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Role of fungal burden in risk stratification of HIV-negative patients with *Pneumocystis* pneumonia: A 12-year, retrospective, observational, multicenter cohort

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ABSTRACT

Objectives: This study aimed to explore the role of fungal burden in risk stratification of patients without HIV-negative patients with *Pneumocystis* pneumonia (PCP).
Methods: This was a retrospective analysis of the characteristics associated with 30-day mortality in patients who were positive for *P. jirovecii* using polymerase chain reaction in bronchoalveolar lavage fluid between 2006 and 2017 in a multicenter cohort from Central Norway. The fungal burden was indicated by the cycle threshold (C_T) values from semiquantitative real-time polymerase chain reaction targeting the β-tubulin gene.
Results: We included 170 patients with proven or probable PCP. The all-cause 30-day mortality was 18.2%. After adjusting for host characteristics and premorbid corticosteroid use, a higher fungal burden was associated with a higher risk of dying: adjusted odds ratio 1.42 (95% confidence interval 0.48-4.25) for a C_T value 31-36, increasing to odds ratio 5.43 (95% confidence interval 1.48-19.9) for a C_T value $\leq$30 compared with patients with a C_T value $\geq$37. The Charlson comorbidity index (CCI) improved the risk stratification: patients with a C_T value $\geq$37 and CCI $\leq$2 had a 9% mortality risk compared with 70% among those with a C_T value $\leq$30 and CCI $\geq$6. Comorbid cardiovascular disease, solid tumors, immunological disorders, premorbid corticosteroids, hypoxemia, abnormal leukocyte counts, low serum albumin, and C-reactive protein $\geq$100 were also independently associated with 30-day mortality. The sensitivity analyses did not suggest selection bias.
Conclusion: Fungal burden may improve the risk stratification of patients without HIV-negative patients with PCP.

Introduction

The epidemiology of *Pneumocystis jirovecii* is evolving. In countries without universal health access and coverage, this opportunistic fungus contributes significantly to the disease burden of the

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HIV/AIDS epidemic [1]. In constrast, we see an increasing incidence of non-HIV *Pneumocystis* pneumonia (PCP) resulting from iatrogenic immunosuppression in high-income countries [2,3]. Increased administration of chemotherapy and immunomodulatory drugs has led to improved survival in patients with cancers and immunological disorders and those undergoing transplantations but at the cost of a larger population at risk of opportunistic infections [4].

PCP patients without HIV infection are often older than the traditional patients with AIDS [2,3,5]. Old age is *per se* associated with a hampered immune system and less regenerative power. More so, owing to concurrent lifestyle changes, comorbidities are likely of increasing importance in opportunistic infections such as PCP. The risk of dying from non-HIV PCP is high and ranges from 20% to almost 90%, depending on the disease severity and underlying disease [6]. Conventional risk estimation tools for community-acquired pneumonia may underestimate the disease severity and are inadequate for non-HIV PCP [7].

Severe infections in patients who are immunocompromised warrant high-quality microbiological diagnostic strategies. Accordingly, polymerase chain reaction (PCR), capable of detecting very low organism quantities, represents a cornerstone in patients without HIV infection [8]. However, the detection of low *P. jirovecii* levels in lower airway samples is an interpretive challenge because it may be due to colonization and not pneumonia [9].

The European guidelines for diagnosing non-HIV PCP emphasize quantitative PCR results in case of negative immunofluorescence [10]. Yet, disease severity in relation to *P. jirovecii* burden remains a key knowledge gap in this heterogenous population. Indeed, the critical threshold for developing full-blown PCP varies according to host predisposition beyond their HIV status [8]. We hypothesized that the fungal burden estimated by semiquantitative real-time PCR in bronchoalveolar lavage fluid (BALF) is associated with the outcome of patients without HIV-negative patients with PCP. To address this hypothesis, we conducted a multicenter study in Central Norway in patients with proven or probable PCP.

Methods

Setting and inclusion

Central Norway comprises approximately 700,000 inhabitants. There are seven local hospitals in the health region (Kristiansund, Levanger, Molde, Namsos, Orkdal, Volda, and Ålesund). St. Olavs Hospital, Trondheim University Hospital is the only tertiary referral hospital. During the study period, St. Olavs Hospital performed the microbiological diagnostics for *P. jirovecii* for all the hospitals in Central Norway. Adults who are HIV-negative (aged ≥16 years) who were admitted to one of the hospitals in the region and had a positive semiquantitative real-time PCR (range 22-40 cycles) for *P. jirovecii* in BALF between 2006 and 2017 were screened for eligibility. Patients with other respiratory samples (n = 67), missing cycle threshold (C_T) value (n = 63), or who did not meet the 2021 criteria for proven or probable PCP were excluded (n = 22) [11]. Inclusion of alive patients required active written informed consent (*i.e.*, returning the letter with signed consent by postal mail). The need for consent from the next of kin of deceased patients was waived. HIV status was only available for deceased patients and consenting survivors. In consequence, screening for eligibility (*i.e.*, control of HIV status) could be done for all deceased patients and consenting survivors but not for nonconsenting survivors. The ethical committee allowed the use of demographic and microbiological data of nonconsenting survivors (*i.e.*, not returning a signed letter of consent). Based on the data from the national HIV/AIDS surveillance, there were 19 new AIDS cases in the health region during the study period [12], and approximately one-third of these presented with PCP [13]. We excluded six patients who were HIV-positive during the screening process and we have little reason to believe that there were many who were HIV-positive among the nonconsenters (n = 19). Despite their unknown HIV status, we used the data of the nonconsenters in the subsequently described sensitivity and survival analyses based on their positive *P. jirovecii* PCR in BALF and retrievable C_T value (Supplementary Figure 1).

Data collection

This study was framed in a research protocol formulated in 2017/2018. Hence, we reviewed medical records and collected comprehensive clinical data retrospectively. We extracted the clinical characteristics from the preceding time points that were closest to when the patient underwent testing. Regarding host factors, we registered non-HIV conditions associated with *P. jirovecii*/PCP in the literature or an indication for chemotherapy or immunosuppression, denoted "underlying disease". In addition, we collected data on co-existing comorbidities and assessed the multimorbidity according to the Charlson comorbidity index (CCI) [14]. We converted corticosteroid exposure into the equivalent dose in methylprednisolone expressed as milligrams per day [15]. To ascertain date of death, we obtained death dates from the Norwegian Population Register.

The study population consisted of proven or probable PCP cases according to the 2021 European Organization for Research and Treatment of Cancer/Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) criteria [11]. Supplementary Table 1 shows the case qualification within subgroups of underlying disease. Concerning the microbiological criteria, direct immunofluorescence was performed until 2017, whenever positive controls were available (n = 61/170, 35.9%), whereas β-D-glucan analysis was not used throughout the study period.

Polymerase chain reactionassay and testing for P. jirovecii

The microbiological diagnosis of *P. jirovecii* was done by semiquantitative real-time PCR adapted from Brancart et al. [16]. The assay targets the β-tubulin gene of *P. jirovecii*, present in a single copy, which results in higher C_T values than the more frequently used multicopy targets (*e.g.*, the mitochondrial large subunit) [17]. The *P. jirovecii* PCR results were reported to clinicians as negative/positive, with a comment about low concentration of *P. jirovecii* if the C_T value was high (*i.e.*, ≥37). Testing for *P. jirovecii* was based on the treating physician's clinical suspicion and decision. BAL procedures were performed by pulmonologist, following a standardized protocol, regardless of hospital and intensive care unit admission.

Polymerase chain reaction-protocol

Respiratory tract samples that were viscous were pretreated with Sputolysin (dithiothreitol, volume 1:2) for 10 minutes for liquefication of mucoid fluids before DNA extraction. Next, if the sample volume was >10 ml, 3 to 5 ml was subjected to centrifugation at 3,000 × *g* for 30 minutes. Thereafter, 500 µl of the supernatant was mixed with 50 µl proteinase K and incubated for 15 minutes at 65°C. If the sample volume was <10 ml, the centrifugation step was omitted, and 1 ml of the sample was mixed with 100 ml proteinase K and incubated as described previously. Then, the mixture was spun down, the supernatant was removed, and 500 µl of the precipitate was used for DNA extraction on a NucliSENS easyMAG instrument (bioMérieux), with an eluate volume of 55 µl.

The reagents and PCR instruments used varied during the study period, but all changes were validated to ensure equal quality. Dur-

Table 1
Characterization of study population.

Characteristics	n with available data (%)	n (%)/median (q_1-q_3)
Background history		
Age years	170 (100)	66.5 (58-74)
Male sex	170 (100)	101 (59.4)
Ever smoking	164 (96.5)	96 (58.5)
Charlson comorbidity index	170 (100)	3 (2-6)
Any comorbidity[a]	170 (100)	115 (67.7)
Hypertension	170 (100)	57 (33.5)
Cardiovascular disease and/or chronic heart failure	170 (100)	50 (29.4)
Chronic lung disease	170 (100)	30 (17.7)
Diabetes mellitus	170 (100)	28 (16.5)
Chronic kidney disease	170 (100)	26 (15.3)
Hematologic and/or solid malignancy	170 (100)	22 (12.9)
Underlying disease	170 (100)	
Hematologic malignancy		65 (38.2)
Solid tumor		43 (25.3)
Solid organ transplantation		28 (16.5)
Immunological disorder		25 (14.7)
Chronic lung disease		7 (4.1)
Miscellaneous conditions[b]		2 (1.2)
Iatrogenic immunosuppression preceding 5 years	170 (100)	167 (98.2)
Iatrogenic immunosuppression at presentation	170 (100)	150 (88.2)
Methylprednisolone-equivalent dose, mg/day	168 (98.8)	1.5 (0-8)
Clinical presentation		
One cardinal symptom[c]	170 (100)	39 (22.9)
Three cardinal symptoms[c]	170 (100)	60 (35.3)
O_2 saturation %[d]	146 (85.9)	89 (85-93)
Cycle threshold value bronchoalveolar lavage fluid, median	170 (100)	35 (32-37)
Leukocyte count $\times 10^9$/l	168 (98.8)	7.2 (4.3-9.9)
Neutrophil count $\times 10^9$/l	137 (80.6)	4.8 (2.4-7.3)
Lymphocyte count $\times 10^9$/l	86 (50.6)	0.65 (0.40-1.1)
Serum albumin g/l	127 (74.7)	33 (28-36)
C-reactive protein mg/l	167 (98.2)	72 (38-138)
Lactate dehydrogenase U/l	105 (61.8)	307 (226-379)
Ground glass opacities/infiltrates on thoracic computed tomography	153 (90.0)	140 (91.5)
Course and outcome		
Antipneumocystis treatment	170 (100)	158 (92.9)
Dose reduction/premature discontinuation	157 (92.4)	60 (38.2)
Documented side effects[e]	158 (92.9)	98 (62.0)
Intensive care unit admission	170 (100)	43 (25.3)
Any ventilation support	170 (100)	46 (27.1)
Any complication[f]	170 (100)	62 (36.5)
Cumulative 30-day mortality	170 (100)	31 (18.2)

[a] "Any comorbidity" also included rheumatic conditions (n = 7) and chronic liver diseases (n = 1).
[b] Miscellaneous conditions included statin-induced myositis exposed to corticosteroids (n = 1) and no definite diagnosis at presentation (n = 1).
[c] Cardinal symptoms included cough, dyspnea, and fever.
[d] Thirty-nine patients were receiving supplemental oxygen when O_2-saturation was measured.
[e] Among patients treated for antipneumocystis, the side effects included arrythmias, bone marrow suppression, nausea/vomiting, liver toxicity, nephrotoxicity, and skin reactions.
[f] Any complication included acute respiratory distress syndrome/respiratory failure (n = 40, 23.5%), superinfection (n = 27, 15.9%), hemodynamic failure (n = 19, 11.2%), renal failure (n = 17, 10.0%), and pneumothorax (n = 3, 1.8%).

ing the main part of the study period, the following procedure and reagents were used: 5 µl of eluate was added to 10 µl of PerfeCTa multiplex quantitative PCR supermix with uracil-*N*-glycosylase, 0.5 µl of each primer (12 µM) and probe (8 µM), and 3.5 µl molecular grade water. BALFs, considered critical patient samples, were extracted, and amplified in duplicates. Amplification reactions were carried out either on a CFX96 real-time system (Bio-Rad), Chromo4 system (Bio-Rad), or LightCycler 2.0 instrument (Roche), with the following cycling conditions: 45°C for 5 minutes; 95°C for 3 minutes; and then 40 cycles of 95°C, 60°C, and 72°C for 10 seconds each.

A cloned PCR product was used as an external positive control, and molecular grade water was used as a negative control in all PCR runs. To control the sample quality, a separate real-time PCR targeting a human 237 base pair intergenic region of chromosome 20 was run, as previously described [18]. All samples were positive for this human target with a C_T value $\leq$37 and no samples were excluded due to nonamplification during the study period. The protocol did not include any ulterior extraction control.

The laboratory participated in the QCMD PCP DNA EQA Program with excellent scores for the core samples during the study period.

Retrievability of cycle threshold values

We collected the C_T values from the log of the PCR instruments, retrospectively. Some of the PCR instruments were replaced before the initiation of this study, resulting in missing C_T values. Because the retrievability of the C_T values merely depended on which machine the analyses were run, we considered the missing pattern "random".

Statistical analyses

We present continuous and categorical variables as medians with first (q_1) and third (q_3) quartiles and proportions with percentages (%), respectively. For comparisons, we used the Wilcoxon rank-sum, chi-square, or Fisher's exact test, as appropriate. Because some of the independent variables had missing data, we specified "n (%) with data" in the tables.

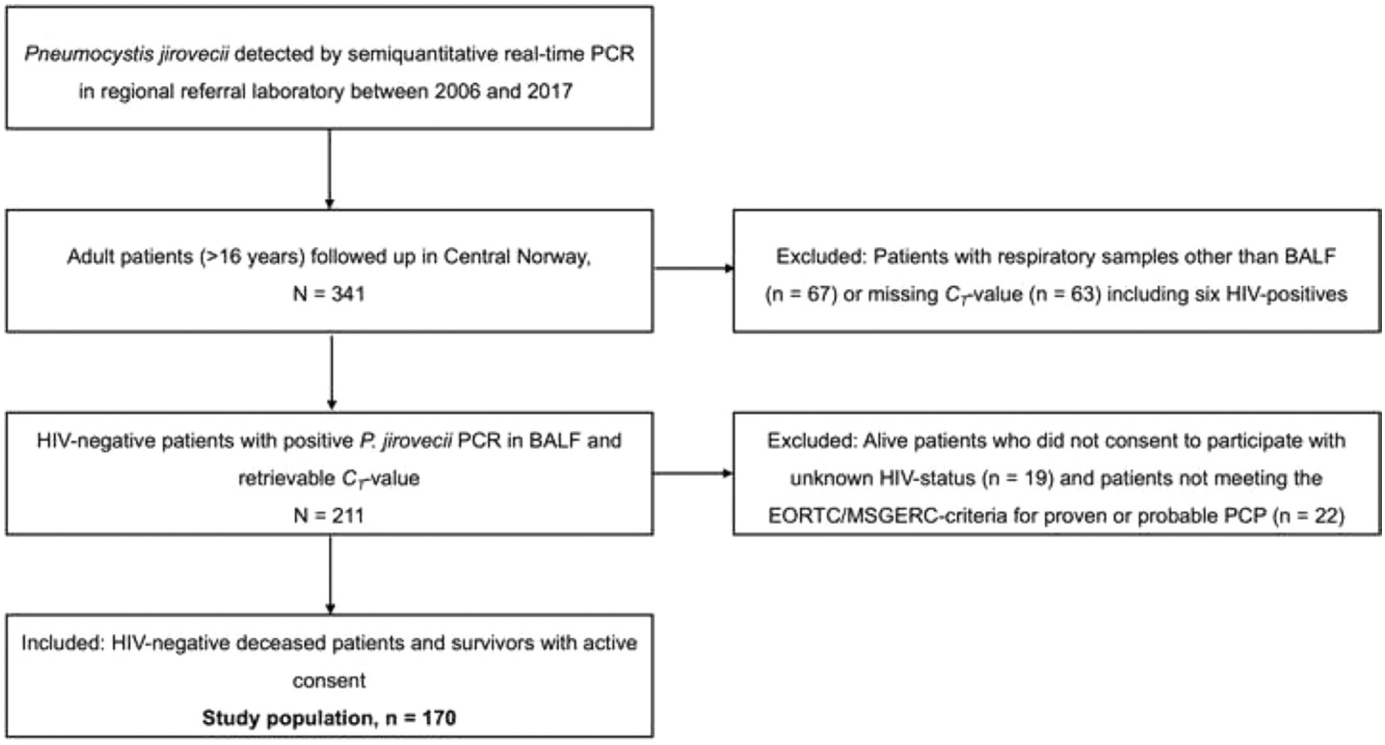

Figure 1. Flowchart of study design. Adult patients without HIV with positive *Pneumocystis jirovecii* semiquantitative real-time PCR in the regional referral laboratory from 2006 to 2017 were screened for eligibility. Patients with respiratory samples other than BALF, missing C_T value, or who did not meet the European Organization for Research and Treatment of Cancer/Mycoses Study Group Education and Research Consortium 2021 criteria for proven or probable PCP were excluded [11]. Inclusion of survivors required active consent (*i.e.*, returning signed information letter by postal mail), whereas all eligible deceased patients were recruited. HIV status was available in consenting survivors and deceased patients, and six patients with HIV were excluded during the screening process. BALF, bronchoalveolar lavage fluid; C_T, cycle threshold; EORTC/MSGERC, European Organization for Research and Treatment of Cancer/Mycoses Study Group Education and Research Consortium; PCP, *Pneumocystis* pneumonia; PCR, polymerase chain reaction.

We used a logistic regression analysis to examine risk factors for 30-day mortality. For continuous variables, we assessed the linearity of the logit with "LOWESS plots" with respect to clinical cutoffs to determine the best fit. We performed univariable and multivariable analyses. In the latter, we included potential confounders based on *a priori* knowledge and performed separate analyses for each exposure variable (Supplementary Table 2). We opted for this approach to let the existing literature guide the selection of covariates. In the models with C_T value as the exposure variable, we included the following covariates based on their potential relationship with fungal burden: age, sex, premorbid corticosteroid exposure, and comorbid chronic lung disease. Underlying disease was excluded due to multicollinearity. The effect estimates of these covariates are also reported in Table 2a. The results are expressed as odds ratios (ORs) with 95% confidence intervals (CIs) generated from the Wald test. We used the "margins" command to determine probability of death within 30 days.

To see whether the trends of the study population were representative, we performed the survival analyses with the data available for all patients with positive *P. jirovecii* PCR in BALF and a retrievable C_T value (N = 211) with the C_T value as the only exposure variable. These analyses included the 19 nonconsenters with unknown HIV status, and the EORTC/MSGERC criteria were not taken into consideration. We applied the Kaplan–Meier method and used the log-rank test for comparisons after verifying the proportional hazard assumption. Next, we performed Cox regression analyses to obtain the adjusted estimates. Available characteristics (*i.e.*, age and sex) were included as covariates. To account for changes in the incidence and lethality over time, we also tested the inclusion of the year of diagnosis. The results are expressed as hazard ratios (HRs) in Table 2b.

Sensitivity and subgroup analyses

To reduce bias from treatment disparity, we performed the analyses restricted to the patients receiving antipneumocystis treatment, with fungal burden as independent variable. Similarly, we performed subgroup analyses in patients with a C_T value $\leq$37 to study the association between the C_T value and outcome within this spectrum. The subgroup analyses were performed *post hoc*.

To assess the nonparticipation bias, we compared the consenters with nonconsenters. Next, we performed sensitivity analyses applying inverse probability weighted regression adjustment. In brief, we calculated the inverse probability of inclusion based on the data available, regardless of consent: age, sex, and hospital (university vs local). We truncated high weights above the 90th percentile. For comparison, we report the weighted estimates of the sensitivity analyses in conjunction with the unweighted (crude) estimates in Tables 2a and 3. The sensitivity analyses were planned per protocol.

All *P*-values were two-sided, and we considered values below 0.05 statistically significant. We used STATA/MP (version 15.1; College Station, TX, USA) to perform all statistical analyses.

Results

Description of study population

Between 2006 and 2017, the regional referral laboratory diagnosed 274 cases with positive *P. jirovecii* PCR in BALF in Central Norway, of whom 211 had a retrievable C_T value. We included 170 patients without HIV (101 males, 59.4%) in the study population (Figure 1). According to the EORTC/MSGERC criteria, 34 (20.0%)

Table 2
Fungal burden indicated by C_T value from semiquantitative real-time PCR and risk of 30-day mortality in patients without HIV with *Pneumocystis* pneumonia.

a) Logistic regression analyses in study population.

Risk factor	n with available data (%)	n category	Events, n (%)[a]	Crude and inverse probability weighted OR[b]							
				Univariable				Multivariable			
Covariates[c,d]				Crude OR (95% CI)	*P*-value	Weighted OR (95% CI)	*P*-value	Crude OR (95% CI)	*P*-value	Weighted OR (95% CI)	*P*-value
C_T value from PCR in BALF	170 (100)				0.03		0.04				
≤30		28	10 (35.7)	4.44 (1.41-14.0)	0.01	4.30 (1.35-13.6)	0.01	5.43 (1.48-19.9)	0.01	5.39 (1.36-21.5)	0.02
31-36		88	15 (17.0)	1.64 (0.60-4.53)	0.34	1.63 (0.59-4.51)	0.35	1.42 (0.48-4.25)	0.53	1.42 (0.48-4.17)	0.52
≥37		54	6 (11.1)	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-
Age, per year								1.02 (0.98-1.06)	0.32	1.02 (0.98-1.07)	0.26
Male sex								1.35 (0.54-3.38)	0.52	1.39 (0.54-3.62)	0.50
Methylprednisolone-equivalent dose, mg/day											
0								1 (ref.)	-	1 (ref.)	-
1-7								0.60 (0.17-2.16)	0.43	0.59 (0.18-1.96)	0.39
8-19								3.58 (1.11-11.5)	0.03	3.70 (0.99-13.8)	0.05
≥20								2.21 (0.68-7.21)	0.19	2.31 (0.68-7.80)	0.18
Chronic lung disease								2.75 (0.97-7.76)	0.06	2.78 (0.88-8.82)	0.08

b) Cox regression analyses in all patients with positive *P. jirovecii* PCR in Central Norway between 2006 and 2017 and retriable C_T value.

Risk factor	n with available data (%)	Crude hazard ratios[b]			
		Univariable		Multivariable[b]	
Covariates[c,e]		HR (95% CI)	*P*-value	HR (95% CI)[d]	*P*-value
C_T value from PCR in BALF per unit increase	211 (100)	0.90 (0.83-0.97)	<0.01	0.89 (0.83-0.96)	<0.01
Age, per year	211 (100)	1.28 (0.85-1.91)	0.24	1.38 (0.92-2.07)	0.12
Male sex	211 (100)	1.27 (0.65-2.48)	0.48	1.07 (0.54-2.11)	0.85

Abbreviations: BALF, bronchoalveolar lavage fluid; CI, confidence interval; C_T, cycle threshold; HR, hazard ratio; OR, odds ratio; PCR, polymerase chain reaction.

[a] Events n (%) refers to the number of deaths within 30 days with "n category" of the same row as denominator.

[b] To account for nonparticipation affecting the study population, we performed sensitivity analyses applying inverse probability weighed regression adjustment in the logistic regression analyses. We report both unweighted (crude) and weighted effect estimates (ORs). The Cox regression survival analysis in all patients with positive *P. jirovecii* in Central Norway between 2006 and 2017 and retrievable C_T value was not affected by nonparticipation. Thus, we only report crude effect estimates (HRs).

[c] Covariates were included based on *a priori* knowledge and drawing of direct acyclic graphs. In the Cox regression analyses inclusion of covariates was also based on data availability of nonconsenting survivors.

[d] Please refer to Table 3 for the univariable effect estimates (ORs) of the covariates of the logistic regression analyses.

[e] We tested inclusion of year of diagnosis to account for change over time, but inclusion resulted in less than 10% change in the effect estimates disproving confounding.

Table 3
Clinical demographic characteristics and risk of 30-day mortality in patients who are HIV-negative patients with PCP.

Risk factor	n with available data (%)	n category	Crude and inverse probability weighted odds ratios[b]							
			Events, n (%)[a]	Univariable			Multivariable[c]			
				OR (95% CI)	*P*-value	Weighted OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	Weighted OR (95% CI)	*P*-value
Age, per year	170 (100)	-	-	1.03 (0.99-1.07)	0.10	1.03 (1.00-1.07)	0.08				
Sex	170 (100)										
Male sex		101	20 (19.8)	1.30 (0.58-2.92)	0.52	1.42 (0.63-3.21)	0.39				
Charlson comorbidity index	170 (100)				0.06		0.03				
≤2		61	8 (13.1)	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-
3-5		58	8 (13.8)	1.06 (0.37-3.04)	0.91	1.12 (0.39-3.23)	0.83	0.91 (0.31-2.67)	0.87	0.92 (0.31-2.77)	0.89
≥6		51	15 (29.4)	2.76 (1.06-7.19)	0.04	3.16 (1.21-8.27)	0.02	2.49 (0.95-6.56)	0.07	2.71 (1.02-7.19)	0.05
Comorbidities	170 (100)										
Hypertension		57	11 (19.3)	1.11 (0.49-2.52)	0.80	1.15 (0.51-2.62)	0.73	0.92 (0.39-2.17)	0.84	0.92 (0.39-2.14)	0.84
Cardiovascular disease/congestive heart failure		50	15 (30.0)	2.79 (1.25-6.21)	0.01	3.03 (1.36-6.78)	<0.01	2.40 (1.03-5.59)	0.04	2.47 (1.00-6.10)	0.05
Chronic lung disease		30	10 (33.3)	2.83 (1.16-6.90)	0.02	3.08 (1.26-7.51)	0.01	2.44 (0.98-6.08)	0.06	2.52 (1.02-6.23)	0.05
Diabetes mellitus		28	5 (17.9)	NA	-	NA	-	NA	-	NA	-
Chronic kidney disease		26	4 (15.4)	NA	-	NA	-	NA	-	NA	-
Malignancy		22	4 (18.2)	NA	-	NA	-	NA	-	NA	-
Any comorbidity[d]		115	24 (20.9)	1.81 (0.73-4.50)	0.20	1.91 (0.77-4.78)	0.17	1.46 (0.56-3.79)	0.44	1.46 (0.58-3.68)	0.42
Underlying disease and corticosteroid exposure[e]											
Underlying disease	170 (100)				0.04		0.05				
Hematologic malignancy		65	6 (9.2)	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-
Solid tumor		43	11 (25.6)	3.38 (1.14-9.99)	0.03	3.75 (1.26-11.1)	0.02	3.52 (1.17-10.5)	0.03	3.79 (1.26-11.4)	0.02
Solid organ transplantation		28	3 (10.7)	1.18 (0.27-5.09)	0.82	1.22 (0.28-5.30)	0.79	1.12 (0.26-4.90)	0.88	1.13 (0.25-5.03)	0.88
Immunological disorder		25	8 (32.0)	4.63 (1.41-15.2)	0.01	4.52 (1.37-14.9)	0.01	4.53 (1.33-15.4)	0.02	4.34 (1.33-14.2)	0.02
Chronic lung disease		7	2 (28.6)	3.93 (0.62-24.8)	0.15	4.37 (0.69-27.8)	0.12	3.22 (0.50-20.6)	0.22	3.36 (0.53-21.2)	0.20
Methylprednisolone-equivalent dose, mg/day	168 (98.8)				<0.01		<0.01				
0		83	10 (12.0)	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-
1-7		38	4 (10.5)	0.86 (0.25-2.93)	0.81	0.88 (0.26-3.03)	0.84	0.62 (0.12-3.30)	0.57	0.63 (0.16-2.48)	0.50
8-19		24	10 (41.7)	5.21 (1.83-14.9)	<0.01	5.61 (1.96-16.1)	0.001	4.26 (1.34-13.5)	0.01	4.38 (1.37-14.0)	0.01
≥20		23	7 (30.4)	3.19 (1.06-9.66)	0.04	3.40 (1.12-10.3)	0.03	2.30 (0.70-7.51)	0.17	2.30 (0.72-7.39)	0.16

(*continued on next page*)

Table 3 (*continued*)

Risk factor	n with available data (%)	n category	Crude and inverse probability weighted odds ratios[b]							
			Events, n (%)[a]	Univariable				Multivariable[c]			
				OR (95% CI)	*P*-value	Weighted OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	Weighted OR (95% CI)	*P*-value
Clinical and laboratory findings											
Cough	170 (100)	100	17 (17.0)	0.82 (0.37-1.80)	0.62	0.79 (0.36-1.75)	0.57	0.84 (0.33-2.14)	0.72	0.83 (0.34-2.02)	0.69
Dyspnea	170 (100)	126	25 (19.8)	1.57 (0.60-4.12)	0.36	1.55 (0.59-4.08)	0.38	1.03 (0.32-3.29)	0.96	1.03 (0.32-3.29)	0.97
Fever	170 (100)	131	22 (16.8)	0.67 (0.28-1.61)	0.37	0.69 (0.28-1.65)	0.40	1.10 (0.35-3.44)	0.87	1.05 (0.38-2.88)	0.92
Three cardinal symptoms[f]	170 (100)	60	9 (15.0)	0.71 (0.30-1.65)	0.42	0.70 (0.30-1.63)	0.40	0.78 (0.27-2.23)	0.65	0.77 (0.27-2.22)	0.62
O_2 saturation ≤89.5%[g]	146 (85.9)	78	24 (30.8)	4.59 (1.75-12.1)	<0.01	4.66 (1.76-12.3)	<0.01	3.64 (1.26-10.5)	0.02	3.65 (1.26-10.5)	0.02
Leukocytes × 10^9/l	168 (98.8)				<0.01		<0.01				
≤3.4		26	2 (7.7)	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-
3.5-10.0		103	15 (14.6)	2.05 (0.44-9.57)	0.36	2.07 (0.44-9.75)	0.36	1.71 (0.33-8.89)	0.53	1.72 (0.37-7.99)	0.49
≥10.1		39	14 (35.9)	6.72 (1.38-32.8)	0.02	6.88 (1.40-33.8)	0.02	7.01 (1.23-39.8)	0.03	7.16 (1.41-36.4)	0.02
Neutrophils, per 10^9/l	137 (80.6)	-	-	1.24 (1.11-1.38)	<0.001	1.24 (1.10-1.39)	<0.001	1.26 (1.10-1.44)	0.001	1.26 (1.07-1.49)	<0.01
Lymphocytes ≤0.9 × 10^9/l	86 (50.6)	59	18 (30.5)	2.52 (0.76-8.36)	0.13	2.59 (0.77-8.66)	0.12	6.02 (1.18-30.8)	0.03	5.93 (1.21-29.0)	0.03
Serum albumin, per g/l	127 (74.7)	-	-	0.89 (0.83-0.96)	0.001	0.89 (0.83-0.96)	0.001	0.86 (0.78-0.94)	0.001	0.85 (0.76-0.96)	<0.01
C-reactive protein ≥100 mg/l	167 (98.2)	64	21 (32.8)	5.10 (2.16-12.1)	<0.001	5.50 (2.32-13.0)	<0.001	6.00 (2.24-16.1)	<0.001	6.37 (2.32-17.5)	<0.001
Lactate dehydrogenase ≥249 U/l	105 (61.8)	67	12 (17.9)	1.16 (0.40-3.40)	0.78	1.20 (0.41-3.52)	0.75	1.23 (0.32-4.78)	0.76	1.24 (0.33-4.61)	0.75
Thoracic computed tomography findings	153 (90.0)										
Ground glass opacities/infiltrates		119	21 (15.0)	0.97 (0.20-4.70)	0.97	0.91 (0.19-4.44)	0.91	1.53 (0.24-9.74)	0.66	1.45 (0.25-8.30)	0.68
Crazy paving pattern		42	8 (19.0)	1.51 (0.59-3.87)	0.40	1.47 (0.57-3.80)	0.42	1.30 (0.40-4.23)	0.66	1.34 (0.42-4.29)	0.62
Crazy paving pattern vs ground glass opacities/infiltrates		42	8 (19.0)	1.54 (0.59-4.04)	0.38	1.51 (0.57-4.00)	0.40	1.12 (0.33-3.83)	0.81	1.15 (0.35-3.81)	0.81

Abbreviations: CI, confidence interval; NA, not applicable; OR, odds ratio; ref., reference.

[a] Events n (%) refers to the number of deaths within 30 days with "n category" of the same row as denominator.

[b] To account for nonparticipation affecting the study population, we performed sensitivity analyses applying inverse probability weighed regression adjustment in the logistic regression analyses. We report both crude (unweighted) and weighted effect estimates (ORs).

[c] We performed separate multivariable analyses for each exposure variable of interest and adjusted for confounders identified based on *a priori* knowledge. Please refer to Supplementary Table 2 for the respective covariates.

[d] "Any comorbidity" also included rheumatic conditions (n = 7) and chronic liver diseases (n = 1).

[e] We excluded patients with miscellaneous conditions (n = 2) from the analyses of immunosuppressive condition due to nonhomogeneity.

[f] Cardinal symptoms included cough, dyspnea, and fever.

[g] Thirty-nine patients were receiving supplemental oxygen when O_2 saturation was measured.

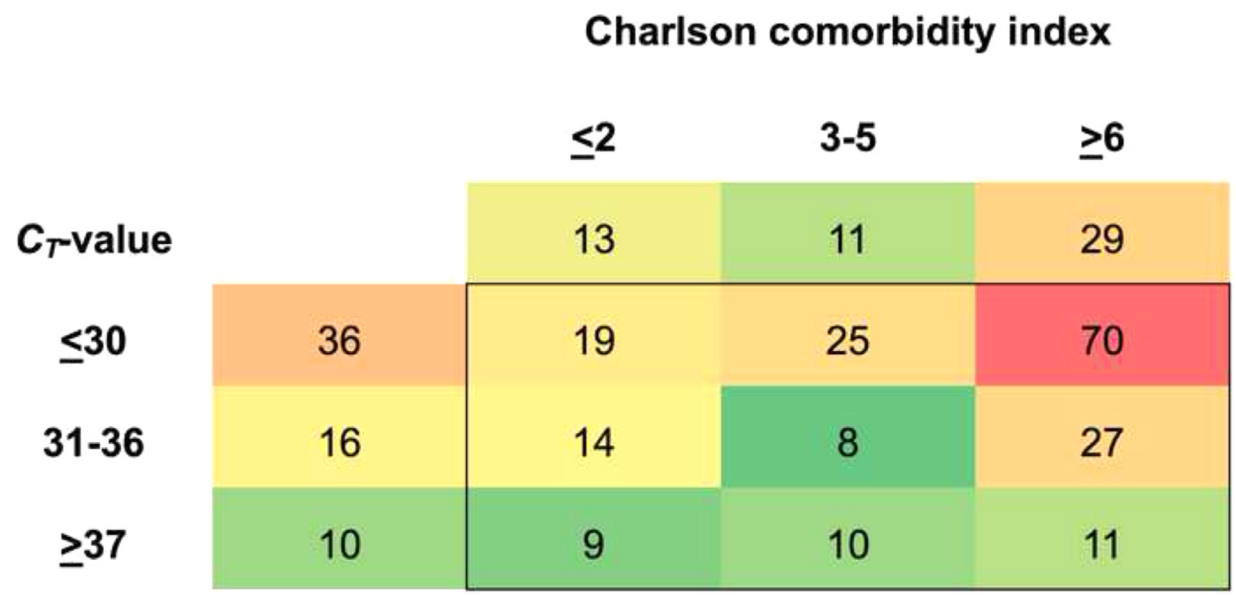

Figure 2. Mortality risk in patients with PCP. Heat map illustrating 30-day mortality (in %) in the study population of 170 patients without HIV within subgroups of Charlson comorbidity index, C_T value from semiquantitative real-time polymerase chain reaction for *P. jirovecii* detection in bronchoalveolar lavage fluid, and their interaction (framed in black). We adjusted for age, sex, and nonparticipation bias through inverse probability weighting. Retrospectively, 34 (20.0%) patients met the European Organization for Research and Treatment of Cancer/Mycoses Study Group Education and Research Consortium criteria for proven PCP, whereas 136 (80.0%) were classified as probable PCP cases. C_T, cycle threshold; PCP, *Pneumocystis* pneumonia.

patients had proven and 136 (80.0%) patients had probable PCP. Table 1 provides the patient characteristics. Antipneumocystis treatment was initiated in 158 (92.2%) patients and was significantly associated with fungal burden: those receiving treatment had a median (q_1-q_3) C_T value of 35 (32-37) compared with 37.5 (36.5-40) of the untreated patients ($p < 0.001$). The overall 30-day mortality was 18.2% (n = 31/170). Although not significant, patients who had comorbid diseases had a higher mortality rate than those with no comorbidity besides their underlying disease: 20.9% (n = 24/115) versus 12.7% (n = 7/55) (p = 0.29).

Concerning nonparticipation bias, we observed no significant skewness according to age, sex, C_T value, hospital, or period between consenters and nonconsenters (Supplementary Table 3).

Fungal burden and risk of 30-day mortality

Fungal burden, reflected by the C_T value from PCR analysis in BALF, was significantly associated with death: OR 1.64 (95% CI 0.60 to 4.53) for C_T values 31-36, increasing to OR 4.44 (95% CI 1.41 to 14.0) for C_T values ≤30 compared with patients with a C_T value ≥37 (Table 2a). In line with the univariable analyses, the multivariable analyses showed that a C_T value ≤30 was independently associated with 30-day mortality, whereas this was not the case for C_T values 31-36: adjusted OR 1.42 (95% CI 0.48 to 4.25) for C_T values 31-36, increasing to OR 5.43 (95% CI 1.48 to 19.9) for C_T values ≤30, compared with patients with a C_T value ≥37. The sensitivity analyses did not undermine this association (Table 2a). The association between fungal burden and mortality risk also held when restricting the analyses to patients receiving antipneumocystis treatment, regardless of adjustment for confounders and weighting (Supplementary Table 4). The same was true for patients with a C_T value ≤37 (Supplementary Table 5). In the latter, both C_T values 30-33 and C_T values ≤30 were significantly associated with higher odds of dying compared with C_T values 34-37.

Consistent with the findings mentioned previously, higher fungal burdens were associated with mortality when analyzing all patients with positive *P. jirovecii* PCR in BALF and a retrievable C_T value between 2006 and 2017 in Central Norway (N = 211) (Supplementary Figure 2). In this population with a median (q_1-q_3) C_T value of 36 (33-37), the adjusted HR for the 30-day mortality risk was 0.89 per C_T value (95% CI 0.83-0.96, $p < 0.01$) within the range of C_T values from 22 to 40 cycles (Table 2b).

Other risk factors for 30-day mortality

Regarding background characteristics, neither age nor sex predicted mortality (Table 3). Multimorbidity, reflected by CCI, was associated with 30-day mortality: OR 1.06 (95% CI 0.37-3.04) for CCI 3-5, increasing to OR 2.76 (95% CI 1.06-7.19) for CCI ≥6 compared with patients with a CCI ≤2. Cardiovascular comorbidity, including congestive heart failure and comorbid chronic lung disease, distinctly increased the mortality risk. Furthermore, underlying disease was associated with mortality: patients with solid tumors and immunological disorders had significantly higher odds of dying compared with those with hematologic malignancies. Moreover, premorbid corticosteroids seemed to increase the mortality risk in a dose-response relationship. Comorbid cardiovascular disease, premorbid corticosteroids, solid tumors, and immunological disorders were independently associated with death in the multivariable analysis. The sensitivity analyses did not suggest substantial bias (Table 3).

Regarding clinical presentation (Table 3), O_2 saturation <90% and severe host response, reflected by C-reactive protein (CRP) ≥100 mg/l and leukocytosis with higher neutrophil counts, increased the mortality risk significantly in the univariable analyses. The same was true for low serum albumin. O_2 saturation <90% and abnormal leukocyte counts, including lymphopenia, CRP ≥100 mg/l, and low serum albumin, were independently associated with death in the multivariable analysis. The sensitivity analyses did not indicate bias (Table 3).

The probability of dying within 30 days from PCR detection was strongly associated with the *P. jirovecii* burden and CCI score combined in the study population (Figure 2). Patients with low burdens (C_T value ≥37 and CCI ≤2) had a 9% risk of dying compared with 70% for those with high burdens (C_T value ≤30 and CCI ≥6). When separated, the spectrums of mortality risk were comparable:

from 10% to 36% for decreasing C_T values and from 13% to 29% for increasing CCI scores, respectively. We observed similar trends for patients with C_T values ≤37 (Supplementary Figure 3).

Discussion

In a population of 170 HIV-negative patients with proven or probable PCP, we studied the association of the fungal burden indicated by the C_T value, clinical demographic characteristics, and laboratory markers with 30-day mortality. Although a C_T value ≤30 was significantly associated with 30-day mortality, this was not the case for higher C_T values ≥31. Other factors significantly associated with mortality in multivariable analysis were comorbid cardiovascular disease, solid tumors, immunological disorders, premorbid corticosteroids, oxygen saturation <90%, leukocytosis with higher neutrophil counts, lymphopenia, lower serum albumin, and CRP >100 mg/l.

Patients who are immunocompromised who present with fever and lung-specific manifestations, including acute respiratory syndrome, require multimodal workups, including PCR analysis to exclude PCP. Survival depends on a prompt antimicrobial treatment, yet the management of patients who test positive on PCR can be challenging owing to diagnostic gray zones (*i.e.*, PCP vs colonization), heterogeneity among patients without HIV, and propensity for side effects. Increased awareness and lowered threshold for PCR testing for *P. jirovecii* in high-income countries magnify this dilemma. Considering this, we assessed the role of fungal burden in clinical risk stratification. Before this study, Liu et al. had reported associations between C_T values and in-hospital and 60-day mortality in 84 patients without HIV using a PCR targeting the major surface glycoprotein [5]. However, C_T value was not an independent predictor of 60-day mortality in their study [5]. Importantly, they only included patients with C_T values ≤35, resulting in a relatively small sample size [5]. Our findings support the hypothesis that fungal burden estimated by real-time PCR is associated with the outcome in the acute phase of infection. The survival analyses comprising all patients with a positive PCR and a retrievable C_T value appeared to confirm this association.

We also show how fungal burden and multimorbidity increase the mortality risk in a synergistic manner (Figure 2). Besides the compromised physiology to recover, reduced resilience against side effects and risk of interactions due to polypharmacy may play a role in this context [19]. The risk stratification included patients with low fungal burdens. Despite the retrospective restriction to proven and probable PCP cases, we cannot exclude that the positive PCR reflected colonization in some of these. In such patients, the pathogenic role of *P. jirovecii* is not completely understood [20,21]. Therein, the vast implications of colonization (e.g., role in lung diseases, precursor state for PCP, and risk of transmission) rather than the immediate risk of dying from infection may favor treatment, but this remains debated [1,6,22].

We found that markers of acute inflammation and hypoxemia were independently associated with infection severity. These observations resonate with previous studies [23–26] and the hypothesized pathophysiology attributing the high mortality in patients without HIV to a deleterious hyperinflammatory host response [6]. Clusters of differentiation 4^+ lymphocytes orchestrate the defense against *P. jirovecii,* and depletions or alterations predispose patients to PCP [27]. Relatedly, we found associations between lymphopenia and increased mortality risk, agreeing with the pooled data in a recent meta-analysis [28]. Although, these biomarkers may ameliorate risk stratification, the feasibility depends on availability and awareness, as underscored by the missing data herein.

The Fungal PCR Initiative has taken important steps toward the standardization of real-time PCR assays for *P. jirovecii* detection [17]. Recognizing this, certain laboratory aspects merit attention. We used C_T values as an indication of fungal burden, which, in contrast to absolute quantitation (*i.e.*, copies/ml), reflects a semi-quantitative estimate. Combined with a single-copy target (*i.e.*, β-tubulin), this hinders direct comparison of results with other studies. Furthermore, the human target used as an internal control in conjunction with the *P. jirovecii* PCR cannot quantitate nor exclude inhibition or extraction problems and therefore entails a certain risk of false negative results [10,29]. Lastly, we were unable to control for differences in the sample volume.

The strengths of this study lie in its regionwide multicenter nature, large sample number, and focus on readily assessable characteristics. All the same, it harbors certain limitations. First, we were unable to include all patients who were still alive. We addressed this with the sensitivity analyses, but we cannot exclude unmeasured confounding from variables such as underlying disease. Second, some of the independent variables had missing data, which we handled by complete case analyses. In addition, to strive for homogeneity, we restricted the analyses to patients with a retrievable C_T value from the PCR performed in BALF, the diagnostic gold standard [10]. These limitations represent potential selection bias. Third, only 28 patients (16.5%) had high fungal burdens (*i.e.*, C_T values ≤30), precluding the subgroup analyses in this category. Forth, owing to the retrospective design, neither the interval between disease onset and PCR analysis nor the treatment protocols were standardized ahead. Moreover, insufficient data hampered the adjustment for coinfections, respiratory insufficiency, and treatment delay. Finally, we might have missed eligible patients because the PCR targeted a single-copy gene and did not include a recommended internal control, as addressed previously.

In conclusion, our results suggest that fungal burden may be useful in the risk stratification of patients without HIV-negative patients with PCP.

Declarations of competing interest

The authors have no competing interests to declare.

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Ethical approval

The Regional Committee for Medical and Health Research Ethics has approved this study (REC-North, reference number 2017/2419).

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Author contributions

SG participated in study design, data collection, statistical analysis, interpretation of the data, and wrote the first draft of the

manuscript. BOÅ and TR participated in data interpretation, statistical analysis, and drafting of the manuscript. LH participated in data interpretation and drafting of the manuscript. JEA and JKD supervised and participated in study design, data collection, interpretation of the data, and drafting the manuscript. All authors have read and approved the manuscript.

Availability of data and materials

The dataset generated and/or analyzed during this study are not publicly available because of ethical and privacy concerns regarding individual study participants but are available from the corresponding author on reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2023.06.013.

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