Diploma thesis

The Influence of Dialysis Treatment on Serum (1→3)-β-D-Glucan Levels

submitted by

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Graz, am 23.06.2016 *Eva Jaindl eh*

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Abstract

Purpose

The purpose of this study was to evaluate whether hemodialysis (HD), hemodiafiltration (HDF) or peritoneal dialysis (PD), using state of the art techniques and devices, are causing false positive serum (1→3)-β-D-glucan (BDG) test results.

Methods

The study was conducted partially in vivo and partially in vitro.

The prospective clinical study enrolled 32 patients with chronic kidney disease (CKD) and without signs of invasive fungal infections (IFIs).

Ten of those patients had no ongoing dialysis and were included to eliminate the possibility of CKD as a confounder for elevated BDG levels.

Twelve of the patients had ongoing HD/HDF therapy. In this group BDG was measured in serum and dialysate at various time points during dialysis treatment.

Ten of the patients received PD and were tested only once for concentrations of BDG in serum and dialysis solution.

In the in vitro study, dialysis fluid was spiked with high concentrations of BDG. During a simulated HD session, samples for BDG determination were drawn at various time points.

BDG results less than 60 pg/ml were interpreted as negative, 60 to 79 pg/ml as intermediate, greater than or equal to 80 pg/ml as positive. The lower detection limit was 15.4 pg/ml.

Results

All patients with CKD and without dialysis had negative serum BDG results.

The majority (71 out of 72) of serum samples obtained from HD/HDF patients were BDG negative, resulting in a specificity of 0.99.

Only one out of ten serum samples of PD patients yielded a positive result, whereas BDG determination was negative in the remaining nine samples with a specificity of 0.90.

All dialysis fluid samples were BDG negative.

In the laboratory bench study, the dialyzer did not release BDG, nor did BDG from the BDG spiked dialysate leak into the simulated patient's blood circulation.

Conclusion

Based on these data, it can be concluded that serum BDG determination seems to be a reliable diagnostic tool for IFI assessment in patients with ongoing HD/HDF or PD using state of the art techniques.

Zusammenfassung

Fragestellung

In dieser Studie wurde untersucht, ob Hämodialyse (HD), Hämodiafiltration (HDF) und Peritonealdialyse (PD), unter Verwendung von Materialien und Geräten die den gängigen Standards entsprechen, zu falsch erhöhten Serumspiegeln von (1→3)-β-D-Glukan (BDG) führen.

Methodik

Die Studie bestand aus einem klinischen und einem in vitro Teil.

An der prospektiven klinischen Studie nahmen insgesamt 32 Patienten mit chronischer Niereninsuffizienz, jedoch ohne klinischen Zeichen und Symptome für invasive Pilzinfektionen teil.

Zehn dieser Patienten erhielten keine Dialysetherapie und wurden untersucht, um die chronische Niereninsuffizienz selbst als potenzielle Ursache falsch positiver BDG Ergebnisse auszuschliessen.

Zwölf der Patienten befanden sich in HD oder HDF Behandlung. In dieser Gruppe wurde BDG im Serum und in Dialysatproben zu verschiedenen Zeitpunkten im Verlauf von zwei aufeinanderfolgenden Dialysesitzungen bestimmt.

Die restlichen zehn Patienten waren unter PD Therapie und wurden einmal einer BDG Messung in Serum und Dialysat unterzogen.

Für die in vitro durchgeführte Studie wurde Dialysat mit hohen BDG Konzentrationen versetzt und Proben zu verschiedenen Zeitpunkten während einer simulierten Dialysebehandlung gesammelt.

BDG Ergebnisse wurden wie folgt interpretiert: weniger als 60 pg/ml als negativ, 60 bis 79 pg/ml als intermediär und grösser gleich 80 pg/ml als positiv. Der kleinste messbare Wert in diesem Rahmen war 15.4 pg/ml.

Ergebnisse

Alle Serumproben der Patientengruppe mit chronischer Niereninsuffizienz und ohne Dialysetherapie waren BDG negativ.

Der Grossteil (71 von 72) der Serumproben von der HD/HDF Gruppe war BDG negativ, entsprechend einer Spezifität von 0.99.

Nur eine Serumprobe der PD Gruppe war BDG positiv, während die BDG Bestimmung in den restlichen neun Proben negativ blieb, resultierend in einer Spezifität von 0.90. Alle Dialysatproben waren BDG negativ.
In der in vitro Studie, konnten weder ein Austritt von BDG interferierenden Substanzen aus der Dialysemembran, noch ein Übertritt von BDG aus den BDG positiven Lösungen in die simulierte Patientenzirkulation festgestellt werden.

Conclusio

Basierend auf den erhobenen Daten kann man davon ausgehen, dass HD, HDF und PD keine falsch positiven BDG Ergebnisse verursachen. Folglich kann BDG in Patienten mit Nierenersatztherapie, die den heutigen Standards entspricht, als zuverlässiger Biomarker für invasive Pilzinfektionen verwendet werden.

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Abbreviations

AIDS	acquired immune deficiency syndrome
BAL	bronchoalveolar lavage
BDG	(1→3)-β-D-glucan
CAPD	continuous ambulatory peritoneal dialysis
CKD	chronic kidney disease
CM	cryptococcal meningitis
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
CSF	cerebrospinal fluid
CTA	cellulose triacetate
Cu	cellulose
DNA	deoxyribonucleic acid
EORTC/MSG	European Organization for Research and Treatment of Cancer/Mycoses Study Group
FMC	Fresenius medical care
GFR	glomerular filtration rate
GN	glomerulonephritis
HD	hemodialysis
HDF	hemodiafiltration
HF	hemofiltration
HIV	human immunodeficiency virus
HSCT	hematopoietic stem cell transplantation
IA	invasive aspergillosis

IC	invasive candidiasis
ICU	intensive care unit
IFI(s)	invasive fungal infection(s)
IQR	interquartile range
LAL	limulus amebocyte lysate
MRC	modified regenerated cellulose
NPV(s)	negative predictive value(s)
PCP	*Pneumocystis* pneumonia
PD	peritoneal dialysis
PMMA	polymethylmethacrylate
pNA	p-nitroanilide
PPV	positive predictive value
SD	standard deviation
SIRS	systemic inflammatory response syndrome
SP	synthetic polysulfone
spp.	species

1 Introduction

1.1 The role of fungi as human pathogens

The rate of IFIs has increased since the 1980s, mainly due to higher numbers of immunocompromised, critically ill patients and more invasive treatment strategies. Diagnosis and treatment of deep-seated mycoses and fungemias remain challenging, leading to a high morbidity and mortality in these patients (1). Besides, the treatment of IFIs is associated with a high financial burden for healthcare systems (2, 3).

Out of an approximately 150.000 known species of fungi, about 50 species have been frequently isolated as human pathogens. Another 300 species have been discovered as opportunistic pathogenic fungi, that are able to cause disease in the immunocompromised organism (4).

1.1.1 Morphology and metabolism

Fungi are eukaryotic organisms that depend on organic substrates for their growth and reproduction (5). In contrast to typical animal cells, most fungi have thick and rigid cell walls (Figure 1) which are composed of polysaccharides, including chitin (0,6-9%), which is a polymer of N-acetylglucosamine, fibrillar β-(1→3)- and β-(1→6)-glucans (40-60%), superficial phosphomannoproteins and galactomannan, respectively (6-25%). The cell wall does not only provide the fungal cell with mechanical strength, integrity and protection, its components also enable adherence to the host's epithelium. The inner cell membrane consists mainly of ergosterol. Apart from its wall the fungal cell is very similar to the human cell which explains the toxic side effects of certain systemic antifungal agents (4, 6-8).

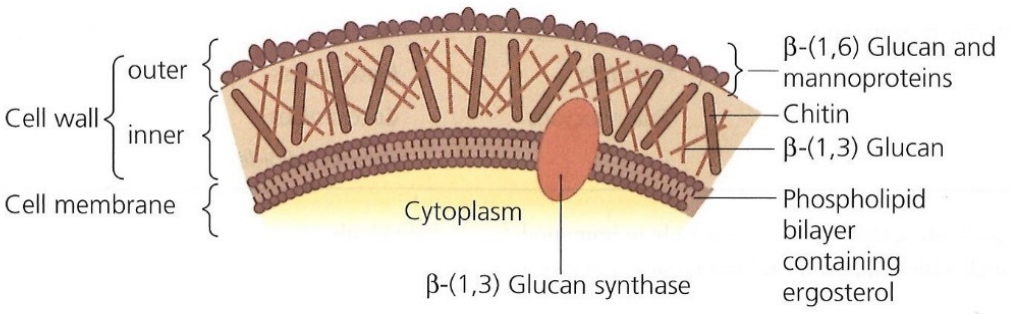

Figure 1. Fungal cell wall structure. Reprinted from (9).

1.1.2 Classification of mycoses

According to the site of infection, mycoses are classified as superficial, subcutaneous, cutaneous or systemic infections (7).

Pathogenic fungi can be distinguished by their virulence. Primary pathogenic fungi are able to infect immunocompetent patients, whereas opportunistic pathogens have a low virulence and thus need hosts that are in an immunocompromised state to cause a severe infection (5, 7).

Systemic infections caused by primary pathogenic fungi, including *Blastomyces*, *Histoplasma* and *Coccidioides* are endemic in parts of Central America and mostly transmitted by inhalation. In Central Europe, IFIs caused by opportunistic fungi play the major role. The predominant fungi responsible for these infections are *Candida* species (spp.) and *Aspergillus* spp. (1, 7).

1.2 The immune system and host factors predisposing for IFIs

Immunity. The mechanisms of host defence against fungi are not yet totally understood but they seem to be quite similar to those against bacteria (10). The first barrier that protects the host against fungi are intact skins and mucosae. If fungi succeed to break through this barrier, neutrophils and macrophages will be activated in order to phagocyte the invaders and produce anti-fungal defensins. T cell activation and differentiation of Type 1 helper cells will be initiated so that they can limit the extent of infection and produce cytokines that stimulate macrophage hyperactivation and activation of natural killer cells. Further mechanisms contributing defense against fungi are the complement system and, probably in a limited way, antibodies as part of the humoral response (11, 12). Additionally, platelets have been shown to have a growth inhibiting effect on *Aspergillus* which may be of particular interest in patients suffering from pancytopenia (13).

Certain health conditions and medical treatments can weaken these essential mechanisms of the immune system. The affected patients are therefore prone to develop IFIs that are associated with high mortality rates (14).

Risk groups for IFIs. Classical risk factors include infections by the human immunodeficiency virus (HIV) and the acquired immune deficiency syndrome

(AIDS), leukaemia, malign lymphoma, hematopoietic stem cell transplantation (HSCT) and the long-term therapy with corticosteroids, zytostatics and immunosuppressants. Furthermore, IFIs have been increasingly reported in non-neutropenic patients, e.g. with underlying respiratory diseases (15) and critically ill patients, treated in intensive care units (ICUs) (16, 17). Invasive procedures, parental nutrition, broad-spectrum antibiotics and dialysis therapy all increase the risk for severe IFIs (14).

1.2.1 Hematological diseases and HSCT

Among patients with hematological malignancies, especially those with acute myeloid leukaemia and myelodysplastic syndromes, are highly vulnerable to IFIs. Long-term neutropenia, which is the main risk factor, can be caused by the hematological malignancy itself or its treatment, particulary by intense chemotherapy. Common infections include invasive aspergillosis (IA), *Pneumocystis jirovecii* pneumonia (PCP) and candidemia (14, 18).

Allogenic stem cell transplantation and associated complications, i.e. graft-versus-host disease, increase the risk for IFIs, most commonly IA and invasive candidiasis (IC) for longer periods of time, even after aplasia (19). While the incidence of mucormycosis still remains low, it has recently become the third common fungal infection in this risk group (20). Through prophylaxis with posaconazole the incidence of breakthrough IFIs can be reduced to approximately 2% (21, 22).

1.2.2 Solid organ transplantation

Infections are among the leading causes of death in solid organ recipients. In the management of these infections it must be considered that the responsible pathogens vary between different points in time after transplantation, strongly influenced by the immunosuppressive regimen (23). Concerning fungal infections, IC was reported to be the most frequent among all organ types, except in lung transplant recipients who where more susceptible to develop IA. Besides, *Cryptococcus* and other molds than *Aspergillus* count to the common fungal pathogens (24). The incidence rates for different organ types vary depending on the type of transplantation procedure and the center. Pappas et al. reported the highest one-year cumulative IFI incidence in small bowel transplant recipients (11.6 %),

followed by lung recipients (8.6 %), liver (4.7 %), heart (4 %), pancreas (3.4 %) and kidney (1.3 %) recipients (25).

1.2.3 ICUs

Malnutrition, chronic obstructive pulmonary disease (COPD), liver failure or therapy with corticosteroids make severely ill patients without classical risk factors and neutropenia vulnerable for invasive infections. *Candida* spp. are the predominant fungal pathogens in the ICU setting, associated with a mortality rate of approximately 30% (26), followed by *Aspergillus* spp. which are showing an increasing incidence in this risk group (27). Leleu et al. demonstrated that IC caused prolonged ICU stays and, in comparison to controls, an elevated relative risk for death of 2.27 in these patients (28).

1.2.4 Renal diseases

CKD is associated with systemic inflammation and immune deficiency, the latter resulting in an increased incidence and severity of infections (29).

Uremia is thought to play the major role in the development of the immune defects as it leads to metabolic and nutritional abnormalities (30). Pathologic changes of metabolism and hemodynamics seem to alter also the intestinal microbiota, resulting in leakiness of the intestinal barrier, increasing its permeability for endotoxins and thus, contributing to the chronic systemic inflammation in these patients (31, 32).

Additional risk factors for IFIs in kidney patients are invasive diagnostic and therapeutic procedures, dialysis itself, as well as the use of broad-spectrum antibacterial agents (30).

Through dialysis therapy, the life expectancy of patients with CKD could be increased but, on the other hand it is known that a dialysis-related release of cytokines causes a chronic inflammatory activation and therefore further weakens the immune system (33). The intermittent hypoperfusion of the intestines during dialysis treatment is discussed to damage the mucosal barrier, consecutively enabling intestinal pathogens (e.g. gram-negative bacteria or fungi) to enter the blood stream, causing systemic infections (32).

As patients on continuous ambulatory peritoneal dialysis (CAPD) are concerned, fungi-caused peritonitis is rare but associated with a high CAPD drop-out rate (34, 35).

According to the United States renal data system, 79% of fungal infections in chronic dialysis patients are caused by *Candida* spp., followed by *Cryptococcus* (6%) and *Coccidoides* (4%) (30, 36).

Diagnostics of infections are particularly challenging in patients receiving dialysis because signs and symptoms are often unspecific and have a delayed onset compared to patients without CKD and dialysis (37).

1.2.5 HIV and AIDS

Approximately 50% of worldwide AIDS-related deaths are caused by IFIs, a majority by cryptococcal meningitis (CM). Patients with a low CD4 T cell count (< 100 cells/mm^3) are highly susceptible to infections caused by the basidiomycete *Cryptococcus neoformans*. PCP is the second leading opportunistic infection in HIV patients in North America, Europe and Australasia. Disseminated histoplasmosis and penicillosis are other life threatening fungal infections occuring in advanced HIV patients. The prevalence of orophangeal and oesophageal candidiasis increases greatly with a CD4 T cell count < 200 cells/mm^3 (38-40).

1.3 The most important opportunistic fungal pathogens and their predominant sites of invasive infections

1.3.1 *Candida* spp.

Candida spp. are the most prevalent opportunistic fungal pathogens, causing 70-90% of all IFIs. While *Candida albicans* is still predominat, infections caused by non-albicans species are increasing (1). Non-albicans species that are frequent human pathogens are *C. glabrata, C. tropicalis, C. parapsilosis, C. krusei, C. guilliermodii, C. pseudotropicalis, C. lusitaniae* and *C. dubliniensis*. As part of the human microbiome, *Candida* spp. colonize the skin and mucosal surfaces including the gastrointestinal, respiratory and the female genital tract (1, 41). Besides, an exogenous acquisition can occur through human-to-human transmission and is even thought to be possible through the hospital environment (41). Indwelling

intravenous catheters are among the main factors that enable *Candida* to enter the bloodstream. Furthermore, disruption of the gastrointestinal mucosal barrier, e.g. after abdominal surgery or through chemotherapy-induced intestinal mucositis, is considered to be a high risk factor for the development of invasive infections. Invasive organ infections include *Candida* meningitis, pneumonia, endocarditis, urinary tract, peritoneal and ocular candidiasis as well as arthritis and osteomyelitis (1, 41). *Candida* peritonitis is a possible complication of PD (30).

1.3.2 *Aspergillus* spp.

Aspergillus spp. are the predominant pathogens regarding invasive mold infections. They occur ubiquitious worldwide, most commonly in organic matter (1, 42).

The most frequent *Aspergillus* spp. causing IFIs is *A. fumigatus*, which is also the most pathogenic species, followed by *A. flavus, A. terreus* and the less common *A. niger* (1).

Infection is usually acquired by inhalation of *Aspergillus* conidia, with impaired mucosae and deficient host defence in the respiratory tract as predisposing factor, but in rare cases also by local tissue invasion after injury or invasive procedures. Pulmonary aspergillosis, occuring after conidial germination and invasion of the pulmonary vasculature, is the most common manifestation of IA. Other invasive syndromes caused by *Aspergillus* spp. include cerebral aspergillosis which has the highest mortality rate, cutaneous aspergillosis and *Aspergillus* endophthalmitis (1, 14, 42). The mortality rate of patients with Aspergillus infection is high, varying from 30% to 95%, according to the population (14).

1.3.3 Other filamentous fungal pathogens

***Mucorales* spp.** Invasive infections caused by *Mucorales* spp. are generally uncommon but their importance has increased recently (1, 43). The most frequently isolated species are *Rhizopus, Lichtheimia* (former *Absidia* and *Mycocladus*) and *Mucor* (20, 44).

The spores of *Mucorales* spp. occur ubiquitious in the enviroment and infection is acquired by inhalation in most cases, less frequently through traumatically or iatrogenically impaired skin (45).

They grow angioinvasive and provoke most commonly rhinocerebral infections, but also pulmonary, gastrointestinal and disseminated infection or mycosis of the central nervous system (CNS) (1, 45).

***Fusarium* and *Scedosporium* spp.** *Fusarium* and *Scedosporium* spp. are filamentous fungal pathogens with a low incidence but a high mortality in severly ill patients (1, 14, 46).

1.3.4 *Cryptococcus* spp.

Cryptococci can be found globally in the environment, but nowadays invasive infections occur primarily in the sub-saharian area where the prevalance of HIV/AIDS is particularly high and access to combined antiretroviral therapy, diagnostics and treatment of IFIs are limited (38, 39).
The major pathogenic *Cryptococcus* spp. are *Cryptococcus neoformans* and *Cryptococcus gattii*. While the fungus is acquired by inhalation, the CNS is its predilection site and the lungs are only the second common site for cryptococcosis (1, 47).

1.3.5 Pneumocystis jirovecii

Infections by the yeast *Pneumocystis jirovecii* occur worldwide. The microorganism is usually transmitted from human to human by aerosols. The main clinical manifestation is interstitial plasma cell pneumonia, ending in respiratory failure if untreated. Other possibly affected organs are the liver, spleen, lymphnodes and bone marrow (14, 38, 40).

1.4 Diagnostic approaches

Although diagnostic tools for IFIs in immunocompromised patients have recently been a field of intensive investigation, the outcome of these patients could only be slightly improved. Early diagnostics and initiation of antifungal treatment are essential to decrease the mortality caused by IFIs. Diagnostic strategies require a combined evaluation of clinical signs and symptoms, as well as diagnostic tools, i.e. radiological findings, histological, chemical and microbiological results. Additionally, a whole variety of new biomarkers have been investigated within the last years to

overcome the limitation of cultural based diagnostic methods, i.e. low sensitivity and long turnaround time. (1, 14).

1.4.1 EORTC/MSG

In 2008, the European Organization for Research and Treatment of Cancer (EORTC) and the Allergy and Infectious Diseases Mycoses Study Group (MSG) published the revised criteria for diagnosis of IFIs (48).

The criteria for each category are summarized in Figure 2.

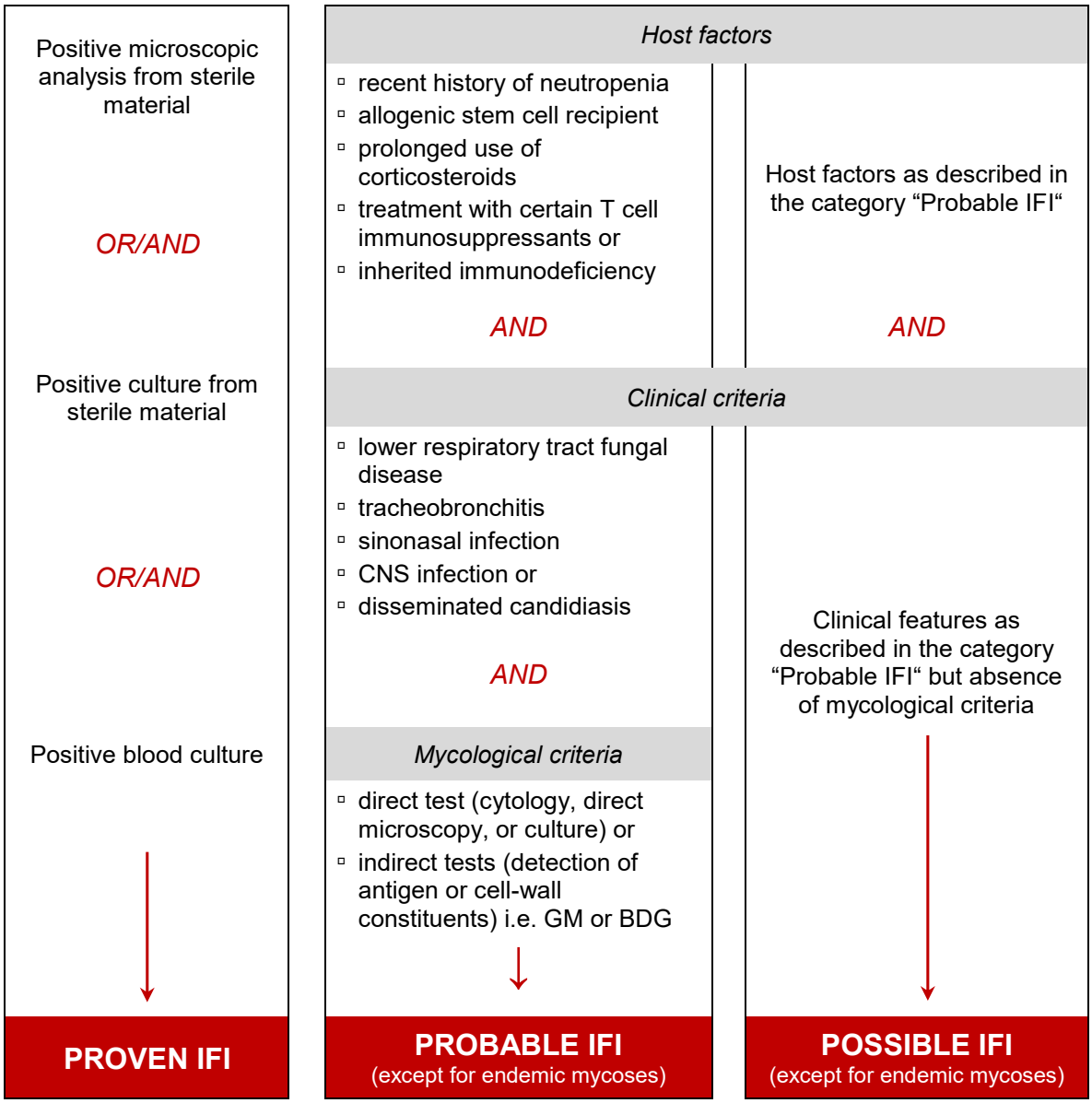

Figure 2. Summary of the criteria for each category. Adapted from (48).

1.4.2 Clinical findings and diagnostic tools

Clinical signs and symptoms. Symptoms of IFIs are generally unspecific and similar to other infections. They include fever > 38.5°C or hypothermia < 36°C and raised levels of inflammatory markers in blood tests (14). Criteria of the systemic inflammatory response syndrome (SIRS) are described in Chapter 3.1.2.

Microbiological culture. The microbiological culture is still considered gold standard and essential for the identification of fungal pathogens, which is important not only for a specific antifungal management, but also for epidemiological reasons. Unfortunately, the time to results can be several days. Besides, sensitivity of cultures remains low and there may be contraindications for the biopsy of sterile sites, i.e. the lung in critically ill patients (2, 3), e.g. because of thrombocytopenia (49).

Cytopathology and histopathology. Microscopic visualization of fungal structures in clinical samples, i.e. bronchoalveolar lavage (BAL), is enabled using 10-15% potassium hydroxide. The use of fluorochromes, i.e. Blankophor increases sensitivity (14). Histopathology is the only way to definitely prove invasive growth of fungi and consequently IFI. Fungal pathogens can be visualized but distinction of morphological features and thus, specification may be difficult and lead to misdiagnosis (2, 14).

Radiological findings. While conventional chest X-rays findings, i.e. infiltrations, cavities or pleural effusion are unspecific, high-resolution computer tomography scans provide high predictive values for early diagnosis of pulmonary infections and features for staging (2, 14). The halo-sign, defined as a "macronodule, surrounded by a perimeter of ground-class opacity" (50), is an early sign of IA and occurs in a majority of concerned patients, but can also be found in mucormycosis or nocardiosis. The air-crescent sign may be observed in later stages of pulmonary aspergillosis. However, negative HR-CT scans cannot exclude the presence of fungal pulmonary infections (14, 50).
Other radiological tests for the detection of IFIs include ophthalmoscopy and ultrasonography of the abdomen and liver (51).

1.4.3 Antigen based biomarkers

Several tests that detect fungal antigens in serum, cerebrospinal fluid (CSF) and urine have become an important part of the diagnostic work-up of IFIs, but should not be used as single detection tool (14):

- **Galactomannan-Antigen test:** When *Aspergillus fumig*atus and other *Aspergillus* spp. grow in tissues, they release galactomannan, a cell-wall polysaccharide. An immunoenzymatic assay named PA-EIA Platelia *Aspergillus* was reported to have an especially high sensitivity and specificity in the detection of galactomannan, although differences of sensitivity depending on the infection site could be observed (52, 53). This test provides early detection of IA infection in serum, BAL and CSF, even before the appearance of first radiological and clinical signs (48, 52). On the other hand, it is limited by the assay's turnaround time that can vary from less than a day to several days depending on the center, as well as possible cross-reactivity and false positive results in patients treated with beta-lactam antibiotics (14, 54). Besides, serum GM detection requires angioinvasive growth of *Aspergillus* in lung tissue. This can be the case in severely neutropenic patients, e.g. hematological patients with ongoing myeloablative therapy, but is often absent in other risk groups for IA development, e.g. COPD patients (55). In patient cohorts without neutropenia, GM performance is limited in serum and therefore needs to be performed in BAL fluid for IA diagnosis (56).

- **Lateral-Flow-Device test:** This immuno-chromatographic assay is based on the detection of an extracellular glycoprotein in BAL fluid and serum, that is secreted by *Aspergillus* during active grow, by a specific monoclonal antibody (JF5) (57). Promising results in the rapid point-of-care diagnosis of pulmonary IA in patients with different underlying diseases, i.e. hematological malignancy, solid organ transplant or respiratory disease could be shown recently (15, 54). This test however, is not yet included in the EORTC/MSG criteria and needs to be further evaluated (54).

- **Mannan/Anti-Mannan test:** The polysaccharide mannan can be found as major component of the cell wall of *Candida* spp. and circulates in serum during infection where it is highly immunogenic (58, 59). The combined detection of this antigen and *Candida* antibodies were reported to have a high sensitivity and specificity for IC, up to 15 days before positive results in blood culture, whereas sensitivity and specificity remained low if only one test, either detection of antigen or antibodies was applied (58, 60, 61). As *Candida* antibodies occur ubiquitious in human sera it is not possible to use them for distinction between colonization, superficial and IC in most cases, especially if mannanaemia is persistent (60, 62).

- **BDG test:** The BDG assay is a panfungal test that is able to detect most fungal pathogens (63). This test will be described in the following Chapter 1.5.

To summarize, the quoted antigen based tests provide rapid results but must be combined with classical methods. The GM-Antigen test as well as the Lateral-Flow-Device can be used for the detection of invasive infections caused by *Aspergillus* spp., the Mannan/Anti-Mannan test is able to detect *Candida* spp., while the BDG test is the only currently available panfungal biomarker (1).

Reverse transcription polymerase chain reaction (PCR). PCR seems to gain importance in diagnostics of fungal disease but it is still limited because of a lack in commercially available standardized assays. It is based on the molecular biological analysis of specific fungal DNA sequences (64).

1.5 BDG as fungal biomarker

As mentioned in Chapter 1.1.1, BDG is a key component of the cell wall of most clinically important fungi and is released into the circulatory system or body fluids during IFIs. It can therefore be used as biomarker for deep mycoses and fungemias (65, 66).

Several assays have been developed for this purpose. They are based on a BDG-triggered activation of the coagulation system of either the American (Limulus polyphemus) or Japanese (Tachypleus tridentalus) Horseshoe Crab. In this study the Fungitell® (former Glucatell) assay (Associates of Cape Cod, East Falmouth, MA, USA) was used, which is the only one approved by the US Food and Drug Administration (FDA). Other currently commercially available tests that are clinically used for BDG detection are the Fungitec-G® test (Seikagaku Biobusiness, Kogyo, Tokyo, Japan), Wako® test (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and Maruha® test (Maruha-Nichiro, Foods Inc., Tokyo, Japan) (67, 68).

Fungi that cannot be detected by BDG assays due to their low amount or even absence of BDG in their cell wall, are *Cryptococcus neoformans* and *Mucorales* spp. (63). However, Rhein et al. critizised that previous studies evaluating BDG performance involved only small numbers of cryptococcosis infections and were focused on determination in serum samples. In a recent study they reported that BDG is detectable in CSF and, with a much lower sensitivity and specificity, even in serum of patients with HIV-associated CM (69).

1.5.1.1 The chemical structure of BDG and its role in the enviroment

BDG (Figure 3) consists of long linear chains of β-(1→3)-D-linked glucose molecules. It is not only found as the predominant glucan component of fungal cell walls (Figure 1), but is also produced by algae (laminarin), bacteria (curdlan), plants (callose) and further organisms. Hence, BDG and subsequently, human exposure to it, is omnipresent (66). In various fungal cell walls polymers of glucans with different linkages, i.e. β-(1→6)-glucans, mixed β-(1→3)- and β-(1→4)-, α-(1→3)-, and α-(1→4)-linked glucans have been found (8).

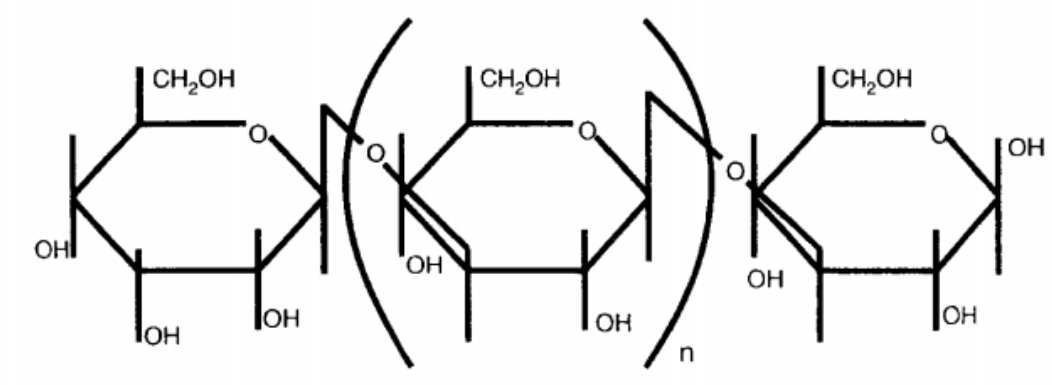

Figure 3. BDG structure. Reprinted from (70).

1.5.1.2 Historical background of BDG testing

In 1956, Frederick Bang and collegues from the Johns Hopkins Marine Biological Laboratory in Woods Hole, MA, USA discovered that an injection of endotoxin into the bloodstream of the arthropodic North American Horseshoe Crab (Figure 4) induced an extensive clotting reaction (71). Further investigations showed that the clotting cascade, called limulus amebocyte lysate (LAL), was initiated by Factor C, a serine protease zymogen, that was released by ameba-like blood cells (72). LAL based pyrogen tests soon became an important evaluation tool used for the approval of pharmaceutical solutions or medical devices (67). Later, another coagulation causing pathway that was not initiated by bacterial endotoxin, but by BDG was discovered. The responsible serine protease zymogen was named Factor G (73).

Serum BDG tests were approved for clinical use in Japan in 1995, in the USA in 2004, while in Europe, they were not included in the EORTC/MSG criteria until 2008 (48).

Figure 4. Horseshoe Crab shell at the Nature Center, Wellfleet Bay Wildlife Sanctuary, MA, USA.

1.5.1.3 The principles of BDG measurement by LAL

As the LAL cascade (Figure 5) is sensitive not only to BDG, but also to endotoxin, the elimination of Factor C is essential for a BDG specific reaction. This is achieved through specific preparations of the reagent used in BDG assays, making only the Factor-G mediated side of the pathway possible. If the serum sample contains BDG, Factor G is activated, that further converts a proclotting to an active clotting enzyme. This clotting enzyme then cleaves p-nitroanilide (pNA) from the chromogenic

substrate Boc-Leu-Gly-Arg-pNA. Through this cleavage pNA turns from colourless to a yellow colour. The rate of optical density increase is determined and interpreted against a standard curve to obtain an estimated concentration of BDG (65, 66). This description refers to the colorimetric method of BDG measurement, as it is applied in the Fungitell assay.

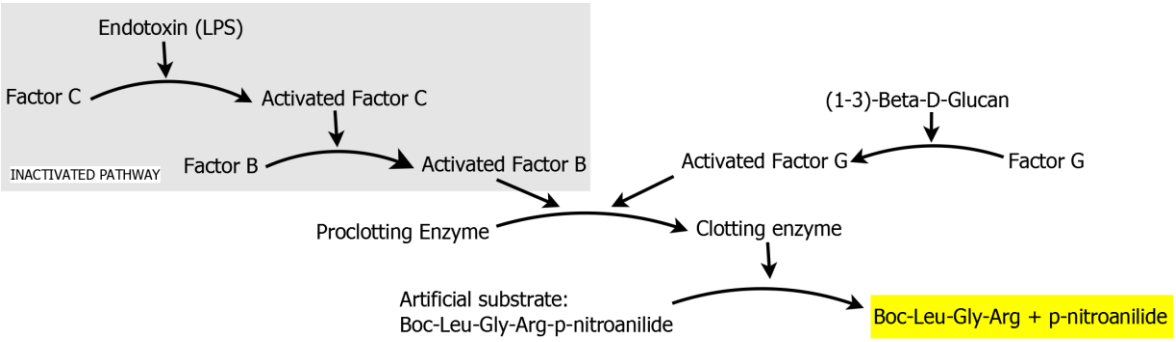

Figure 5. LAL pathway. Adapted from (70).

The original test kits were designed for manual testing and only feasible for the BDG determination of a couple of samples at once, in case of the Fungitell assay, a number of 21 samples (65). In order to enable rapid and cost-efficient single-sample testing, Prüller and collegues adopted the Fungitell assay for automation on a BCS XP coagulation analyzer (74). This method will be further described in Chapter 3.2.1.

1.5.1.4 Applicability and limitations of BDG testing

Pathogenic fungi that produce BDG and can therefore be detected by the referring tests, include *Candida* spp., *Aspergillus* spp., *Fusarium* spp., *Trichosporon* spp., *Saccharomyces cerevisiae*, *Acremonium* spp., *Coccidoides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii* and *Pneumocystis jirovecii* (63). A positive test result suggests a probable IFI in case of underlying risk factors, according to the EORTC/MSG criteria (48) as shown in Chapter 1.4.1. Studies on the performance of BDG assays reported high positive (PPV) and even higher negative predictive values (NPVs) (75-78). The remarkable NPVs, in a metaanalysis by Lamoth and collegues, of 94.6% (75), seem to be of particular interest, as overtreatment with antimycotics in severly ill patients is frequent and can result in unnecessary side effects and a substantial financial burden for health care institutions (79, 80).

However, there are confounding factors and substances that were reported to lower the accuracy of BDG test results and the specificity (65):

- **Infection sites and fungal pathogens:** Factors that can reduce the ability to detect BDG include certain tissue locations and encapsulation that reduce BDG contribution to the bloodstream as well as no or low BDG production by *Cryptococcus, Mucormycetes* and the yeast phase of *Blastomyces dermatidis*, as already mentioned (63, 81-83).

- **Iatrogenic exposure:**
  - Exposure to BDG containing materials during surgery, i.e. gauze or cellulosic membranes can lead to falsely elevated BDG for up to several days (84, 85).
  - The use of cellulose containing depth filters within the process of preparing blood products, i.e. immunoglobulins are causing high BDG levels in these products and are therefore considered a source for false positive beta-D-glucanemia (86). Lo Cascio et al. could recently show that blood components that were produced with synthetic filters seem not to have confounding influence on BDG testing (87). The influence of HD on BDG will be mentioned separately, in Chapter 1.5.1.5.
  - Several broad-spectrum antibiotics that are commonly used in critically ill and immunocompromised patients, i. e. piperacillin-tazobactam, can lead to positive BDG results due to introduction of galactomannan antigens in the manufacturing process. These findings were limited to a few batches (88, 89).

- **Certain infections:** False positive BDG results were also reported in patients with *Enterococcus faecalis* bacteremia (90).

- **Interfering substances:** Several sample conditions can change the optical density of the serum, independently from BDG. According to the manufacturer's instructions for use of the Fungitell test these interfering sample conditions include hemolysis, sample turbidity caused by lipemia, the presence of visually apparent bilirubin and turbid serum (65). Pickering et al. reported that high concentrations of bilirubin and triglycerides caused false negative and hemolysis false positive BDG results (78).

Furthermore, potential, but yet unproven sources of contamination have been found. It remains unclear if mucositis is a cause of falsely elevated BDG (91). The exposure to echinocandins is suspected to decrease BDG levels as their therapeutical mechanism is based on the inhibition of BDG synthesis (92). Polyenes were observed to let BDG levels increase, whereas in this certain study, echinocandins seemed not to influence serum BDG concentration (93). Treatment with antitumor polysaccharides is also suspected to falsely elevate serum BDG (78) and there might be factors for false positivity that are not yet known (94).
A high-dose oral intake of BDG supplements, that have been commercialized for alleged immune system stimulating effects (95), seems not to interfere with BDG determination, but studies in patients with a leaky intestinal barrier are lacking (96).

1.5.1.5 The background of dialysis techniques and how various dialysis membranes influence serum BDG levels

Principles of dialysis. Dialysis is based on the exchange of solute substances between the blood compartment of the patient and a suitable dialysis fluid across a semipermeable membrane (97). Dialysate is produced by the dialysis machine from tap water that is specially treated and enriched with electrolytes and buffer substances (98). In HD, the solutes are moved by passive diffusion down a concentration gradient, while in hemofiltration (HF) the movement of water and solutes is achieved by filtration down a pressure gradient. HDF combines these two principles (97, 99). PD uses the peritoneum as semipermeable membrane. Dialysis fluid containing a high amount of osmotic molecules and electrolytes and buffers is infused through a catheter to the peritoneal cavity. The exchange of substances is based on diffusion, ultrafiltration and convection (99, 100).

Dialysis membranes. Various materials have been used for dialysis membranes (Table 1). Nowadays, synthetic and modified cellulose membranes have replaced cellulosic membranes that are known to trigger an inflammatory reponse and therefore have a low biocompatibility (97, 101).

	Unmodified cellulose	Modified cellulose	Synthetic
low-flux	- Cuprammonium rayon (Cuprophan) - Cuprammonium cellulose	- Haemophan - Cellulose diacetate (Dicea, Diaphan, Althane)	- Polysulfone (Biosulfan, PS, Helixone) - Polycarbonate (Gambrane)
high-flux		- Cellulose triacetate (Tricea)	- Polysulfone (Biosulfan, PS, Helixone) - Polyamide (Polyflux) - Polyethersulfone (Syntra) - Polyacrylonitrile (AN69) - Polymethylmethacrylate (PMMA)

Table 1. Materials of dialysis membranes. Adapted from (97).

BDG as fungal biomarker in patients on dialysis. The first reports about LAL reactive, but non-pyrogenic extracts that were released by unmodified cellulose hollow fiber dialyzers, date back to the 1980ies (102-105). A research group of Yamagami et al. was one of the first who demonstrated that cellulosic β-D-glucan was responsible for the cross-reaction with Factor G from the LAL cascade (106). Recent studies confirmed that unmodified cellulose membranes caused falsely elevated BDG levels in the absence of IFIs, but BDG results for modified cellulosic membranes are conflicting. Kato et al. determined highly elevated BDG levels in patients receiving dialysis via modified regenerated cellulose membranes (MRC), whereas patients with synthetic polysulfone (SP) membranes remained BDG negative. Furthermore, they observed a gradual decrease of BDG levels in patients who where switched from MRC to SP dialyzers (107). In a study by Kanda et al. BDG levels increased after dialysis using Cellulose (Cu) membranes, but remained negative in patients with Cellulose triacetate (CTA) and polymethyl methacrylate (PMMA) membranes (108) (Table 2).

Filter material		False positive BDG levels	Source
Unmodified cellulose	Not further specified	Yes	(104)
	Cuprophan	Yes	(105)
	Not further specified	Yes	(108)
Modified cellulose	MRC (modified cuprophan)	Yes	(107)
	Cellulose acetate	No	(105)
	CTA	No	(108, 109)
Synthetic	PS	Yes*	(110, 111)
	PS	No	(107, 109)
	PMMA	No	(108)
PD	-	No	(112, 113)
*the authors suspected other confounding factors than dialysis for the false BDG positivity			

Table 2. Various filter materials and their influence on BDG measurement. Adapted from (101).

In conclusion, the frequently cited recommendation that BDG determination for IFI diagnostics should not be applied in dialysis patients seems to refer primarly to those with unmodified cellulose dialyzers and cannot be generalized. The influence of synthetic membranes on BDG levels seems to be negligible but needs to be further investigated (101, 107, 108).

1.5.1.6 The performance of BDG testing in various risk populations for IFIs

At the end of this chapter, the results of studies quoted below will be summarized in Table 3.

- **Hematological patients**

  IA or candidemia. In the third European Conference on Infections in Leukemia meeting, evidence for BDG detection in serum of hematological high risk patients was rated to be moderate. Therefore it was recommended to use BDG testing in conjunction with clinical, microbiological and radiological findings (68). These recommondations are based on the ECIL-3-meta-analysis of the diagnostic performance of BDG antigenemia in IA or candidemia by Lamoth et al. that included six high-quality cohort-studies (114-118) focused on hemato-oncological patients. For two consecutive tests, the cumulative estimates of sensitivity and specificity were 50% and 99%, respectively (75).

PCP. For the use of BDG determination in diagnosis and monitoring of PCP in hematological patients the same recommendations as for HIV/Aids patients as described above can be applied (119).

- **HSCT**

  IA and IC. Koo et al. observed a high test specificity (93%) but limited sensitivity (43%) among patients with HSCT and suggested BDG detection as diagnostic adjunct (120). In a study on BDG performance focused on HSCT patients only, Reischies and collegues reported a remarkable NPV of 99% and suggested BDG testing as promising tool to rule out IFIs in this risk group (121).

- **Solid organ transplantation**

  The performance of BDG testing in solid organ transplant recipients has not yet been part of extensive investigations. A study in lung transplant recipients considered the high NPV of 97% as the most relevant feature, as sensitivity (63%), specificity (66%) and especially PPV (9%) remained low in this population (110).

  Another study on the preemptive treatment of IFIs based on BDG levels after liver transplantation, observed similar values on the test performance (122).

- **ICUs**

  IC. As *Candida* spp. are considered one of the most frequent pathogens causing infections in ICU patients, there is a certain need for reliable and fast biomarkers, i.e. BDG (123). Posteraro et al. could demonstrate the usefulness of BDG measurement to detect IC early in ICU patients. In this certain study the sensitivity, specificity, PPV and NPV for single sample BDG testing reached 93.7%, 93.6%, 75.0%, and 98.6%, respectively. BDG elevation occurred 24 to 72 hours before blood cultures turned positive in candidemic patients (76). Similar observations of increased BDG levels in ICU patients, days before IC diagnosis were made in other studies (124, 125).

  IC and IA. A longterm evaluation in ICU patients documented the course of BDG levels that were persistently high and decreased slowly in proven IFI. In contrast, BDG levels of patients not affected by IFIs, started to increase at day 14, but not up to serum levels observed in patients with proven IFI. This shows once more

the necessity to be aware of confounding factors and the conjunction of several diagnostic tools (126).

Prattes et al. found evidence for the usefulness of BDG as marker for antifungal treatment stratification at the ICU. Depending on BDG results, antifungal therapy was either started, continued or discontinued in the participating patients. In about one third of patients who had empirically received antifungal treatment, this therapy could be safely discontinued (77). This finding is of high importance, regarding the frequent antifungal treatment in ICU patients without evidence for IFIs (79).

- **Renal diseases**

  Specific studies on the applicability of BDG determination in renal diseases are lacking, whereas several studies regarding the influence of dialysis membranes have been conducted and were already mentioned above. As fungal pathogens are seldomly responsible for peritonitis in patients on PD, there are just individual cases decribed on BDG testing in this patient population. However, BDG measurement in peritoneal fluid seems to be useful in the detection of fungal peritonitis (112).

- **AIDS/HIV**

  CM. The potential of BDG testing in CSF obtained from HIV patients with CM was mentioned above. In the cited study of Rhein et al. BDG was detectable in CSF with a high sensitivity of 89% and specificity of 85%. Moreover, it was observed that BDG in CSF normalized rapidly after initiating antifungal therapy. In conclusion, the group suggested BDG measurement in CSF as a diagnostic adjunct for the evaluation of treatment efficacy (69).

  PCP. BDG detection was reported to be a useful tool in diagnosis of PCP in HIV-infected patients, especially in those who are not able to undergo BAL. As the serum level does not correlate with disease severity it cannot be used for monitoring the response to antifungal therapy (119, 127).

Study population	Type of IFI	Test medium	Assay (cut-off)	Sensitivity	Specificity	PPV	NPV	Source
Hematological malignancies	IA or IC	serum or plasma	Fungitell (60-80 pg/mL); Fungitec-G (20 pg/mL); Wako/Maruha (11 pg/mL)	50% [a]	99%[a]	84%[a]	95% [a]	(75) Metaanalysis
HSCT	IC or IA or PCP/other IFIs	serum	Fungitell (80 pg/ml)	43%[b] 64%[c]	93% [b] 91% [c]			(120)
HSCT	IA or other IFIs	serum	Fungitell (80 pg/ml)	81%	98%	65%	99%	(121)
Lung transplant recipients	IA or IC	serum	Fungitell (60 pg/ml) Fungitell (80 pg/ml)	64% 63%	9% 66%	14% 9%	50% 97%	(110)
Liver transplant recipients	IA or IC or PCP	plasma	Fungitec G test (40 pg/ml)	58%	83%	35%	93%	(122)
Non-neutropenic ICU patients	IC	serum	Fungitell (80 pg/ml)	94%	94%	72%	99%	(76)
Surgical and medical ICU	IC	serum	Fungitell (80 pg/ml)	100%[d]	75%[d]	30%[d]	100%[d]	(111)
Surgical and medical ICUs	IA or IC	serum	Glucatell (40 pg/ml)	52%	76%	46%	80%	(126)
Aids/HIV	CM	CSF	Fungitell (80 pg/ml)	89 %	85 %	92%	79%	(69)
		serum		79%	61%	61%	79%	(69)
	PCP	serum	Fungitec G MK (23.2 pg/ml)	96%	88%			(127)
Aids/HIV and hematological malignancies	PCP	serum	Fungitell (60 pg/ml) Fungitell (80 pg/ml) Fungitell (100 pg/ml)	100% 100% 100%	85.7% 89% 96.4%			(119)

[a] Estimated values for two consecutive tests
[b] Initial BDG assay for IFIs
[c] Highest BDG level for IFIs at end of hospitalization
[d] Two sequential BDG detections for optimal assay performance
Abbreviations: IFIs, invasive fungal infections; PPV, positive predictive value; NPV, negative predictive value; HIV, humane immunodeficiency virus; CM, cryptococcal meningitis; CSF, cerebrospinal fluid; PCP, *Pneumocystis jirovecii* pneumonia; IA, invasive aspergillosis; IC, invasive Candidiasis; HSCT, hematopoietic stem cell transplantation; ICU, intensive care unit;

Table 3. Performance of BDG testing in the above cited studies.

1.6 Antifungal prophylaxis and treatment strategies

There are three main groups of drugs used for antifungal therapy in clinical routine:

- Azoles (fluconazole, voriconazole, posaconazole, isavuconazole, itraconazole)
- Polyenes (liposomal amphotericin B)
- Echinocandins (caspofungin, anidulafungin, micafungin) (1, 14)

Factors that must be considered in the selection of antifungal agents include the fungal spectrum, potential adverse effects, interactions with other drugs, types of application, clinical efficacy and epidemiology. Moreover, it is influenced by the site of infection, age, clinics and comorbidities of the patient and the severity of disease (1, 14).

As diagnosis remains challenging and mortality increases rapidly if onset of antifungal management is delayed, there is a practice of prophylaxis at time of high-risk, as well as empirical therapy of suspected IFIs in neutropenic patients (14, 128, 129).

Figure 6 shows an example how biomarkers, i.e. BDG assays are integrated into antifungal treatment strategies (130).

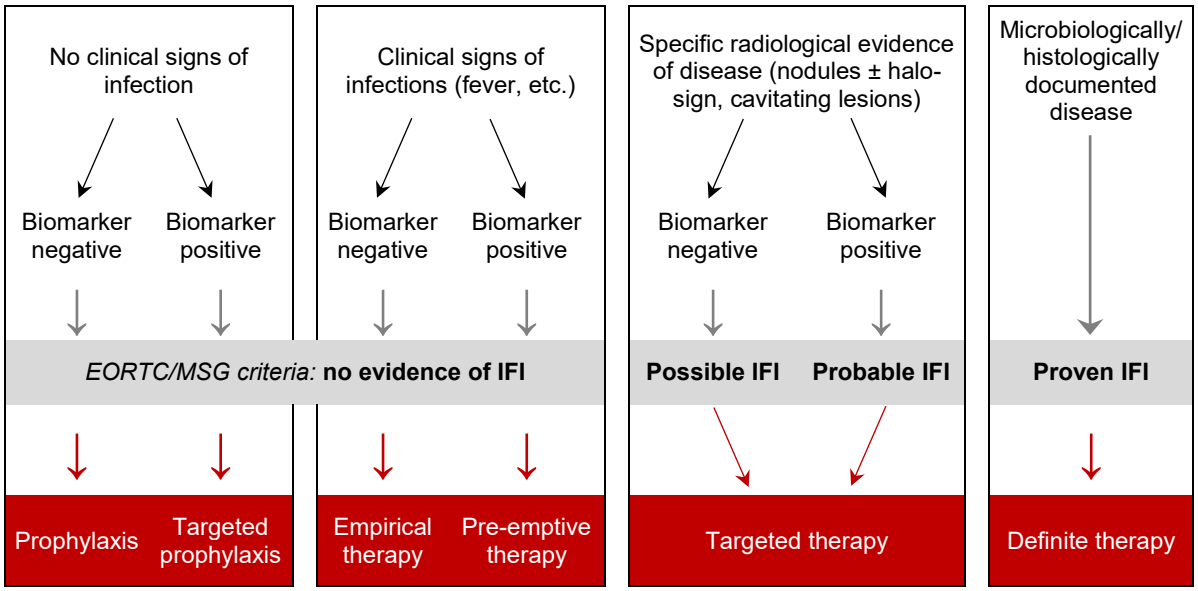

Figure 6. Progression of directed therapy, with IA as an example. Adapted from (130).

2 Study hypothesis and aims

2.1 Study hypothesis

Serum BDG levels are not influenced and not falsely elevated due to HD, HDF or PD using state of the art techniques and devices.

2.2 Study aims

A. In vivo

The primary objective of the in vivo part of the study was to examine, whether dialysis therapy (HD/HDF or PD) caused increase of serum BDG levels in patients without evidence for IFIs.

In patients receiving HD or HDF treatment, the influence of synthetic membranes (e.g. Polyflux, Gambro; FX series, Fresenius Medical Care (FMC)) on serum BDG was evaluated.

Secondary objectives of the study included an assessment of the number of false positive BDG samples in patients receiving dialysis treatment, the kinetics of serum BDG in those patients, the influence of PD, as well as the influence of CKD itself on serum BDG.

B. In vitro

The primary objective of the in vitro part of the study was to demonstrate that standard polysynthetic dialysis membranes are neither releasing nor leaching BDG during HD.

3 Methods

A. In vivo

We conducted a prospective, observational cohort study in patients receiving various types of dialysis treatment and a group of patients with CKD that were not on dialysis. Only patients without signs and symptoms for IFIs were enrolled. BDG determination was conducted in blood and dialysate samples, applying a special sampling scheme for each study group.

3.1 Participants

During the inclusion period, all consecutive adult patients receiving HD, HDF or PD at the Clinical Division of Nephrology, Department of Internal Medicine, Medical University of Graz, Austria were screened for study eligibility criteria using their electronic medical records. Furthermore, patients with CKD but without dialysis were screened.

3.1.1 Inclusion Criteria

- Age ≥ 18 years
- CKD stage 5d receiving HD, HDF or PD treatment at the Division of Nephrology, Department of Internal Medicine, Graz, Austria
- CKD stage 3a, 3b, 4 or 5 not treated by dialysis
- Signed informed consent

3.1.2 Exclusion Criteria

- Patients who had surgery within the last seven days
- Patients who were diagnosed with IFI within the last two months
- Solid organ transplantation in past medical history
- Active malignoma
- Intake or requirement of listed drugs
    - Systemic antifungals
    - Beta-lactam antibiotics
    - Immunoglobulins

- Blood-derived products
- Signs and symptoms of ongoing systemic infection
  - Body temperature > 38.5°C
  - Presence of two or more of the SIRS criteria
    - → Body temperature > 38°C or < 36°C
    - → Heart rate > 90 beats/minute
    - → Respiratory rate > 20/minute
    - → $paCO_2$ < 33 mm Hg
    - → WBC < 4,000 cells/mm³ or > 12,000 cells/mm³ or > 10 % immature bands
  - C-reactive protein (CRP) level > 5 mg/l and WBC > 12,000 cells/mm³

The inclusion and exclusion criteria were controlled using a prepared questionnaire and checklist on each inclusion day. The questionnaire is attached at the end of the manuscript.

3.1.3 Data collection

Test kits for each patient including the questionnaire and check list, a blood test order form for our clinical laboratory and defined blood collection tubes were prepared.

A case report form was created using Microsoft Excel for collection of the participant's data, including demographic data (birth date, age on inclusion day, gender), the patient's current diseases and medications, details of the dialysis settings, signs and symptoms of potential ongoing systemic infections, results of routinely performed laboratory tests and study related measurements.

This study was conducted in accordance with the Declaration of Helsinki, 1996, Good Clinical Practice, and applicable local regulatory requirements and law.

3.2 Test methods (in vivo)

3.2.1 BDG measurement

BDG levels were determined using the Fungitell® assay (48, 65) with an adopted BDG protocol suitable for use on a routine BCS XP® coagulation analyzer (74) (Figure 7) at the Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz.

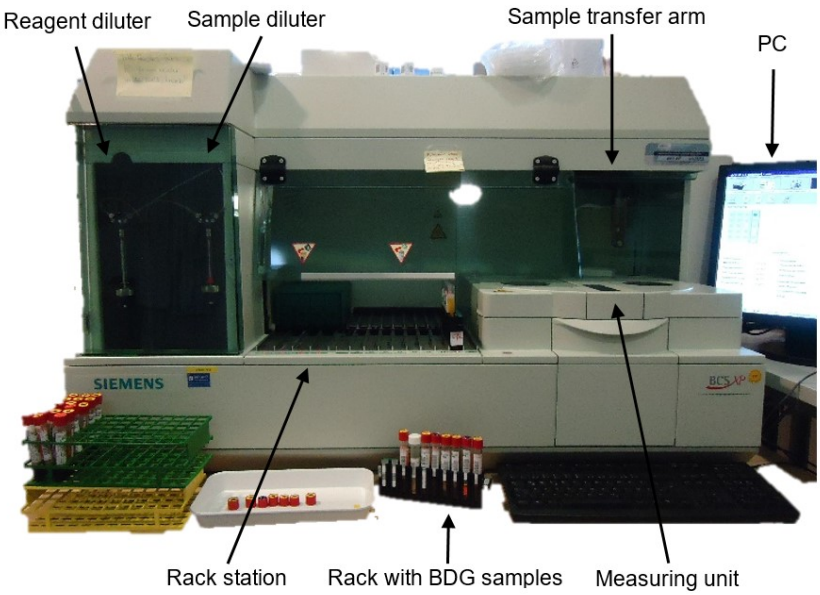

Figure 7. BCS XP® coagulation analyzer at the Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz.

The Fungitell reagent was prepared following the instructions of the manufacturer, aliquoted and stored at -70°C. The reagent used for serum pretreatment that dissociates triple-helix into single-stranded BDG, was produced by combining equal volumes of 0.25 M potassium chloride (KCl) with 1.2 M potassium hydroxide (KOH). The BDG standard that was provided by the original Fungitell kit was used as positive control and distilled water as negative control (65, 74).

If the sample was collected between 8 am and 4 pm, serum and dialysate BDG testing were performed immediately. If samples were collected between 4 pm and 8 am, the blood collection tubes were stored at 7° Celsius without being opened for a maximum of 16 hours before testing.

After centrifugation of the serum samples, the sample tubes, controls, thawed Fungitell reagent and serum pretreatment agent were placed on the rack station and the program for fully automated BDG measurement was run.

According to the instructions for use of the Fungitell® assay (65), BDG values greater than or equal to 80 pg/mL were interpreted as positive, values from 60 to 79 pg/mL as indeterminate and values lower than 60 pg/mL as negative result. The lower detection limit by the BCS XP® coagulation analyzer was 15.4 pg/ml, the upper detection limit 4000 pg/ml (74).

For retrospective analysis or retesting, remaining serum and dialysis fluid samples were labeled with unique codes and stored at -70° Celsius.

BDG determination was conducted by the medical technical assistants who usually perform those laboratory tests at the institution and they were blinded on the origin of the samples.

3.2.2 Routine procedures

Prior to sample collection and dialysis treatment, a questionnaire and check list was used for each patient to take medical history and to evaluate the inclusion and exclusion criteria.
In order to detect potential signs and symptoms of systemic infections, body temperature, respiratory rate, blood pressure and heart rate were measured by the dialysis nurses.
Blood tests indicative for systemic infections including leucocytosis, leucopenia, neutrophilia, and elevated CRP were performed as part of the routine laboratory measurements.

3.2.3 Sample collection

Blood samples were drawn by the study investigators themselves or by instructed dialysis nurses under supervision of an investigator, using Vacuette® blood collection tubes. In order to avoid potential contamination from gauze or other

cellulose materials that were used for disinfection before vascular puncture, samples for BDG testing were drawn after the EDTA samples.

In the following the various sampling schemes for each study subgroup will be described.

3.2.3.1 Subgroup 1: Patients suffering from CKD but without ongoing dialysis treatment

In order to exclude renal failure as a potential confounder for elevated serum BDG levels, patients suffering from CKD but not receiving dialysis treatment were included in this study. Blood collection was performed within the monthly routine check-up at the outpatient clinic.

Assessment list	Visit 1
	Baseline investigations
Informed consent	X
Inclusion/exclusion criteria	X
Routine check of vital signs	X
Routine laboratory tests	X
Blood samples for study reasons	X

Table 4. Schedule of assessements for patients with CKD but without dialysis.

After the questionnaire and clinical examination, a routine EDTA sample and serum sample were taken. Then, an additional serum sample for BDG determination was taken (Table 4).

3.2.3.2 Subgroup 2: Patients undergoing HD or HDF

HD and HDF patients received dialysis using the following dialysis equipment:

- 4008H HD machines (FMC Schweinfurt, Germany)
- Polysulfone filters (Diasafe®, FMC)
- Solid sodium bicarbonate (BiBag, FMC) and liquid acid concentrates (SW240A, B Braun, Melsungen, Germany)
- Standard blood lines

- Standard dialyzers: Polyflux 17L, Polyflux 170H or Polyflux 210H (Polyamix membrane material: Polyarylethersulfone, Polyvinylpyrrolidone and Polyamide), Helixone FC Cor Diax 1000 or Helixone FC Cor Diax 8000 (Helixone membrane material: Polysulfone)

Samples were collected at two consecutive dialysis sessions applying the described time schedule (Table 5).

Assessment	Visit 1				Visit 2	
	Baseline	t0	t30	t180	Baseline	t0‘
Informed consent	X					
Inclusion/exclusion criteria	X				X	
Routine check of vital signs	X				X	
Routine laboratory tests	X				X	
Blood samples		X	X	X		X
Dialysate in- and outflow samples		X	X	X		

Table 5. Schedule of assessment for patients on HD/HDF.

During the first session, labeled as Visit 1, blood and dialysate samples were taken right before the start of HD/HDF (t0), after 30 minutes (t30) and after 180 minutes (t180) of treatment. At the second session (Visit 2) blood samples were collected just once – right before dialysis (t0‘). Patients usually receive dialysis every second or third day, thus the second session is considered the treatment two or three days after the first sample collection.

Further details of sample collection are depicted in Figure 8 and Table 6.

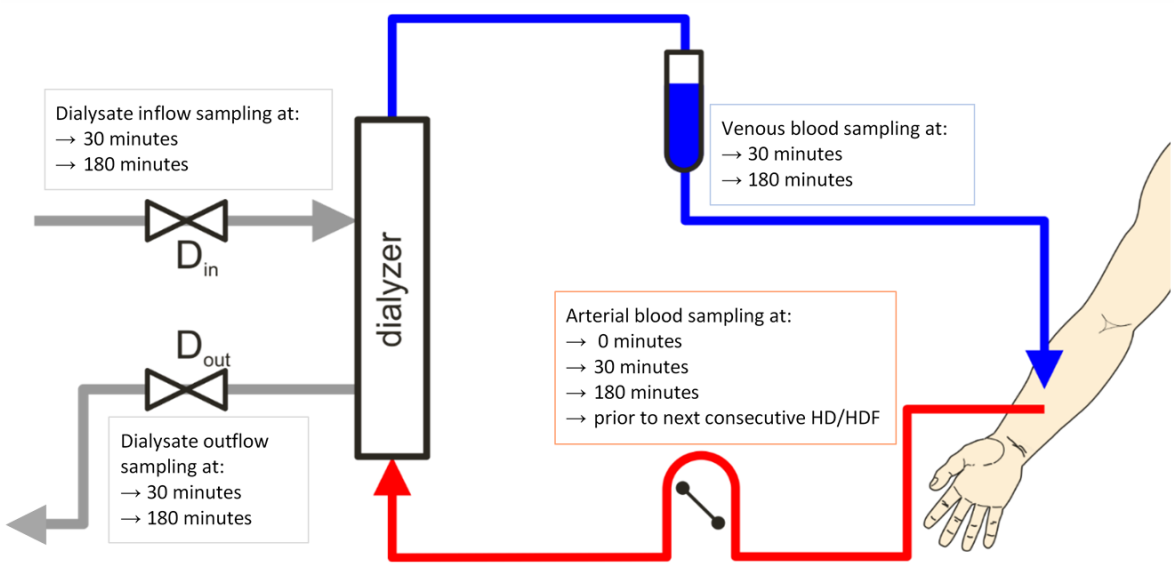

Figure 8. Schedule of sample collection in HD/HDF patients.

The description “arterial blood” refers to the patient's blood pumped towards the dialysis machine, while “venous blood” means the already dialyzed blood being returned to the patient. “Dialysate inflow” refers to the fresh dialysate and “dialysate outflow” refers to the used dialysate that has already been in contact with the patient's blood via the dialyzer's membrane.

	Blood samples					Dialysate samples	
Point of Time	**Serum arterial**	**Serum venous**	**EDTA arterial**	**EDTA venous**	**Lithium-heparine**	**Dialysate inflow**	**Dialysate outflow**
t0	X		X		X		
t30	X	X	X	X		X	X
t180	X	X	X	X		X	X
t0‘	X		X		X		

Table 6. Blood and dialysate sampling in detail.

At Visit 1, after puncture of dialysis shunts, one arterial serum, one EDTA and one Lithiumheparine tube were collected from the participants and used for t0 BDG determination and further blood investigations.

Thirty and 180 minutes after initiating the dialysis session, one EDTA and one serum sample were taken from each the arterial and venous lumen of the dialysis catheter.

Additionally, one sample from both the fresh and used dialysate (dialysate in- and outflow, respectively) were drawn.
At Visit 2, one arterial EDTA, one Lithiumheparine and one serum tube were drawn only once before dialysis (t0‘).

3.2.3.3 Subgroup 3: Patients undergoing PD

Patients on PD were included at a routine appointment at the nephrology outpatient clinic.

Assessment	Visit 1
	Baseline
Informed consent	X
Inclusion/exclusion criteria	X
Routine check for vital signs	X
BDG sample from the dialysis bag	X
Routine laboratory tests	X
Blood samples	X
Sample of dialysis solution outflow	X
Sample of dialysis solution inflow	X

Table 7. Schedule of assessment for patients on PD.

As shown in Table 7, one serum sample and one sample from dialysis outflow solution were drawn for BDG testing.
Additionally, dialysis inflow solutions were tested to exclude potential BDG contamination.

3.3 Statistical methods

Statistical analysis was performed using IBM SPSS statistics version 22 (IBM Corp., Chicago, IL, USA).

Descriptive statistics included measures of median and 25% to 75% interquartile range (IQR) for continuous variables and absolute numbers and percentages for categorical variables.

Normal distribution of variables was assessed using the Kolmogorov-Smirnov test.

For the analysis of differences in continuous variables between all three subgroups, the non-parametric Kruskal-Wallis test was used. In case of statistically significant results the Mann-Whitney U test was applied for follow-up comparisons between two subgroups. The non-parametric Friedman test was used to determine changes in BDG levels between the four various time points during HD/HDF dialysis treatment.

P-values < 0.05 were considered statistically significant.

Specificity with 95% confidence intervals (CI) were calculated for BDG performance.

B. In vitro

3.4 Test methods (in vitro)

The in vitro part of the study was conducted at the Institute of Physiology, Medical University of Graz, Austria. Simulating a HD session using state of the art materials and devices, the potential leaching and leaking of BDG from a BDG-contaminated dialysate compartment into an initially BDG-free blood compartment was examined in an experimental setup.

3.4.1 Materials

The following dialysis equipment was used for the experiments:

- 4008H HD machine (FMC)
- Polysulfone filters (Diasafe®, FMC)
- Solid sodium bicarbonate (BiBag, FMC) and liquid acid concentrates (SW240A, B Braun)
- Standard blood lines
- Standard high-flux dialyzer (Polyflux 210H, Gambro AB, Hechingen, Germany)

Three different solutions containing high concentrations of BDG were produced, using the following BDG sources:

- Overnight cultures of *Candida albicans* (*Candida albicans* reference strain ATCC 90028), grown in tryptic soy-broth (TSB, Becton Dickinson, Le Pont de Claix, France)
- Extracts from non-sterile cellulose swabs (Profümed, Grimmenstein, Austria), suspended in dialysate
- Carboxymethyl modified BDG (CM-curdlan, Megazyme International, Wicklow, Ireland) with a molecular mass of approximately 1.10^6 D (g/mol), extrapolated from colloid osmotic pressure measured in dilute solutions (131)

BDG measurements in the obtained solutions had concentrations in the range of 300 to nearly 4000 pg/mL.

3.4.2 Setup and procedure

The dialysis machine's hot citric acid disinfection programme was run before each experimental dialysis session.

Simulating a standard dialysis treatment, the length of each session was set to four hours, the blood compartment flow rate to 300mL/min and the dialysate flow rate to 500 mL/min. The applied dialysis mode was HD without ultrafiltration. Dialysate conductivities were set at 14 mS/cm and dialysate temperature at 37°C.

The dialysate compartment was perfused and recirculated by the BDG spiked solutions, using a 5 L reservoir. For perfusion of the blood compartment, ultrapure dialysate that was produced by the dialysis machine within one single-pass setup was used (Figure 9).

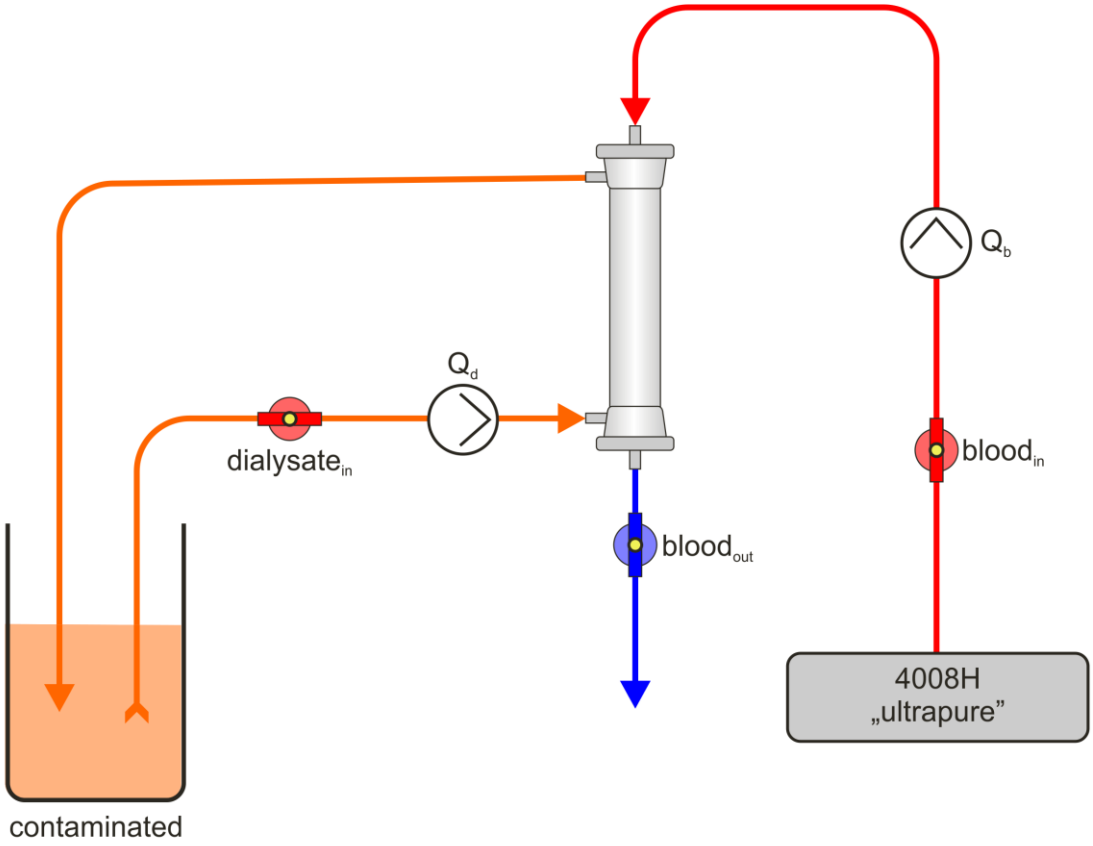

Figure 9. Setup for the simulation of a HD session.

Abbreviations: Q_b, Blood compartment; Q_d, Dialysate compartment; Sampling ports: blood$_{in}$, blood$_{out}$, and dialysate$_{in}$.

3.4.3 Sample collection for BDG testing

As in the in vivo study's sample collection, samples were drawn using Vacuette® blood collection tubes.

Potential cellulose born contamination was prevented by avoiding cellulose containing swabs for disinfection.

Samples for BDG determination were drawn from the sampling ports (Figure 9) at baseline (0) and in intervals of one hour (1, 2, 3, 4) from the in- and outflow of the dialyzer blood compartment ($blood_{in}$, $blood_{out}$) and from the recirculating and BDG spiked solution of the dialyzer's dialysate compartment ($dialysate_{in}$).

BDG levels were tested at the Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, in the same way and with the same cut-off levels as described in Chapter 3.2.1.

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4 Results

A. In vivo

4.1 Participants

4.1.1 Subgroup 1: Patients suffering from CKD but without ongoing dialysis

Between March and April 2015, all adult patients with renal failure but without ongoing dialysis, who had presented at the Division of Nephrology, Department of Internal Medicine, Graz for a routine check-up, were screened for our participation criteria. We collected blood samples from the first ten patients who met the inclusion criteria, had no exclusion criteria and agreed to give informed consent. All enrolled individuals of this subgroup were included in the data analysis.

4.1.2 Subgroup 2: Patients undergoing HD or HDF

In April 2015, all adult patients receiving HD or HDF at our Division of Nephrology were screened for in- and exclusion criteria.

We assessed 34 patients for eligibility (Figure 10). Of those assessed, 22 received HD and 12 received HDF treatment.
From these patients, 15 HD and five HDF patients did not meet inclusion criteria or met exclusion criteria as described in Figure 10.
Two HDF patients were not included in analysis because chemical laboratory tests showed highly elevated levels of lipids and triglycerides.
Thus five HD and seven HDF patients were included in data analysis.

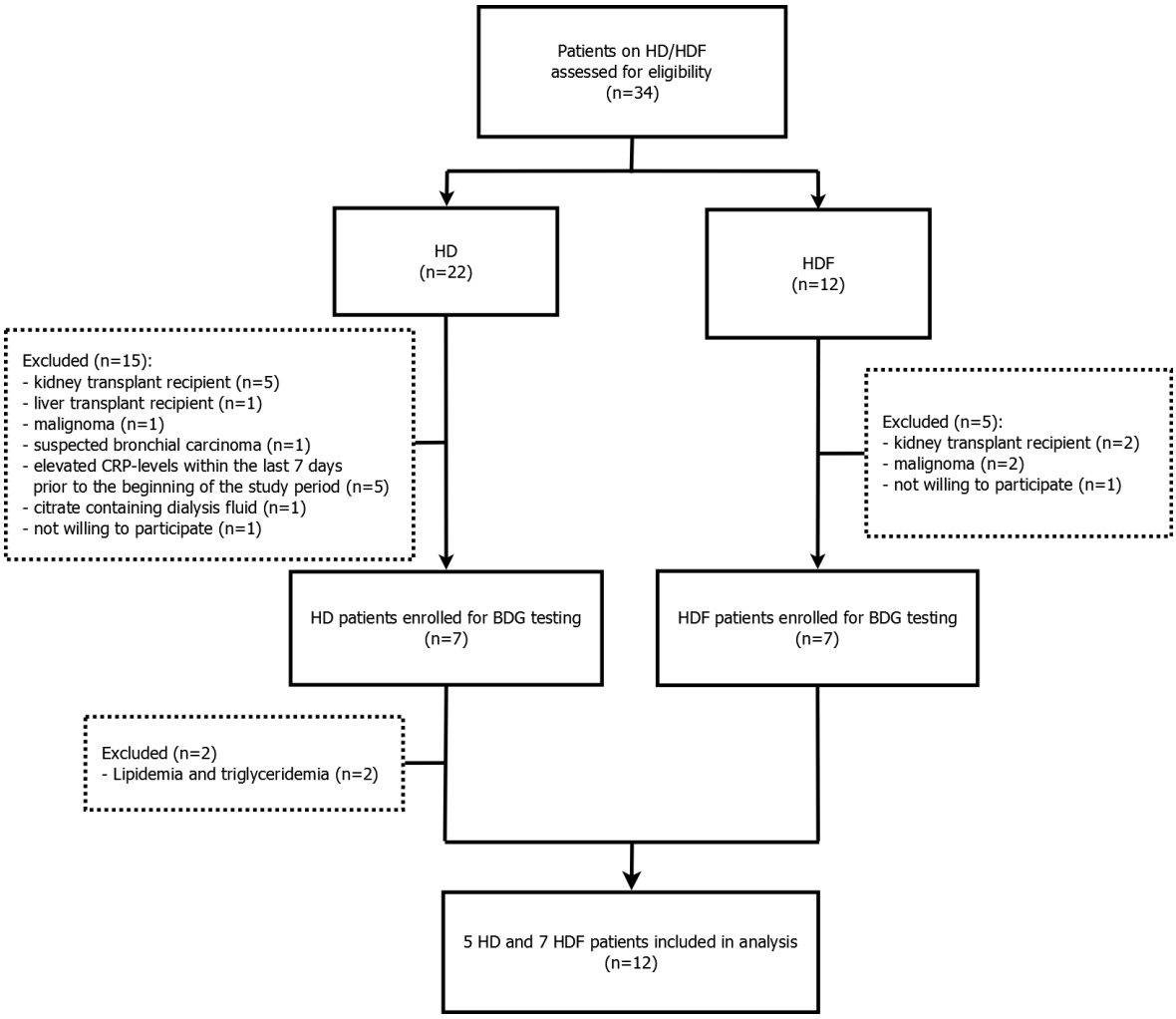

Figure 10. Enrollment flowchart of HD/HDF patients.

4.1.3 Subgroup 3: Patients undergoing PD

In August 2015 all adult patients receiving CAPD were screened and the first ten who met our criteria were included in the study.

4.1.4 Demographic and clinical characteristics

Demographic and clinical characteristics of the participants are listed in Table 8.

Parameter	Subgroup 1: Patients without dialysis (N=10)	Subgroup 2: Patients on HD/HDF (N=12)	Subgroup 3: Patients on PD (N=10)
Age, years	66.5 (44-86)	63.5 (41-78)	61 (28-72)
Female sex, n (%)	2 (20)	6 (50)	4 (40)
CKD – stage, n (%)			
III	2 (20)	-	-
IV	4 (40)	-	-
V	4 (40)	12 (100)	12 (100)
Dialysis vintage, months	-	9.5 (1 – 73)	21 (5 – 47)
HD	-	7	-
HDF	-	5	-
Materials of Dialysis Membrane, n (%)			
Polyamix*	-	10 (83.3)	-
Helixone**	-	2 (16.7)	-
Dialyzer, n (%)			
Polyflux 17L	-	6 (50)	-
Polyflux 170H	-	3 (25)	-
Polyflux 210H	-	1 (8.3)	-
Helixone FC Cor Diax 1000	-	1 (8.3)	-
Helixone FC Cor Diax 8000	-	1 (8.3)	-
GFR (ml/min), (IQR)	18.2 (10.7 – 27.8)	6.5 (5.5 – 7.3)	5.9 (4.2 – 6.5)
Creatinine (mg/dl), (IQR)	3.4 (2.2 – 4.7)	7.2 (6.4 – 8.6)	8.3 (6 – 11.1)
Leucocytes (G/l), (IQR)	7.7 (7.3 – 8.7)	6.6 (5.6 – 7.9)	6.5 (4.9 – 8.3)
CRP (mg/l), (IQR)	2 (1.6 – 6.5)	3.4 (2.1 – 5.2)	1.6 (1 – 9.1)
Primary renal diagnose, n (%)			
Renovascular Disease	3 (30)	2 (16.7)	1 (10)
Diabetes mellitus	1 (10)	1 (8.3)	2 (20)
Primary glomerulo-pathy/-nephritis	1 (10)	3 (25)	4 (40)
Interstitial nephropathy	-	-	1 (10)
Cystic kidney disease	1 (10)	2 (16.7)	-
Multisystem disease	2 (20)	3 (25)	1 (10)
Other	2 (20)	1 (8.3)	1 (10)

Median and range displayed, besides otherwise noted.
*= Polyarylethersulfone, Polyvinylpyrrolidone and Polyamide blend
**= Polysulfone

Table 8. Baseline characteristics of patients.

Age. The median age of patients without dialysis was 66.5 years (range 44 to 86), of patients on HD or HDF 63.5 years (range 41 to 78) and of patients on PD 61 years (range 28 to 72). The distribution of age showed no significant difference across the three subgroups (p=0.455).

Gender. The group without dialysis consisted of two female (20%) and eight (80%) male patients. The HD/HDF group consisted of six women (50%) and six men (50%). Four patients on PD (40%) were female and six (60%) male (Figure 11).

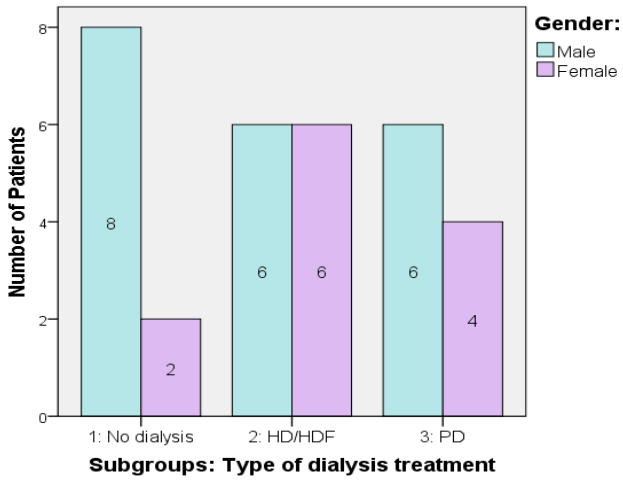

Figure 11. Gender distribution in the three subgroups.

CKD stage. In the group without dialysis two patients (20%) had CKD stage III, four (40%) stage IV and four (40%) stage V. All patients (100%) of the HD/HDF and the PD group had CKD stage V.

Dialysis vintage. There was no significant difference in the duration in months since when the patients first had received dialysis between the HD/HDF group (median 9.5, range 1 to 73) and the PD group (median 21, range 5 to 47) (p=0.093).

Details on dialysis membranes and dialyzers of subgroup 2: HD/HDF. In ten patients (83.3%) Polyamix membranes (Polyarylethersulfone, Polyvinylpyrrolidone and Polyamide) were used.

Two patients received dialysis using Helixone membranes (Polysulfone). In six patients (50%) a Polyflux 17L dialyzer was used. Three patients (25%) received dialysis via a Polyflux 170H dialyzer, one patient (8.3%) each via a Polyflux 210H, Helixone FC Cor Diax 1000 or Helixone FC Cor Diax 8000.

Baseline kidney parameters:

GFR. The baseline GFR in ml/min was significantly higher in patients without dialysis (median 18.2, IQR 10.7 to 27.8) compared to patients on HD/HDF (median 6.5, IQR 5.5 to 7.3) and PD (median 5.9, IQR 4.2 to 6.5) ($p<0.001$). Across dialysis receiving patients the distribution of GFR was not significantly different ($p=0.381$) (Figure 12).

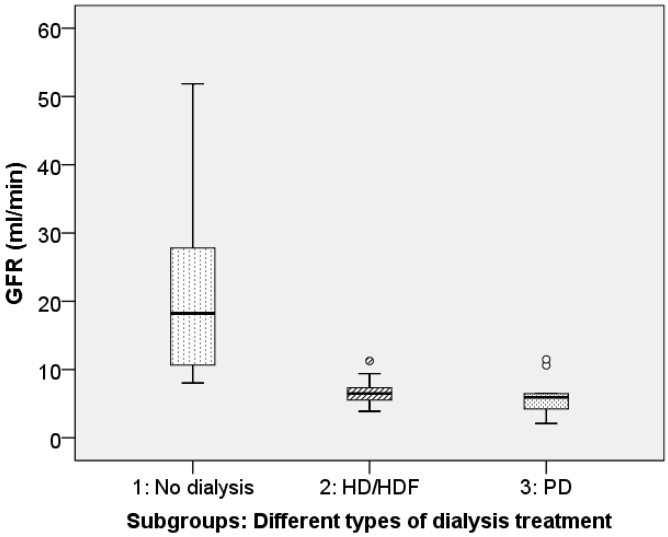

Figure 12. Comparison of baseline GFR between the three subgroups.

Creatinine. Creatinine levels in mg/dl were significantly lower in patients without dialysis (median 3.4, IQR 2.2 to 4.7) than in patients on HD/HDF (median 7.2, IQR 6.4 to 8.6) and PD (median 8.3, IQR 6 to 11.1) ($p<0.001$). There was no significant difference in the distribution of creatinine levels across dialysis receiving patients ($p=0.481$) (Figure 13).

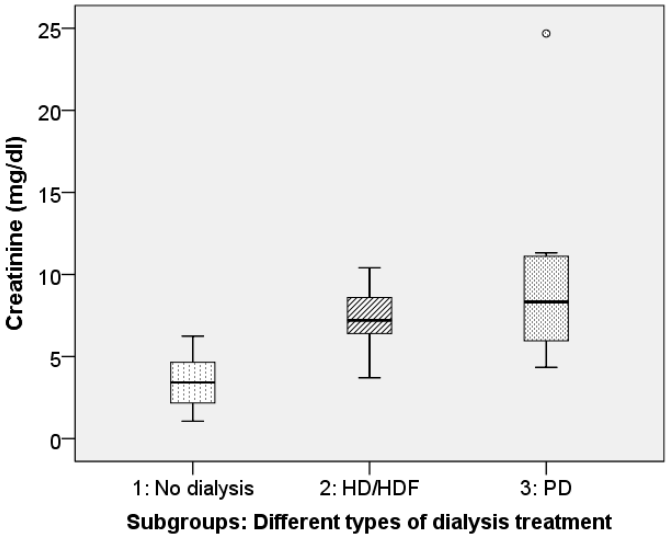

Figure 13. Comparison of baseline creatinine between the three subgroups.

Baseline parameters for inflammation:

Leucocytes. The distribution of leucocyte levels (G/l) was not significantly different across patients without dialysis (median 7.7, IQR 7.3 to 8.7), patients on HD/HDF (median 6.6, IQR 5.6 to 7.9) and patients on PD (median 6.5, IQR 4.9 to 8.3) (p=0.293) (Figure 14).

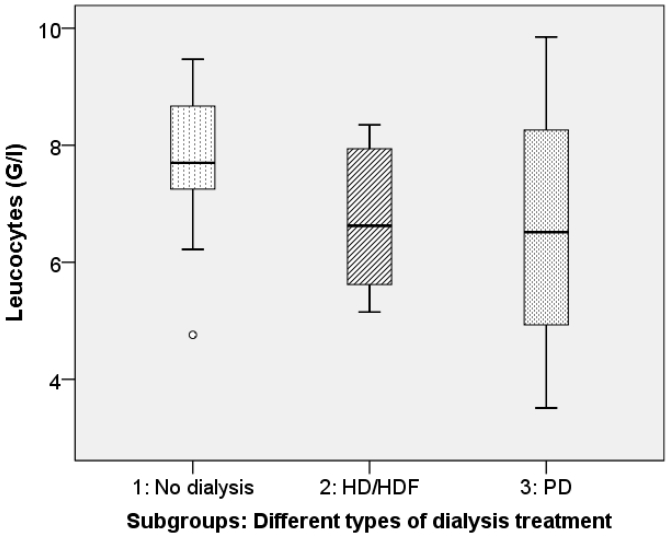

Figure 14. Comparison of baseline leucocytes levels between the three subgroups.

CRP. Also the distribution of CRP levels (mg/ml) was not significantly different across patients without dialysis therapy (median 2, IQR 1.6 to 6.5), patients on HD/HDF (median 3.4, IQR 2.1 to 5.2) and patients on PD (median 1.6, IQR 1 to 9.1) (p=0.540) (Figure 15).

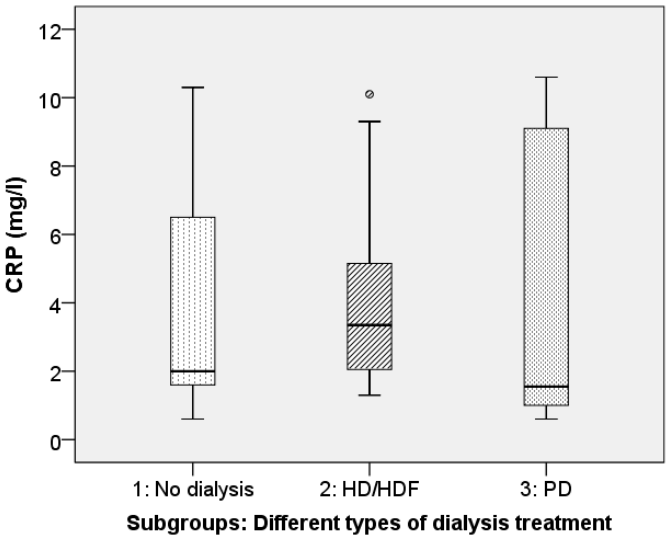

Figure 15. Comparison of baseline CRP levels between the three subgroups.

Classification of primary kidney diseases:

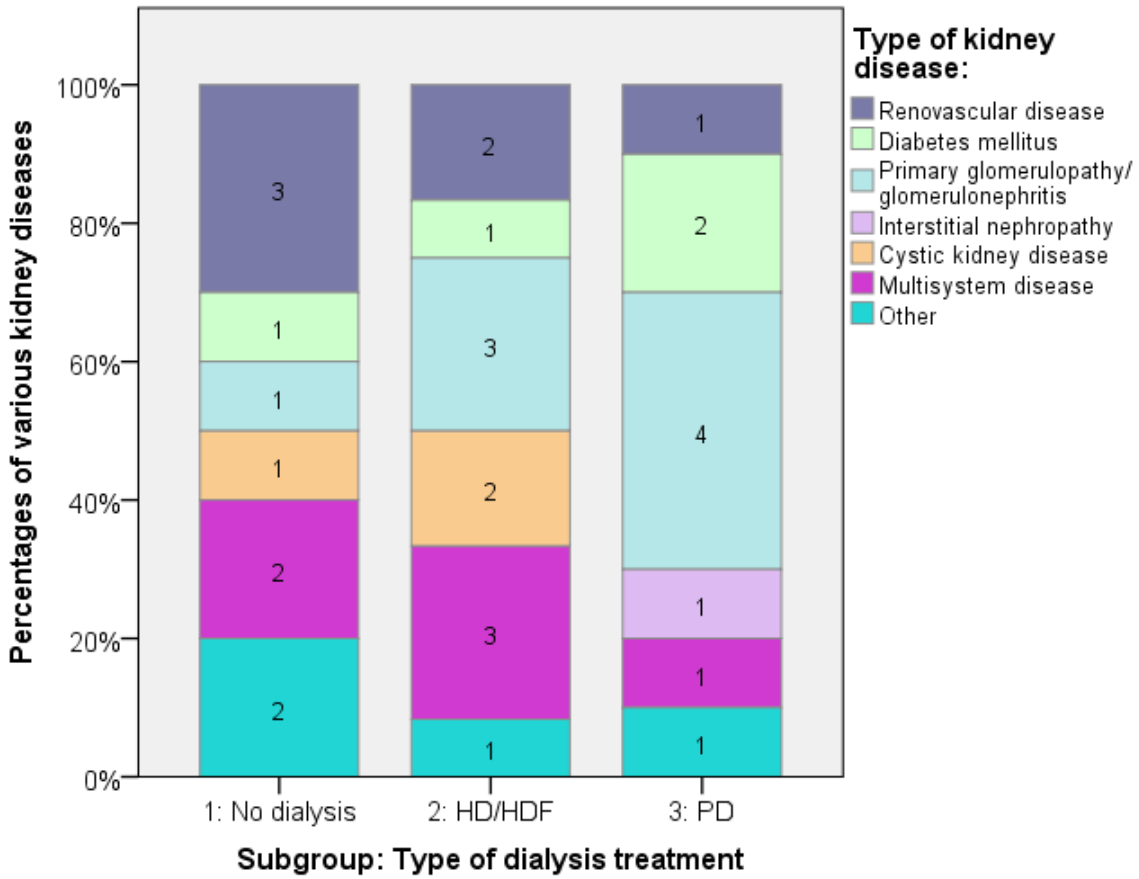

Figure 16. Distribution of primary causes of CKD with absolute frequencies displayed inside bars.

Subgroup 1. Primary renal diagnoses in patients without dialysis were as follows: renovascular disease (30%), diabetes mellitus (10%), primary glomerulopathy/glomerulonephritis (GN) (10%), cystic kidney disease (10%), multisystem disease (20%) and other (20%).

Subgroup 2. Underlying kidney diseases in HD/HDF patients were renovascular disease (16.7%), diabetes mellitus (8.3%), primary glomerulopathy/GN (25%), cystic kidney disease (16.7%), multisystem disease (25%) and other (8.3%).

Subgroup 3. Patients on PD had renovascular disease (10%), diabetes mellitus (20%), primary glomerulopathy/GN (40%), interstitial nephropathy (10%), multisystem disease (10%) and other (10%) as primary kidney disease.

4.2 BDG results (in vivo)

4.2.1 Subgroup 1: Patients suffering from CKD but without ongoing dialysis

In subgroup 1, all serum samples (100%) resulted in negative BDG levels (<15.4 pg/ml).

4.2.2 Subgroup 2: Patients undergoing HD or HDF

Seventy-one out of 72 serum samples obtained from HD/HDF patients yielded a negative BDG result, resulting in a specificity of 0.986 (95% CI 0.925 to 0.998) (Table 9, Figure 17, 18).

Patient No.	Visit 1					Visit 2
	BDG_t0_art	BDG_t30_art	BDG_t30_ven	BDG_t180_art	BDG_t180_ven	BDG_t0'
001	15.40	15.40	15.40	15.40	15.40	15.40
002	15.40	15.40	15.40	15.40	15.40	34.18
003	30.30	15.40	15.40	48.30	15.40	15.40
004	15.40	15.40	15.40	15.40	15.40	15.40
005	15.40	15.40	15.40	21.00	49.30	54.30
006	15.40	15.40	15.40	15.40	15.40	15.40
007	15.40	15.40	15.40	56.79	31.50	15.40
008	15.40	15.40	15.40	15.40	15.40	15.40
009	15.40	15.40	15.40	15.40	38.83	43.60
010	15.40	15.40	15.40	40.48	27.10	15.40
011	44.87	15.40	15.40	15.40	15.40	15.40
012	40.56	15.40	15.40	15.40	15.40	146.15
Mean ± SD	21.19 ± 10.96	15.40 ± 0	15.40 ± 0	24.15 ± 15.19	22.49 ± 11.64	33.45 ± 37.95
Median (IQR)	15.4 (15.4 - 22.85)	15.40	15.40	15.40 (15.40 – 30.74)	15.40 (15.40 – 29.30)	15.40 (15.40 – 38.89)
All BDG values are displayed in pg/ml						

Table 9. Serum BDG levels at the different time points in detail, mean ± standard deviation (SD), median (interquartile range (IQR)). The only positive result displayed in red colour.

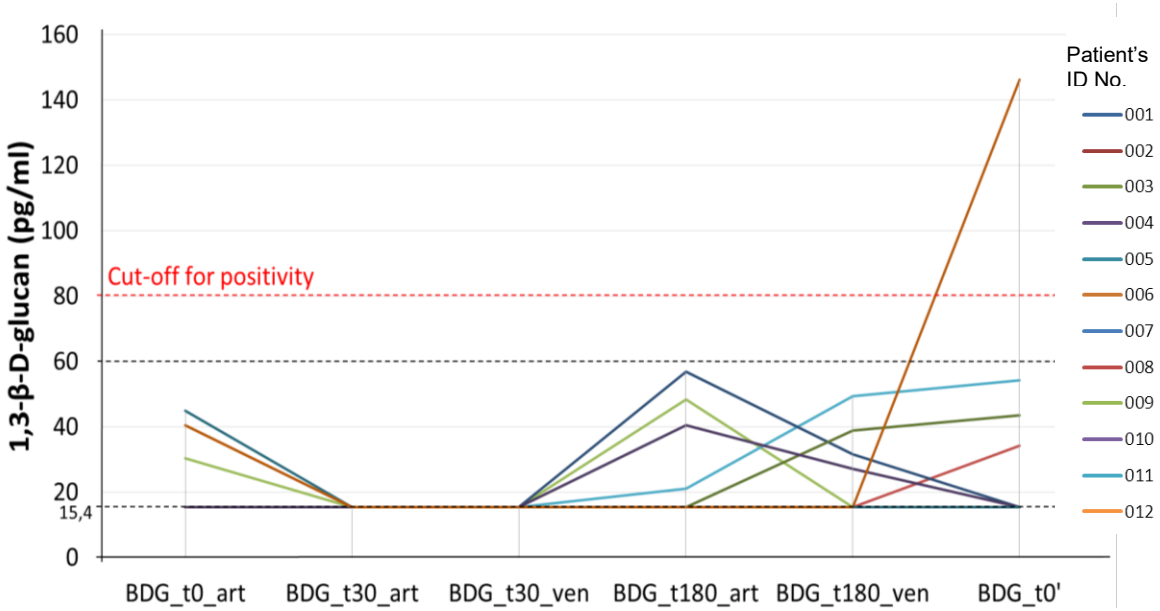

Figure 17. Serum BDG levels at the different time points during HD and HDF.

The results of the Friedman Test revealed no significant difference in serum BDG levels across the four time points (V1: right before dialysis, 30min, 180min after starting dialysis, V2: right before dialysis) (p=0.071).

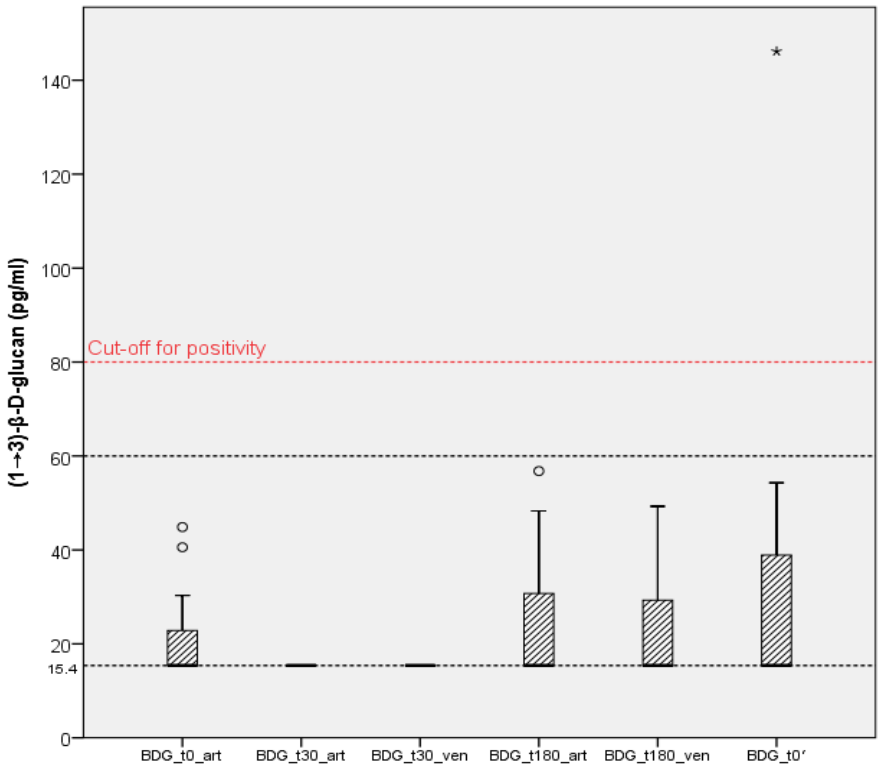

Figure 18. Boxplots for serum BDG levels at the different time points.

BDG determination in dialysate samples resulted in a median value of 15.4 pg/ml (IQR 15.4 to 15.4 pg/ml). There was no significant difference in BDG levels across dialysate levels at the two time points of measuring (V1: 30min, 180min after starting dialysis) (p=0.145).

4.2.3 Subgroup 3: Patients undergoing PD

Nine out of ten patients of the PD group had negative BDG results, one patient had a positive result of 93.1 pg/ml (Figure 19).

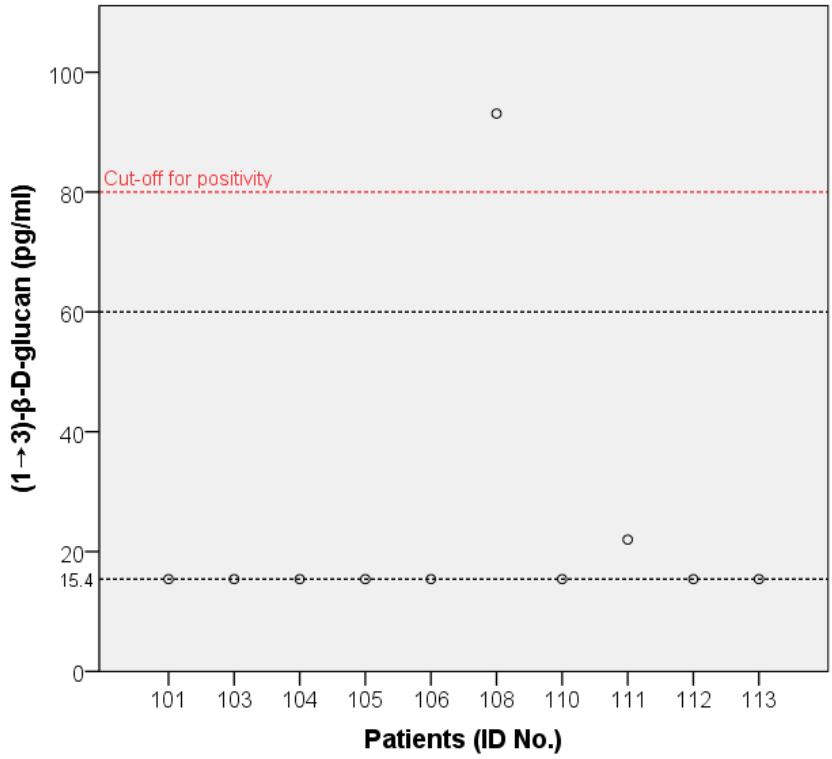

Figure 19. BDG results of PD patients.

All dialysate samples of those patients were negative (<15.4pg/ml). CAPD dialysate solutions that were tested included Extraneal® PD Solution, Nutrineal® PD4 1.1% amino acid solution, Physioneal® 40 with 1.36%, 2.27%, and 3.86% glucose, all from Baxter (Deerfield, IL, USA), and Balance® with 1.5%, 2.3%, and 4.25% glucose, all from FMC (Bad Homburg, Germany).

B. In vitro

4.3 BDG results (in vitro)

Despite the high concentrations of BDG solutions that circulated in the dialysate compartment, BDG remained negative in the blood compartment at all measurement times during the course of simulated HD treatment (Table 10).

Solution	Sample	$Blood_{in}$	$Blood_{out}$	$Dialysate_{in}$
	Time (h)	BDG (pg/mL)	BDG (pg/mL)	BDG (pg/mL)
***Candida* supernatant**	0	<15.4	30.1	3389.8
	1	<15.4	<15.4	3297.5
	2	<15.4	<15.4	3250.9
	3	<15.4	<15.4	3214.4
	4	<15.4	<15.4	3218.3
Cellulose supernatant	0	<15.4	<15.4	2050.4
	1	<15.4	<15.4	2371.6
	2	<15.4	<15.4	2153.7
	3	<15.4	<15.4	1760.8
	4	<15.4	<15.4	1832.7
CM-curdlan	0	<15.4	<15.4	393.4
	1	<15.4	<15.4	323.4
	2	<15.4	<15.4	390.1
	3	<15.4	<15.4	367.8
	4	<15.4	<15.4	398.8

Table 10. BDG levels measured in the three different BDG solutions at various time points

BDG determination in those samples obtained from the $Blood_{in}$ port was below the lower detection limit at all times, fluid samples from the $Blood_{out}$ port remained negative (<60 pg/ml) during the HD sessions.

The samples drawn from the dialysate compartment contained very high concentrations of BDG, close to the assay's upper detection limit of 4000 pg/ml. A slight decrease of BDG within the test solution containing *Candida* and Cellulose supernatant ($Dialysate_{in}$) could be observed during the course of the HD session (Figure 20).

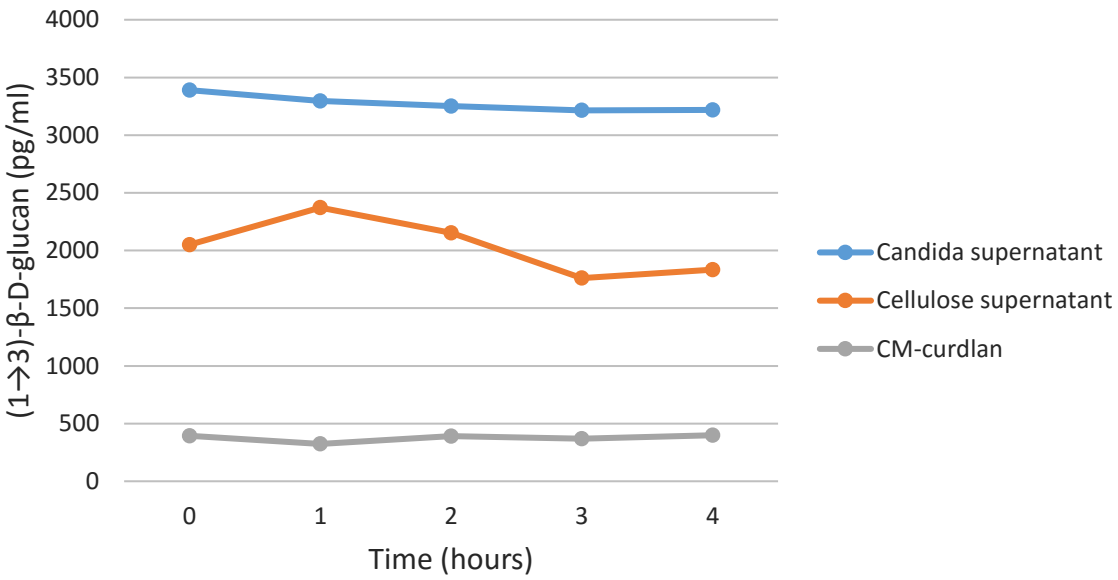

Figure 20. BDG levels measured in the dialysate compartment at the different time points during simulated HD.

5 Discussion

In this prospective study we examined the influence of HD, HDF and PD using state of the art materials and devices on serum BDG levels. Our clinical investigations and in vitro experiments could demonstrate that dialysis does not cause false positive BDG levels in the abscence of IFIs.

BDG is usually not present in mammalian serum or plasma and can only be found in negligibly small amounts in organs, except of the intestines and lungs where larger amounts can be detected, as shown in rat models by Nakao et al. (132). However, *Candida* colonization of the skin and the gastrointestinal, respiratory and genito-urinary tract is common in healthy individuals (1, 41). As a translocation of endotoxic lipopolysaccharide, a major cell wall component of gram negative bacteria, from the gastrointestinal tract into the circulatory system was observed in CKD patients (133), a translocation of fungal components from the intestines into the bloodstream was suspected to be a cause of falsely elevated BDG results in these patients. As no previous data exist, showing that in CKD patients without IFI BDG is not elevated, we measured BDG in these patients to exclude CKD as confounding factor for beta-D-glucanemia and could demonstrate that CKD per se seems not to be a cause of elevated serum BDG levels.

In our study, HD and HDF using state of the art dialysis equipment including synthetic dialyzer membranes (Polyamix™, Gambro and Helixone™, FMC) were shown not to cause false positive BDG test results. This could be demonstrated through BDG tests at two consecutive HD/HDF sessions in 12 patients without IFIs. Baseline serum BDG levels were negative in all patients. All of the samples obtained during the course of the first HD/HDF session, more precisely 30 and 180 minutes after initiation of dialysis treatment, from arterial and venous bloodlines as well as dialysate in- and outflow, were BDG negative. At the consecutive HD/HDF session, when there was only one baseline serum sample drawn for BDG determination before dialysis treatment, one patient had a positive serum BDG result (146 pg/ml). This patient did not develop an IFI in follow-up consultations, even in abscence of systemic antifungal therapy, thus the positive BDG result was interpreted as false positive. There are various potential confounding factors other beside dialysis that could have led to positivity in this patient, i.e. contamination at the time of blood

collection or later during the processing of the serum sample for BDG measurement (65, 78) but its actual cause remains unknown.

The high specificity of 99% in this study population was reenforced by the results of the simulated HD sessions. There was neither BDG release from the polysynthetic membrane (Polyamix™, Gambro) nor leakage of BDG from the BDG spiked solutions into the patient's simulated circulatory system, despite the very high BDG concentrations of up to nearly 4000 pg/ml in the dialysate compartment. The minimal BDG decrease in the dialysis solution produced from *Candida albicans* and in the solution from cellulose supernatant during the course of the experimental dialysis session may be explained due to adsorption of high molecular mass moieties to the outside of the dialyzer fibers. Another possible explanation is a potential degradation and break-up of large molecular mass fractions to moieties below 6 kD that cannot be detected by the Fungitell assay. In the third tested solution made from carboxymethyl modified BDG there was no BDG decrease. To our knowledge this is the first study that conducted an in vitro HD session in order to investigate the influence of synthetic membranes and BDG spiked dialysate on BDG levels in the simulated patient's compartment. This method ensured an unbiased result regarding potential patient-related BDG confounding factors.

Overall, these findings met our expectations as it was cellulosic material from no longer used cellulose dialysis membranes that was considered as confounder on BDG diagnostic performance. Since the 1980ies several studies could demonstrate that cellulose containing dialysis membranes release LAL reactive material (102-106). Due to their low biocompatibility, cellulosic membranes were replaced by synthetic materials (97, 101).

Our clinical data from HD/HDF patients are in accordance with findings by Kato and collegues who compared the influence of MRC and PS membranes on plasma BDG levels in patients without IFIs. They observed that MRC membranes caused substantial increase of serum BDG whereas PS membranes did not cause elevated BDG levels (107). Kanda and collegues had similar observations concerning BDG measurement in 27 HD patients with synthetic PMMA membranes before and after dialysis. BDG detection remained negative with this kind of membranes, as well as when the participants received HD using CTA membranes, while the patients had highly positive results when Cu membranes were used and also the filling liquid in Cu membranes was tested positive for BDG (108).

Two studies by Held and collegues also found that modern polysulfone and CTA membranes do not influence serum BDG levels (90).
In contrast, two studies reported about elevated serum BDG levels in HD patients using synthethic membranes. In a study on the utility of BDG testing in lung transplant recipients by Alexander et al., seven out of 12 patients who had HD therapy within the last seven days had BDG levels > 60 pg/ml. Citing the study of Kato et al., they concluded that HD treatment was not likely to be the cause for these elevated BDG results, but did not quote other possible confounders at this point. Precise information on the individual BDG results are missing in this paper, so that the number of patients with intermediate and true positive results remains unclear (110). Hanson et al. conducted a study on BDG based preemptive antifungal therapy in 64 ICU patients, of whom five patients received HD. They observed an association of HD with elevated BDG levels, but with the study of Kanda et al. as reference also this research group suspected other causes than HD for BDG elevation (111). Based on our data, we support the suspicion of these authors that other reasons than ongoing HD may have caused the elevated BDG levels.

Furthermore, we could demonstrate that PD does not influence serum BDG testing. Only one out of ten serum samples obtained from patients on PD yielded a positive result (93.1 pg/ml), whereas BDG determination was negative in the remaining nine samples as well as in all samples from dialysate solutions of those patients (<15.4 pg/ml). The patient with a positive result had suffered from peritonitis caused by coagulase-negative staphylococci that was treated with intraperitoneal administration of vancomycin and cefepime two weeks prior to serum sampling. Peritonitis may cause damage of the intraabdominal barrier with subsequent translocation of fungal and/or bacterial fragments and probably even antibiotics into the circulatory system. This is of particular interest in this patient, as cefepime was shown to contain a substantial amount of BDG (89).

Last but not least, the previously mentioned potential translocation of fungal components from the intestines into the circulatory system of CKD patients, that could have been enhanced by a hypoperfusion of the intestines during HD/HDF treatment or through peritoneal dialysis (32), could not be observed in our study

populations, as there was no significant increase of serum BDG levels during HD/HDF sessions.

There are a few limitations that must be considered when interpreting the results of our study.
Only synthetic membranes have been investigated in this study. Thus, no conclusions on the reliability of serum BDG determination in patients undergoing dialysis using cellulose containing membranes can be drawn. Even though these membranes are not used any longer in most countries, they may still be used for dialysis in developing countries.
Moreover, only patients without IFIs were included in this study. Therefore, our results and the high specificity could overestimate the performance of the BDG assay in these patients.
As PD is concerned, we only tested patients with CAPD while there were no participants receiving automated PD or continuous cycling PD.
A limiting factor of the in vitro study was that just one type of dialysis membrane was tested.

In conclusion, dialysis treatment seems not to be a factor that confounds the diagnostic performance of serum BDG measurement. Positive BDG levels in dialysis patients need to be interpreted in context with other clinical findings and should trigger further investigations to either exclude or confirm IFIs.

Our data are of high clinical relevance considering the immunocompromised state and vulnerability to invasive infections of dialysis patients (29, 30, 33). Reliable biomarkers may help to diagnose or exclude suspected infections and therefore help to avoid unnecessary antifungal treatment, the associated side effects and high costs, and ultimately decrease the high mortality according to IFIs among these patients.

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7 Attachments

7.1 Questionnare/check list

ID No.

Anamnesebogen „The Influence of Extracorporeal Blood Purification Methods on Serum 1,3-Beta-D-Glucan Levels“

Name, Vorname		Geschlecht	◯ M ◯ W
Einschlussdatum Datum v1 Datum v2		Geburtsdatum	
Dialyseart	◯ HD ◯ HDF	◯ PD ◯ IA	◯ PP ◯ keine Dialyse
Indikation zur Dialyse		Ätiologie	
CKD Stadium		Seit wann Dialyse? [Datum, zumindest Jahr]	
Weitere Details zur Dialyse			
Andere relevante Erkrankungen			
Besteht momentan eine antibiotische Behandlung?	◯ Ja ◯ Nein	Wenn ja, welche? Wenn ja, seit wann?	
Parenterale Ernährung	◯ Ja	◯ Nein	

Weitere Ausschlusskriterien

OP < 7 Tage	◯ Ja ◯ Nein	Invasive Mykose < 2 Monate	◯ Ja ◯ Nein
St. p. SOT	◯ Ja	◯ Nein	
Verbotene Medikamente	◯ Systemische Antimykotika ◯ Beta-Laktam Antibiotika ◯ Immunglobuline ◯ Blutprodukte (außer IA/PP) ◯ keine der gelisteten verbotenen Medikamente		
Zeichen und Symptome einer systemischen Infektion	◯ Ja ◯ Nein	[Details siehe Untersuchungen]	

Anamnese und Untersuchung auf Hinweise einer systemischen Infektion

Anamnese

Fieber	
Gewichtsverlust	
Nachtschweiss	

Vitalparameter

Fieber	°C
Atemfrequenz	/min
RR	mmHg
Herzfrequenz	/min

Gewicht

Gewicht	Vor Dialyse	kg
	Nach Dialyse	kg

Sampling Schema HD/HDF

T0 arteriell

O Serum mit Gel O EDTA klein O Lithium Heparin mit Gel

Beleg-

T30 arteriell

O Serum mit Gel O EDTA klein O Harnröhrchen (Dialysat)

Beleg-

T30 venös

O Serum mit Gel O EDTA klein O Harnröhrchen (Dialysat)

Beleg-

T180 arteriell

O Serum mit Gel O EDTA klein O Harnröhrchen (Dialysat)

Beleg-

T180 venös

O Serum mit Gel O EDTA klein O Harnröhrchen (Dialysat)

Beleg-

T0‘ arteriell

O Serum mit Gel O EDTA klein O Lithium Heparin mit Gel

Beleg-

7.2 Publications

Parts of this study were presented at the following meetings:

- Annual congress 2016 of the Austrian Society for Infectious Diseases and Tropical Medicine, Saalfelden, Austria (ÖIK 2016)
- American Society for Microbiology's Interscience Conference of Antimicrobial Agents and Chemotherapy (ICAAC) and International Society of Chemotherapy's International Congress of Chemotherapy and Infection (ICC) 2015, San Diego, California, USA
- Congress on Trends in Medical Mycology (TIMM) 2015, Lisbon, Portugal