Diploma Thesis

Parallel detection of (1→3)-β-D-Glucan in corresponding same day urine and serum samples obtained from patients with hematological malignancies

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1 Abbreviations

BDG = 1,3-beta-D-glucan
IFI = invasive fungal infections
IA = invasive aspergillosis
HSCT = hematopoietic stem cell transplant
GM = galactomannan
GIT = gastro intestinal tract
ICU = intensive care units
PAMPs = pathogen associated molecular patterns
PRRs = pattern recognition receptors
TLRs = toll-like receptors
CLRs = c-type lectin receptors
DCs = dendritic cells
GvHD = graft versus host disease
IFD = invasive fungal disease
CSF = cerebrospinal fluid
MALDI-TOF = matrix-assisted laser desorption ionization-time of flight mass spectrometry
PCR = polymerase-chain-reaction
MRI = magnetic resonance imaging
CNS = central nervous system
FGD = 18F-fluorodeoxyglucose
PET = positron emission tomography
LDF = lateral-flow device
HR-CT = high resolution computer tomography
IPA = invasive pulmonal aspergillosis
PPV = positive predictive value
NPV = negative predictive value
DOR = diagnostic odds ratio
ROC = receiver-operating-characteristic-curve
AUC = area under the curve
CI = confidence interval
IQR = interquartile range
2 Abstract

2.1 German

_Hintergrund:_ Der BDG Test für Serum ist ein diagnostischer Marker für invasive Pilzinfektionen, welcher für Patienten mit hämatoonkologischen Erkrankungen eingesetzt wird. Jedoch ist die aktuelle Datenlage für die Testung von BDG aus Urin limitiert.

_Methoden:_ Serum- und Urinproben wurden jeweils am selben Tag, zweimal pro Woche, gleichzeitig gewonnen. Insgesamt wurden 241 Urinproben von 60 Patienten mit dem BDG Fungitell® Assay getestet. Zusätzlich, wurde eine Urin BDG/Kreatinin Ratio eingesetzt, um den Bias durch die Urinverdünnung zu verringern.

_Resultate:_ 19 der Proben hatten positive, korrespondierende Serum BDG (>200 pg/ml), 33 intermediäre (80-200 pg/ml), und 189 negative Serum BDG (<80 pg/ml) Werte. Sensitivität, Spezifität, positiver prädiktiver Wert, negativer prädiktiver Wert (bezogen auf den IFI Status des Patienten) ergaben: 32%,84%, 50%,70% für den Cut-off von 80 pg/ml und 20%, 95%, 67%, 70% für den Cut-off von 200 pg/ml. Die Urin BDG/Kreatinin Ratio erhöhte die Sensitivität auf 47-81% ohne signifikanten Verlust der Spezifität (49%-70%). In der Gruppe mit BDG Serum Werten > 200 pg/ml zeigte sich eine bessere Korrelation (BDG Urin: r=0.453; p=0.051; Urin BDG/Kreatinin Ratio: r=0.722; p=0.000), verglichen mit den Gruppen mit intermediären und negativen BDG Serum Werten.

_Fazit:_ Insgesamt zeigte sich eine gute Korrelation in allen Gruppen, die Verwendung der Urin BDG/Kreatinine Ratio führte zur Verbesserung der Korrelation und der klinische Performance.
2.2 English

Background: Serum 1,3-beta-D-glucan (BDG) testing is an diagnostic marker for invasive fungal infections (IFI) applied for patients with hematological malignancies. Though for the application of BDG urine testing, currently limited data exists.

Methods: Serum and urine samples were collected on the same day, twice a week in parallel, from adult patients with underlying hematological malignancies. A total of 241 urine samples from 60 patients were investigated with the BDG Fungitell® assay. In addition, a BDG/Creatinine ratio to exclude urine dilution bias was introduced.

Results: 19 had positive corresponding serum BDG (>200 pg/ml), 33 intermediate (80-200 pg/ml), and 189 negative serum BDG (<80 pg/ml). Sensitivity, specificity, positive predictive value and negative predictive value (according to the patients state of IFI) were: 32%, 84%, 50%, 70% when using an 80 pg/ml urine cut-off, and 20%, 95%, 67%, 70% using a 200 pg/ml cut-off. With the urine BDG/Creatinine ratio, the results showed higher sensitivities ranging from 47-81% without significant loss of specificity, which ranged from 49% to 70%. Correlation was superior in the groups >200 pg/ml (BDG urine: r=0.453; p=0.051; urine BDG/Creatinine ratio: r=0.722; p=0.000) compared to the negative and intermediate ranges.

Conclusion: Overall correlation of same-day urine BDG and serum BDG was moderate. In general correlation and the clinical performance was superior when the urine BDG/Creatinine ratio was applied, therefore urine BDG testing in urine could be improved by introducing a BDG/Creatinine ratio.
3 Introduction

Within the last twenty years the incidence of invasive fungal infections (IFI) has increased, which is also a consequence of the higher complexity of the health care system (1). Especially hematological patients represent the most frequent patient group for IFI (2) with Candida spp. as the most common fungi causing infections, followed by the Aspergillus spp. In contrast to the hematopoietic stem cell transplant (HSCT) recipients in which the invasive Aspergillosis (IA) is the most frequent fungal infection (59.2 %) (3).

The gold standards for diagnosing invasive candidiasis or deep-seated candidiasis are blood cultures or sterile collected cultures of infected tissues, however there are some critical disadvantages including the long turnaround time up to 8 days for a positive culture and a determination of the fungal species (4). These difficulties in the diagnostic process consequently lead to an overtreatment of patients in risk for IFI, since time is precious leading to the next problem the high toxicity and costs of antifungal therapy (5).

Therefore in the last years finding a more effective way to diagnose IFIs using non-cultural diagnostic methods has gained importance. One of these new diagnostic features are 1,3-beta-D-glucan (BDG) and the galactomannan (GM) immunoassay, which is used for the diagnosis of invasive IA, and recent studies have shown that those tests to be helpful for the early diagnosis of IFI (6, 7) and led to the implementation of serum BDG testing in patients with high risk of fungal infections in recent clinical guidelines (8, 9).

Data for testing of BDG in urine are scarce although it may bear some advantages compared to serum testing like the frequent, easy and noninvasive specimen collection, which may increase sensitivity (10, 11). The study by Raggam et al, with a small amount of urine samples (n=80) tested, showed a moderate correlation to the serum samples (11) therefore our aim was to evaluate BDG testing in urine in a larger patient cohort and to compare results obtained from BDG urine testing with BDG obtained from serum testing. Additionally the establishment of a clinically relevant cut-off value for BDG in urine was of interest together with elimination of the bias due to urine-creatinine excretion.
3.1 Candida spp.

3.1.1 Morphology

The *Candida* spp. consists of a stable cell wall with up to 80-90% of highly complex carbohydrates and proteins. The outer layer is mainly built of O- and N-linked mannose polymers. The internal cell wall mainly consists of skeletal polysaccharides like chitin and BDG (12, 13). The cell wall composition depends on the form of the fungi. Only 2 % chitin is present in yeast cells, while the amount in hyphal cells is 2-5 times higher. The BDG content of the inner layer of yeast and hyphal cells is similar (20-40%) but the distribution is different, hence the yeast cells expose more BDG on the cell surface than the hyphal cells (13).

3.1.2 Epidemiology

*C. albicans* can colonize the healthy oral, vaginal and gastrointestinal mucosa and the skin. *C. albicans* is a commensal organism and about 80 % of all humans are colonized, especially the gastrointestinal tract (GIT) (12, 14). *Candida* spp. is the most frequent cause of invasive fungal infections (IFI). The estimated incidence amounts 2.7 cases per 100.000 habitants in Austria (15). In year 2014, 178 proven candidemias were detected in participating centers of the AURES report. At least 23% of them occurred at the Medical University of Graz. Referring to the AURES report of 2014 *C. albicans* caused 58 % of all candidemias, followed by *C. glabrata* 23 %, *C. parapsilosis* 6%, *C. tropicalis* 4%, *C. krusei* 0.6% *C. dubliniensis* 1%, and *C. orthopsilosis* 1% (16). The most invasive infections caused by *Candida* spp. occur at Intensive Care Units (ICU) (29%), followed by internal departments (25%), hematological-oncological departments (5%) and surgical departments (22%) and other departments (8%). 2013 an increase at hematological-oncological departments could be observed to (16%), from 2007-2012 the rate was only 1-8% (16).

3.1.3 Pathogenesis

3.1.3.1 Detection by the innate immune system

The first contact of the fungal cell wall is the innate immune system, which includes antimicrobial peptides, the complement system and immune cells. The cell wall components of the fungi, pathogen associated molecular patterns
(PAMPs) are detected by pattern recognition receptors (PRRs) which are present on the surface of innate immune cells like phagocytes. PRRs identify microbial patterns like carbohydrates (β-glucans), glycoproteins (mannans), nucleic acids, glycolipids, and lipoproteins (17, 18).

There are three major classes of PRRs, which play a role in the immune response to Candida spp. The first group consists of the Toll-like receptors (TLRs). They are most present on mononuclear phagocytes. The binding of microbial molecules leads to the production of pro-inflammatory cytokines like TNF alpha and IL-12, and cross-talk with adaptive immune cells for antigen presentation. Important for the immune response to C. albicans are TLR2, TLR4 and TLR9 (13, 18).

The next important group are C-type lectin receptors (CLRs) which are present on dendritic cells (DCs), further on macrophages and neutrophils (19). Dectin-1 recognizes BDG ligand uptake and is responsible for phagocytosis, pro-inflammatory cytokine production and the cooperation with TLRs. Dectin-2 helps maturing inflammatory monocytes and recognizes mannose rich structures; additionally it induces TNF α production in response to C. albicans (13, 18).

3.1.3.2 Colonization vs. Invasion

Submucosal neutrophil granulocytes, macrophages, DC and T-helper cells are essential for controlling the fungal pathogens. The most important virulence factor is the changing into the hyphae form and elongating hyphae. Active penetration by thigmotropism is responsible for the invasion into the intercellular space and the blood system (12, 20).

Assumingly there is a threshold for the amount of fungal pathogen, which can be controlled and immunotolerated by the host. When the threshold is reached, thus the host may get infected, therefore the immune system of the host must be able to discriminate between the commensal colonization and the pathogenic invasion state of C. albicans (20). Hyphae predominate at the primary site of infiltration of epithelial cell layers and tissues, whereas yeast cells are generally found either in the epithelial surface or emerging from penetrating hyphae that are infiltrating tissues.
3.1.3.3 Endocytosis

Knowingly the mucosal surfaces of healthy individuals can be colonized with C. albicans. By a small number of yeast cells no epithelial cell damage is induced. The point of invasion is reached when C. albicans steps over the epithelial cell border by inducing endocytosis. Yeast and hyphae forms are able to induce endocytosis, however hyphae are superior in sensing endocytosis (13, 19, 20).

3.1.3.4 Direct penetration

Likewise both forms are able to penetrate the tissue directly. The hyphae form more than the yeast form (13). Invasive growing is regulated by secretorial aspartyl proteinases and phospholipid in the GIT and at the skin (12). The gut is one of the frequent ways by which Candida enters the bloodstream, they invade through translocation or after large abdominal surgery. Other ways are any kind of intravascular catheters, mainly central-venous types. The colonization leads to the building of a biofilm and the fungus is spread from the biofilm in the blood system. The resulting candidemia can lead to secondary located infections in the lung, liver, spleen and kidneys. Further the fungus can lead to candiduria by entering the urinary tract. In most of the cases the infections stays localized at the urinary tract or leads to secondary candidemia (21, 22).

3.1.4 Risk factors

An increased risk for ICI can be found in patients with solid or hematological neoplasm and neutropenia, human immunodeficiency virus (HIV)-infection, diabetes mellitus, burn injuries, critical ill patients and patients under intensive care (23). T cell suppressive therapy, recent allogeneic stem cell transplantation (SCT), prolonged neutropenia (0.5x10^9 neutrophils/L [<500 neutrophils/mm3] for >10 days) and prolonged corticosteroid use is additionally associated with development of invasive mold infections and/or IFI. Other factors may be indwelling catheters, chronic graft versus host disease (GvHD), total body irradiation and the resulting GIT toxicity, bacteremia, use of broad spectrum antibiotics and infection with the cytomegalovirus (24).
3.2 Aspergillus spp.

3.2.1 Morphology
The cell wall of A. fumigatus is constructed of polysaccharides. There are two groups of polysaccharides alkali insoluble polymers which represent the fibrillar core of the cell wall and the second group consists of alkali-insoluble polymers which are responsible for linking the fibrils (25). The fibrillar core of the cell wall is composed of a branched β1,3 glucan (4% of β(1,6) branch points) to which chitin/chitosan, β(1,3)/β(1,4)-glucan and β(1,5) galacto-α(1-2)/α(1-6) mannan (GM) are covalently bound. The alkali-soluble amorphous fraction is mainly composed of linear chains of α(1-3)-glucans with intra-chain α(1-4) linked glucose units every hundred α(1-3)-linked glucose units, galactomannan and galactose-amino-galactan (GAG). The cell wall core structure is a branched β(1,3)-glucan bound to chitin through a β(1,4)linkage is common to the vast majority of fungal cell walls whereas the composition of the alkali-soluble fraction is most often class-specific or genus-specific (26).

3.2.2 Epidemiology
After Candida spp., Aspergillus spp. is the most common cause for IFI. Especially in immunocompromised patients, IA is an important cause of morbidity and mortality. An epidemiology study of the PATH Alliance registry investigated the epidemiology of IA. Hematological malignancies (48.3%) are the most frequent underlying diseases followed by solid organ transplant (29.2%) and HSCT (27.9%). Identified isolates included A. fumigatus (72.6%), A. flavus (9.9%), A. niger (8.7%) and A. terreus (4.3%). The lung was the most frequently affected organ. However 10 % of the patients had multiple infections (3). In Austria the AURES report 2014 showed, that from 204 tested specimens 74% were tested positive for Aspergillus spp., especially A. fumigatus with 79% (16).

3.2.3 Pathogenesis
Aspergillus spp. is found airborne and easily enters the human body by inhalation. Less common is the way through the GIT or the skin. In healthy human the fungi does not cause infection. Immunocompromised patients with neutropenia caused by chemotherapy, underlying hematological disease or immunosuppression to
treat GvHD, allows the fungi to get invasive. The life cycle of *A. fumigatus* starts with conidia, and when reaching the alveoli, the conidia can germinate and transform into the hyphae form. In this form the fungi can invade the parenchyma (27).

3.2.4 Detection by the innate immune system
Mainly alveolar macrophages recognizes conidia by specific PRRs identifying the fungi and trigger the immune response (28). In the recognition of *A. fumigatus* there are three PRRs known which are important.

TLR 2 and 4 are associated with the triggering of a pro-inflammatory response through *A. fumigatus*. Both conidia and hyphae of *A. fumigatus* trigger cytokine production in macrophages with the help of TLR2. Additionally conidia are able to stimulate macrophages by TLR4. TLR3 and TLR9 are intracellular TLRs, epithelial cells manage their protection against *A. fumigatus* through TLR3 and TLR9, by recognizing fungal RNA and DNA (28). C-type leptin receptors are expressed at human airway epithelium, DCs and the gut. The extracellular part of CLRs has carbohydrate recognition domains. Dectin-1 recognizes BDG and plays a part in the identification of *A. fumigatus* and furthermore leads to pro-inflammatory cytokine production through the activation of TLR2. Moreover the Mannose receptor and Dectin-2 may play a role in the inflammatory response to *A. fumigatus* (28). TLRs and Dectin-1 help the host cells to differentiate between resting conidia, germinating conidia and the hyphae of *A. fumigatus* (17).

3.2.5 Risk factors
Generally all factors, which weaken the immune system, are to be considered as risk factors. For example, hematological malignancies, allogenic hematopoietic stem-cell transplantation, immunosuppressive therapy after solid-organ transplantation, advanced AIDS.

3.3 Definitions of Invasive of Fungal Disease (IFD)
These definitions were developed from the Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperation Group and the National Institute of Allergy and Infectious Disease Mycoses Study Group
(EORTC/MSG) Consensus Group, to simplify the process of shaping uniform groups of patients for clinical and epidemiologic research in the field of IFI. Host factors include the risk and clinical-pathological features. These criteria are designed for clinical research but also can be used in clinical practice. Especially in patients with three or more host factors present and/or a combination of prolonged neutropenia and T cell suppressants clinicians should be aware (29).

After the revised definitions of the (EORTC/MSG) Consensus Group there are three classifications: proven, probable and possible invasive fungal infection, every group includes special criteria. The criteria are defined as follows bellow (30).

3.3.1 Host Factors for Risk of IFD
Host Factors are characteristics by which individuals are predisposed to IFI. Following host factors were defined by the EORTC/MSG Consensus group:

- Recent history of neutropenia (0.5x10⁹ neutrophils/L [<500 neutrophils/mm³] for >10 days) temporally related to the onset of fungal disease
- Receipt of an allogenic stem cell transplant
- Prolonged use of corticosteroids (excluding among patients with allergic bronchopulmonary aspergillosis) at a minimum dose of 0.3 mg/kg/day of prednisone equivalent for > 3 weeks
- Treatment with other recognized T cell immunosuppressants such as cyclosporine, TNF-α blockers, specific monoclonal antibodies, or nucleoside analogues during the past 90 days
- Inherited severe immunodeficiency (such as chronic granulomatous disease or severe combined immunodeficiency)

3.3.1.1 Categories of IFD
The EORTC/MSG Consensus group defined three steps, which indicate the possibility to the diagnosis of IFI. The following criteria for proven, probable or possible IFD are borrowed from the Revised Definitions of IFD the EORTC/MSG Consensus Group.
3.3.1.2 Proven IFD

To achieve the level of proven IFI the fungus must be detected by microscopic analysis, cultures of sterile material, blood cultures or serological analysis for the cryptococcal antigen in cerebrospinal fluid (CSF).

Microscopic analysis includes histopathologic, cytopathologic or direct microscopic examination. For culture from sterile material the specimen must be collected from a sterile site and a clinically or radiologically abnormal site in case of molds. For yeast the specimens should be obtained from a normally sterile site showing a clinically or radiological abnormality compatible with an infectious event. Blood cultures showing growth of a mold or yeasts fulfill the criteria of proven IFD. Only in case of disseminated cryptococcosis a serological analysis is permitted for defining proven IFD.

3.3.1.3 Probable IFI

The status of probable IFD needs the presence of a host factor, a clinical criterion, and a mycological criterion.

I. Host Factors
   See 2.3.1

II. Clinical criteria
   - Lower respiratory tract fungal disease
     - The presence of 1 of the following 3 signs on CT:
       - Dense, well-circumscribed lesions(s) with or without a halo sign
       - Air-crescent sign
       - Cavity
     - Tracheobronchitis
       - Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis
   - Sinunasal infection
     - Imaging showing sinusitis plus at least 1 of the following 3 signs:
       - Acute localized pain (including pain radiating to the eye)
       - Nasal ulcer with black eschar
• Extension from the paranasal sinus across bony barriers, including into the orbit
• CNS infection
  • 1 of the following 2 signs:
    • Focal lesions on imaging
    • Meningeal enhancement on MRI or CT
• Disseminated candidiasis
  • At least 1 of the following 2 entities after an episode of candidemia within the previous 2 weeks:
    • Small, target-like abscesses (bull’s-eye lesions) in liver or spleen
    • Progressive retinal exudates on ophthalmologic examination

III. Mycological criteria
• Direct test (cytology, direct microscopy, or culture)
  • Mold in sputum, bronchoalveolar lavage fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following:
    • Presence of fungal elements indicating a mold
    • Recovery by culture of a mold (e.g., Aspergillus, Fusarium, Zygomycetes, or Scedosporium species)
• Indirect tests (detection of antigen or cell-wall constituents)
  • Aspergillosis
    • Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF
  • Invasive fungal disease other than cryptococcosis and zygomycoses
    • BDG detected in serum

3.3.1.4 Possible IFD
To achieve the status of possible IFD the patients must meet the host factors sufficiently and must be showing appropriate host factors and with adequate clinical signs compatible with IFI but without meeting the mycological criteria.
3.4 Diagnosis

3.4.1 Pre-analytic considerations and choice of adequate sample material

Pre-analytical errors often lead to discrepant irrelevant results. Appropriate sample type, sample collection, and transport issues to the clinical laboratory is crucial. If needed, all samples can be refrigerated for a short time, except for dermatological samples like skin, hair and nails, blood and CSF. Samples often contain contaminating bacteria or fungi that could lead to false results, by overgrowing the actually sprout/germ. There is always the possibility of contamination, through the physiological skin flora of the patient, the airways of the patient or in the laboratory. Therefore the culture medium should contain antibacterial antibiotics to avoid overgrowth, e.g. cycloheximide, combinations of gentamicin and chloramphenicol or ciprofloxacin (31, 32).

3.4.1.1 Serum samples

For microbiological serology, gel tubes with clot activator are used. Serum tubes should not be centrifuged within 30 minutes after blood collection and incubated in an upright position, with a speed of 1500 gravity (2000-3000 revolutions per minute) over 10 minutes. If not tested immediately, serum samples should be stored at 4°C, if storage exceeds more than 48 hours, freezing of samples at -20°C is required (31, 33).

3.4.1.2 Blood cultures

For blood cultures three separately taken punctures are recommended within 24 h, including anaerobic and aerobic flasks. The samples should be kept at 37°C, if possible, or at least at room temperature. The maximum storage- and transportation time should not exceed 16 hours (31, 33).
3.4.1.3 Respiratory tract secretions and bronchoalveolar lavage (BAL) samples

**Sputum:**
Before collection, the mouth cavity should be rinsed with tap water, except for the detection of mycobacteria. The sputum must be coughed up into a sterile beaker and should not be contaminated by saliva.

**Sputum induction:**
Sputum induction is used when no spontaneous sputum can be produced. Therefore 20-25 ml sterile hyperosmolar (3%) saline solution is inhaled through a ultrasonic nebulizer (34).

**Endotracheal aspirate:**
Before taking the samples a changing of the tracheal tube with a sterile catheter is necessary. Then the secret should be aspirated, with an endotracheal suction system from the deep segments of the bronchial tree (Figure 1).

![Endotracheal suction system](image)

**Figure 1:** Endotracheal suction system (35)

**Bronchial aspirate:**
The bronchial secrete is also aspirated with the bronchoscope as described above.

**BAL:**
First of all, secrete from upper respiratory tract must be aspirated before entering the lower respiratory tract with the bronchoscope.

Via the bronchoscope, a certain amount of 0,9 % saline solution is injected into the lower respiratory tract followed by an aspiration of the previously injected liquid as much as possible (Figure 2). In case of taking samples from immunocompromised patients, with the intention to detect an obligate pathogen, the first portion is kept. Normally the first portion is discarded.
Lung biopsy:
Normally the biopsy is taken intraoperative or post mortem by site-specific excavation of lung tissue contaminated with fungi.
For all samples obtained from the respiratory tract the storage- and transportation period should not exceed two hours and 12 hours when refrigerated (31).

3.4.1.4 Urine samples
Midstream urine:
For proper collection, the first urine portion is discarded and the second portion should be collected in a sterile beaker (Figure 3). The use of urine beakers with an included transfer unit is recommended to avoid contamination.
Disposable urine catheter:
The disposable urine catheter (Figure 4) is inserted and urine is collected, the first urine portion must be discarded as well.

Figure 4: Disposable urine catheter (38)

Long-term urine catheter:
The specimen is collected through the needle-free puncture of the allocated puncture side of the permanent urine catheter (Figures 5,6), after previous disinfection.

Figure 5: Long-term urine catheter collection system (39)
Bladder puncture:

Today bladder puncture is especially used in urinalysis or urine culture of neonates or children younger than 2 years. Other indications are phimosis, chronic infection of the urethra or peri-urethral glands, urethral stricture and urethral trauma (41).

3.4.2 Diagnosis of *Candida* spp. as causative agent of IFI

The diagnosis of invasive fungal infections is always a combination of different diagnostic tools (42). For invasive candidiasis blood cultures remain the gold standard, in case of the deep-seated candidiasis sterile collected cultures of the infected tissue side are considered to be gold standard. The gold standard for deep-seated candidiasis is culture from steriley collected biopsy samples enabling the direct detection of the fungi. The biopsy is an invasive procedure, which poses a risk for these patients, being in serious conditions anyway increasing the risk for the development of an IFI (4).

3.4.2.1 Laboratory methods for detection of *Candida* spp.

3.4.2.1.1 Direct Detection

3.4.2.1.1.1 Microscopic examination

Microscopy techniques include fresh and stained examination of microbiological samples, as well as histopathological studies. Microscopic examination has a few disadvantages. First of all a sufficient amount of fungal structures in the sample must be constituted for analyzing. At this point the infection with the fungi is already at a progressive stage. Further the sensitivity depends on augmentation and the number of fields examined (43). The microscopically detection of *Candida* spp. can be performed from sputum, bronchial secrete, urine, stool, blood-cultures,
biopsy material, liquor. The patients sample is transferred to an microscope slide and stained using the lactophenol cotton blue colorization in general (32, 42). This technique is helpful because results can be obtained at the same day but the differentiation of the species is not always possible, therefore further microbiological methods are necessary.

3.4.2.1.1.2 Culture
Blood cultures still are the gold standard for diagnosing candidemia. Unfortunately cultures showing a sensitivity of approximately 50% (4). The whole procedure from collection of the blood culture until the identification of the species needs a minimum of 2 days (44). One study showed that the time varies according to the species.

*C. albicans* needs about 32 h for the first positive results for yeast detection and about 84 h for final identification and susceptibility testing. *C. glabrata* isolates need two to three times more until the final report (45).

3.4.2.1.1.3 Identification
After growth, the species must be identified. This can be performed by the microscopic examination as described above, or conventional used identification measures.

3.4.2.1.1.4 Germ tube test
This is an easy, cheap and fast method for the identification of *C. albicans*, already at the day of cultivation. About 90% of the *C. albicans* can be identified with this method. If they are negative, further tests like chromogenic culture plates are needed for identification. Serum (human, sheep) with suspected *Candida* spp. colonies from culture plates is incubated for 2 to 3 hours at 35°-37°C and then evaluated with the microscope (Objective 1:40) (46, 47).

3.4.2.1.1.5 Chromogenic culture
This test is used for the identification of further *Candida* spp. or germ tube test negative *C. albicans*. These plates contain different chromogenic substrates, which facilitate the identification of different *Candida* spp., which include carbohydrates, amino acids, phosphates or other substances. Different enzymes of the various *Candida* spp. interact with this substrates and show a characteristic
colony color (12, 32). For example: *C. albicans* / *C. dubliniensis*: green, *C. tropicalis*: blue, *C. krusei*: rose, dry, *C. glabrata*: purple. The time period until the colorization is visible accounts up to 3 days. For *C. glabrata* additional tests are needed, because other yeast species tend to turn purple (46).

### 3.4.2.1.1.6 Sugar fermentation test

The fermentation test of glucose, maltose, sucrose, lactose, galactose, rhamnose und trehalose is another method, which can be used. The cultivated yeast is mixed with the sugar in a glass tube and incubated for two to three days. A positive reaction corresponds to gas development. *C. albicans* for example is positive for glucose and maltose, variable for galactose, trehalose and sucrose and negative for lactose and rhamnose (46).

### 3.4.2.1.1.7 Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF):

For this test a single colony out of the culture media is applied on the target plate. The specimen is ionized and separated according to the mass and the ionization grade, every species has his characteristically spectrum. Several studies investigated the time to identification, costs and precision of this test. The study of Tan et al. shows that for non-albicans yeast the diagnosis is made 2 days earlier compared to the conventional methods and low consumable costs for this method are lower. Only for *C. albicans* MALDI-TOF shows no advantage because the germ-tube test is faster in the identification and it is cheaper in consideration of the costs (48).

### 3.4.2.1.2 Molecular detection

For *Candida* spp. several commercially available, IVD-CE marked real-time-polymerase chain reaction (PCR) assays including SepsiT (Molyzm) for yeast fungi only or the panfungal MycoReal assay are suitable for routine diagnostics (49). Real-time PCR shows advantages like the fast turnaround time of 2 hours and they surpass culture in sensitivity. The study of Schabereiter-Gurtner et al. developed novel real-time PCR assays that among others allow the simultaneous detection and identification of several *Aspergillus* and *Candida* species and the detection of different species in general. The results showed a very high sensitivity of the real-time PCR assays (50).
PCR remains a promising method but they still are poorly standardized lacking international reference material making results better comparable, and therefore not recommended in the EORTC/MSG Guidelines (49, 51).

3.4.2.1.3 Non-cultural Detection

3.4.2.1.3.1 1-3-β-D-Glucan Detection

BDG is present in the cell wall of most fungi, though it is not highly specific for *Candida* spp. Several detection assays have been developed based on activation of the coagulation cascade by BDG. The Fungitell® assay is colorimetric for qualitative detection of BDG in the serum. Based on a modification of the Limulus Amebocyte Lysate (LAL) pathway (Figure 7). The Fungitell® reagent eliminates factor C, and only reacts with BDG at the factor G induced side of the pathway. BDG activates factor G. The activated factor G transforms the pro-clotting enzyme into the clotting enzyme. The clotting enzyme separates pNA from Boc-Leu-Gly-Arg, which is a chromogenic peptide substrate, the created chromophore absorbs at 405 nm. Fungitell® assay measures the rate of the increase of the optical density. The results are compared to a standard curve. The BDG concentration in the sample can be yielded by the produced estimates. The limitations of the test are that a few fungi species produce low levels of BDG, like *Cryptococcus* spp., or even don’t produce BDG like zygomycetes. False positive results were found in patients who were treated with fractionated blood products like serum albumin and immunoglobulin or in specimens exposed to glucan-containing gauze (52).

![Figure 7: Limulus Amebocyte Lysate Pathway as used in the Fungitell® assay (53)](image)

The Fungitell® assay shows a diagnostic sensitivity for IFI diagnosis of approximately 77% and a diagnostic specificity of about 85% (54). Since 2008, the
serum BDG test has been implemented to the diagnostic criterion for IFI in the revised EORTC/MSG guidelines (30).

### 3.4.2.1.4 Medical imaging

#### 3.4.2.1.4.1 Magnetic resonance imaging (MRI) of the central nervous system (CNS)
In the case of cerebral infestation of Candida infection, the MRI can show numerous micro abscesses of less than 3mm, enhancement is seen often, rarely seen is infarction or hemorrhage. In the T1 sequence the lesions are hypo intense, whereas the enhancement in the T2 sequence can vary (55).

#### 3.4.2.1.4.2 High-resolution computer tomography (HR-CT)
Signs of pulmonary candidiasis are small nodular lesions without cavitation, however they are less specific compared to the findings in HR-CT in invasive pulmonal aspergillosis (IPA) (56).

#### 3.4.2.1.4.3 $^{18}$F-fluorodeoxyglucose (FDG) - positron emission tomography (PET)
Lesions caused through disseminated candidiasis can show increased FDG avidity (57). Advantages of the FDG-PET scan are that lesions in the whole body can be detected; additionally one case report showed that the lesions could be detected at a very early stage of infection (58). It has the same sensitivity than conventional methods. In some cases with hepato-splenic candidiasis even more lesions in liver and spleen could be detected compared to conventional methods (59). Furthermore the FDG-PET can be used for staging and therapy monitoring in case of IFI (56).

### 3.4.3 Diagnosis of *Aspergillus* spp. as causative agent of IFI
The diagnosis of IA continues to be challenging. Blood-cultures are often negative and lacking sensitivity. The gold standards to diagnose IA are respiratory specimens obtained by sputum induction, BAL and proven tissue invasion by histopathology (17, 60).
3.4.3.1 Laboratory methods for detection of *Aspergillus* spp.

3.4.3.1.1 Direct detection

3.4.3.1.1.1 Microscopic examination

As a first orientating diagnostic examination, microscopy of fresh and stained material is meaningful; however these methods have limitations since identification of type of species needs further cultivation and resistance testing for choosing optimal antifungal therapy. Routinely, cotton blue colorization is used for identification, for histological sections, haematoxylin and eosin, periodic acid–Schiff and silver stains are used (43).

3.4.3.1.1.2 Culture

Culture, tissue invasion and histopathology provide the most certain evidence of IA. Though these methods are insensitive, only 25-50% are tested positive, besides biopsy may be difficult in high-risk patients (60). Although contamination may occur through airways, in high-risk patients already single colonies must be taken seriously. For culturing *Aspergillus* spp. in general Sabouraud-glucose agar is used. Other culture media are conventional agars like blood agar or chocolate agar, malt extract agar, cornmeal agar, potato agar and heart infusion agar can be used for further differentiation. The incubation temperature ranges 30° to 37°C (46).

3.4.3.1.1.3 Identification

3.4.3.1.1.4 Microscopic examination after culturing

Identification of most species can be made through microscopic examination. For identification the examination of the anamorphic stage is important. The conidiophores are evaluated after length, breadth, surface and color, conidia after form, surface and size (42).

3.4.3.1.1.5 MALDI-TOF

For the identification of *Aspergillus* spp. it is also possible to use MALDI-TOF. Still it remains more difficult and should only be performed in laboratories, which are specialized for this procedure. Besides most of the species can be identified through microscopic examination (46).
3.4.3.1.2 Molecular detection

The PCR shows a high sensitivity and specificity, it rather can be used as screening test or confirmation test in high-risk patients. However, the PCR is still not implemented in the guideline since lacking of standardization. In addition, the lower limit of quantitation is not necessarily associated with clinical relevant infective dose of the pathogen making the differentiation between colonization and invasion difficult (50). At present there are several real-time-PCR assays commercially available for Aspergillus spp. MycAssay Aspergillus (Mycnostica) and the Aspergillus spp. Q-PCR Alert Kit (Nanogen) for detection of samples from the respiratory tract, LightCycler SeptiFast Test (Roche Diagnostics) for determination of A. fumigatus in blood samples, and MycoReal Aspergillus (ingenetix) can identify five different Aspergillus species (A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus) (50, 61).

3.4.3.1.3 Non-cultural Detection

3.4.3.1.3.1 Serum GM testing

GM is a component of the cell wall of Aspergillus spp. The Platelia enzyme immunoassay (EIA; BioRad Laboratories, Marnes-la-Coquette, France) is a one-stage colorimetric immuno-enzymatic sandwich microplate assay, which can be applied for serum samples and BAL fluid. The assay uses rat EBA-2 monoclonal antibodies, which are directed against Aspergillus GM. The heat-treated samples and the conjugate (Anti-GM monoclonal antibody/ peroxidase labeled) are added to the wells, which are coated with monoclonal antibodies, and incubated. In the presence of GM antigen, a monoclonal antibody-GM-monoclonal antibody/peroxidase complex is formed and results in a color change from blue into yellow. The optical density of specimens and controls is estimated with a spectrophotometer set at 450 and 620/630 nm wavelength. There are a few limitations of the test to consider, whereas contamination is of utmost importance (62). Recent studies showed that GM determination can be promising referred to an earlier adequate antifungal therapy and consequently can have a positive influence on the patients survival (63). Another useful option for GM-testing is, therapy monitoring to identify early breakthroughs in patients on antifungal therapy. The GM test used with BAL specimens uses a different cut-off and
showed a higher sensitivity and specificity for diagnosing invasive pulmonary aspergillosis than serum. Therefore the BAL fluid GM test is the current gold standard for diagnosing IPA (61).

3.4.3.1.3.2 Point of Care Test Lateral-Flow Device
A novel promising diagnostic method is the Aspergillus Lateral-Flow Device (LFD). The test uses a mouse monoclonal antibody to detect a glycoprotein antigen and can be used for BAL or serum specimens. This LFD test is easy to use and has a fast turnaround time. One study compared different diagnostic tests, including the LDF, which showed beside the GM immunoassay and the PCR the highest sensitivity (80%) and specificity (95%) (61, 64, 65).

3.4.3.1.3.3 Volatile compounds
Volatile organic compounds (VOCs) are produced by Aspergillus spp. and can be detected in the breath of infected individuals, however this method yet has not sufficiently underwent clinical investigation (60).

3.4.3.1.4 Medical imaging
3.4.3.1.4.1 MRI of CNS
Multiple brain lesions in immunocompromised patients may be caused by fungal infection showing characteristic patterns, which may help to differentiate between a bacterial or fungal infection through Aspergillus spp. Reduced diffusion caused by the high viscosity and cellularity of fungal pus, can be an early indicator for fungal infection. Sometimes, radiographic appearance of Aspergillosis can be typically ring-like. All in all MRI is a helpful diagnostic tool to confirm the diagnosis, regarding the clinic and other diagnostic tests (55).

3.4.3.1.4.2 HR-CT
Remains one of the most important diagnostic tools in IA because specific signs in computer tomography (CT), like the halo sign or the air-crescent sign with parallel clinical symptoms can lead to the diagnosis. According to the practice guidelines for the diagnosis and management of aspergillosis a CT is recommended, in patients with any clinical evidence of IPA (66).
3.4.3.1.4.3 FDG-PET

Lung malignancy can show similar pattern, in this case further histopathologic clarification is necessary. FDG-PET is helpful in differentiating between IPA and non-IPA and in therapy monitoring of patients with IPA (57).
3.5 Urine diagnostic relevant for our investigation

3.5.1 Microscopically detection:

3.5.1.1 Sediment:

Yeast:
The appearance can be filamentary, tubular, colorless, oval and from different size. In the urine sediment pseudomycelia (build by Candida spp. through longitudinal grow of the sprouting blastospores) can be detected microscopically (67). Candida spp. is the most frequent yeast detected in urinary sediment. It is often seen in urine from women with vaginitis, which leads to a contamination of the urine. Additionally squamous epithelial cells, bacteria, and leucocytes are found. However Candida spp. can cause real urinary tract infections in patients with risk factors like diabetes mellitus, structural abnormalities of the urinary tract, indwelling and long-term catheters, prolonged antibiotic treatment, or immunosuppression. If one of these factors is present, Candida spp. detection in the urine could be a sign of colonization or even of an invasive infection of the urinary tract (68, 69).

3.5.2 Culture

Urine cultures are considered as gold standard for the diagnosis of urinary tract infections. The examination includes the inhibitor test, quantitative culture, identification of the pathogen and susceptibility testing. As culture media for example blood agar or selective culture media for enterobacteria like MacConkey and cytosine lactose electrolyte deficient (CLED) agar are recommended. For Candida spp. Chromoagar plates (for identification) and Sabourad-glucose agar for Aspergillus spp. are used. The incubation durance for bacteria is about 16-24, if a fungal infection is expected the durance is about 48 h. The inhibition test is performed with agar plates, which are covered with spores of Bacillus subitilis. Every inhibition of the growing is a detection of an inhibition substance, like antibiotics and chemotherapeutics. The valid cutoffs for an urinary tract infection may not be applicable as the antibiotics may have reduced the number of the pathogen (70).
3.5.3 Urine-Creatinine

Creatinine concentration or clearance, serve as measuring tool for the integrity of 24-hour urine. The renal creatinine clearance depends primarily from the muscle mass, and is only elevated little by meat based diet or muscle activity. Therefore the clearance is relatively constant. At the determination of urine metabolites creatinine is often used as reference quantity (69).

3.6 Aims of the study and study design

The following aims of our study were defined:

- To compare results obtained from parallel testing of BDG in urine testing and serum
- To evaluate the diagnostic value of BDG in urine
- To establish a cut-off value for BDG in urine together with correction of influence of urine creatinine

The study design comprised the following procedures and population:

- From August 2012 to December 2013, serum samples (n= 241) and midstream urine samples (n= 241) were collected from adult hospitalized patients with hematological malignancies (n=60)
- The study adhered to the Declaration of Helsinki 1996, Good Clinical Practice
- The study protocol was approved by the local ethics committee of the Medical University Graz, Austria (protocol number 23–343). All participants gave written informed consent
- The samples were collected from patients above 18 years of age at the risk for IFI, with routine BDG screening and without urinary catheters, were identified and screened by clinical rounds, chart reviews, and surveys of electronic documents including microbiological test results
- Patients’ medical records were reviewed individually by using a standardized data collection template in order to collect demographic information and clinical data on outcomes of therapy and adverse events, and mycological laboratory test results
3.6.1 Sample collection

Serum and urine samples were collected in parallel, twice a week on the same days. Serum samples were collected together with the routine blood collection using a 9 ml VACUETTE® serum tubes. The patients were instructed regarding the procedure of midstream-urine collection prior sampling. Then, urine samples were collected with using a VACUETTE® sterile urine collection beaker (Figure 8) with an integrated urine transfer unit and evacuated with a 6 ml sterile VACUETTE® urine tube on the morning and stored at -70°C within 2 h after sample collection for retrospective testing.

Figure 8: Sterile urine collection beaker (71)
4 Methods

4.1 Demographic Data

A total of 60 patients at risk for IFI according to the EORTC criteria were included. From those 60 patients a total of 241 urine samples together with corresponding serum samples were obtained for this study:

- 27 urine and serum sample-pairs were obtained from 11 patients with probable IFI
- 56 urine and serum samples-pairs were obtained from 30 patients with possible IFI
- A total of 82 urine and serum sample-pairs could be classified as IFI samples
- The remaining 159 urine and serum sample pairs from 49 patients could be classified as no-IFI samples
- The majority of urine and serum sample-pairs (202/241) were obtained from patients receiving mold-active antifungal prophylaxis or therapy

<table>
<thead>
<tr>
<th>Characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>81 ± 21</td>
</tr>
<tr>
<td>No. females/ No. males</td>
<td>24/36</td>
</tr>
<tr>
<td>Underlying Disease</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>24</td>
</tr>
<tr>
<td>NHL</td>
<td>5</td>
</tr>
<tr>
<td>MDS</td>
<td>4</td>
</tr>
<tr>
<td>MM</td>
<td>8</td>
</tr>
<tr>
<td>ALL</td>
<td>3</td>
</tr>
<tr>
<td>OTHERS</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 1: Demographic Data of the study population. ALL, Acute Lymphocytic Leukemia; AML, Acute Myeloid Leukemia; MDS, Myelodysplastic Syndrome; MM: Multiple Myeloma; NHL, Non-Hodgkin Lymphoma; Others: Follicular Lymphoma, Aplastic Anemia, Burkitt lymphoma, Hodgkin Lymphoma, Myelofibrosis, Myelodysplasia

4.2 Laboratory testing

Serum samples were tested prospectively for BDG using the Fungitell® assay (Associates of Cape Cod, USA) with the routine screening. The native urine samples (n=241) were tested retrospectively in October 2014, using the Fungitell® assay. The samples were tested after vortexing but without any pretreatment.

The measurement of BDG is based on the Limulus test BDG activates factor G, a serineprotease zymogen of the Limulus amebocyte lysate, which is extracted from
amebocytes of horseshoe crab species. This in turn activates a coagulation cascade. The activity of this reaction can be measured with use of colorimetric or turbidimetric methods (52). The serum and urine specimens were prepared following the manufacturer’s instructions.

4.2.1 Standard protocol of the Fungitell® assay

First, preparation of glucan standard is performed using one vial of glucan standard and mixed with the same volume of reagent grade water, which results in 100 pg/ml solution. After that, preparation of serum alkaline pre-treatment reagent (0.25 M potassium hydroxide and 1.2 M potassium chloride, combined 1:1) is made. This reagent converts triple-helix glucans into single-stranded glucans, which are more reactive, and further it inactivates the serine proteases and serine-inhibitors in serum that can give a false positive and a false negative result, due to the higher pH. After vortexing the samples, an adding of 20 μl of serum pre-treatment reagent to all specimens to test is and they are incubated for 10 minutes at 37°C degrees. 25 μl of the five standards and 25 μl of the blank are transferred to the microplate. When the incubation ended, 100 μl Fungitell® reagent to each specimen (negative controls, standards and samples) is added. The assay is monitored at 405 nm kinetically for 40 min, in an incubating plate reader. BDG concentration >80 pg/ml are considered as positive and < 60 pg/ml are considered to be negative. Values between 60 and 80 pg/ml are considered as grey zone.

4.2.2 Automated protocol of the Fungitell® assay

The Fungitell® assay was adapted for automation, using a routine BCS XP coagulation analyzer according to the manufacturer’s instructions. The Fungitell® reagent was reconstituted as recommended by the manufacturer. The assay included aqua bi-dest as a negative- control and glucan-standard as a positive control within every run together with urine and serum specimens (7, 52).

4.2.3 Urine creatinine testing

Urine creatinine levels were determined with Cobas 8000 automated analyzer (Roche Diagnostics, Rotkreuz, Switzerland), using the Jaffé Generation 2 assay for urine.
4.3 Statistical methods

The statistical analyses were performed using the Statistical Package for Social Sciences version 23.0 (SPSS Inc., Chicago, IL, USA) and Windows Excel 2008. Urine BDG values were compared to serum BDG values obtained at the same day.

4.3.1 Sensitivity, specificity, PPV, NPV

Sensitivity, specificity, PPV, NPV) and the diagnostic odds ratio (DOR) for BDG-urine positivity were calculated based on urine BDG levels 80pg/ml, 200pg/ml and 500pg/ml. 80 pg/ml was taken referring to the recommendations for the serum BDG cut-off for positivity. The calculation was performed in two ways- first, we considered patients with probable and possible IFI as positive and second, only patients with probable IFI were considered as positive and patients with possible IFI and no IFI as negative. The optimal cut-offs for urine samples were defined using the DOR method.

4.3.2 Urine BDG/Creatinine Ratio

For improving the diagnostic performance of BDG urine detection, a formula, which takes the urine dilution into account, was implemented:

\[ \text{BDG - Creatinine ratio } \mu g/ml = \frac{\text{Urine BDG } pg/ml \div \text{Creatinine of urine mg/dl}}{1000} \]

To find the optimal cut-off the Receiver-Operating-Characteristic-Curve (ROC-Curve) was used. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using different cut-offs. The Urine BDG/ Creatinine ratio was estimated with the IFI status (probable IFI only positive factor; probable/possible IFI was the positive factor) the following cut-offs of 0,06 µg/ml, 0,08 µg/ml 0,1 µg/ml were yielded.

4.3.3 Correlation analysis

For correlation analysis between serum and corresponding urine samples, samples were grouped regarding serum level-ranges (group 1, BDG serum levels <80 pg/ml, n=189; group 2, BDG serum levels ranging from 81 – 200 pg/ml, n=33;
group 3, BDG serum levels >200 pg/ml, n=19). The correlation was calculated once for the BDG urine levels and again for the urine BDG/Creatinine ratio. Correlation between serum and urine BDG results was calculated using Spearman-Rho correlation analysis. And for group 3 the Pearson-Correlation was used.
5 Results

5.1 Diagnostic and clinical sensitivities, specificities, PPV, NPV and DOR

5.1.1 Urine BDG

189 serum samples were negative, 33 in the intermediate range from 80-200 pg/ml and 19 were above 200 pg/ml.

From the 189 urine samples corresponding to the negative serum samples, 155 were also found in this range whereas 24 were tested within 80-200 pg/ml and 10 were found above 200 pg/ml (Table 2).

From the 33 urine samples corresponding to those within 80-200 pg/ml in serum, three were also found in this range whereas 28 were tested negative and two were found above 200 pg/ml (Table 2).

From the 19 urine samples corresponding to those above 200 pg/ml in serum, 12 were also found in this range whereas one sample was tested within 80-200 pg/ml and 6 were found negative (Table 2).

<table>
<thead>
<tr>
<th>Serum BDG levels</th>
<th>&lt; 80 pg/ml</th>
<th>80-200 pg/ml</th>
<th>&gt;200 pg/ml</th>
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</thead>
<tbody>
<tr>
<td>Urine BDG levels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 80 pg/ml</td>
<td>155/189</td>
<td>23/33</td>
<td>10/19</td>
</tr>
<tr>
<td>80-200 pg/ml</td>
<td>28/189</td>
<td>3/33</td>
<td>2/19</td>
</tr>
<tr>
<td>&gt;200 pg/ml</td>
<td>6/189</td>
<td>1/33</td>
<td>12/19</td>
</tr>
</tbody>
</table>

Table 2: Same-day urine and serum samples according to groups formed based on BDG serum levels

When the same cut-offs, which are used for serum were applied for the urine samples, the sensitivity was found to be 38% and the specificity showed 80%, if only the probable IFI were considered as positive. If Probable and possible IFI were considered as positive, the sensitivity was found to be 32% and the specificity showed 84%; however, as expected, the higher cut-offs lead to a higher specificity and lower sensitivity (Table 3).
<table>
<thead>
<tr>
<th>Urine cut-off</th>
<th>IFI Status</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>DOR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 80 pg/ml</td>
<td>prvs. po+no</td>
<td>38%</td>
<td>80%</td>
<td>19%</td>
<td>92%</td>
<td>2.57(1,1-6,1)</td>
</tr>
<tr>
<td>&lt; 80 pg/ml</td>
<td>pr+po vs no</td>
<td>32%</td>
<td>84%</td>
<td>50%</td>
<td>70%</td>
<td>2.38(1,3-4,5)</td>
</tr>
<tr>
<td>&gt;200 pg/ml</td>
<td>pr vs po+no</td>
<td>31%</td>
<td>93%</td>
<td>33%</td>
<td>92%</td>
<td>5.53(2,1-14,7)</td>
</tr>
<tr>
<td>&gt;200 pg/ml</td>
<td>pr+po vs no</td>
<td>20%</td>
<td>95%</td>
<td>67%</td>
<td>70%</td>
<td>4.58(1,9-11,2)</td>
</tr>
</tbody>
</table>

Table 3: Sensitivity, specificity, PPV, NPV, DOR plus 95% confidence interval (95% CI) for different BDG urine cut-offs for urine samples related to IFI status (no, possible (po), probable (pr))

5.1.2 Urine BDG/Creatinine ratio

When applying the urine BDG/Creatinine ratio, the results showed higher sensitivities ranging from 47-81% without significant loss of specificity, which ranged from 49% to 70%, according to the clinical classifications of IFI (Table 4)

<table>
<thead>
<tr>
<th>Urine cut-off</th>
<th>IFI Status</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>DOR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,06 µg/ml</td>
<td>pr vs. po+no</td>
<td>81%</td>
<td>53%</td>
<td>16%</td>
<td>95%</td>
<td>3.68(1,3-10,1)</td>
</tr>
<tr>
<td>0,06 µg/ml</td>
<td>pr+po vs no</td>
<td>67%</td>
<td>49%</td>
<td>40%</td>
<td>75%</td>
<td>1.95(1,2-3,4)</td>
</tr>
<tr>
<td>0,06 µg/ml</td>
<td>pr vs no</td>
<td>81%</td>
<td>49%</td>
<td>21%</td>
<td>94%</td>
<td>4.04(1,5-11,3)</td>
</tr>
<tr>
<td>0,08 µg/ml</td>
<td>pr vs po+no</td>
<td>65%</td>
<td>58%</td>
<td>16%</td>
<td>93%</td>
<td>2.65(1,1-6,2)</td>
</tr>
<tr>
<td>0,08 µg/ml</td>
<td>pr+po vs no</td>
<td>53%</td>
<td>60%</td>
<td>41%</td>
<td>72%</td>
<td>1.72(1,0-3,0)</td>
</tr>
<tr>
<td>0,08 µg/ml</td>
<td>pr vs no</td>
<td>65%</td>
<td>60%</td>
<td>21%</td>
<td>91%</td>
<td>2.88(1,2-6,9)</td>
</tr>
<tr>
<td>0,1 µg/ml</td>
<td>pr vs po+no</td>
<td>62%</td>
<td>68%</td>
<td>19%</td>
<td>94%</td>
<td>3.36(1,5-7,8)</td>
</tr>
<tr>
<td>0,1 µg/ml</td>
<td>pr+po vs no</td>
<td>47%</td>
<td>70%</td>
<td>45%</td>
<td>72%</td>
<td>2.11(1,2-3,7)</td>
</tr>
<tr>
<td>0,1 µg/ml</td>
<td>pr vs no</td>
<td>62%</td>
<td>70%</td>
<td>25%</td>
<td>92%</td>
<td>3.81(1,6-9,0)</td>
</tr>
</tbody>
</table>

Table 4: Sensitivity, specificity, PPV, NPV, DOR plus 95%CI for different urine BDG/Creatinine ratio cut-offs for urine samples related to IFI status (no, po, pr)
5.2 ROC-Curve Analysis

5.2.1 BDG urine compared to Urine BDG/Creatinine ratio

ROC curve analysis revealed an AUC of 0,683 (p 0,005; 95% CI 0.550 – 0.786) for BDG urine and an AUC of 0,728 (p <0,001; 95% CI 0.622 – 0.835), when urine BDG/Creatinine ratio was used (Figure 9).

![ROC Curve](image)

Figure 9: ROC Curve according to IFI status showing BDG in urine vs. urine BDG/Creatinine ratio

5.3 Correlation analysis

5.3.1 Correlation between same day BDG in serum and urine samples

No significant correlation of BDG levels was found between serum and urine samples in group 1 (r=0.015; p=0.835) and in group 2 (r=0.163; p=0.366).

In group 3 a non-significant positive correlation for BDG between serum and urine samples could be observed (r=0.332; p=0.165); in addition, in this group the Pearson correlation was calculated (r=0.453; p=0.051), (Figure 10).
5.3.2 Correlation between same day BDG in serum and urine

**BDG/Creatinine ratio**

No significant correlation was found between for urine BDG/Creatinine ratio and serum BDG in group 1 samples ($r=0.031; p=0.672$) and in group 2 samples ($r=0.077; p=0.670$). In group 3 a significant positive correlation for urine BDG/Creatinine ratio between serum and urine samples could be observed ($r=0.549; p=0.015$); in addition, in this group the Pearson correlation was calculated ($r=0.722; p=0.000$).

5.4 BDG urine levels according to IFI status

Urine BDG levels were prone to be higher in samples obtained from patients with probable IFI in comparison with samples obtained from patients without IFI (median 40,96 interquartile range (IQR) 25-75; 15,28 – 285,51 vs. median 15,38 IQR 25-75% 15,38 –54,00), with significant difference ($p =0,001$ Mann-Whitney-U Test), (Figure 11).
Figure 11: BDG urine levels according to IFI status
5.5 Urine BDG/Creatinine levels according to IFI status

Urine BDG/Creatinine ratio levels were prone to be higher in samples obtained from patients with probable IFI in comparison with samples obtained from patients without IFI (median 0.1304 IQR 25-75; 15,28 – 285,51 vs. median 0,0572 IQR 25-75% 15,38 –54,00), with significant difference (p =0,001 Mann-Whitney-U Test), Figure (12).

![Figure 12: urine BDG/Creatinine ratio according to state of IFI](image-url)
6 Discussion

6.1 Pre-analytical issues
While easy access to sample material may demonstrate a clear advantage, pre-analytical influences e.g. iatrogenic contamination during collection procedures, which may lead to false positive results, should be considered disadvantageous. In this study sample collection was standardized using the VACUETTE® sterile urine reservoirs with integrated urine transfer units and the patients were instructed clearly to minimize the pre-analytical errors.

6.2 Advantages regarding urine as sample material
Non-invasive, frequent and easy sample collection would account for possible future home testing when point-of-care tests are available and may increase the sensitivity.

6.3 Sensitivity and specificity for urine BDG
Calculation of clinical and diagnostic sensitivities and specificities for identification of IFI are strongly influenced by the insensitivity of all current diagnostic assays as this also does refer to BDG testing. Hence, it has to be noted that BDG serum value used as the “gold standard” has its limitations for calculating the performance of the urine assay. The performance of both tests mainly differ due to origin of sample materials – while urine sample may become positive because of impaired renal function with decrease in creatinine clearance, while serum levels will remain negative and the other round when excessive urine dilution leads to false negatives in urine specimens while serum samples may in fact be true positives. Data obtained from a metanalysis study showed the sensitivity of BDG testing in serum seems to be lower than the specificity (54). The same trend clearly could be observed for BDG testing in urine, with poor sensitivities ranging from 12% to 37% depending on the associated serum BDG ranges defined in this study; while obtained specificities ranged from 82% to 92% according to the serum BDG ranges defined in this study. As a consequence of these findings, BDG cut-offs used for serum cannot be applied for urine samples since validated reference values are lacking.
6.4 Urine BDG/Creatinine ratio

Because the target analyte concentration in urine strongly depends on the grade of urine dilution, BDG as target analyte was adjusted to renal excretion function, which is reflected by urine creatinine concentration. In this study, the urine BDG/Creatinine ratio was used to improve the diagnostic value resulting in a higher specificity with also a trend for higher sensitivity. The calculated urine BDG/creatinine cut-off value of 0.06 µg/ml turned out to be the most powerful to safely rule out IFI in urine testing of BDG, revealing an NPV of 95%. This cut-off further was associated with 81 % sensitivity and 53 % specificity. The quite low PPV of 15% can be explained through the high rate of patients receiving mold-active prophylaxis, nevertheless, a NPV of 95% is promising for rapid rule out severe IFI infections and for discontinuing antifungal treatment.

6.5 Correlation

In a previous study (11), BDG values were found to correlate better between serum and urine at high positive ranges, while BDG values within negative- and intermediate ranges showed poor correlation. Therefore it seems to be useful to apply a higher cut off when testing BDG in urine samples. Correlation analysis performed with the urine BDG/Creatinine ratio showed better correlation, especially in the high positive group, although AUC obtained in ROC curve analysis was not significant. This correlation was mainly expended to the high amount of samples in the no-IFI group, which were high positive (> 500 pg/ml). This most likely account to the pre-analytical influence regarding urine specimen collection is more susceptible for contamination then collection of serum samples. In general the results achieved with the urine BDG/Creatinine ratio lead to significant positive correlation between serum and urine BDG values. This finding indicates when using urine BDG testing, normalization against urine-creatinine and calculating urine BDG/Creatinine ratio is advantageous.

6.6 Limitations

The pre-analytical errors still cannot be prevented, even though the patients of this study were instructed clearly on the collection procedure, since the patients could not be controlled during the collection procedure.
A further limitation may be the small sample size of probable cases and missing proven cases, therefore an adequate prediction about the clinical performance cannot be made.

The urine BGD/Creatinine ratio may be influenced by high meat diets, rhabdomyolyses, extensive exercise and other conditions, which can lead to elevated urine creatinine levels, and the ratio might be not useable for these kind of samples. The composition of urine samples is not as stable as serum samples. Besides from the urine dilution, other influencing factors like the fluctuating urine pH-values may lead to false positive or negative BDG urine values.
7 References


37. Sample is transferred from urine beaker to urine tube: greiner-bio-one; 2015 [Sample is transferred from urine beaker to urine tube].


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Detection of (1→3)-β-D-glucan in same-day urine and serum samples obtained from patients with haematological malignancies.

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