Dissertation

Mould Infections in Patients with Hematological Malignancies: Novel Diagnostic Approaches, Standardized Urine Testing and Comparison with Serum Results

submitted by

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Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt und abgefasst und jene Personen und Institutionen, die am Zustandekommen der Forschungsdaten beteiligt waren, namentlich genannt habe. Andere als die angegebenen Quellen habe ich nicht verwendet und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen habe ich als solche kenntlich gemacht. Die Arbeit an der Dissertation und daraus entstandener Publikationen wurde gemäß den Regeln der „Good Scientific Practice" durchgeführt.
Graz, am 13.07.2017

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "*Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz*"
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Dissertation

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1 Abbreviations

A

AIDS - Acquired immunodeficiency syndrome

AML - Acute myeloid leukemia

AUC - Area under the curve

B

BAL - Bronchoalveolar lavage

BDG - 1,3-β-D-glucan

C

CGD - Chronic granulomatous disease

CI - Confidence interval

CT - Computer tomography

D

DNA - Deoxyribonucleic acid

E

EDTA - Ethylenediaminetetraacetic acid

EIA - Enzyme immunoassay

ELISA - Enzyme-linked immunosorbent assay

EORTC - European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group

Et al - et alii/et aliae

F

FDA - US Food and Drug Administration

G

GM - Galactomannan

GvHD - Graft versus host disease

HIV - Human immunodeficiency virus

Hrs – hours

I

IA - Invasive aspergillosis

ICU - Intensive care unit

IDSA - Infectious Diseases Society of America

IFI - Invasive fungal infection

IQR - Interquartile range

M

Mab - Monoclonal antibody

N

NPV - Negative Predictive Value

O

OD - Optical Density

ODI - Optical Density Index

P

PCR - Polymerase chain reaction

POC - Point of Care

PPV - Positive Predictive Value

R

RCT – Randomised controlled trial

ROC - Receiver Operating Characteristics

S

SCT - Stem cell transplantation

spp - species pluralis

TNF-α - Tumor necrosis factor α

V

VC - Validation cohort

WNS - White nose syndrome

2 Figures

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4 Abstract

4.1 Introduction

Recent studies on the diagnostic performance of urine galactomannan (GM) determination for invasive aspergillosis (IA) have not taken into account urine dilution. Aim of this study was to evaluate whether a ratio of urine GM (optical density index (ODI)) divided by urine creatinine (mg/dl) could improve the diagnostic performance of urine GM determination.

4.2 Methods

This prospective study was conducted at the Medical University Hospital of Graz, Austria, between September 2014 and August 2015. Urine samples from hematological patients at risk for invasive fungal infections (IFI) were collected twice weekly, and GM (Platelia®) and creatinine levels were determined from urine samples. To normalize the urine GM levels against the urine creatinine levels the GM-creatinine ratio was calculated (GM level*100/creatinine level). We evaluated the new urine GM-creatinine ratio clinically by comparing it to the conventional urine GM (ODI). Cut-offs were determined by Youdens Index and diagnostic performance was analyzed for the GM-creatinine index and the conventional GM ODI compared between IA vs. no IA cohorts and IA vs. validation cohorts.

4.3 Results

632 samples from 71 patients were analyzed for this study. Ten patients (34 samples) were included in to the IA cohort, 61 patients (598 samples) in the cohort with no evidence for IA; 48 patients (48 samples) from an outpatient department served as a validation cohort (VC). With a cut-off at 0.26 Sensitivity, Specificity, Negative Predictive Value (NPV), Positive Predictive Value (PPV) were found to be 79.4%, 68.6%, 98.3%, 12.6%, respectively. When the IA cohort was compared to the no IA cohort ROC curve analysis showed that the area under the curve (AUC) value for the GM-creatinine ratio was 0.801 and therefore higher than the AUC for the conventional GM ODI at 0.746. The differences between the AUC values were bigger when the IA cohort was compared to the VC with 0.792 for the conventional urine GM ODI and 0.913 for the urine GM-creatinine ratio.

4.4 Conclusion

The urine GM-creatinine ratio allows better interpretation of detectable GM levels from urine samples and improves the diagnostic performance.

5 Abstract in German

5.1 Einleitung

Bisherige Studien über die diagnostische Leistung für invasive Aspergillose durch Galaktomannnan (GM) Bestimmung aus dem Urin haben die Urinverdünnung nicht berücksichtigt. Ziel dieser Studie war es zu evaluieren, ob die Formel: Galaktomannan (ODI) im Urin multipliziert mit 100 und geteilt durch den Kreatiningehalt (mg/dl) im Urin, die diagnostische Leistung von GM Bestimmung aus dem Urin verbessen kann.

5.2 Methodik

Diese prospektive Studie wurde zwischen September 2014 und August 2015 an der Medizinischen Universität Graz durchgeführt. Zweimal wöchentlich wurden von hämatologischen Patienten mit Risiko für invasive Pilzinfektionen Galaktomannan (Platelia) und Kreatininwerte aus Urinproben bestimmt. Um den Urin GM-Wert gegen den Urin Kreatininwert zu normalisieren wurde eine GM/Kreatinin Ratio berechnet (GM-Wert x 100/ Kreatininwert). Wir haben die neue GM/Kreatinin Ratio klinisch evaluiert indem wir die diagnostische Leistung mit der des konventionellen GM Wertes (optische Dichtebestimmung) verglichen haben. Cut-offs wurden durch den Youdens Index berechnet und die diagnostische Leistung von der konventionellen Galaktomannan Bestimmung und die neu eingeführte GM/Kreatinin Ratio wurde zwischen in zwei Kohorten verglichen (Invasive Aspergillose vs. keine Invasive Aspergillose und Invasive Aspergillose vs. einer Validierungskohorte).

5.3 Resultate

632 Proben von 71 Patienten wurden in die Studie eingeschlossen. 10 Patienten (34 Proben) wurden der IA Kohorte zugeteilt, 61 Patienten (598 Proben) der Kohorte ohne IA zugeteilt und 48 Ambulanzpatienten (48 Proben) dienten als eine Validierungskohorte. Mit einem Grenzwert bei 0,26 waren Sensitivität, Spezifität, Negative Prädiktive Wert (NPV), Positive Prädiktive Wert (PPV) jeweils 79,4%, 68,6%, 98,3%, 12,6%. Eine ROC Curve Analyse für den Vergleich zwischen IA Kohorte und der Kohorte ohne IA zeigte eine Fläche unter der Kurve von 0,801 für die neuen GM/Kreatinin Ratio und von 0,746 für die konventionelle GM

Bestimmung. Die Unterschiede zwischen den Werten für die Fläche unter der Kurve vergrößerten sich noch wenn die IA Kohorte mit der Validationskohorte verglichen wurde mit 0,792 für die konventionelle GM Bestimmung und 0,913 für die GM/Kreatinine Ratio

5.4 Konklusion

Die in dieser Studie untersuchte GM/Kreatinine Ratio ermöglicht eine bessere Interpretation von messbaren GM Werten im Urin und trägt dazu bei, die diagnostische Leistung von GM Bestimmung im Urin zu verbessern.

6 Introduction

6.1 Fungi and fungal infections

Fungi compose one of the six kingdoms of eukaryotic organisms in the biological taxonomic ranking. Scientists and biologists have often used the term "hidden kingdom" for fungi to describe how little is still known about fungal species. It is believed that fewer than ten percent of the estimated 1.5 million fungal species have been formally identified (1).
On one hand, fungi are indispensable to life on this planet through their ability to break down complex organic matter and recycle essential nutrients back into the environment, their symbiotic associations with plants and animals, and as food sources for humans and animals alike. On the other hand, fungi are known to be able to cause devastating infections and are the only group of organisms that have repeatedly caused extinction of other living organisms on earth (2). In the past devastating fungal plant infections, were known to have altered the course of human history. The most important example being “the great famine” caused by the late blight affecting irish potato plants in the nineteenth century, which induced a period of mass starvation and emigration (Figure 1).
However, it seems that also at present fungal infections become more important again. In April 2012 Nature titled its current issue „Fear of Fungi“, reviewing the recently emerging fungal pathogens that are causing extinction and are threatening natural ecosystems and food security (3).

Figure 2, showing starving irish children at Skibbereen during the great famine, West Cork, in 1847. From a series of illustrations by Cork artist James Mahony (1810-1879), commissioned by Illustrated London News 1847. Free of known copyright restrictions in the EU.PDM.

Fungal-caused animal infections were at present not recognized to be threatening animal species to extinction. However, recently emerging fungal infections have caused the highest rate of extinctions ever witnessed in animals (3). The two most severe fungal infections are threatening bat and amphibian populations at present (3). The fungus *Batrachochytrium dendrobatidis*, which infects the skin of many amphibians species, resulted in the loss of 40% of amphibian species in parts of America in recent years (4). It has been reported that half of all amphibian species are in decline worldwide due to this fungal infection (Figure 2, on the right). The white-nose syndrome (WNS) caused by ascomycete fungus *geomyces destructans* in bats, was detected ten years ago and has caused a reduction in bat populations in the eastern USA by 80% (5) (Figure 2, on the left).

Figure 3, showing on the left a little brown bat with white-nose syndrome in Greeley Mine, Vermont, March 26, 2009. By Marvin Moriarty/ U.S. Fish and Wildlife Service Headquarters, distributed under a CC-BY 2.0 licence no changes were made. On the right a chytrid-infected frog. By Forrest Brem, published in Gewin V (2008) Riders of a Modern-Day Ark. PLoS Biol 6(1): e24. https://doi.org/10.1371/journal.pbio.0060024. distributed under a CC-BY 2.0 licence no changes were made.

6.2 Fungal infections in humans: an emerging threat

Of the nearly 1400 human pathogens only about 20 percent are fungal species and less than twelve fungal species are able to cause life-threatening diseases (6). Compared to bacterial, viral and parasitic infections fungal infections have played a neglectable part of human infections in history, whereas fungi are the predominant pathogen species in plants. The vast majority of fungal species is not capable to cause infections in the animal kingdom including humans (2). Especially the higher body temperature of mammals, which exceeds the

thermotolerance of many fungi, has been made responsible for the relative resistance of humans against fungal infections (2). Bats are affected with the WNS during hibernation and can be cured by raising their body temperature. It was even discussed that the difference in basal body temperature and the resulting natural resistance against fungal infections posed an evolutionary selective advantage for mammals compared to ectothermic animals. This advantage probably facilitated the emergence of mammals at the end of the cretaceous. Fungal infections posed a selective pressure that favored mammals compared to reptiles (7). The severity of infection in humans caused by fungi range from mild superficial infections in otherwise healthy patients, to life-threatening invasive infections in already severely ill immunosuppressed patients.

Publications suggest that fungal infections in humans are globally emerging."Emerging" infections are characterized by a recent increase in incidence or virulence or if a new host population is affected.Thirteen factors were identified and published in 2003 to influence the emergence of infectious diseases (8) :

- microbial adaption and change
- human susceptibility to infection
- climate and weather
- changing ecosystems
- human demographics and behavior
- economic development and land use
- international travel and commerce
- technology and industry
- breakdown of public health measures
- poverty and social inequality
- war and famine
- lack of political will
- intent to harm

Many of the above listed factors are currently relevant, like increases in travel and climate changes (9) (10). One example for emerging fungal infections in humans is the 1999 outbreak of *Cryptococcus gattii* infections on Vancouver Island, Canada, an environmental *fungus* which was previously restricted to tropical and subtropical regions (11).

While many factors, like the ones mentioned above, are currently discussed to contribute to the emergence of fungal infections, the most important factor leading to the emergence of fungal infections in humans started only in the second half of the 20th century. Advances in medical care and new therapeutical options, like high dose chemotherapy, immunosuppressant therapy, the options for organ transplantation and elaborated intensive care have led to the survival of patients with malignancies, trauma-patients, human immunodeficiency virus (HIV)-patients, premature neonates and also patients at a very high age. These advances have saved many lives, but they also created a growing population of patients with diminished immune reaction, who are at a higher risk for infections in general. This increased survival time elongates the time of patients being at risk for IFI, which also contributes to the increasing incidence of IFI (Figure 3).

This rise of a population of patients at risk for invasive fungal infections has actually led to the increasing incidence of fungal infections throughout the world. In the United States, deaths due to mycoses have increased from the 10th most common infectious disease cause of death to the seventh between 1980 and 1997, in this time period death due to invasive aspergillosis showed a four-fold increase (12). Cases of sepsis caused by fungal infections have increased more than 200% between 1979 and 2000 (13) and mucormycoses-related hospitalizations doubled between 2000 and 2013 in the United States (14).

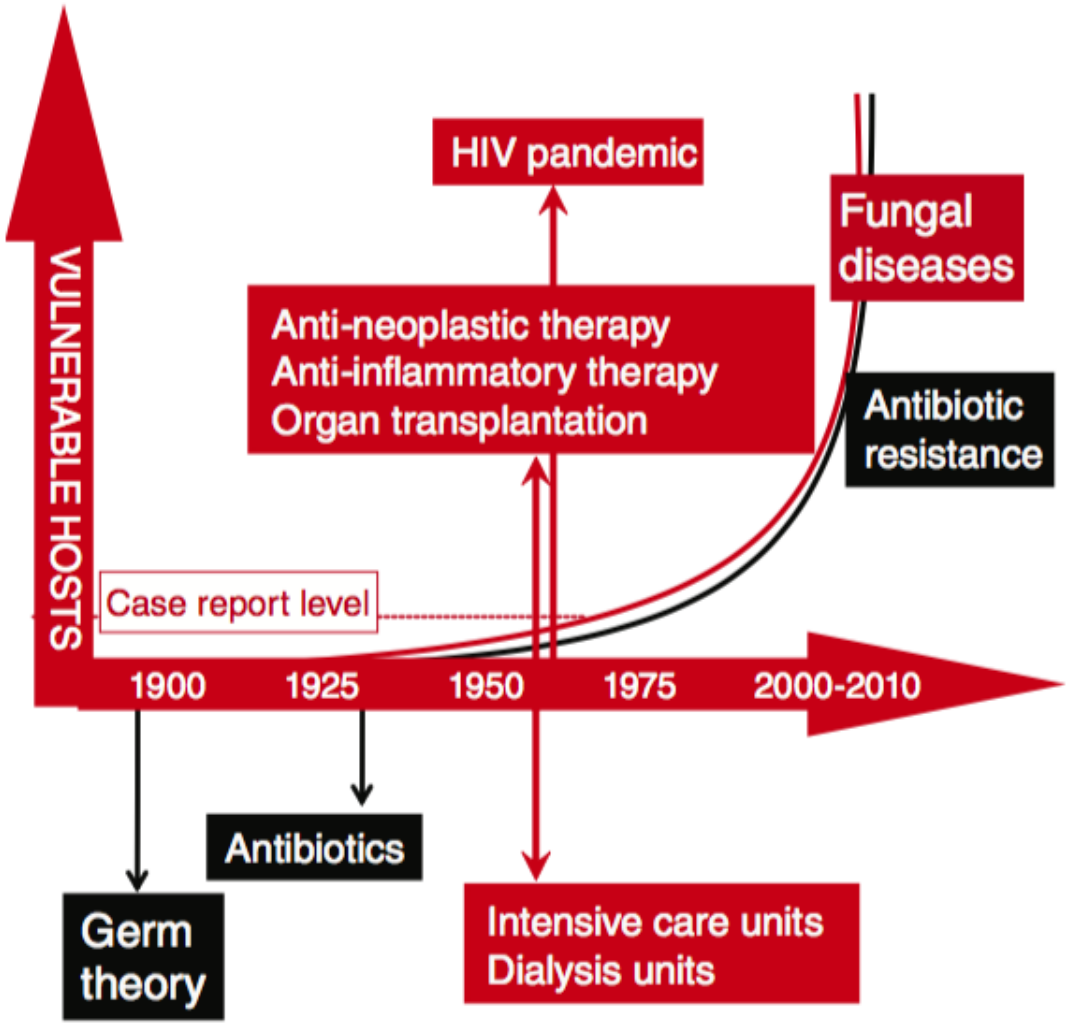


Figure 4, showing the increase of fungal diseases over time, from: Fungal Diseases: An Emerging Threat to Human, Animal, and Plant Health. The National Academy of Sciences. License provided by the National Academy Press: 4167730504159 (2).

Given that many pathogenic fungal species, such as *Aspergillus spp.*, surround us permanently the immune status is the most important factor influencing the development of life-threatening, self-limiting or no infection at all, after contact with a fungal pathogen. Three major factors of immune defense play a role in the defense against infectious fungi: first intact skin and mucosal barriers, second phagocytosis provided by the innate immune system and third, the cell-mediated immune defense. When these immune defenses systems are weakened due to underlying diseases or due to external influences like side effects of medical therapy, higher risk for fungal infections occur (15) (16) (17).

When only the skin or mucosal barrier are damaged by trauma or surgery, fungal infections in immunocompetent patients of the skin and soft tissue are generally localized and present a good outcome (18). However, fungal infections in patients with debilitation of the innate

immune system and/or the cell-mediated immune system, are considerably more severe. Patients with chronic granulomatous disease (CGD), are at risk for fungal infections, as this disease is associated with phagocytic dysfunction. Phagocytosis has been reported to be of utmost importance in the defense against fungal pathogens (19). The importance of cell-mediated immune defense against fungal infections is demonstrated by the rising incidence of specific fungal infections associated with the declining level of CD4+ T lymphocytes in HIV positive/ acquired immunodeficiency syndrome (AIDS) patients (20). The cell-mediated immune defense is also impaired in patients treated with immunosuppressive therapies, like glucocorticoids, tumor necrosis factor-α antagonists or infliximab, after solid organ transplantation or after stem cell transplantation, to prevent tissue rejection and Graft versus Host disease (GvHD) (21) (22) (23). Severe and prolonged neutropenia (<100 neurophils/μL), mostly caused by high dose myeloablative chemotherapy in patients with hematological malignancies is the most important risk for invasive fungal infections, especially for invasive aspergillosis (24). Some of these risk factors for IFI illustrate how medical advances also lead to a growing population threatened by IFI (23) (21).

6.3 *Aspergillus spp.* and Aspergillosis

6.3.1 The role of *Aspergillus spp.*

The genus *Aspergillus* consists of a few hundred species, which surround us ubiquitously. The name *Aspergillus* is derived from the Latin word aspergere (to sprinkle) based on Pier Antonio Micheli´s observation in 1729 (Figure 4). Micheli, who was the director of the public gardens in Florence, noticed the resemblance between the sporulating head of an *Aspergillus* fungi and the holy water sprinkler, called aspergillum used in catholic churches. Also, the German name for *Aspergillus* "Gießkannenschimmel" leans on the resemblance of its sporulation head with the head of a watering pot (Figure 5). Rudolf Virchow was the first to publish a detailed microscopic description of *Aspergillus spp.* in 1856 (25).

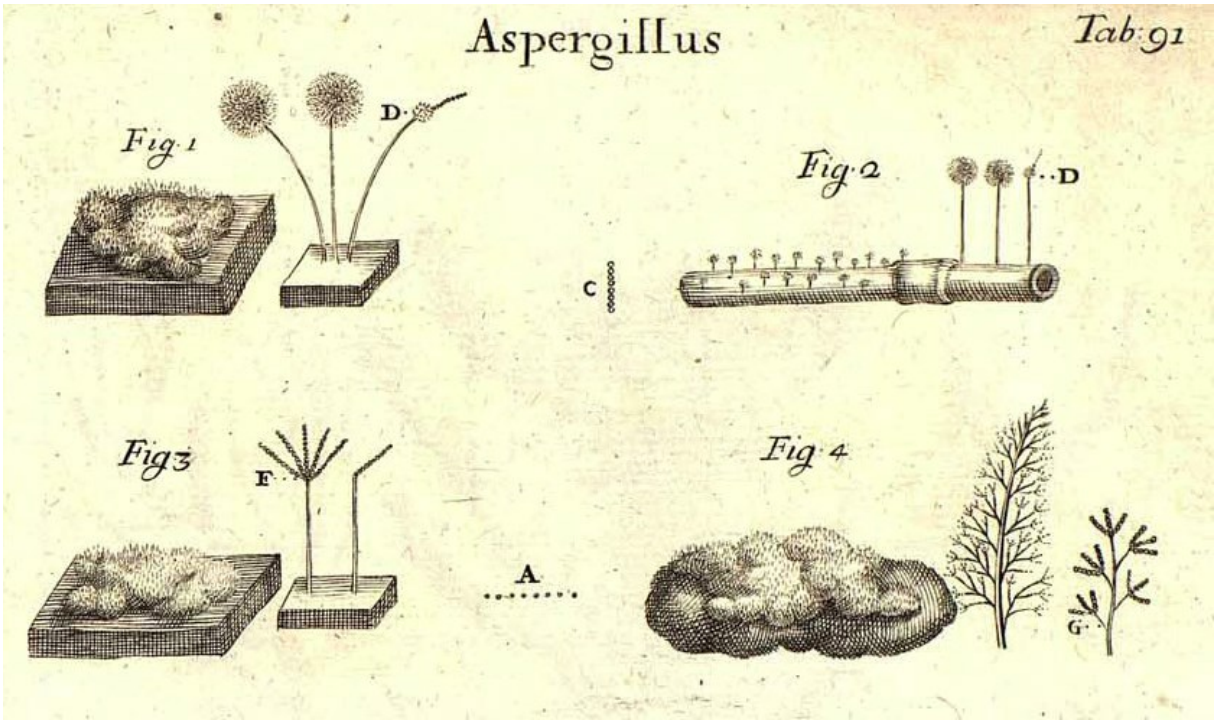


Figure 5, showing the first published drawing of *Aspergillus* by Micheli in "Nova Plantum Genera" from 1729. Free of known copyright restrictions in the EU.PDM.

Figure 6, showing a sporulating head of *Aspergillus* fungi, by James Scott/Medmyco, distributed by a Creative Commons licence 1.0, no changes were made.

Aspergilli are saprophytes which grow abundantly on decaying vegetation, where they degrade complex plant polymers as an efficient recycler in nature. Different from most other fungi, *Aspergillus spp.* are equipped with pronounced thermotolerance ranging between 12° and 65°C (26) for *Aspergillus fumigatus*. This thermotolerance enables *Aspergillus spp.* to survive on living mammal-tissue, in contrast to most other fungi who do not have this tolerance at their disposal and are thus unable to infect mammals.

Aspergillus spp. were long thought to be part of fungi imperfecti, which differentiate from other fungi by only asexual reproduction. However, also teleomorph forms of *Aspergillus spp,* which describes a sexual form of reproduction, were described in population genetic studies (27). The discovery of also sexual reproduction in *Aspergillus spp.* is of great importance given the opportunity to perform classical genetical analysis in studys to analyze pathogenicity and fungicide resistance (28). Genetic studies show for example how local antifungal pressure can influence the genetic evolution of *Aspergillus spp ,*which is crucial for the better understanding of , for example, azole resistance (29)

An *Aspergillus* lifecycle commonly starts in form of a conidia, which originates from a sporulating *Aspergillus*-head. When the air-transported conidia attach to a solid or fluid surface it may start to germinate, depending on temperature, pH and humidity. Through mitosis, branching hypha start growing and when enough nutrients are available a sporulating head with new conidia starts to form.

Like other fungi, *Aspergillus spp.* take in nutrients by secreting acids and enzymes into their direct environment, which degrade complex polymers. Their components are then absorbed by the fungi, the *fungus* thus digests its food prior to the intake (30). These secreted enzymes are one of the reason for tissue damage in case of infection by *Aspergillus spp,* however*,* they are also used in industrial food processing. *Aspergillus niger* is used in the industrial production of citric acid, amylases, pectinases and phytases; *Aspergillus terreus* in the production of lovastatin, a cholesterol-lowering drug and *Aspergillus oryzae* plays an important role in Asian food production for the fermentation of soybeans into soy sauce and rice into sake (31).

6.3.2. Spectrum of human diseases caused by *Aspergillus spp.*

Given the ubiquitous existence of *Aspergillus spp.*, inhalation of *Aspergillus* conidia occurs frequently. It was reported that each of us inhales more than 100 *Aspergillus* conidia daily, as the Aspergillus air concentrations indoors and outdoors variates between 1-100 conidia/m^3 (32). However, inhalation of these spores has most of the time no consequence for any healthy organism. *Aspergillus spp.* contact with the human organism may result in a variety of clinical syndromes, depending on the effectiveness of immune response provided by multiple cellular functions that include clearance of inhaled conidia, creation of secondary inflammation and killing of invasive hyphae. Thus, the consequence of contact with

Aspergillus spp. ranges from harmless colonization, to invasive aspergillosis, a rapidly progressing and frequently fatal disease. The severity of clinical consequence resulting from contact with *Aspergillus* spores mostly depends on the grade of underlying immunosuppression, higher immunosuppression correlating with increased risk of invasive disease.

Contrary to IA, which is described below, chronic pulmonary disease caused by *Aspergillus spp*. develops over a longer time and occurs mostly as a complication of underlying respiratory disorders, like COPD or tuberculosis, without immunosuppression. Forms of chronic pulmonary aspergillosis includes chronic cavitary pulmonary aspergillosis which is the most common form and which, if untreated, can progress to chronic fibrosing pulmonary aspergillosis (33). Aspergilloma are also classified as chronic pulmonary aspergillosis of the lung. They form spheric fungal masses in preexisting cavities of the lung, due to chronic lung disease such as bullous emphysema, sarcoidosis or tuberculosis, and are difficult to access with antifungal treatment and often require surgical excision (34). Aspergilloma can also form in the paranasal sinuses without tissue invasion (35).

Allergic bronchopulmonary aspergillosis is a chronic allergic response to *Aspergillus spp.* exposure, which occurs in immunocompetent patients, who are, mostly affected by pulmonary diseases such as asthma or cystic fibrosis. Symptoms consists of worsening of pulmonary function due to recurrent exacerbations, pulmonary infiltrates, bronchiectasis and can result in early pulmonary fibrosis, which is associated with significant poorer prognosis (36) (37).

Apart from the above mentioned diseases, *Aspergillus spp.* produce toxins which are dangerous for humans. Serious illness and death can be the result of consumption of crops contaminated with aflatoxins, a highly toxic carcinogen with immunosuppressive properties produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin intoxications are a common problem in developing countries (38).

6.3.3. Invasive Aspergillosis

6.3.3.1 Pathophysiology

IA results from inhalation of *Aspergillus* conidia by an immunodeficient host, and in case of disease progression, subsequent hematogenous dissemination. As *Aspergillus fumigatus* has been described to produce smaller spores compared to other *Aspergillus* species it is more likely to cause IA due to the facilitated access to pulmonary alveoli (39). Other ways of

infection, for example through damaged skin barrier, resulting in IA have been reported but are very rare and almost only occurs in immunosuppressed hosts (40).
The first line of defense against inhaled *Aspergillus* conidia is ciliary clearance, which ideally results in removal of conidia before they reach the alveoli (31). *Aspergillus fumigatus* conidia are more likely to avoid ciliary clearance due to their smaller size (39).
When conidia reach the alveoli, pulmonary macrophages constitute the first line of innate phagocytic host defense against inhaled conidia. They play a major role in preventing invasive disease and are not only able to ingest and kill *Aspergillus* conidia, but they are also responsible for the induction of secondary inflammation (41). Pathogen recognition receptors expressed on the surface of macrophages recognize and bind to pathogen-associated molecular patterns expressed on the surface of fungal conidia, which results in the activation of macrophages. The activated macrophages induce the production and secretion of proinflammatory cytokines like interleukins and TNF-α. The secretion of these inflammatory mediators from alveolar macrophages results in neutrophil recruitment and activation of cellular immunity (42). However, with a lower number of vital pulmonary macrophages resulting in absence of adequate pulmonary host defense, the inhaled *Aspergillus* conidia may germinate, which describes the transformation from conidia into hyphae, the tissue invasive form of moulds. In case of sufficient neutrophil count of the host, neutrophils which reach the site of infection are able to kill conidia that evaded alveolar macrophages and can even kill *Aspergillus* hyphae by producing fungicidal proteins and reactive oxygen species (15) (16). However, the specific and non-specific immune defense is interfered by toxins and proteases, which are secreted by the *Aspergillus* cells (43). These fungal factors increase the virulence by inhibition of phagocytosis and impairment of functional T-cell response (44).

6.3.3.2 Risk factors for invasive aspergillosis

Like for other IFI the most important risk factor for IA is immunodeficiency and IA occurs within various forms of underlying immunosuppressant diseases. The severe immunodepression, that results from myeloablative chemotherapy in from of prolonged and profound neutropenia (<100 neutrophils/μL) has been described to put patients at highest risk for IA (24). These aggressive chemotherapies are most often used for patients with acute myeloid leukemia (AML) and in preparation for hematopoietic stem cell transplantation (HSCT) (45). However, a study published in 2016 reported that only in one third of analyzed invasive aspergillosis cases prolonged neutropenia was found prior to infection, when

prolonged neutropenia was defined as <500 absolute neutrophil count, for at least 10 days (46). Despite the fact that the 2008 modified diagnostic criteria for IA were designed only for cancer patients, many studies report on cases of invasive aspergillosis in patients, who are immunocompromised due to other underlying diseases (47). Cases of invasive aspergillosis are, however, less frequent in patients who are immunocompromised due to other immunosuppressant diseases (21) (48) (49).

IA occurs in forms of inherited immunodeficiencies such as chronic granulomatouse disease, an inherited disorder of the NADPH oxidase complex leading to deficient phagocytosis (50). Also acquired immunodeficiency in AIDS patients represents a risk factor for IA. Cases of invasive aspergillosis were also reported in patients who receive immunosuppressant therapies after solid organ transplantation or for other reasons (23). Patients with choric pulmonary diseases, who are at risk for other forms of infections caused by *Aspergillus spp*. also can develop invasive diseases. In intensive care unit (ICU) patients, who don't suffer from inherited or acquired immunodeficiencies cases of IA were reported (51).

6.3.3.3 Treatment and prevention

Given the high mortality of IA, clinical strategies in the management of high risk patients focus on prevention of IA with anti mould prophylaxis and in case of suspected infection on initiation of treatment as early as possible. Two strategies of initiation of treatment are discussed in the literature, empiric treatment in febrile neutropenic patients or preemptive treatment based on results of biomarker screening tests (galactomannan and β-D-glucan) (52). In 2016 the guidelines of the Infectious Diseases Society of America (IDSA) for treatment of aspergillosis were published and replaced the guidelines published in 2008. Between these two publications new therapy options were studied and more data on the diagnostic performance of non-culture-based biomarkers became available and were included into the new version (53). When IA is suspected initiation of antifungal therapy must not be delayed and should be started at the same time with the ongoing diagnostic evaluation. It was reported that early initiation of antifungal therapy improves the outcome when IA is strongly suspected, as it takes too much time to wait for definite diagnostic evaluation and diagnostic performance of available test are limited. Only very few randomised control trials (RCT) were conducted analyzing optimal therapy options for IA and studies are limited by the few numbers of proven IA cases (54).

Today relevant guidelines recommend i.v.voriconazole for the first line therapy of IA. I.v. Voriconazole should be administered on the first day of therapy with a loading dose of 6mg/kg every 12hrs , which is to be continued with 4mg/kg every 12hrs. Alternative first line therapy options include liposomal amphotericin B and isavuconazole (53) (54).
Initial IA treatment with voriconazole has been recommended since in 2002 a better survival outcome and less side effects were published from a RCT, for the patients treated with voriconazole compared to patients treated with amphotericin B deoxycholate for IA (55). Amphotericin B deoxycholate is now no longer recommended for the treatment of IA given the occurrence of severe side effects. Only lipid derivates of amphotericin B should be considered in salvage therapy or when voriconazole is not available or cannot be administered (53) (54). Amphotericin B acts cytocidal due to extraction of ergosterol from fungal lipid bilayer membranes, by building extramembranous aggregates (56). Voriconazole belongs to the group of triazole antifungals, which inhibit the synthesis of ergosterol, resulting in loss of fungal cell wall integrity. After the study in 2002, comparing voriconazole and amphotericin B deoxycholate, a posthoc ananylsis in 2015 of this study was published, now including the 2008 revised EORTC diagnostic criteria, which however did not result in changes of therapy recommendations (57). In 2007 RCT-results were published, on the comparison of two different dosages of liposomal amphotericin B in the first 14 days of therapy for patients with IA, study results favored the lower dosage (58). A posthoc analysis of this study published in 2011, including the 2008 revised EORTC diagnostic criteria did not result in changes of recommendations regarding liposomal amphotericin B usage (59). In 2015 a RCT was published which analyzed first line antifungal combination therapy for IA patients, comparing voriconazole with anidulafungin versus voriconazole with placebo. Anidulafungin belongs to the antifungal group of echinocandins, which inhibit the synthesis of 1,3-β-D-glucan (60). Only in a subgroup analysis of this study a better outcome was found for the patient group with combination therapy resulting in conservative recommendations regarding initial combination therapy for IA in guidelines (54) (53). Also in 2015 the RCT was published, comparing the new triazole isavuconazole to the commonly used voriconazole as first line therapy against IA. It was found that isavuconazole shows the same efficacy as voriconazole against IA but provides a better safety profile (61).
The empiric therapy approach was introduced when diagnostic methods for invasive fungal infections were limited and patients with neutropenic fever often suffered from undiagnosed fungal infections. The administration of an antifungal agent in case of neutropenic fever in patients with hematologic malignancies is still common, however there are no placebo-

controlled studies that have analyzed the effect of preemptive therapy now that Posaconazole prophylaxis is widely used (62). With better diagnostic methods commonly used as screening for patients at risk the preemptive therapy approach was suggested to replace the empiric therapy approach. (63).
In case of accessible tissue affected by IA surgical measures were reported to be of help to decrease fungal burden by removing non-vascularized, necrotic tissue, which is not reached by antifungal therapy. However, surgical options are often limited by the overall state of the patient and high risk of bleeding (64).
The usage of posaconazole as anti mould prophylaxis is recommended in high risk patients, since in 2007 a large RCT was published, which demonstrated the superiority of oral posaconazole prophylaxis in comparison to the prophylaxis with fluconazole or itraconazole in the prevention of IA in hematological patients (53) (65).

6.4 Diagnosis of invasive aspergillosis

In the past, physicians had to rely on morphological differences between microbial species to identify a causative organism under the microscope. In fungal diagnostics mycologists used phenotypical characteristics to identify *Aspergillus spp.* However, some *Aspergillus* species are not distinguishable based on phenotypic typing, as phenotypic characteristics are largely subjective and unstable because the appearance of *Aspergillus spp.* under the microscope varies depending on cultivation media and temperature (66).
Today molecular methods have replaced the microscopic diagnostic approach and tremendously expanded the number of recognizable *Aspergillus* species. The ability to differentiate between *Aspergillus spp.* is clinically relevant, given that subspecies of *Aspergillus* show variable antifungal susceptibilities and pathogenity in vivo (21).
Today histopathologic demonstration of fungal hyphae invading human tissue combined with positive culture results of *Aspergillus spp.* still provides definitive evidence of invasive aspergillosis (47). Histopathologic tissue analysis, however, requires tissue samples gained from invasive biopsy techniques. Biopsy techniques for invasive aspergillosis often require bronchoscopy with transbronchial biopsy, computed tomography-guided transthoracic needle biopsy or, most invasive, video-assisted thorascopic surgery. Given that most patients with invasive aspergillosis are neutropenic and weakened by their underlying disease, puncture of *Aspergillus* suspicious lesions in the lungs would often put them under unreasonably risk for

uncontrollable bleeding and additional infection. Thus, the first diagnostic approach to *Aspergillus spp.* infections involves the use of non-invasive techniques. The new molecular diagnostic analysis requires samples such as serum, urine, sputum and bronchoalveolar lavage fluid, which can be collected from patients easily.

The clinical consequence of each diagnostic finding (i.e. the initiation of further diagnostic steps or the immediate administration of antifungal therapy) depends on clinical parameters of the patient and the nature of the applied diagnostic test. In the last decades the decision making process for diagnosis and treatment of invasive aspergillosis has become facilitated by the introduction of established guidelines based on diagnostic definitions (53) (54) (67) (47). Since *Aspergillus* spores are ubiquitously and we inhale them constantly, positive culture results from the airway are not obligatory associated with disease. Thus, for the definite diagnosis of invasive aspergillosis, *Aspergillus spp.* need to be detected either by direct isolation or indirect through biomarkers also the patient has to be at risk for invasive aspergillosis and present clinical symptoms (47). The probability of *Aspergillus spp.* being the causative agent of an infection is a function of the patient's risk factors and the patient's clinical presentation. Invasive aspergillosis is generally seen as difficult to diagnose and a scale of certainty of diagnosis: with "possible", "probable" or "proven" invasive aspergillosis, is always also indicated. This scale of certainty of diagnosis is based on the 2008 revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC) diagnostic criteria (47).

6.4.1 Imaging

As the route of infection occurs mainly over inhalation of *Aspergillus spp* conidia, IA mostly affects the lungs first; hence, imaging of the chest plays an important role in the diagnosis of invasive aspergillosis. Imaging of the chest should be initiated especially when neutropenic patients present with fever, pleuritic chest pain and hemoptysis. However, chest x-ray is insensitive in detecting early stages of invasive aspergillosis and magnetic resonance imaging (MRI) does not add diagnostical value for chest imaging; typical lesions for IA can best be seen in chest computer tomography scans (CT).
Signs of IA on CT scans were included in the 2008 diagnostic criteria proposed by the EORTC, and include dense, well-circumscribed lesions (nodules) with or without a surrounding halo of ground-glass gray attenuation, air-crescent sign, and cavity formation

(47). Chest CT should be performed early after onset of fever in high risk neutropenic patients as improved survival rates were reported for this strategy (53). Localized lesions on chest CT scans also help to guide further invasive diagnostic procedures such as BAL or CT guided biopsies to the region of interest. Horger et al. reported, after investigating 45 patients with invasive aspergillosis, that the most commonly found lesions in chest CT scans in these patients were: <1 cm nodules, followed by unspecific consolidations, larger nodules and peribronchial infiltrates. They also found early halo signs in 82% of patient´s chest CT scans (68). The halo sign represents a central tissue necrosis due to Aspergillus hyphae and surrounding hemorrhage, these signs coalesce with time and can only been seen within the first 10 days of angioinvasion (69). The air crescent sign can be seen in 50% of cases with invasive aspergillosis. In case of an air crescent sign, a crescent-shaped area of radiolucency around a parenchymal consolidation or nodular opacity can be seen. The air crescent sign has been reported to be associated with improvement of the immune response and has been reported with better survival rates (70).
When IA is suspected and chest CT scan shows suspicious central pulmonary lesions, bronchoscopy with bronchoalveolar lavage should be performed. Samples should be send for histophathology, culture and biomarker analysis. However peripheral pulmonary lesions can be best reached by transbronchial or percutaneous biopsy, when the patient´s condition allow for these interventions (53).

6.4.2 Histopathology

Diagnostic accuracy of histopathology for IA is limited (53). Affected lung tissue samples, gained by transthoracic percutaneous needle aspiration, video-assisted thoracoscopic biopsy or bronchial lavage from a patient with suspected IA can be dyed after tissue fixation with Gomori methenamine silver or periodic acid-Schiff staining technique, or directly with Calcofluor or Blankophor, to detect *Aspergillus*-like features (71). Under the microscope mould species can be seen as narrow septet hyaline hyphae with dichotomous angle 45° branching (Figure 6). When tissue invasion by fungal hyphae can be seen under the microscope it provides evidence for definitive invasive fungal infection, “proven” disease according to EORTC criteria. However, other filamentous fungi like *Scedosporium app.* and *Fusarium spp.* can have similar appearance to *Aspergillus spp.,* thus the definite causative agent cannot be identified by histopathology (47) (72). When cavities or sinuses are affected by the infection vesicles with conidia can be observed. Only a few hints can be gained from histopathology regarding the *Aspergillus* species, in *Aspergillus niger* infection calcium

oxalate crystals can be seen in respiratory specimen and *Aspergillus terreus* produces round to pear shaped aleurioconidia next to lateral hyphal walls. It is important to confirm the genus and species of the causative fungal agent by another diagnostic technique since the required treatment for causative fungi may differ (73).

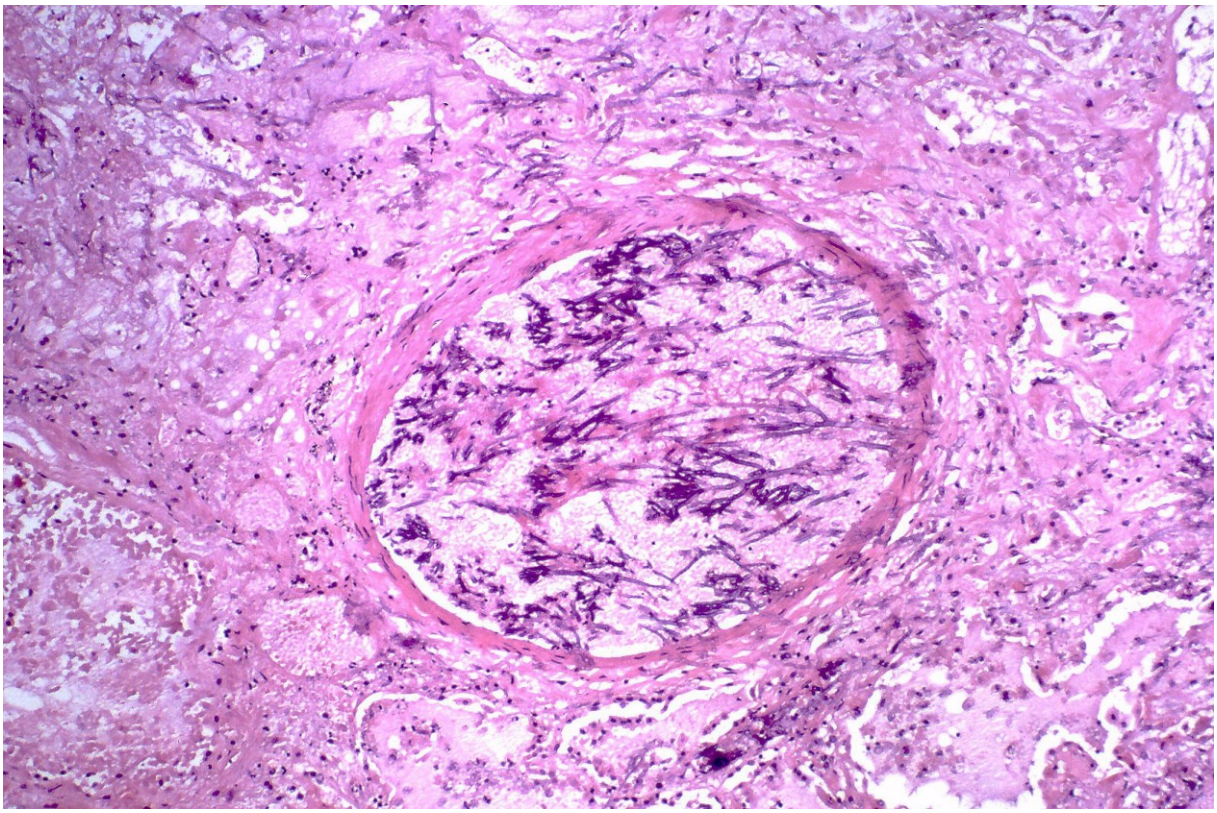


Figure 7, showing angioinvasion of Aspergillus spores. Picture by Jale Rosen distributed by Creative Commons licence 2.0. No changes were made.

6.4.3 Culture of *Aspergillus spp*.

Culture of fungi has not been established as a key method for diagnosing invasive infection, especially since slow sporulating species are difficult to diagnose with culture and positive results may reflect colonization only (74).

Studies have observed that patients with proven invasive aspergillosis have frequently negative culture results. Kontoyiannis et al. and Neofytos et al. showed in surveillance studies that 25% to 50% of patients with evidence for invasive aspergillosis have had negative culture results, which demonstrates how insensitive cultural methods for diagnosing invasive aspergillosis are (75) (76).

When cultural techniques are successful and specimen chosen for culture contain *Aspergillus spp.*, it is a rapidly growing *fungus* which can be seen to have grown massively overnight

(Figure 7), sometimes lifting up the lit of the cultural dish. The formation of a sporulating head is helpful to identify *Aspergillus spp.* by culture more easily.
When only cultural methods where available *Aspergillus lentus*, a slow sporulating species, has been regarded as non-pathogen due to its slow sporulating phenotype, which was not possible to grow in a cultural dish (77).
The study published by Hovath et al. discussed the positive predictive value of respiratory-tract culture results for invasive aspergillosis from different patient populations with probable or proven invasive aspergillosis. The positive predictive value was highest (72%) in hematopoietic cell transplant recipients, patients with underlying hematological malignancies and granulocytopenic patients. The positive predictive value was lower (58%) in patients after solid organ transplantation and patients receiving glucocorticoid therapy, and in HIV positive patients the positive predictive value was even lower (14%) (78).
The positive predictive value also depended on the specimen sent for cultural analysis and was highest in bronchoalveolar lavage fluid samples. This might be due to the fact that invasive aspergillosis is more often in patients with radiological findings, who will be further analyzed by bronchoscopy. Culture results should not be interpreted alone. Clinical and radiographic findings help to separate true- from false-positive culture results.

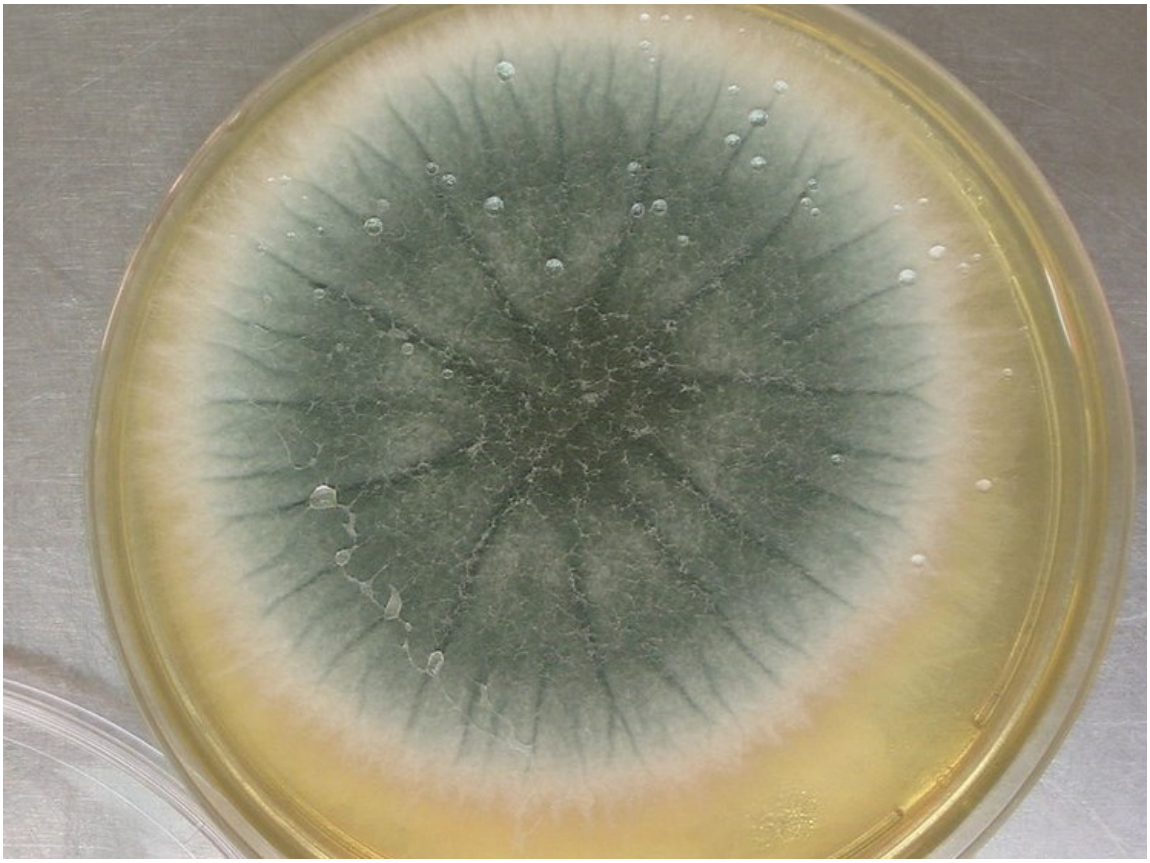


Figure 8, showing *Aspergillus fumigatus* in culture. Picture by Jankaan nl, distributed by Creative Commons licence 3.0. No changes were made.

6.4.4 Biomarker diagnostics

Diagnostical methods for invasive aspergillosis based on biomarker detection were introduced to overcome the lack in specificity and sensitivity in cultural and histological methods. After the introduction of biomarker technologies it was tried to detect different *Aspergillus spp*. antigens from different body fluids by different modes of detection (radioimmunoassay, enzyme-linked immunoassay (monoclonal antibody (Mab EBA1/Mab EBA2/ Mab476)) counter-immuno-electrophoresis, latex agglutination = Pastorex®).
The biomarkers used in clinical routine today for diagnosing invasive aspergillosis are galactomannan and 1, 3-β-D-glucan (β-D-glucan /BDG), which both can be detected with different assays and their diagnostical performance was studied intensively. However, research groups are still working on the detection and establishment of new biomarker-test for invasive aspergillosis, which allow for point of care testing and improved sensitivity and specificity (79).

6.4.4.1 1, 3-β-D-glucan

B-D-glucan is a cell wall component of most pathogenic fungi.
B-D-glucan detection has been described as a panfungal test, as β-D-glucan is produced by many different fungal species, and β-D-glucan can be detected in patients with candidiasis, aspergillosis and *Pneumocystis jirovecii* pneumonia. However, it is generally negative in patients with fungal infections caused by *Cryptococcus spp.* or *Zygomycetes spp.* such as: *Mucor, Rhizopus* and *Absidia* (80). The Fungitell® assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA) is a commercial, US Food and Drug Anministration (FDA) approved test, which was also used in this study, that detects β-D-glucan by measuring the activation of Factor G through horseshoe crab substrates, the same principle used in the limulus assay top detect endotoxin (81).

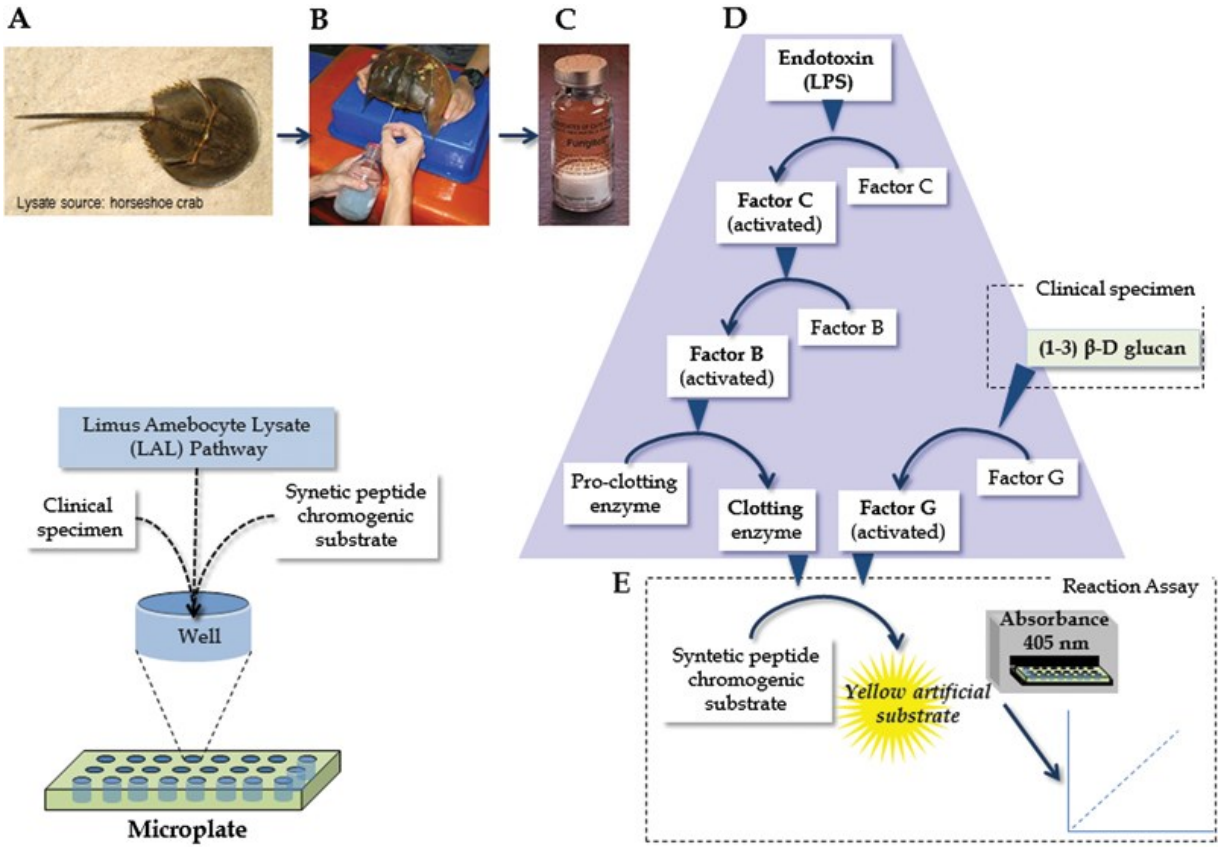


Figure 9, showing the steps of the limulus assay for detection of β-D-glucan. From "Role of Non-Culture-Based Tests, with an Emphasis on galactomannan Testing for the Diagnosis of Invasive Aspergillosis"Miceli et al, license provided by Thieme: 4167721335989.No changes were made.(81)

Figure 8 showing the steps of the limulus assay for detection of β-D-glucan. A) The horseshoe crab (Limulus polyphemus). B) Hemolymph is taken from the crab´s pericardium and the animal is returned to the water. Limulus amebocyte lysate is gained by centrifugation and lysis of Hemolymph fluid. C) Limulus amebocyte lysate is stored as white powder, ready for usage in the Fungitell assay. D) The modified Limulus amebocyte lysate pathway. E) The BDG assay is performed in microplates and results are given after analysis in an incubation reader (81).

After spectrophotometer analysis, optical density values are converted into β-D-glucan concentrations. Results < 60 pg/mL are interpreted as negative, as intermediate when results are between 60 pg/mL and 79 pg/mL and as positive when results are > 80pg/mL. However, these commonly used cut-off values were defined for patients treated for hematological malignancies to identify breakthrough invasive candidiasis, therefore optimal cut-off values may differ for diagnosis of invasive aspergillosis (82). While in clinical routine detection of β-D-glucan is mostly used from serum samples and bronchoalveolar lavage fluid samples, many studies have been published that report of β-D-glucan detection by the Fungitell® assay

from different body fluids like cerebrospinal fluid and urine and in different patient populations (83) (84) (80) (85). The diagnostic performance of β-D-glucan detection for invasive fungal infections was mostly studied for serum samples and substantial heterogeneity was reported for sensitivity and specificity values. The pooled sensitivity and specificity of β-D-glucan for the diagnosis of invasive fungal infection in a meta-analysis published in 2011 was 77% and 85%, respectively (86). Another meta-analysis published one year later reported lower sensitivity and higher specificity rates, with 50% and 99%, respectively (87).
Many variables have been published to influence the detection of β-D-glucan and result in false positive results, caused by gauze packing of serosal surfaces, intravenous therapy with immunoglobulins, chemotherapy, albumin, amoxicillin-clavulanic acid and infections with pseudomonas aeruginosa, cellulose used in membranes for hemodialysis or in filters used for intravenous therapy (88) (89). However, β-D-glucan levels are not wrongly elevated using state of the art renal replacement therapy (90). B-D-glucan is recommended in high risk patients for invasive aspergillosis, often used additionally to GM detection (53).
Sulahian et al. compared the diagnostic performance of the β-D-glucan assay with the galactomannan enzyme immunoassay (EIA) for invasive aspergillosis by analyzing the sera of 105 patients with invasive aspergillosis. He found higher specificity values for the galactomannan EIA with 97% versus 82%, however higher sensitivity values for the β-D-glucan assay with 49% versus 81% (91).

6.4.4.2 Galactomannan

galactomannan is a specific cell wall component of most *Aspergillus* and *Penicillium* species. It is a heat stable heteropolysaccharide built of a mannan core with side chains containing galactofuranosyl, varying in lengths and position (Figure11). The mannan core has been described to be non-immunogenic, however the side chains are immunoreactive and therefore the key part of the molecule regarding serological detection. The beta (1, 5)-linked galactofuranose side chain residues of the GM molecule offer the epitope for antibody binding. In addition, the EB-A2 monoclonal antibody, which is a component of the Platelia® assay, used in this study, derived from rats, is directed towards these epitopes. Four of these epitopes are required for antibody binding and therefore for successful GM detection (92).

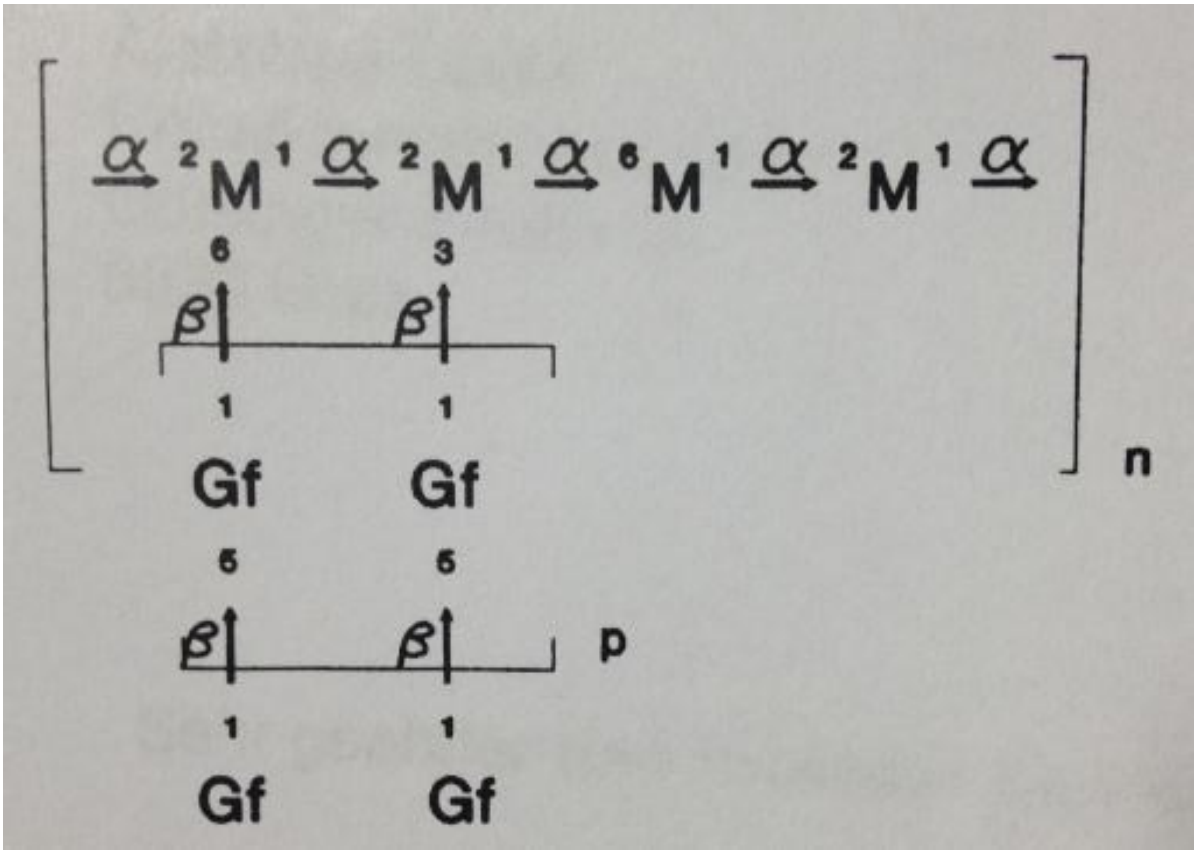


Figure 10, showing the chemical structure of a galactomannan molecule, from "Chemical and immunological characterization of the extracellular galactomannan of Aspergillus fumigatus", Latge et al. Infect. Immun. 1994 (92).

The exact composition of GM varies not only between fungi genera and strains but also between conditions under which GM can be produced, extracted and purified (92).
The GM molecule was early identified as a potential antigen for biomarker diagnostics in invasive aspergillosis. Early detection systems for GM used the latex agglutination, which were however low in sensitivity and were replace by newer detection methods using a double sandwich enzyme immunoassay, which were also used in this study (Platelia®) (93).
The detection of GM for diagnostic purposes by the Platelia® enzyme-linked immunosorbent assay (ELISA) method has been used in the clinical routine for decades, and has been approved by the FDA for serum and bronchoalveolar lavage fluid samples.
The result of the test is given as a ratio of the optical density of each sample to the optical density of a threshold control (provided with each test kit) called the optical density index (ODI). In the early years of using the Platelia® ELISA relatively high cut-off values (1-1.5 ODI) were chosen to avoid high rates of false positive results. However, it was later shown in studies that lower cut-off values (0.5-0.7 ODI) resulted in better diagnostic performance (94) (95).

The diagnostic performance of GM detection from serum samples has been studied intensively. However, the reported diagnostic performance of the GM detection using the Platelia® ELISA from serum varies due to inconsistency in patient population, chosen cut-off values and administration of antifungal medication. A meta-analysis which included 27 studies and 4284 patients reported a diagnostic performance for the Platelia® ELISA for proven invasive aspergillosis of 71% sensitivity and 89% specificity. However, when also probable cases were considered as true positives the sensitivity fell to 61% and the specificity increased to 93% (96). The effect of antifungal therapy was not taken into consideration, as it was only reported in four of the included studies.

Because it was reported in many studies that the GM detection test in patients with invasive aspergillosis was positive before the onset of clinical symptoms and radiological findings, many centers initiated GM screening routines for all patients at risk for invasive aspergillosis to allow for early administration of antifungal therapy (97) (98) (99). Due to the initiation of antimould prophylaxis for patients at risk for invasive aspergillosis the incidence rate of invasive aspergillosis decreased in this patient cohort. The lower incidence rate of invasive aspergillosis in this patient cohort then resulted in decrease of sensitivity of GM screening. It was therefore discussed that GM detection should only be considered for high risk patients or patients with suspected invasive aspergillosis as prevalence is higher in cases of clinical disease suspicion (53) (100) (101) (102).

GM detection was not only discussed as a tool for initial diagnosis of invasive aspergillosis but also as a tool to monitor therapy response in patients with proven invasive aspergillosis. A review analyzing 27 studies which included patients with proven or probable invasive aspergillosis showed that patients were significantly more likely to die and to have aspergillosis verified by autopsy when serial GM testing results were persistently positive after initial diagnosis (103). Also decrease of GM values after initial diagnosis in patients with invasive aspergillosis were reported to indicate lower risk of all-cause mortality (104).

A wide range of possible causes for false positive GM detection results were reported which have to be considered when interpreting low specificity values. The antibiotic preparations piperacillin-tazobactam and amoxicillin-calvulanic acid have been reported to have caused false positive results (105). Possible cross reactions with food and bacteria can also cause false positive results in the setting of damaged intestinal mucosa, possibly resulting from mucositis caused by chemotherapy or graft-versus-host diseases (106). Infections caused by other fungi than *Aspergillus spp.,* which also carry GM or other cross reacting antigens, such

as *Fusarium spp., Penicillium spp.* or *Histoplasma capsulatum,* have also been reported to have caused false positive test results (107) (108) (109).

6.4.4.3 GM detection from urine samples

Almost four decades ago in 1978, the first study on indirect *Aspergillus fumigatus* antigen detection from urine samples from experimentally infected rabbits was published. The used antisera X12 detected a not further identified carbohydrate containing *Aspergillus*-antigen 60–120 hrs after i.v. injection of conidia from serum and urine samples by counter-immuno-electrophoresis (110). This diagnostic approach was again studied by Dupont in 1987 also in experimentally infected rabbits with *Aspergillus fumigatus* and from patients diagnosed with IA. They rated GM detection from urine samples for both humans and rabbits as more promising than detection from serum samples (111). Dupont also studied radiolabeled *Aspergillus fumigatus* GM intravenously injected into rabbits and found that 35% of the injected dose was renally excreted by 24 hrs (112). In 1990 a study was published by Haynes et al, in which the detection of *Aspergillus fumigatus* antigen from urine samples from IA patients using the Mab EB-A1 was described. They used gel electrophoresis for *Aspergillus* antigen detection and proposed this antigen to be valuable for further development of Aspergillus biomarker tests from urine samples (113). In a study by Ansorg et al. from 1994, it was once more concluded that urine testing is more reliable than serum testing for the detection of *Aspergillus* galactomannan. The authors used the latex agglutination test in serial urine and serum samples from 26 bone marrow transplant recipients. Diagnostic performance was better from urine samples compared to serum samples and antigenuria preceded antigenemia (114). However, in 1995 the rat IgM Mab EB-A2, (which is used in the Platelia® assay used in this study) was first used to analyze serial urine and serum samples from immunosuppressed leukemic patients with suggested or proven IA. The authors found lower specificity in urine samples compared to serum samples. Diagnosis of IA was obtained later with urine samples compared to serum samples. However, urine dilution was not taken into consideration (115). In a study published five years later, also using the Mab EB-A2 (Platelia®), diagnostic performance of GM detection from serum samples was also found to be superior to detection from urine samples. However, diagnostic performance of urine testing improved by ten-fold concentration of urine samples but remained inferior to serum testing (116).

In 2004, the first review was published analyzing the diagnostic utility of GM detection from different body fluids other than serum. It was concluded that the diagnostic value of urine

testing remains unclear, reporting of equal numbers of studies that concluded urine to be a better specimen than serum and studies that found serum to be better than urine. However, due to the low number of proven IA cases in these studies, different techniques of urine pretreatment and GM detection as well as different study designs, comparability of study results was not given and the diagnostic value of GM testing from urine samples could not be evaluated. In addition, animal models of IA were subject of criticism since intravenously administrated conidia lead to high fungal burden of the kidney, which might result in unrealistic high urinary excretion of GM compared to animal models in which infection was conducted via aerosol inhalation (117).

In 2012 Dufresne et al. selected a new antibody (Mab476) which detects a different epitope of GM than the EBA2 (Platelia®) from urine by immunoassay and as a point-of-care test. However, a urine inhibitor on the immunoassay was detected which could be abrogated by dialysis or desalting of urine samples. Only in 4 out of 11 urine samples from probable/proven IA patients antigenuria was detected. Not enough patient samples were included in this study to analyze diagnostic performance characteristics for the point-of-care test and the new immunoassay (118).

Fisher et al. published in 2012 a prospective multicenter study in which he included neutropenic pediatric patients to evaluate diagnostic performance of GM detection form urine and serum samples, which were tested after storage at -70°C by the Platelia® ELISA. Urine samples were not pretreated in this study. Due to the low incidence of probable and proven IA cases in the study cohort results cannot be used to evaluate diagnostic performance of Platelia® Elisa from urine samples in pediatric patients. Urine testing showed a high rate of false positive results, however the only case of probable IA was detected by urine testing, and missed by serum testing. GM ODI cut-off for urine was used at 0.5, authors suggested an increase to 1.0 to reduce the number of false positive results (119).

Study, classification of disease	No. of patients	Study design	Underlying condition(s)	Pretreatment of the urine sample	Galactomannan detection method	No. of patients positive for galactomannan	No. of patients who were cured/ no. who died
Dupont et al., 1987 [37]							
Proven	13	NR	Hematologic malignancies, other hematologic diseases, breast cancer, inflammatory diseases, BMT	Dialysis	RIA/ELISA	6/7[a]	5/8
Suspected	8	NR	Hematologic malignancy, aplastic anemia, CGD	Dialysis	RIA/ELISA	0/0[a]	NR
Haynes et al., 1990 [38]: proven or probable	3	Prospective	Hematologic malignancy, BMT	Filtration	Immunoblot	2	NR
Rogers et al., 1990 [26]: proven	8	Prospective	Hematologic malignancy, inborn errors of metabolism, solid tumor, aplastic anemia, congenital immunodeficiency	Dialysis	Inhibition ELISA	44%	NR
Ansorg et al., 1994 [30]							
Proven	4	Retrospective	Hematologic malignancy, BMT	Centrifugation	Pastorex	3	4/4
Probable	3	Retrospective	Hematologic malignancy, BMT	Centrifugation	Pastorex	2	1/2
Suspected	9	Retrospective	Hematologic malignancy, BMT	Centrifugation	Pastorex	9	7/2
Stynen et al., 1995 [16]							
Proven	3	Retrospective	Hematologic malignancy	Centrifugation	Platelia	2	0/3
Probable	2	Retrospective	Hematologic malignancy, aplastic anemia	Centrifugation	Platelia	2	0/2
Possible	2	Retrospective	Hematologic malignancy, aplastic anemia	Centrifugation	Platelia	1	0/2
Salonen et al., 2000 [42]							
Proven[c]	5	Prospective	Hematologic malignancy	Centrifugation	Platelia	2/2[b]	0/5
Probable[c]	1	Prospective	Hematologic malignancy, BMT	Centrifugation	Platelia	0/0[b]	0/1
Possible[c]	15	Prospective	Hematologic malignancy, SCT	Centrifugation	Platelia	5/10[b]	9/6

NOTE. BMT, bone marrow transplantation; CGD, chronic granulomatous disease; NR, not reported; Pastorex, latex agglutination test; Platelia, sandwich ELISA; SCT, stem cell transplantation

[a] RIA result/ELISA result.

[b] Unconcentrated urine/concentrated urine sample.

[c] Classification according to the European Organization for Research and Treatment of Cancer Mycoses Study Group consensus definitions [45].

Table 1, showing studies analyzing galactomannan detection from urine samples since 1987, from „Utility of Aspergillus Antigen Detection in Specimens Other than Serum Specimen“ Klont et al, license provided by Oxford University Press (117).

In 2012 Garcia et al. proposed also to elevate the cut-off from 0.5 to 1.5 ODI (Platelia® ELISA) for urine and serum GM detection in a study on IA in dogs to improve specificity while sensitivity was not altered. In this study cohort of 67 dogs, eight were culture proven diagnosed with IA and median GM ODI levels from urine samples were significantly higher in the IA group compared to the control group. In this study urine testing showed good diagnostic potential, with sensitivity of 88% and specificity of 92%. Urine samples were not pretreated and concentration of urine was not taken into consideration though (120).
In a study published in 2014 much lower ODI cut-off values (0.1) for GM detection from urine samples were chosen, which increased the sensitivity to 47% but held specificity values at 86% (121).
It some studies it was tried to improve the diagnostic performance of GM testing from urine samples by concentrating the urine samples (116). However, no study which analyzed the diagnostic potential of GM testing from urine samples took variations of the urinary flow rate into consideration. It seems possible that in the studies, described above, some urine samples were low concentrated after high water intake, and were thus false negative tested. Sensitivity of GM detection from urine samples might improve by normalizing the urine samples against creatinine.

6.4.4.4 Creatinine normalization for urine diagnostic approaches

The major disadvantage of biomarker testing from urine samples is the variability in concentration from void to void. The concentration of any urinary solute (biomarker) varies from sample to sample due to the renal capacity to regulate water reabsorption from urine over more than an order of magnitude. Given a constant production and excretion rate of any biomarker, oliguria will increase and polyuria will decrease its absolute concentration levels in urine samples. Thus, due to the variability of urinary concentration, any biomarker concentration in urine depends not only on its renal excretion rate but also on the urinary flow rate (122) (123) (124).
Urinary biomarker concentration variations due to the changing water content can be eliminated by calculating the urinary excretion rate. The biomarker concentration in urine is multiplied by the volume of the void and divided by the accumulating time of the void in the bladder, assuming the bladder is completely emptied with each void and the entire sampling void volume is known (125). This approach requires vast knowledge, which makes it unpractical for clinical use.

When biomarkers are measured from urine samples, different methods have been reported to standardize biomarker levels, such as urine osmolality, collage, albumin, cystatin C or ratio of protein biomarker to total protein content in the sample (126). Standardized normalization methods are, however, lacking and the creatinine adjustment is being most widely used (127) (128).

Creatinine is produced by the human organism as a breakdown product from creatinine phosphate from muscle cells, at a nearly constant rate when metabolism, renal function and muscle mass are stable. Thus, urinary creatinine concentration is inversely proportional to urine flow rate. creatinine is removed from the organism mainly over the kidneys by glomerular filtration and a little by proximal tubular secretion. Tubular reabsorption of creatinine is almost not happening. Urine concentration normalization against creatinine requires a linear relationship between the biomarker of interest and urine creatinine concentrations, a constant creatinine excretion rate between different individuals and within one individual (123).

7 Hypothesis and aim of this study

Hypothesis: The diagnostic performance of GM detection from urine samples for IA versus no IA improves significantly when urine dilution is taken into account versus conventional measurement of GM from urine samples.

H_0: The GM/creatinine ratio does not improve the diagnostic performance of GM testing from urine samples for IA versus no IA compared to conventional measurement of GM in urine samples.
H_1: The GM/creatinine ratio improves the diagnostic performance of GM testing from urine samples for IA versus no IA compared to conventional measurement of GM in urine samples.

To evaluate whether a ratio of urine GM and creatinine could improve the diagnostic performance of urine GM determination, the following points were investigated:

- Analysis of the relation between GM concentration and creatinine concentration in serial dilution of urine.
- Calculation of a ratio of GM and creatinine values from tested urine samples
- Analysis of the relation between conventional GM ODI values from serum and urine and the newly introduced GM/creatinine.
- Comparison of median values of the GM/creatinine ratio from urine samples between different patient cohorts.
- Evaluation of the diagnostic performance of GM/creatinine ratio comparing different patient cohorts.

8 Material and Methods

8.1 Study Design

We performed this single center, prospective observational study between September 2014 and August 2015 at the University Hospital of Graz, Austria, to evaluate the influence of urine dilution on the diagnostic performance of GM detection from urine samples, evaluated by the urine GM/creatinine ratio, in patients with underlying hematological malignancies at risk for IA (129).

The study protocol was approved by the local ethics committee at the Medical University of Graz (Graz, Austria) (EC no. 23-343) and was registered at ClinicalTrials.gov (registration no. NCT01576653)

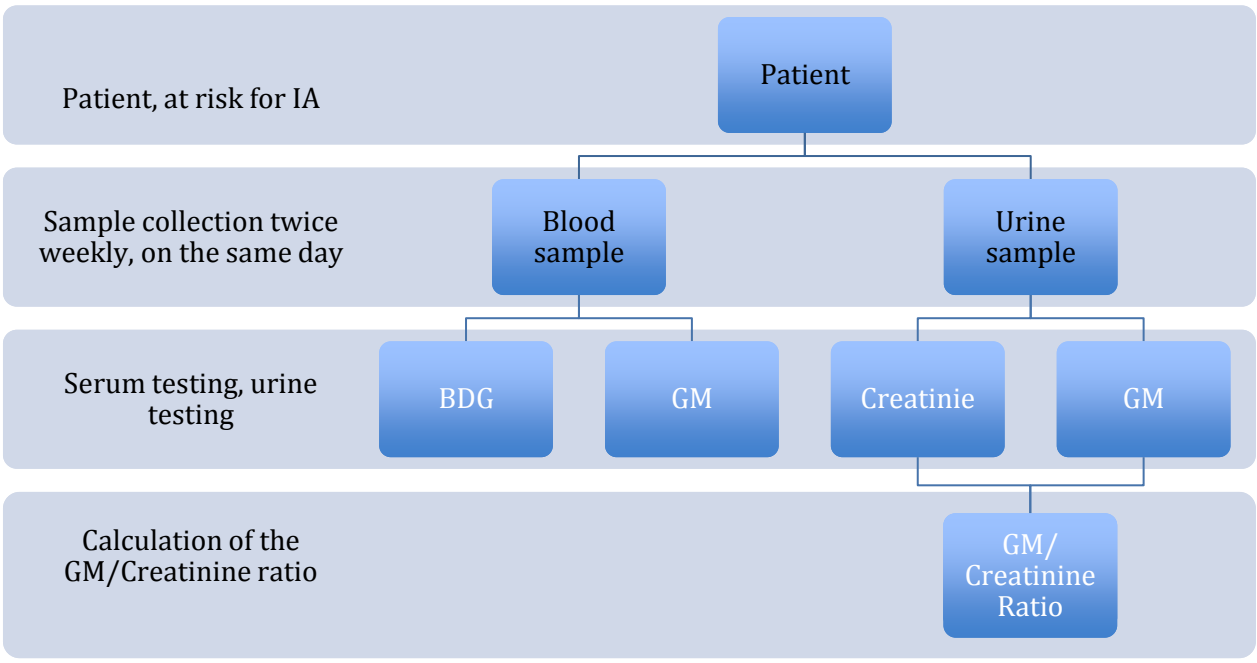


Figure 11, showing the study sample-collection-scedule for included study patients. Twice weekly (on the same day), collection of serum and urine samples. Serum sample analyzis for BDG and GM. Urine sample analyzis for GM and creatinine. Calculation of the GM/creatinine ratio.

8.1.1 Patients inclusion

Patients at risk for invasive aspergillosis, that were included in this study were hospitalized to the hematological or bone marrow transplantation ward of the University Hospital of Graz. Patients were included from September 2014 to July 2015. The patients signed an informed consent after they discussed the study protocol with a physician and had a chance to ask all remaining questions. They were informed that twice weekly morning mid-stream urine samples were collected for the study and that additional blood samples were taken, when BDG or GM screening was not scheduled for the patient on days when urine samples were collected (Figure 10). Blood samples collected for this study were taken during routine blood sample collection so that no additional puncture of the vain was performed. Patients from the validation cohort, that contributed urine samples to the study came for routine check-ups to an outpatient department of an internal medicine ward of the University Hospital of Graz (129).

8.1.2. Inclusion criteria

For this study, two groups of patients were investigated; hospitalized hematological patients at risk for IA and non-hematological patients of an outpatient, internal medicine department.
The following hematological patients were considered for inclusion:
adult patients over 18 years of age, admitted to the hematology or bone marrow transplantation ward of the University Hospital of Graz, with actual or expected risk for IFI according to 2008 revised EORTC criteria. In this study, risk for IFI was mostly caused by prolonged neutropenia, secondary to high dose chemotherapy for leukemia or myeloablative chemotherapy in preparation for allogeneic stem cell transplantation, or immunosuppression therapy after allogeneic stem cell transplantation.
The following non-hematological patients were considered for inclusion:
Non-hematological patients, who were scheduled for urine testing during a routine check-up at an outpatient, internal medicine department. Patients, who had their samples analyzed by the Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, were asked to contribute remains of their urine samples to the study and were classified as validation cohort without risk for IFI. No blood samples were collected from these patients (129).

8.1.3 Exclusion criteria

For hematological patients exclusion criteria were as follows:

- Under 18 years of age
- Denied consent or withdrawal of consent
- Not at risk for IFI
- Urine catheter

For patients from the outpatient department exclusion criteria were as follows:

- Under 18 years of age
- Denied or withdrawal of consent
- Urine catheter

8.1.4 Sample collection

All hematological patients included into this study underwent a fungal infection screening routine, which consisted of blood and urine samples taken twice a week on the same day.
On Mondays and Fridays blood samples were taken for serum GM and BDG testing.
Urine collection cups were labelled with patient´s name and date of urine collection and were placed the evening before sample collection in each patient´s bathroom.
Mid-stream morning urine was collected in cups and transferred to the laboratory. From each urine sample six aliquotes were transferred to1.8mL Nunc® (Thermo Scientific) tubes, using a standard pipette with unfiltered pipette tips. Four aliquotes à 500µL and two aliquotes à 1.8mL were labeled with the patient´s study identification number and date of the sample collection. Labeled Nunc® tubes were then collected in boxes for storage. These were stored at -70°C for 2 to 6 months until urine creatinine and GM testing was performed. The position of each Nunc® tube in storage boxes was documented in Excel files, with patient´s identification number and date of sample collection, to allow for easy relocation of any specific sample (129).

8.2 GM testing from serum and urine samples

Urine and serum samples were tested for GM according to the same protocol (130), however serum samples were tested in the clinical routine on the same day as sample collection, whereas urine samples were stored at -70°C and tested after complete sample collection. The Platelia® Aspergillus test assay (EIA; Bio-Rad Laboratories, Marnes-la-Coquette, France), an immunoenzymatic sandwich microplate assay for the detection of aspergillus galactomannan antigen, was used for both urine and blood samples.

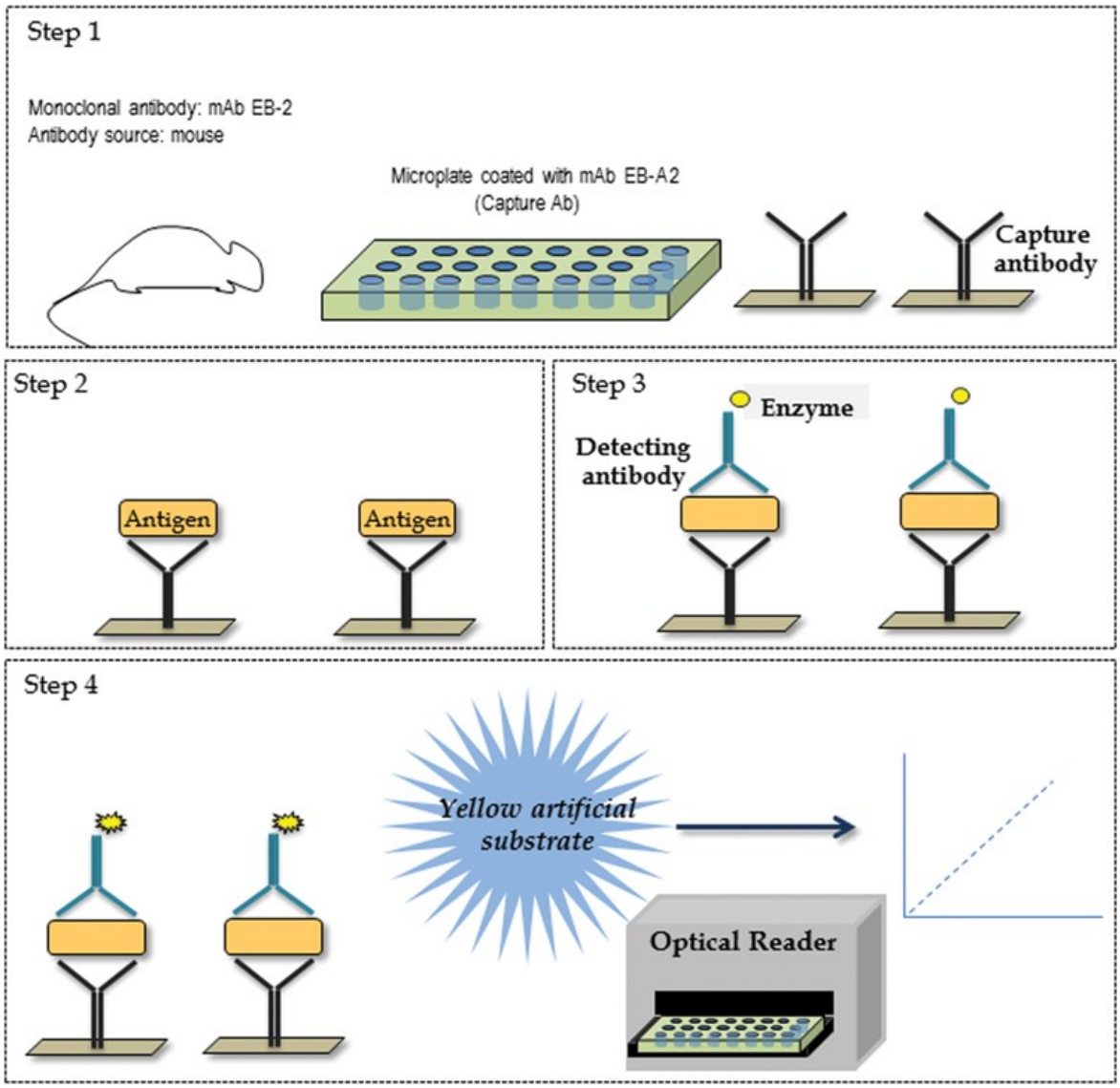


Figure 12, showing the steps of the Aspergillus galactomannan ELISA immunoassay. From "Role of Non-Culture-Based Tests, with an Emphasis on galactomannan Testing for the Diagnosis of Invasive Aspergillosis"Miceli et al, license provided by Thieme: 4167721335989.No changes were made (81).

Figure 11, showing the steps of the Aspergillus galactomannan (Platelia®) ELISA immunoassay. Step 1: The capture antibody (murine monoclonal antibody (mAb) EB-A2) is used to cover the microplate well floors. Step 2: Samples (urine or serum) are added to the wells. Step 3: The detecting antibody (labeled anti-mAb EB-A2) is added to the wells. A "mAb–antigen–labeled anti-mAb complex" is formed, when the sample is positive. Step 4: Positive samples containing the "mAb-antigen–labeled anti-mAb complex" are revealed by formation of a blue color when the substrate solution is added. Results are given after analysis by an optical reader (81).

EBA-2 is the monoclonal antibody used in this test, which is directed against *Aspergillus* GM (131). EBA-2 is coated on the floor of the wells to bind the antigens in the sample (if present) and EBA-2 is also used to detect antigens bound to the well floor.

In the first step of the GM detection analysis, Ethylenediaminetetraacetic acid (EDTA) is used to precipitate proteins that could interfere with the test and to dissociate immunocomplexes which might be present in the sample (Figure 13). When the deproteinized sample is given into the micro well plate GM (if present in the sample) connects with the EBA-2 antibody at the bottom of the well and forms a monoclonal antibody-GM-monoclonal antibody peroxidase sandwich complex with added reagents (Figure 14). After unbound material is washed away (Figure 15) a chromogen solution reacts with the sandwich complexes and turns positive samples, i.e. with GM present, blue (Figure 16). This enzyme reaction is stopped before spectrophotometer analysis by adding an acidic solution, which turns positive blue samples yellow (Figure 17). At 450 and 620/630 wavelength the optical density (absorbance) is measured (Figure 18).

Material/Equipment	Contents/ Purpose
Micro well strip plate	Microplate, 96 wells coated with Anti-GM monoclonal Antibodies EBA-2
Plate sealers	Adhesive sheet for microplate
Absorbent paper	Cleaning
Disposable gloves	Protection (acidic solution used)
Protective glasses	Protection (acidic solution used)
Pipettes with disposable tips	For 50µL, 100µL, 300µL and 1000µL
Tubes	1.5 mL able to support heating to 120°C with airtight lits
Centrifuge	Suitable for 1.5mL tubes
Heat block	Suitable for 1.5mL tubes
Agitator	Mixing reagents in tubes
Microplate incubator	Providing 38°C
Automated microplate washer	Wash unbound material from Microplate
Microplate reader	Equipped with 450 nm filters

Table 2, showing laboratory material and equipment used for galactomannan testing from urine and serum samples.

Reagent	Contents	Purpose	Number in text
Washing solution	- tris NaCl buffer (pH 7,4) - 2% v/v Tween® 20 - <1,5% v/v ProClin ™ 300	Used in the automated microplate washer (12)	14
Negative Control Serum	- Negative serum - Negative for anti-HIV-1, anti HIV-2, anti-HCV antibodies and HBs Ag - < 1.5% v/v Pro Clin ™ 300	Providing a negative sample for absorbance comparison	15
Cut-off Control Serum	- Serum containing GM - Negative for anti-HIV-1, anti HIV-2, anti-HCV antibodies and HBs Ag - < 1.5% v/v Pro Clin ™ 300		16
Positive Control Serum	- Serum containing GM - Negative for anti-HIV-1, anti HIV-2, anti-HCV antibodies and HBs Ag - < 1.5% v/v Pro Clin ™ 300	Providing a positive sample for absorbance comparison	17
Conjugate	- Anti GM monoclonal Antibody/peroxidase labeled - < 1.5% v/v Pro Clin ™ 300	Binds to GM antigens bound to the Micro well floor if sample is positive (containing GM)	18
Sample Treatment Solution	-EDTA acidic solution	Removes unbound material	19
Chromogen TMB Solution	- 3,3´,5,5´-tetramethylbenzidine <0,1% v/v	Colors GM-Antibody Complexes blue	20
Stopping Solution	-1N sulphuric acid solution (H2SO4)	Turns positive samples from blue to yellow	21

Table 3, showing reagents used for galactomannan testing from urine and serum samples.

The proposed test protocol of the manufacturer for serum and bronchoalveolar lavage (BAL) testing was applied for the analysis of urine samples in this study (130).
The reagents from the testing kit (Table 3) were first brought to room temperature for 30 minutes after they were taken from the refrigerator-storage at 2-8°C.
In the first step of the procedure 300µL of each urine sample were transferred to individual 1.5mL polypropylene tubes (Figure 12). Tubes were labelled with patient´s identification number and date of the sample. 300µL of the negative control serum (Table 3, number 15), the positive control serum (Table 3, number 17) and the cut-off control serum (Table 3, number 16), provided by the test kit, were also each transferred to 1.5mL polypropylene tube and tubes were labelled accordingly.

Figure 13, showing 1.5 mL polypropylene tubes with urine samples ready for galactomannan testing.

Then 100µL of the sample treatment solution (Table 3, number 19) was added into each of the 1.5mL polypropylene tubes for deproteinization of the study samples. The tubes were then mixed thoroughly on an electrical vortex machine and closed tightly to prevent emerging of sample fluid during heating. The tubes were placed into the heat block and were left for 6 min at 120°C. To finalize the deproteinization the tubes were centrifuged for 10 min at 10000g (Figure 13). In the following steps only the supernatant above the precipitated protein was used for further analysis.

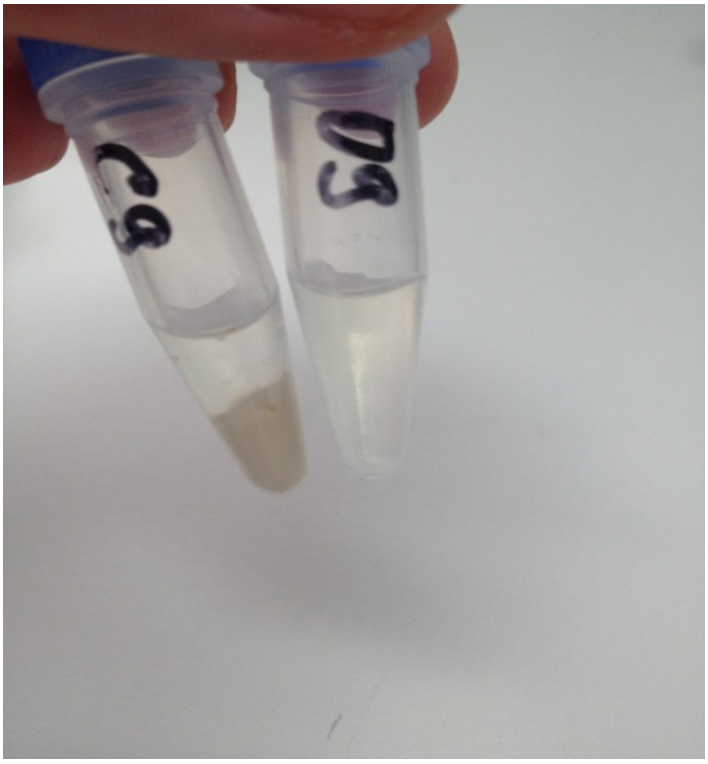


Figure 14, showing 1.5 mL polypropylene tubes after centrifugation of urine samples for deproteinization, the tube on the left containing protein sediment.

In the next step the Conjugate solution (Table 3, number 18) (Anti-GM Monoclonal Antibody) was first mixed and then 50µL from the Conjugate (blue color) were pipetted into each slot of a 90 well plate (Figure 14). According to a 90 well chart, prepared to identify which sample was analyzed in which well, 50µL of the Positive Control Serum (Table 3, number 17), 50µL of the Cut-off Control Serum (Table 3, number 16) and 50µL of the Negative Control Serum (Table 3, number 15) were pipetted (changed pipette tips between each reagent) into their wells.

Figure 15, showing the 90 well plate beeing filled with the blue conjugate solution, which contains the anti-GM monoclonal antibody, which binds to GM antigens on the micro well floor if the sample is positive.

According to the 90 well chart 50µL of each urine sample were transferred into each well. The plate was then covered with a sticky foil ensuring that the entire surface of the microwell plate was covered watertight to avoid evaporation. The sealed plate was then incubated in a dry microplate incubator for 90min at 37°C.

After incubation the plate sealing foil was removed and the microwell plate was placed into the microplate washer (Figure 15), where first all fluid contents of the wells were aspirated into a waist container, leaving the bound immunocomplexes to the floor of the wells. The wells were then washed 5 times in the microplate washer to remove all remaining unbound material.

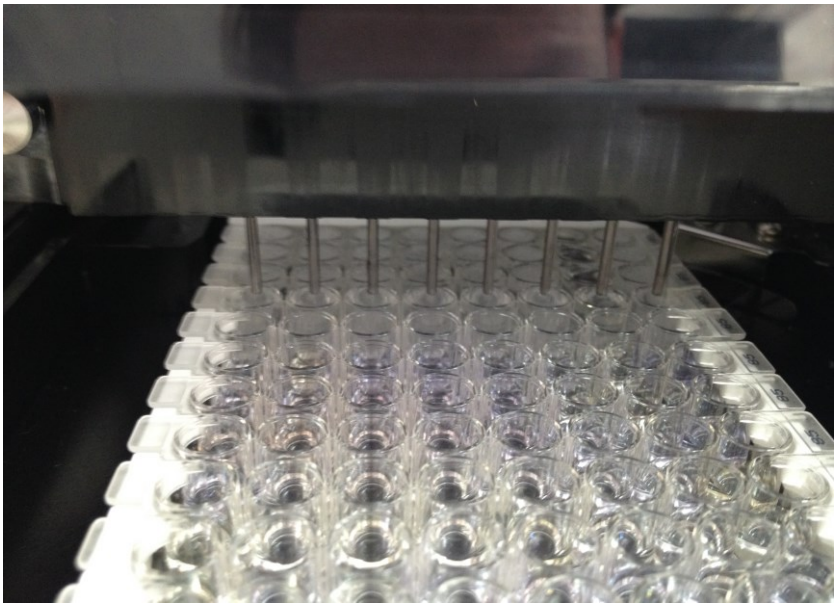


Figure 16, showing unbound material beeing washed from the 90 well plate.

After the washing step, 200µL of the Chromogen TMB Solution (Table 3, number 20) was quickly added to the "empty" wells with a mulipiette and present GM-antibody complexes were colored blue (Figure 16). The micro well plate was then for 30min incubated, in a dark drawer at room temperature (18-25°C).

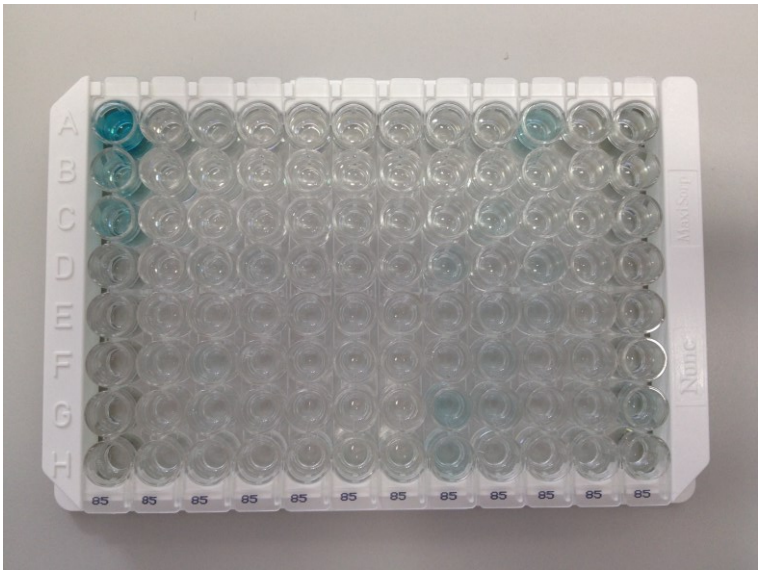


Figure 17, showing the 90 well plate after the final incubation step, with positive samples in light blue colour.

After this final incubation 100µL of the acidic Stopping Solution (Table 3, number 21) was added to each well, to stop the coloring process, positive samples turned from blue to yellow (Figure 17).

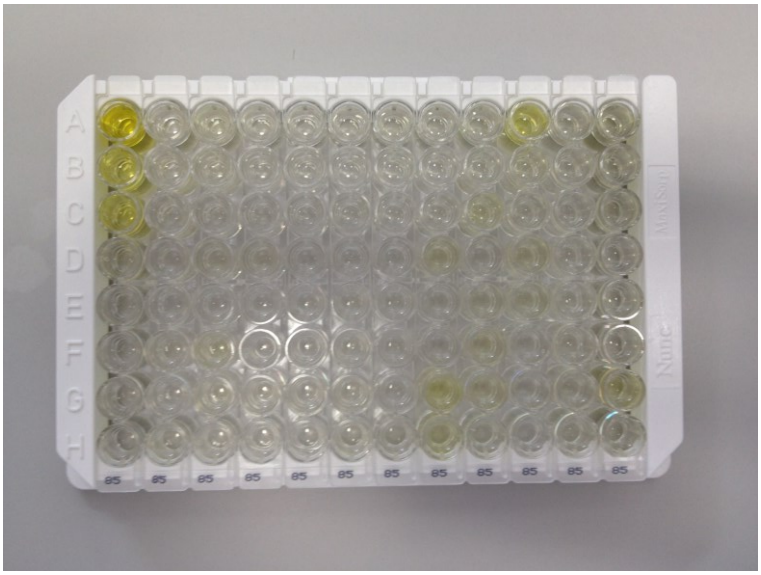


Figure 18, showing the 90 well plate after the stopping solution was added and positive samples turned from blue to yellow.

Finally, the micro well was placed in the microplate reader and samples were analyzed at 450nm (reference filter of 620/6030nm) (Figure 18). After analysis a result sheet was printed, which indicated GM optical density index (ODI) for each sample, according to the well position on the plate (Figure 19).

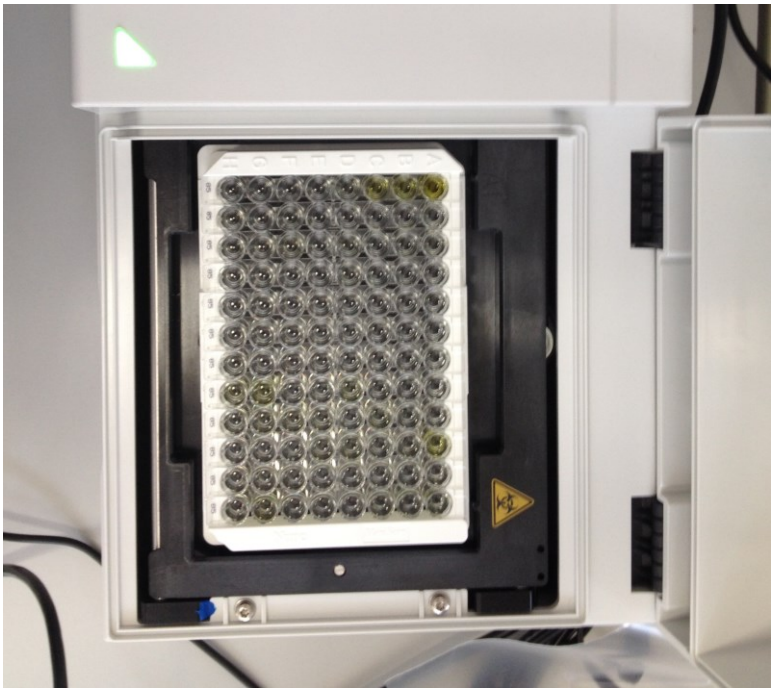
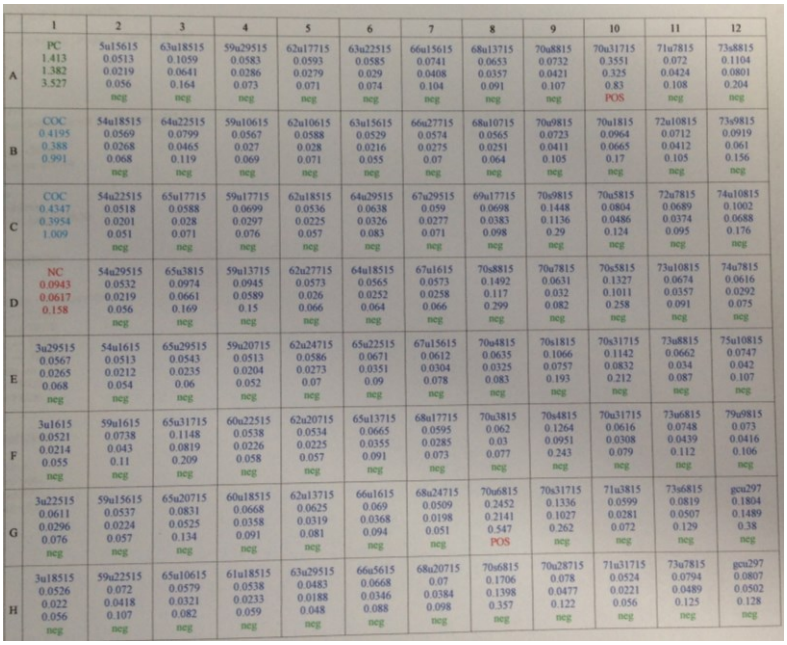


Figure 19, showing the 90 well plate in the microplate reader for optical density analysis.



	1	2	3	4	5	6	7	8	9	10	11	12
A	PC 1.413 1.382 3.527	5u15615 0.0513 0.0219 0.056 neg	63u18515 0.1059 0.0641 0.164 neg	59u29515 0.0583 0.0286 0.073 neg	62u17715 0.0593 0.0279 0.071 neg	63u22515 0.0585 0.029 0.074 neg	66u15615 0.0741 0.0408 0.104 neg	68u13715 0.0653 0.0357 0.091 neg	70u8815 0.0732 0.0421 0.107 neg	70u31715 0.3551 0.325 0.83 POS	71u7815 0.072 0.0424 0.108 neg	73s8815 0.1104 0.0801 0.204 neg
B	COC 0.4195 0.388 0.991	54u18515 0.0569 0.0268 0.068 neg	64u22515 0.0799 0.0465 0.119 neg	59u10615 0.0567 0.027 0.069 neg	62u10615 0.0588 0.028 0.071 neg	63u15615 0.0529 0.0216 0.055 neg	66u27715 0.0574 0.0275 0.07 neg	68u10715 0.0565 0.0251 0.064 neg	70u9815 0.0723 0.0411 0.105 neg	70u1815 0.0964 0.0665 0.17 neg	72u10815 0.0712 0.0412 0.105 neg	73s9815 0.0919 0.061 0.156 neg
C	COC 0.4347 0.3954 1.009	54u22515 0.0518 0.0201 0.051 neg	65u17715 0.0588 0.028 0.071 neg	59u17715 0.0699 0.0297 0.076 neg	62u18515 0.0536 0.0225 0.057 neg	64u29515 0.0638 0.0326 0.083 neg	67u29515 0.059 0.0277 0.071 neg	69u17715 0.0698 0.0383 0.098 neg	70s9815 0.1448 0.1136 0.29 neg	70u5815 0.0804 0.0486 0.124 neg	72u7815 0.0689 0.0374 0.095 neg	74u10815 0.1002 0.0688 0.176 neg
D	NC 0.0943 0.0617 0.158	54u29515 0.0532 0.0219 0.056 neg	65u3815 0.0974 0.0661 0.169 neg	59u13715 0.0945 0.0589 0.15 neg	62u27715 0.0573 0.026 0.066 neg	64u18515 0.0565 0.0252 0.064 neg	67u1615 0.0573 0.0258 0.066 neg	70s8815 0.1492 0.117 0.299 neg	70u7815 0.0631 0.032 0.082 neg	70s5815 0.1327 0.1011 0.258 neg	73u10815 0.0674 0.0357 0.091 neg	74u7815 0.0616 0.0292 0.075 neg
E	3u29515 0.0567 0.0265 0.068 neg	54u1615 0.0513 0.0212 0.054 neg	65u29515 0.0543 0.0235 0.06 neg	59u20715 0.0513 0.0204 0.052 neg	62u24715 0.0586 0.0273 0.07 neg	65u22515 0.0671 0.0351 0.09 neg	67u15615 0.0612 0.0304 0.078 neg	70u4815 0.0635 0.0325 0.083 neg	70s1815 0.1066 0.0757 0.193 neg	70s31715 0.1142 0.0832 0.212 neg	73u8815 0.0662 0.034 0.087 neg	75u10815 0.0747 0.042 0.107 neg
F	3u1615 0.0521 0.0214 0.055 neg	59u1615 0.0738 0.043 0.11 neg	65u31715 0.1148 0.0819 0.209 neg	60u22515 0.0538 0.0226 0.058 neg	62u20715 0.0534 0.0225 0.057 neg	65u13715 0.0665 0.0355 0.091 neg	68u17715 0.0595 0.0285 0.073 neg	70u3815 0.062 0.03 0.077 neg	70s4815 0.1264 0.0951 0.243 neg	70u31715 0.0616 0.0308 0.079 neg	73u6815 0.0748 0.0439 0.112 neg	79u9815 0.073 0.0416 0.106 neg
G	3u22515 0.0611 0.0296 0.076 neg	59u15615 0.0537 0.0224 0.057 neg	65u20715 0.0831 0.0525 0.134 neg	60u18515 0.0668 0.0358 0.091 neg	62u13715 0.0625 0.0319 0.081 neg	66u1615 0.069 0.0368 0.094 neg	68u24715 0.0509 0.0198 0.051 neg	70u6815 0.2452 0.2141 0.547 POS	70s31715 0.1336 0.1027 0.262 neg	71u3815 0.0599 0.0281 0.072 neg	73s6815 0.0819 0.0507 0.129 neg	gcu297 0.1804 0.1489 0.38 neg
H	3u18515 0.0526 0.022 0.056 neg	59u22515 0.072 0.0418 0.107 neg	65u10615 0.0579 0.0321 0.082 neg	61u18515 0.0538 0.0233 0.059 neg	63u29515 0.0483 0.0188 0.048 neg	66u5615 0.0668 0.0346 0.088 neg	68u20715 0.07 0.0384 0.098 neg	70s6815 0.1706 0.1398 0.357 neg	70u28715 0.078 0.0477 0.122 neg	71u31715 0.0524 0.0221 0.056 neg	73u7815 0.0794 0.0489 0.125 neg	gcu297 0.0807 0.0502 0.128 neg

Figure 20, showing the result sheet given by the microplate reader with optical densitiy values for the positive control sample, negative control samples and each urine sample.

8.3 BDG testing from serum

From hematological study patients' blood serum samples were screened twice weekly for BDG. BDG detection from serum was performed with other samples in the clinical routine at the Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, using the Fungitell® assay (Associates of Cape Cod, East Falmouth, MA, USA).The Fungitell® assay is based on the modified Limulus amebocyte coagulation pathway. It was adapted for automatic sample testing as described in a study by Prüller at al, using the BCS® XP (Siemens Healthcare Diagnostics, Marburg, Germany) coagulation analyzer, which allows for both single and large scale testing with 1-hour time to result (132). As indicated by the manufacturer of the Fungitell® assay, BDG serum concentrations of ≥80pg/mL were considered to be positive, while a concentration of <60pg/mL was considered to be negative. Twice weekly BDG serum levels of study patients were used to evaluate the actual probability of an invasive aspergillosis infection according to EORTC criteria.

8.4 Creatinine testing from urine

creatinine levels in urine samples of study patients were analyzed, to evaluate the grade of urine dilution. creatinine levels were retrospectively determined from 500µL urine aliquotes stored at -70°. creatinine detection from urine samples was done using the Cobas® 8000 modular analyzer, Roche, Switzerland, at the Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz.
This creatinine detection method is based on the Jaffe reaction (133). This reaction is performed by adding Picric acid to urine samples, where in the alkaline medium a red color forms. The velocity of the color change is directly proportional to the creatinine concentration in the urine sample, which is measured at 520 nm (134). Results are given in mg/dL.

8.5 GM-Creatinine Ratio

After all urine samples were tested for GM and creatinine, as described above, the GM-creatinine ratio was calculated. The GM ODI level of a sample was multiplied by 100 and divided by the sample's creatinine level (129).

GM-creatinine ratio = (GM ODI x 100)/creatinine level

8.6 Data collection

Study patients were followed prospectively after enrollment and demographical data, receipt of antifungal medication, state of neutropenia, fungal specific serological results, chemotherapy and chest CT scan results were documented weekly.
Demographical data were collected from patients regarding, sex, age, body weight, height and underlying disease. When serum concentrations of antifungal medication were measured in clinical routine, levels were documented.
Chemotherapy was classified in high dose and low dose chemotherapy, regarding the expected drop of neutrophils and the resulting immunodeficiency during and after the therapy was administered. It was also documented why chemotherapy was indicated, whether the patient was going through induction therapy with a curative attempt, palliative attempt or as preparation for stem cell transplantation.
It was also documented whether patients were receiving immunosuppressant therapy such as Ciclosporin or Mycophenolat-Mophetil.
For each patient the latest CT scan of the chest was inspected for signs of fungal infections defined by the revised EORTC criteria in 2008 and documented. CT scans were inspected for halo signs, air-crescent signs, cavities, nodules, nodules close to the pleura, and infiltrations on one side or both sides (135).

8.7 Classification of patients according to EORTC criteria

Based on the patient´s actual neutrophil count, overall clinical state, serum GM and BDG levels and latest chest CT findings, patients were classified twice weekly, for each day of sample taking, according to 2008 revised EORTC criteria (47) into:

- No evidence for IA
- Possible IA
- Probable IA
- Proven IA.

In 2008, the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycosis Study Group (EORTC/MSG) updated diagnostic criteria for immunosuppressed

patients at risk for IFI (47). The first version of these criteria were published in 2002 and were restricted to cancer patients and patients receiving hematopoietic stem cell transplantation, they were intended for the evaluation of invasive fungal infections in clinical studies only (67). During the six years between these two versions the diagnostical options for IFI improved. Serological markers, GM and BDG testing were introduced and found their way in the diagnostic routine for IFI. Computer tomography of the chest has also been found to be of help in the early diagnostic of IFI. Therefor these diagnostical tools were included in the revised EORTC criteria. In both EORTC criteria versions patients are classified into four groups: "no IFI", "possible IFI", "probable IFI" and "proven IFI", however in the 2008 revised criteria the "probable" group has been expanded and the "possible IFI" group has been diminished. Also in the 2008 revised version the groups "probable IFI" and "possible IFI" were restricted to apply only for patients that are immunocompromised. However, the group "proven IFI" can be applied to any patient (47).

It was criticized that with the EORTC criteria version from 2002 to many dubious cases could be included into the "possible IFI" group, merely based on broad-spectrum antibiotic treated, persistent neutropenic fever with unspecific pulmonary infiltrates. In contrast patients with the same clinical profile but with far more specific pulmonary abnormalities such as halo signs or air-crescent signs were also only classified as "possible IFI" (136). This situation showed the need for a clearer definition of the criteria to distinguish between patients with a dubious clinical profile from the more likely cases, in the absence of mycological evidence. The revised criteria demand now, that a patient has to show signs that are highly likely to be caused by a fungal etiology (like halo or aircrescen sign), when mycological evidence is lacking, in order to classify him or her to the "possible IFI" group. The classification to the "probable IFI" and "proven IFI" group in the revised criteria is based on: host factors, clinical manifestations and mycological evidence (47).

Now that GM testing, BDG testing, detection of fungal DNA by PCR and high-resolution chest CT are available in the clinical routine, these advances were included into the revised criteria. It was also taken into consideration that a larger group of patients are at risk for invasive fungal infections which were not covered within the 2002 criteria, such as recipients of solid organ transplants and patients with primary immunodeficiency.

8.7.1 EORTC criteria

For a patient to be classified to the group of "probable IFI" one host factor, a clinical criterion and a mycological criterion has to be present. Patients that meet only a host factor and a clinical criterion but no mycological criteria are to be considered as "possible IFI" (47).

8.7.1.1 Host factors:

- Recent history of neutropenia, temporally related to the onset of fungal disease ($<0.5+10^9$ neutrophils/L for >10 days)
- Receipt of an allogeneic stem cell transplant
- Prolonged use of corticosteroids (excluding among patients with allergic bronchopulmonary aspergillosis) at a mean dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks
- Treatment with other recognized T cell immunosuppressant, such as cyclosporine, TNFα blockers, specific monoclonal antibodies (such as alemtuzumab), or nucleoside analogues during the past 90 days
- Inherited severe immunodeficiency (such as granulomatous disease or severe combines immunodeficiency)

8.7.1.2 Clinical criteria:

- Lower respiratory tract fungal disease
- The presence of one of the following three sings on chest CT:
    - dense, well-circumscribed lesion(s) with or without a halo sign
    - air-crescent sign
    - cavity
- Tracheobronchitis
    - tracheobronchial ulceration, nodule, pseudo membrane, plaque or eschar seen on bronchoscopy analysis
- Sinonasal infection
    - imaging showing sinusitis plus at least one of the following three sign
        - 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
    - one of the following two signs
        - focal lesion on imaging
        - meningeal enhancement on MRI or CT
- Disseminated candidiasis
    - at least one of the following two entities after an episode of candidemia within the previous two weeks
        - small, target-like abscesses (bull´s-eye lesions) in liver or spleen
        - progressive retinal exudates on ophthalmologic examination

8.7.1.3 Mycological criteria:

- Direct test (cytology, direct microscopy or culture)
    - Mould in sputum, bronchoalveolar lavage fluid, bronchial brush, or sinus aspirate samples, indicated by one of the following:
        - presence of fungal elements indicating a mould
        - recovery by culture of a mould (e.g. *Aspergillus, Fusarium, Zygomycetes* or *Scedosporium* species)
- Indirect test (detection of antigen or cell-wall constituents)
    - Aspergillosis
        - GM antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF
    - Invasive fungal disease other than cryptococcosis and zygomycoses
        - B-D-glucan detected in serum

To be classified to the group of "proven IFI" fungal species must be detected by microscopy or by culture from sterile material or blood. The difference between the direct tests that lead to the diagnosis of “possible” IFI or to the diagnosis of “proven” IFI is that only tissue sample, which are sterile, can lead to a diagnosis of “proven” IFI (47).
Sterile tissue samples obtained by needle aspiration or biopsy have to show hyphae or melanized yeast-like forms and signs of associated tissue damage due to infection when examined by direct microscopy, histopathology or cytopathology. To diagnose a "proven”

IFI by cultural methods a mould or black yeast has to grow in culture from a specimen, which was gained by a sterile procedure from a normally sterile body site. Also this site has to present clinically or radiologically abnormalities due to an infectious process. However, cultural growth from non-sterile fluids like bronchoalveolar lavage fluid, a cranial sinus cavity or urine are not considered of a "proven" IFI patient.
Blood culture can also be used for cultural methods and lead to the diagnosis of a "proven" IFI, however growth of Aspergillus spp. from blood culture has to be always considered as contamination. Serological methods are not applicable (47).

8.8 Statistical analysis

For statistical analysis, IBM SPSS Statistics (version 22; IBM Corp., Amrok, NY, USA) and R 3.2.0 (creatinine.r-project.org) were used.
Study patients were grouped according to EORTC criteria into the IA cohort (possible, probable and proven IA cases), which were then compared to the cohort of patients with no evidence for IA and to patients from the validation cohort.
Correlation between conventional GM ODI levels from serum and urine samples and GM-creatinine ratio levels was evaluated using Spearman-Rho correlation analysis. Cut-offs for conventional GM ODI and for the GM-creatinine ratio in urine samples were determined by Youdens Index (137). Median and interquartile ranges of conventional GM ODI levels from urine samples and GM-creatinine ratio from urine samples were displayed in boxplots, compared between IA cohort versus no IA cohort and between IA cohort versus validation cohort.
For cut-off determined in this study, diagnostic performance was evaluated by sensitivity, specificity, Negative Predictive Value (NPV) and Positive Predictive Value (PPV). Receiver operating characteristics (ROC) curves analyses were performed and areas under the curve (AUC) displayed. A p value < 0.05 was considered to be statistically significant.

9 Results

9.1 Pre-Test: Creatinine as a marker for urine dilution

Before including patients in this study we analyzed one urine sample highly positive for GM in order to investigate the relation between GM and creatinine concentrations in serial dilution.

The urine sample was diluted in five steps (1:10, 1:25, 1:50, 1:75 and 1:100), and GM and creatinine levels were determined at every dilution stage (Figure 20).

It was found that the two analytes showed proportional concentration reduction. In order to normalize urine dilution in biomarker diagnostics using creatinine, it is required that the biomarker (GM) and creatinine show proportional concentration changes (129). Thus, these results were a requirement for our study.

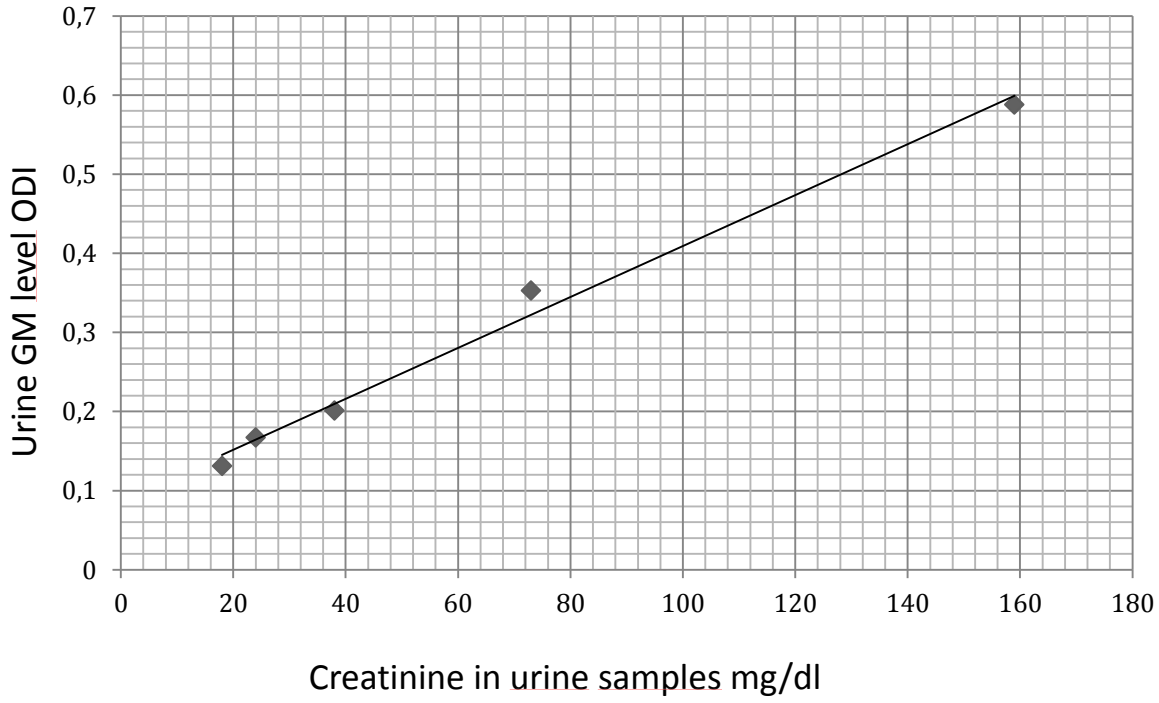


Figure 21, showing the concentration changes of urine galactomannan and urine creatinine in serial dilution. From Reischies et al,"Urine galactomannan-to-creatinine Ratio for Detection of Invasive Aspergillosis in Patients with Hematological Malignancies". *J Clin Microbiol,* License of reproduction provided by American Society for Microbiology

9.2 Demographical Data

A total of 71 patients at risk for IA were included in this study, 37 (52.1%) thereof were male and 34 (47.9%) were female, with a median age of 55 years (IQR: 46-62) Figure 21 shows the age distribution of study patients at time of sample collection. Patients were treated for the following diseases: 30/71 (42.3%) acute myeloid leukemia, 9/71 (12.7%) acute lymphocytic leukemia, 8 (11.3%) Non-Hodgkin lymphoma, 7 (9.8%) myelodysplastic syndrome, 7 (9.8%) multiple myeloma, and 10 (14.1%) other hematologic malignancies (Figure 22). 34 (47.9%) patients received allogeneic stem cell transplantation (SCT) before or during collection of study samples. 12 (16.9%) patients received autologous SCT and 25 (35.2%) study patients had not received SCT while the study was conducted. The majority of patients 61 of 71 (85.9%) received antifungal medication during the time of sample collection (Table 4).

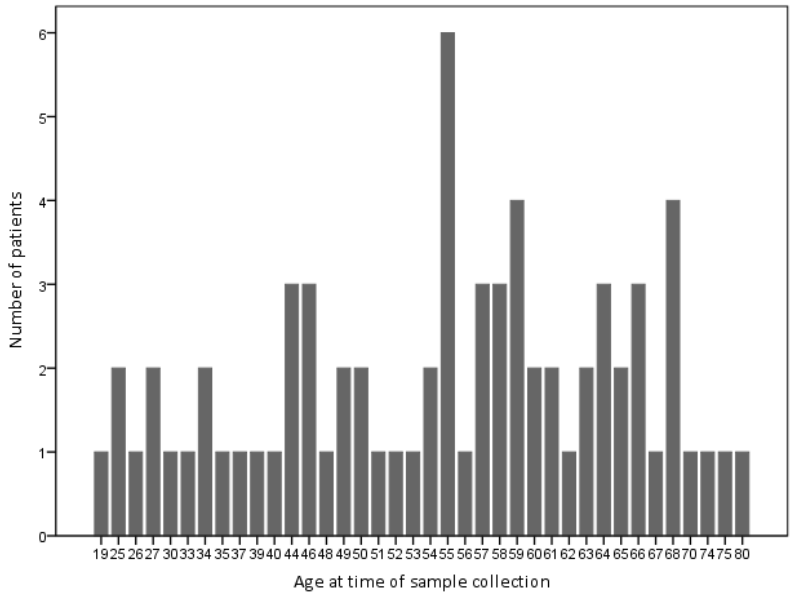


Figure 22, showing patients age distribution at time of sample collection.

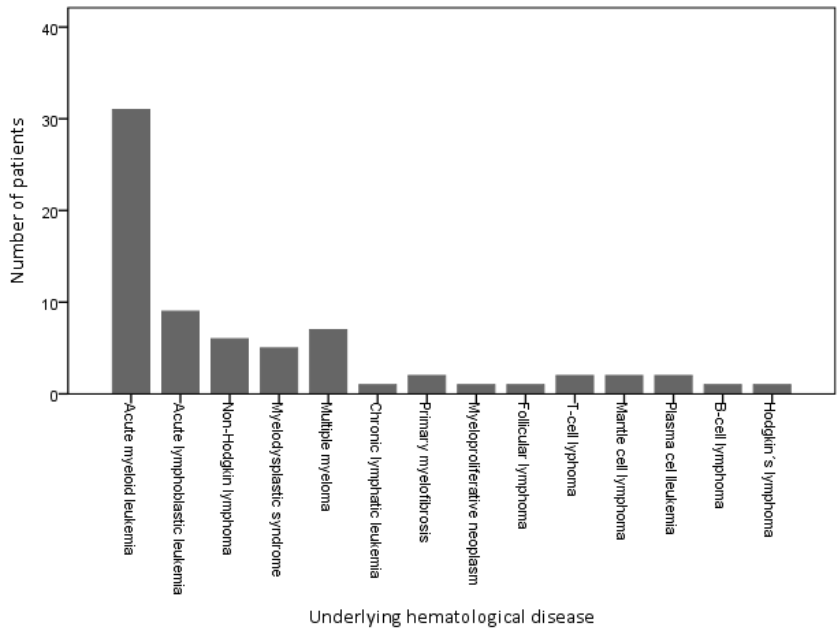


Figure 23, showing the distribution of underlying diseases of study patients.

Patients	
Total	71
Sex	
Female	34 (47.9%)
Male	37 (52.1%)
Age - median	55 (IQR: 46-62)
Underlying hematological disease	
Acute myeloid leukemia	30 (42.3%)
Acute lymphocytic leukemia	9 (12.7%)
Non-Hodgkin lymphoma	8 (11.3%)
Myelodysplastic syndrome	7 (9.8%)
Multiple myeloma	7 (9.8%)
Other hematological malignancies	10 (14.1%)
Stem cell transplantation recipients	
Allogeneic	34 (47.9%)
Autologous	12 (16.9%)

Table 4, showing demographical data of study patients.

9.3 EORTC classification of patients and samples

Of the 71 study patients 61 (85.9%) showed no evidence of IA and contributed 598 urine samples for analysis, four patients out of 71 (5.6%) had possible IA and contributed 15 urine samples, five patients out of 71 (7.0%) had probable IA and contributed 16 urine samples and one patient out of 71 (1.4%) had proven IA and contributed 3 urine samples. Figure 23 shows the distribution of study patients classified according to EORTC criteria. The latter 10 patients with possible, probable and proven IA were included in the IA cohort for further analysis. Additional 48 patients from an outpatient department, who came for routine urine testing, were included in the validation cohort and contributed 48 urine samples. Of the 680 urine samples analyzed for this study 598 came from patients with no evidence for IA, 34 came from the 10 patients included into the IA cohort and 48 samples came from patients in the validation cohort. Table 5 shows the distribution of patients and study samples classified according to EORTC criteria. 32 out of 34 (94%) samples from the IA cohort and 419 out of 598 urine samples in the no-IA cohort were collected from patients who were receiving mould-active antifungal prophylaxis or therapy

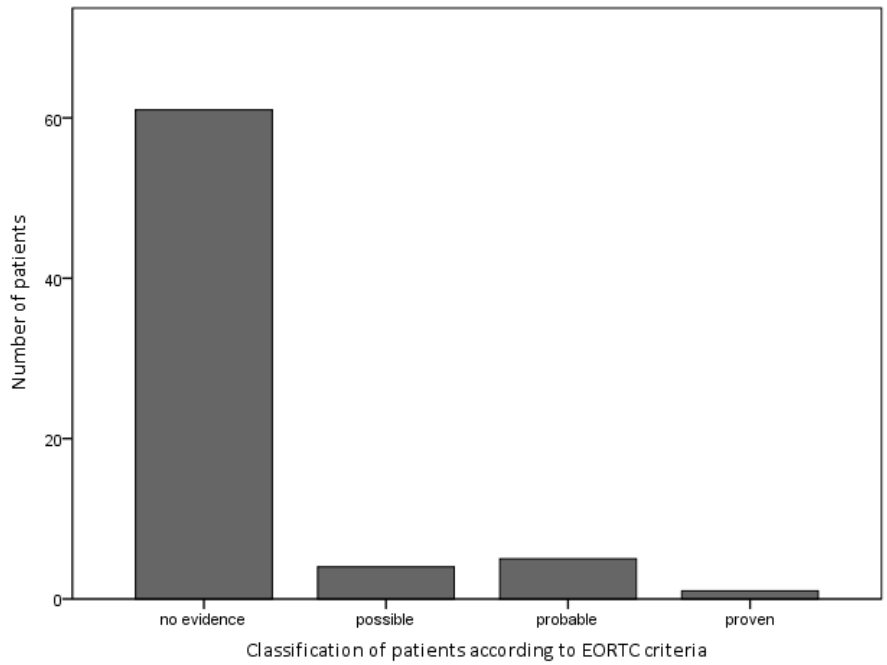


Figure 24, showing the classification of study patients according to EORTC criteria.

EORTC	Patients	Samples	Cohort
Total	71 (100%)	632	-
No evidence	61 (85.9%)	598	no IA
Possible	4 (5.6%)	15	IA
Probable	5 (7.0%)	16	
Proven	1 (1.4%)	3	
Validation Cohort	48	48	VC

Table 5, showing distribution of patients and samples classified according to EORTC criteria.

9.4 Diagnostic performance of the galactomannan-to-creatinine ratio

The diagnostic performance of the new galactomannan-to-creatinine ratio (GM/creatinine ratio) from urine samples was investigated by comparing patients with possible, probable and proven IA, all classified in the IA cohort, with patients without signs of invasive aspergillosis and patients from the validation cohort.

Median levels of the GM/creatinine ratio were compared with the median levels of the conventional galactomannan Optical Density Index (GM ODI) between these patient groups. Sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) were calculated for the GM/creatinine ratio at a cut-off at 0.1 and for conventional galactomannan ODI levels at 0.25.

Ideal cut-offs were determined by Youndes index and were 0.26 for the urine GM/creatinine ratio. Sensitivity, specificity, NPV and PPV, for the IA cohort versus the no IA cohort, per sample, were found to be: 79%; 70%; 98.4%;13%, respectively (137).

The diagnostic performance values for sensitivity and NPV increased when only probable and proven cases were included in the IA cohort, without possible cases, versus the no IA cohort: sensitivity, 84%; specificity, 70%; NPV, 99.3%; PPV, 8%.

9.4.1 Comparison between patients with signs for invasive aspergillosis and patients without signs for invasive aspergillosis

Median levels of urine galactomannan were compared with median levels of the calculated GM/creatinine ratio between the patient cohort without signs for invasive aspergillosis (no IA) and the patient cohort with possible, probable and proven invasive aspergillosis (IA).
Median galactomannan levels from urine samples were 0.11 (IQR: 0.08 to 0.16) in the patient cohort with invasive aspergillosis and 0.07 (IQR: 0.06 to 0.09) in the patient cohort without evidence for invasive aspergillosis, p value= <0.001.
Median GM/creatinine ratio from urine samples were 0.46 (IQR: 0.27 to 1.41) in the patient cohort with invasive aspergillosis and 0.17 (IQR: 0.09 to 0.30) in the patient cohort without evidence for invasive aspergillosis, p value= <0.001 (Table 6).
Difference of median values between the two patient cohorts were more distinct for GM/creatinine ratio values than for conventional GM ODI values.
Differences in medians of galactomannan and the GM/creatinine ratio are depicted in the boxplots figures (Figure 25 and Figure 26) (129).

	IA	IQR	no IA	IQR	p
Median GM ODI	0.11	0.08 - 0.16	0.07	0.06 - 0.09	**<0.001**
Median GM/crea ratio	0.46	0.27 - 1.41	0.17	0.09 - 0.30	**<0.001**

Table 6, comparing the GM ODI median values and the GM/creatinine ratio median values of the patients with IA and without IA

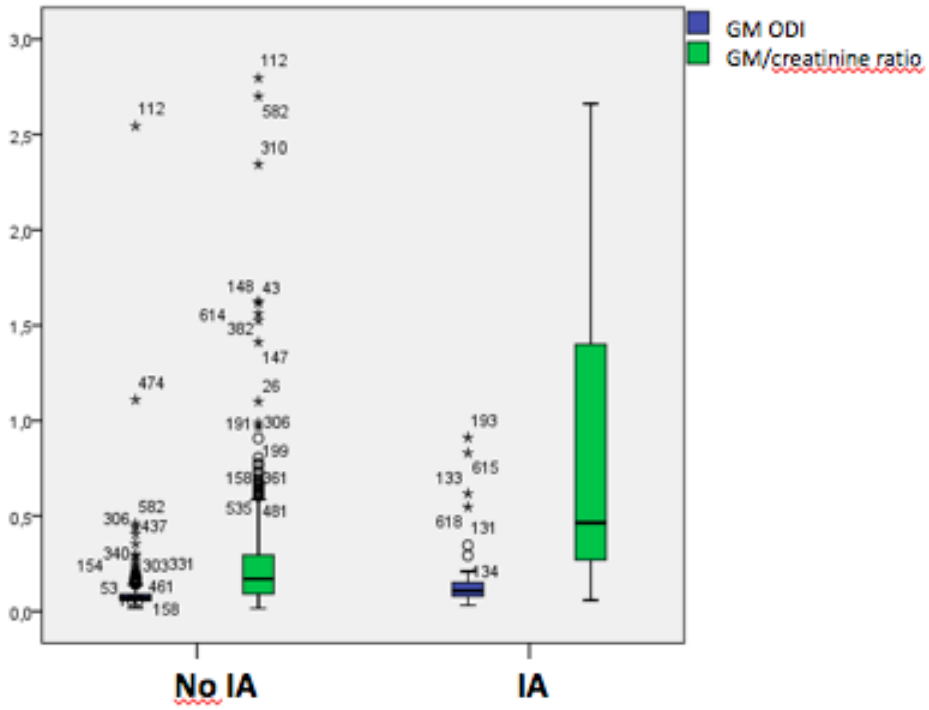


Figure 25, depicting the median values of the GM ODI (blue) and the GM/creatinine ratio (green) of the patients with IA and without IA. From Reischies et al,"Urine galactomannan-to-creatinine ratio for Detection of Invasive Aspergillosis in Patients with Hematological Malignancies". *J Clin Microbiol,* License of reproduction provided by American Society for Microbiology.

9.4.2 Comparison between patients with signs for invasive aspergillosis and patients from the validation cohort

Median levels of urine galactomannan were compared with median levels of the calculated GM/creatinine ratio between the patient cohort with signs for invasive aspergillosis (IA) and the validation cohort (VC).

Median galactomannan levels from urine samples were 0.11 (IQR: 0.08 to 0.16) in the IA patient cohort and 0.06 (IQR: 0.05 to 0.08) in the validation patient cohort, p value= <0.001.

Median GM/creatinine ratio from urine samples was 0.46 (IQR: 0.27 to 1.41) in the patient cohort with invasive aspergillosis and 0.07 (IQR: 0.04 to 0.14) in the validation patient cohort, p value= <0.001 (Table 7) (129).

Difference of median values between the two patient cohorts were more distinct for GM/creatinine ratios than for conventional GM ODI values.

	IA	IQR	Validation Cohort	IQR	p
Median GM ODI	0.11	0.08 - 0.16	0.06	0.05 - 0.08	**<0.001**
Median GM/crea ratio	0.46	0.27 - 1.41	0.07	0.04 - 0.14	**<0.001**

Table 7, comparing the GM ODI median values and the GM/creatinine ratio median values of the patients with IA and the validation cohort.

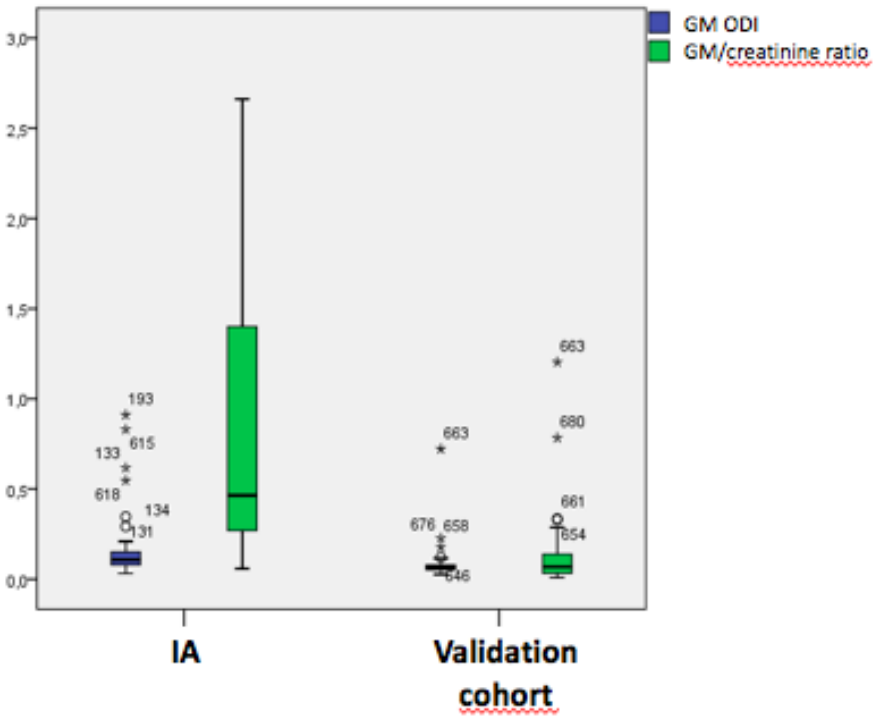


Figure 26, depicting the GM ODI median values (blue) and the GM/creatinine ratio median values (green) of the patients with IA and the validation cohort. From Reischies et al,"Urine Galactomannan-to-Creatinine Ratio for Detection of Invasive Aspergillosis in Patients with Hematological Malignancies". *J Clin Microbiol,* License of reproduction provided by American Society for Microbiology.

9.4.3 Correlation analysis between urine GM/creatinine ratio, urine GM ODI and serum GM ODI values.

Spearman-Rho correlation analysis was performed between GM/creatinine ratio values from urine and the conventional GM ODI values from urine and for conventional GM ODI from serum.

A strong correlation was detected between GM/creatinine ratio values and conventional GM ODI values from urine samples (r: 0.712, p<0.001)

However, weaker correlations were found between GM ODI values from serum and both urine GM/creatinine ratios (r = 0.157, P < 0.001) and conventional urine GM ODI values (r = 0.180, P < 0.001).

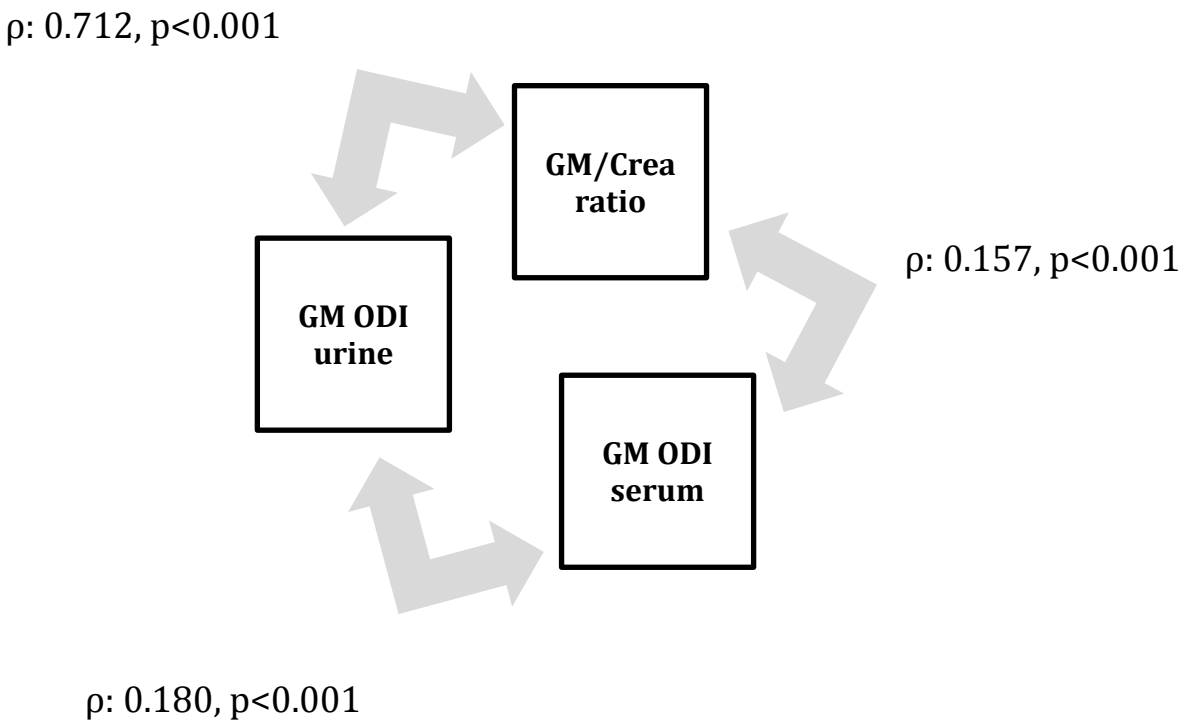


Figure 27, showing correlation values between urine GM/creatinine ratio, urine GM ODI and serum GM ODI values.

9.4.4 Diagnostic performance and area under the curve

The accuracy of the GM/creatinine ratio in differentiating IA (n = 34) samples from samples without IA (n = 598) and in differentiating IA samples (n =34) from samples from the validation cohort (n = 48) was tested with Receiver Operating Characteristic (ROC) curve analysis. Findings were compared to results of ROC curve analysis for the accuracy of the conventional GM ODI levels from urine in differentiating samples from different patient cohorts. ROC curve analysis was also performed for serum GM levels for differentiating IA samples from samples without IA.

When differentiating between IA samples and no IA samples area under the curve (AUC) values were found to be lower, for conventional urine GM ODI with a value of 0.746 (95% confidence interval (CI), 0.655 to 0.837), and for serum GM with a value of 0.749 (95% CI, 0.657 to 0.841), compared to the higher AUC value for the newly introduced urine GM/creatinine ratio with 0.801 (95% CI, 0.719 to 0.882; $P < 0.001$) (Figure 27).

Also in differentiating between IA samples and samples from the validation cohort AUC values were higher, at 0.913 (95% CI, 0.853 to 0.973; $P < 0.001$) for GM/creatinine ratio compared to AUC values of conventional urine GM ODI, at 0.792 (95% CI, 0.689 to 0.895; $P < 0.001$) (Figure 28) (129). Thus the GM/creatinine ratio was found to offer the best accuracy to differentiate between samples from the validation cohort and the IA cohort.

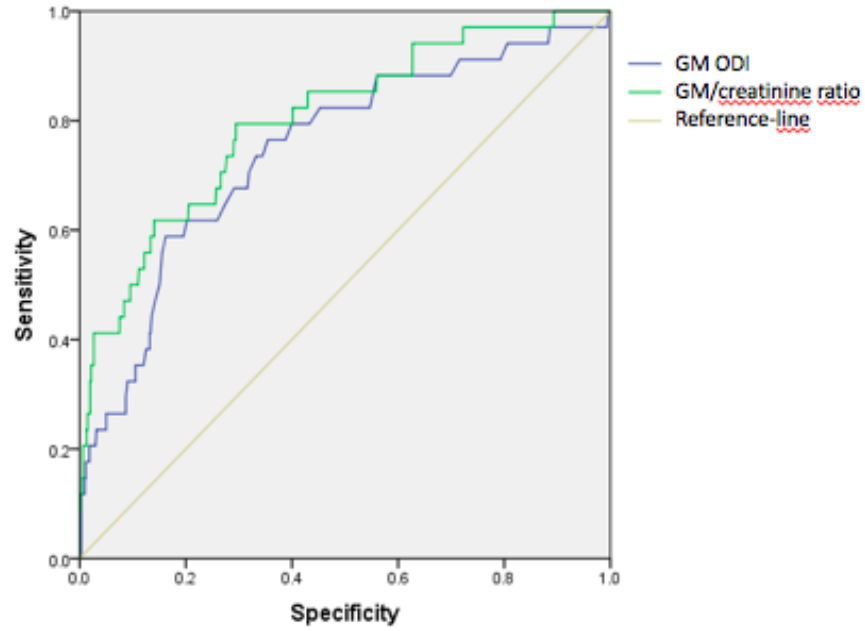


Figure 28, showing the ROC curves of the conventional GM ODI values and the GM/creatinine ratio in differentiating between samples of patients with IA and without IA. From Reischies et al,"Urine Galactomannan-to-Creatinine Ratio for Detection of Invasive Aspergillosis in Patients with Hematological Malignancies". *J Clin Microbiol,* License of reproduction provided by American Society for Microbiology.

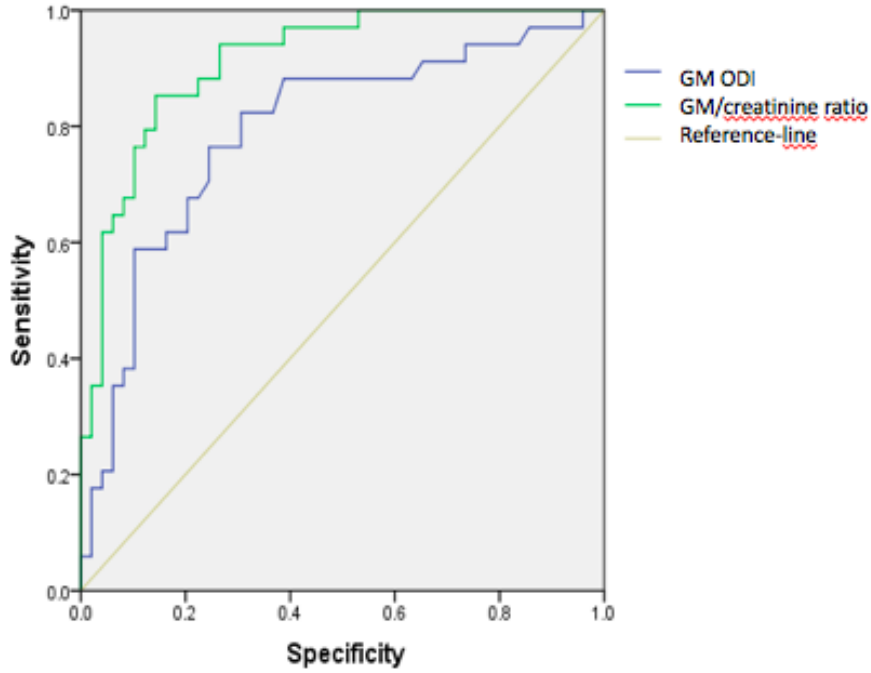


Figure 29, showing the ROC curves of the conventional GM ODI values and the GM/creatinine ratio in differentiating between samples of patients with IA and of the validation cohort. From Reischies et al,"Urine Galactomannan-to-Creatinine Ratio for Detection of Invasive Aspergillosis in Patients with Hematological Malignancies". *J Clin Microbiol,* License of reproduction provided by American Society for Microbiology.

9.5 Summary of results

We performed a prospective study on the diagnostic performance of GM detection from urine samples in patients with hematological malignancies. The urine dilution was taken into account by calculating the GM/creatinine ratio. We found the following:

- creatinine and GM concentrations decreased direct proportionally in serial dilution of urine.

- Conventional urine GM ODI values correlated significantly with GM/creatinine ratio values and GM ODI values from serum.

- Median values of the GM/creatinine ratio differed significantly between patients with IA (possible/probable/proven) and without IA and between patients with IA (possible/probable/proven) and the validation cohort.

- The optimal cut-off for the diagnostic performance of the GM/creatinine ratio was at 0.26, as calculated by the Youdens Index

- Per sample sensitivity, specificity, NPV and PPV were 79%, 70%, 98.4% and 13% for patients with IA (possible/probable/proven) vs patients with no evidence for IA and 84%; 70%; 99.3%; 8% for patients with IA (probable, proven) vs patients with no evidence for IA, respectively, at a 0.26 cut-off for the GM/creatinine ratio.

- AUC of the GM/creatinine ratio for differentiating patients with IA(possible/probable/proven) vs patients with no evidence for IA was 0.801 and for differentiating patients with IA (possible/probable/proven) vs patients from the validation cohort the AUC was 0.913.

Given the significant improvement of diagnostic performance of GM detection from urine samples in differentiating between IA patients from no IA patients when urine dilution is taken into account using the GM/creatinine ratio, the H_0 is rejected.

10 Discussion

This represents the first study to evaluate the confounding factor of urine dilution when testing GM from urine samples for diagnosis of IA (129). When biomarker detection methods for IA were first analyzed, biomarker detection from urine samples were commonly studied. Despite initial promising results on IA biomarker detection from urine samples (117) (111) subsequent studies focused on IA biomarker detection from serum samples and only GM detection from serum has been introduced to the 2008 revised EORTC diagnostic criteria for IA (47). In recent studies GM screening from serum for patients at risk for IA was criticized due to the poor diagnostic performance resulting from lower disease incidence in the presence of effective anti mould prophylaxis (100) (102). Despite the lower sensitivities, GM determination in serum and BAL remain the gold standard for biomarker diagnostic driven diagnosis of IA, because performance of all other available diagnostic tests are impacted by antimould prophylaxis as well (53). Improving the diagnostic performance of GM detection from urine samples might allow patients and physicians to benefit from advantages of urine testing. Urine sample collection compared to serum sample collection is noninvasive and reduces the risk of infection at the vein puncture site, which is important to consider for the often severely ill and mostly immunocompromised patients at risk for IA. Screening for GM from urine samples compared to screening from serum specimen, would allow for reduction of blood taking. Also anemia, resulting from frequent collection of blood samples, could be reduced by urine screening (138). Urine samples are also easier to collect compared to blood samples, which means that, once urine point-of-care (POC) tests for GM detection have been established, patients could collect samples themselves and test from home. Urine testing also allows for more frequent examination of large sample volumes, which may increase the sensitivity of GM detection. In contrast to blood sample collection, however, collection standards are often lacking for urine samples in biomarker diagnostic and can influence the test results. The least variability in urine protein concentration occurs in first morning urine, however, the longer time spend in the bladder was discussed to might lead to increased proteolysis (126). Influences of urine collection standards on GM detection were not described in any previous study and mostly morning urine samples were collected. Collection standards for urine samples would help to improve the comparability and repeatability of biomarker diagnostic studies from urine samples in the future.

The diagnostic performance of GM detection, using the Platelia® ELISA from human and animal urine samples was previously investigated by only five studies (115) (116) (120) (119) (121). Before the Platelia® ELISA was available GM detection from urine samples was first described in 1987 and in four subsequent studies, however methods of GM detection e.g. the latex agglutination test, were not comparable (112). When the Platelia® ELISA was introduced and commercially on the market, it was reported to provide a higher sensitivity for serum samples and was then also used in studies analyzing urine samples.

In 1995 Stynen et al. were the first to report on GM detection from urine samples using the Platelia® ELISA (115). They tested 222 centrifuged urine samples from immunocompromised patients without evidence for IA in order to study GM optical density (OD) background values in urine (and serum) samples to calculate a suitable cut-off value. Interestingly, they reported on much lower background GM OD levels in urine compared to serum samples. Results of the GM detection immunoassay were reported as obtained or were converted into galactose or GM concentrations as deduced from a calibration curve, different to more recent publications and our own study, in which conventional GM values from urine samples are given as an index (ODI), calculated with a negative controle value, which is given in each assay kit. In the 222 urine samples GM OD levels were between 0 and 0.3 with an average GM OD of 0.03. They calculated the cut-off for GM OD from urine by adding four standard deviations to the average OD value, resulting in 0.19. Urine samples were available from seven patients with possible or probable IA in that study. Thereof, GM OD was positive in urine samples of five patients, which results in a per patient sensitivity of 71%. GM OD urine levels were lower than comparable serum levels and GM was always earlier positive in serum than in urine samples. No information was given on antifungal medication of the patients during collection of study samples. Although specific values for sensitivity and specificity were not reported, the authors suggested serum to be the better specimen for GM detection given the low specificity in urine testing. False-positive results in 8% of urine samples (8 of 97 samples) were reported. Urine dilution was not taken into account (115).

Five years later, in 2000, Salonen et al. were the first to investigate the effect of urine concentration on the diagnostic performance of GM detection from urine samples (116). They prospectively investigated urine samples, from patients at risk for IA. Urine samples were centrifuged and tested twice, first directly after centrifugation and again after 10-fold

concentration by evaporation. The cut-off for urine samples was also determined by adding four standard deviations to the mean of GM OD values of 20 negative urine control samples which resulted in a cut-off of 0.3 OD, which was higher compared to the cut-off proposed by Stynen et al. in the study described above (i.e.,0.19 OD). Salonen et al. also calculated an optical density index (ODI) by preparing negative and positive control samples, the OD value of each urine sample was then divided by the mean of OD value of the 2 prepared negative and positive control samples. When all patients with proven, probable and suspected IA, were analyzed as cohort with IA, sensitivity per patient was: 7/21 (33%) for non-concentrated urine samples. However, sensitivity was improved considerably to 12/21 (57%) when concentrated urine samples were tested (116). This means that false negatives urine samples of five patients seemed to have been too diluted for conventional urine GM detection and were corrected by concentrating the urine samples. This correction possible could have also been obtained by calculation of the GM/creatinine ratio.

In the early 2000s these findings of Stynen et al. and Salonen et al. were discussed to be helpful to decide on when to start treatment with Amphotericin B in patients at risk for IA. In both studies no information is given on whether patients received antimould treatment at the time of sample collection. The EORTC diagnostic criteria used in our study were not available then. However, the certainty of diagnosis of IA was also already indicated by a scale of probability and similar to the EORTC diagnostic criteria patients were classified as suggested, probable and proven. Patients, which were classified as suggested, probable and proven in both studies would have also been classified into the IA cohort in our study (129). In both studies sensitivity and specificity values were given as per patient, as both groups often analyzed only one sample per patient, an important difference from our study, in which patients at risk were screened twice weekly and many consecutive samples were collected and analyzed (129). Thus, sensitivity and specificity values were given as per sample in our study (129) (115) (116). The Salonen study was the first to demonstrate that taking the urine dilution into account for GM detection, results in better diagnostic performance (116). However, it has to be considered that GM molecules might degenerate during the concentration process.

In the following decade no studies were published on GM detection from urine samples and this diagnostic approach has taken a back seat until Garcia et al. published an animal study in dogs on GM detection from urine and serum with promising results.

Published in 2012, Garcia et al. analyzed the diagnostic performance of GM detection from urine and serum samples in dogs (120). A very good performance of GM detection from urine samples was reported in this study. When interpreting the results, however, important differences regarding the study design have to be taken into consideration. Dogs were classified in three groups according to their diagnosis. Dogs with culture proven IA and localized pulmonary aspergillosis were classified into the „Asper +"group. From this group urine samples of nine dogs with culture proven systemic aspergillosis were available for analysis. This group was compared to the „Asper – like" group and the control group. The „Asper – like" group comprised dogs that were diagnosed with diseases with similar clinical presentations to IA, including other fungal infections (but no aspergillus infections). The control group consisted of dogs which were diagnosed with diseases, which showed no similar clinical presentation to systemic IA infection. However, dogs with the diagnosis of localized sinonasal Aspergillus infection were also included into the control group. GM results were given as GM ODI and cut-off was chosen at 0.5, 1.0 and 1.5.
They found, without taking urine dilution into account, significant differences when comparing the median GM ODI values from urine samples between the „Asper +"group and the other two groups. This finding is different to our results, as we found only minimal differences when comparing median GM ODI values from the IA cohort and the non-IA cohort (Table 6 and Figure 24) (129).
Per dog sensitivity for urine GM ODI was 7/8 (88%) with all samples showing GM ODI values >4.0 GM ODI. In the "Asper – like" group 3 out of 25 (12%) dogs had false positive urine samples. Dogs in the "Asper – like" group were treated due to paecilomycosis, bacterial pneumonia and penicilliosis. In the control group two of 34 (6%) dogs had false positive urine samples. The two dogs were treated for nailbed mycosis and gastric helicobacteriosis and inflammatroy bowel disease. Comparing the diagnostic performance of GM detection from serum and urine specimens in this study, better performance was found for serum specimens with a sensitivity of 92% compared to 88% sensitivity for urine specimen. However, higher overall specificity values were found for urine testing (92%) compared to serum testing (84%) (120).
After these promising animal-study results were published, Fisher et al. were the first to publish a study on GM detection from urine samples in humans in over a decade (119). Fisher et al. conducted a prospective, multicenter study in, in which 213 neutropenic, pediatric patients at risk for IA were enrolled for consecutive GM detection from serum, BAL and urine samples. Samples were tested twice and only consecutive positive samples were

considered positive (119). Urine concentration was not taken into consideration. The cut-off value for urine samples was defined at 0.5 GM ODI and 1.0 GM ODI. Despite the large patient cohort, however, the validity of the study was limited, due to the low incidence of IA cases. Of the 213 patients only one was retrospectively considered to have had "probable" IA, and 24 patients were classified as „possible" IA, according to EORTC criteria (47). Nevertheless, diagnostic performance of GM detection from urine samples was better compared to GM detection from serum, as the only probable IA case was detected by urine testing only and the serum sample of that patient remained negative. The urine sample tested positive for GM one week before chest CT detected pulmonary nodules. Thus, it was discussed in this study that GM detection from urine samples should be further investigated, especially the optimal approach for processing urine specimens. Also authors concluded that further research should be focused on decreasing the rate of false positive results while preserving the sensitivity. Fisher et al. found little difference in median values of GM ODI from urine samples when comparing patients classified as possible IA and patients without evidence for IA. These results are congruent with our findings, that differences in median GM ODI values from urine between the validation cohort or patients without evidence for IA and patients classified into the IA cohort were small (129). By calculating the GM/creatinine ratio we found, however, that the differences in mean values increased considerably between these cohorts (Figure 24 and 25, Table 6 and 7).
A large proportion of the pediatric patients (93.4%) analyzed in this study were receiving antimould prophylaxis, probably due to which the incidence of invasive aspergillosis was very low (1/213). As reported by Duarte et al. GM screening in these patient cohorts results in high rates of false positive results and a low positive predictive value (100).
We did not perform duplicate testing in our study, different to Fisher et al. who found that, when diagnostic performance was calculated using the first testing only, sensitivity, PPV, NPV remained stable with a moderate reduction in specificity (119). Thus, considering the higher costs, duplicate testing might only be considered to reduce high false positive rates but not to increase sensitivity.

In 2014 Duettmann et al. published a study, which was also conducted at the Medical University Hospital of Graz (121) and had a comparable study design to ours (129). Serum and urine samples were collected form hematological patients twice weekly and GM Platelia® ELISA assay was performed from these samples. Urine samples were analyzed without any further pretreatment, however, and as an important difference to our study urine

concentration was not taken into account and only half of the analyzed samples were collected during neutropenia. Different cut-off values were analyzed for urine testing at 0.2 ODI, 0.15 ODI and 0.1 ODI. Best diagnostic performance was found for a cut-off at 0.1 ODI. Diagnostic performance was also given as per sample, since multiple urine and serum samples were analyzed from each patient. 55% of the patients received antifungal prophylaxis. With six false negatives and 26 potentially false positive urine samples, sensitivity was 71.4%, specificity 88.2%, PPV 36.6%, NPV 97%. Different to our study Youdens index identified a very low optimal cut-off value of 0.1 ODI, which reflects the importance in urine testing to be able to detect very low concentrations of GM, to obtain the best diagnostic performance (137). However, high background GM OD levels have been reported for urine samples and thus choosing low cut-off values comes with the downside of risking a high rate of false positives, resulting in low specificity (115). The cut-off at 0.1, however, was chosen below the negative control value of most test kits (negative control values in our study were: median 0.179 (IQR: 0.147-0.219)) resulting in difficulties to interpret these results clinically. In our study calculating the GM/creatinine ratio made many urine samples, with detectable GM levels below the negative control value (median GM ODI in IA cohort: 0.10750, (IQR: 0.7900-0.15550)), clinically interpretable (median GM-creatinine Index in IA: 0.4641 (IQR: 0.2690-1.4111)) (129).

10.1 Interpreting our results relative to clinical care decisions.

Our results show improvement of the diagnostic performance of GM detection from urine samples when the GM/creatinine ratio was calculated. In particular, the excellent AUC value determined by ROC curve analysis, for differentiating IA samples from validation cohort samples, at 0.913 (95% CI, 0.853 to 0.973; $P < 0.001$) may argue for the superiority of the use of this ratio (129). The ROC curve analysis demonstrates several things: first it shows the trade-off between specificity and sensitivity; any increase in sensitivity will result in a decrease in specificity. An ideal diagnostic test would result in a curve, that follows the left hand border and then the top border of the diagram, thus a test with low diagnostic accuracy results in a curve that comes close to the 45° diagonal of the diagram. An excellent diagnostic test has a high value for the AUC, so a perfect test would result in AUC value of 1 and an AUC value of 0.5 represents a worthless test (Figure 27 and 28) (139).
However, similar to many other comparable studies we also found high rates of false positive results. The clinical consequence drawn from biomarker testing methods with a high rate of

false positives may result in a higher number of patients receiving unnecessary therapy. However, given the severity of IA, unnecessary therapy seems rather tolerable than having patients being treated too late or not at all when diagnosis is missed. The NPV of >98% of the GM/creatinine ratio may, however, be a useful measure for ruling out breakthrough IA and offers valuable support in deciding on de-escalation of antifungal therapy, thus reducing side effects and costs. The low PPV in our study of 13% reflects the problem of patient cohorts in which the disease prevalence is low, even if the test has good sensitivity and specificity values (102) (100).

10.2 Study design and detection methods influencing GM detection from urine samples

An explanation for positive GM results from urine samples, when serum samples remain negative, might be the very rare case of Aspergillus infection of the kidneys and Aspergillus urinary tract infection. It has been published that in patients with GM antigenuria, kidney involvement was proven by autopsy (114) (119). This is of special importance to consider when working with animal models of experimental systemic aspergillosis, in which conidia are often injected intravenously resulting in higher rates of fungal infection of the kidneys than when infection occurs over inhalation (112) (111).

Pretreatment of urine samples prior to GM testing was discussed in several studies. In the application of point-of-care tests (POC), urine pretreatment has been reported to be of use. For example, in the design of a lateral flow device test, which works over antibody-antigen interactions, a pretreatment (desalting or dialysis) removes possible inhibitors and seems to be of advantage for POC tests. However, urine sample pretreatment other than centrifugation and concentration was not analyzed for ELISA detection (116) (119) (118).
Given the fact that the GM molecule is very variable in size and composition (GM isolated from urine samples has been described to vary between <10-70kDA (111)), it has to be taken into consideration that the GM molecule is likely to be degraded during physiological kinetics or during analytical procedures. The immunocompetent galactofuranosyl residues are acid-sensitive. However, acidic removal of complexing antibodies is a necessary step during the ELISA assay (140). Degradation of a GM molecule in the bloodstream might lead to increased additional tubular renal filtration and therefore lead to higher urine GM levels than the GM blood level would let one expect and which would then be interpreted as "false positive". It has also been published, that the binding of the EB-A2 antibody requires at least four galactofuranosyl side chains epitopes of the GM molecule. So it might be arguable that

intensive degraded GM molecules are no longer detected by the Platelia ELISA and sensitivity then may be compromised (97). Future studies should focus on identifying optimal sample pretreatment for GM detection from urine samples.

11 Limitations

The main limitations of our study is the relatively small number of samples from probable and proven IA cases. The limited number of probable and proven IA patients, was also reported as a limitation by many other studies, analyzing the diagnostic performance of biomarker detection tests or prophylaxis and treatment of IA (53) (119) (121).

Many publications have discussed the reproducibility of GM detection from serum samples after storage. It has been reported that after storage of serum samples GM was no longer detectable (141) (142) (143) (144). The storage of urine samples within this study at -80°C might have also reduced initial positive GM samples. However, further studies need to evaluate the reproducibility of GM testing from urine samples, especially since the sample type seems to be of importance, as reproducibility of GM detection from BAL samples has been reported to be more reliable than from serum samples (145).

Finally, the proposed normalization of urine samples using the urine creatinine concentration might fail in conditions with elevated urine creatinine levels, such as rhabdomyolysis, high-meat diets and extensive exercise (146).

12 Conclusion

Our results show that diagnostic performance of GM detection from urine samples can be improved by taking urine dilution into account. The high negative PPV may help to reduce unnecessary empirically driven started antifungal medication in patients who do not have IA and would profit from a reduction of medication because of the side effects. Use of the proposed urine GM/creatinine ratio may be a simple reliable approach and warrants further investigations as a diagnostic tool for IA in patients with hematological malignancies.

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14 References

1. Hawksworth DL. The fungal dimension of biodiversity: Magnitude, significance and conservation. Mycol Res. 1991;95(6):641–55.
2. Olsen L, Choffnes ER, Relman DA, Pray L, Forum on Microbial Threats, Board on Global Health, et al. Fungal Diseases: an Emerging Threat to Human Animal and Wildlife Health: Workshop Summary. Washington (DC): The National Academy of Sciences Engineering and Medicine; 2011.
3. Fisher MC, Henk DA, Briggs CJ, Brownstein JS, Madoff LC, McCraw SL, et al. Emerging fungal threats to animal, plant and ecosystem health. Nature. 2012 Apr 11;484(7393):186–94.
4. Frick, CREATININE.F., et al.,. An Emerging Disease Causes Regional Population Collapse of a Common North American Bat Species. Sci 2010 329 5992 P 679 - 682.
5. Blehert, D. S.et al. Bat white-nose syndrome: an emerging fungal pathogen? Sci 323 227 2009.
6. Casadevall, A. Determinants of virulence in the pathogenic fungi. Fungal Biology Reviews 21:130–132.
7. Casadevall A. Fungi and the Rise of Mammals. PLOS Pathog. 2012 Aug 16;8(8):e1002808.
8. Istitute of Medicine. Microbial Threats to Health: Emergence, Detection, and Response. Washington DC: The National Academies Press; 2003.
9. Raftery AE, Zimmer A, Frierson DMW, Startz R, Liu P. Less than 2 °C warming by 2100 unlikely. Nat Clim Change. 2017 Jul 31;
10. Cutler SJ. Refugee crisis and re-emergence of forgotten infections in Europe. Clin Microbiol Infect. 2016 Jan;22(1):8–9.
11. Galanis E, MacDougall L, Kidd S, Morshed M. Epidemiology of Cryptococcus gattii, British Columbia, Canada, 1999–2007. Emerg Infect Dis. 2010;(16(2)):251–7.
12. McNeil MM, Nash SL, Hajjeh RA, Phelan MA, Conn LA, Plikaytis BD, et al. Trends in Mortality Due to Invasive Mycotic Diseases in the United States, 1980–1997. Clin Infect Dis. 2001 Sep;33(5):641–7.
13. Martin GS, Mannino DM, Eaton S, Moss M. The Epidemiology of Sepsis in the United States from 1979 through 2000. N Engl J Med. 2003 Apr 17;348(16):1546–54.
14. Vallabhaneni S, Benedict K, Derado G, Mody RK. Trends in Hospitalizations Related to Invasive Aspergillosis and Mucormycosis in the United States, 2000–2013. Open Forum Infect Dis. 2017 Jan 1;4(1).
15. Ben-Ami R, Lewis RE, Kontoyiannis DP. Enemy of the (immunosuppressed) state: an update on the pathogenesis of Aspergillus fumigatus infection: Review. Br J Haematol. 2010 Jul 7;406–17.
16. Nauseef WM. How human neutrophils kill and degrade microbes: an integrated view. Immunol Rev. 2007 Oct;219(1):88–102.
17. Phadke, Mehrad B. Cytokines in host defense against Aspergillus: recent advances. Med Mycol. 2005;(May 43):Suppl 1:S173-6.
18. Breen JO. Skin and Soft Tissue Infections in Immunocompetent Patients. Am Fam Physician. 2010 Apr 1;81(7):893–9.
19. Erwig LP, Gow NAR. Interactions of fungal pathogens with phagocytes. Nat Rev Microbiol. 2016 Feb 8;14(3):163–76.
20. Brown GD, Meintjes G, Kolls JK, Gray C, Horsnell CREATININE. AIDS-related mycoses: the way forward. Trends Microbiol. 2014 Mar;22(3):107–9.
21. Gregg KS, Kauffman CA. Invasive Aspergillosis: Epidemiology, Clinical Aspects,

and Treatment. Semin Respir Crit Care Med. 2015 Oct;36(5):662–72.
22. Lortholary O, Gangneux J-P, Sitbon K, Lebeau B, de Monbrison F, Le Strat CREATININE, et al. Epidemiological trends in invasive aspergillosis in France: the SAIF network (2005–2007). Clin Microbiol Infect. 2011 Dec;17(12):1882–9.
23. Pappas PG, Alexander BD, Andes DR, Hadley S, Kauffman CA, Freifeld A, et al. Invasive Fungal Infections among Organ Transplant Recipients: Results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin Infect Dis. 2010 Apr 15;50(8):1101–11.
24. Caira M, Candoni A, Verga L, Busca A, Delia M, Nosari A, et al. Pre-chemotherapy risk factors for invasive fungal diseases: prospective analysis of 1,192 patients with newly diagnosed acute myeloid leukemia (SEIFEM 2010-a multicenter study). Haematologica. 2015 Feb 1;100(2):284–92.
25. Virchow R. Beiträge zur Lehre von den beim Menschen vorkommenden pflanzlichen Parasiten. Archiv für pathologische Anatomie und Physiologie und für klinische Medizin. Archiv für pathologische Anatomie und Physiologie und für klinische Medizin.; 1856.
26. Kozakiewicz Z, Smith D. Physiology of Aspergillus. In 1994. (Biotechnology handbooks - 7: Aspergillus.).
27. David M. Geiser. Sexual structures in Aspergillus : morphology, importance and genomics. 2009;Medical Mycology(47):S21–6.
28. O'Gorman CM, Fuller HT, Dyer PS. Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus. Nature. 2009 Jan 22;457(7228):471–4.
29. Ashu EE, Hagen F, Chowdhary A, Meis JF, Xu J. Global Population Genetic Analysis of *Aspergillus fumigatus*. Mitchell AP, editor. mSphere. 2017 Feb 22;2(1):e00019-17.
30. de Vries RP, Visser J. Aspergillus Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. Microbiol Mol Biol Rev. 2001 Dec 1;65(4):497–522.
31. Dagenais TRT, Keller NP. Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis. Clin Microbiol Rev. 2009 Jul;22(3):447–65.
32. Brakhage AA, Langfelder K. Menacing mold: the molecular biology of Aspergillus fumigatus. Annu Rev Microbiol. 2002;(56):433–55.
33. Denning DW, Cadranel J, Beigelman-Aubry C, Ader F, Chakrabarti A, Blot S, et al. Chronic pulmonary aspergillosis: rationale and clinical guidelines for diagnosis and management. Eur Respir J. 2016 Jan;47(1):45–68.
34. Roy PM, Khanna S, Mehta CREATININE, Khan AZ. Aspergilloma of the Lung: Strategy to Prevent Endobronchial Spillage. Innov Phila Pa. 2016 Oct;11(5):373–5.
35. Grosjean P, Weber R. Fungus balls of the paranasal sinuses: a review. Eur Arch Oto-Rhino-Laryngol Off J Eur Fed Oto-Rhino-Laryngol Soc EUFOS Affil Ger Soc Oto-Rhino-Laryngol - Head Neck Surg. 2007 May;264(5):461–70.
36. Reddy A, Greenberger PA. Allergic Bronchopulmonary Aspergillosis. J Allergy Clin Immunol Pract. 2017 Jun;5(3):866–7.
37. Agarwal R, Chakrabarti A, Shah A, Gupta D, Meis JF, Guleria R, et al. Allergic bronchopulmonary aspergillosis: review of literature and proposal of new diagnostic and classification criteria. Clin Exp Allergy. 2013 Aug;43(8):850–73.
38. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr. 2004;(80):1106–22.
39. Stevens DA, Moss RB, Kurup VP, Knutsen AP, Greenberger P, Judson MA, et al. Allergic Bronchopulmonary Aspergillosis in Cystic Fibrosis—State of the Art: Cystic Fibrosis Foundation Consensus Conference. Clin Infect Dis. 2003 Oct;37(s3):S225–64.
40. Tatara AM, Mikos AG, Kontoyiannis DP. Factors affecting patient outcome in primary cutaneous aspergillosis: Medicine (Baltimore). 2016 Jun;95(26):e3747.
41. Morrison BE, Park SJ, Mooney JM, Mehrad B. Chemokine-mediated recruitment of

NK cells is a critical host defense mechanism in invasive aspergillosis. J Clin Invest. 2003 Dec 15;112(12):1862–70.
42. Bhatia S, Fei M, Yarlagadda M, Qi Z, Akira S, Saijo S, et al. Rapid Host Defense against Aspergillus fumigatus Involves Alveolar Macrophages with a Predominance of Alternatively Activated Phenotype. PLOS ONE. 2011 May 1;6(1):e15943.
43. Heinekamp T, Schmidt CREATININE, Lapp K, Pähtz V, Shopova I, Köster-Eiserfunke N, et al. Interference of Aspergillus fumigatus with the immune response. Semin Immunopathol. 2015;37(2):141–52.
44. Stanzani M, Orciuolo E, Lewis R, Kontoyiannias D, Martins S, St John L. Aspergillus fumigatus suppresses the human cellular immune response via gliotoxin-mediated apoptosis of monocytes. Blood. 2005 Mar 15;105(6):2258–65.
45. Perfect JR, Hachem R, Wingard JR. Update on Epidemiology of and Preventive Strategies for Invasive Fungal Infections in Cancer Patients. Clin Infect Dis. 2014 Nov 15;59(suppl_5):S352–5.
46. Abers MS, Ghebremichael MS, Timmons AK, Warren HS, Poznansky MC, Vyas JM. A Critical Reappraisal of Prolonged Neutropenia as a Risk Factor for Invasive Pulmonary Aspergillosis. Open Forum Infect Dis. 2016 Feb 12;3(1).
47. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, et al. Revised Definitions of Invasive Fungal Disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis. 2008 Jun 15;46(12):1813–21.
48. Hidron AI, Gongora MC, Anderson AML, DiazGranados CA. Prolonged survival of a patient with AIDS and central nervous system aspergillosis. Med Mycol. 2009 Jan;47(3):327–30.
49. Barton RC. Laboratory Diagnosis of Invasive Aspergillosis: From Diagnosis to Prediction of Outcome. Scientifica. 2013;2013:1–29.
50. Segal BH, Romani LR. Invasive aspergillosis in chronic granulomatous disease. Med Mycol. 2009;47 Suppl 1:S282-290.
51. Taccone FS, Van den Abeele A-M, Bulpa P, Misset B, Meersseman CREATININE, Cardoso T, et al. Epidemiology of invasive aspergillosis in critically ill patients: clinical presentation, underlying conditions, and outcomes. Crit Care. 2015;19(1).
52. Groll AH, Tragiannidis A. Recent advances in antifungal prevention and treatment. Semin Hematol. 2009 Jul;46(3):212–29.
53. Patterson TF, Thompson GR, Denning DW, Fishman JA, Hadley S, Herbrecht R, et al. Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America. Clin Infect Dis. 2016 Aug 15;63(4):e1–60.
54. Tissot F, Agrawal S, Pagano L, Petrikkos G, Groll AH, Skiada A, et al. ECIL-6 guidelines for the treatment of invasive candidiasis, aspergillosis and mucormycosis in leukemia and hematopoietic stem cell transplant patients. Haematologica. 2017 Mar;102(3):433–44.
55. Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann J-CREATININE, et al. Voriconazole versus Amphotericin B for Primary Therapy of Invasive Aspergillosis. N Engl J Med. 2002 Aug 8;347(6):408–15.
56. Anderson TM, Clay MC, Cioffi AG, Diaz KA, Hisao GS, Tuttle MD, et al. Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol. 2014 Mar 30;10(5):400–6.
57. Herbrecht R, Patterson TF, Slavin MA, Marchetti O, Maertens J, Johnson EM, et al. Application of the 2008 Definitions for Invasive Fungal Diseases to the Trial Comparing Voriconazole Versus Amphotericin B for Therapy of Invasive Aspergillosis: A Collaborative Study of the Mycoses Study Group (MSG 05) and the European Organization for Research

and Treatment of Cancer Infectious Diseases Group. Clin Infect Dis. 2015 Mar 1;60(5):713–20.
58. Cornely OA, Maertens J, Bresnik M, Ebrahimi R, Ullmann AJ, Bouza E, et al. Liposomal Amphotericin B as Initial Therapy for Invasive Mold Infection: A Randomized Trial Comparing a High-Loading Dose Regimen with Standard Dosing (AmBiLoad Trial). Clin Infect Dis. 2007 May 15;44(10):1289–97.
59. Cornely OA, Maertens J, Bresnik M, Ebrahimi R, Dellow E, Herbrecht R, et al. Efficacy outcomes in a randomised trial of liposomal amphotericin B based on revised EORTC/MSG 2008 definitions of invasive mould disease: Re-categorising AmBiLoad results. Mycoses. 2011 Sep;54(5):e449–55.
60. Marr KA, Schlamm HT, Herbrecht R, Rottinghaus ST, Bow EJ, Cornely OA, et al. Combination Antifungal Therapy for Invasive Aspergillosis: A Randomized Trial. Ann Intern Med. 2015 Jan 20;162(2):81.
61. Maertens JA, Raad II, Marr KA, Patterson TF, Kontoyiannis DP, Cornely OA, et al. Isavuconazole versus voriconazole for primary treatment of invasive mould disease caused by Aspergillus and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial. The Lancet. 2016 Feb;387(10020):760–9.
62. Marr KA. Empirical Antifungal Therapy — New Options, New Tradeoffs. N Engl J Med. 2002 Jan 24;346(4):278–80.
63. Morrissey CO, Chen SC-A, Sorrell TC, Milliken S, Bardy PG, Bradstock KF, et al. Galactomannan and PCR versus culture and histology for directing use of antifungal treatment for invasive aspergillosis in high-risk haematology patients: a randomised controlled trial. Lancet Infect Dis. 2013 Jun;13(6):519–28.
64. Reischies F, Hoenigl M. The role of surgical debridement in different clinical manifestations of invasive aspergillosis. Mycoses. 2014 Sep;57 Suppl 2:1–14.
65. Cornely OA, Maertens J, Winston DJ, Perfect J, Ullmann AJ, Walsh TJ, et al. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. N Engl J Med. 2007 Jan 25;356(4):348–59.
66. Balajee SA, Marr K. Phenotypic and genotypic identification of human pathogenic aspergilli. Future Microbiol. 2006 Dec;1(4):435–45.
67. Ascioglu S, Rex JH, Pauw B de, Bennett JE, Bille J, Crokaert F, et al. Defining Opportunistic Invasive Fungal Infections in Immunocompromised Patients with Cancer and Hematopoietic Stem Cell Transplants: An International Consensus. Clin Infect Dis. 2002 Jan 1;34(1):7–14.
68. Horger M, Hebart CREATININE, Einsele CREATININE, Lengerke C, Claussen CD, Vonthein R, et al. Initial CT manifestations of invasive pulmonary aspergillosis in 45 non-HIV immunocompromised patients: association with patient outcome? Eur J Radiol. 2005 Sep;55(3):437–44.
69. Greene RE, Schlamm HT, Oestmann J-CREATININE, Stark P, Durand C, Lortholary O, et al. Imaging findings in acute invasive pulmonary aspergillosis: clinical significance of the halo sign. Clin Infect Dis Off Publ Infect Dis Soc Am. 2007 Feb 1;44(3):373–9.
70. Gotway M, Dawn S, Caoili E, Reddy G, Araoz P, Webb CREATININE. The radiologic spectrum of pulmonary Aspergillus infections. - PubMed - NCBI. J Comput Assist Tomogr. 2002;(26(2)):159–73.
71. Andreas S, Heindl S, Wattky C, Moller K, Ruchel R. Diagnosis of pulmonary aspergillosis using optical brighteners. Eur Respir J. 2000 Feb 1;15(2):407–11.
72. Lee S, Yun NR, Kim K-CREATININE, Jeon JH, Kim E-C, Chung DH, et al. Discrepancy between histology and culture in filamentous fungal infections. Med Mycol. 2010 Sep;48(6):886–8.
73. Guarner J, Brandt ME. Histopathologic Diagnosis of Fungal Infections in the 21st Century. Clin Microbiol Rev. 2011 Apr;24(2):247–80.

74. Cuenca-Estrella M, Bassetti M, Lass-Flörl C, Rácil Z, Richardson M, Rogers TR. Detection and investigation of invasive mould disease. J Antimicrob Chemother. 2011 Jan;66 Suppl 1:i15-24.
75. Neofytos D, Horn D, Anaissie E, Steinbach CREATININE, Olyaei A, Fishman J, et al. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry. Clin Infect Dis Off Publ Infect Dis Soc Am. 2009 Feb 1;48(3):265–73.
76. Kontoyiannis DP, Marr KA, Park BJ, Alexander BD, Anaissie EJ, Walsh TJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin Infect Dis Off Publ Infect Dis Soc Am. 2010 Apr 15;50(8):1091–100.
77. McClenny N. Laboratory detection and identification of Aspergillus species by microscopic observation and culture: the traditional approach. Med Mycol. 2005 May;43 Suppl 1:S125-128.
78. Horvath J, Dummer S. The use of respiratory-tract cultures in the diagnosis of invasive pulmonary aspergillosis. Am J Med. 1996 Feb;100(2):171–8.
79. Prattes J, Heldt S, Eigl S, Hoenigl M. Point of Care Testing for the Diagnosis of Fungal Infections: Are We There Yet? Curr Fungal Infect Rep. 2016 Jun;10(2):43–50.
80. McCarthy MW, Petraitiene R, Walsh TJ. Translational Development and Application of (1→3)-β-d-Glucan for Diagnosis and Therapeutic Monitoring of Invasive Mycoses. Int J Mol Sci. 2017 May 24;18(6):1124.
81. Miceli MH, Maertens J. Role of Non-Culture-Based Tests, with an Emphasis on Galactomannan Testing for the Diagnosis of Invasive Aspergillosis. Semin Respir Crit Care Med. 2015 Oct;36(5):650–61.
82. Pickering JW, Sant CREATININE, Bowles CAP, Roberts WL, Woods GL. Evaluation of a (1→3)-β-d-Glucan Assay for Diagnosis of Invasive Fungal Infections. J Clin Microbiol. 2005 Dec;43(12):5957.
83. Reischies FMJ, Prattes J, Woelfler A, Eigl S, Hoenigl M. Diagnostic performance of 1,3-beta-D-glucan serum screening in patients receiving hematopoietic stem cell transplantation. Transpl Infect Dis. 2016 Jun;18(3):466–70.
84. Reischies FMJ, Prattes J, Pr?ller F, Eigl S, List A, CREATININE?lfler A, et al. Prognostic potential of 1,3-beta-d-glucan levels in bronchoalveolar lavage fluid samples. J Infect. 2016 Jan;72(1):29–35.
85. Raggam RB, Fischbach LML, Prattes J, Duettmann CREATININE, Eigl S, Reischies F, et al. Detection of (1→3)-β-D-glucan in same-day urine and serum samples obtained from patients with haematological malignancies. Mycoses. 2015 Jul;58(7):394–8.
86. Karageorgopoulos D, Vouloumanou E, Ntziora F, Michalopoulos A, Rafailidis P, Falagas M. β-D-glucan assay for the diagnosis of invasive fungal infections: a meta-analysis. - PubMed - NCBI. Clin Infect Dis. 2011;(15;52(6)):750–70.
87. Lamoth F, Cruciani M, Mengoli C, Castagnola E, Lortholary O, Richardson M, et al. Beta-Glucan Antigenemia Assay for the Diagnosis of Invasive Fungal Infections in Patients With Hematological Malignancies: A Systematic Review and Meta-Analysis of Cohort Studies From the Third European Conference on Infections in Leukemia (ECIL-3). Clin Infect Dis. 2012 Mar 1;54(5):633–43.
88. Marty FM, Koo S. Role of (1,3)-beta-D-glucan in the diagnosis of invasive aspergillosis. Med Mycol. 2009 Jan;47(s1):S233–40.
89. Prattes J, Raggam RB, Vanstraelen K, Rabensteiner J, Hoegenauer C, Krause R, et al. Chemotherapy-Induced Intestinal Mucosal Barrier Damage: a Cause of Falsely Elevated Serum 1,3-Beta-d-Glucan Levels? Warnock DW, editor. J Clin Microbiol. 2016 Mar;54(3):798–801.

90. Prattes J, Schneditz D, Prueller F, Jaindl E, Sauseng N, Hoenigl M, et al. 1,3-beta-d-Glucan testing is highly specific in patients undergoing dialysis treatment. J Infect. 2017 Jan;74(1):72–80.
91. Sulahian A, Porcher R, Bergeron A, Touratier S, Raffoux E, Menotti J, et al. Use and Limits of (1-3)- -D-Glucan Assay (Fungitell), Compared to Galactomannan Determination (Platelia Aspergillus), for Diagnosis of Invasive Aspergillosis. J Clin Microbiol. 2014 Jul 1;52(7):2328–33.
92. Latgé JP, Kobayashi CREATININE, Debeaupuis JP, Diaquin M, Sarfati J, Wieruszeski JM, et al. Chemical and immunological characterization of the extracellular galactomannan of Aspergillus fumigatus. Infect Immun. 1994 Dec;62(12):5424–33.
93. Verweij PE, Rijs AJ, Pauw BED, Horrevorts AM, Hoogkamp-Korstanje JA, Meis JF. Clinical evaluation and reproducibility of the Pastorex Aspergillus antigen latex agglutination test for diagnosing invasive aspergillosis. J Clin Pathol. 1995 May;48(5):474.
94. Maertens J, Theunissen K, Verbeken E, Lagrou K, Verhaegen J, Boogaerts M, et al. Prospective clinical evaluation of lower cut-offs for galactomannan detection in adult neutropenic cancer patients and haematological stem cell transplant recipients. Br J Haematol. 2004 Sep;126(6):852–60.
95. Marr KA, Balajee SA, McLaughlin L, Tabouret M, Bentsen C, Walsh TJ. Detection of Galactomannan Antigenemia by Enzyme Immunoassay for the Diagnosis of Invasive Aspergillosis: Variables That Affect Performance. J Infect Dis. 2004 Aug;190(3):641–9.
96. Pfeiffer CD, Fine JP, Safdar N. Diagnosis of Invasive Aspergillosis Using a Galactomannan Assay: A Meta-Analysis. Clin Infect Dis. 2006 May 15;42(10):1417–727.
97. Hope CREATININE, Walsh T, Denning D. Laboratory diagnosis of invasive aspergillosis. Lancet Infect Dis. 2005 Oct;5(10):609–22.
98. Maertens J, Verhaegen J, Lagrou K, Eldere JV, Boogaerts M. Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. Blood. 2001 Mar 15;97(6):1604–10.
99. Sulahian A, Boutboul F, Ribaud P, Leblanc T, Lacroix C, Derouin F. Value of antigen detection using an enzyme immunoassay in the diagnosis and prediction of invasive aspergillosis in two adult and pediatric hematology units during a 4-year prospective study. Cancer. 2001;(15;91(2)):311–8.
100. Duarte RF, Sanchez-Ortega I, Cuesta I, Arnan M, Patino B, Fernandez de Sevilla A, et al. Serum Galactomannan-Based Early Detection of Invasive Aspergillosis in Hematology Patients Receiving Effective Antimold Prophylaxis. Clin Infect Dis. 2014 Dec 15;59(12):1696–702.
101. Cornely OA. Editorial Commentary: Galactomannan Testing During Mold-Active Prophylaxis. Clin Infect Dis Off Publ Infect Dis Soc Am. 2014 Dec 15;59(12):1703.
102. Marr KA, Laverdiere M, Gugel A, Leisenring CREATININE. Antifungal Therapy Decreases Sensitivity of theAspergillus Galactomannan Enzyme Immunoassay. Clin Infect Dis. 2005 Jun 15;40(12):1762–9.
103. Miceli M, Grazziutti M, Woods G, Zhao CREATININE, Kocoglu M, Barlogie B, et al. Strong Correlation between Serum Aspergillus Galactomannan Index and Outcome of Aspergillosis in Patients with Hematological Cancer: Clinical and Research Implications. Clin Infect Dis. 2008 May;46(9):1412–22.
104. Koo S, Bryar JM, Baden LR, Marty FM. Prognostic Features of Galactomannan Antigenemia in Galactomannan-Positive Invasive Aspergillosis. J Clin Microbiol. 2010 Apr 1;48(4):1255–60.
105. Mattei D, Rapezzi D, Mordini N, Cuda F, Lo Nigro C, Musso M, et al. False-Positive Aspergillus Galactomannan Enzyme-Linked Immunosorbent Assay Results In Vivo during Amoxicillin-Clavulanic Acid Treatment. J Clin Microbiol. 2004 Nov 1;42(11):5362–3.

106. Asano-Mori CREATININE, Kanda CREATININE, Oshima K, Kako S, Shinohara A, Nakasone CREATININE, et al. False-positive Aspergillus galactomannan antigenaemia after haematopoietic stem cell transplantation. J Antimicrob Chemother. 2007 Dec 19;61(2):411–6.
107. Tortorano AM, Esposto MC, Prigitano A, Grancini A, Ossi C, Cavanna C, et al. Cross-Reactivity of Fusarium spp. in the Aspergillus Galactomannan Enzyme-Linked Immunosorbent Assay. J Clin Microbiol. 2012 Mar 1;50(3):1051–3.
108. Huang CREATININE-T, Hung C-C, Hsueh P-R. Aspergillus galactomannan antigenemia in penicilliosis marneffei: AIDS. 2007 Sep;21(14):1990–1.
109. Wheat LJ, Hackett E, Durkin M, Connolly P, Petraitiene R, Walsh TJ, et al. Histoplasmosis-Associated Cross-Reactivity in the BioRad Platelia Aspergillus Enzyme Immunoassay. Clin Vaccine Immunol. 2007 May 1;14(5):638–40.
110. Lehmann PF, Reiss E. Invasive aspergillosis: antiserum for circulating antigen produced after immunization with serum from infected rabbits. Infect Immun. 1978 May;20(2):570.
111. Dupont B, Huber M, Kim SJ, Bennett JE. Galactomannan antigenemia and antigenuria in aspergillosis: studies in patients and experimentally infected rabbits. J Infect Dis. 1987 Jan;155(1):1–11.
112. Bennett J, Friedmann M, Dupont B. Receptor-mediated clearance of Aspergillus galactomannan. J Infect Dis. 1987;(155(5)):1005–10.
113. Haynes KA, Latge JP, Rogers TR. Detection of Aspergillus antigens associated with invasive infection. J Clin Microbiol. 1990 Sep;28(9):2040.
17. Ansorg R. CREATININE von HE Rath PM. Aspergillus antigenuria compared to antigenemia in bone marrow transplant recipients. -
115. Stynen D, Goris A, Sarfati J, Latgé JP. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J Clin Microbiol. 1995 Feb;33(2):497.
116. Salonen J, Lehtonen O, Teräsjärvi M, Nikoskelainen J. Aspergillus antigen in serum, urine and bronchoalveolar lavage specimens of neutropenic patients in relation to clinical outcome. Scand J Infect Dis. 2000;(32(5)):485–90.
117. Klont RR, Mennink-Kersten MASH, Verweij PE. Utility of Aspergillus Antigen Detection in Specimens Other than Serum Specimens. Clin Infect Dis. 2004 Nov 15;39(10):1467–74.
118. Dufresne SF, Datta K, Li X, Dadachova E, Staab JF, Patterson TF, et al. Detection of Urinary Excreted Fungal Galactomannan-like Antigens for Diagnosis of Invasive Aspergillosis. Litvintseva AP, editor. PLoS ONE. 2012 Aug 10;7(8):e42736.
119. Fisher BT, Zaoutis TE, Park JR, Bleakley M, Englund JA, Kane C, et al. Galactomannan Antigen Testing for Diagnosis of Invasive Aspergillosis in Pediatric Hematology Patients. J Pediatr Infect Dis Soc. 2012 Jun;1(2):103.
120. Garcia RS, Wheat LJ, Cook AK, Kirsch EJ, Sykes JE. Sensitivity and Specificity of a Blood and Urine Galactomannan Antigen Assay for Diagnosis of Systemic Aspergillosis in Dogs. J Vet Intern Med. 2012 Jul 1;26(4):911–9.
121. Duettmann CREATININE, Koidl C, Troppan K, Seeber K, Buzina CREATININE, Wölfler A, et al. Serum and urine galactomannan testing for screening in patients with hematological malignancies. Med Mycol. 2014 Aug;52(6):647–52.
122. Stiegel MA et al. Kidney injury biomarkers and urinary creatinine variability in nominally healthy adults. - PubMed - NCBI [Internet]. [cited 2017 Mar 20]. Available from: Biomarkers. 2015;20(6-7):436-52. doi: 10.3109/1354750X.2015.1094136
123. Waikar SS, Sabbisetti VS, Bonventre JV. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. Kidney Int. 2010 Sep;78(5):486.
124. Tang KWA, Toh QC, Teo BW. Normalisation of urinary biomarkers to creatinine for clinical practice and research – when and why. Singapore Med J. 2015 Jan;56(1):7.

125. Rigas M, Okino M, Quackenboss J. Use of a pharmacokinetic model to assess chlorpyrifos exposure and dose in children, based on urinary biomarker measurements. Toxicol Sci. 2001;(61(2)):374–81.
126. Thomas CE, Sexton CREATININE, Benson K, Sutphen R, Koomen J. Urine Collection and Processing for Protein Biomarker Discovery and Quantification. Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol. 2010 Apr;19(4):953–9.
127. Reid CN, Stevenson M, Abogunrin F, Ruddock MW, Emmert-Streib F, Lamont JV, et al. Standardization of Diagnostic Biomarker Concentrations in Urine: The Hematuria Caveat. PLoS ONE. 2012;7(12).
128. Barr DB, Wilder LC, Caudill SP, Gonzalez AJ, Needham LL, Pirkle JL. Urinary Creatinine Concentrations in the U.S. Population: Implications for Urinary Biologic Monitoring Measurements. Environ Health Perspect. 2005 Feb;113(2):192.
129. Reischies FMJ, Raggam RB, Prattes J, Krause R, Eigl S, List A, et al. Urine Galactomannan-to-Creatinine Ratio for Detection of Invasive Aspergillosis in Patients with Hematological Malignancies. Warnock DW, editor. J Clin Microbiol. 2016 Mar;54(3):771–4.
130. BioRad. PLATELIA Aspergillus. Instruction for use.Cat no. 62794. Marnes--Coquette FranceBio-RAd. 2013;
131. Stynen D, Sarfati J, Goris A, Prévost MC, Lesourd M, Kamphuis CREATININE, et al. Rat monoclonal antibodies against Aspergillus galactomannan. Infect Immun. 1992 Jun;(60(6)):2237–45.
132. Pruller F, Wagner J, Raggam RB, Hoenigl M, Kessler CREATININE, Truschnig-Wilders M, et al. Automation of serum (1->3)-beta-D-glucan testing allows reliable and rapid discrimination of patients with and without candidemia. Med Mycol. 2014 Jul 1;52(5):455–61.
133. Küme T, Sağlam B, Ergon C, Sisman AR. Evaluation and comparison of Abbott Jaffe and enzymatic creatinine methods: Could the old method meet the new requirements? J Clin Lab Anal. 2017 Feb;e22168.
134. Delanghe JR, Speeckaert MM. Creatinine determination according to Jaffe—what does it stand for? NDT Plus. 2011 Apr;4(2):83–6.
135. Prasad A, Agarwal K, Deepak D, Atwal S. Pulmonary Aspergillosis: What CT can Offer Before it is too Late! - PubMed - NCBI. J Clin Diagn Res. 2016;(10(4)):TE01-5.
136. Borlenghi E, Cattaneo C, Capucci MA, Pan A, Quaresmini G, Franco F, et al. Usefulness of the MSG/IFICG/EORTC diagnostic criteria of invasive pulmonary aspergillosis in the clinical management of patients with acute leukaemia developing pulmonary infiltrates. Ann Hematol. 2007 Mar;86(3):205–10.
137. Youden WJ. Index for rating diagnostic tests. Cancer. 1950;3(1):32–5.
138. Thavendiranathan P, Bagai A, Ebidia A, Detsky AS, Choudhry NK. Do Blood Tests Cause Anemia in Hospitalized Patients? J Gen Intern Med. 2005 Jun;20(6):520–4.
139. Kester ADM, Buntinx F. Meta-analysis of ROC Curves. Med Decis Making. 2000 Oct;20(4):430–9.
140. Mennink-Kersten MASH, Donnelly JP, Verweij PE. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. Lancet Infect Dis. 2004 Jun;4(6):349–57.
141. Oren I, Avidor I, Sprecher CREATININE. Lack of intra-laboratory reproducibility in using Platelia Aspergillus enzyme immunoassay test for detection of Aspergillus galactomannan antigen: Letter to the Editor. Transpl Infect Dis. 2012 Feb;14(1):107–9.
142. Furfaro E, Mikulska M, Miletich F, Viscoli C. Galactomannan: testing the same sample twice? Transpl Infect Dis. 2012 Aug;14(4):E38–9.
143. Johnson G, Sarker S-J, Hill K, Tsitsikas D, Morin A, Bustin S, et al. Significant Decline in Galactomannan Signal during Storage of Clinical Serum Samples. Int J Mol Sci.

2013 Jun 24;14(7):12970–7.
144. Dufresne SF, Beauchemin S, Lavallee C, Laverdiere M. Instability of Aspergillus Galactomannan in Stored Clinical Samples. J Clin Microbiol. 2014 Dec 1;52(12):4435–6.
145. Wheat LJ, Nguyen MH, Alexander BD, Denning D, Caliendo AM, Lyon GM, et al. Long-Term Stability at -20 C of Aspergillus Galactomannan in Serum and Bronchoalveolar Lavage Specimens. J Clin Microbiol. 2014 Jun 1;52(6):2108–11.
146. Torres PA, Helmstetter JA, Kaye AM, Kaye AD. Rhabdomyolysis: Pathogenesis, Diagnosis, and Treatment. Ochsner J. 2015 Spring;15(1):58.