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## **Risk factors and new markers of pulmonary fungal infection**

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# Chapter 5

## **$\beta$ -D-GLUCAN AND S-ADENOSYLMETHIONINE SERUM LEVELS FOR THE DIAGNOSIS OF *PNEUMOCYSTIS* PNEUMONIA IN HIV-NEGATIVE PATIENTS: A PROSPECTIVE STUDY**

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## Abstract

**Objective:** To prospectively assess the diagnostic utility of S-adenosylmethionine (AdoMet) and (1→3)- $\beta$ -D-glucan ( $\beta$ -D-glucan) serum markers for Pneumocystis pneumonia (PCP) in HIV-negative patients.

**Methods:** HIV-negative, immunocompromised patients suspected of PCP based on clinical presentation and chest imaging were included. PCP was confirmed or rejected by results of direct microscopy and/or real time PCR on broncho-alveolar lavage (BAL) fluid. Measurement of serum  $\beta$ -D-glucan and AdoMet was performed on serum samples collected at enrollment and during follow-up. Both serum  $\beta$ -D-glucan and AdoMet were assessed for diagnostic accuracy and correlation with clinical and laboratory parameters.

**Results:** In 31 patients enrolled (21 PCP-positive, 10 PCP-negative), AdoMet levels did not discriminate between patients with and without PCP. Elevated serum  $\beta$ -D-glucan was a reliable indicator for PCP with a sensitivity of 0.90 and specificity of 0.89 at the 60 pg/ml cut-off. In PCP-positive patients  $\beta$ -D-glucan serum levels decreased during treatment and inversely correlated with Pneumocystis PCR cycle threshold values in BAL fluid.

**Conclusions:** The level of serum  $\beta$ -D-glucan – but not AdoMet – was diagnostic for PCP within the clinical context and may serve as marker for pulmonary fungal load and treatment monitoring.

## Introduction

*Pneumocystis pneumonia* (PCP), caused by *Pneumocystis jirovecii* is an important cause of morbidity and mortality in patients with human immunodeficiency virus (HIV) infection and other conditions associated with immunosuppression [1]. The diagnosis of PCP is based on microscopy methods (silver, giemsa and immunofluorescent staining) and real-time PCR performed on broncho-alveolar lavage (BAL) samples obtained from patients with a compatible clinical picture [2]. Microscopy techniques are limited by their sensitivity and time demanding procedures. Currently used real time PCR methods to detect *P. jirovecii* yield high sensitivity but might lack the required specificity by detecting *P. jirovecii* also in patients who are colonized but do not suffer from PCP [3-6]. Furthermore, the need for both sensitive and specific serum tests for PCP becomes particularly evident when invasive diagnostic procedures cannot be performed due to a patient's clinical condition. Hence, a number of serum markers, including (1 $\rightarrow$ 3)- $\beta$ -D-glucan ( $\beta$ -D-glucan) and S-adenosylmethionine (AdoMet) levels were recently studied for their ability to discriminate between PCP and other pulmonary conditions [7-10].

The polysaccharide  $\beta$ -D-glucan is one of the major components of the cyst wall of *P. jirovecii*, but is present also in the cell wall of other fungal pathogens e.g. *Aspergillus fumigatus* and *Candida* spp. [11, 12]. Its potential as a discriminative marker in serum was first proposed after studies in PCP infected rats as well as in human case series [10, 13]. Watanabe et al. recently demonstrated the high potential of  $\beta$ -D-glucan as a discriminative marker for PCP in a study with HIV infected patients [14]. Yet, prospective data about its use for diagnosing PCP in solid organ transplant recipients and patients with other causes of immunodeficiency is limited. Alternatively, being proposed as a useful biochemical marker in 2003, the measurement of AdoMet levels in serum was recently re-introduced as a promising diagnostic test for PCP in patients infected with HIV, yielding a sensitivity and specificity of >90% [7, 15]. In the cell's metabolism, AdoMet serves as an essential intermediate substance e.g. for methylation reactions and polyamine synthesis. In contrast to almost all other micro-organisms capable of causing disease in humans, *Pneumocystis* spp. seem to depend on exogenous AdoMet although conflicting data were published [16, 17]. Contrary to high intracellular concentrations, extracellular concentrations are low and may be depleted during PCP [17].

The question has remained whether the observations in HIV-infected individuals with regard to the accuracy of these new serum markers for PCP can be extrapolated to the HIV-negative population. HIV-related PCP and non-HIV related PCP are known to be different in terms of clinical characteristics [18, 19]. Several studies demonstrated that a higher load of *P. jirovecii* is present in the lungs of patients with HIV as compared to patients with PCP due to other underlying disorders [20, 21]. Despite the lower amount of antigen, the inflammatory response of the immune system appeared to be more severe in HIV-negative immunocompromised individuals with PCP, which is thought to account for the more severe clinical

picture and higher mortality reported in this group [19, 22]. In this study we prospectively assessed whether serum AdoMet, its most direct metabolite adenosylhomocysteine (AdoHcy), the AdoMet/AdoHcy ratio and  $\beta$ -D-glucan, would be reliable indicators for the diagnosis of PCP in HIV-negative immunocompromised individuals. In addition, the correlation of serum AdoMet and  $\beta$ -D-glucan levels with real-time PCR results as well as with other biochemical and clinical parameters were evaluated.

## Methods

### Patients

In this prospective observational study, consecutive HIV-negative, adult immunocompromised patients suspected of having PCP based on presentation and chest imaging were enrolled during admission in the Leiden University Medical Center, a tertiary care and teaching hospital in The Netherlands. Videobronchoscopy was performed and a segment of an involved lung zone was lavaged using 20 ml aliquots. The diagnosis of PCP was confirmed or rejected by results of direct microscopy methods and/or real-time PCR of the dihydropteroate synthetase (DHPS) gene of *P. jirovecii* on the BAL fluid [23]. Patients who were thereafter considered PCP negative served as a control group. None of the case- or control patients had other proven invasive fungal infections. Demographical characteristics and data about medical history, symptoms at clinical presentation, treatment and disease outcome were extracted from the medical files. Levels of Lactate dehydrogenase (LDH) and leukocyte count in BAL fluid were acquired from the hospitals' electronic database. PCR cycle threshold values (ct-values) were obtained from the database of the Department of Medical Microbiology. The time of diagnosis was defined as the date when microbiological evidence was first obtained, i.e. the date of the BAL procedure. Data were anonymously noted on case record forms (CRFs) and a database was constructed. The study was approved by the institutional review board of the Leiden University Medical Center and all patients provided written informed consent for participation in the study. Because in our hospital performing a bronchoscopy is part of the standard work-up protocol for immunocompromised patients presenting with pneumonia in which atypical or fungal micro-organisms are suspected to be the cause, informed consent for bronchoscopy was obtained separately by the lung physician as part of clinical routine.

### Sample collection and measurement of S-adenosylmethionine and (1 $\rightarrow$ 3)- $\beta$ -D-glucan

Serum samples were prospectively collected around the time of diagnosis and during one or more time points during the first week of follow-up. Per protocol, blood was drawn for study purposes when venipuncture was performed for other clinical reasons. AdoMet and AdoHcy

were measured by a method adapted from Gellekink et al. [24, 25]. In short, after withdrawal and rapid centrifugation of EDTA-blood for determination of AdoMet and AdoHcy, plasma aliquots were stored at -80°C until the time of analysis. AdoMet, AdoHcy and their ratio were determined using liquid chromatography mass spectrometry (LC-MS/MS). After thawing of the non-acidified plasma samples, portions of 10  $\mu$ l were injected on a 50 x 2.1 mm Atlantis C-18 column (Waters) and eluted in a gradient of methanol in aqueous acetic acid (0.1%). The retention times were 0.6 min (AdoMet) and 1.4 min (AdoHcy). Standards were dissolved in 1 mmol/L HCl; pool sera were AdoMet/AdoHcy depleted by solid phase extraction (SPE) and spiked with the calibrator. Calibration curves for AdoMet and AdoHcy were linear to 500 nmol/l. For the measurement of  $\beta$ -D-glucan, a commercial  $\beta$ -D-glucan assay (Fungitell®, Associates of Cape Cod, Massachusetts, USA) was used. After thawing of samples, the test was performed according to the manufacturers' instructions. All serum tests were performed in batches at the end of the study in a blinded fashion, i.e. the laboratory staff performing the serum tests was unaware of the clinical condition of the patients and outcome of the BAL examinations (which were performed in an other laboratory and by different personnel).

### Statistical analysis

Comparisons between groups were performed by use of the Mann-Whitney U test for continuous variables and chi-square and Fishers exact tests for categorical variables. Data were expressed as medians with ranges or interquartile ranges (IQR). Potential correlations between variables were analyzed by use of Kendall's tau-b and Spearman's rho rank correlation tests. Receiver operating characteristic curves were constructed to assess diagnostic accuracy. A p-value of  $p < 0.05$  was considered significant. All calculations were performed using the SPSS statistical software package for Windows version 17.0.

## Results

### Study population

Thirty-one consecutive immunocompromised patients suspected of having PCP were enrolled over a 12 month period from March 2005 to March 2006. The majority of patients (65%) were at risk due to receiving a solid organ transplantation (liver transplant: 1, kidney transplant: 19). The baseline characteristics of case and control patients are presented in table 1. None used PCP chemoprophylaxis at presentation. The diagnosis of PCP was ascertained in 21 and rejected in 10 patients. Patients with a solid organ transplantation were overrepresented in the group with PCP (81% versus 30%). No other differences were found between groups. Four out of the 21 patients with PCP were admitted to the ICU and two patients

**Table 1.** Characteristics of study patients with and without *Pneumocystis pneumonia*

	Patients with PCP (n=21)	Patients without PCP (n=10)	p-value*
Male/female ratio	11/10	8/2	
Age (years), median (IQR)	57 (50-62)	59 (55-70)	0.19
Diagnostic methods (BAL fluid), n (%)			
Microscopy positive	13 (62)	0	
PCR positive	20 (100) <sup>†</sup>	0	
Underlying condition, n (%)			
Solid organ transplantation	17 (81)	3 (30)	
Hematological malignancy	3 (14)	5 (50)	
Other <sup>‡</sup>	1 (5)	2 (20)	
Laboratory findings, median (IQR)			
LDH (U/L)	794 (526-1078)	525 (418-599)	0.05
CRP (mg/L)	114 (48-218)	154 (60-217)	0.62
PaO <sub>2</sub> (kPa)	8.3 (7.0-9.2)	8.3 (7.1-9.4)	0.72
Outcome of PCP			
Length of hospitalisation (days), median (IQR)	11 (7-20)	-	
ICU admission, n (%)	4 (19)	-	
30-day mortality, n (%)	2 (10)	-	

Note: PCP: *Pneumocystis pneumonia*; IQR: inter quartile range; LDH: lactate dehydrogenase; CRP: c-reactive protein. ‡: The patient with PCP and 2 patients without PCP were at risk due to Cushing's syndrome or received medication to treat autoimmune disease respectively. †: PCR was not performed in 1 case, but microscopy was positive. \*: p-value calculated by Mann Whitney U test.

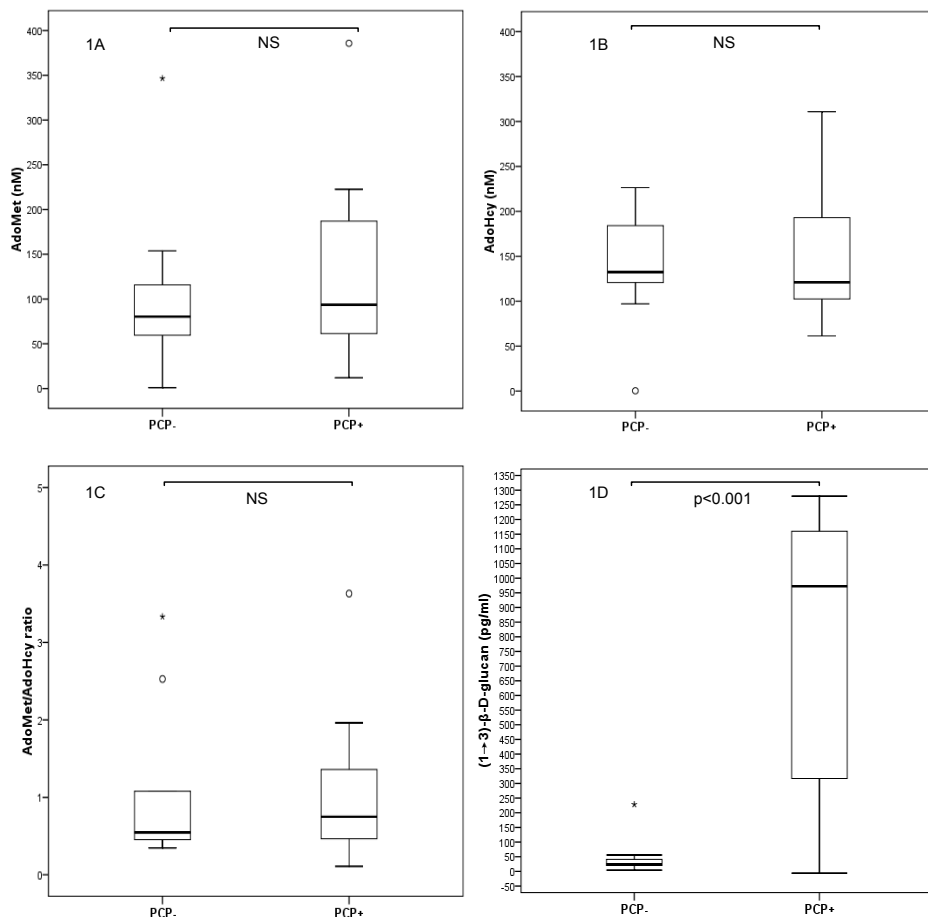
died within the first 30 days following PCP diagnosis. All ascertained PCP cases received Trimethoprim-Sulfamethoxazole (TMP-SM) 2400 mg b.i.d. and steroid treatment according to local guidelines. Among the patients without PCP, 3 had community acquired pneumonia, 5 were diagnosed with lung fibrosis and interstitial pneumonitis related to their underlying disease, 1 patient had a pulmonary infection with *Mycobacterium malmoense* and 2 patients recovered spontaneously while no definite diagnosis was made. Empirical treatment for PCP was given for a short time (i.e. usually <48 hours) to 6 of these patients while awaiting the results of the BAL examination. None of the control patients developed PCP at a later time point.

### Comparisons of serum markers at time of diagnosis

#### *AdoMet*

The baseline serum AdoMet concentrations as well as the AdoHcy levels and AdoMet/AdoHcy ratios are shown in figure 1A-C. At initiation of empirical treatment for PCP (which coincided with the timing of the BAL) the median AdoMet level in patients with confirmed PCP was 93.6 nM (IQR 61.1-188.4 nM; range 12.0-385.8 nM) as compared to 80.3 nM (IQR 58.2-125.3

**Figure 1.** Whisker box-plots of serum levels of AdoMet, AdoHcy, AdoMet/AdoHcy ratio and  $\beta$ -D-glucan in patients with and without *Pneumocystis pneumonia* at time of suspected diagnosis.



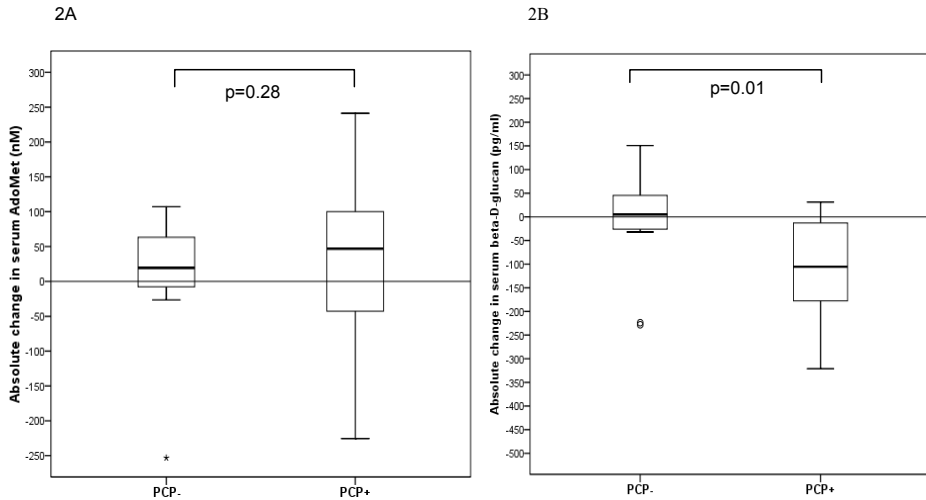
nM; range 1.0-346.6 nM) in the control group ( $p>0.05$ ; figure 1A). AdoHcy serum levels and AdoMet/AdoHcy ratio were also not significantly different when compared between patients with and without PCP. Follow-up measurement of serum AdoMet levels after a median of 3 days (IQR 2-4 days) of treatment did not show a significant change in patients with PCP as compared to patients without PCP (figure 2A).

### *$\beta$ -D-glucan*

Serum levels of  $\beta$ -D-glucan were significantly higher in patients with PCP as compared to patients without PCP as depicted in figure 1D. The median serum  $\beta$ -D-glucan level in patients with confirmed PCP was 956.9 pg/ml (IQR 281.6-1171.5 pg/ml; range 0-1279 pg/ml) and 25.3 pg/ml (IQR 14.9-48.4 pg/ml; range 4.5-228.3 pg/ml) in the control group ( $p<0.001$ ). A second  $\beta$ -D-glucan serum level measured at a median of 3 days (IQR 2-4 days) showed an



**Figure 2.** Absolute changes in serum AdoMet and  $\beta$ -D-glucan levels between time of diagnosis and follow-up at day 3 in patients without PCP and in patients with PCP on treatment.

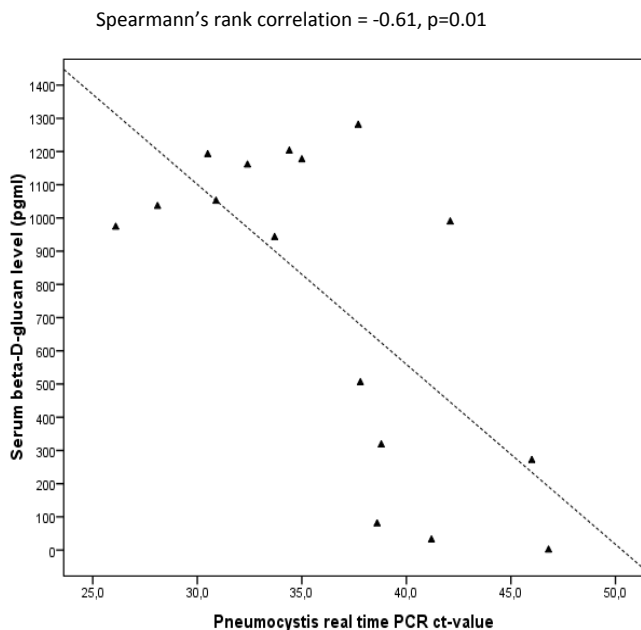


average decrease of 92.9 pg/ml during treatment in patients with PCP compared to a 13.1 pg/ml increase in patients without PCP (figure 2B). At the standard cut-off point of 80 pg/ml,  $\beta$ -D-glucan yielded a sensitivity of 0.86 and specificity of 0.89. When the cut-off point was attenuated to 60 pg/ml, receiver operating characteristic curves showed that the  $\beta$ -D-glucan test performed optimally as an indicator for PCP (AUC 0.89 95%CI 0.75-1.0,  $p=0.001$ ), with a sensitivity and specificity of 0.90 and 0.89 respectively.

### Correlation of $\beta$ -D-glucan and AdoMet with clinical and laboratory parameters

The median PCR ct-value in of patients with PCP was 34.7 (range 26.1 to 46.8). In this group the  $\beta$ -D-glucan serum levels tended to correlate with the PCR cycle threshold value in BAL fluid (Kendall's tau-b correlation coefficient  $r=-0.30$ ,  $p=0.07$ ; Spearman's rho  $=-0.47$ ,  $p=0.04$ ). This correlation was more profound when the analysis was repeated for the group with a solid organ transplant only (Kendall's tau-b correlation coefficient  $r=-0.40$ ,  $p=0.03$ ; Spearman's rho  $=-0.61$ ,  $p=0.01$ ; figure 3). Neither serum  $\beta$ -D-glucan levels nor ct-values of the Pneumocystis PCR (regarded as both dependent on detection of constituents of *P. jirovecii*) were linked with LDH or CRP in serum at time of BAL. For AdoMet no significant correlations were found between the serum levels and the above variables. Death, ICU admission or length of hospital stay (i.e. time from start of TMP-SM to discharge) was not predicted by  $\beta$ -D-glucan or AdoMet serum levels.

**Figure 3.** Scatter plot of  $\beta$ -D-glucan levels in serum by *Pneumocystis* real-time PCR cycle threshold value in solid organ transplant recipients with PCP.



## Discussion

In this prospective clinical study of immunocompromised patients not infected with HIV, we found that serum  $\beta$ -D-glucan - but not AdoMet - was a reliable indicator for PCP. In addition, we detected a significant correlation between the quantity of *P. jirovecii* DNA detected in BAL fluid and the serum  $\beta$ -D-glucan level. Furthermore, follow-up levels of  $\beta$ -D-glucan significantly decreased over a relatively short time during treatment. However, the median absolute value measured after a median of 3 days of treatment still remained far above the upper limit of normal, indicating that a clinical response observed at the bedside is of at least equal importance. At present, the kinetics of serum  $\beta$ -D-glucan during PCP are incompletely understood. Previously reported data indicate that, in patients with PCP, the elevated serum  $\beta$ -D-glucan levels only slowly return to normal over a period of several weeks after the start of adequate treatment [26]. Nevertheless, limited data from two small studies investigating the kinetics of serum  $\beta$ -D-glucan suggest that a decreasing  $\beta$ -D-glucan level correlates with the clinical recovery of the patient [27]. Clearly, this aspect of the clinical use of the  $\beta$ -D-glucan assay needs further prospective evaluation.

In HIV infected individuals the clinical relevance of serum  $\beta$ -D-glucan was convincingly addressed recently [14]. However, studies have demonstrated that relatively lower loads of *P. jirovecii* exist during PCP in the lungs of immunocompromised patients without HIV [20, 21]. In a study performed by Nakamura et al., serum  $\beta$ -D-glucan levels were also significantly

**Table 2.** Recent studies of  $\beta$ -D-glucan for the diagnosis of *Pneumocystis pneumonia* in HIV-negative immunocompromised patients.

1 <sup>st</sup> Author (ref)	Year	Journal	N HIV- (N HIV+) <sup>‡</sup>	Assay used	Design/Main Results
Desmet (8)	2009	J Clin Microbiol	12 (16)	F	Retrospective, case control study: Hematological and HIV-infected patients; serum $\beta$ -D-glucan yielded a sensitivity of 94% and specificity of 100% at a 100 pg/ml cut-off level.
Del Bono (32)	2009	Clin Vacc Immunol	8 (8)	F	Prospective, case control study: $\beta$ -D-glucan is a reliable marker for diagnosing PCP. No difference between HIV and non-HIV PCP.
Nakamura (28)	2008	Intern Med	16 (19)	G	Retrospective study: $\beta$ -D-glucan was a reliable diagnostic marker for PCP. The detection rate of $\beta$ -D-glucan in non-HIV PCP was lower than in HIV related PCP.
Persat (12)	2008	J Clin Microbiol	4 (16)	F	Part of larger retrospective study, investigating the use of the $\beta$ -D-glucan assay in a broad spectrum of fungal infection
Marty (33)	2007	Ann Intern Med	13 (3)	F	Case series: $\beta$ -D-glucan testing may be useful as a noninvasive diagnostic tool for PCP.
Tasaka (9)	2007	Chest	44 (13)	G	Case-control study: HIV-negative patients mainly had collagen-, or hematological diseases. Serum $\beta$ -D-glucan was a reliable marker for the diagnosis of PCP
Shimizu (34)	2005	Clin Exp Rheum	15 (0)	G	Case series: all patients had connective tissue disease, $\beta$ -D-glucan testing may be useful as a noninvasive diagnostic tool for PCP.

**Note:** HIV: human immunodeficiency virus; G: G-test (Seikagaku Corporation, Tokyo, Japan); F: Fungitell test (Associates of Cape Cod, Massachusetts, USA); PCP: *Pneumocystis pneumonia*; ‡: Due to the mixed population in most  $\beta$ -D-glucan clinical studies, the number of included patients that were HIV-negative (HIV-) and HIV positive (HIV+) are given in column 4.

lower in patients with PCP due to other underlying causes than HIV [28]. These observations question whether serum  $\beta$ -D-glucan levels can be used to reliably aid in the diagnosis of PCP in HIV negative immunocompromised patients. Due to limited clinical data yet available, this issue has not been completely clarified. Table 2 shows an overview of recent English language medical literature on  $\beta$ -D-glucan as a serum marker for PCP in patients without HIV. Although numbers of included patients are small and most of the reported data were obtained retrospectively, these earlier observations concur with the findings of our study. In contrast to the study of Tasaka et al. (n=44), the population of our study is dominated by solid organ transplant recipients [9].

Despite previous investigations claiming AdoMet to be a both highly sensitive and specific marker for PCP in HIV positive patients, this could not be confirmed in our study of a HIV-negative population with PCP. Knowledge about AdoMet as a marker for PCP in the clinical setting is currently driven by one research group only and the method in general is laborious, requires costly, advanced technical equipment and would be hard to sustain in its present form as a test performed in daily hospital routine. High performance liquid chromatography (HPLC) was used in preceding studies investigating the association between serum AdoMet and PCP [7, 29]. In this study we applied the method of stable-isotope dilution liquid chro-

matography–electrospray injection tandem mass spectrometry, a more advanced and now commonly used method for measuring AdoMet and AdoHcy [24, 30, 31]. The mechanism by which AdoMet would be useful as a marker for PCP is its depletion from the serum during infection with *P. jirovecii*. Since lower pulmonary fungal loads are present in HIV-negative patients with PCP, this may influence the serum AdoMet level in a way that its reliability as a diagnostic test for PCP becomes severely compromised. Moreover, because AdoMet is the product of the human body's own metabolism, other factors e.g. malnourishment, general clinical condition and other variables may easily affect the level of serum AdoMet. For example, it was recently reported that serum folate correlated with AdoMet and that body mass index was the strongest determinant of AdoMet and AdoHcy in multivariable analyses [25].

## Summary and Conclusions

Although AdoMet at first appeared a most promising candidate marker, it failed to discriminate between HIV-negative immunocompromised patients with and without PCP. In contrast, serum  $\beta$ -D-glucan levels  $>60$  pg/ml showed high diagnostic accuracy for PCP in HIV-negative immunocompromised patients without other fungal infections. Given the correlation with the PCR cycle threshold value (i.e. a quantitative surrogate for presence of *P. jirovecii* DNA) and significant decrease of the serum  $\beta$ -D-glucan level after treatment initiation, its additional purpose in both research and clinical settings may be that of an indicator of pulmonary load of *P. jirovecii*. Due to the relatively slow decrease of serum  $\beta$ -D-glucan over time, clinical judgment probably should prevail in estimating the response to treatment. Though, whether in special circumstances (e.g. in case of suspected TMP-SM resistance) the trend of the serum  $\beta$ -D-glucan level may be of additional value, remains to be researched. Due to the 'panfungal' nature of  $\beta$ -D-glucan, its use for the purpose of diagnosing PCP is clearly limited during concurrent infection with other fungal pathogens [12]. The serum  $\beta$ -D-glucan test must therefore not be used as a single test for PCP but interpreted within the clinical context. Thus, a primary suspicion of PCP above other fungal infections (supported by clinical signs and symptoms as well as chest imaging) seems warranted. Under this condition the serum  $\beta$ -D-glucan level may be used as a test with both high sensitivity and specificity for PCP in HIV-negative immunocompromised patients.

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