* causing 121/149 episodes of fungemia. In patients with hematological malignancies more NAC were found than in patients with solid tumors (19). The total-mortality after 30 days was 39%. *C. glabrata* associated infections showed the highest mortality followed by *C. tropicalis. C. tropicalis* is the second or third most common *Candida spp.* and predisposes disseminated infections with high mortality in immuno-compromised patients (26). Before 1990, *C. tropicalis* and *C. krusei* were found more frequently in hematological patients, while *C. glabrata* was found in patients with solid tumors (19). Two other prospective studies in Italy showed the increase of *C. glabrata* (29, 30). This increase may be associated with the use of azole antifungal agents. In that study, *C. albicans* only caused 37.5 % of candidaemias in haematologic patients, while it caused 79.4% of candidaemias in intensive-care patients (31).

Candida spp. can cause different types of diseases and early diagnosis remains a challenge. Symptoms are unspecific, blood cultures become positive only late (30). The detection of candida in skin-mucous-membrane smears, sputum, BAL, urine and stool are not indicative of an infection, but of colonization (1). Early clinical manifestations are present in patients with sepsis (27). The late or the non-treatment of patients with invasive candidiasis is an autonomous prophet of death (32).

1.4.3 Cryptococcosis

C. neoformans is the most popular pathogen causing cryptococcosis. It is a yeast species just as *C. albicans*, and acquired by inhalation. *Cryptococcosis* is difficult to diagnose because symptoms, such as pleural symptoms, fever or cough, are non-specific. Meningitis is a common symptom in HIV-seropositive patients (27). Other than the mentioned manifestations in the CNS, the lung, prostate and eye, *C. neoformans* is responsible for further manifestations in different organs (e.g. hepatitis, sinusitis, oesophagitis and pyelonephritis). In the severely immune-compromised host, cryptococcosis can occur. Beside the endocarditis, which is rarely seen, bone involvement and cryptococcal peritonitis are also reported. Furthermore, cryptococcal infections can occur in any organ of the human body (7, 28).

Host factors for cryptococcosis are Mb.Hodgkin, Lymphoma, high-dose therapy of glucocorticoids, decreased T-cell immunity and HIV-infections (26). Invasive cryptococcosis shows the following microbiological criteria (24):

- Cryptococcal antigen in blood samples
- Direct microscopic/cytological evaluation of Cryptococcus in sputum or BAL
- Fungal elements in sterile body fluids detected by cytologic/ direct microscopy.

1.4.4 Aspergillosis

There are more than 200 *Aspergillus spp.* worldwide. Only a few of them are pathogenic to humans and cause invasive infections. The most important species are *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus fumigatus*, which are commonly isolated (27).

Beside the previously mentioned risk-factors, HSCT plays a major role in acquiring IA. Marr et al. revealed that risk for acquiring IA was as high as 13% in patients with allogeneic and autologous bone marrow transplantation (33). Also the use of Infliximab is considered to be a risk factor (34). It is assumed that most patients get the infection outside the hospital. In hospital, it is very important to ensure building activity which is a major source of infection. The presence of plants and food (unpeeled fruits) in hospital rooms also present an enormous risk (19).

A study of Panackal et al. showed a connection between climate and geography concerning IA. In the 10 year study, period allogeneic HSCT patients in two different departments (Seattle and Houston) were compared. The study started in January 1992. During the study period a decrease of incidence of IA in allogeneic HSCT patients was observed in the non-summer months, while in the summer an increase was evident. The IA rate in Seattle was associated with climatic aspects. High temperatures and low precipitation encouraged the spore counts. In Houston there was a lower count of spores, which was independent of the climate (35).

Aspergillus spp. reaches the respiratory tract through inhalation and causes pneumonia (18). Invasive pulmonary aspergillosis is a major threat in patients with hematological malignancies (7). Other preferred sites are the paranasal sinuses, the skin, soft tissue, the CNS, endocardium and the eyes (18). Osteomyelitis can also be caused by *Aspergillus spp*. Symptoms are non-specific. The most common ones are: dyspnoea, subfebrile temperature despite antibiotic therapy and pleural pain (26). *Aspergillus spp.* are able to invade other organs for instance liver and heart. Neurological deficits, cerebral infarcts and abscesses may also be caused by invasive aspergillosis (7).

1.5 Diagnosis of IFI

Because of the high morbidity and mortality associated with IFI, it is very important to provide clarity and uniformity in diagnosis and treatment. The EORTC and the MSG released guidelines for clinical and epidemiology research and in addition for diagnosis and treatment. These definitions should help to provide clarity and uniformity in defining and treating IFI and foster exchange between researchers. The guidelines were revised in 2008 (23).

To establish a definitive diagnosis of IFI, invasive diagnostic procedures are needed. The right identification of the fungus is important for the consecutive antifungal therapy, the duration of therapy and surgical interventions (36). In the EORTC definitions, three levels of probability for diagnosis of IFI are determined

and tailored to immuno-compromised patients with cancer and HSCT recipients (23). The three levels are called "**proven"**, "**probable"** and "**possible".**

1.5.1 Proven

The **proven** level comes into force by detection of a fungus by histological analysis or culture*. C. neoformans*, for instance, is detected by his capsular antigen in the CSF (23).

1.5.2 Probable and possible

The **probable** and **possible** levels are based on three elements (23):

- Host factor
- Clinical signs and symptoms
- Microbiological criteria

In the case of the probable level, a host factor, clinical features and mycological evidence are needed. The possible level is based on adequate host factors and clinical evidence in correspondence with IFD, but without any mycological support (23).

1.5.3 Laboratory detection of IFI

In the diagnosis of IFI, it is very important to collect the species orderly and transport them rapidly to the laboratory. In the laboratory diagnosis of IFIs, there are three methods for detection (37):

- Direct microscopy
- Culture
- Serology

In the direct microscopy, fungal elements make it easier to identify the species.
The culture needs prolonged incubation time and a temperature of 28°C and 37° C to isolate common filamentous fungi and yeasts. Serological tests are available and are used for antibody and antigen detection of *Candida spp., Aspergillus spp.* and *Cryptococcus spp.*. Galactomannan testing (GM) in BAL is a well-proven

diagnostic tool and can be used in addition to microbiologic and radiologic findings to identify IA (37, 38).

1.5.4 Diagnostic procedures

To get to a definite diagnosis of invasive fungal infections some diagnostic steps are important (36):

a) Signs and symptoms
b) Microscopy
c) Culturing techniques
d) Antigen and antibody detection
e) Imaging procedures

Re a) Signs and symptoms

The symptoms of IFI are very unspecific. Unexplained fever and pulmonary infiltrates in the course of broad-spectrum antibiotic-treatment and relapsing febrile episodes are just a few of the non-specific symptoms of IFI. It is very difficult to distinguish bacterial and bloodstream infections from each other because they cannot be clinically differentiated (36).

Cough, pleural pain and hemoptysis are suspicious of pulmonary aspergillosis while sinusitis with necrotic lesions is associated with mould infection.
Moulds also cause IFI in the CNS. It is very important to execute further diagnostic procedures to assure IFI (36).

Re b) Microscopy

Filamentous fungi are detected by direct microscopy. The microscopy enables the detection of the hyphal diameter, the presence of septa or ramification pattern. If there is a suspicion of IFI, it is advisable to examine mycological culture as well as microscopy (36). In many cases it is possible to make a definitive diagnosis by direct microscopy. Because of the less sensitivity of direct microscopy it is important to prove the diagnosis with culturing techniques afterwards (2).

Re c) Culturing techniques

Most pathogenic fungi are identified by culture and common fungi such as *A. fumigatus* and *C.albicans* produce characteristic colonies within a few days. The isolation of *Candida spp*. from clinical samples is easier than the isolation of moulds. Sterile body fluids (blood, CSF, pleural effusion) provide reliable results. Retrospective autopsy studies found out that only 50 % of the patients with disseminated candidiasis show positive blood cultures during their lifetime. For clinical diagnosis of invasive fungal infections, blood cultures (three pairs from peripheral veins and central lines) are needed regularly. The disadvantage of blood cultures in detecting invasive fungal infections is the low sensitivity for moulds. The highest detection rate is obtained by two to three aerobic/anaerobic blood culture bottles with 10 ml of blood each (2, 36). The sputum of a patient with neutropenia > 7days and clinical signs tailored to IFI should be taken as a possible indication for fungal pneumonia. In spite of the physiological appearance of yeast on the skin and the upper and lower gastrointestinal tract, a positive sputum or bronchoalveolar lavage (BAL) fluid is suspicious of contamination or colonization until the contrary is proven. Culturing techniques of BAL fluid, sputum or other fluid compartments are very important for the early diagnosis of IA. Patients who are at high risk for IFI should dispense blood samples which have to be cultured for fungi. Fungi which were recovered should be identified to find out their species (36, 39).

Re d) Antigen and antibody detection in blood and CSF

In the diagnosis of invasive candidiasis and aspergillosis, various antibody and antigen test methods are available. Also in the diagnosis of *C. neoformans*, antigen testing is a commonly accepted method. The cryptococcal meningitis in the immuno-compromised host is mainly detected by cryptococcal antigen in blood or CSF. The testing should always be done in both samples (36).

The ELISA test for detecting *Candida*-mannan-antigen in neutropenic patients is a sensitive tool, but a definition of the clinical value has to be found. Because of the wide-ranging sensitivity (17%-90%) of antibody tests, it is more useful to link antigen and antibody tests (36). The test has yet, however, not been established in hematological patients. The Galactomannan antigen testing (Platelia®

Aspergillus) shows a specificity of 90%-100% and a sensitivity of 80%-100% for diagnosing invasive aspergillosis (36). Another test is the 1->3-ß-D-Glucans (BG) test (35). BG is part of the fungal cell wall and the availability of this component in the bloodstream walks along with IFI (40).

Technique was improved through years to detect IA early. The main focus is on circulating markers, including genomic fungal DNA and fungal cell wall components (1->3 β-D-glucan) (41). In different laboratories, ELISA is used for the detection of *Aspergillus* antigens. This special test is used in patients with hematological malignancies, neutropenia and HSCT (42). This method is established in many European countries and is one of the non-invasive additional diagnostic tests. In the context of screening-programs, blood-serum of patients with high-risk was tested. With the Platelia© Aspergillus test it is possible to discover Galactomannan (43).

Galactomannan is part of the cell wall of *Aspergillus spp*. It is a polysaccharide and is released by growing hyphae (44). This major aspergillar element is set free during ongoing invasive disease (45). Contrary to the Platelia© Aspergillus, histo-pathological examinations of deep tissue infections are considered to be gold standard. These kinds of examinations are very difficult to manage in the immuno-compromised host. Positive and dubious results have to be proven twice to avoid false-positive ones. These false-positive results can occur through contamination of the samples by ubiquitous *Aspergillus* spores (43). The Platelia-test provided good results in the detection of IA. Different studies showed a higher sensitivity (80-100%) and a higher specificity (90-100%) than former procedures (46). A Cochrane review by Leeflang et al. 2008 was obtained to evaluate the diagnostic accuracy of GM detection in serum for the diagnosis of IA. In several studies, different sensitivities and specificities were evaluated. In twelve studies the overall sensitivity was 78% (61% to 89%) and the overall specificity was 82% (72% to 88%). In twelve other studies the overall sensitivity was 75% (59% to 86%) and the mean specificity was 91% (84% to 95%), while in another 17 studies the overall sensitivity was 64% (50-77%) and the mean specificity was 95% (91% to 97%) (48).

Serological tests have to be controlled in short intervals and are recommended twice a week. In the neutropenic-phase, antigen-testing is the preferred method (46). A study of Pfeiffer et al. indicated that the usefulness of GM testing is higher in patients with hematological malignancies or HSCT than in patients with solid-organ transplantation (47). It is assumed that GM is released by different *Aspergillus spp*. and some other moulds, but has no specificity for *Aspergillus fumigatus* (36). GM tests remain negative in IFIs caused by *Zygomycetes*, *Fusarium spp*. or *Candida spp*. To evaluate the outcome of IA in patients with hematological malignancies, a noninvasive, objective and reproducible marker as the GMI is needed. This assay seems to fulfill the opportunity to monitor the outcome (49). Because of the intermittent distribution of GM-antigen, it is very important to control serological tests at least every second to third day (7). The ELISA test is also used for the detection of IA in other body fluids, for instance liquor and bronchial lavage (46).

In addition to the GM, there is another test for the diagnosis of IFI. The so called Fungitell assay is a test which detects the (1 ->3)-ß-D-Glucan (BG). (1 ->3)-ß-D-Glucan is a cell wall component of a huge number of fungi. Yet, many fungi, including *Candida spp*., *Aspergillus spp*. and *Pneumocystis jiroveccii* produce 1-3-β-D-Glucan, *Zygomycetes spp.* and *C.neoformans* do not have this component. BG activates factor G of the horseshoe crab coagulation cascade (50). The presence of BG in the blood circulation may be associated with IFI (61). The use of BG still remains a sparsely defined, additional tool for the diagnosis of IFI in patients with compatible clinical symptoms (51).

Re e) Imaging procedures

In conventional chest X-rays, early signs of invasive aspergillosis are not detected because signs of IA occur late. Therefore, early diagnosis by conventional chest X-rays is difficult. Typical infiltration patterns (“halo-sign”, macronodules) of IA are caught early by high-resolution computed tomography (HRCT) or thin section CT (36, 39).

1.5.5 Diagnosis of IA

To confirm the diagnosis of IA a tissue biopsy showing invasion with hyphae has to be done. Proven IA also requires positive culture for *Aspergillus*.

Because of the *Aspergillus* hyphae, which are 3-6 µm, hyaline septate and acute angle branched, it is possible to differentiate from mucormycosis but not from other opportunists, including *Fusarium*. The suspicion of probable invasive Aspergillosis can also be confirmed by BAL culture or BAL/Serum GM test. Patients with a low risk for IA and without any clinical illness, but with a positive culture for Aspergillus should be observed carefully (25).

HRCT is a fixed value in the diagnostic of IA and should be performed early in patients with non-specific pulmonary symptoms. Typical alterations in the CT are (52):

- "halo-sign"
- "Crescent-of-air-sign"
- Little nodular infiltrates

A pulmonary nodule or mass in the CT is surrounded by an area, which reminds of ground-glass attenuation (53). The appearance of the halo-sign in neutropenic patients is suspicious of invasive aspergillosis (54). Crescent-of-air sign occurs through air between the devitalized tissue and the parenchyma (55). Air-crescent sign and cavitations are seen before or after the diagnosis of neutropenia. In high risk patients, the use of HRCT should be preferred for the diagnosis of IA (36).

1.6 Treatment of invasive fungal infections in hematological patients

The treatment of invasive fungal infections in patients with hematologic malignancies is still a major problem and represents a challenge. The most important aspect is the deficiency of adequate methods for early diagnosis (56). There are four therapeutic possibilities in the management of IFI in patients with cancer (57):

a) Prophylaxis
b) Empirical treatment

c) Pre-emptive treatment
d) Treatment of established IFI

Re a) Patients at risk are treated prophylactically to avoid any possibility of fungal infection. In this case, patients do not show any sign of disease. The main aspect of prophylaxis is to accomplish a decrease in the incidence and mortality of IFI (57).

Re b) It is very important to treat neutropenic patients with persistent fever and no response to broad-spectrum antibiotics with anti-fungals. The patients do not show any radiological (57).

Re c) Neutropenic patients with persistent fever, significant colonization, positive radiological signs and antigen/PCR findings (probable IFI) are treated pre-emptively. The early acting can save lives (57).

Re d) Treatment of established IFI means to fight against the proven aspergillosis or IC (57).

1.6.1 General antifungal therapy

The detection of Nystatin in 1949 lead to a development of specifically-acting antifungals. There are three groups of antifungals for the treatment of invasive fungal infections (58):

- Echinocandins (Caspofungin)
- Triazole (Posaconazole, Voriconazole, Itraconazole)
- Polyene (liposomal Amphotericin B)

Echinocandins

Caspofungin is an agent of the Echinocandin-group. Echinocandins show an interaction with the Beta-1, 3-D-Glucan-Synthase, which occurs in the fungal cell wall. Because of this interaction, the building of Beta-1,3-Glucan is stopped. Beta-1,3-Glucan as well as ergosterol are not components of the human cells (58) .

Triazoles

The agents Posaconazole, Itraconazole, Fluconazole and Voriconazole are part of the Triazoles group. Azoles block the activity of lanosterol-C-14-alpha-Demythelase by linking on the enzyme's heme group, followed by a strong decrease of the egosterol synthesis. There is an accumulation of toxic sterols which are integrated into the cell-membrane. The fungal-cell loses its ability for reproduction (41).

Polyenes

Liposomal Amphotericin B is part of the polyenes. This group forms pores in the cell-membrane and initiate fungal cell's lysis by the outflow of monovalant cationes and low-molecule substances (41).

1.6.2 Activity spectrum of antifungal agents

Echinocandins: clinical efficacy for moulds, yeasts, inhibition of the synthesis of the cell wall (58)

Indication: refractory IA, IC (58)

- Caspofungin: refractory IA, IC (58)
- Anidulafungin: refractory IC (58)

Triazoles: clinical efficacy for moulds, yeasts, dermatophytes, inhibition of the biosynthesis of ergosterol (58)

Indication: invasive aspergillosis, other mould infections (58)

- Posaconazole: clinical efficacy for moulds, yeasts and dermatophytes
  Indication: invasive aspergillosis, other mould infections (58)
- Voriconazole: clinical efficacy for moulds, yeasts and dermatophytes
  Indication: invasive aspergillosis, invasive candidiasis (58)
- Itraconazole: clinical efficacy for moulds, yeasts and dermatophytes
  Indication: dermatophyte infections (58)
- Fluconazole: clinical efficacy for yeasts and dermatophytes; lack of efficacy for moulds (58)
  Indication: skin mycoses; candidiasis (58)

Polyenes:

- Liposomal Amphotericin B: clinical efficacy for moulds, yeast; lack of efficacy for dermatophytes (58)
  Indication: IA, IC, Zygomycosis (58)

Casopfungin (Cancidas ©) is approved for antifungal treatment in non-neutropenic patients (27). The fungicidal activity of Caspofungin is directed against the whole *Candida spp* and aims to eliminate candidiasis (27, 57). A study of Mora-Duarte et al showed the same effect of AmB-D in candidiasis. The only difference between Caspofungin and AmB-D was the better tolerance of Caspofungin (59). Caspofungin also has a fungistatic effect against *Aspergillus spp*. and is used in patients with IA who are intolerant to other antifungal agents as salvage therapy (57). In febrile neutropenic patients, Caspofungin is also used as an empirical antifungal agent. A trial of Walsh et al. 2004 compared liposomal amphotericin B and caspofungin. The randomized trial involved over 1000 neutropenic patients. In this group, Caspofungin showed a better effect and a higher tolerance (27).

Anidulafungin (Ecalta©) is approved for antifungal treatment in patients with invasive candidiasis. A study of anidulafungin compared with fluconazole was performed. In this study, anidulafungin with a dosage of 200 mg on day 1 and then 100mg/day was matched with fluconazole with a dosage of 800 mg on day 1, followed by 400 mg/ day. The success rate of anidulafungin (75%) was higher than of fluconazole (60 %) (27%). Anidulafungin has an “in vitro” activity against *Aspergillus spp*., but not an “in vivo” one (25).

Posaconazole (Noxafil©) represents an oral azole and belongs to the new generation of broad spectrum azoles. In vitro Posaconazole shows activity against many fungi with medical importance (*Aspergillus spp., Candida spp., Fusarium spp., Zygomycetes*). In vivo, Posaconazole shows a high activity against IA and IC (60). Posaconazole is taken as a prophylaxis in patients at high risk for IFI (e.g GVHD patients, prolonged neutropenia). 200 mg of Posaconazol three times daily reduces the risk of fungal infections in allogeneic HSCT recipients with GVHD and patients with prolonged neutropenia after myelosuppressive chemotherapy for

AML or MDS. Posaconazol proved superior to fluconazole in the prophylaxis of fungal infections in patients with GVHD (25).

Voriconazole (Vfend©) was developed and licensed for the therapy of IA. A randomized unblended comparative trial showed a better survival outcome with voriconazole than with amphotericin B. Voriconazole has the European license for treatment of invasive *Candida* infections which are resistant to fluconazole. Voriconazole is considered the gold standard treatment for IA in all guidelines (27). In patients with documented IA, voriconazole is therefore the antifungal agent of choice (57).

Fluconazole (Diflucan©) plays an important role in the prophylaxis treatment and is one of the most studied antifungal agents. A daily dose of 400 mg fluconazole in HSCT recipients and allogenic bone marrow patients reduce the incidence and mortality of IFI (57). The controlled trial of Goodmann JL et al.1992 and the prospective, randomized, double-blind study of Slavin et al. 1995, showed a decrease of morbidity and mortality of allergenic HSCT recipients through fluconazole (60). Fluconazole also has an effect on GVHD (57). Fluconazole is also used for prophylaxis in patients with neutropenia. An important aspect is the presence of fever in these patients. The major drawback of fluconazole is the lacking efficacy against moulds (60). In non-neutropenic patients with candidaemia, fluconazole has the same effect as amphotericin B (27). In several studies, break-through infections were observed. This effect happens because of the ineffectiveness of fluconazole against moulds and *C.krusei*. The activity against *C.glabrata* depends on the dose (57). An empirical therapy with fluconazole in neutropenic patients with suspected fungal infections is contraindicated because of its limited effect on moulds (61).

Compared to fluconazole, itraconazole (Sporanox©) shows a broader activity spectrum. This spectrum contains activity against *Aspergillus spp*. In their meta-analysis of randomized controlled trials, Vardakas KZ et al. showed that prophylaxis with itraconazol in neutropenic-patients with hematological malignancies is superior to fluconazole (60). Itraconazole reduces break-through IFIs. In the incidence and mortality due to fungal infections, itraconazole has the

same effect as fluconazole. In allogeneic HSCT recipients, there is no benefit for itraconazole compared to fluconazole. The usage of itraconazole, in combination with other drugs, leads to several cases of toxic death and is not recommended for prophylactic use (57).

The indication for L-AmB (Ambisome©) is severe systemic and/or deep mycoses. L-AmB is also used as an empirical therapy in neutropenic patients with fever in whom antibiotics have failed (27). In general, Amphotericin B has the broadest spectrum of activity. The oral application reduces the incidence of superficial fungal infections, but not the invasive ones. In the management of IFI it is important to take the lipid based forms of Amphotericin B. The lipid forms show less toxicity than the Amphotericin B deoxycholate (57). The paper of Walsh et al. 1999 showed the same effectiveness of L-AmB and AmB-D concerning the empirical treatment of neutropenic sepsis. The study also established fewer side effects of L-AmB (27).
Cornely et al. have shown the superiority of Voriconazole over amphotericin B deoxycholate in their AmBiLoad Trial. Voriconazole shows a lot of drug interactions and no activity against *Zygomycetes*. Amphotericin B deoxycholate has a broad activity against *Aspergillus spp.*, *Zygomycetes* and other moulds, but has a low effect in immune-suppressed patients and serious toxicities. The better solution of treatment of immune-suppressed patients is the use of liposomal amphotericin B. Liposomal amphotericin B is safer than the deoxycholate forms and can be used in higher doses (62).

2 Material and methods

During the study period, 729 patients were admitted to the study centre. Of these, 129 patients with hematological malignancies and antifungal therapy were identified at the Department of Internal Medicine, Division of Hematology, Medical University of Graz .The duration of the study was seven months, from April 2010 until October 2010. The study was supervised by ao.Univ.-Prof Dr. Robert Krause and Dr. Martin Hönigl.

The type of study was prospective and the patients were observed twice a week at the Division of Hematology. All patients were observed during their inpatient stay. No data of the outpatient department were collected. The research questions dealt with the epidemiology of invasive fungal infections. IFIs were defined according to EORTC criteria 2008. Furthermore, the crucial question was the impact of the GM (Platelia©) assay usage on epidemiology of IFI. GM testing was accomplished twice a week at the Division of Hematology.

2.1 Study aims

- Evaluating epidemiology of IFI in patients with hematological malignancies
- Evaluating the host factors according to the EORTC guidelines 2008
- Evaluating risk factors for IFI
- Accurate evaluation of underlying disease, chemotherapy, neutropenia, antifungal therapy, imaging methods, GM-antigen testing
- Evaluating GM-antigen testing as a sensitive tool in the early diagnosis of IFI
- Comparison of patients with antifungal therapy and possible, probable or proven IFI, with patients under antifungal therapy but without IFI

2.2 Methods

In this study, patients with hematological malignancies were observed prospectively twice a week at the Division of Hematology, Medical University of Graz. This was an observational survey of patients with evidence of IFI and

patients receiving antifungal therapy. Medical reports of the patients were reviewed. Data of 729 cases during their inpatient stay were collected. The data were evaluated from the medical records and transmitted on case report forms (CRF). The collection of data was simplified by the use of these CRF. The CRF enabled an accurate recording of data and had three pages (AIHM 1-AIHM 3). Not all of these three pages were used for every patient, because not every patient had an antifungal therapy or fulfilled EORTC/MSG criteria for IFI. The classification of proven, probable and possible is predetermined by the EORTC guidelines 2008 and is a very important tool of this study.

To accompany the patients during their whole inpatient stay, data were completed by the use of MEDOCS. MEDOCS is the database of the Medical University of Graz, Leopold Auenbrugger, which contains the medical history of all patients undergoing medical treatment at the University hospital. MEDOCS is a SAP (System Applications and Products in Data Processing, Walldorf, Germany) based system, which is used for patient administration, cost calculation and storage of data in all departments and divisions of the Medical University of Graz. All the data were collected anonymously in an electronic database.

For all 729 cases, a CRF was set up. On page 1 sex, age, date of inpatient stay, underlying diseases and room of care were evaluated first. Room of care means standard room, which is located at the normal-care unit and heap-filtered room, which is a room at the bone-marrow transplantation unit, where air is filtered of bacteria, fungi and aerosols. The chemotherapy was checked to evaluate the schema – high-dose or low-dose/palliative. The use of corticosteroids and the duration was also considered. The question concerning HSCT was also part of the CRF - it was important to differ between allogeneic and autologous. Depending on the time of SCT, we differentiated recent SCT (<6 months before episode/admittance) and previous SCT (>6 months before episode/admittance). Another important part of the CRF was the use of T-cell suppressants, and if yes, which one. Neutropenia (< 500/mm$^{3)}$ evaluated and also its duration. Results of bronchoalveolar lavage (BAL) and bronchoscopy (BSC) were evaluated. One of the crucial questions of this study was the usage of GM testing and GM results. Microbiological examinations were also evaluated and also biopsies of the lung/or

other organs were questioned. Imaging methods especially CT of the thorax or the paranasal sinus were evaluated. If a CT was done, specific signs of IFI were checked. All these points were on page 1 of the CRF. Page 1 was filled out for every patient.

On page 2, antifungal agents were evaluated (n=129). Some of the patients got more than one antifungal agent during their inpatient stay. Dosage and duration of therapy with Caspofungin / Cancidas©, Voriconazole / Vfend©, LipAmpho B / Ambisome©, Posaconazole / Noxafil©, Itraconazole / Sporanox©, Fluconazole / Diflucan©. Anidulafungin / Ecalta©, Micafungin / Mycamine© and others, were evaluated. It was very important for the outcome of this study to evaluate the cause of therapy carefully. The causes for antifungal therapy were prophylactic, empirical, preemptive and directed. In case of prophylaxis, the respective reason was evaluated (AML,HSCT and GVHD). The question in the CRF to AML, HSCT and GVHD was important too. If patients fulfilled EORTC/MSG criteria for IFI (n=28), page 3 was completed.

Page 3 contained date of diagnosis and the IFI criteria set out in the EORTC guidelines. Patients were categorized into three groups - **probable, possible and proven**.

The category possible is consisting of three clinical criteria - specific sign, sinusitis plus clinical findings and unspecific sign plus clinical findings. Specific signs mean the presence of typical changes in the imaging, for instance dense, well-circumscribed lesions with or without halo sign, air-crescent sign or cavity. Another criterion is the presence of sinusitis and clinical signs, for instance acute localized pain or nasal ulcer. Also unspecific signs and clinical findings are evaluated in this category. Another important part of the category possible is represented by the host criteria. Those involve allogeneic HSCT, neutropenia, prolonged use of corticosteroids and T-cell suppressants.

The category probable is also based on the same clinical criteria and host criteria as the category possible plus, in addition, a microbiological criterion.
Microbiological criteria contain:

- Culture
- Antigen detection (GM)

Details are depicted in Table 2.

The category proven contains the fungal species and the question of organ involvement. Details are depicted in table 1.

In case of IFI whether or not, the infection fulfilled criteria of break-through infection, as well as therapy response and the outcome after 12 weeks were additionally evaluated.

2.3 Study population

2.3.1 Definitions

The whole study population contained 729 patients. Every patient represented one case. Every case was observed accurately and followed up by its medical reports. If a patient changed the medical unit from the normal care unit to the bone-marrow transplantation unit, and new antifungal therapy was prescribed, the patient counted as a new case. Some of the 729 cases were readmitted. A patient readmitted with prolonged antifungal therapy (as a prophylactic therapy) was still counted as one case. The only reason for establishing a new case in patients readmitted was a change in the antifungal therapy.

In this study, 729 cases with hematological diseases were evaluated. Among the 729 cases, 129 cases with hematological malignancies were treated with antifungal agents. There are 85 male and 44 female cases in this population.

In the course of the study, it was possible to evaluate the host factors and to classify the patients into the categories, probable, possible and proven. Another aim of the study was the evaluation of host factors. Three groups (group A, B, C) were defined in which the host factors were evaluated. In group A, B and C, all important host factors for invasive fungal infections were evaluated and the groups were compared among each other. These host factors were prolonged

neutropenia > 10 days, treatment with T-cell suppressants > 90 days, prolonged use of corticosteroids < 21 days and allogeneic HSCT. Group A contained cases with probable/proven IFI, group B contained patients with antifungal therapy but without IFI and group C contained patients without systemic antifungal therapy and without IFI. Group A included 28 patients, group B 101 patients and group C included 197 patients. Patients of group C, who conformed to the host factor criteria, were collected as one inpatient stay. Longest inpatient stay was evaluated in case of readmittance. By means of these drafts, host factors were evaluated.

2.3.2 Criteria of including and excluding

Including criteria

Study population includes all patients above 18 years with systemic antifungal treatment at the Division of Hematology, Medical University of Graz.

Excluding criteria

Study population excludes all patients below 18 years with long term prophylactic antifungal treatment at the Division of Hematology, Medical University, who were recorded before the start of the study.

Table 1: Criteria for proven IFI (23)

Analysis and specimen	Moulds	Yeasts
Microscopic analysis: sterile material	Histopathologic, cytopathologic, or direct microscopic examination of a specimen obtained by needle aspiration or biopsy in which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage	Histopathologic, cytopathologic, or direct microscopc examination of a specimen obrained by needle aspiration or biopsy from a normally sterile site (other than mucosa membranes) showing yeast cells- for example, *Cryptococcus* species indicated by encapsulated budding yeasts or *Candida* speciesshowing pseudohyphae or true hyphae.
Culture		
Sterile material	Recovery of a mould or „black yeast“ by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consitent with an infectious disease process, excluding BAL fluid, a cranial sinus cavity specimen, and urine	Recovery of a yeast by culture of a sample obtained by a sterile procedure (including a freshly placed, 24 hours ago, drain) from a normally sterile site showing a clinical or radiological abnormality consitent with an infectious disease process
Blood	Blood culture that yields a mould (e.g *Fusarium* species) in the context of a compatible, infectious disease process	Blood culture that yields yeast (e.g., *Cryptococcus* or *Candida* species) or yeast-like-fungi (e.g., *Tricosporon* species)
Serological analysis: CSF	Not applicable	Cryptococcal antigen in CSF indicates disseminated cryptococcosis

Table 2: Criteria for probable IFI (23)

Host factors[a]	Clinical criteria[b]	Mycological criteria[c]
Recent history of neutropenia (<0.5 x 10^9 neutrophils/L [!500 neutrophils/mm3] for 110 days) temporally related to the onset of fungal disease Receipt of an allogeneic stem cell transplant Prolonged use of corticosteroids (excluding among patients with allergic bronchopulmonary aspergillosis) at a mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks Treatment with other recognized T cell immunosuppressants, such as cyclosporine, TNF-a 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)	Lower respiratory tract fungal disease[c] 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[d]: 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	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)[e] *<u>Aspergillosis</u>* *Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF* *<u>Invasive fungal disease</u> other than cryptococcosis and zygomycoses* *b-d-glucan detected in serum*

NOTE: Probable IFD requires the presence of a host factor, a clinical criterion, and a mycological
criterion. Cases that meet the criteria for a
host factor and a clinical criterion but for which mycological criteria are absent are considered possible IFD.
a Host factors are not synonymous with risk factors and are characteristics by which individuals predisposed
to invasive fungal diseases can be recognized. They are intended primarily to apply to patients given treatment for malignant disease and to recipients of allogeneic hematopoietic stem cell and solid-organ transplants. These host factors are also applicable to patients who receive corticosteroids and other T cell suppressants as well as to patients with primary immunodeficiencies.
b Must be consistent with the mycological findings, if any, and must be temporally related to current episode.
c Every reasonable attempt should be made to exclude an alternative etiology.
d The presence of signs and symptoms consistent with sepsis syndrome indicates acute disseminated disease,
whereas their absence denotes chronic disseminated disease.
e These tests are primarily applicable to aspergillosis and candidiasis and are not useful in diagnosing
infections due to Cryptococcus species or Zygomycetes (e.g., Rhizopus, Mucor, or Absidia species). Detection of nucleic acid is not included, because there are as yet no validated or standardized methods.

3 Results

A total of 729 cases with hematological malignancies were admitted to the Division of Hematology, Medical University of Graz. 129/729 (18%) cases with hematological malignancies and antifungal treatment were evaluated during seven months in 2010. Out of 129 cases, 28 fulfilled criteria of possible, probable or proven IFI.

3.1 Gender distribution of study population

85/129 patients with antifungal therapy were male (66%), 44/129 were female (34%). The median age of the 129 patients was 53.5 years.

*Fig.*1: Gender distribution

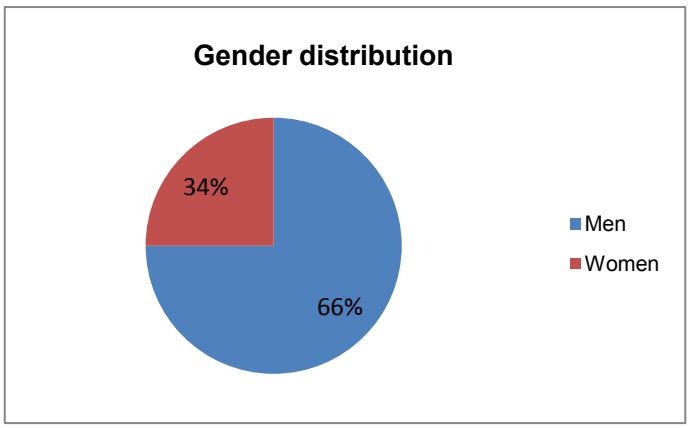
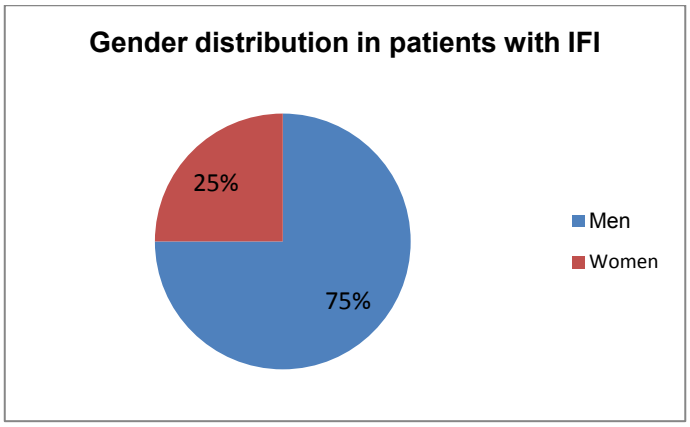


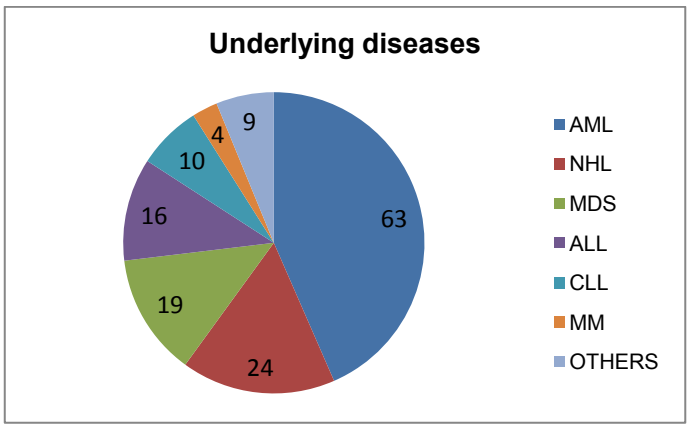
*Fig.*2: Gender distribution in patients with IFI



Concerning the 28 patients with IFI, 21 were male (75%) and seven female (25%) The median age of patients with IFI was 53.6 years. Details are depicted in *Fig*.2.

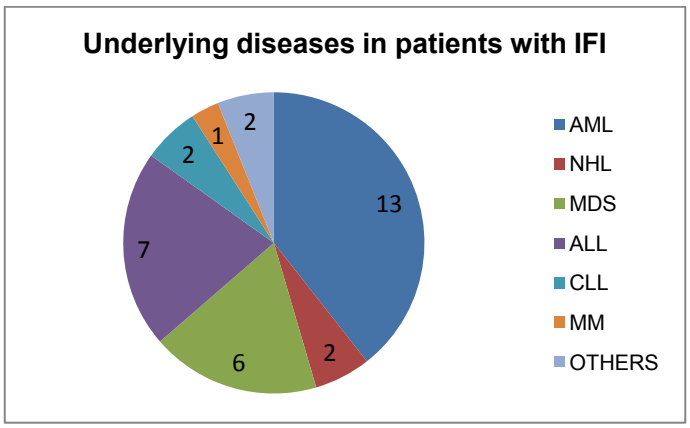
3.2 Underlying diseases

Fig.3: Underlying diseases



In this study, the leading underlying disease was AML (63/129 patients; 49%), followed by NHL (24/129; 19%) and MDS with (19/129; 15%) patients. 16/129 (12%) had ALL, 10/129 (8%) CLL and 4/129 (3%) MM. Other underlying hematooncological diseases were present in 9/129 (7%) patients. Underlying diseases in patients with IFI are depicted in *Fig*.4

Fig.4: Underlying diseases in patients with IFI

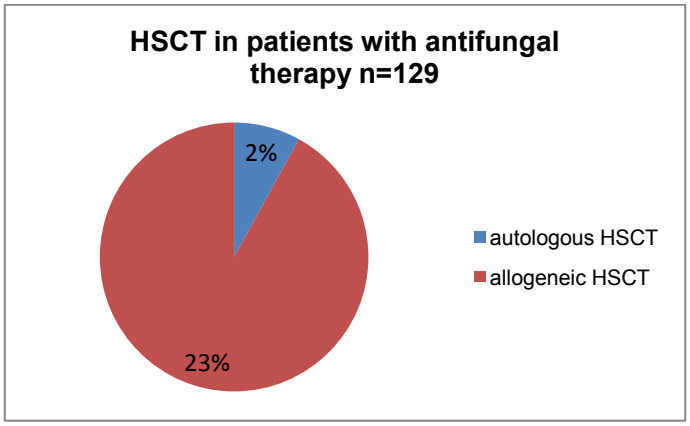


The most common underlying disease in patients with IFI was AML (13/28 patients; 46%), followed by ALL (7/28 patients; 25%) and NHL (2/28 patients; 7%). 6/28 (21%) patients had MDS and 2/28 (7%) patients had CLL. Other underlying hemato-oncological diseases such as MM or others were present in 1/28 (4%) and 2/28 (7%) patients.

3.3 HSCT

30/129 patients with antifungal therapy (23%) underwent allogeneic HSCT of whom 6/129 (5%) underwent recent allogeneic HSCT and 24/129 (19%) patients underwent previous allogeneic HSCT. 2/129 patients (2%) underwent previous autologous HSCT.

*Fig.*5: HSCT in patients with antifungal therapy



3.4 Chemotherapy

81/129 patients (63%) with antifungal therapy were treated with a high-dose chemotherapy, whilst 17/129 (37%) patients were treated with a low-dose/palliative chemotherapy. The percentage of chemotherapy in patients with antifungal therapy is depicted in Figure 6. 20/28 (71%) patients with IFI were treated with a high-dose chemotherapy and 3/28 (29%) patients with IFI were treated with a palliative (low-dose) chemotherapy. The percentage of chemotherapy in patients with IFI is depicted in *Fig.*6 and *Fig.*7.

*Fig.*6 Chemotherapy in patients with antifungal therapy

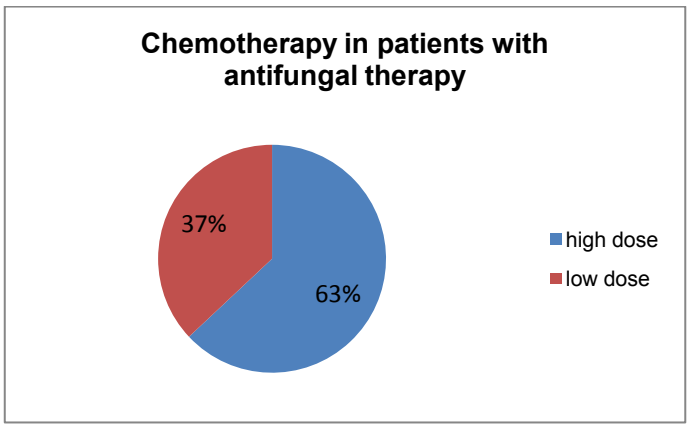
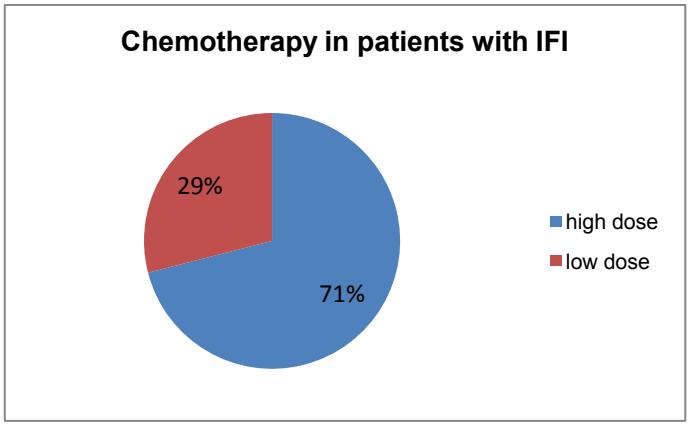


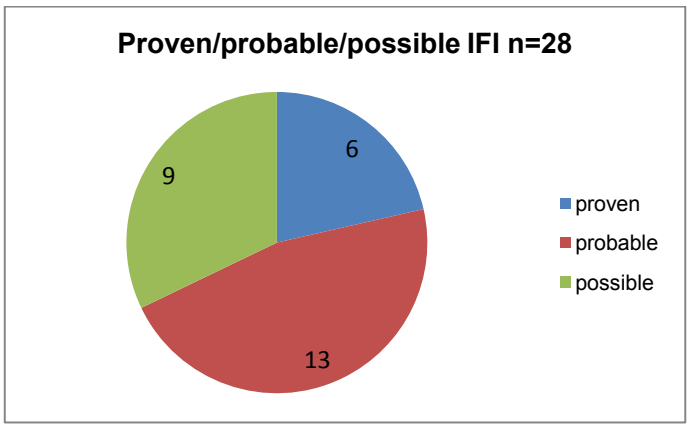
*Fig.*7 Chemotherapy in patients with IFI



3.5 Invasive fungal infections

Out of the 729 patients, 28/729 (3,8%) had an invasive fungal infection according to the EORTC criteria. These 28 patients were assigned into the categories proven (6/28; 21%), probable (13/28; 46%) and possible (9/28; 32%). Results are depicted in *Fig.*8.

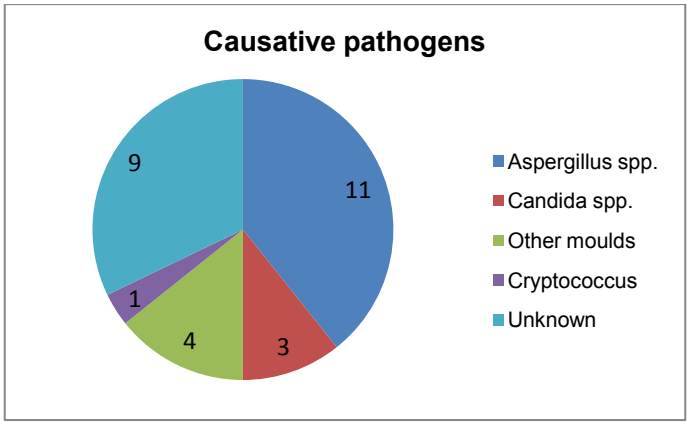
Fig.8: Proven/probable/possible IFI



Most of IFIs were caused by moulds, followed by yeasts. In the case of moulds, *Aspergillus spp*. was found in 11/28 patients (39%). Other moulds affected 4/28 patients (14%). 9/15 IMIs were only diagnosed by GM testing. 9/28 patients (32%; all possible IFIs) developed IFI with an unknown pathogen. These unknown pathogens were *Fusarium solani, Absidia spp., Geosmithia argillacea and Penicillium spp.*

In patients with yeast infections, *Candida spp*. were found in 3/28 patients (11%). *Candida albicans* was found in 2/3 patients while *Candida parapsilosis* was found in 1/3 patients. *Cryptococcus neoformans* was found 1/28 patients (4%). Results are depicted in *Fig*. 9.

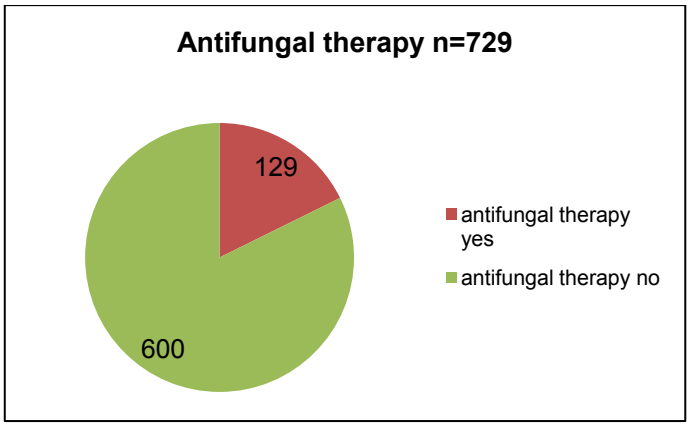
Fig. 9: Causative pathogens



3.6 Antifungal therapy

129/729 patients received an antifungal therapy. Results are depicted in Figure 10.

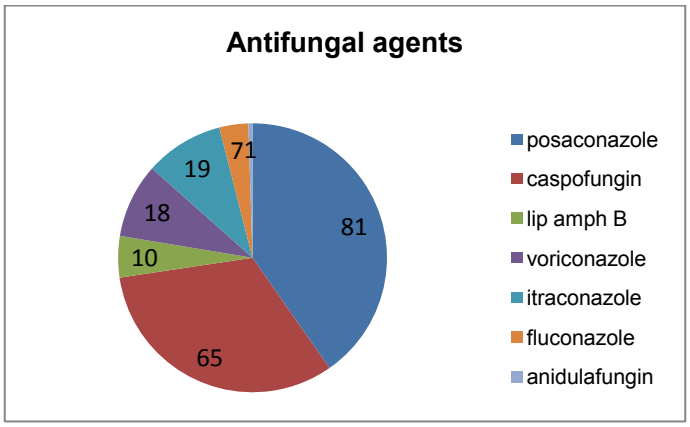
*Fig.*10: Antifungal therapy



In the course of this study, patients with antifungal therapy were treated with seven different antifungal agents. Some of the patients were treated with more than one of these agents.

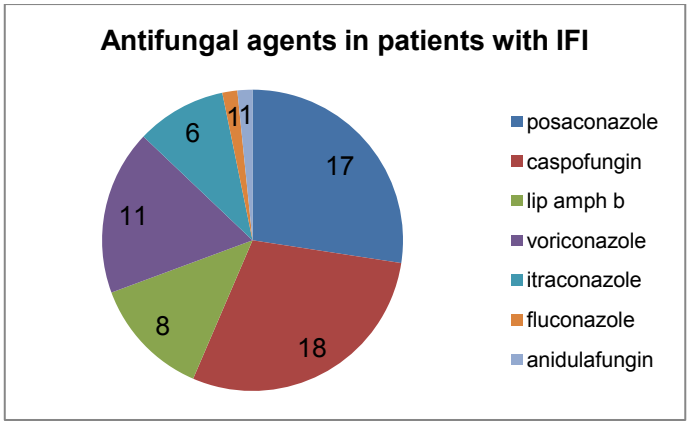
The agent prescribed most frequently was posaconazole (81/129; 63%), followed by caspofungin which was prescribed in 65/129 patients (50%). Itraconazole was used in 19/129 (15%) patients and voricoanzole in 18/129 patients. Lip amph B was prescribed in 10/129 (8%) patients and fluconazole in 7/129 (5%) patients. Anidulafungin was prescribed only in 1/129 patients (1%). Antifungal agents are depicted in *Fig.*11.

*Fig.*11: Antifungal agents



Patients with IFI were treated with antifungal agents as follows: The most commonly used agent was caspofungin in 18/28 patients (64%), followed by posaconazole in 17/28 patients (61%). Voriconazole was used in 11/28 patients (39%), lip amphotericin B in 8/28 patients (29%). Itraconazole was used in 6/28 patients (21%), fluconazole and anidulafungin were prescribed in one patient (4%) each. Results are depicted in *Fig*.12.

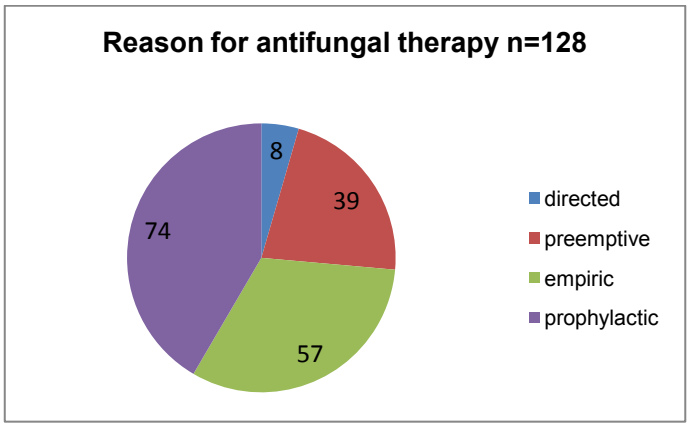
Fig.12: Antifungal agents in patients with IFI



3.7 Reason for antifungal therapy

The reasons for the 129 patients to receive antifungal therapy were classified into directed, preemptive, empirical and prophylactic. Some patients fulfilled more than one criterion. The most important cause for receiving antifungal agents in 74/129 (57%) patients was prophylactic. An empirical therapy was done in 57/129 patients (44%). 39/129 (30%) patients got a preemptive therapy, while 8/129 were treated in a directed approach. Results are depicted in *Fig.*13.

Fig.13:Reason for antifungal therapy



3.8 Host factors

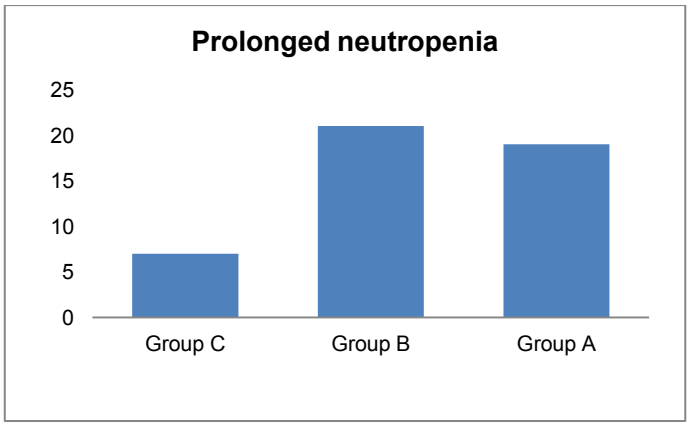
Host factors according to EORTC/MSG were evaluated in patients with possible/probable/proven IFI (group A n=28), patients with systemic antifungal therapy but without IFI (group B; n=101) and patients without systemic antifungal therapy and without IFI (group C n=197).

3.8.1 Prolonged neutropenia

Prolonged neutropenia means <500 neutrophils/mm^3 for >10days.

Prolonged neutropenia occurred in 19/28 (68%) patients in group A and in 21/101 patients in group B. 7/197 patients (4%) in group C had prolonged neutropenia as a host factor. Results are depicted in *Fig*.14.

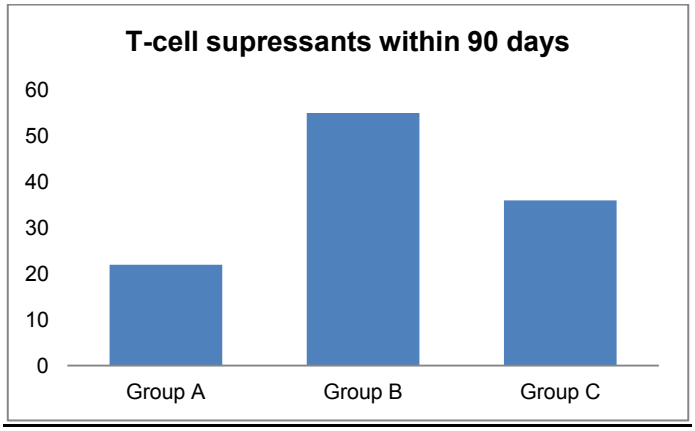
*Fig.*14: Prolonged neutropenia



3.8.2 Treatment with T-cell supressants >90 days

In group A, 22/28 patients (79%) were treated with T-cell supressants within 90 days before diagnosis. 55/101 (54%) patients of group B and 36/197 (18%) of group C had a T-cell suppressants therapy. Results are depicted in *Fig*.15.

Fig.15: T-cell suppressants within 90 days

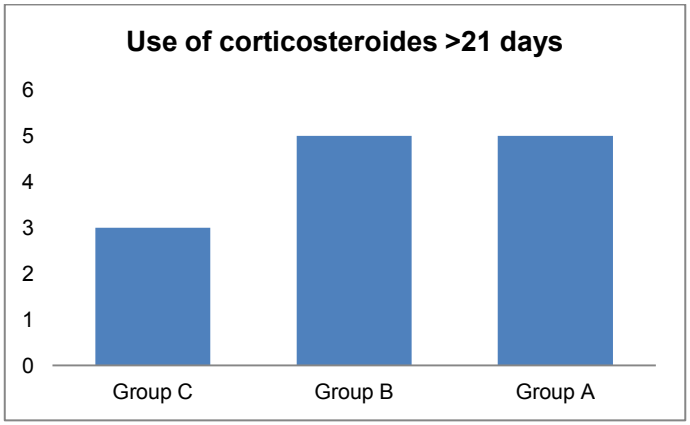


3.8.3 Prolonged use of corticosteroids

Prolonged use of corticosteroids is defined as a minimum dose of 0.3 mg/kg/day of prednisone >21 days.

In group A, 5/28 patients (18%) had a prolonged use of corticosteroids. In group B, 5/101 patients (5%) used corticosteroids >21 days while 3/197 (2%) patients of group C were treated over 21 days with corticosteroids. Resultes are depicted in *Fig.*16.

Fig.16: Use of corticosteroides



3.8.4 HSCT

Allogeneic HSCT

5/28 patients (18%) in group A, 1/101 patients in group B and 0/197 patients in group C underwent recent allogeneic HSCT while 6/28 patients in group A (21%), 18/101 patients (18%) in group B and 17/197 (9%) in group C underwent previous allogeneic HSCT.

3.9 Outcome of patients with IFI

23/28 patients (82%) improved while on antifungal therapy. The rate of 12 weeks survival was 75% (21/28 patients) while a break-through infection was observed in 4/28 patients (14%) with IFI.

3.10 Room of Care

During their inpatient stay, the patients were separated in different units.
In group A, 18/28 patients (64%) stayed at the normal care unit in a standard room, while 10/28 patients (36%) stayed at the bone marrow transplantation unit in a hepa-filtered room. In group B, 86/10 (85%) patients stayed at the normal care unit in a standard room and 15/101 (15%) patients stayed in a hepa-filtered room. In group C, 188/197 (95%) patients were hosted in a standard room and 9/197 (5%) in a HEPA-filtered room at the bone marrow transplantation unit.
Results are depicted in *Fig*.17 and *Fig*.18.

Fig.17: Room of care-standard room

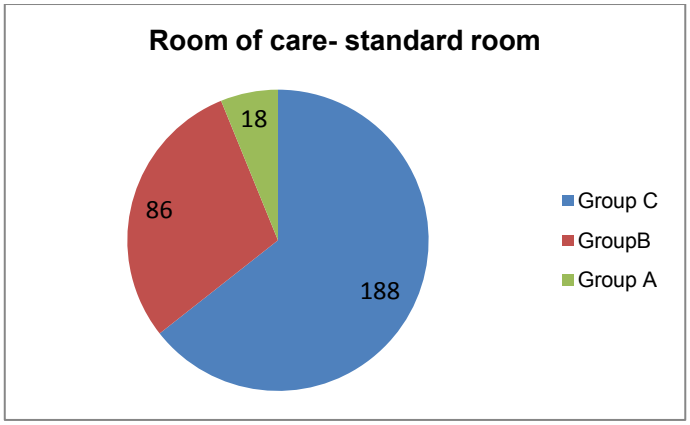
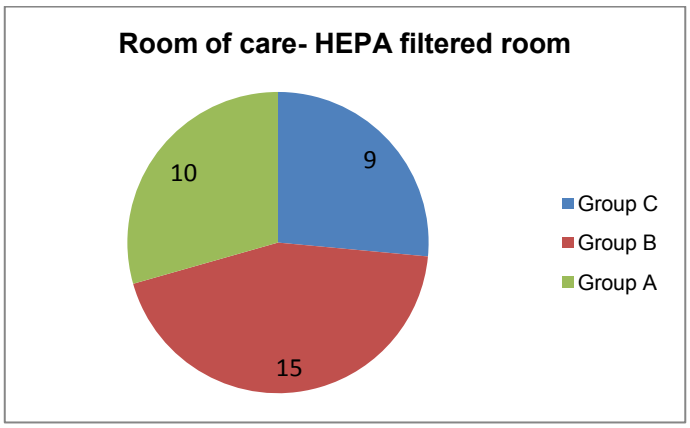


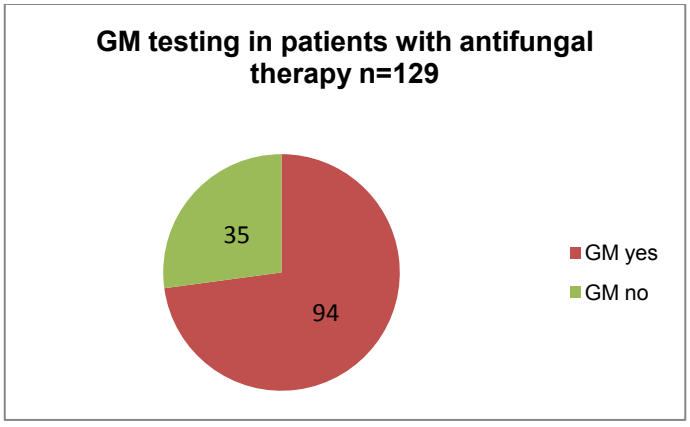
*Fig.*18: Room of care-HEPA filtered room



3.11 Galactomannan testing

In 94/129 patients (73%) with antifungal therapy, GM testing was performed.
In 10/94 cases (11%) GM resulted positive. In 9/10 patients (90%) probable IA was diagnosed. 1/10 patients (10%) had proven *Geosmithia argillacea* infection.
GM index results were frequently >20 in this patient. Overall, in 7/10 cases GM was >0,7 and in three other cases between 0.5 and 0.7. 84/94 cases showed a negative GM level. Possible false negative results of GM due to the use of antimicrobilogical agents were net observed in this study population. 9/24 patients (38%) had possible IMI, 13/24 patients (54%) probable and 2/24 (8%) proven IMI. 9/15 cases of probable and proven IMI were diagnosed just by GM. This result shows the impact of GM in the diagnosis of IMI. Without GM testing, more than half of all probable and proven IMIs would have been possible
Results are depicted in *Fig.19*.

*Fig.*19. GM testing in patients with antifungal therapy



3.12 IMI

24/729 patients (3%) developed IMI. 9/24 patients (38%) had possible IMI, 13/24 patients (54%) probable and 2/24 (8%) proven IMI. 9/15 Without GM testing, more than half of all probable and proven IMIs would have been possible. Results are depicted in *Fig.*20 and *Fig.*21.

*Fig.*20: IMI

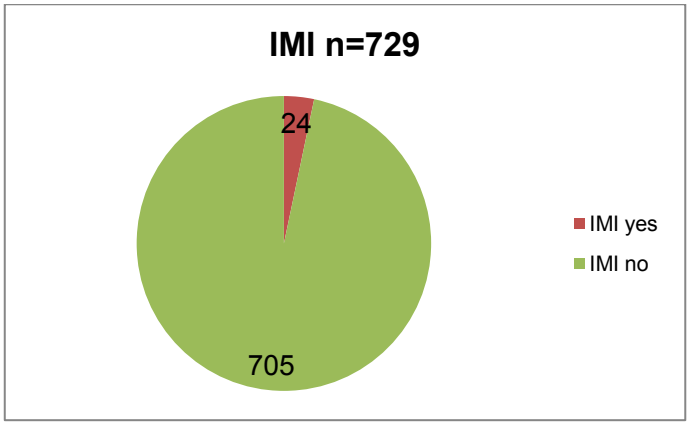
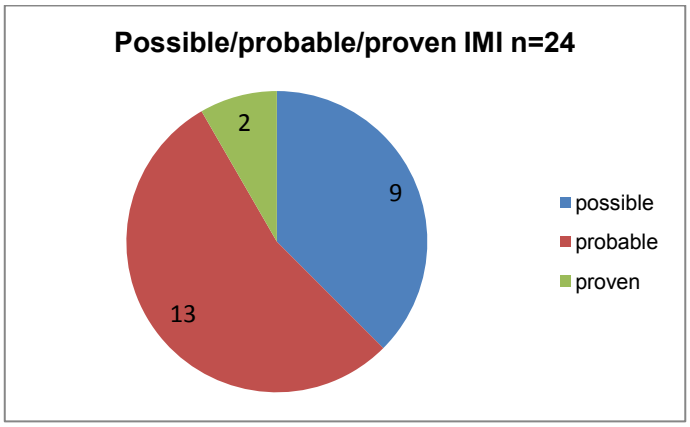
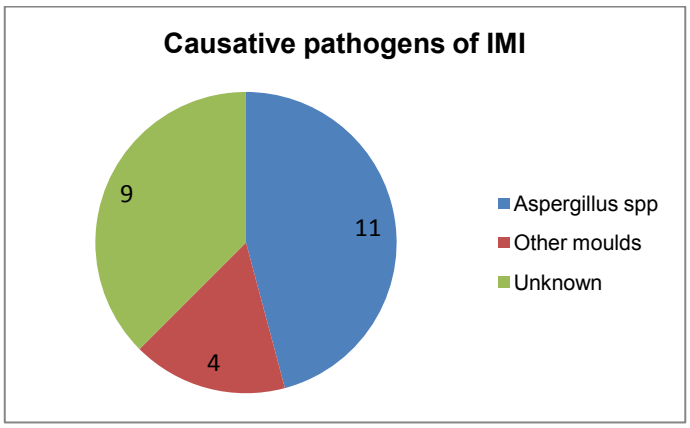


Fig.21: Possible/probable/proven IMI



The most important IMI was IA, occuring in 11/24 (46%) cases followed by other moulds in 4/24 patients. 9/24 patients had possible to IMI due to unknown causative pathogens.

*Fig.*22: Causative pathogens of IMI



3.12.1 Outcome of patients with IMI

Four of 24 patients with IMI had a breakthrough infection and six other of the 24 showed a fatal outcome. Five of these six people died within four weeks whilst one other died after 10 weeks.

4 Discussion

We conducted a prospective observational study in patients with hematological malignancies and found a rate of 18% had received systemic antifungal therapy and 4% had IFI according to EORTC/MSG criteria. *Aspergillus* was the leading cause followed by other moulds and *Candida spp*. Hoenigl et al. conducted a comparable study at the same clinical center with similar duration in 2007 before GM testing was introduced. 690 patients were included in that study. That study dealt with the epidemiology of IFI in patients with hematological malignancies and antifungal therapy. 117 of the patients were treated with antifungal agents. The most widespread disease in the 117 patients with antifungal therapy was AML. AML occurred in 52/117 patients (44%). The rarest disease in 2007 was MM with 7/117 patients (63). In the study presented here, AML also was the most frequent underlying disease in 49% of patients. In both study populations, IFIs occurred in AML patients, compared to other underlying hematological malignancies.

In the study of Hoenigl et al. in 2007, 23/117 patients with antifungal therapy underwent HSCT. 11/23 underwent autologous HSCT, whilst 12/23 patients underwent allogeneic HSCT. 8/12 allogeneic HSCT patients had an IFI, whilst 0/11 patients with autologous HSCT had an IFI (63). In our study, 24/129 patients with antifungal therapy underwent HSCT. 6/24 patients underwent autologous HSCT and 18/24 underwent allogeneic HSCT. 1/6 patients with autologous HSCT and 5/18 allogeneic HSCT recipients had an IFI.

Another prospective study was set up by the Transplant Associated Infections Surveillance Network in 2009, surveying the epidemiology of IFI in patients with HSCT. The study population contained 875 HSCT recipients. During the study, 983 proven and probable IFI were detected. 184/875 (21%) patients had received autologous HSCT and 689/875 (78%) were allogeneic HSCT recipients (64). In the study by Hoenigl and colleagues, 24/117 patients with antifungal therapy had possible IFI, 6/117 probable and 4/117 proven IFI. In general, 34/117 patients had an IFI (63).

In our study 6/28 patients with IFI were proven, 13/28 patients probable and 9/28 possible IFI. We therefore found markedly increase of probable and proven IFIs after introduction of GM test when results are compared to the study in 2007. Also probable and proven IMIs increased markedly, whereas the number of possible IFIs decreased dramatically in 2010 when compared to 2007. Therefore, overall rate of IFI decreased slightly in 2010 when compared to 2007. One of the reasons for this decrease may be the prophylactic use of posaconazole in patients with high risk. Posaconazole has an effect on IA and IC. Another important tool for the earlier diagnosis of IFI was the implementation of the GM-testing, which was used in our study twice a week. The majority (69%) of probable IMI cases in this study had mycological criteria fulfilled by GM test only and were therefore only probable because of the implementation of Platelia© GM testing. Otherwise these cases would have been classified as possible IFI.

GM is a product of the cell wall of *Aspergillus spp.*, or rather a polysaccharide, which invades the blood circulation of the immune-compromised host (44). The sandwich ELISA (Platelia© Aspergillus) and its sensitivity and specificity were explored by Maertens et al. in large autopsy-controlled trials. These trials showed a sensitivity of 92.6% and a specificity of 95.4% (65). In contrast to Maertens et al., Pfeiffer et al. showed a sensitivity of 64% and a specificity of 93% in their meta-analysis. Overall performance of GM testing varies between centers and studies. (66). Among the studies that reported poor performance of GM testing, is a study from North Carolina where the use of GM test was not helpful in diagnosis of IA. In eighty-six patients (not all hematological malignancies), 234 test were performed. The rest of the sixty-six patients had just one test. In two patients with biopsy-proven IA, multiple galactomannan tests were negative, whilst in one patient with heel skin punch and diagnosed IA, two of the tests were negative too. Four patients had six positive galactomannan tests. Just one case of AML with positive GM, showed pathology in the chest computed tomography. The study, however, may have been too small to assess the benefit of GM-testing as multiple GM testing was only performed in 28 patients (42). A study of Sun Hee Park et al. at the Catholic University Korea focused on prolonged neutropenic patients with AML under chemotherapy who had proven/probable IA. The study aimed at the analysis of GM levels concerning the outcome of the patients. There were 58 patients with

AML in the study population of whom five were proven and 53 were probable to IA. All patients were neutropenic with a mean duration of 31 days. The outcome at week six was good whilst at week twelve the outcome of patients with a GM test, which normalized during these weeks, was much better than in patients with persistently positive GM values independent of resolved neutropenia or response to chemotherapy. In summary, GM values showed a strong correlation in the outcome of neutropenic patients with IA. Sun Hee Park et al. showed the usefulness of GM as a representative marker concerning the outcome of patients with IA in their study. They also advised to integrate GM tests into the therapeutic monitoring (67). In their study conducted from January 2001 to December 2005, Cordonnier et al explored the GM index in severely neutropenic patients compared to other patients. In the course of the study, Cordonnier et al found a significantly higher GM index in severely neutropenic patients. They also found out that a negative result or low GM index does not exclude the presence of IA (66).
Many centers in Europe use GM as a screening tool in allogeneic HSCT recipients. As one of the first studies performed on the clinical value of GM test, Maertens et al. showed the usefulness of GM detection in patients with allogeneic HSCT especially under corticosteroid treatment from 1997 to 2001. Overall, the use of GM makes sense in the presence of coexisting conditions which disguise the diagnosis of IA (68).

A retrospective study in Neuilly, France with 41/1231 hematological patients who were considered to be at high risk for IA was conducted. In this study, Galactomannan antigen detection and PCR were compared. 281 serum samples of the 41 patients during their risk period due to IA were collected. 22/41 were probable to IA according to the mycological criteria. 15/22 (87 samples) patients showed a positive GM-test, whilst 12/22 (20 samples) patients showed a positive PCR. 19/20 PCR samples were also positive for GM (69).

Just 1/19 patients without IA showed three false-positive GM-testing results.
It was observed that both GM-testing and PCR were probable to be positive before death. This French study determined that PCR is not more sensitive than GM-testing, but both techniques together can help to achieve an early diagnosis of IA (67).

In our study, only GM- testing was done. In 94/129 patients with antifungaltherapy, Platelia© Aspergillus was performed. 10/94 cases showed a positive result. 9/10 patients had probable IMI, whilst 1/10 cases had proven *Geosmithia* infection.

As in the French study, it is possible to establish a connection between probable and proven IMI to the use of Platelia galactomannan. Without the test many cases of probable IMI could not be detected as possible. Our study showed an increase of IMI after the implementation of Platelia© Aspergillus. The possible, probable and proven results of the study from 2007 mentioned above may have been caused by the lack of GM-testing. It can be seen that 24/117 patients in this study were possible IFI under the same conditions as the patients in our study. Yet, in the study of 2007, it was difficult to categorize possible cases into proven and probable. The GM-testing may have helped to detect probable and proven cases according to the microbiological criteria.

In our study, posaconazole was the most frequently used antifungal agent (81/129 patients). During the study of 2007 at the University of Graz, caspofungin was the most frequently used antifungal agent and posaconazole was used in 38/117 cases (63). Fluconazole as an antifungal agent was used in 7/117 cases in 2007 and in 7/128 cases in our study. Ullmann et al. performed a study in 2007 with a total of 600 patients comparing posaconazole and fluconazole prophylaxis in patients with severe GvHD. 301/600 patients were treated with posaconazole and 299/600 were treated with fluconazole. The occurence of IFI under posaconazole was 5.3% and 9.0% under fluconazole therapy. In this study, posaconazole showed better results concerning proven and probable IA. Posaconazole also reduced the mortality rate in terms of fungal infections (70) . The study of Ullman et al. therefore underscored the impact of posaconazole when used as a prophylaxis in GVHD patients.

Itraconazole was used in our study in 19/129 patients with antifungal therapy and in 6/28 patients with IFI. Cornely et al. compared posaconazole and fluconazole/itraconazole prophylaxis in patients with prolonged neutropenia after induction chemotherapy for AML/MDS in 602 patients, of whom 304 received

posaconazole, 298 patients received fluconazole or itraconazole. Because of the treatment with posaconazole the proven/probable cases of IFI amounted to seven cases. The group of fluconazole or itraconazole contained 25 patients. In general, the study showed a better prevention of IFI under posaconazole treatment (60).

The study of Hoenigl et al. in 2007 showed different results concerning the rational for antifungal therapy when compared to this study (63). In that study, 52/117 patients with antifungal therapy were treated prophylactically, whilst in our study, 74/129 patients were treated that way (63). Also the empirical treatment differed significantly. In our study, 57/129 patients were treated empirically. As a standard of care, empirical antifungal therapy is established in patients with hematological malignancies and neutropenia, who are in a febrile phase despite broad-spectrum antibacterial treatment. Treatment costs and toxicity would be reduced by reserving antifungals in this stadium (71). The primary focus in the study of 2007 was on the preemptive therapy (50/117 patients), whilst empirical therapy was performed in 31/117 cases (63). We had 39/129 patients with preemptive therapy. A possible reason may be the study of Cordonnier et al. which suggests that preemptive treatment increases the incidence of invasive fungal disease and that empirical treatment may provide better survival rates for patients receiving induction chemotherapy (71).

Directed therapy in our study population was administred in 8/129 patients and in the study of Hoenigl et al. 4/117 (63). The second most frequently used antifungal agent in our study was caspofungin. Caspofungin is used in patients without neutropenia for antifungal treatment. Caspofungin primarily has an effect on *Candida spp*., but also acts as a fungistatic agent against *Aspergillus spp*. In the study of Hoenigl et al., caspofungin was the most frequently used antifungal agent (63). A study of Herbrecht et al between May 2005 and February 2008 in allogenic HSCT patients showed a favorable outcome under caspofungin therapy. There were 42 patients registered at 13 centers. 11/42 patients had proven/probable IA and 31/42 had possible IA. 13/31 possible cases changed in the process of the study to proven/probable IA. The dose of Caspofungin on the first day was 70 mg. On day two patients got 50 mg/day for up to twelve weeks. 12 weeks were the maximum treatment duration. The dose was matched with the patient's weight.

The study found that caspofungin was well tolerated and led to a favorable outcome in the first-line therapy of IA. The mortality rate was 21% at week six and 50% at week twelve (72).

As we can see, caspofungin has a big influence on the outcome of IA. The general survival rate in our study was 21/28 cases within 12 weeks. This survival rate may be due to the early antifungal management and the earlier diagnosis of IFI because of the new EORTC/MSG criteria and the implementation of the Platelia© Aspergillus.

In our study, *Aspergillus spp*. was the most common causal pathogen (11/28 patients). *Candida spp*. were found in 3/28 cases, followed by *Cryptococcus neoformans* in 1/28 cases. The number of unknown causative pathogens (meaning possible IFI) was 9/28 patients, whilst other moulds occurred in 4/28 patients. The study of Hoenigl et al. in 2007 induced *Candida spp*. as the leading pathogen in 7/34 cases, followed by *Aspergillus spp*. in 2/34 patients (63).
24/28 patients in our study developed an IMI whilst in the study of Hoenigl et al. 27/34 patients suffered from an IMI (63). Another five-year retrospective study of Hoenigl et al. 2012 at the Medical University of Graz also showed a clear rise of *Aspergillus spp*. as a causative pathogen (73).

Concerning host factors in our study, group A (patients with IFI) contained 7/28 patients with three or more host factors present. Group B contained 13/101 patients and group C contained just one patient with three or more host factors present. The most common host factors in group A were the use of T-cell suppressants in 22/28 cases and prolonged neutropenia in 19/28 cases. These results demonstrated a similarity to the study of Hoenigl et al. 2012. In this study, prolonged neutropenia and the use of T-cell suppressants represented the most common host factors in group A (29/58 patients). Group B in our study contained 38/101 patients with prolonged neutropenia, group C just had 7/197 patients with neutropenia >10 days. Both studies therefore showed that prolonged neutropenia is a major host factor for the development of IFI. 22/28 patients in group A, 55/101 patients in group B and 36/197 patients in group C received T-cell suppressants within 90 days. In the study of Hoenigl et al., 35/44 patients with IMI and 69/116

patients with antifungal therapy received T-cell suppressants (73). As we can see, T-cell suppressants may be therefore a very important factor for establishing IFI.

Compared to a previous study at the same institution, it was possible to establish, because of the GM testing, a significantly higher number of probable and proven IFI. This special diagnostic tool enabled a more exact classification of probable IFI according to the EORTC/MSG criteria.

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6 Curriculum vitae

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Education

October 2003- present Medical University of Graz
1995-2003 Privatgymnasium Sacré Coeur Graz
1991-1995 Volksschule Sacré Coeur Graz

Professional experiences

October 2011-present Practical Year: 10 weeks neuro surgery, 10 weeks Internal medicine, 5 weeks general medicine, 5 weeks psychiatry

August 2011 Hospital Barmherzige Brüder Graz Eggenberg, Division of Neurology and Psychiatry
Clibical traineeship for 4 weeks

February 2011 Hospital Barmherzige Brüder Graz Eggenberg; Divison of Neurology and Psychiatry
Clinical traineeship for 2 weeks

September-October 2010 Hospital Barmherzige Brüder Graz Eggenberg, Division of Neurology and Psychiatry
Clinical traineeship for 4 weeks

August-September 2010 Hospital Barmherzige Brüder Graz Eggenberg, Division of Internal medicine
Clinical traineeship for 2 weeks

September 2009 Hospital Elisabethinen Graz, Division of Anesthesiology
Clinical traineeship for 2 weeks

October 2006 UKH Graz
Clinical traineeship for 2 weeks

July 2004 LSF Graz
Internship at the Stroke Unit for 4 weeks

December 2003 LKH Graz, Division of Internal medicine
Internship Division of Rheumatology for 3 weeks

Published papers

Hoenigl, M; Raggam, RB; Salzer, HJ; Valentin, T; Valentin, A; Zollner-Schwetz, I; Strohmeier, AT; Seeber, K; Wölfler, A; Sill, H; Krause, R
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German native
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Medicine, dogs, sports