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Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
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Trondheim, November 2023

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Pneumocystose i Midt-Norge – karakterisering av sykdomsbyrde og identifikasjon av diagnostiske og prognostiske faktorer

I denne avhandlingen har jeg studert soppmikroben *Pneumocystis jirovecii. P. jirovecii* kan gi livstruende lungebetennelse i individ med svekket immunforsvar, såkalt Pneumocystose. Pneumocystose utgjør en økende trussel for kreftpasienter, organtransplanterte og personer som tar immundempende medisiner for en rekke ulike sykdommer. På grunn av stigende forventet levealder og medisinske fremskritt i behandlingen av en rekke sykdommer er denne risikopopulasjon i rask vekst.

Målet med studiene var å karakterisere sykdomsbyrden og risikoprofiler assosiert med Pneumocystose i et norsk pasientutvalg og identifisere diagnostiske og prognostiske faktorer.

Studiene er basert på anonymiserte journalopplysninger fra pasienter som fikk påvist *P. jirovecii* i en luftveisprøve på St. Olavs hospital, Trondheim Universitetssykehus i perioden 2006 til og med 2017. Vi rekrutterte pasienter fra alle sykehusene i Helse Midt-Norge RHF.

I den første studien inkluderte vi 296 pasienter. Hele 98 % hadde andre underliggende sykdommer enn HIV-infeksjon som gjorde dem utsatt infeksjon med *P. jirovecii.* Flertallet led av kreft etterfulgt av autoimmune sykdommer, organtransplantasjon og kroniske lungesykdommer. Kun tre pasienter mottok forebyggende behandling. Pasientene debuterte med uspesifikke symptom som feber, tørrhoste og tungpust. Dødeligheten var 21.6 % på sykehus. Antallet pasienter testet for *P. jirovecii* og antallet med positiv test økte i studieperioden. I den andre studien fant vi at påvisningsmetoden for *P. jirovecii*, semi-kvantitativ nukleinsyreamplifisering, var moderat god til å skille lungebetennelse fra bærerskap. Nøyaktigheten økte ved å studere pasienter i undergrupper etter underliggende sykdom. I den tredje studien fant vi at høy soppmengde i en nedre luftveisprøve var assosiert med dødelig utfall. Dødeligheten var særlig høy blant dem med flere samtidige sykdommer (komorbiditeter) og høy soppmengde.

Pneumocystose ser ut til å være på fremmarsj i Norge og er assosiert med høy sykelighet og død. Sykdommen utgjør en risiko for en stadig eldre, mer heterogen og kompleks pasientpopulasjon. Forebyggende tiltak og økt bevissthet er nødvendig for å bremse utviklingen og redusere sykdomsbyrden.

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Capoterra, July 2023
Stine Grønseth

List of publications

This thesis is based on the following papers. They will be referred to by their roman numerals.

Paper I: Grønseth S, Rogne T, Hannula R, Åsvold BO, Afset JE, Damås JK. Epidemiological and clinical characteristics of immunocompromised patients infected with Pneumocystis jirovecii in a twelve-year retrospective study from Norway. BMC Infect Dis. 2021 Jul 7;21(1):659. doi: 10.1186/s12879-021-06144-1.

Paper II: Grønseth S, Rogne T, Hannula R, Åsvold BO, Afset JE, Damås JK. Semiquantitative Real-Time PCR to Distinguish Pneumocystis Pneumonia from Colonization in a Heterogeneous Population of HIV-Negative Immunocompromised Patients. Microbiol Spectr. 2021 Sep 3;9(1):e0002621. doi: 10.1128/Spectrum.00026-21.

Paper III: Grønseth S, Rogne T, Heggelund L, Åsvold BO, Afset JE, Damås JK. Role of fungal burden in risk stratification of HIV-negative patients with *Pneumocystis* pneumonia: A 12-year retrospective observational multicenter cohort. International Journal of Infectious Diseases, 2023, ISSN 1201-9712. doi: 10.1016/j.ijid.2023.06.013.

List of abbreviations

α:	Alfa
β:	Beta
γ:	Gamma
A-a-gradient:	Alveolar-arterial-gradient
AIDS:	Acquired immunodeficiency syndrome
ALI:	Acute lung injury
AMP:	Antimicrobial peptide
APACHE:	Acute Physiology and Chronic Health Evaluation
AUC:	Area under the curve
BALF:	Bronchoalveolar lavage fluid
BLAST:	Basic local alignment tool
BMI:	Body mass index
BUN:	Blood urea nitrogen
CAP:	Community acquired pneumonia
CAR:	C-reactive protein-albumin ratio
CAT:	Corticosteroids adjunctive treatment
CD:	Cluster of differentiation
CDC:	Centers for disease control and prevention
Cdc2:	Cell division cycle-2
CCI:	Charlson comorbidity index
CHF:	Congestive heart failure
CI:	Confidence interval
CMV:	Cytomegalovirus
CPP:	Crazy paving pattern
CRP:	C-reactive protein
C_T:	Cycle threshold
DHFR:	Dihydrophosphate reductase
DHPS:	Dihydropteroate synthase
DIF:	Direct immunofluorescence microscopy
DNA:	Deoxyribonucleic acid
ECIL:	European Conference on Infections in Leukaemia
ECOG:	Eastern Cooperative Oncology Group
EORTC:	European Organization for Research and Treatment of Cancer
FCR:	Fludarabine, cyclophosphamide, and rituximab
FN:	False negative
FP:	False positive
FiO_2:	Fraction of inspired oxygen
GAFFI:	Global Action for Fungal Infections
GGO:	Ground glass opacities
HAART:	Highly active antiretroviral therapy
HIV:	Human immunodeficiency virus
HR:	Hazard ratio
HRCT:	High resolution computed tomography
HSCT:	Hematopoietic stem cell transplantation

IBD:	Inflammatory bowel disease
ICU:	Intensive care unit
IFN:	Interferon
IL:	Interleukin
ITS-2:	Internal transcribed spacer-2
IV:	Intravenous
Kex-1:	Kexin-like serine protease
KL-6:	Krebs von den Lungen-6
LDH:	Lactate dehydrogenase
LIFE:	Leading International Fungal Education
LRS:	Lower respiratory tract specimen
MAC:	Membrane attack complex
MIQE:	Minimum information for publication in quantitative real-time PCR experiments
Mt-LSU:	Mitochondrial large subunit
MGW:	Molecular graded water
MSG:	Major surface glycoprotein
MSGERC:	Mycoses Study Group Education and Research Consortium
MSIS:	"Meldingssystem for meldepliktige sykdommer»
Mt-SSU:	Mitochondrial small subunit
NA:	Not available/assessed
NIPH:	Norwegian Institute of Public Health
NPV:	Negative predictive value
OR:	Odds ratio
PAMP:	Pathogen-associated molecular pattern
PaO_2:	Partial pressure of oxygen
PCP:	*Pneumocystis* pneumonia
PEEP:	Positive end expiratory pressure
PJP:	*Pneumocystis jirovecii* pneumonia
PCR:	Polymerase chain reaction
PPV:	Positive predictive value
PRR:	Pathogen recognizing receptor
PS:	Performance score
RA:	Rheumatoid arthritis
R-CHO(E)P:	Rituximab-cyclophosphamide, doxorubicin, vincristine, (etoposide), and prednisolone
ROC:	Receiver operating characteristic
rRNA:	Ribosomal ribonucleic acid
SAPS:	Simplified Acute Physiology Score
SARS-CoV-2:	Severe Acute Respiratory Virus Corona Virus-2
SLE:	Systemic lupus erythematosus
SOFA:	Sequential Organ Failure Assessment
SOT:	Solid organ transplantation
SP-D:	Surfactant protein-D
Spp.:	Species (plural)
Th:	T-helper
TMS:	Trimethoprim sulfamethoxazole
TN:	True negative
TNF:	Tumor-necrosis-factor
TP:	True positive
TS:	Thymidylate synthase
URS:	Upper respiratory tract specimen
WHO:	World Health Organization

Summary in Norwegian

Bakgrunn

Pneumocystis jirovecii er en opportunistisk soppmikrobe som kan gi livstruende lungebetennelse (pneumocystose) hos immunsvekkede pasienter. Pneumocystose er først og fremst kjent som en AIDS-definerende sykdom hos personer med fremskredet HIV-infeksjon. Takket være effektiv anti-retroviral behandling for HIV er denne sykdomsbyrden i nedgang i høyinntekstland som Norge. Moderne krefthandling, immundempende medisiner, vellykkede organtransplantasjoner og generell økning i forventet levealder gjør derimot at HIV-negative risikopopulasjoner er i rask vekst. Det finnes forebyggende behandling for pneumocystose som er både trygg og kostnadseffektiv. Ved sykdom er prognosene best når målrettet antimikrobiell behandling startes opp raskt. Utfordringen er å gjenkjenne pneumocystose klinisk og stille riktig diagnose. Det overordnete målet for dette prosjektet var å undersøke sykdomsbyrden assosiert med pneumocystose i en norsk helseregion og pasientutvalg. I tillegg ville vi identifisere diagnostiske og prognostiske faktorer assosiert med pneumocystose.

Metoder

Alle tre artiklene er basert på journalopplysninger fra pasienter som fikk påvist *P. jirovecii* i en luftveisprøve mellom i 2006 og 2017 ved St. Olavs hospital, Universitetssykehuset i Trondheim. Pasientene ble rekruttert fra hele Midt-Norge. Inklusjon av overlevende pasienter krevde aktivt samtykke (dvs. retur av signert samtykkebrev via posten). Vi samlet inn opplysninger om demografi, underliggende sykdommer, medisinbruk, symptom, funn, radiologi, forløp og utfall. Alle data ble anonymisert og registrert i et skreddersydd elektronisk skjema. Deretter brukte vi statistiske metoder for å analysere dataene. I første artikkel beskrev vi pasientpopulasjon, deres forløp og epidemiologiske trender. I andre artikkel studerte vi påvisningsmetoden for *P. jirovecii,* semikvantitativ nukleinsyreamplifikasjon, og hvorvidt denne kan skille mellom lungebetennelse og bærerskap. I tredje artikkel identifiserte vi faktorer assosiert med økt risiko for å dø innen 30 dager etter påvisning av *P. jirovecii* i en nedre luftveisprøve.

Resultat

I den første studien inkluderte vi 296 pasienter med påvist *P. jirovecii* i luftveisprøve i Midt-Norge mellom 2006 og 2017. Blant disse hadde 98 % (n = 290/296) andre underliggende

sykdommer enn HIV-infeksjon. Majoriteten led av kreftsykdommer etterfulgt av autoimmune sykdommer, status gjennomgått organtransplantasjon, kroniske lungesykdommer og HIV-infeksjon. Blant de HIV-negative var inntak av systemiske kortikosteroider på en rekke ulike indikasjoner en viktig fellesnevner. Kun tre pasienter mottok forebyggende behandling på sykdomstidspunktet. Pasientene presenterte med uspesifikke symptom som feber, hoste og tungpust. Dødeligheten på sykehus var 21.6 %. Vi fant en årlig økning i testing for *P. jirovecii* og antall positive tester i studieperioden. I den andre studien fant vi at påvisningsmetoden for *P. jirovecii* var moderat god til å skille bærerskap fra reell lungebetennelse i HIV-negative pasienter. Nøyaktigheten til testen ble bedre ved å studere pasientene i undergrupper etter underliggende sykdom. I den tredje artikkelen fant vi at høy soppmengde i en nedre luftveisprøve var assosiert med økt risiko for å dø innen 30 dager. Dødeligheten var særlig høy blant dem som led av flere samtidige sykdommer slik som hjerte- og karsykdommer inkludert hjertesvikt. Andre risikofaktorer for død var kreftsvulster, autoimmune grunnsykdommer, systemiske kortikosteroider, inflammatorisk vertsrespons, alvorlig respirasjonssvikt og lave nivåer av albumin og sirkulerende lymfocytter i blodet.

Konklusjon

Pneumocystose ser ut til å være på fremmarsj i Norge og er assosiert med høy sykelighet og død. Sykdommen rammer stadig eldre og mer komplekse pasientpopulasjoner. Forebyggende tiltak og strategier for å øke bevisstheten og redusere sykdomsbyrden er nødvendig.

Summary in English

Background

Pneumocystis jirovecii is an opportunistic fungus and the causative agent of *Pneumocystis* pneumonia (PCP) in humans. PCP is foremost known as an AIDS-defining illness in people with advanced HIV-infection. However, with the advent of highly active antiretroviral therapy this disease burden is declining in industrialized countries with universal health care. In contrast, we observe an increasing incidence of non-HIV PCP owing to longer survival and more aggressive therapies applied to cancers, immunological disorders, solid organ transplantation, and chronic lung diseases. PCP can be life-threatening. Prompt diagnosis and initiation of antimicrobial treatment improve survival but are challenged by unspecific clinical presentation and manifestations. Chemoprophylaxis to high-risk individuals is both safe and cost-effective. The overall aim of this Ph.D.-project was to investigate the burden of PCP in a Norwegian healthcare setting and identify diagnostic and prognostics predictors for PCP.

Methods

All three papers in this thesis are based on review of electronic hospital records. We included adults with positive *P. jirovecii* PCR in a respiratory specimen between 2006 and 2017 at St. Olavs hospital, Trondheim University Hospital, the only tertiary referral center in Central Norway. Inclusion of survivors required active consents. We collected comprehensive clinical and epidemiological characteristics and registered de-identified data in a tailored electronic form. Next, we used statistic methods to analyze the data. In the first study, we described the patient population, their clinical course, and epidemiological trends. In the second study, we assessed the in-house semiquantitative real-time PCR's ability to differentiate between PCP and colonization in HIV-negative patients. In the third study, we identified factors associated with 30-day mortality in HIV-negative patients with proven or probable PCP.

Results

In the first study we included 296 patients from Central Norway Health Authority with positive *P. jirovecii* between 2006 and 2017. All but six patients had non-HIV underlying conditions. Cancers combined accounted for 61.5 %, followed by immunological disorders, solid organ transplantations, and chronic lung diseases. Premorbid exposure to systemic corticosteroids alone or in combination with other therapies was a common denominator in

73.5 %. Only three patients were receiving chemoprophylaxis at presentation. The majority presented with at least two cardinal symptoms whereas hypoxia, cytopenias, and radiological manifestations compatible with PCP constituted the main objective findings. In-hospital mortality was 21.6 % (n = 64/296). We found an annual increase in both the number of PCR tests performed and positive cases from the introduction of PCR in 2006 to 2017. In the second study, the in-house PCR assay showed a sensitivity of 71.3 % and a specificity 77.1 % for distinction between non-HIV PCP and colonization. Stratification according to underlying condition improved the discrimination, likely due to intrinsic and extrinsic host heterogeneity within non-HIV PCP. In the third study, we found that high fungal burdens, indicated by low cycle threshold-values from semiquantitative real-time PCR in bronchoalveolar lavage-fluid, were independently associated with higher 30-day mortality. The risk of dying was especially high in those with high degree of multimorbidity in addition to high fungal burdens. Comorbid cardiovascular disease, solid tumors, immunological disorders, premorbid corticosteroids, severe hypoxemia and inflammatory host response, low serum-albumin, and lymphopenia were also independently associated with 30-day mortality.

Conclusion

The incidence of PCP seems to be increasing in Norway in patients exposed to chemotherapy and immunosuppressants. The morbidity and mortality attributed to *P. jirovecii* is substantial. This evolution calls for strategies to increase awareness and administration of prophylaxis to reduce the disease burden.

1 Introduction

1.1 Modern immunosuppressive era

Iatrogenic immunosuppression represents a double-edged sword in the era of modern medicine. While immunosuppressive drugs improve the quality of life and social outcomes of numerous individuals living with chronic immunological disorders and transplant recipients, they also increase the number of people living with secondary immunodeficiencies [1]. Simultaneously, population ageing in industrialized countries is accompanied by increased incidence of cancers [2]. Oncological treatment regimens tend to fail at discriminating malignant from healthy cells with rapid turnovers, including immune cells [3]. Thus, immunosuppression is a feared and common side effect. Indeed, infectious complications remain a major cause of morbidity and mortality in cancer patients [4]. Collectively, longer survival, development of new drugs, broader indications, and more aggressive treatment to patients with chronic conditions are resulting in a larger population with significant immune defects [5-7].

Against this backdrop, opportunistic infections foremost associated with the human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) reemerge [5]. Infection with the atypical fungus *Pneumocystis jirovecii* represents one of these. *Pneumocystis* pneumonia (PCP) is recognized as a severe and potentially fatal complication in patients exposed to iatrogenic immunosuppression and chemotherapy [1]. The epidemiology of PCP is evolving rapidly. The advent of highly active anti-retroviral therapy (HAART) has caused a decline in the incidence of HIV-associated PCP, especially in industrialized countries with universal health care access and coverage [8]. Meanwhile, several studies evidenced in this thesis indicate that non-HIV PCP cases are on the rise. Increased prescription of immunosuppressive drugs and an increase in the number of individuals undergoing solid organ transplantations (SOT), receiving hematopoietic stem cell transplantation, and surviving cancer, likely contribute to this trend. Besides increasing individual susceptibility to infections, provision of favorable grounds for disease transmission and reservoirs for pathogens, represent major safety concerns of modern immunosuppression [9].

Optimization of current prevention strategies requires precise knowledge about the epidemiology of *P. jirovecii*. Prior to this project, the disease burden of PCP in Norway was largely unknown. Concurrently, data from the Norwegian Prescription Database showed that the number of users of antineoplastic drugs (category L01) and immunosuppressive drugs (category L04A), corticosteroids excluded, more than doubled in Norway from 2006 to 2017 (**Figure 1**) [10]. These patients constitute a heterogeneous population with a prolonged life-expectancy. While the medications represent a cornerstone in controlling their underlying conditions, an increased risk of infections arises as the reverse of the medal. This development and reports from comparable countries implicated that PCP could represent a substantial and mounting public health threat in Norway. With this project, we sought to characterize the epidemiology of PCP in immunosuppressed individuals in a Norwegian health care setting and investigate diagnostic, therapeutic, and prognostic aspects. The study period comprised 12 years, from 2006 to 2017. To provide background information on relevant topics and the status quo in 2017 when this project initiated, the following paragraphs cover the immune system, secondary immunodeficiencies including HIV-infection, *P. jirovecii,* and PCP. Furthermore, in the second section of the background chapter an overview of the relevant literature published up to the end of 2017 is presented.

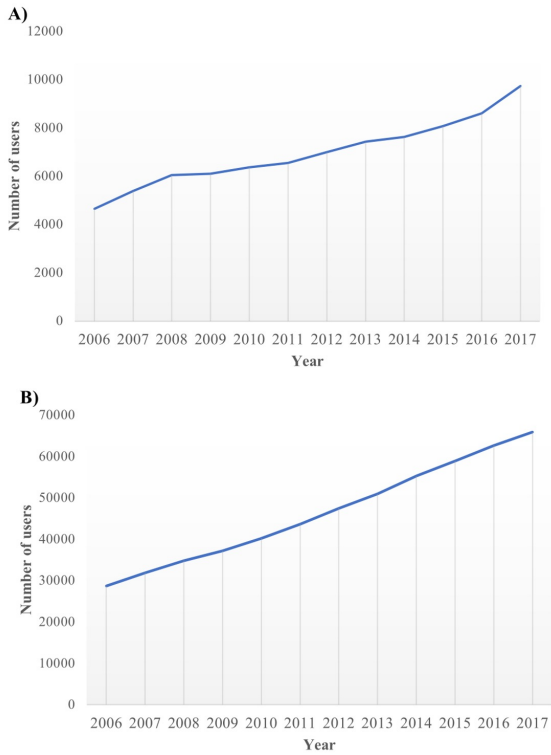

Figure 1. Graphic depiction of data from the Norwegian Prescription Database showing the number of users of antineoplastic (A) and immunosuppressive agents (B) in Norway from 2006 to 2017 [10]. Both sexes and users aged 15 years or older are included from the entire country. Note differing y-axes.

1.2 Basic overview of the immune system

To provide an insight into the mechanism of iatrogenic immunosuppression, the following section will provide an overview of the immune system, the target of immunosuppressive drugs. The immune system is constituted by two levels of defense, innate and adaptive immunity, both addressed here.

1.2.1 Innate immunity

The innate immune system exerts the first line of defense together with natural barriers such as skin, mucous membranes, and respiratory cilia. Macrophages, dendritic cells, and granulocytes constitute the innate immune system [11]. The latter includes neutrophils, eosinophils, and basophils. Collectively, the innate immune cells inhibit and delay the growth of microorganisms in the initial phase of infection until the adaptive immune system becomes activated. The cells derive from a common myeloid progenitor cell in the bone marrow [12], making them susceptible to any nocuous influence affecting the myeloid tissue.

The innate immune cells have three principal mechanisms of action: recognition, presentation, and mobilization of the adaptive immune systems. Recognition of pathogens is possible through pathogen-recognizing receptors (PRRs), present in macrophages, neutrophils, and dendritic cells [11]. Binding of so-called pathogen-associated molecular patterns (PAMPs) to the receptors, triggers an array of antimicrobial immune responses involving inflammation and secretion of molecules to alert and orchestrate the adaptive immune system [11]. Functionally, there are three types of PRRs, namely signaling, endocytic and secreted receptors [13]. For instance, activation of membrane-bond toll-like receptors (TLRs), induces intracellular signaling cascades [13]. The net downstream effects include translocation and activation of nuclear-factor kappa B-pathways, resulting in gene expression of pro-inflammatory cytokines, chemokines, and receptors involved in stimulation of adaptive immune cells [13]. Cytokines such as tumor necrosis factor-alfa (TNF-α) and interleukin-6 (IL-6) act as means of intercellular communication in addition to having organ specific effects [14]. TNF-α and IL-6 are both subjects of iatrogenic immunosuppression.

Endocytic receptors, present in the cell membrane, mediate phagocytosis of the PAMP-receptor complex [13]. The macrophage mannose receptor is critical for ingestion and

eradication of *Pneumocystis*, and mutations in the receptor gene results in increased susceptibility to PCP [13].

Antimicrobial peptides (AMPs) belong to the class of secreted receptors, together with other molecules like C-reactive protein secreted during the acute-phase response of infection [13]. Their main function is opsonization, that is binding to microbial cell walls to tag them for recognition by phagocytes and the complement system for killing. The latter, constitutes a component of innate immunity [12]. Activated complement generates three primary effects: anaphylatoxins involved in allergic reactions, opsonins for tagging, and assembly of the terminal membrane attack complex (MAC) which lysis opsonized pathogens or damaged "self-cells" [12].

1.2.2 Adaptive immune system

The adaptive immune system consists of B-and T-lymphocytes. In common, these act as a second line of defense, requiring more time to react, but when they do so, they develop immunologic memory to provide a more rapid and robust immune response in case of a secondary infection with the same pathogen. Both B-and T-cells receptors are complex multi-component molecules resulting from intricate genetic arrangements and intended errors. This evolution assures a receptor diversity greater than the number of receptor genes coding the various proteins to recognize all potential pathogens [12, 15].

T-cells originate from hematopoietic stem cells that have migrated to the thymus and develop into cluster of differentiation (CD)4^+ T-helper cells or cytotoxic $CD8^+$ T-cells [11, 12]. Mature T-cells recognize antigens through interaction between their membrane bound T-cell receptor and major histocompatibility complex (MHC)-molecule on antigen presenting cells [12]. $CD8^+$ T-cells recognize endogenous antigens presented on MHC class-1, expressed by all nucleated cells [11]. $CD8^+$ T-cell activation results in killing of infected or cancerous cells [12]. Oppositely, $CD4^+$ T-cells interact with MHC class-II, exclusively expressed by macrophages, dendritic cells, and B-cells, and recognize exogenous peptides originating from phagocytosis [11]. Upon activation, the $CD4^+$ T-cells replicate and differentiate into situation-dependent lineages based on the cytokine milieu produced by the antigen presenting cell [11]. The arising sub-population of so-called T-helper (Th) cells conserve the antigen specificity of the progenitor cell but secrete different constellations of cytokines to mediate distinct effector

functions [15]. These include enhancement of microbe killing (Th1-cells), production of antibodies and expulsion of helminths (Th2-cells), induction of inflammatory responses (Th17-cells), and dampening of immune responses (regulatory Th-cells) [11]. The cytokine IL-23 secreted by Th17-cells is implicated in the autoimmune disease psoriasis, and novel psoriasis drugs target IL-23 [16].

B-cells arise from hematopoietic stem cells in the bone marrow and are primarily responsible for a humoral response with secretion of antigen-specific antibodies, which are involved in neutralization of viruses, opsonization of pathogens, antibody-mediated cytotoxicity, and activation of the complement system [12, 15]. Rituximab, a CD20-antibody directed against $CD20^+$ B-cells, is used in treatment of both autoimmune diseases and lymphoproliferative malignancies and causes depletion of this cell line [11, 17].

Natural killer cells (NK)-cells also descend from common lymphoid progenitor cells in the bone marrow [12]. They respond quickly to transformed cell (e.g., cancer cells) or virus-infected cells and act by cytolytic antibody-dependent killing [12].

1.3 Secondary immunodeficiencies

1.3.1 Definition and distinctions

There is a distinction between primary and secondary immunodeficiencies: Primary immunodeficiencies are congenital and caused by genetic defects, while secondary immunodeficiencies are acquired during life. The latter can arise from infectious agents, metabolic diseases, drugs, environmental conditions, and extremes of ages [3]. The following section will cover causes of secondary immunodeficiencies, namely iatrogenic immunosuppression, non-iatrogenic host factors, and HIV-infection.

1.3.2 Immunosuppressive drugs - classification and mechanisms of action

Immunosuppressive drugs are prescribed for numerous conditions, including rheumatic diseases, autoimmune disorders of the skin, central nervous system and gastrointestinal tract, hematologic disorders, cancers, and to SOT recipients to prevent graft rejection. Here follows a brief overview since there is a clear association between certain infections and the pharmacological mechanism of action at a cellular and molecular level.

Classic immunosuppressive drugs

Prednisone has become one of the most important anti-inflammatory drugs since it was first isolated in 1950 and used in clinical trials for rheumatoid arthritis (RA) [17]. van Staa et al. estimated the prevalence of oral corticosteroid use to be between one and two percent in the adult population in Great Britain [18]. Corticosteroids inhibit the immune system both quantitatively and qualitatively by influencing protein synthesis in the target cell [19]. They affect virtually every cell type involved in immune responses and put the host at increased risk of various viral, bacterial, fungal, and parasitic infections [19]. Importantly, at high doses (usually defined as >20 mg for 4 weeks) corticosteroids cause a depletion of circulating T-lymphocytes, due to alterations in lymphocyte kinetics through inhibition of IL-2, a principal T-cell growth factor an T-cell regulator, amongst other mechanisms [3, 11]. As a result, the immune surveillance of dormant intracellular pathogens such as *Mycobacterium tuberculosis,* latent viral pathogens of the *Herpesviridae* family, including cytomegalovirus (CMV), as well as cell-mediated immunity to fungi like *P. jiroveci* diminishes [5, 9]. Exposure to corticosteroids may also result in diminished response to passive immunization, making vaccines less effective [11]. Because of this unspecific immunosuppression and several other side effects, many steroid-sparing drugs have emerged [17]. For example, cytotoxic agents, originally conceived to control cancerous cell growth and ablate the bone marrow for transplantation [3]. Later, the indications have extended to autoimmune and immunological disorders and graft rejection prophylaxis, exploiting the proliferative nature immune cells have in common with cancer cells [3]. The alkylating agent cyclophosphamide, and the antimetabolites methotrexate, mycophenolate, azathioprine, and 6-mercaptopurine are among the most common drugs for these applications are [3, 17]. Other compounds such as sulfasalazine, leflunomide, and hydroxychloroquine interfere with deoxyribonucleic acid (DNA)-synthesis and inhibit both B-and T-cell proliferation [3]. Depending on the dose, they may also impair cellular and humoral memory from previous antigen sensitizations [3]. Toxicity to hematopoietic and nonhematopoietic cells with development of cytopenias and disruption of skin and mucosal barriers represents the major drawback of these agents [3, 4]. Most of these effects occur in a dose-dependent manner, and cytopenias caused by myelosuppression is the most important dose-liming effect [4]. These considerations are especially important in oncology owing to higher doses and shorter treatment intervals, often in combination with radiotherapy [4, 13].

Lastly, tacrolimus and ciclosporins suppress T-cells via calcineurin-inhibition which in turn hampers production of IL-2 [11]. Other agents with similar mechanisms of action and immune selectivity include sirolimus and pimecrolimus [3]. Compared to corticosteroids and cytotoxic drugs, the advantage of these drugs is sparing of macrophages and neutrophils [3].

Biologic drugs

The term "biologic drugs" is used to classify proteins produced with biological methods, in difference from chemically synthesized drugs (e.g., methotrexate) [17]. Characteristically, biological drugs target pro-inflammatory cytokines, including IL-1-β, TNF-α, IL-6, their receptors or surface proteins expressed on immune cells (e.g., CD20 on B-cells). The advantage is increased immune specificity. Over the last decade, the indications for prescribing biologic drugs have increased dramatically. These include, RA, spondylarthritis, inflammatory bowel disease (IBD) and dermatological conditions such as psoriasis as mentioned above [6, 17]. Inhibitors of TNF-α are the most widely used biologic agents. TNF-α is potent pro-inflammatory cytokine secreted by macrophages, T-cells, B-cells, and dendritic cells [17]. While inhibition reduces chronic pathological inflammatory response in autoimmune diseases, it also neutralizes the important anti-infective action of TNF-α. More specifically, TNF-α is essential in fighting intracellular bacterial infections (e.g., *Listeria, Legionella, Salmonella*), viruses and fungi [20]. In addition, TNF-α contributes to formation and maintenance of granulomas, a strategy to control pathogens that are difficult to eradicate, such as mycobacteria [20].

1.3.3 Other causes of impaired immunity

Global life expectancy has increased markedly since the beginning of the third millennium [21]. With respect to immune function and morbidity, longevity has several implications. First, ageing is one of many host factors that elicit an immunosuppressive effect *per se,* so-called immunosenescence [22]. Quantitative and qualitative alterations resulting in a less robust and effective function of both the innate and adaptive immune system characterize this phenomenon [3, 22]. Second, the risk of developing cancer increases with ageing as the genetic instabilities from exposure to carcinogens accumulate and immune surveillance decreases [23]. Aside from extensive treatment-related immunosuppression in oncology, malignant processes such as lymphoproliferative malignancies, contribute to the secondary immunodeficiency [11]. In solid tumors, obstruction from tumor progression, diagnostic and

surgical procedures, and insertion of medical devices may increase the susceptibility to infection due to stasis and damaged epithelial linings [4]. Concerning other extrinsic factors, viral infections other than HIV, including influenza virus and CMV, are associated with lymphopenia, T-cell anergy, and phagocytic defects [3, 24]. The latter is also present in autoimmune disease like RA and systemic lupus erythematosus (SLE) [24]. Lastly, common non-communicable diseases such as cirrhosis, diabetes mellitus, chronic uremia, nephrotic syndrome, and malnutrition, compromise immune function [3, 11]. Collectively, these conditions represent physiological and pathological factors contributing to the "net state of immunosuppression" [9].

1.3.4 HIV and AIDS

Subtypes and epidemiology

HIV type 1 and 2 are the major human AIDS-viruses and origin from cross-species zoonosis from African primates [25]. HIV-1 is more pathogenic, transmits more easily and predominates with a worldwide distribution, though different subtypes prevail across continents [26]. In contrast, HIV-2 progresses more slowly to AIDS and is restricted to West-Africa where it is endemic [3]. According to UNAIDS, an estimated 36.7 million people were living with HIV at the end of 2017 [27]. The advent HAART has led to an increasing prevalence due to higher life expectancies of people living with HIV [26]. The incidence has declined by almost 50 % since a peak in 2006, to approximately 1.8 million new cases in 2017 [27]. HIV/AIDS still represent a major disease burden, especially in Sub-Saharan Africa, harboring more than half of the world's population living with HIV [27].

HIV-transmission occurs through sexual contact, parenteral inoculation (e.g., contaminated needles), and vertically from infected mothers to fetus or newborns [25]. In developing countries, unprotected heterosexual intercourse is the primary mode of transmission [26]. Gender inequalities characterize the HIV-epidemic, and most women are infected by men [26, 27]. Sex-workers, intravenous drug-users, immigrants, and prisoners represent other vulnerable groups [26, 27]. Globally, about two thirds of the infected people have access to HAART [27]. Discrimination, stigma, and laws criminalizing drug-use and homosexuality continues to be informal barriers impeding universal HAART-access [26].

Clinical aspects

The diagnosis of HIV-infection is usually made by detection of antibodies against the HIV protein p24 with a sensitive ELISA or commercial combination test detecting both antigen and antibodies [3, 28]. Positive tests require confirmation by Western Blot detecting several antibodies or PCR for detection of HIV DNA and RNA [3, 28].

HAART targets viral entry into host cells as viral replication. The reverse transcriptase is prone to errors promoting emergence of genetically diverse strains giving origin to antiviral resistance. Combining drugs from various classes limits resistance and represented a historical breakthrough [25]. Genotyping identifies HIV mutations that confer resistance to tailor the treatment regimens [3, 28].

Progressive CD4+ T-cell depletion ultimately resulting in AIDS constitute the natural progress of HIV-infection. Clinically, tree phases can be distinguished [26]. First, the acute phase, characterized by a peak in viremia from dissemination from the mucosal membranes and rapid loss of $CD4^+$ T-cells. Acute HIV-infection may be accompanied by flu-like symptoms, rash, and lymphadenopathy. Unspecific symptoms, high viral loads, and host unawareness makes this phase a potential driver of the epidemic. A middle chronic phase follows, with low-grade viral replication in the lymph nodes due to the host's inability to eradicate the virus. Host and pathogen factors determine how long this phase lasts [3]. Lastly, when the $CD4^+$ T-cell count becomes less than 200 cells/mm^3, the hallmark manifestations, termed AIDS-defining diseases, occur. These consist of opportunistic infections, secondary neoplasm, many of which caused by oncogenic viruses, diarrhea and weight loss, and HIV encephalopathy with dementia [26, 29]. Major HIV/AIDS-pathogens beyond *P. jirovecii* include *M. tuberculosis,* viruses belonging to the *Herpesviridae* family (e.g., herpes simplex, varicella zoster, and CMV), and fungi like *Candida* spp. and *Cryptococcus* neoformans [26, 28].

Recent discoveries regarding HIV pathogenesis have evidenced a marked increase in immune activation, also in individuals on HAART [26]. This state has been implicated in development of non-communicable comorbidities in people living with HIV.

HIV/AIDS in Norway

In Norway, the incidence of HIV saw a rise at beginning of the new millennium followed by a decline owing to decreased transmission among men who have sex with men [28]. Together with immigrants they represent the major risk groups in Norway [28]. The declining trend has been attributed to increased testing, prompt HAART initiation, access to preventive measures like pre- and post-exposure prophylaxis [28]. In 2017, between 4500 and 5000 people were living with HIV in Norway, more men than women [28].

1.4 *P. jirovecii*

1.4.1 Brief history, classification, and current epidemiology

Pneumocystis is a unicellular yeast-like fungus and the causative agent of PCP [30]. Historically, Chagas first identified *Pneumocystis* in the lungs of rats and guineapigs in 1909, but he misclassified them as a morphologic form of the protozoan *Trypanozoma cruzii* [31]. Within a few years, *Pneumocystis* became recognized as a separate organism, and renamed *Pneumocystis carinii* [32]. The "protozoan hypothesis" remained until 1988, when modern DNA analysis demonstrated that *Pneumocystis* is a fungus [32]. However, several traits such as its lacking chitin and ergosterol in the cell wall, the latter a key target for anti-fungals, response to pentamidine, an anti-protozoa agent, and very difficult growth *in vitro,* makes *Pneumocystis* an atypical fungus [32, 33] (**Figure 2).**

During the Second World War, Jirovec and Vanek identified *P. carinii* as a cause of "plasma cell pneumonia" in malnourished and premature infants [34]. When a human-specific species was recognized in 2011, it was named *P. jirovecii* in honor of Jirovec, while *P. carinii* was reserved for the rat-specific species [34]. This discovery disproved a pre-existing hypothesis of PCP being a zoonotic disease transmitting across species [35]. Indeed, each species is characterized by different genetics and exhibits stringent host specificity, which is unprecedented in other fungi [36].

Figure 2. Diagram summarizing specific traits that make *Pneumocystis* spp. atypical fungi.

PCP is an opportunistic infection and causes substantial morbidity and mortality in individuals with impaired immunity [30]. Before 1980, it was uncommon and cases of PCP appeared sporadically, including outbreaks in Iranian orphanages in the 1950's [31]. With the emergence of HIV/AIDS-epidemic in the early 1980's, the burden increased drastically, and PCP soon became the most common AIDS-defining disease in developed countries [8, 37]. Later, the introduction of prophylaxis to HIV patients with $CD4^+$ T-cells below 200 cells/mm^3 from 1989 and, above all, the advent of HAART in the mid 1990's, caused a substantial decline in the incidence [37]. In Europe, the incidence fell from 4.9 cases per 100 000 person years before 1995 to 0.3 cases per 100 000 person years after 1998 [38]. Despite this evolution, *P. jirovecii* remains a leading opportunistic pathogen in HIV-infected individuals who are not receiving or responding to HAART, or are unaware of their HIV-status, especially in the third world where HIV is endemic [30]. Moreover, industrialized countries may expect a growing burden of non-HIV PCP cases due to increased and more aggressive administration of chemotherapy and immunomodulatory drugs [5]. Worldwide, the total number of PCP cases was estimated to be approximately 500 000 cases in 2017 [39]. In Norway, PCP is not a notifiable disease unless it occurs in a patient with HIV/AIDS, hence the incidence in HIV-negative individuals is unknown [28]. According to national health

registries, PCP is the leading opportunistic infection in HIV-infected people, and the initial AIDS-defining disease in one third of all AIDS cases [40]. Recent epidemiological trends are addressed more in detail in **Section 1.7 Previous literature related to the papers in this thesis.**

1.4.2 Acquisition and transmission

Inability to sustained culture of *Pneumocystis* spp. outside host lungs has hampered basic research on life cycle and identification of its infectious form [41]. *Pneumocystis* enters the host via the airborne route, but how *Pneumocystis* transmits and PCP is acquired, remain key knowledge gaps [41]. Today, two postulated mechanisms exist: Prevalence studies showing close to universal seropositivity in infants have led to a hypothesis of endogenous reactivation from latent infection in immunocompromised hosts [34]. The alternative hypothesis states that PCP results from new exposure, either from interhuman transmission or a common environmental source [30]. The mechanisms are not mutually exclusive and may co-exist [42]. Several outbreak reports and detection of *P. jirovecii* DNA in patient rooms and surroundings support the latter theory [41]. Also, recurrent infection in HIV-infected individuals attributed to genetically distinct strains, favors new exposure rather than reactivation [43]. Concurrent genotype switching in both nuclear and mitochondrial gene loci, argued against selection of mutations occurring during the first disease episodes, which could have been an alternative explanation behind the findings [43]. Moreover, molecular genotyping studies showing genetic homogeneity between strains isolated from patients developing PCP in clusters, suggest nosocomial transmission [44]. Based on mounting evidence, a growing fear is that health workers exposed to *P. jirovecii* may serve as transient vectors between infected and susceptible patients, respectively [45]. So far, this issue is considered and managed differently. The Center for Disease Control (CDC) in the United States advices placing PCP patients in separate rooms to protect other immunocompromised patients [46]. The French guidelines are stricter and advocate droplet isolation [47]. In contrast, the consensus group releasing guidelines for patients with malignancies in 2014 deemed the evidence insufficient to mandate isolation of cancer patients with PCP [48]. In Norway, there are no official infection control guidelines to prevent nosocomial transmission.

1.4.3 Colonization and its implications

In contrast to immunocompromised hosts, infection in immunocompetent individuals usually result in asymptomatic colonization, that is the presence of *P. jirovecii* in persons without signs and symptoms of acute pneumonia or in persons in whom respiratory manifestations may be related to an alternative diagnosis [34, 49]. The reported prevalence of colonization varies across studies due to different diagnostic techniques (i.e., microscopy, PCR, and serology), respiratory specimens, demographics, and geographic locations [50].

There are several host related risk factors for colonization, for instance age, underlying condition, such as chronic lung diseases, malignancy or HIV-infection, pregnancy due to altered immune status, smoking, and medications like corticosteroids and TNF-α-inhibitors [34] (**Figure 3**). However, some reports suggest that colonization also occurs in healthy immunocompetent individuals [34]. Indeed, in an autopsy study, mainly performed on individuals succumbing a violent death, Ponce et al. found *P. jirovecii* in the lungs of 64.9 % (n = 50/77) of the victims using both PCR and microscopy [51]. Other studies have reported lower prevalence in the general population, likely reflecting heterogeneity in the above-mentioned factors [50].

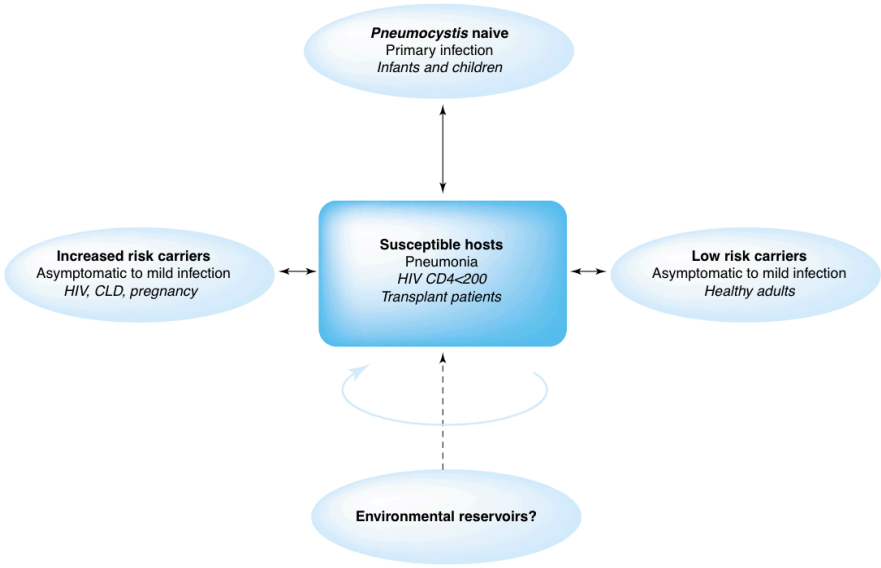

Figure 3. Potential sources and routes of exposure preceding PCP. Bold text reflects the general groups that may be infected with examples in italics. The dotted line from environmental reservoirs represents the unconfirmed nature of this route of exposure. CLD, chronic lung disease. Figure reprinted with permission from [52].

The clinical implications of colonization are vast. First, colonization likely precedes PCP, though the duration of the incubation period remains undefined and depends on host factors [53, 54]. Therefore, colonization should be considered a risk factor for developing PCP, especially in combination with compromised immunity [55]. In healthy individuals, a normal immune response eradicates the fungi and colonization appears to be a transient phenomenon [56, 57]. Second, colonized individuals, including children, may serve as fungal reservoirs and vectors in transmission networks [52, 55]. Thus, treatment may reduce the human reservoir of *P. jirovecii* [53, 54]. On the other hand, prophylaxis prescribed to colonized individuals may promote antimicrobial resistance arising from new mutations, especially in the sulfa drug target (i.e., dihydropteroate synthase (DHPS)) [50]. Lastly, the association between colonization and specific diseases have raised hypotheses of a pathogenic role for *P. jirovecii* [34]. In the case of chronic pulmonary obstructive disease (COPD), fungal tropism for the lungs combined with immunogenicity argue for a primary role, while structural damages and immunosuppression from smoking and corticosteroids, could explain secondary colonization [34]. Colonization has also been implicated as a co-factor in sudden infant death, cystic fibrosis, lung cancer, and interstitial lung disease to mention a few [34, 50].

1.4.4 *Pneumocystis* biology

All life cycles of *Pneumocystis* spp. have been observed in lungs, and extrapulmonary disease is rare [57]. *Pneumocystis* grows extracellularly attached to alveolar pneumocytes type 1, and are thought to be host obligate, which would explain the inability to culture organisms on artificial media outside host lungs [35]. Also, no viable form has been identified in the environment, providing additional evidence for this theory [35, 57]. Two distinct life forms can be appreciated with microscopy during infection, namely cysts (8 μm in diameter) and trophozoites (1-4 μm in diameter) [30]. The trophic form results from asexual reproduction [30]. Sexual reproduction occurs when trophozoites conjugate into diploid precysts, which in turn mature and rupture to release new trophozoites [30]. During infection, the trophozoites dominate over the cyst form in an approximate 10:1 ratio [53]. Cysts seem to be the infectious propagule based on animal studies [35]. The antifungals echinocandins target β-D-glucan and are active against the cystic form, whereas β-D-glucan is absent in trophozoites [35]. While echinocandins may prevent transmission through destruction of cysts, they appear insufficient in clearing infection [34].

Once *Pneumocystis* attaches to pneumocytes, fungal cell wall components bind to the extracellular matrix surrounding the epithelium, which in turn activate signal pathways promoting growth and proliferation [41]. *Pneumocystis* likely replicates at low levels in the alveolar space of hosts with intact immune systems [35]. *Pneumocystis* evades host immunity through active antigen variation and masking of β-D-glucan, a known PAMP and immune activator [58]. In this context, the host seems to tolerate transient or residing fungi in a commensal-like relationship [35, 57]. However, any immune compromise may perturbate this equilibrium permitting proliferation of *Pneumocystis* [35, 55] (**Figure 4)**. Release of β-D-glucan from organism death in states of high organism burden appears to increase and contribute to deleterious an immune activation described below [58].

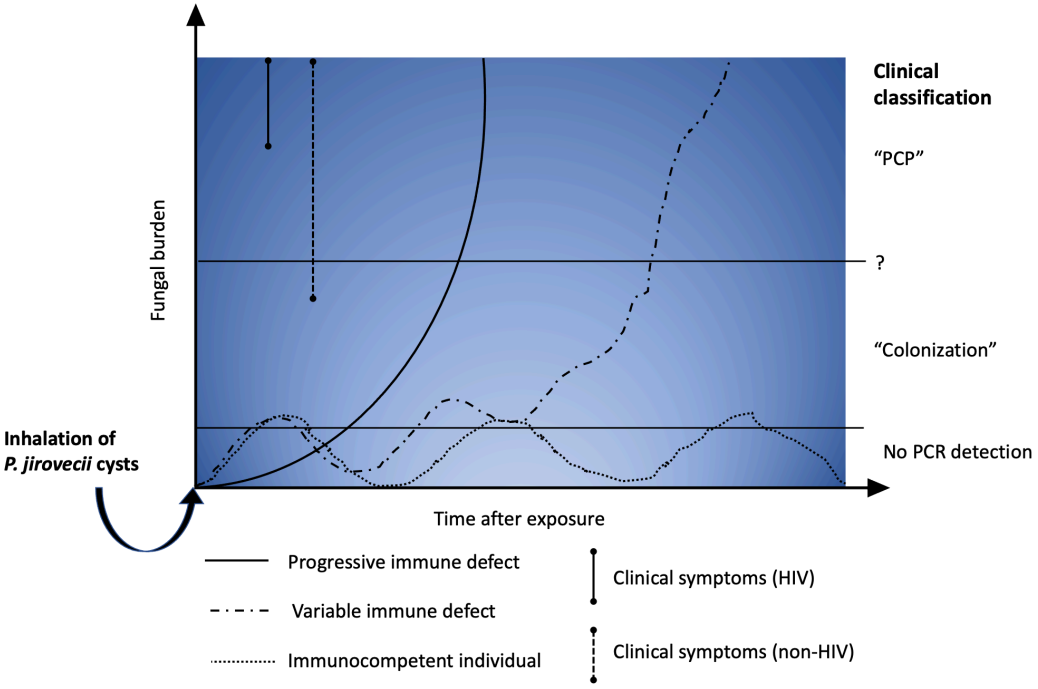

Figure 4. Natural course of infection according to immune status. Upon exposure, *P. jirovecii* multiplies in low levels in the alveolar space in the lungs. In the initial phase PCR may be negative. Depending on the host's immune status, the evolution of fungal proliferation may differ. Rapid evolution from exposure to clinical manifestation, i.e., PCP, typically occurs in advanced HIV-infection or individuals exposed to immunosuppressive regimens, though at high (solid line) and low burdens (dotted line), respectively. Variable fungal loads eventually sufficient to cause PCP can be observed in cancer patients submitted to courses of chemotherapy. Lastly, low levels of *P. jirovecii* without clinical manifestations of PCP are typically detected by PCR in immunocompetent individuals with or without risk factors for colonization. In these cases, this state may represent a transient carriage or precede PCP development depending on the host subsequent immune status. Regardless of outcome, these individuals may represent reservoirs and disease vectors for interhuman transmission. Figure adapted from [55].

1.4.5 Possible advances for basic research

In 2011, Schildgren et al. reported a novel mechanism to grow *P. jirovecii* using differentiated pseudostratified CuFi-8 cells in a three-dimensional air-liquid interface culture system [59]. However, another research group failed to reproduce the results [60]. Any sustainable culture method for *ex vivo* studies would enhance knowledge about *Pneumocystis* spp., their life cycle, and potentially new treatment targets.

1.4.6 Host response to infection

In vitro studies have shown that attachment of *Pneumocystis* to pneumocytes is insufficient to disrupt the barrier function and cause the lung injury observed in severe PCP [30]. It appears that severe pneumonitis and mortality correlate with neutrophilic lung inflammation rather than organism burden [61]. Here, follows a brief overview of the immune response to *Pneumocystis.*

Upon inhalation, *Pneumocystis* must first overcome the mucociliary clearance in the upper airways. Smoking is known to impair this defense mechanism, which might contribute to increased risk of colonization in smokers [50]. Once the fungi reach the lower airways, degradation by alveolar macrophages represents the main clearance mechanism [56]. In response to fungal proliferation, macrophagic uptake occurs through multiple receptors systems including binding of the major surface glycoprotein (MSG) to the mannose receptor [53]. Opsonic proteins, like immunoglobulin G, enhance phagocytosis [53]. Besides phagocytic effector functions, recognition of immunogenic cell wall components such as mannose and β-D-glucan leads to transcription of proinflammatory cytokines and chemokines to recruit other immune cells [53].

Considering the adaptive immune system, $CD4^+$ T-cells play an essential role for eradication, particularly through activation and stimulation of macrophages and development of immunological memory [30]. PCP occurs almost exclusively in HIV-infected individuals when $CD4^+$ T-cells fall below 200 cells/mm^3, underscoring their critical role [56]. $CD4^+$ T-cells proliferate in response to *Pneumocystis* antigens and generate interferon-gamma (IFN-γ) [53]. IFN-γ recruits more macrophages, and induces their production of TNF-α, superoxides and reactive nitrogen species [30]. The latter two are pivotal for killing ingested organism, while TNF-α induces production of cytokines and chemokines and recruits monocytes,

lymphocytes, and neutrophils [36]. Relatedly, antibody-mediated neutralization of TNF-α delays *Pneumocystis* clearance in animal models [30]. In humans, prescription of TNF-α inhibitors to RA-patients is associated with PCP, particularly when combined with other immunosuppressants [20, 42].

Regarding cytotoxic $CD8^+$ T-cells, animal studies suggest that depletion does not increase susceptibility to PCP, nor do $CD8^+$ T-cells succeed in controlling infection in absence of $CD4^+$ T cells [34]. However, mice depleted of both $CD4^+$ and $CD8^+$ T-cells exhibit significantly higher fungal burdens compared to those lacking $CD4^+$ T-cells alone, suggesting that $CD8^+$ T-cells aid clearance [34]. $CD8^+$ T-cells has also been implicated in the immune mediated inflammation responsible for impaired lung function [62]. A more recent animal study demonstrated that the effector function may depend on $CD8^+$ T-cell subset, that is cytotoxic type 1 $CD8^+$ T-cells enhanced macrophage-mediated killing, whereas type 2 $CD8^+$ T-cells promoted lung injury [63].

Lastly, B-cells elicit the humoral defense against *Pneumocystis* and function as antigen-presenting cells to naïve T cells [64]. Both functions contribute to successful infection control [34]. The importance of antibodies has been confirmed directly in experimental animal studies, while the observations that most healthy adults are seropositive, provide indirect evidence [34]. Moreover, neutralization of plasma cells from administration of rituximab is associated with increased risk of PCP in RA- and lymphoma-patients [42]. The function of antigen-presentation by B-cells was highlighted in an animal model by Lund et al. [65]. In this study, activation of $CD4^+$ T-cells was compared between wildtype mice and mice with MHC class II knocked-out B-cells [65]. In the lungs of latter, activated $CD4^+$ T-cells were fewer, and the fungal burden remained higher in the knock-out mice [65].

1.4.7 Double-edge nature of immune response

In animals without HIV-infection, researchers have found an inverse relationship between *P. carinii* burden and the level of circulating $CD4^+$ or $CD8^+$ T-cells [66]. Undoubtably, organism clearance requires effective innate and adaptive immune responses. However, severe PCP is attributed to massive neutrophil influx causing alveolar damage and impaired gas exchange through release of proteases, oxidants, and cationic proteins [36]. Indeed, Limper et al. observed that oxygenation and survival appeared to be inversely correlated with neutrophil

influx in the alveoli [61]. This was especially evident in HIV-negative PCP patients who despite lower fungal burdens, showed higher neutrophil counts in bronchoalveolar lavage fluid (BALF) and had higher mortality compared to AIDS-patients [61]. Mice with severe combined immunodeficiency (SCID) lacking functional B- and T- cells show normal oxygenation until late stages of disease, while immune reconstitution through intact spleen cells results in an intense T-cell mediated inflammatory response [62]. Similarly, initiation of HAART in HIV patients undergoing PCP-treatment may provoke a paradoxical worsening of PCP characterized by deteriorated respiratory status, so-called immune reconstitution syndrome [53]. These observations collectively indicate that cellular immune responses drive the disease progression to severe lung injury [53]. That said, a murine model suggested that higher *Pneumocystis* burdens produce pro-inflammatory cytokines and surfactant changing the surface tension, ultimately leading to gas exchange failure and respiratory deterioration [67]. Hence, the pathophysiology behind deleterious PCP is likely multifactorial.

1.5 PCP

1.5.1 Risk factors for PCP

General considerations

In general, most patients at risk of PCP harbor a defect in the T-cell arm of immunity, whether from HIV-infection, non-HIV immunocompromising diseases, or iatrogenic immunosuppression, particularly corticosteroids [42, 68]. PCP occurs less frequently in patients with immunodeficiencies restricted to humoral immunity or B-cells [68, 69].

HIV-infection

With respect to HIV-infection, $CD4^+$ T-cells below 200 cells/mm^3 is the principal risk factor whether it results from unawareness of seropositivity status, ineffective, poor or no adherence to HAART [8].

HIV-negative patients

Regarding non-HIV patients, at least three sub-populations can be distinguished. The first group comprises cancer patients. According to consensus guidelines by Cooley et al., cancer patients at risk include heavily pre-treated patients, especially those with myelo- and lymphoproliferative malignancies (collectively referred to as patients with hematological malignancy), patients with relapsed disease, and those receiving high doses of corticosteroids,

other chemotherapeutics including monoclonal antibodies resulting in prolonged lymphopenia (e.g., alemtuzumab), or combinations of these (e.g., rituximab-cyclophosphamide, doxorubicin, (etoposide), vincristine, and prednisolone (R-CHO(E)P)) [48]. PCP has also been reported in association with high dose methotrexate, the fludarabine, cyclophosphamide, and rituximab-regimen (i.e., FCR), gemcitabine, cytarabine to mention a few [48, 53]. Regarding hematological patients, those with acute lymphoblastic leukemia and those undergoing allogenic stem cell transplantation are at particular risk, while autologous stem cell transplantation entails inferior risk [48]. PCP has been reported in patients undergoing chemotherapy for a vast diversity of solid tumors, however, patients with both primary and secondary brain tumors appear to have distinctly higher risk [48, 68]. In the latter, corticosteroid use is implicated, but cranial radiotherapy and the chemotherapeutic agent temozolomide also increase the risk of lymphopenia and subsequent PCP in this population [42, 48, 68].

Second, between five and 15 % of SOT-recipients not receiving prophylaxis present with PCP [42]. The risk varies according to transplant organ, transplant center and immunosuppressive treatment [42]. The risk is highest during first six months and up to a year following transplantation during periods of intensified immunosuppression such as lymphocyte-depleting antibody therapies or bolus corticosteroids to treat graft rejection [70]. The timeline is reset with any episode of graft rejection or intensified immunosuppression [70].

Lastly, individuals affected by inflammatory or autoimmune disorders such as RA, spondylarthritis, SLE, vasculitidies, IBD, and chronic lung diseases, requiring immunosuppression to control their disease, constitute the third group [42, 68]. Besides the intensity of immunosuppression and use of corticosteroids, higher ages and co-existing lung disease seem to increase the risk of developing PCP in this heterogenous population [53].

In 2014 Fillâtre et al. published incidence rate estimates based on data from a tertiary care center in France using positive direct examination on BALF to define PCP cases [71] (**Figure 5)**. According to this retrospective study, patients with polyarteritis nodosa and granulomatosis with polyangiitis had the highest risk with 93.2 and 71.9 PCP cases per 100 000 person years, respectively [71]. For more common diseases such as RA, the incidence rate was estimated to 30 cases per 100 000 person years [71]. Lung cancer showed the lowest incidence [71]. Central nervous system cancer was associated with intermediate

risk, while hematological cancers involved intermediate to high risk [71]. Incidence in IBD-patients was not reported, but in a retrospective monocenter study from the United States it was estimated to be in the lower range, that is 10.6 PCP cases per 100 000 person years based on hospital discharge data [72]. In a sub-group analysis, the risk was almost six-fold higher in those with any prescription for immunosuppressive medication compared to those without: 32 vs. 5.5 cases per 100 000 person years, respectively [72].

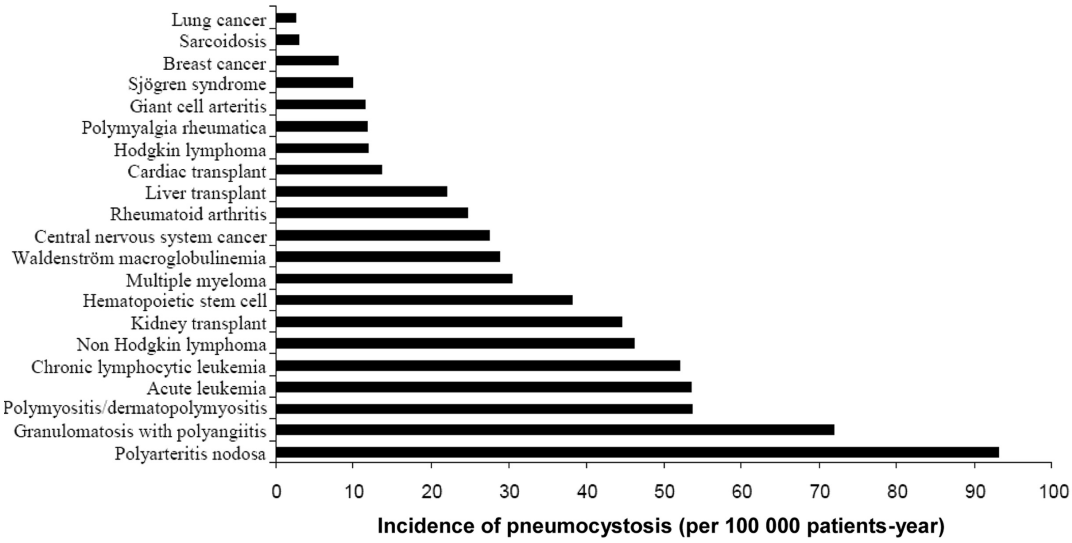

Figure 5. Incidence rate of PCP according to underlying condition estimated over a 10-year period in a monocenter study from Rennes, France. Figure reprinted with permission from [71]. Cases were defined as positive direct microscopic examination performed on BALF. Patients with isolated positive PCR were excluded.

1.5.2 Clinical and radiological diagnosis

Definitions regarding diagnostic tests

The following section will include terms to describe diagnostic validity, as defined in **Table 1** and **Table 2**, respectively.

	Diseased	**Healthy**	**Total**
Positive test	True positive (TP)	False positive (FP)	TP+FP
Negative test	False negative (FN)	True negative (TN)	FN+TN
Total	TP+FN	FP+TN	

Table 1. Definitions of possible diagnostic test results in a population of healthy and diseased individuals.

Term	Definition	Formula	Note
Sensitivity	Probability of resulting positive given presence of disease	$Sensitivity = \frac{TP}{TP + FN}$	Depend only on test characteristics.
Specificity	Probability of resulting negative given absence of disease	$Specificiity = \frac{TN}{TN + FP}$	
Negative predictive value (NPV)	Probability of having disease given positive test	$NPV = \frac{TN}{TN + FN}$	Depend on test characteristics and disease prevalence in sample population.
Positive predictive value (PPV)	Probability of not having disease given negative test	$PPV = \frac{TP}{TP + FP}$	

Table 2. Definition of terms used to describe the validity of a diagnostic test.

Clinical presentation

Identification of patients with PCP relies on clinical suspicion. Manifestations include cough, dyspnea, fever, tachypnea, tachycardia, and hypoxia, sometimes accompanied by a history of weight loss and fatigue [69]. Due to non-specific characteristics, clinicians may mistake PCP for pneumonia of bacterial, viral, or other fungal etiologies, malign process, sarcoidosis, or non-infectious interstitial pneumonitis [42]. Together with pulmonary embolism and cardiothoracic events, these conditions represent the main differential diagnoses. PCP can provoke a systemic inflammatory response and impair hemodynamics similar to that seen in patients with bacterial sepsis [73]. However, such reactions are rare, and shock and extra-respiratory symptoms could point towards co-infections or another diagnosis [42, 73]. Importantly, the presentation varies according to the degree of immunosuppression, and more markedly with respect to the patients' HIV-status [30, 69]. In HIV-infected individuals, PCP usually initiates insidiously with fever, non-productive cough, and dyspnea [42]. In contrast, non-HIV PCP typically has a fulminant onset of severe pneumonitis which is more frequently complicated by respiratory failure and acute respiratory distress syndrome (ARDS) [30, 69]. Lung examinations range from normal (even in cases with hypoxemia) to diffuse bilateral crackles [42]. Arterial blood gas enables calculation of the alveolar-arteria (A-a)-gradient to confirm intrapulmonary cause (i.e., widening gradient >20 mm Hg) and objective determination of disease severity. Partial pressure of oxygen (PaO_2) equal or greater than 70 mm Hg (9.4 kPa) in room air or A-a-gradient inferior to 35 mm Hg defines mild to moderate PCP, while partial pressure of oxygen inferior to 70 mm Hg in room air or A-a-gradient greater than 35 mm Hg defines moderate to severe PCP [42]. This grading system is used to guide treatment (see section **1.6 Management and outcome**). Miller's grading system taking

into account signs and symptoms, oxygenation and radiologic findings, represents an alternative [74].

Biological findings

No biomarker can rule in nor rule out PCP. In HIV-negative patients, there is no universal cutoff for $CD4^+$ T-cells, but low counts and lymphopenia are compatible with PCP [42]. Elevated lactate dehydrogenase (LDH) reflects lung inflammation and injury but is not specific [30].

Radiological features

Plain chest X-ray is the initial imaging modality of choice and may reveal bilateral interstitial infiltrates [30, 69]. The distribution is typically perihilar in the early phase or mild cases but becomes more homogenous and diffuse to form a "butterfly pattern" if the disease progresses [30, 42]. Less common features include cysts or pneumatoceles, solitary or multiple nodules, pneumothorax, and upper-lobe distribution in patients receiving aerosolized pentamidine as prophylaxis [30].

High resolution computed tomography (HRCT) or thin section CT is indicated, especially after performing a negative or inconclusive chest X-ray because it enhances disease detection and etiological differentiation in immunocompromised patients [75]. In a study based on 51 patients assessed for HIV-PCP, the sensitivity and specificity of HRCT were 100 % and 89 %, respectively [76]. Extensive ground glass opacity (GGO) with predilection for the apical and central lung regions is the principal finding in PCP, reflecting interalveolar fibrin, debris, and organisms [77]. However, the GGO pattern (e.g., mosaic, central with peripheral sparing, diffuse and homogenous) may vary according to underlying condition, including HIV-status, and immunosuppression [53, 77]. As the disease advances, consolidations and thickening of septa, with or without intralobular lines superimposed on GGO to form a so-called "crazy paving" pattern (CPP), may be appreciated (**Figure 6)** [77]. CPP reflects severe lung injury and is more frequent in HIV-negative patients in whom it tends to evolve more rapidly [42, 77]. In contrast, pulmonary cysts predominate in HIV-PCP compared to non-HIV PCP (56 % vs. 3 %, respectively) and predispose for spontaneous pneumothorax [78]. Solitary or multiple nodules are occasionally observed and reflect granulomatous disease [77] Lymphadenopathy, three-in bud sign, cavitations and pleural effusions are considered rare findings and may represent differential diagnoses [77, 78]. The GGO pattern usually disappears with initiation

of adequate anti-*pneumocystis* treatment, and CT may be useful to monitor disease evolution and treatment efficacy [78].

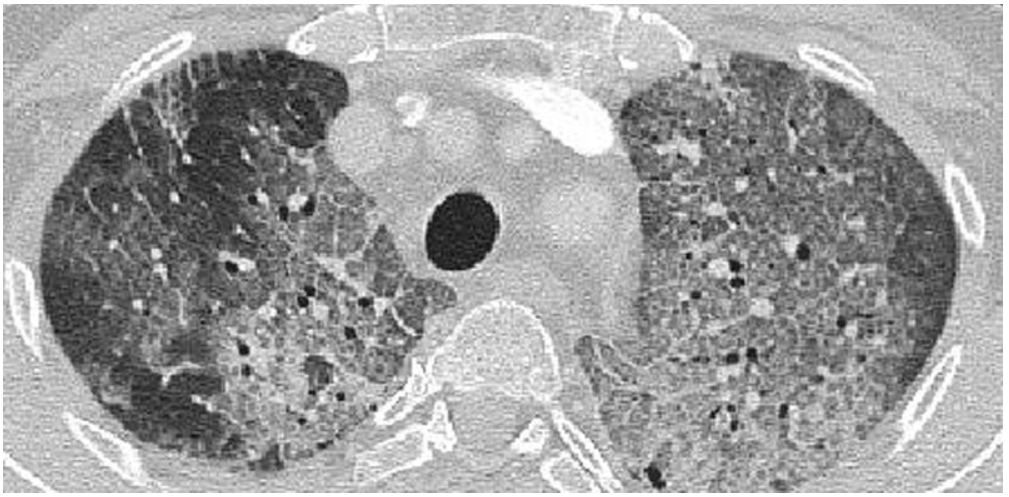

Figure 6. HRCT scan showing CPP in a lymphoma-patient with PCP. Figure reprinted with permission from [78].

1.5.3 Microbiological diagnosis

Respiratory specimens

Pneumocystis has tropism for the lungs, and there is an increasing organism gradient from the upper to the lower respiratory tract [36]. Considering this, open lung biopsy used to be the gold standard [79]. Today, BALF, a semi-invasive alternative to biopsies, is the optimal specimen for microbiological analysis, especially in HIV-negative patients harboring low fungal burdens [53]. Lavaging two or more areas of the lung, may increase the diagnostic yield [79]. However, bronchoscopy and BAL can induce transient hypoxemia [79]. Therefore, in patients with moderate to severe hypoxemia from pneumonia, it is best to lavage only the involved area, usually the upper lobe [79]. Induced sputum obtained by inhalation of hypertonic saline, also represents a “lower respiratory tract specimen” (LRS) and is an acceptable first step to avoid invasive sampling [80]. However, due to inferior sensitivity, a negative analysis performed on induced sputum does not allow exclusion of PCP, especially in HIV-negative patients [80]. This is clearly advocated in the fifth diagnostic European Conference on Infections in Leukeamia (ECIL)-guidelines [80]. Other specimens include expectorate, nasopharyngeal swabs and aspirates, oral washing, all considered “upper respiratory tract specimens” (URSs), and tracheal aspirates. Since the organism concentration

increases towards the alveoli, the respiratory specimens rank as follows with respect to diagnostic yield: BALF over induced sputum over URSs [80]. Accordingly, detection of *P. jirovecii* in URSs is suggestive of PCP in patients with high pre-test probability (i.e., compatible manifestations and compromised immunity), but the NPV is unsatisfactory [80].

Microscopy

Before the introduction of molecular approaches, microscopic identification of cysts or trophozoites upon staining with dyes or antibodies was the usual microbiological technique to confirm a diagnosis of PCP [30, 42]. Today, microscopic detection remains the gold standard for "proven PCP", and preferred techniques include methenamine silver stain for cysts, the modified Giemsa stain for all life cycles, non-specific calcofluor fluorescent stain, and immunofluorescent stain using specific monoclonal antibodies [81]. High availability and affordability represent advantages of microscopy, whereas low and variable sensitivity between 60-97 % depending on respiratory specimen (i.e., URS vs. LRS and quality), staining method, HIV-status, laboratory experience, and prior prophylaxis and HAART are major drawbacks [42, 81]. Of all the stains, immunofluorescent stains using monoclonal antibodies offers the highest sensitivity and specificity [82]. However, the NPV remains unsatisfactory in HIV-negative patients harboring lower fungal burdens. Thus, negative microscopy examinations warrant molecular confirmation to rule out PCP regardless of staining method [80].

Polymerase Chain Reaction

The first polymerase chain reaction (PCR)-assays for detection of *P. jirovecii* emerged in the 1980's [30]. Now, there is a vast repertoire of assays mainly distinguished by their output results, that is conventional end-point PCR vs. quantitative real-time PCR. The former is further divided into single round vs. nested PCR. **Table 3** provides an overview of the different PCRs. Gene targets also differ across assays, and a distinction is made between single- and multi-copy genes. Multi-copy genes, such as the mitochondrial large and small sub-units of ribosomal RNA (mt-LSU and mt-SSU) respectively, are present in several copies, and increase the sensitivity [83]. However, varying copy-numbers is an issue with respect to inter-strain comparisons and establishment of cut-offs. Dihydropholate reductase (DHFR) and thymidylate synthase (TS) are examples of single-copy genes [83]. In a meta-analysis, Fan and colleagues reported a pooled sensitivity, specificity, and area under the curve (AUC) of 98.3 % and 91.0 %, and 0.98, respectively, for the diagnosis of PCP using

any PCR technique on BALF [84]. In sub-group analyses the sensitivity and specificity were all above 90 % and 80 %, respectively [84]. Despite indisputable advantages, PCR raises some practical issues. First, because of discordant results, that is patient specimens with positive PCR and negative staining results [83, 84]. Second, because the high sensitivity of PCR compromises the capability of distinguishing colonization from PCP [42]. The main pitfall is to retain a diagnosis of PCP when the positive result represents colonization or even airborne contamination from the environment via another infected patient [85]. Real-time PCR assays, enabling estimation of organism burden, could resolve these problems, but have suffered from lack of standardization and inability to define universal cutoffs to define PCP [80]. Even when HIV-status is taken into consideration, there are gray zones in which fungal burdens overlap in patients with PCP and colonization [86]. This could reflect a continuous progression from carriage to active infection [80]. For these reasons, no recommendation about the preferential use of real-time PCR over immunofluorescence staining was made in the ECIL-guidelines from 2014 [80].

The minimum information for publication in quantitative real-time PCR experiments (MIQE) states clear guidelines for validation of real-time PCR assays [87]. These include considerations on PCR controls to obtain reliable results. For instance, to lower the risk of false negative results, assays should include a so-called negative control to detect PCR inhibition. Most commercial controls are based on alien DNA added at low concentrations in the specimen before DNA-extraction. Successively, its presence is checked in a duplex PCR together with the target gene. Control of the entire real-time process (i.e., extraction and replication) represents an advantage of this principle [88]. Alternatively, one can use known concentrations of DNA to detect switches in C_T-values caused by inhibition [87]. Use of human DNA as extraction and inhibition control is deemed inadequate because of the presence of much higher quantities of human DNA compared to target DNA [80].

Measurement of β-D-glucan and KL-6 in serum

Two serum markers, Krebs von den Lungen-6 (KL-6) and β-D-glucan, have been evaluated as diagnostics for PCP. KL-6, a glycoprotein expressed on pneumocytes, can enter the serum during infectious lung diseases. One study showed serum-elevation of KL-6 in HIV-associated cases, but generalizability to non-HIV PCP is yet to establish [89]. β-D-glucan is a major cell wall polysaccharide in many human-pathogenic fungi besides *P. jirovecii* [42].

Measurement of β-D-glucan in serum has been proposed to diagnose PCP since high serum-levels have been detected in patients with PCP [81]. Owing to high sensitivity, it may be used to exclude PCP or as an ancillary test when quantitative PCR and immunofluorescent microscopy produce discordant results [80]. However, it is not sufficient alone to diagnose PCP since other fungal infections also induce elevation of β-D-glucan in serum [80]. **Figure 7** summarize the fifth ECIL-guidelines for diagnosing non-HIV PCP.

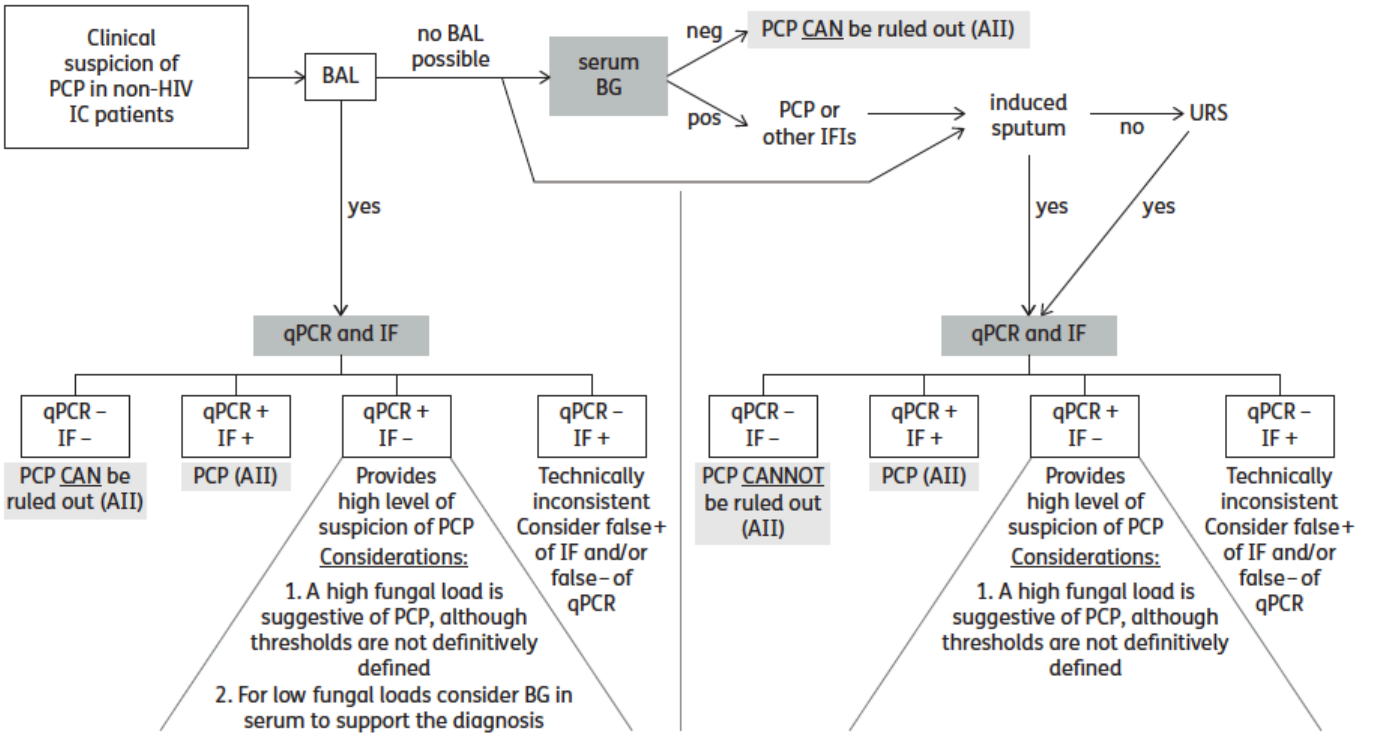

Figure 1. Flow chart for the diagnosis of *Pneumocystis* pneumonia in non-HIV immunocompromised (IC) patients. Biological tests are highlighted in dark grey and recommendations in light grey. BG, β-D-glucan; A-II, level of recommendation; IFI, invasive fungal infection.

Figure 7. Summary of the guidelines to diagnose non-HIV PCP from the Fifth European Conference on Infections in Leukaemia. Figure reprinted with permission from [80].

	Conventional end-point PCR	Real-time PCR
Principle	PCR consists of repeated replication of pre-defined genome segments. PCR runs on thermocyclers varying the temperature according to the step of the reaction: 1) Denaturation of the template into single strands (about 95°C). 2) Annealing of primers to each original strand for new synthesis (about 55 °C) 3) Extension of the new DNA strands from the primers (about 72°C). The ingredients in conventional PCR include template DNA, nucleotides, buffer solution, distilled water and Taq polymerase. The copies produced after the extension step, so-called amplicons, are re-amplified with the same primers, leading to an exponential replication of the template. After the end of amplification, gel electrophoresis is used to analyze the amplified PCR products.	Set-up and thermocycles like end-point PCR, but presence of fluorophores (probe or intercalating dye) producing a fluorescent signal permits real-time monitoring of template amplification. Fluorescence emission is monitored with each round of amplification in the exponential phase and is proportional to the amount of PCR amplicon. The amplification curves of positive tests appear above a threshold line indicating the maximum level of background fluorescence. A typical PCR is carried out over 40 cycles. The C_T-value is the number of cycles at which the signal emitted by a PCR reaches the threshold line and a low C_T-value corresponds to a high target load, and *vice versa.* Positive specimens are characterized by crossing the background threshold within an established cut-off and exponential amplification curves.
Subtypes	**Single round PCR:** One set of primers and one amplification reaction. **Nested PCR:** Two sequential amplification reactions, each with a different pair of primers. The second pair corresponds to a target harbored within the target of the first run.	**Quantitative**: Results are expressed as an absolute quantity (e.g., copy/mL). **Semiquantitative:** Results are expressed as C_T-values but can be converted into copy number equivalents of a plasmid containing the amplicon by generation of a standard curve.
Pros/Cons	**Pros:** Increased sensitivity compared to microscopy of stained specimens . **Cons:** Poor discrimination between PCP and colonization. Risk of crossover contamination. Labor-intensive and time-consuming due to time spent on preparing and running agarose gels and multiple PCRs (nested PCR only). Expensive in resource-limited settings.	**Pros:** Increased sensitivity and accuracy and wide dynamic range (potentially between 1 and 10^{11} copies). Closed tube format eliminates risk of cross-contamination. Rapid and cost-effective. Improved capacity for differentiation between PCP and colonization. Multiplex PCR possible. **Cons:** Expensive in resource-limited settings. Fluorophores may influence the validity.

Table 3. Overview of different PCRs used to detect *P. jirovecii*.

1.6 Management and outcome

1.6.1 Indication for treatment

With reference to the paragraphs above, reasoned clinical suspicion of PCP in immunocompromised patients is always an indication for treatment [90, 91]. Initiation should not be deferred by diagnostic procedures such as BAL, since *P. jirovecii* remains detectable in bronchial secretions for many days after systemic treatment [92]. For the same reason, sequential bronchoscopic examinations to monitor the course of the infection and treatment are not recommended [69].

1.6.2 Antimicrobials

First-line treatment

One of *Pneumocystis'* distinctive features is the presence of cholesterol in the cell membrane instead of ergosterol, which is the target of typical antifungal agents, namely ketoconazole and amphotericin B [93]. Several other antimicrobials are available for the treatment of PCP, and most are anti-folate inhibitors [69]. The rationale relies on *Pneumocystis'* dependency of new synthesis of folic acid [93]. The combination trimethoprim-sulfamethoxazole (TMS) is the most efficient drug in treating severe PCP [30]. Collectively, the two anti-folic components of TMS, trimethoprim and sulfamethoxazole, inhibit DHFR and DHPS, respectively [93]. TMS is the recommended first-line therapy regardless of disease severity [42]. Adverse effects occur frequently, and patients with known sulfa-allergies are intolerant to this first-line combination [30]. Desensitization should be considered, except in cases of known history of immediate hypersensitivity or severe skin reactions [48]. Severe adverse reactions are less common in non-HIV patients compared to HIV patients [48].

Administration and duration

The daily recommended dose of trimethoprim is 15-20 mg/kg plus 75-100 mg/kg sulfamethoxazole [42]. For patients with moderate to severe PCP, intravenous (IV) treatment is recommended to assure sufficient drug absorption, while patients with mild and moderate disease may receive oral or IV administration [42, 48]. Recommended duration of curative PCP-treatment is 21 days for patients with HIV, and 14 days for non-HIV patients, though extended treatment should be considered for the latter population in cases of severe immunosuppression, high fungal load, or delayed clinical improvement [42].

Treatment failure and second-line treatment

An early and reversible clinical deterioration is frequently observed within the first 3 to 5 days of treatment in absence of corticosteroids adjunctive treatment (CAT) [42]. Accordingly, treatment failure is defined as a lack of improvement or worsening of respiratory function documented by reduced arterial oxygen saturation after at least 4 to 8 days of anti-PCP treatment [48]. Therefore, changes in the treatment regimen should be awaited until the end of day 5 to 8, and concurrent non-infectious and infectious etiologies should be ruled out by repeating thoracic CT and BAL [42, 91]. Patients who experience treatment failure at the end of the first week of oral therapy should be switched to IV administration, likewise, patients on IV therapy should receive an alternate IV regimen [42]. Equal second-line regimens for severe PCP include pentamidine IV or a combination of oral or IV clindamycin and oral primaquine [48]. Pentamidine used to be the principal drug available for PCP since the 1950s, and it has demonstrated equal efficacy compared to TMS, but since it is associated with both minor and severe adverse effect it is no longer the drug of choice [69]. Dapsone plus trimethoprim is an alternative first-line regimen for mild to moderate disease in case of sulfa-allergy, although there is 20 % cross-reaction between sulphonamides and dapsone. Consequently, it is contraindicated in cases where there is a history of immediate hypersensitivity or severe reactions [48]. Patients should be tested for glucose-6-phosphate dehydrogenase deficiency before receiving primaquine and dapsone [48]. Oral atovaquone is considered third-line therapy [48]. Regardless of antimicrobial agents, it is of utmost importance to check all co-medications for drug-drug interactions in patients treated for PCP [91].

1.6.3 Supportive treatment and monitoring

Supportive treatment includes administration of supplemental oxygen, hydration, nutritional sustenance, and intubation with mechanical ventilation when called for [69]. Regarding disease monitoring, besides the abovementioned resolution of CT-findings, LDH usually declines in conjunction with clinical improvement [69].

1.6.4 Role of adjunctive corticosteroids

CAT has documented effect in lowering mortality in HIV PCP patients with resting hypoxemia at admission [94]. The rationale for CAT is to suppress excessive inflammation associated with killing of *P. jiroveci* organisms [53, 69]. The advantageousness of CAT for

non-HIV patients is not well-established and studies have produced conflicting results [42]. The guidelines available in 2017 stated that CAT may be appropriate in patients with moderate to severe PCP, but the indication must be evaluated on case-by-case basis [48].

1.6.5 *P. jiroveci* resistance to antimicrobials

Molecular techniques have permitted identification of mutations in the DHPS gene that encodes for the DHPS enzyme inhibited by dapsone and sulfamethoxazole in individuals exposed to sulfa-containing drugs [30, 48]. The prevalence varies greatly in countries in the developed world and populations studied (e.g., HIV-infected vs. non-HIV), from 0 % in Sweden, 20 % in Denmark, and 69 % in the United States [95]. Studies undertaken to investigate whether there is an association between DHPS polymorphisms and outcome, failure of either PCP prophylaxis, treatment, or both, have so far produced conflicting results [30]. Most patients infected with mutant strains respond clinically to full-dose TMS therapy [30]. Therefore, identification of DPHS mutations is not recommended nor performed as a routine-analysis in clinical laboratories [48, 69].

1.6.6 Chemoprophylaxis

Choice of drug

TMS is the drug of choice for primary chemoprevention of PCP regardless of HIV-status [48, 96] However, there are associated limitations with this drug, including documented hypersensitivity, renal impairment, drug interactions, myelosuppression, and gastrointestinal disturbance [48]. Some of the adverse effects require monitoring, specifically with respect to kidney and bone marrow function and electrolytes. TMS as a first-line agent is strengthened by the potential advantage of being active against other infectious complications (such as common bacterial infections, listeriosis, nocardiosis, and toxoplasmosis) [42, 96]. Alternative agents include oral dapsone or atovaquone, and aerosolized pentamidine if TMS intolerance develops [69]. Each drug has its own list of potential adverse effects and limitations. There are various regimens in terms of dosing and frequency of administration [96].

Prevention of HIV-associated PCP

In the context of HIV-infection, the indication for chemoprophylaxis against PCP is primarily determined by the number of $CD4^+$ T cells, and primary PCP-prophylaxis should be given when the count is less than 200 cells/mm^3, or if there is a history of oropharyngeal

candidiasis, including patients receiving HAART and pregnant women [30]. Patients with a history of PCP should receive lifelong secondary prophylaxis, unless reconstitution of the immune system results from HAART, as demonstrated by a rise in $CD4^+$ T cells [30]. Cessation of primary or secondary prophylaxis is indicated when the $CD4^+$ T cell count has remained above 200 cells/mm^3 for at least three consecutive months [30].

Prevention of non-HIV PCP

A Cochrane review from 2014 concluded that TMS is highly effective and safe in non-HIV immunocompromised patients with a number needed to treat to prevent PCP of 19 (95 % confidence interval (CI) 17-42) [97]. Moreover, the authors reported 85 % reduction in incidence (RR 0.15, 95 % CI 0.04-0.62) and 83 % reduction in PCP-related mortality (RR 0.17, 95 % CI 0.03-0.94), respectively [97]. Concerning indications, the fifth ECIL-guidelines for PCP-prevention from 2016 largely reflects the populations at high risk of developing PCP [96]. High dose corticosteroids constitute a main indication for prophylaxis together with other lymphocytotoxic agents [96]. With regards to other immunocompromised non-HIV patients, the risk associated with the underlying condition as well as the specific immunosuppressant should be weighted to determine the indication [42]. The recommendations for SOT-recipients are often updated since new risk groups regularly emerge [42]. In general, PCP-prophylaxis is recommended for all SOT-recipients at least the first 6 to 12 months following transplantation, though individual risk factors and transplanted organ may modify this generalization [98].

For other non-HIV groups, there are more uncertainties regarding duration of prophylaxis, especially for patients with autoimmune or inflammatory diseases [42]. In brief, prophylaxis should be administered alongside immunosuppressants or chemotherapy, and probably continue for a period after cessation for the immune system to reconstitute [48]. For patients successfully treated for an episode of PCP, secondary prophylaxis should be maintained as long as immunosuppression is prescribed [69, 91]. As implied above, the management of colonization is debated. Eventual benefits from prophylaxis must be balanced against the risk of severe side effects which appear to be rare [97].

1.6.7 Prognosis

PCP remains a serious and potentially life-threatening infection in immunocompromised patients, especially in those without HIV-infection. The case fatality rate depends on the underlying condition, most markedly on the HIV-status [93]. The risk of dying ranges from 20 to almost 90 % in patients without HIV, depending on the disease severity [42]. In comparison, it is estimated below 20 % in AIDS-patients unless mechanical ventilation is required [30]. Moreover, patients with malignancies are at greater risk of death than SOT-recipients or those with connective tissue disorders [30]. Besides more severe inflammatory host response, the high morbidity and mortality rate observed in HIV-negative individuals likely reflect intrinsic differences in the immune impairment, advanced age, pre-existing medical comorbidities, and a more fulminant presentation [69]. Irrespectively of underlying condition, development of acute respiratory failure is the major factor influencing prognosis [93]. Respiratory failure may be accompanied by ARDS. This syndrome is characterized by neutrophil infiltration and increased permeability in the alveoli from epithelial and endothelial cell damage resulting in stiff lungs and hypoxemia [99]. In short, ARDS is defined as respiratory distress occurring within one week of a known clinical insult or worsening respiratory symptoms, bilateral opacities on chest imaging in combination with hypoxemia and absence of hydrostatic oedema (Berlin definition from 2011) [100]. Prognostic factors are reviewed more in detail in the next section.

1.7 Previous literature related to the papers in this thesis

1.7.1 General considerations

Here follows a brief review of the relevant literature for this thesis. Articles published up to the end of 2017 are presented in this section. Articles published after 2017 are addressed in the discussion. Since the literature for paper I and paper III largely overlapped, the literature is described collectively. Heterogeneity in case definition, inclusion criteria, microbiological detection method (microscopy vs. PCR, and varying PCR assays), setting (any ward vs. intensive care unit (ICU)), and endpoints hamper direct inter-study comparisons, and must be taken into consideration.

1.7.2 Paper I and III

Search strategy

Work with paper I begun during Research program in 2017, and the paper was drafted in Autumn 2020. For paper III, the work begun in Autumn 2021, and the final draft was submitted in October 2022. Search for relevant literature was performed in PubMed. The search syntax included the following: "PCP", "PJP", "*Pneumocystis jirovecii* pneumonia", "*Pneumocystis carinii* Pneumonia", "Prognostics", "Predictors", "risk factors", "Epidemiology", "Epidemiological", "HIV-negative" and "without HIV". Relevant articles in PubMed were cross-referenced in Web of Science to review reference lists and citing articles, respectively. Studies restricted to HIV-positive patients and pediatric cases were disregarded. Moreover, studies focusing on heterogenous non-HIV populations (i.e., not restricted to patients RA, leukemia etc.) with or without inclusion of HIV patients were preferred. Articles in English were included regardless of year of publication. In September 2022 during work with this thesis, an updated search that included "fungal burden" in the syntax was made.

Brief review

The literature search for paper I and paper III resulted in studies performed in North America, Asia including the Middle East, and Europe (**7.1 Literature paper I and III**). Retrospective observational monocenter studies based on case-reviews dominate, and many have suffered from small sample numbers (<100 participants). However, a few longitudinal studies published before 2018 reported on PCP epidemiology in the general population, namely from the United States, France, and the United Kingdom [101-103]. The former described

declining trends in PCP-related deaths, attributed to fewer HIV-associated PCP deaths [103]. Oppositely, the European studies reported increasing fatality trends, in addition to increasing incidences [101, 102]. The latter trends resulted from increases in non-HIV cases [101, 102]. This evolution was supported by several center-based studies [104-113]. Some authors have speculated whether the increasing incidence of non-HIV PCP results from nosocomial transmission [113, 114].

The study populations have reflected the prevalence of the underlying condition including HIV, regional HAART-coverage, center-function (e.g., transplant center or not), and risk associated with the specific underlying conditions as illustrated by Fillâtre et al. in [71]. That said, hematological malignancies have appeared to predominate. Lacking prescription of prophylaxis to patients at risk has been evidenced across continents [106-111, 115-128]. Regarding non-HIV patients, premorbid systemic corticosteroid exposure alone or in combination with chemotherapy or immunosuppressants has been highlighted repeatedly [106, 108, 109, 115-120, 122-125, 127-138]. However, the observational nature of these studies precludes causal inference. In comparison, PCP upon exposure to inhalation corticosteroids has only been reported sporadically, and always in patients with lung disease with or without compromised immunity [139].

Numerous researchers have compared PCP in HIV vs. non-HIV patients and pointed out demographic, clinical, and prognostic differences [89, 103, 104, 107, 116, 122, 126, 129, 133, 137, 140-143]. Higher age, more fulminant and severe onset, susceptibility to diagnostic and therapeutic delays, greater need of mechanical ventilation, higher frequency of complications, and worse outcomes in patients without HIV have been common traits.

Regarding risk assessment, Asai et al. found that conventional risk tools used for community acquired pneumonia (CAP) underestimate disease severity in non-HIV PCP [120]. Relatedly, numerous studies have focused on identifying independent predictors of mortality in mixed HIV and non-HIV populations [102, 122, 127, 138, 144], pure non-HIV populations [106, 118, 123, 124, 135, 145], and mixed/pure ICU-populations [117, 125, 134, 137, 146, 147]. In 2017, Liu et al. published a meta-analysis on risk factors for mortality from PCP in non-HIV patients based on pooled data from 13 studies [148]. They found that higher age, female sex, solid tumor, dyspnea, high LDH, low serum-albumin, longer interval from symptom onset to treatment, respiratory failure, ICU-admission, co-infection, chemotherapy, and invasive

ventilation were significantly associated with increased mortality [148]. In contrast, hematological malignancies, SOT, and use of immunosuppressive agents were significantly associated with lower mortality [148]. They found no association with mortality and autoimmune diseases, cough, fever, neutropenia, lack of prophylaxis, CAT, nor preceding corticosteroid therapy, respectively [148]. Besides these exposure variables, two studies have investigated whether genotype is linked to outcome, without finding any association [118, 138]. However, small samples might have influenced the results (50 and 82 patients, respectively). The studies focusing on the effect of CAT have produced conflicting results [132, 147, 149, 150]. An inherent bias in clinical practice towards administration of corticosteroids to patients with severe PCP denotes a hinder which can be managed by propensity matching, but this was not the case in neither of these studies. Until 2019 and the study by Liu et al. [151], there was to my knowledge no one who had studied whether fungal burden is associated with the outcome in non-HIV patients.

Nordic studies

At the end of 2017, only a few studies were available from Nordic countries. These studies constituted a natural framework for comparison due to similar demographics and health systems. First, in 2005 Mikaelsson et al. published a 10-year retrospective monocenter study from Gothenburg, Sweden [126]. In their study of 118 cases in 108 patients, HIV-infection and SOT were the primary underlying conditions. However, a majority of 75 % were HIV-negative. Among 64 cases of proven non-HIV PCP, all had received chemotherapy or immunosuppressive treatment. Of these, 29.7 % required mechanical ventilation, 21.9 % died. Noteworthy, Mikaelsson et al. found no increase in non-HIV PCP between 1991 and 2001, despite increasing number of cytotoxic treatments. The authors suggested that more widespread prescription of prophylaxis to patients at risk explained this finding. Second, in 2009 Overgaard et al. published a three-year retrospective monocenter study from Copenhagen, Denmark [108]. In their study population of 50 HIV-negative patients, hematological malignancies constituted the primary underlying conditions. Overall mortality was 14 %. This study showed an increasing trend in cases from 2002-2004. Lastly, data on PCP from Norway was limited beyond the article on PCP in the “Smittevernveileder” of the Norwegian Institute of Public Health (NIPH) [40] and a case series on six B-cell lymphoma patients treated with the R-CHOEP-14 regimen from 2007 [152].

In 2013, the Leading International Fungal Education (LIFE) launched an initiative to estimate the burden of fungal infections in country by country [39]. Estimates from Denmark were published in 2012, partly based on the study from Overgaard et al. (i.e., for non-HIV cases), and resulted in an incidence of 1.5 per 100 000 person years of PCP [153]. A selection of studies, primarily from European and industrialized countries, is summarized in **7.2 Burden of PCP in selected countries**. This includes Norwegian and Swedish estimates published in 2018 and 2019, respectively [154, 155].

1.7.3 Paper II

Search strategy

Work with paper II begun in 2020 and the paper was drafted in Autumn 2021. The search strategy and selection were similar to the ones described above, but the search syntax included the following: "PCP", "PJP", "*Pneumocystis jirovecii* pneumonia", "*Pneumocystis carinii* Pneumonia", "Differentiation", "Discrimination", "real-time PCR", "Colonization", "Epidemiological", "HIV-negative" and "without HIV". In January 2023 during work with this thesis, an updated search was made.

Brief review

Before 2018, several groups had established and evaluated real-time PCR protocols for detection of *P. jirovecii* in respiratory specimens (**7.3 Literature paper II**).The studies exhibit important heterogeneity in study populations (mixed HIV and non-HIV or pure non-HIV), respiratory specimens (BALF and/or induced sputum, or mixed), the PCR target (e.g., mitochondrial rRNA, DHPS, DHFR, MSG, β-tubulin, etc.), PCR assay (commercial or in-house), the quantification method of fungal load (copies/mL, copies/tube, or C_T-value), PCP case criteria, reference method (e.g., microscopy, clinical and/or radiological etc.), PCR platforms (automated or manual), and the expression of results. This hampers direct comparisons.

A majority of the studies has also assessed the performance of quantitative PCR cut-offs to differentiate between PCP and colonization, primarily in retrospective [49, 88, 156-170] followed by prospective [54, 86, 171-178] and mixed studies [179-181]. The rationale is to assess whether real-time PCR can guide treatment decisions to spare colonized patients from anti-*pneumocystis* treatment with its associated side effects. Several studies have evidenced

significant differences in fungal loads between patients with positive and negative microscopic examinations and the categories of “proven PCP” and “colonization”. While the diagnostic accuracy for distinction between “proven PCP” and “colonization” has been high, it has resulted moderate for distinction between “probable/possible PCP” and “colonization”. Indeed, several studies have proposed gray zones between cut-offs to provide 100 % sensitivity (lower cut-off) and 100 % specificity (upper cut-off), respectively [49, 86, 157-161, 166, 168, 170-175, 177-179]. In a minority of the studies, overlapping spectrums in fungal burden have precluded establishment of cut-offs for discrimination [168, 171], and patients may have PCP despite extremely low fungal burdens in BALF at the detection limit of the most sensitive assays targeting multi-copy genes [182]. Higher fungal burdens in HIV-associated PCP compared to non-HIV PCP has been confirmed in studies with mixed populations emphasizing the need for stratification [86, 158]. Of note, Montesinos et al. evidenced that fungal burden varies according to predisposition beyond HIV-status [88]. Furthermore, Robert-Gangneux et al. found that patients with isolated positive PCR had different underlying risk factors than patients with microscopy-proven PCP [171]. Positive microscopic examination was *per se* significantly associated with lower C_T-values, reflecting higher fungal burdens [171]. Hematological malignancy was significantly associated with having an isolated positive PCR [171]. Difficulties in establishment of singular cut-offs, particularly for non-HIV PCP, could be ascribed to the heterogeneity since the threshold for PCP may differ according to underlying condition [88, 158, 159, 182]. Most studies have concorded that a negative real-time PCR performed in an LRS allows ruling out PCP given positive results for the internal and specimen process controls (i.e., exclusion of inhibition and extraction defects, respectively). For patients with fungal burden in the gray zone, Alanio et al. proposed that patients with high clinical probability should be treated [173]. Contrarily, those with low clinical probability could be resampled for induced sputum and followed by blood markers such as serum-β-D-glucan and be considered for prophylaxis. Relatedly, two studies have demonstrated that quantitation of β-D-glucan in serum may improve the discrimination when it is performed as an ancillary test in conjunction with PCR [49, 172]. Commercial assays have not proven superior for *P. jirovecii* detection nor for PCP diagnosing [162, 164, 167, 183]. Regarding external validity, Linssen et al. found excellent quantitative and qualitative agreement between three centers with correlation coefficients >0.84 despite use of different assays [168].

With respect to respiratory specimens, BALF specimens have predominated. Alanio et al. reported no significant difference in fungal loads between induced sputa and BALFs, however, they had access to both specimens in five cases only [173]. In contrast, two studies reported inferior validity in induced sputa [181, 184]. Fujisawa et al. suggested that induced sputum could represent an acceptable first step to avoid invasive sampling in respiratory distressed patients [163], though BALF remains the gold standard with the highest NPV. Regardless of specimen, differences in specimen volume remains an important bottleneck. Bandt et al. showed that normalization to host DNA can overcome this limitation, though quantification of *P. jirovecii* DNA may be sufficient for diagnostic purposes and more feasible in clinical settings [166].

2 Aims

The overall aim of this Ph.D.-project was to investigate the disease burden of PCP in a Norwegian healthcare setting and identify diagnostic and prognostics predictors for PCP. In **paper I**, we sought to characterize the population infected with *P. jirovecii*, their clinical course and outcome, and study epidemiological aspects to identify eventual implications for future management. To describe the evolution over the 12-year period, we also wanted to report trends in incidence and testing in Central Norway. In **paper II**, we aimed at identifying diagnostic predictors for PCP. In particular, we wanted to assess whether the in-house semiquantitative real-time PCR assay used to detect *P. jirovecii* at St. Olavs hospital could be used to discriminate PCP from colonization in HIV-negative patients. Finally, the aim of **paper III** was to identify prognostic factors in non-HIV patients with PCP. Knowing that fungal burden may vary according to underlying condition, we had a particular focus on the prognostic implications of this observation.

2.1 Specific objectives

Specific objectives included (answered in paper number):

1. Describe the diagnostic and epidemiological trends of *P. jirovecii* in Central Norway in a 12-year multicenter retrospective study (paper I)

2. Characterize symptoms and clinical course in immunosuppressed patients with PCP in a 12-year multicenter retrospective study (paper I)

3. Identify diagnostic predictors of PCP in immunosuppressed patients in a 12-year multicenter retrospective study (paper II)

4. Identify prognostic markers for survival from PCP in immunosuppressed patients in a 12-year multicenter retrospective study (paper III)

3 Materials and methods

3.1 Materials

3.1.1 Setting and population base

Norway is an industrialized country with a gross domestic product per capita of 75 496.8 United States dollars in 2017 [185]. Universal health coverage is provided by tax-based public services [186]. Municipalities organize primary health care, while the national government provides specialized health care through four state-owned regional health authorities [186, 187]. All three papers comprised in this thesis are primarily based on retrospective review of electronic health records of patients admitted to any of the hospitals belonging to Central Norway Health Authority. In 2016, it offered services to approximately 700 000 citizens, making it the third largest health authority in terms of population coverage [187]. The region of Central Norway comprises two counties, Trøndelag and Møre og Romsdal. They include both rural and urban populations. County demographics and determinants of health are fairly representative of

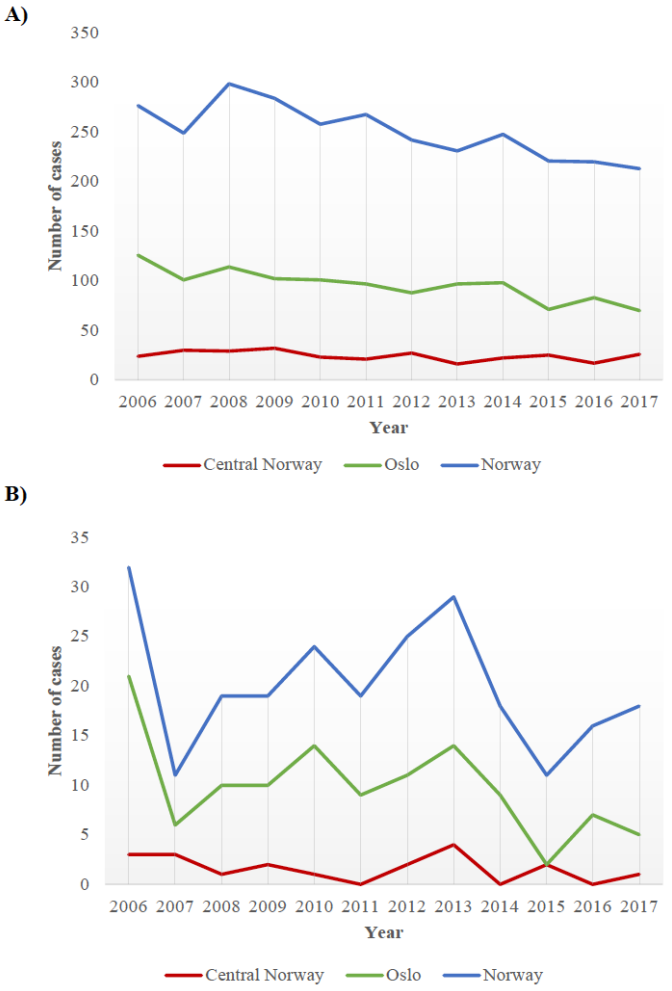

Figure 8. New HIV-infections **A)** and AIDS cases **B)** notified to the Norwegian Surveillance System of Communicable Disease [194]. Oslo (green) and Norway (in blue) are shown for reference in comparison with Central Norway (in red). Note varying y-axes.

Norway overall with a few exceptions (**Table 4**). Considering the relevance for this thesis, **Figure 8** shows the incidence of HIV and AIDS cases in Central Norway during the study period. Oslo, the capital with 666 759 inhabitants in 2017, and Norway with 5 258 317 inhabitants the same year, are shown for reference [188].

		Central Norway			Norway overall	Stat. sign.[1]	Ref.
Index[2]	**Year**	**Sør-Trøndelag**	**Nord-Trøndelag**	**Møre og Romsdal**			
		Trøndelag[3]					
Population >80 years, prediction (%)	2025	4.6*	5.7*	5.5*	4.8	X	[189-191]
Low-income households (%)	2014	8.5*	11 %	8.9*	12	X	
Income differences (P90/P10)[4]	2014	2.6	2.5	2.6	2.8	X	
Drop-outs senior high school (%)	2015	21	19*	19 *	22	X	
Higher education (%)	2015	34.4	25.4	26.6	32.2	NA	
Physical activity >2.5 hours/week (%)	2015	60 *	56	58	54	X	
Daily smokers (%)	2012-2016					X	
16-44 years		6.2 *	9.6	8.6	9.3		
45-74 years		16	16	16	17		
Obesity, 17 years (%)	2015	22	29 *	25 *	23	X	
Good self-reported health (%)	2015	82	81	83	80	X	
Life expectancy (years)	2009-2015					X	
Men		80.1*	79.7	80.3*	79.6		
Women		83.8	84.0	84.6*	83.7		
Prescription for diabetes type 2 medication per 1000	2015	33*	37*	33	35	X	
Users of antineoplastic/ immunomodulators per 1000	2017	23.8		23	21	NA	[10]
Unemployment (%)	2015	2.5	2.6	2.9	2.9	NA	[192]
Immigrants (1st/2nd degree) (%)	2016	11.7	7.8	11.9	16.3	NA	[193]

Table 4. Selected demographics and determinants of health in Central Norway.

[1] X Tested for significant difference. NA, not tested for significant difference.
[2] *Significantly different from Norway overall. Green, yellow, and red color indicate that the county's performance is significantly better, insignificant, or significantly worse than the national level.
[3] Sør-Trøndelag and Nord-Trøndelag were fused to one county in 2018.
[4] Ratio between the income of the 90th and 10th percentiles, respectively.

3.1.2 Study design and inclusion

St. Olavs hospital is situated in Trondheim, the largest city of Central Norway. As the only tertiary care and university hospital in the health authority, St. Olavs hospital receives specimens for selected microbiological analyses, including detection of *P. jirovecii*, from all the seven local hospital in the region (i.e., Kristiansund, Levanger, Molde, Namsos, Orkdal, Volda, Ålesund). From 2006, semiquantitative real-time PCR has been the principal detection method for *P. jirovecii* at the Department of Medical Microbiology. Therefore, to screen patients for eligibility, we identified adults aged 16 years or older who had been admitted to any of the hospitals in Central Norway and resulted PCR positive for *P. jirovecii* (i.e., C_T-value $\leq$40) in any respiratory specimen. The study period was between 2006 and 2017. Only primary episodes were considered. Identification of patients was performed retrospectively through linkage with the Laboratory Information System using the 11-digit personal identification number (i.e., "birth number"), that is unique for every Norwegian citizen. Inclusion of alive patients required active consent (i.e., returning information letter with signed consent by postal mail). The need for consent from next of kin or legal guardians of deceased patients was waived. HIV-status was only available for deceased patients and consenting survivors. Based on national HIV/AIDS surveillance, there were 19 new AIDS cases in Central Norway between 2006 and 2017 [194], and approximately one third of these presented with PCP (i.e., 6.3 estimated new HIV/AIDS-associated PCP cases during the study period) [28]. We considered these data in the below-described survival and sensitivity analyses.

Study populations

The study populations varied across the papers according to specific inclusion criteria (**Figure 9**). In **paper I,** all adults who had resulted PCR positive for *P. jirovecii* in a respiratory specimen and had been hospitalized in Central Norway between were 2006 and 2017 were eligible for inclusion. In **paper II**, we excluded HIV-positive patients and those who had not undergone thoracic CT during the diagnostic work-up. Lastly, in **paper III**, HIV-negative patients with retrievable C_T-value from PCR analysis in BALF who met the 2021 European Organization for Research and Treatment of Cancer/Mycoses Study Group Education and Research Consortium (EORTC/MSGERC)-criteria for proven or probable PCP were included [195]. For **paper III**, we got approval from the ethical committee to access certain demographic and microbiological data of non-consenters to perform the sensitivity analyses described below. During this data extraction, we examined the data of the non-consenters

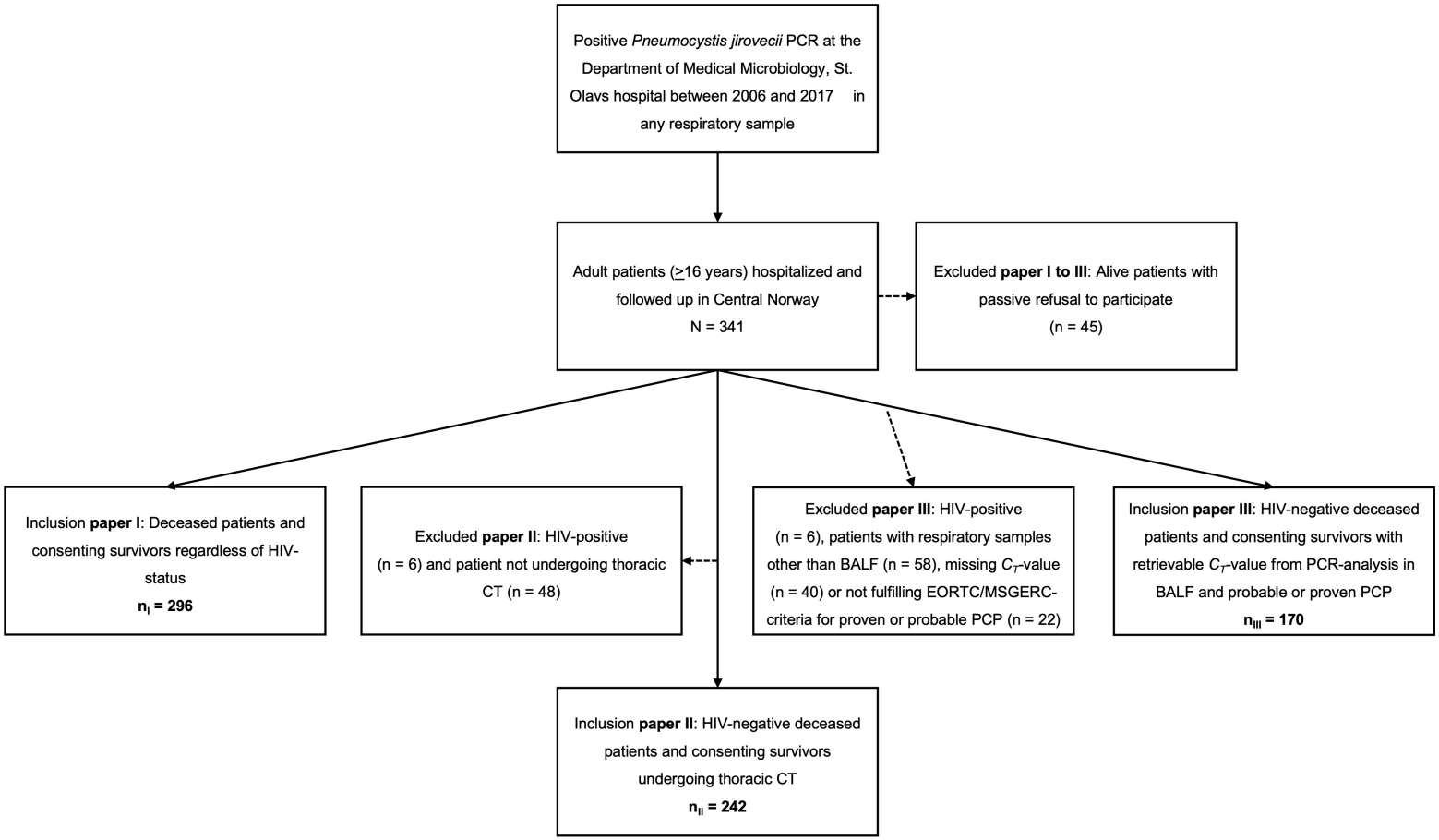

Figure 9. Flowchart of study populations in paper I to III. Dashed arrows indicate exclusion of patients.

more thoroughly. In retrospect, we discovered that some of these only had positive direct immunofluorescence (DIF) microscopy examination and no PCR result. Therefore, they were not eligible for inclusion in any of the studies. Moreover, one HIV patient who had been transferred from a local hospital to St. Olavs hospital was registered twice with six months apart. Hence, the actual number of HIV patients were six and not seven in **paper I**. The figures and statistics presented in this thesis are updated according to these corrections.

Testing for P. jirovecii and respiratory specimens

During the study period, respiratory specimens were mainly collected as BALFs (n = 274/341, 80.4 %), followed by expectorates (n = 47/341, 13.8 %), induced sputa (n = 11/341, 3.2 %), tracheal aspirates (n = 4/341, 80.4 %), biopsies (n = 3/341, 0.9 %), and nasopharyngeal swab specimens (n = 2/341, 0.6 %). 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 eventual ICU-admission.

3.1.3 Data collection and registration

Upon inclusion, we reviewed the health records of eligible patients. Patients were de-identified and data were registered in a form designed for this study in Epi Info (version 7.2.2.6™; Centers for Disease Control and Prevention, Atlanta, GA, United States) . Prior to initiating, we ran a pilot comprising 30 patients to optimize the form. The final data collection included comprehensive epidemiological, clinical, and biological data. Regarding clinical manifestations, we collected those documented at the preceding time points closest to when the patient underwent testing for *P. jirovecii.* For radiological data, we assessed the reports of the respective radiologists. We registered singular comorbidities and weighted multimorbidity according to the age-adjusted and original Charlson comorbidity indices (CCI) (**paper I and II,** and **paper III**, respectively) (See **Appendix 7.3**) [196, 197]. Concerning iatrogenic exposures, we recorded all immunosuppressants and chemotherapies administered the five years preceding presentation. We converted systemic corticosteroids into the equivalent dose in methylprednisolone expressed as milligrams (mg) per day [198] and differentiated exposure patterns (i.e., daily, intermittent, and none). For anti-*pneumocystis* treatment, we collected regimen, duration, and associated side effects as far as documented in the records. Moreover, we registered any adjuvant or overlapping corticosteroids in addition to other

antimicrobials administered after the detection of *P. jiroveci*, (i.e., antibiotics, antifungals, and antivirals). Likewise, we recorded management, mainly admission to an ICU and ventilation support. With respect to complications, we registered those documented by the treating physicians in the charts and discharge data (key characteristics summarized in **Table 5**). With respect to ascertainment of outcome, in-hospital mortality was defined as death recorded in the charts or hospital discharge data. To determine mortality upon discharge, we used automatic linkage of hospital records with the Norwegian Population Register using the "birth number" after assuring sufficient follow-up (i.e., at least 180 days).

Complication	**Clinical characteristics**
Respiratory failure/ARDS	Cyanosis, dyspnea, tachypnea, crackles, hypoxemia (O_2-saturation $\leq$94 % and/or PaO_2 $\leq$9.5 kPa) [199], with or without bilateral infiltrates on chest X-ray/thoracic CT.
Superinfection	Infection diagnosed upon positive PCR test for *P. jirovecii* and treated with antimicrobials.
Hemodynamic failure	Need for vasopressor to maintain mean arterial pressure >65 mm Hg.
Renal failure/acute kidney injury	Rise in creatinine and/or reduction in urine output.
Pneumothorax	Punctured lung verified by chest X-ray and/or thoracic CT.

Table 5. Key characteristics of recorded complications.

Microbiological data

During the study period, PCR results were reported in the Laboratory Information System as positive/negative, sometimes accompanied by a comment about the fungal burden, especially if the C_T-value was high (i.e., $\geq$37) (**Table 6**). C_T-values from PCR analysis for *P. jirovecii* were not reported in the Laboratory Information System during the study period. Therefore, we collected C_T-values from the log of the PCR instruments in retrospect. Some of the PCR instruments were replaced before the initiation of the study, resulting in missing C_T-values. Since retrievability of C_T-values merely depended on which machine the analyses were run, we considered the missing pattern "random". PCR replaced DIF microscopy definitely in 2017. Before this DIF microscopy was performed in conjunction with PCR, whenever a positive control was available. Accordingly, we registered DIF microscopy data as "missing", "positive", "negative", and occasionally "unfeasible". We also documented data on microorganism that were co-identified with *P. jirovecii.* To assess the clinical relevance, we

searched for other data to support or disprove the clinical relevance, such as results from PCR analysis in EDTA whole blood and/or microscopic examination of lung biopsy to differentiate viral shedding from overt CMV-pneumonitis [200].

***C*T-value**	**Fungal burden**	**Signal**
≤29	High	Strong positive
30-36	Moderate	Moderate positive
37-38	Low	Weak positive
39-40	Very low near detection limit	Very weak positive

Table 6. Comment about *P. jirovecii* burden in case of positive semiquantitative real-time PCR.

3.2 Methods

3.2.1 Case definitions and study variables

Paper I

In **paper I**, we primarily sought to describe epidemiological characteristics and trends of *P. jirovecii* over 12 years in Central Norway. Secondarily, we classified the patients as having PCP ("PCP^+"), being colonized ("PCP^-"), or as "undetermined" (i.e., missing data) since the main inclusion criteria, positive PCR, is not necessarily synonymous with PCP. The classification was performed *post hoc* and based on the ECIL-criteria [80] and C_T-values from previous studies [86, 181] (**Figure 10A**). The principal objective of this categorization was to determine whether the characteristics and trends of the PCR positive population overall were representative or not.

Paper II

In **paper II**, the PCP case definition was used as a reference method in evaluation of the in-house PCR assay's ability to distinguish PCP from colonization. Besides available DIF microscopy results, the case definition was based on multimodal clinical criteria (**Figure 10B**). Importantly, C_T-values were not considered in this process. In addition, we aimed at identifying predictors for PCP with PCP or colonization (i.e., "PCP^+" or "PCP^-") as dichotomous outcome (i.e., dependent) variable. Host factors (i.e., demographics, comorbidities, underlying condition), iatrogenic exposures, C_T-value from semiquantitative PCR analysis in BALF or tracheal aspirate, clinical presentation (symptoms, objective

findings), biochemistry (e.g., leukocyte profile, LDH), and thoracic CT-findings, constituted the exposure (i.e., independent) variables. These variables were chosen based on being readily available and assessable during the diagnostic workup and their association with PCP as documented in the literature. We used logistic regression models, and the variables having p-values <0.10 were subsequently analyzed in multivariable analyses. In the latter, we included confounders as covariates. By definition, confounders are associated with both the exposure and the outcome, but not a consequence of neither of these [201]. In **paper II**, confounders were identified by *a priori* knowledge and drawing of direct acyclic graphs (DAGs). We opted for this approach to let the existing evidence guide the selection of covariates.

Paper III

In **paper III** we also used regression models and 30-day mortality constituted the outcome variable. Again, we put emphasis on availability during selection of exposure variables in conjunction with review of the literature. In **paper III**, all the exposure variables were examined in both uni- and multivariable analyses. The latter included confounders based on *a priori* knowledge and drawing of DAGs. In the models with C_T-value as exposure variable, we included the following covariates based on their potential relationship with fungal burden: age, sex, premorbid corticosteroids, and chronic lung disease. Underlying condition was excluded from the models due to multicollinearity.

3.2.2 Microbiological detection of P. jirovecii

Semiquantitative real-time PCR assay

The in-house semiquantitative real-time PCR assay, based on TaqMan, was adapted from Brancart et al. [202]. The assay targets the highly conserved β-tubulin gene of *P. jirovecii*, present in a single copy which results in higher C_T-values compared to more frequently used multi-copy targets (e.g., mt-LSU and mt-SSU). The *P. jirovecii* quantitation is linear in the range from 10^2 to 10^6 DNA-copies/mL [202]. The reported limit of detection is 50 copies/mL (about 1 copy per reaction) with a 100 % detection rate for ≥100 copies/mL [202]. Its specificity for *P. jirovecii* has been demonstrated empirically by testing DNA from several fungal, bacterial, and viral species without generating any positive signals [202]. Furthermore, *in silico* alignments of the primers (i.e., basic local alignment tool (BLAST) search) do not indicate mismatches in the respective hybridization sites, nor homology with other pathogens.

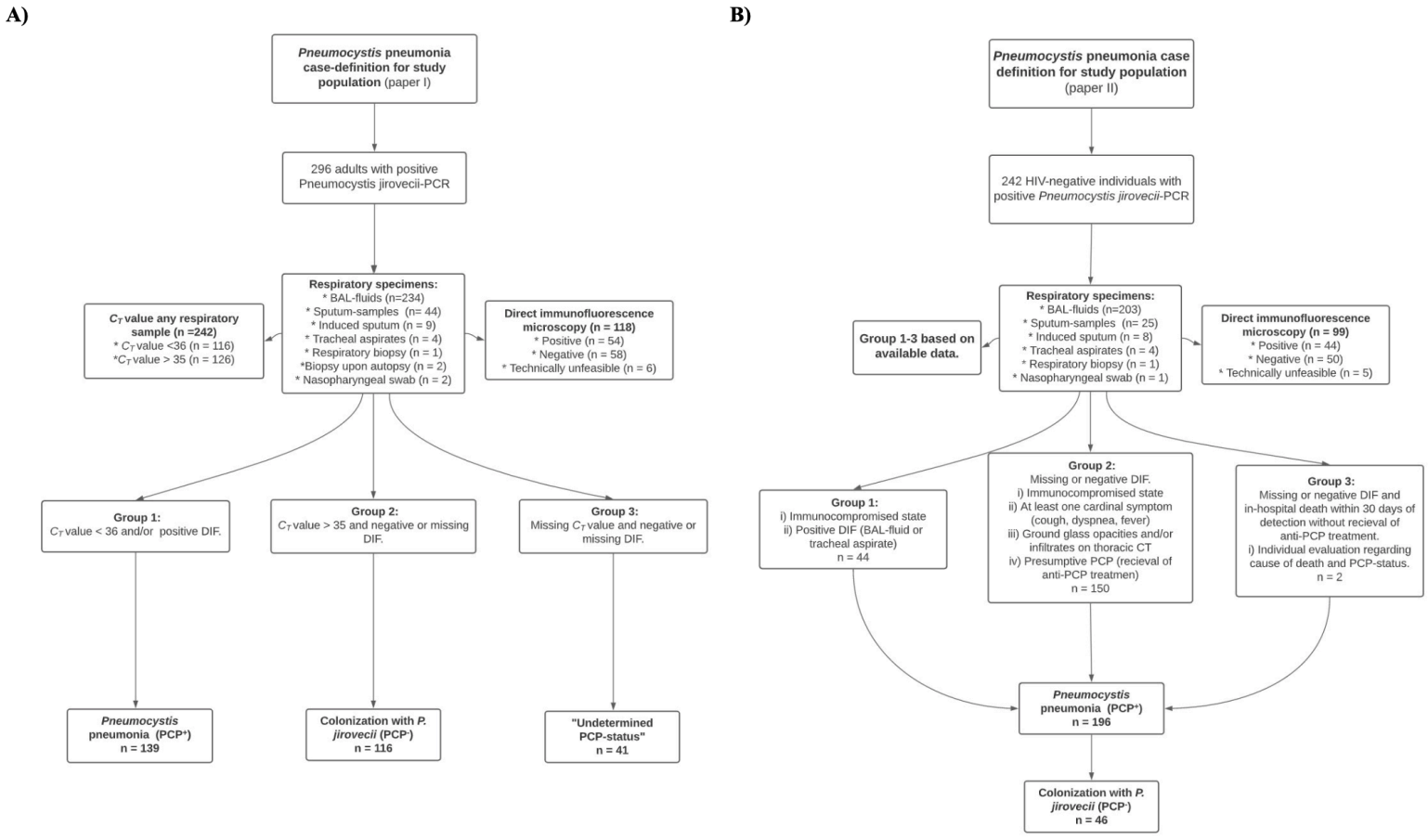

Figure 10. Case definitions and respiratory specimens in **paper I A)** and **paper II B)**, respectively.

PCR protocol

Respiratory tract specimens 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 specimen 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 specimen volume was <10 ml, the centrifugation step was omitted, and 1 ml of specimen was mixed with 100 ml proteinase K and incubated as described above. Then, the mixture was spun down, the supernatant was removed, and 500 µl of precipitate was used for DNA extraction on a NucliSENS easyMAG instrument (bioMérieux) with an eluate volume of 55 µl.

Reagents and PCR instruments used varied during the study period, but all changes were validated to ensure equal quality. During 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 qPCR 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 specimens, 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 specimen quality, a separate real-time PCR targeting a human 237 base pair intergenic region of chromosome 20 was run, as previously described [203]. All specimens were positive for this human target with a C_T-value ≤37 and no specimens were excluded due to nonamplification during the study period. The protocol did not include an ulterior extraction control.

The laboratory participated in the Quality Control for Molecular Diagnostics PCP DNA External Quality Assessment Program with excellent scores for core specimens during the study period.

DIF microscopy

DIF microscopy was performed with MONOFLUO Pneumocystis jirovecii IFA Test Kit #32515 (Bio-Rad). The monoclonal antibodies, conjugated with fluroescin-isothiocyanat, bind to any form of P. jirovecii, i.e., cysts and trophozoites (Figure 11). Specimens from patients with proven PCP were used as positive controls and the test results were purely qualitative.

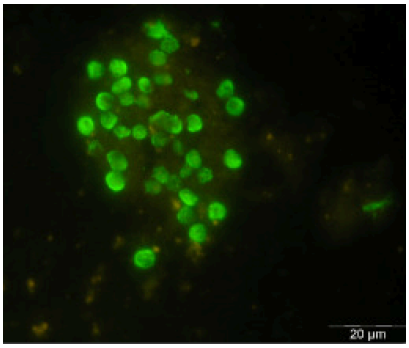

Figure 11. Cluster of *P. jirovecii* cysts in BALF visualized by DIF microscopy. Figure reprinted with permission from [257].

3.2.3 Statistics

Descriptive statistics and univariable comparisons

In all three papers, we presented continuous and categorical variables as medians with first (q_1) and third (q_3) quartiles or means with standard deviation (SD), and proportions with percentages (%), respectively. For comparisons, we used the Wilcoxon rank sum, Chi-square, or Fischer's exact test as appropriate. Since some variables had missing data, we specified the number of observations assessed. In **paper II,** we also used simple linear regression to compare C_T-values across underlying conditions.

Regional incidence estimates in paper I

In **paper I**, we estimated regional incidence rates and accessed the online databank of Statistics Norway to retrieve the number of people aged 16 years or older living in Central Norway during the study period [204]. These statistics represented the denominators. Since PCR was introduced at St. Olavs hospital in late 2006, we calculated estimates from 2007 to 2017. In 2017, Molde hospital, a local hospital in the health authority, also established PCR testing for *P. jirovecii.* For completeness, we included 14 patients with positive PCR in their laboratory in the incidence estimates of that year. We expressed incidence estimates graphically as cases per 100 000 person years with 95 % CIs.

Assessment of discrimination in paper II

In **paper II**, we used receiver operating characteristic (ROC)-curves to assess the validity of the semiquantitative real-time PCR. The area under the ROC-curve (AUC), an equivalent to the C-statistic, represents a summary measure of discrimination. Thus, in this case it quantified the ability of the in-house assay to assign a high probability to patients with PCP, and a low probability to those with colonization. The AUC ranges from 0.5 to 1, and an AUC of 0.5 indicates that discrimination results from chance alone, while 1.0 indicates perfect discrimination. Upon depiction of ROC-curves, we calculated the sensitivity, specificity, PPV and NPV according to different C_T-values as cut-offs. We reported the measures of validity with 95 % confidence intervals.

Logistic regression analyses

In **paper II** and **paper III,** we used logistic regression models to identify risk factors for PCP, and 30-day mortality, respectively. For continuous variables, we assessed the linearity of the logit with "lowess plots" with respect to clinical cut-offs to determine the best fit. We performed univariable and multivariable analyses. In the latter, we included potential confounders as covariates. We expressed results as odds ratios (ORs) with 95 % CI generated from Wald's test. **In paper III,** we used the "margins"-command to determine probability of dying within 30 days after adjusting for age, sex, and non-participation as described below.

Survival analyses

In **paper III**, we also performed survival analyses with the data available for all patients with positive *P. jirovecii* PCR in BALF and retrievable C_T-value to see whether the trends of the study population were representative. These analyses included the 19 non-consenters with unknown HIV-status. We had excluded six HIV-positive patients during the screening process, and we have little reason to believe that there were many left among the 19 non-consenters (i.e., 6.3 estimated cases in Central Norway). EORTC/MSGERC-status was not taken into consideration in these analyses. C_T-value was the only exposure variables. First, we applied the Kaplan Meier-method. Then we used the log-rank test for comparisons after verifying the proportional hazard assumption. Next, we performed Cox-regression analyses to obtain age- and sex-adjusted estimates. To account for changes in the incidence and lethality over time, we tested inclusion of year of diagnosis as covariate. We expressed results as hazard ratios (HRs) with 95 % CI.

Sub-group analyses

In **paper I,** we repeated the descriptive analyses in patients with retrospectively classified as "PCP^{+}". In **paper II**, we compared "PCP^{+}" patients with C_T-values <31 to "PCP^{+}" patients with C_T-values ≥31 to uncover eventual differences between those with high and low fungal burdens, respectively. In **paper III,** we performed analyses restricted to patients receiving anti-*pneumocystis* treatment with fungal burden as exposure variable to reduce bias from treatment disparities. Second, we performed sub-group analyses in patients with C_T-value ≤37 to study the association between C_T-value and outcome within this spectrum. In all three papers, we performed the sub-group analyses *post hoc.*

Sensitivity analyses in paper III

To assess non-participation bias in **paper III**, we compared eligible consenters to non-consenters who were presumed eligible (i.e., retrievable C_T-value from PCR in BALF but unknown HIV-status) (**Figure 12**). Next, we performed sensitivity analyses applying inverse probability weighted regression adjustment. In brief, we calculated the inverse probability of inclusion based on the available data: age, sex, period (before or after 2011), and hospital (university vs. local). Deceased patients were given a weight of one since inclusion was independent of consent. For survivors, the weight was one divided the probability of inclusion. Thus, the weight was close to one for patients with high probability of participation and above one for patients with low probability of participation, respectively. To reduce the effect of outliers, we truncated high weights above the 90th percentile. The sensitivity analyses were planned per protocol. EORTC/MSGERC-status was not considered in these analyses and all patients with retrievable C_T-value from PCR in BALF were considered eligible.

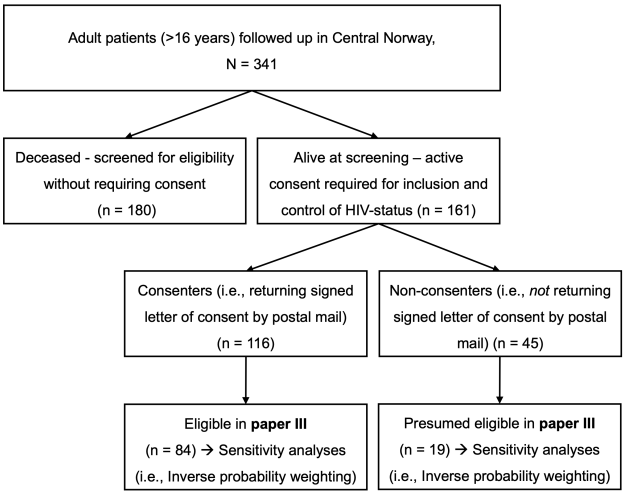

Figure 12. Presentation of recruitment process with respect to consent. Inclusion of alive patients required active consent. In **paper III,** we used these data of 19 non-consenters who were presumed eligible (i.e., retrievable C_T-value from PCR in BALF but unknown HIV-status) to perform sensitivity analyses (i.e., inverse probability weighting). EORTC/MSGERC-status was not taken into account in these analyses and all patients with retrievable C_T-value from PCR in BALF were considered eligible.

Software and statistical significance

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, United States) to perform all statistical analyses.

3.3 Ethics and data protection

The studies reported in this thesis have been approved by the Regional Ethics Committee for Health and Research Ethics (REC-North, reference number 2017/2419/REC North). In addition, the studies have been approved by the Data Access Committee of Nord-Trøndelag Hospital Trust and the Data Protection Officer of Nord-Trøndelag Helse Møre og Romsdal Hospital Trusts, respectively. All data have been managed in accordance with the General Data Protection Regulation adapted by the European Union in 2016.

4 Results

4.1 Paper I

In **paper I**, we included 296 patients with a median age (q_1-q_3) of 66 (59-74) years and male preponderance of 60.5% (n = 179/296). All but six patients had non-HIV underlying conditions, with cancers combined accounting for 61.5 % (n = 182/296) (**Figure 13**). Moreover, 71.6 % (n = 212/296) had at least one comorbidity.

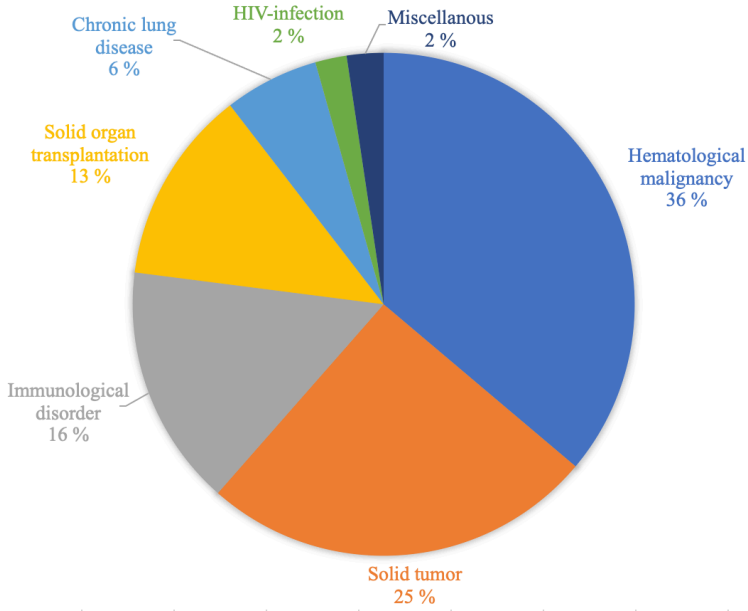

Figure 13. Distribution of underlying conditions in the study population of paper I. Miscellaneous diseases included statin-induced myositis (n = 1), common variable immunodeficiency (n = 1), and no definite diagnosis at the time of presentation (n = 5).

At presentation, 83.8 % (n = 248/296) were receiving iatrogenic immunosuppression or chemotherapy which largely reflected the respective underlying conditions. Premorbid systemic corticosteroids exposure in the two months preceding detection of *P. jirovecii* was a common denominator in 73.5 % (n = 216/294). The indications, doses, and exposure patterns exhibited a great variety. Despite harboring risk factors, only 1.0 % (n = 3/296) of the study population was receiving prophylaxis. A majority of 74.7 % (n = 221/290) presented with at least two cardinal symptoms whereas hypoxia, cytopenias, and radiological manifestations compatible with PCP constituted the main objective findings. Anti-*pneumocystis* treatment was initiated in 87.8 % (n = 260/296). At least one complication occurred in 40.5 % (n = 120/296), and 29.4 % (n = 87/296) received intensive care. In-hospital mortality was 21.6 % (n = 64/296). Compared to 2007, the regional referral laboratory received 3.3-times more specimens for testing in 2017, while the number of positive specimens increased by 1.8. The proportion of positive specimens remained stable (20.8 % $\pm$ 4.7). These trends were accompanied by a rise in the incidence of patients with positive *P. jirovecii* PCR in Central Norway: from 5.0 cases per 100 000 person years in 2006 to 10.8 cases per 100 000 person years in 2017, respectively.

4.2 Paper II

In **paper II**, we included 242 HIV-negative patients. Retrospectively, 196 patients were classified as having PCP, whereas 46 patients were classified as being colonized. Based on 171 patients with BALF or tracheal aspirate, the discrimination of the in-house semiquantitative real-time PCR assay was good. **Figure 14A** shows the ROC-curve which had an AUC of 0.80 (95 % CI 0.73 to 0.88). A C_T-value of 36 corresponded to a sensitivity of 71.3 % (95 % CI 63.7 to 78.9 %) and specificity of 77.1 % (95 % CI 63.2 to 91.1 %). C_T-values ≤30 confirmed PCP with 100 % PPV, while no C_T-value allowed exclusion of PCP with 100 % NPV. We noted a diversity in fungal loads according to underlying conditions: SOT-recipients had significantly lower C_T-values than patients with hematological malignancies, indicating higher fungal burdens in the former. Consequently, we attempted stratification which improved the discrimination (**Figure 14BC**). In SOT-recipients, a C_T-value of 36 resulted in sensitivity of 95.0 % (95 % CI 85.4 to 100 %) and specificity of 83.3 % (95 % CI 53.5 to 100 %). In patients with hematological malignancies a higher cut-off of 37 yielded a sensitivity of 88.5 % (95 % CI 79.8 to 97.1 %) but reduced the specificity to 66.7 % (95 % CI 35.9 to 97.5 %). For the remaining underlying conditions the diagnostic validity appeared inferior. Lastly, premorbid systemic corticosteroids, lower C_T-values, presence of cardinal symptom triad, lower O_2-saturation, abnormal lung auscultation, and CPP on thoracic CT were independently associated with PCP. In contrast, chronic lung diseases predicted colonization.

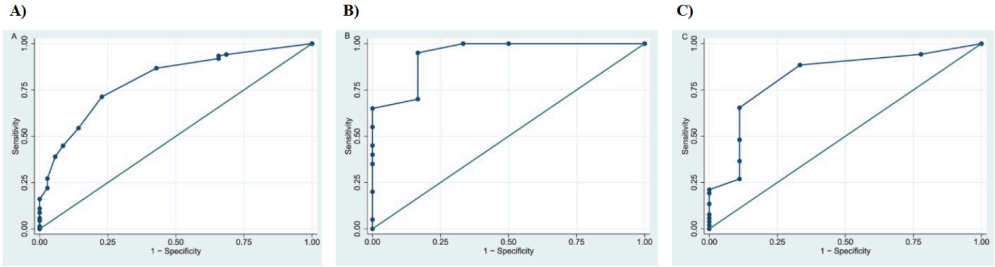

Figure 14. ROC-curves of C_T-values obtained by semiquantitative real-time PCR in BALF and tracheal aspirate specimens in **A)** study population (n = 171), **B)** SOT-recipients (n = 26), and **C)** patients with hematological malignancies (n = 61) for discrimination between PCP and colonization. Figure reprinted with permission from [205].

4.3 Paper III

In **paper III**, we included 170 HIV-negative patients with proven or probable PCP. All-cause 30-day mortality was 18.2 % (n = 31/170). Regarding risk factors of fatal outcome, we found that higher *P. jirovecii* burdens indicated by lower C_T-values, significantly increased the mortality risk. After controlling for host factors and premorbid systemic corticosteroids, this association persisted for C_T-value ≤30 but not for C_T-value >30: adjusted odds ratio (OR) 1.42 (95% CI 0.48 to 4.25) for C_T-value 31-36, increasing to OR 5.43 (95% CI 1.48 to 19.9) for C_T-value ≤30, compared to patients with C_T-value ≥37. The association withheld when restricting the analyses to patients receiving anti-*pneumocystis* treatment and patients with C_T-value ≤37, respectively. In the latter, both C_T-value 30–33 and C_T-value ≤30 were significantly associated with higher odds of dying compared to C_T-value 34–37. The survival analysis of all patients with positive *P. jirovecii* PCR in BALF and retrievable C_T-value between 2006 and 2017 in Central Norway (N = 211) corroborated the association between C_T-value and fatal outcome ulteriorly: adjusted HR for 30-day mortality risk was 0.89 per C_T-value (95 % CI 0.83-0.96, p<0.01). Concerning host factors, comorbid cardiovascular disease including congestive heart failure (CHF), were significantly associated with 30-day mortality after controlling for age and sex. Furthermore, patients with solid tumors and immunological disorders had significantly higher odds of dying compared to those with hematological malignancies in the multivariable analyses. Premorbid corticosteroid exposure was independently associated with the outcome in a dose-dependent manner. Regarding clinical characteristics, O_2-saturation <90 %, and severe host response indicated by leukocytosis with higher neutrophil counts and CRP ≥100 mg/L, were independently associated with 30-day mortality. The same was true for low serum-albumin and lymphopenia. With emphasis on availability and easy risk stratification, we estimated the risk of dying according to fungal burden combined with CCI. Patients with high burdens (C_T-value ≤30 and CCI ≥6) had almost an eight-fold increase in the risk of dying compared to those with low burdens (C_T-value ≥37 and CCI ≤2): 70 % vs. 9 %, respectively. When separated, the spectrums of mortality risk were comparable. We observed similar patterns for patients with C_T-value ≤37. The sensitivity analyses did not indicate substantial selection bias.

5 Discussion

5.1 Summary of main findings

Primarily HIV-negative patients had a positive *P. jirovecii* PCR in Central Norway between 2006 and 2017. Indeed, 98.0 % of the first study population (n = 290/296) had underlying diseases other than HIV. Moreover, 83.3 % had ongoing treatment with iatrogenic immunosuppression or chemotherapy including corticosteroids at presentation. Only 1.0 % was receiving prophylaxis. Our research confirms the unspecific manifestations of these patients, mainly characterized by fever, respiratory distress, cytopenias, often accompanied by radiological evidence of a pulmonary disease process. The rates of ICU-admission (29.4 %) and in-hospital mortality (21.6 %) underscore the morbidity and mortality associated with *P. jirovecii*. Regarding diagnostic and epidemiological trends, we found an annual increase in both the number of PCR tests performed and positive cases from the introduction of PCR in 2006 to 2017. The in-house semiquantitative real-time PCR assay showed a sensitivity of 71.3 % and a specificity 77.1 % for discrimination of non-HIV PCP from colonization. We were unable to establish a cut-off with 100 % NPV for PCP. As exemplified by patients with hematological malignancies and SOT-recipients, stratification improved the discrimination. While lower C_T-values, premorbid corticosteroids, cardinal symptoms, low oxygen saturation, abnormal lung auscultation, and CPP on thoracic CT were independently associated with PCP, chronic lung disease was associated with colonization. Concerning the outcome of patients with EORTC/MSGERC proven or probable PCP [195], we found that C_T-values $\leq$30 independently predicted 30-day mortality, while this was not the case for C_T-values $\geq$31. Cardiovascular disease including CHF, immunological disorders, solid tumors, premorbid corticosteroids, O_2-saturation <90 %, leukocytosis with higher neutrophil counts, lymphopenia, low serum-albumin, and CRP >100 mg/L were also significantly associated with mortality in multivariable analysis. Higher *P. jirovecii* burdens and multimorbidity increased the mortality risk in synergistic manner. So, combined with underlying comorbidities, fungal burden estimated by real-time PCR may improve risk stratification.

5.2 Methodological considerations

5.2.1 Reliability and validity

When evaluating the quality of research, it is central to consider the reliability and validity. Reliability is to what extent you obtain similar results when repeating a measurement, method, or procedure [206]. Random error reduces the precision and undermines the reliability [201]. The magnitude of standard deviations and confidence intervals reflect the random error of estimates [207]. Large standard deviations or wide confidence interval indicate low precision and *vice versa* [207]. Validity is to what extent the observed data is accurately measured and is based on judgement, not a computed statistic [201]. External validity is whether the results can be generalized to other populations, settings, and time periods. Internal validity is a perquisite for external validity and refers to whether the results are legitimate with respect to study design, conduction, and data analyses. Fundamentally, internal validity examines the presence of systematic error (bias), that is deviation from the truth [207]. In the following section, I will discuss whether observed changes in outcome can be uniquely attributed to the exposures and not to other possible causes such as bias, confounding, and chance.

5.2.2 Selection bias

Selection bias occurs when the association between exposure and disease is different among participants and non-participants [201]. For this reason, it is also called participant bias. Inclusion of alive patients required active consent, and as outlined in Material and methods, 45 of 161 (28.0 %) presumed eligible survivors did not consent to participate. That is, they were not available to be screened for inclusion in all three papers and constituted 13.2 % (n = 45/341) of the whole population of patients with positive *P. jirovecii* PCR. This represents an important source of selection bias, in particular "selection of deceased". In paper III, we used data of non-consenters to perform sensitivity analyses. This was of particular importance in paper III, since 30-day mortality was the outcome variable. The sensitivity analyses did not indicate selection bias. However, the inclusion criteria of paper III, namely restriction to BALF specimens and patients with retrievable C_T-value, represented potential sources of selection bias *per se*. To test this hypothesis and evaluate participation bias overall, we successively compared consenters (n = 116) to non-consenters (n = 45) in retrospect. We did not consider respiratory specimen, retrievability of C_T-value, EORTC/MSGERC-

classification, nor HIV-status (i.e., all non-consenters were presumed eligible for inclusion) (refer to **Figure 12**). These analyses indicated that age and sex were associated with participation: Consenters were significantly older than non-consenters (median age (q_1-q_3) 65 years (56-72.5) versus 51 years (32-66), p<0.01), and the male representation was higher in the former group (n (%) 67 (57.8) vs. 19 (42.2), p = 0.08). We observed no skewness according to C_T-value, respiratory specimen, hospital, nor period. While this data is limited and does not give complete profiles of non-participants, we can make some assumptions: 1) Consent to participate was not random. Indeed, women and younger survivors were less likely to participate. This likely disproves healthy participant bias, i.e., that healthier individuals are more prone to participate. 2) The distribution of underlying conditions and associated therapies in the studies may be skewed since some conditions affect more women than men (e.g., autoimmune diseases). 3) Patients who underwent BAL to test for *P. jirovecii* were likely not representative of the overall population who underwent PCR testing. This may be due to the invasiveness of such sampling. The latter concern also applies to paper II. 4) While age was not significantly associated with 30-day mortality in paper III, an analysis of all patients with positive *P. jirovecii* in Central Norway (N = 341) indicates that age was associated fatal outcome (OR 1.02 per year, 95 % CI 1.00 to 1.05). Although women were overrepresented among non-consenting survivors, the same was not true for male sex (OR 1.56, 95 % CI 0.87-2.80). These findings support assumption 3) and resonate with previous studies showing that higher age is associated with fatal outcome [148, 208]. It is important to bear these aspects in mind when interpretating the results, and when comparing our findings to those of other studies.

In paper I, HIV-infection represented a potential source of selection bias due to the stigma associated with HIV/AIDS. However, as outlined in **section 3.1.1**, the national surveillance data contraindicate that this was a major trend. In paper II, exclusion of those who did not undergo thoracic CT might have introduced selection bias since ordering thoracic CT-scans might not have occurred randomly.

5.2.3 Information bias

Information bias, also known as misclassification, observation, or measurement bias refers to incorrect collection, measurement, or reporting of information [201]. Differential information bias affects exposed and unexposed differently in a systematic fashion, and the direction is

unpredictable (i.e., away or towards the null). Random or non-differential information bias affects exposed and unexposed equally and bias the effect estimates towards the null. In all three papers we used case definitions to distinguish PCP from colonization, and eventual misclassifications would have introduced information bias. During the project planning, we evaluated to use the International Classification of Diseases (ICD)-codes for PCP to identify eligible patients. However, such coding is susceptible to inconsistencies and misclassifications. Moreover, it has been found to have low sensitivity [209]. Therefore, we opted for a microbiological criterion. That said, it could have been interesting to compare ICD-codes to our classifications. Owing to similar considerations, we did not use the death certificates to define deaths caused by PCP. Rather, we used 30-day mortality as outcome variable in paper III.

Dichotomizing or categorization of continuous exposure variables in paper II and paper III might have led to misclassification of the exposure. The cut-offs were based on the logit of the variables in combination with clinical discretion. In paper III, the cut-offs for C_T-values were also based on our findings in paper II, namely that a C_T-value $\leq$30 confirmed the diagnosis. While categorization makes the interpretation more intuitive, it may also oversimplify biological associations. More so, the power of the statistical analyses decreases due to categorization into smaller sub-groups. This might have been the case for premorbid corticosteroids in paper III. Methylprednisolone equivalent doses between 8 and 19 mg/day were significantly associated with 30-day mortality, while this was not the case for doses $\geq$20 mg/day. Considering this, one can argue that we should have kept the original form of the continuous variables or operated with both forms.

5.2.4 Incorporation bias

Incorporation bias occurs when the results of the index test are included in the adjudication process and leads to falsely elevated sensitivity and specificity [210]. In paper II, we used multimodal criteria to define PCP which we used as reference method in evaluation of the in-house semiquantitative real-time PCR assay (i.e., index test). For patients with missing or negative DIF, clinical manifestations and whether they had received treatment or not was part of the case criteria. C_T-values were not communicated to the treating physicians, however, the test results (i.e., positive or negative) were sometimes accompanied by a comment about the PCR signal (refer to **Table 6**). This might have introduced indirect incorporation bias in the

adjudication of these patients (n = 150/242, 62.0 %) since the treating physicians likely were more inclined to treat patients with moderate and strong positive results. This in turn might have inflated the significance of our results. The same might be true for symptoms and CPP on thoracic CT for the same patient group since the case definition comprised at least one symptom and GGO.

5.2.5 Detection bias

Detection bias refers to when decreased or increased surveillance of a phenomenon leads to the misperception that the phenomenon is decreasing or increasing in magnitude. In paper I, we found an annual increase in the number of patients with a positive *P. jirovecii* PCR. This finding must be interpretated in light of the concomitant increase in PCR testing. To distinguish real increase from one resulting from increased detection, we also reported the annual proportion of positive specimens. This remained stable which could suggest a real increase since an increase due to detection bias would have lowered this proportion.

5.2.6 Confounding and mediation

Confounding arises when researchers relate an exposure to an outcome but actually measures the effect of a third factor, namely a confounder **(Figure 15)** [201]. Confounding can be handled by randomization, multivariable regression models (paper II and paper III), stratification (paper II and paper III), or restriction (i.e., sub-group analyses) (paper III). Regardless of the

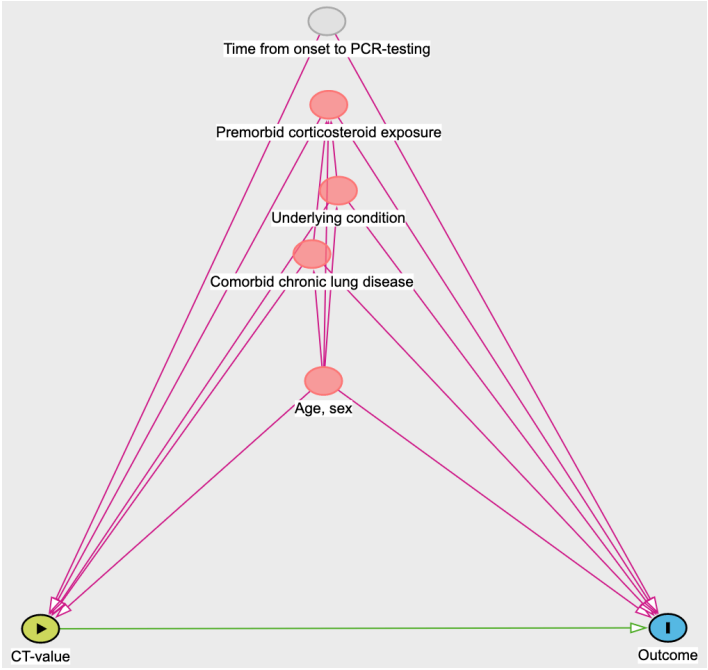

Figure 15. Example DAGs realized with DAGitty. Red arrows indicate confounding. Gray circle indicates unmeasured confounding, here exemplified by time from symptom onset to PCR testing.

approach adopted, inability to identify or measure confounders, results in residual confounding which in turn introduces systematic error [201]. We collected a wide selection of variables, which enabled controlling for these in the regression models. While this reduces the chance of residual confounding, confounders that were unintentionally omitted, were missing, or measured incorrectly, could have caused bias. For instance, in paper III, we studied the association between fungal burden indicated by C_T-value and the risk of dying within 30 days. We found that higher fungal burdens were associated with higher risk of dying. However, we did not have accurate data on the time interval from disease onset to PCR testing precluding adjustment for this variable. Thus, we cannot exclude that delayed testing permitted fungal proliferation resulting in lower C_T-values and poorer outcomes. The same principle applies to delayed treatment initiation, health-seeking behavior, distance to hospital, socioeconomic status, drug adherence to mention a few. Another possible source of confounding regards the population in focus. Immunosuppressed and multimorbid patients are highly susceptible to limitations of care such as “do not resuscitate” or “do not intubate”. This is an example of confounding by indication or disease severity which might have had unpredictable downstream effects that were difficult to measure in retrospect. Such confounding could also influence the observed association between premorbid corticosteroids and outcome (paper II and III), though in this case the confounding may be bidirectional. That is, physicians may be more reluctant to prescribing corticosteroids to older or multimorbid patients owing to their vast side effects. Oppositely, critical, or terminal patients may be more likely to receive corticosteroids to alleviate symptoms and side effects (e.g., in oncology). For these reasons, we included age, sex, and underlying conditions in the models assessing the effect of premorbid corticosteroids.

Mediators are intermediary variables, through which the exposure variable acts indirectly on the outcome variable (**Figure 16**) [206]. When the relationship between the exposure variable and outcome variable only exists through the mediator, it is termed full mediation. This is less common than partial mediation which denotes that the mediator is only paritally responsible for the relationship between the exposure and outcome. In the latter scenario, there is still a relationship between the exposure and the outcome when the mediator is excluded from the model, it is just not as strong. CAT represents a theoretical mediator in the relationship between severe hypoxemia (i.e., O_2-saturation <90 %) and 30-day mortality in paper III since patients with severe PCP are more likely to receive such treatment. As it was difficult to differentiate between CAT and prolonged or continued therapy due to PCP (i.e., overlapping

corticosteroids), we did not include CAT in the regression models. However, it is possible that such inclusion would have modified the relationship between severe hypoxemia and 30-day mortality owing to partial mediation.

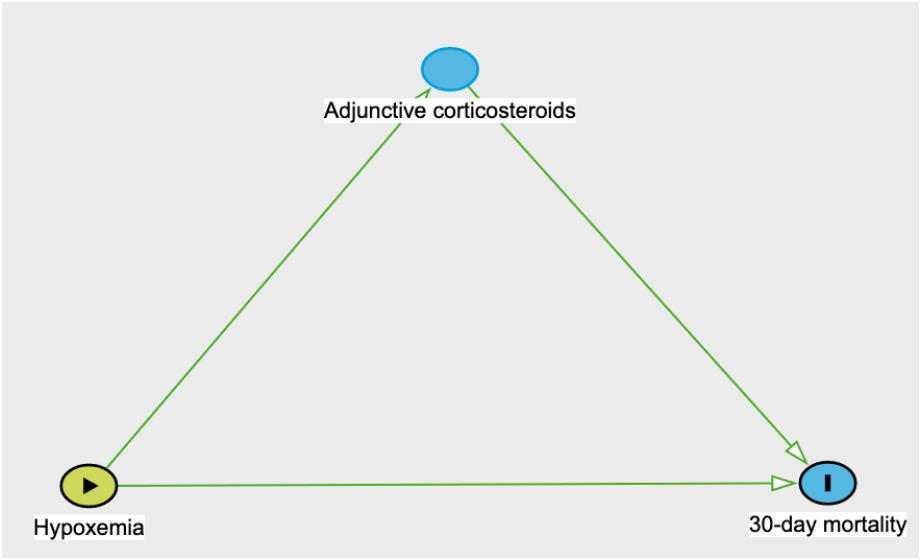

Figure 16. Example DAGs realized with DAGitty, illustrating mediation.

5.2.7 Data validity, missing, and study design

All three paper in this study are primarily based on data collected from electronic health records that were not developed as research tools. This may undermine data validity [209]. First, the external validity may be compromised if such records are not in universal use. We have little reason to believe that this was a major obstacle in our studies since the catchment area corresponded to a public health authority providing specialized health care which includes management of immunocompromised patients. Second, the data availability may depend on economic incentives, documentation practice, and real-time factors influencing the treating physicians such as awareness and workload to mention a few [211]. Moreover, the process of abstracting data from records to a research-ready analytic data set may be subject to inconsistencies, subjectivity, and misinterpretations [209]. For instance, no documentation of a manifestation is not necessarily synonymous with its absence if it was not asked for, not looked for, or merely not documented. Also, special variables require operationalization such as calculating the CCI [209]. Collectively, these steps can introduce selection bias, information bias, and residual confounding [209]. For this reason, we designed, tested, and

tailored a registration form before initiating data collection. For diagnostic test such as DIF, chest X-ray, and thoracic CT we distinguished “negative test result” from “not performed” to avoid downstream selection or information bias. Concerning comorbidities, we relied on a combination of anamnesis and ICD-codes to increase the sensitivity. Ideally, the case reviews should have been performed by independent reviewers blinded to each other’s results, however, this would have compromised the feasibility of the project. Instead, we used plenary discussion in case of ambiguities and in some instances, we asked for second opinions (e.g., new evaluation of CT-scans by a radiologist).

Some of the independent variables had missing data. This is a common problem for retrospective studies and those relying on electronic case records and entails a risk of selection bias [209]. Multiple imputation is a method to overcome this limitation but requires that the mechanism is random. For clinical data originating from case records this was not the case. More so, whereas the missing of C_T-values was random, respiratory sampling was not. Therefore, we handled the missing with complete case analyses, and reported the number of observations rigorously for transparency. While a prospective study design would likely have reduced this limitation, it would have required several years or a larger catchment area to obtain the same sample numbers.

Owing to the retrospective nature, neither the interval between disease onset and PCR analysis, notation of clinical manifestations, nor treatment protocols were standardized *per protocol*. Also, we cannot exclude that practice changed during the 12-year period or varied between hospitals. Lastly, we did not include negative controls, and one may argue that this compromises the validity of the findings [207]. The lack of negative controls also precludes analysis of the representativeness of patients with positive *P. jirovecii* PCR with respect to those who undergo PCR testing but have negative results.

5.2.8 Chance

We did not control for increase in familywise error rate across statistical analyses (e.g., Bonferroni correction) and statistically significant relationships should be interpreted with caution. Especially the sub-groups with limited number of observations had low power to test the respective hypotheses. Nonetheless, we believe that it is unlikely that our findings

arise merely from chance considering the low p-values, mostly narrow confidence intervals, and in several instances dose-dependent associations suggesting a biological gradient.

5.2.9 Microbiological considerations

The current in-house PCR assay was chosen in 2006 for its robustness and clinical utility based on the report from Brancart et al. [202]. The sensitivity and objectivity are superior to those of microscopic examinations, which have been the traditional gold standard for PCP diagnosing. Moreover, the assay is highly reproducible, the minimum detection limit is low (about 50 copies/mL), and the linear range covers five orders of magnitude exceeding the minimum standard. Lastly, the direct 1:1 organism quantification is considered an important advantage of β-tubulin in a clinical microbiological context. Nevertheless, certain aspects warrant attention. First, single-copy genes exhibit lower dynamic range than multi-copy genes. While this increases the specificity for PCP, it also decreases the sensitivity. Second, the in-house PCR assay does not include a recommended internal control (reviewed in section **1.5.3**). While the human target PCR gives an indication of the specimen quality through comparison with anticipated results, it cannot quantitate nor exclude inhibition or extraction problems. Collectively, these concerns entail a risk of false negative results which might have inflicted selection bias during the screening for eligible patients. Third, despite controlled conditions, the cellular content and volume recovered from BAL can vary considerably [212]. Relative quantification enables controlling for the latter, but this was not performed. Thus, we cannot exclude bias from different specimen volumes. Forth, we used C_T-values as an indication of fungal burden which in contrast to absolute quantitation (i.e., copies/mL) reflects a semiquantitative estimate. Recent experience with Severe Acute Respiratory Virus Corona Virus-2 (SARS-CoV-2) demonstrates that C_T-values can vary significantly between and within methods [213]. Although we acknowledge these issues and that our findings await validation, we do not believe that the general significance of the studies is severely biased.

5.2.10 External validity

As shown in section **3.1.1**, the population of Central Norway is fairly representative of Norway except for lower HIV/AIDS burden and lower proportion of immigrants than regions with bigger cities. With caution to these differences, the results can be generalized to the Norwegian population and probably Western European countries with similar population

structures and health care systems. In paper III, we categorized the patients according to the first EORTC/MSGERC-criteria and restricted the population to patients with proven or probable PCP [195]. This enhances extrapolation. However, as outlined above, the generalizability to other microbiological laboratories and PCRs targeting other genes may not be straightforward. Finally, we did not use the updated CCI which accounts for the advancement in technology, management, and treatment of chronic diseases, and their implications for survival [214]. It can be discussed whether this introduces information bias or undermine the external validity. Importantly, the weights did not change for the conditions with the highest prevalence in the study populations including cancers with or without metastases and chronic lung diseases [214].

5.3 Strengths

All three papers are based on hospital records from a whole health authority. With this population-based approach we achieved a regional catchment area covering all levels of specialized health care and included a wide range of underlying conditions. Moreover, we achieved relatively high sample numbers which increased the power in calculations and permitted sub-group analyses. Also, the 12-year study design allowed analysis of temporal trends with regards to epidemiology and testing after the advent of PCR testing for *P. jirovecii* in Central Norway. Data on all specimens sent for *P. jirovecii* also enabled evaluation of detection bias. We collected a wide selection of variables including detailed data on comorbid non-communicable diseases considering their possible attribution to the "net state of immunosuppression" and frailty. Furthermore, in the evaluation of the in-house PCR assay we included patients who are similar to those encountered in clinical practice and evidenced real diagnostic challenges in non-HIV PCP. Finally, in paper II and III, we focused on readily available risk factors for PCP and fatal outcome, respectively, to facilitate clinical guidance and risk assessment.

5.4 Limitations

With reference to section **5.2 Methodological considerations**, the studies presented in this thesis have important limitations. First, we cannot make causal claims due to the observational nature. Second, as discussed in detail above, the retrospective study design and microbiological method might have inflicted selection bias, information bias, and residual confounding. Moreover, hindsight bias is likely to affect all retrospective studies [215]. Third, we only included patients from one health authority and relied on active consent from survivors. Forth, certain analyses would have required larger sample sizes to have sufficient power (e.g., sub-groups analysis of patients with C_T-values $\leq$30 in paper III). Finally, owing to the lack of a valid reference standard to diagnose PCP until 2021, the reference standards for PCP used in paper I and paper II were suboptimal and differed according to sub-groups (refer to **Figure 10**). This entailed a risk of non-differential information bias and incorporation bias (paper II) as discussed above. Prospective adjudication in real-time by experienced infectious disease clinicians blinded to PCR results might have given a better approximation of ground truth. However, neither approach, that is retrospective case definition nor prospective adjudication, assures 100 % specificity for PCP. For instance,

misclassifications may occur in patients colonized with *P. jirovecii* who suffer from respiratory symptoms caused by other pulmonary diseases or infections.

5.5 Discussion of main findings

Observational studies can merely identify statistical associations and not make causal inference. A statistical association does not necessarily equal a causal association [201]. To aid the judgement of causality, one may consider the consistency and strength of the associations, coherence with existing knowledge, and biological plausibility [201]. Therefore, in the following section I will discuss the meaning of our findings and compare them to those of other studies.

5.5.1 Recent epidemiology of *P. jirovecii* in industrialized countries

The literature review in section **1.7.2** indicated that the burden of PCP is shifting towards a rise in non-HIV cases and a decrease in HIV cases in industrialized countries. Our findings from Central Norway seem to support this epidemiological evolution. The most recent evidence from general populations in Europe and the United States [216-218] and two center-based studies from Asia [219, 220], respectively, shows ulterior consistency. **Figure 17** presents the progress of the UNAIDS Fast Track targets of 90 % diagnosed, 90 % on HAART, and 90 % virally suppressed for people living with HIV [221]. Considering this evolution in combination with ageing populations, expansion of immunocompromised populations with increasing life-expectancies, improvements in bronchoscopy and microbiological testing techniques in industrialized countries [5, 218], the shift seems plausible. The latter aspects have especially enhanced non-HIV PCP diagnoses due to lower fungal burdens requiring high-quality LRSs and more sensitive detection methods.

5.5.2 Burden of *P. jirovecii* in industrialized countries

Like in Norway, PCP is generally not a reportable disease *per se,* and incidence estimates rely on observational studies and register data. In paper I, we showed that the incidence of patients with positive *P. jirovecii* PCR in Central Norway doubled from 5.0 cases per 100 000 person years in 2006 to 10.8 cases per 100 000 person years in 2017, respectively. Our estimates are higher than those reported from other industrialized countries [39, 218, 222], including one with Norwegian estimates [155]. In the latter, the burden of PCP was estimated to 5.0 cases per 100 000 person years in 2015 based on data from six microbiological laboratories using DIF microscopy and/or PCR for the diagnosis [155]. The same year, St. Olavs hospital detected a record of 11.0 cases per 100 000 person years in Central Norway, while the

average was 5.8 cases per 100 000 person years. Several reasons may explain these inconsistencies. First, we divided the number of cases by the number of adults aged 16 years or higher. Inclusion of the pediatric population in the denominators while assuming that the number of pediatric cases was negligible would reduce our estimates by approximately 20 %. Second, we reported the number of patients with positive *P. jirovecii* PCR which is not synonymous with overt PCP. A more stringent case definition would reduce our estimates ulteriorly. Third, most comparable studies exhibit low data completeness according to various groups at risk of PCP [153, 154, 223-232], which likely results in underestimates [229]. Forth, some of the discrepancy may be attributed to different time periods and varying degrees of detection bias across countries. In contrast, it seems unlikely that varying nosocomial transmission plays a major role in explaining the differences since strict infection control including droplet isolation is not universally adopted nor advocated [233].

Concerning the distribution of underlying conditions, the study population in paper I is comparable to those of other studies with patients with hematological malignancies dominating [151, 216, 217, 222, 234-240]. Risk of PCP, prevalence of the underlying conditions and prescription of prophylaxis, determine the incidence within sub-populations at risk. For instance, clinicians may encounter more PCP patients suffering from RA than connective tissue diseases combined despite lower estimated risk in the former [71]. Indeed, RA affects between 0.5 to 1 % of the population in industrialized countries [241], while connective tissue diseases and vasculitidies are rare diseases [242].

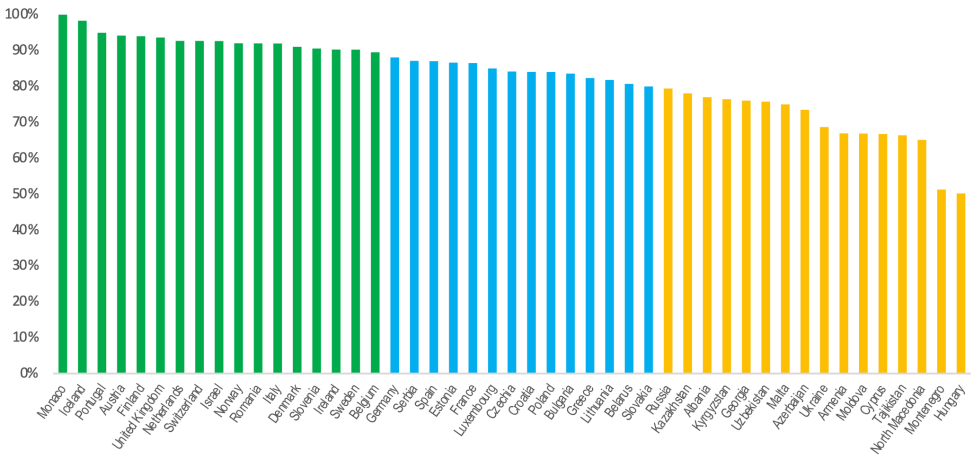

Figure 17. Percentage of people living with HIV who know their status, are on treatment, and are virally suppressed in 47 countries across Europe and Central Asia, reported in 2021. The colors indicate percentual coverage: green (>90 %), blue (80-90 %), and yellow (<80 %), respectively. Figure reprinted with permission from Noori et al. [221].

5.5.3 Consequences and gaps of evolving epidemiology

Our research based on a sample from the third largest health authority in Norway confirms that the morbidity and mortality associated with *P. jirovecii* is substantial despite a non-HIV preponderance. Recent studies have evidenced that non-HIV PCP is associated with higher costs [216, 217], longer hospital stays [216, 217, 243], and as referred above higher mortality compared to HIV PCP [151, 216, 219, 222, 235, 236, 238, 240, 243, 244]. Older patients [151, 216, 217, 219, 220, 222, 236, 243-245], more multimorbidity [216, 219, 245], diagnostic and therapeutic delays [151, 222, 238], and more severe respiratory impairment [236, 245] may contribute to these gaps. In fact, HIV-negative status is not consistently an independent risk factor for death in multivariable analyses [217, 236, 240, 243]. Recently, Pates et al. found a significant increase in PCP patients who were aged 75 years or older between 2012 and 2021 in a nationwide register study from England [218]. Despite being unable to differentiate between HIV and non-HIV PCP, the authors attributed the development to a rise in non-HIV PCP cases [218]. Albeit not statistically significant, our data on all patients with positive *P. jirovecii* PCR between 2006 and 2017 (N = 341) indicate a similar demographic shift (**Figure 18**).

In paper III, we found that cardiovascular diseases including CHF were associated with 30-day mortality and this association persisted when adjusting for age and sex in non-HIV patients with proven or probable PCP. The weighted multivariable analyses showed the same associations for CCI $\geq$6 and comorbid chronic lung diseases. While these findings may seem obvious, they underscore an important implication of the changing demographics in PCP. Further investigations are needed to identify what medications or comorbidities increase the risk of severe respiratory status or mortality in non-HIV PCP, and how to prevent it [102, 246].

Relatedly, McDonald et al. evidenced that present treatment of PCP relies on data from trials conducted 25-35 years ago which undermines its external validity to populations at risk today including older, immunocompromised non-HIV patients with comorbidities and varying degrees of polypharmacy [247]. Furthermore, the choice of dose and duration of treatment are based almost entirely on anecdote [247]. This is important given a systematic review and meta-analysis from 2020 of six observational studies which suggested that lower doses ($\leq$15 mg/kg/day of the trimethoprim component) may represent a better balance between avoidance

of toxicity and clinical cure [248]. Regarding CAT to non-HIV patients, a meta-analysis of 16 retrospective observational studies found that corticosteroids were associated with lower mortality in patients with respiratory failure (OR 0.63, 95 % CI 0.41-0.95) [249]. In contrast, CAT appeared harmful to unselected non-HIV patients without respiratory failure [249]. The authors suggested that CAT should be administered selectively based on patients' PaO_2 preferably adjusted for the fraction of inspired oxygen (i.e., PaO_2/FiO_2). In addition, they called for clinical trials on CAT to non-HIV patients to increase the data validity. It seems plausible that more tailored treatment strategies in combination with increased awareness could reduce some of the prognostic gaps between non-HIV and HIV PCP.

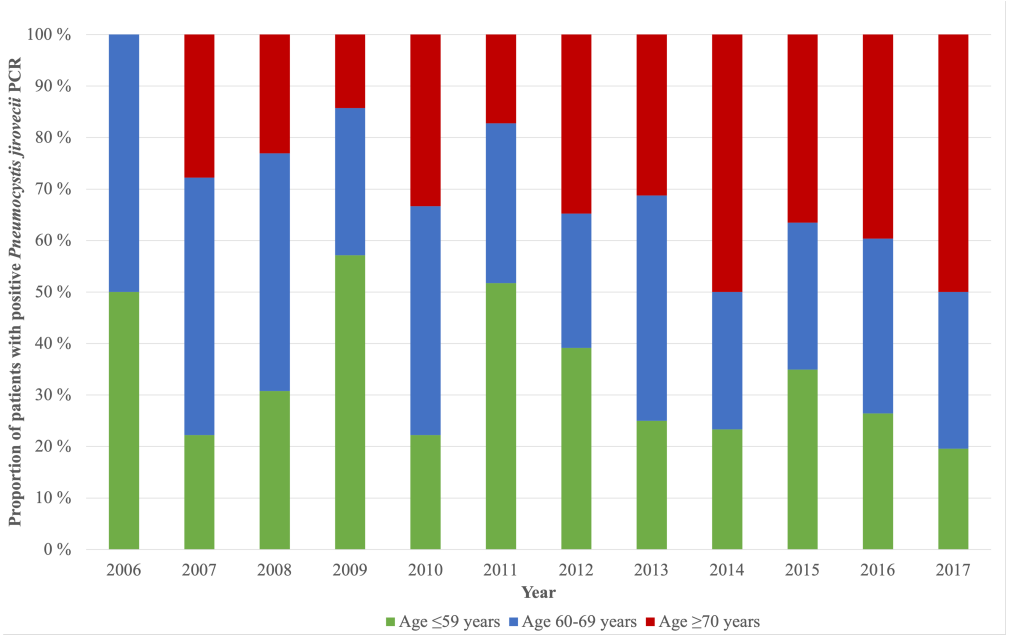

Figure 18. Age distribution of 341 patients with positive *P. jirovecii* PCR within age groups ≤59 years, 60-69 years, and ≥70 years in Central Norway between 2006 and 2017. The shift in age distribution was not statistically significant.

5.5.4 Real-time PCR to distinguish PCP from colonization

In paper II, we evaluated the utility of the in-house semiquantitative real-time-assay in diagnosing non-HIV PCP in LRSs (i.e., BALF and tracheal aspirates). A C_T-value of 36 allowed discrimination of PCP from colonization with a sensitivity of 71.3 % (95 % CI 63.7 to 78.9 %) and specificity of 77.1 % (95 % CI 63.2 to 91.1 %), respectively. This corresponded to an acceptable PPV of 92.4 % (95 % CI 87.3 to 91.1 %), while the NPV was

only 40.9 % (95 % CI 29.0-52.8 %). A C_T-value ≤30 confirmed the diagnosis. In contrast, no cut-off enabled exclusion of PCP with 100 % NPV. As outlined in the background, this is not unique for our study. However, establishment of upper and lower cut-offs to obtain 100 % NPV and PPV, respectively, has predominated. This has also been the case in most studies published after 2017 [250-253]. On the contrary, Damhorst et al. [254] and Aguilar et al. [255] described two different assays both targeting the mtLSU of *P. jirovecii* with sensitivity and specificity >90 % in BAL applying only one cut-off. The former used cyto- and histopathology (not further specified) as reference method [254], whereas the latter used multimodal criteria including the real-time PCR in validation for probable PCP and positive microscopy examination for proven PCP [255]. Both studies included HIV and non-HIV patients without stratifying accordingly [254, 255]. High proportion of HIV patients (43 % and 79.7 % of immunocompromised patients, respectively) may partially explain the high sensitivity and specificity in their respective studies.

5.5.5 Standardization of real-time PCRs for detection of *P. jirovecii*

Considering the heterogeneity within non-HIV PCP and discrepancies between studies on real-time PCR for *P. jirovecii* detection, an important contribution to this field was made by the Fungal PCR Initiative that compared several in-house and commercial *P. jirovecii* PCRs [256]. In this study, targeting whole nucleic acids (i.e., RNA and DNA) and mtSSU provided the earliest C_T-values (i.e., lowest detection threshold) [256]. β-tubulin was found to have the latest C_T-values after mtLSU and MSG [256]. Indeed, a mean difference of seven C_T-values (200-fold variation) was observed between the assay targeting the β-tubulin gene and the those targeting multi-copy genes [256]. Interestingly, the study also confirmed the large variation within real-time PCRs quantifying *P. jirovecii* nucleic acids, even when the same assays were used [256]. This may explain some of the divergencies across studies evaluating real-time PCR to discriminate PCP from colonization. In consequence, the authors argued that interpretative thresholds may be defined in the future if centers agree to use the most sensitive method [256]. Besides targets, sampling method, specimen volume, timing of sampling with respect to treatment, time from sampling to analysis, specimen storage, specific protocols, laboratory reagents used for specimen processing and DNA extraction, amplification techniques, level of expertise of technicians, and different clinical contexts may also influence the results [254, 257]. Future standardization studies should account for some of these factors [256].

5.5.6 Heterogeneity of non-HIV PCP

In paper II, we showed how stratification according to underlying condition improved the discrimination, and we attributed this to heterogeneity within non-HIV PCP. Specifically, SOT-recipients had significantly higher fungal burdens compared to patients with hematological malignancies. Indeed, to improve the NPV in the latter, we increased the cut-off to a C_T-value of 37 which resulted in a sensitivity of 88.5 % (95 % CI 79.8 to 97.1 %). In contrast, the universal cut-off of 36 yielded a sensitivity of 95.0 % (95 % CI 85.4 to 100 %) in SOT-recipients. We hypothesized that these observations resulted from intrinsic and extrinsic host factors including immunological differences and cyclic versus continuous exposure to chemotherapy and immunosuppressants, respectively. Relatedly, Damiani et al. assessed the performance of a β-D-glucan-assay for discrimination of PCP from colonization and made similar observations. The median serum-β-D-glucan of patients with hematological malignancies was significantly lower of those with systemic autoimmune or inflammatory disorders and SOT-recipients and was below the established cut-off defined by the manufacturer [258]. Overall, the assay showed a sensitivity of 87 % (95 % CI 73 to 94 %) [258]. In contrast, in patients with hematological malignancies the same cut-off yielded a sensitivity of only 64 % (95 % CI 35 to 85%) [258]. Moreover, the fungal burden indicated by C_T-values from real-time PCR was significantly different between patients with hematological malignancies and SOT-recipients [258]. The authors speculated whether the high fungal loads observed in SOT-recipient result from altered T-cell functions drawing a parallel to HIV patients in whom $CD4^+$ T-cells level determines the risk of PCP and PCP is characterized by a peak in β-D-glucan and extremely high fungal loads [258]. Conversely, patients with hematological malignancies with B-cell disorder may have preserved $CD4^+$ T-cells activation which could contribute to fungal clearance and therefore explain lower fungal loads and β-D-glucan [258]. This hypothesis, however, does not explain why patients with hematological malignancies develop overt PCP at lower fungal burdens suggesting a lower threshold of immune tolerance. It should be noted that Damiani et al. [258] in line with others [259] observed a poor correlation between C_T-values from real-time PCR and β-D-glucan. Nevertheless, their findings add to the literature evidencing how the diversity within non-HIV PCP has important diagnostic and possibly prognostic implications. **Figure 19** summarizes important issues related to non-HIV PCP.

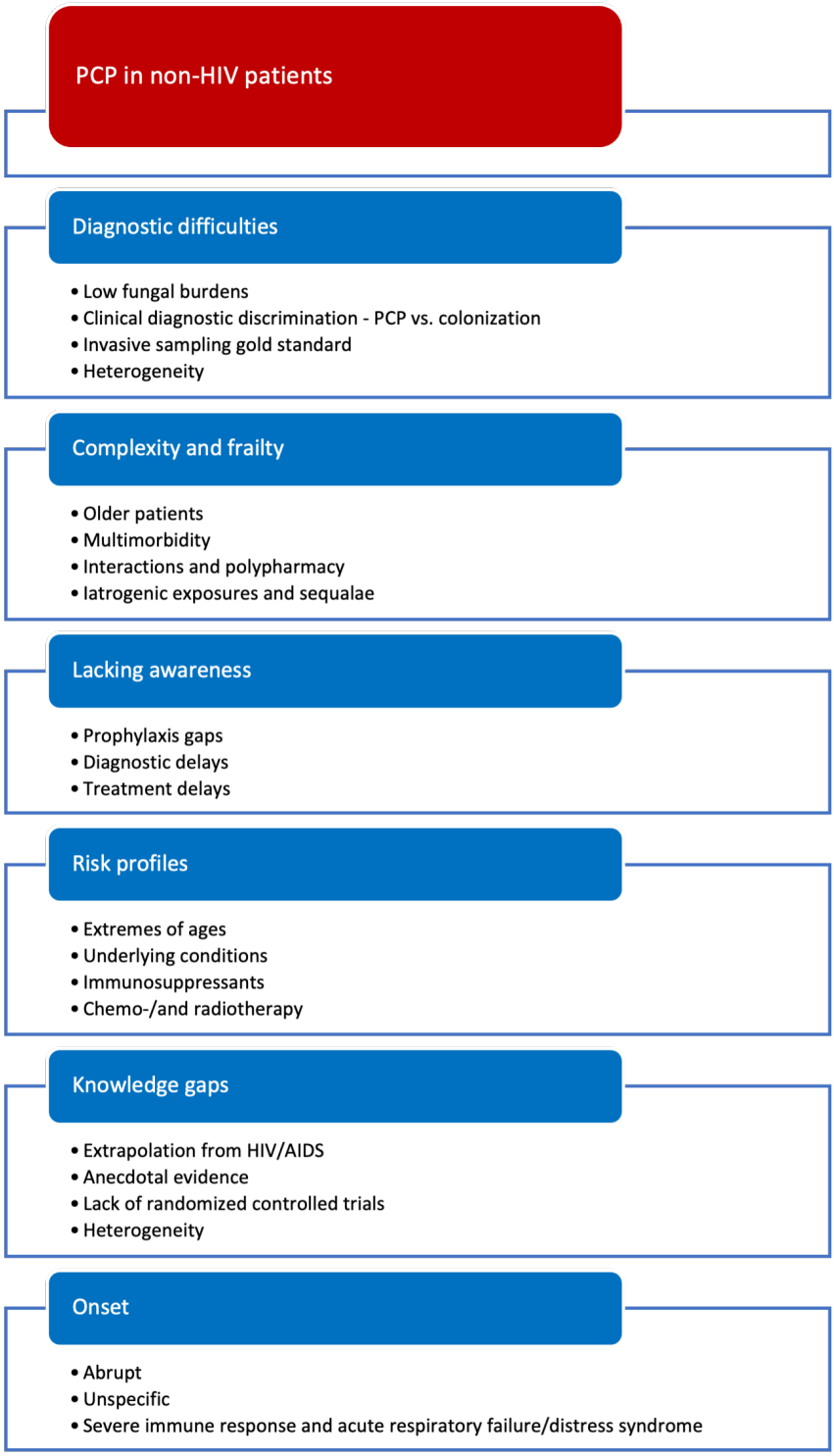

Figure 19. Issues related to PCP in non-HIV patients.

5.5.7 Relationship between fungal burden, host response, and outcome

In paper III, we found that markers of acute inflammation and respiratory impairment, namely leukocytosis with higher neutrophil counts, CRP ≥100 mg/L, and severe hypoxemia were independently associated 30-day mortality. These observations resonate with other studies [260-262], and the hypothesized pathophysiology behind severe non-HIV PCP [30]. Indeed, as outlined in the introduction, deleterious PCP evolution appears more closely related to the extent of lung inflammation than the severity of the organism burden [61]. However, this observation was made comparing BALFs from HIV PCP and non-HIV PCP patients without further distinctions [61]. Until recently, few studies had assessed whether fungal burden estimated by real-time PCR is associated with the outcome in non-HIV PCP. In 2019, Liu et al. reported an association between C_T-value from real-time PCR in BALF and induced sputum and in-hospital and 60-day mortality in 84 non-HIV patients [151]. Their PCR targeted MSG and they only included patients with C_T-values ≤34, resulting in a relatively small but stringent patient sample [151]. C_T-value was not an independent predictor of 60-day mortality, whereas in-hospital mortality was not included as outcome in multivariable analysis [151]. Our study on the outcome of patients with proven or probable PCP, complements their findings. We found that C_T-value ≤30 was independently associated with 30-day mortality, while this was not the case for C_T-value ≥31. Moreover, in the sub-group analysis of patients with C_T-value ≤37, both C_T-value 30–33 and C_T-value ≤30 were significantly associated with higher odds of dying compared to C_T-value 34–37. Our findings support the hypothesis that fungal burden estimated by real-time PCR is associated with outcome in the acute phase of infection. The 30-day survival analysis comprising all patients with positive PCR and retrievable C_T-value appeared to confirm this association with an adjusted HR of 0.89 per C_T-value (95 % CI 0.82-0.96, p<0.01) within the range of C_T-values from 22 to 40 cycles. Beyond 30 days, we believe that host factors are the primary determinants of outcome.

5.5.8 Risk stratification and index of suspicion

In extension to investigating relationship between fungal burden and outcome, we also explored the role of real-time PCR in clinical risk stratification in patients with proven or probable PCP. Based on the available data and emphasis on readily assessable characteristics, we calculated the risk of dying according to fungal burden, CCI, and their interaction. Noteworthy, we found that patients with high burdens (C_T-value ≤30 and CCI ≥6) had almost

an eight-fold increase in the risk of dying compared to those with low burdens (C_T-value ≥37 and CCI ≤2) being 70 % vs. 9 %, respectively. When separated, the spectrums of mortality risk were comparable. We observed similar trends when restricting the analyses to patients with C_T-values ≤37. We concluded that fungal burden combined with underlying comorbidities may improve risk stratification in non-HIV patients with PCP. Relatedly, in the above-mentioned study by Damhorst et al., the authors found that C_T-value had played a role in clinical decisions to initiate or discontinue anti-*pneumocystis* treatment [254]. While C_T-values from PCR in BALF differed significantly among treated and untreated patients, the authors found no correlation with other surrogates of disease severity including CAT, length of stay, ICU, or radiological findings [254]. However, as evidenced above, their study population was mixed which may influence the generalizability to non-HIV PCP [254].

In line with pooled data from a 2021 meta-analysis, we found that lymphopenia was independently associated with increased 30-day mortality risk [208]. It is of utmost importance to consider lymphocyte counts not only diagnostically, but also in risk stratification. To disregard immune status if neutropenia is absent denotes a pitfall in this context. Considering the close relationship between premorbid corticosteroids, lymphocytes, and fungal burden, they likely represent different aspects of severe and potentially fatal immunodepression.

Our research shows how patients at risk of PCP meet a wide range of specialists and the vast spectrum of indications behind chemotherapy and immunosuppressants including corticosteroids. Chen et al. evidenced that 30 of 62 non-HIV patients with proven PCP had received immunosuppressants for less than three months on presentation [220]. As accentuated in the introduction, systemic exposure corticosteroids play a major role in the occurrence of PCP. In paper I, we reported a patient with statin-induced myositis, a disease not associated with PCP *per se.* In his case, the infection was attributed to treatment with high-dose corticosteroids. Even tapering doses represents a major risk factor [42], perhaps due to reappearance of lung inflammation [106]. The unspecific presentation of PCP challenges clinical recognition. In our experience PCP is often mistaken for pneumonia of other etiology or pulmonary embolism due to the fulminant onset and overlapping risk profiles (e.g., in cancer patients). With respect to the latter differential diagnosis, presence of GGO and other signs indicating atypical pneumonia in combination with absence of pulmonary embolisms often raise the suspicion of PCP by the radiologist. This diagnostic delay occurs on the cost of

prompt treatment. Thus, increased awareness of "red flags" such as systemic corticosteroids, chemo- or radiotherapy, and lymphopenia seems inevitable to reduce diagnostic and therapeutic delays, and ultimately improve the outcomes.

5.6 Implications and future research

This thesis sheds lights on the contemporary epidemiology and clinical challenges related to *P. jirovecii* and PCP in a resource rich-setting. Our research also identifies potentials for improvement. The first regards prophylaxis to high-risk patients. Although we did not review the cases with respect to compliance to the current guidelines for prophylaxis, our findings indicate a substantial gap between those who would benefit from prophylaxis and those who receive it. This has also been a recurrent finding in the most recent epidemiological studies from comparable contexts [219, 220, 222, 235, 239, 240, 244, 260, 261, 263]. Hence, strategies to improve awareness of prophylaxis guidelines in immunocompromised patients are warranted. Automatic warnings in digital order systems that pops up when corticosteroids are prescribed above a certain threshold could be a contribution [235]. Even multimorbid patients should be considered for prophylaxis. For instance, in paper III, solid tumors resulted the underlying conditions with the highest mortality risk together with immunological disorders. In our experience, PCP often defines a turning point for cancer patients by shifting the treatment intent from curative to palliative. Second, the continuous emergence of novel immunosuppressants and increasing prescription of existing medications imply that the threat of opportunistic infections like PCP in non-HIV patients will persist and possibly increase. Considering that most incidence estimate comes from epidemiological studies, making non-HIV PCP a notifiable infection could be an important step to increase the knowledge base. The third concern also regards public health surveillance. Strategies to prevent nosocomial transmission should be assessed acknowledging the mounting evidence of interhuman transmission (**Figure 20**) [264]. However, the cost-benefit equation of such implementations must be evaluated by public health expertise. Genotyping studies to investigate the molecular epidemiology in Norway outside and within hospitals would probably aid such discussions. Forth, the retrospective study design based on case reviews has several limitations. Therefore, as an extension to our research, it would be interesting to perform an observational study on PCP, perhaps on national level using hospital discharge data and linkage with national data registries. Center-based studies with prospective inclusion could be equally useful. Finally, new of 2022, is that WHO launched the first global effort to systematically prioritize fungal

pathogens including *P. jirovecii* [265]. The rationale is to drive further research and policy interventions to strengthen the response to fungal infections and antifungal resistance. The future will reveal its success [265].

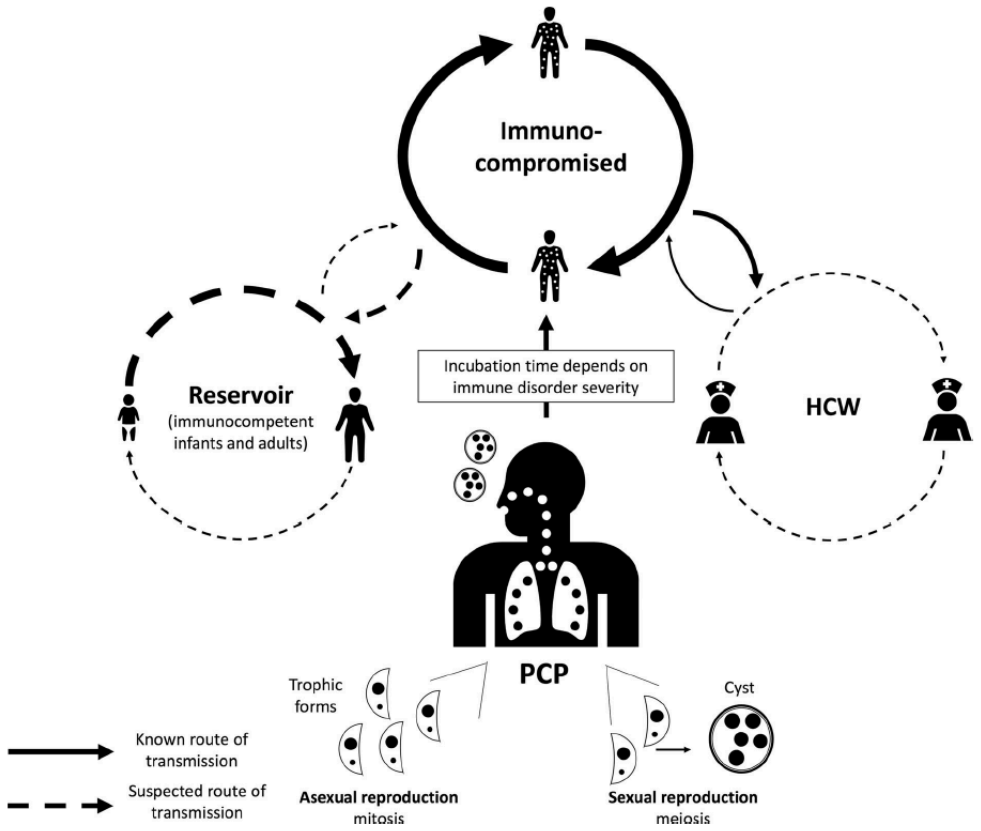

Figure 20. Hypothetical transmission mode of *P. jirovecii* during outbreaks. Routes of transmission evidenced by genotyping are represented by solid lines, and suspected routes of transmission by dotted lines, respectively. The arrows' width represents the hypothetical fungal burden and consequent likelihood of transmission. Figure reprinted with permission from [264].

6 Conclusions

This thesis, based on regional multi-center data, adds information about the disease burden associated with *P. jirovecii* in a Norwegian healthcare setting. In addition, we evidence trends and clinical and diagnostic challenges in a population-based sample of patients with positive *P. jirovecii* PCR over a 12-year period from Central Norway. Our results indicate that this opportunistic fungus primarily affects non-HIV immunocompromised patients exposed to iatrogenic immunosuppression and chemotherapy. The unspecific presentation of PCP requires that clinicians maintain a high index of suspicion, especially in encounters with patients harboring risk factors. Major "red flags" include predisposing underlying conditions, iatrogenic exposures such as high-dose corticosteroids and chemotherapy regimens, absence of prophylaxis, and lymphopenia. We found an annual increase in both the number of PCR tests performed and positive cases from the introduction of PCR in 2006 to 2017. The in-house semiquantitative real-time PCR showed a sensitivity of 71.3 % and a specificity 77.1 % for discrimination of non-HIV PCP from colonization. Stratification of patients according to underlying condition improved the discrimination, suggesting that extrinsic and intrinsic host factors should be accounted for in diagnostic algorithms. With regards to the outcome, we found that higher fungal burdens indicated by C_T-values $\leq$30, cardiovascular disease including CHF, solid tumors, immunological disorders, premorbid corticosteroids, severe hypoxemia and host response, lymphopenia, and low serum-albumin were independently associated with 30-day mortality in non-HIV PCP. The shifting epidemiology of *P. jirovecii* in industrialized countries towards non-HIV preponderance calls for targeted strategies to reduce the negative impact. First, increased awareness of guidelines on prophylaxis is warranted. Finally, we need high-quality clinical trials and prospective studies restricted to non-HIV patients to improve the validity of future evidence to review guidelines and public health policies.

7 Appendix

7.1 Literature paper I and III

Author, year, country[a]	**Design and period**	**Population and inclusion**	**Focus and study objectives**	**Study population(s)**	**Mortality/survival**	**Main findings**
Kovacs et al. [129], 1984, United States	Retrospective monocenter case review: 1979 – 1983 (~4,5 years)	• **HIV** and **non-HIV patients** aged 10 years or older at diagnosis • Morphologically confirmed PCP	Comparison of PCP in HIV vs. non-HIV patients.	85 patients • 46 HIV-positive • 39 HIV-negative	Overall in-hospital mortality: NA Overall *survival* rate according to HIV-status: • HIV-positive: 57 % • HIV-negative: 50 %	Focus of study: • Non-HIV patients had more fulminant onset, higher respiratory rate, and lower oxygenation on presentation, but experienced fewer adverse effects.
Peters et al. [266], 1987, United States	Retrospective monocenter case review: 1976-1983 (8 years)	• Adult and pediatric **HIV** and **non-HIV patients** • Confirmed by microscopy in microbiology or pathology laboratory	Case series describing clinical characteristics, diagnostics, and outcome.	53 patients • 2 HIV-positive • 51 HIV-negative	Overall in-hospital mortality: NA 28-day mortality: 47%	Factors associated with mortality (survivors compared to non-survivors)[5,7]: • Coexisting infection Other/focus of study: • PCP remains a significant and life-threatening complication of diseases or treatments associated with immune suppression
Sepkowitz et al. [130], 1992, United States	Retrospective monocenter case review: 1978 – 1989 (12 years)	• **Non-HIV patients** at a cancer hospital (mean age survivors 36.2±18.6 years), mean age non-survivors 44.9+20.9 years) • Morphologically confirmed PCP	Predisposing factors, attack rate, underlying disease, and outcome of PCP in non-HIV cancer patients.	142 patients	Overall mortality in patients diagnosed ante-mortem (n = 114): 49 %	Factors associated with mortality (survivors compared to non-survivors)[5,7]: • Higher age • Higher leukocyte count on admission • Lower PaO_2 on initial arterial blood gas Other/focus of study: • Patients with CNS-tumors receiving corticosteroids are at risk of PCP and should receive prophylaxis.

Yale et al. [131], 1996, United States	Retrospective monocenter case review: 1985 – 1991 (7 years)	• **Non-HIV patients** (age not specified) • Morphologically confirmed PCP	Predisposing illness and premorbid corticosteroid therapy non-HIV patients with PCP.	116 patients	Overall in-hospital mortality: 34 % Mortality after respiratory failure: 66 % According to primary underlying disease: • Hematologic malignant disorder 34 % • Inflammatory disease 35 % • Solid tumor 53 % • SOT 21 % • Other condition 36 %	Factors associated with *survival* (survivors compared to non-survivors)[5]: • Lower dose of premorbid corticosteroids Other/focus of study: • The vast majority (90.5 %) had received systemic corticosteroids prior to developing PCP without proper prophylaxis.
Pareja et al. [132], 1998, United States	Retrospective monocenter case review: 1989– 1995 (11 years)	• Adult **non-HIV patients** • Morphologically confirmed PCP	Use of CAT in cases of severe PCP in non-HIV patients.	31 patients	Overall in-hospital mortality: 40.0 % According to treatment: • High-dose CAT (>60 mg prednisone daily): 44 % • Low-dose CAT (<30 mg prednisone daily/tapered dose): 36 % According to complications: • Respiratory failure 57.1 % • Respiratory co-pathogen 55.6 %	Factors associated with mortality (survivors compared to non-survivors): • Identification of co-pathogens in respiratory specimen Other/focus of study: • High-dose corticosteroids may accelerate recovery in cases of severe adult non-HIV PCP.
Mansharamani et al. [104], 2000, United States	Retrospective monocenter case review: 1985 – 1995 (11 years)	• Adult **HIV** and **non-HIV patients** • Morphologically confirmed PCP	Management of PCP in non-HIV patients from 1985 to 1995 compared to HIV positive patients.	475 patients (638 cases) • 442 HIV-positive • 33 non-HIV	Overall mortality HIV-positive: 9.6 % • Hospitalized 13.2 % • Intubated 56.2 % Overall mortality non-HIV patients: 39.4 %	Focus of study: • Decline in HIV PCP and increase in non-HIV PCP cases during study period. • Non-HIV PCP represents a significant burden, while HIV PCP outcomes appeared to meliorate except in patient with acute respiratory failure.

					• Hospitalized 40.6 % • Intubated 59.1 %	
Festic et al. [115], 2005, United States	Retrospective case review: 1995 - 2002 (7 years)	• Adult **non-HIV patients** with PCP and acute respiratory failure admitted to **ICU** and treated with positive pressure ventilation • Microbiologically confirmed PCP	Outcome and associated factors of acute respiratory failure in non-HIV PCP patients admitted to an ICU.	30 patients	In-hospital mortality: 67 % Long-term mortality: • 6 months: 77 % • 1-year: 80 %	Factors associated with *mortality* in non-HIV patients (survivors compared to non-survivors)[2,3]: • Delayed intubation • Duration of mechanical ventilation (invasive/non-invasive) • Higher APACHE-III-score day 1 in the ICU • Pneumothorax
McKinnel et al. [116], 2012, United States	Retrospective monocenter case review: 1996 - 2008 (12 years)	• Adult **HIV** and **non-HIV patients** • Pathologically confirmed PCP	Prevention and inpatient management of PCP in non-HIV patients compared to HIV patients.	97 patients • 65 HIV-positive • 32 non-HIV	In-hospital mortality: NA Overall all-cause in-hospital mortality according to HIV-status: • HIV-positive: 19 % • HIV-negative: 27 % 90-day all-cause mortality according to HIV-status: • HIV-positive: 28 % • HIV-negative: 41 %	Focus of study: • Demographics and clinical presentation varied between HIV and non-HIV patients. • Antibiotics treatment was started significantly later to non-HIV PCP patients. • Non-HIV patients were less likely to receive prophylaxis. • CAT did not affect outcome significantly. • Several transplant patients presented with late onset PCP.
Calero-Bernal [128], 2016, United States	Retrospective monocenter case review: 2006 - 2010 (5 years)	• Adult **non-HIV patients** • Symptoms in combination with positive microscopy (silver staining) and/or positive real-time PCR on spontaneous or induced sputum, BALF or biopsy.	Underlying condition, immunosuppressive therapies, and clinical outcome of PCP in HIV-negative patients.	128 patients	In-hospital mortality: NA In-hospital mortality according to ICU-admission: • ICU-admitted: 40 % • Non-ICU: 49 % 28-day mortality: • ICU-admitted: 30 % • Non-ICU: 30 %	Factors associated with *mortality* in (survivors compared to non-survivors)[1,3]: • Need for ventilatory support Other/focus of study: • Patients receiving merely intermittent steroids or those with severe immunosuppressive conditions without iatrogenic factors appears at risk of non-HIV PCP.

Wickrmaseka-ran et al. [103], 2017, United States	Retrospective nationwide database study using death certificate statistics: 1999-2014 (5 years)	• Adult and pediatric **HIV** and **non-HIV patients** • ICD codes for PCP	Study trends in PCP mortality and estimate lost productivity for PCP-associated deaths in the United States during the study period.	• 11 512 PCP-attributed deaths • 8231 deaths directly caused by PCP	Overall in-hospital mortality: NA	Focus of study: • Decline in PCP- deaths, primarily due to fewer HIV-associated PCP-deaths. • Increasing proportion of deaths caused by non-HIV immunocompromising conditions. • Mean age at death lower in HIV-associated cases. • Cancers and immunological disorders positively associated with PCP death in non-HIV patients. • Productivity loss associated with premature PCP-death >12 billion USD.
Wieruszewski et al. [234], 2018, United States	Retrospective monocenter case review: 2000 – 2016 (10.5 years)	• Adult **non-HIV patients** • ICD-9 and 10-codes for PCP and microbiological confirmation (positive PCR/smear for *P. jirovecii*)	Evaluate the impact of early CAT in acute ill non-HIV PCP patients.	323 patients	Overall in-hospital mortality: NA 30-day mortality: 22.9 %	Other/focus of study[4,7,11]: • Early CAT not associated with improved respiratory outcome, reduced mortality, need for mechanical ventilation, ICU-admission nor length of stay.
Mundo et al. [236], 2020, United States	Retrospective monocenter case review: 1996 – 2019 (25 years)	• Adult **HIV** and **non-HIV patients** • Positive DIF in a respiratory specimen	Clinical predictors associated with mortality in a retrospective study.	71 patients • 43 HIV-positive • 28 HIV-negative	In-hospital mortality: NA Overall in- mortality according to HIV-status: • HIV-positive: 16.3 % • HIV-negative: 71.4 % 90-day mortality according to HIV-status: • HIV-positive: 7.14 % • HIV-negative: 59.3 % 1-year mortality according to HIV-status: • HIV-positive: 7.69 % • HIV-negative 76.0 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,3,5]: • Higher age • Atypical symptoms • Higher Aa-gradient • Lower PaO_2/FiO_2 • Mechanical ventilation • ICU-stay • Not receiving CAT Independent risk factors for *mortality*[4**]: • Not receiving CAT • HIV-negative status

Kanj et al. [217], 2021, United States	Retrospective nationwide multicenter case review: 2005 – 2014 (10 years)	• Adult **HIV** and **non-HIV patients** • ICD-codes for PCP	Assess host factors in PCP-related hospitalizations and compare outcomes between HIV and non-HIV patients.	3384 patients • 604 HIV-positive • 2780 HIV-negative	Overall in-hospital mortality: 14.0 % In-hospital- mortality according to HIV-status: • HIV-positive: 5.0 % • HIV-negative: 16.0 %	Focus of study[3,4,5,11,13]: • HIV-associated PCP hospitalizations decreased, whereas it increased for non-HIV immunocompromising conditions. • After adjusting for age, sex, and smoking status, there was no difference in mortality between non-HIV and HIV patients with PCP. • Daily hospitalizations costs were higher for non-HIV patients than HIV patients.
Su et al. [140], 2008, Taiwan	Retrospective monocenter case review: 2004-2006 (~3 years)	• Adult and pediatric **HIV** and **non-HIV patients** • Presence of consistent clinical symptoms, and/or chest radiograph abnormalities in combination with positive nested PCR in expectorated sputum	Elucidate the clinical presentation and outcome of PCP in Taiwan.	49 patients • 15 HIV-positive • 34 HIV-negative	Overall 30-day mortality: 36.7 % 30-day mortality according to HIV-status: • HIV-positive: 6.7 % • HIV-negative: 50.0 %	Factors associated with *mortality* (survivors compared to non-survivors) [3,5]: • HIV-negative status • Lower $CD4^+$ T cell counts
Boonsargnuk et al. [117], 2008, Thailand	Retrospective monocenter case review: 2000-2006 (~7 years)	• Adult **HIV** and **non-HIV patients** with acute respiratory failure admitted to an **ICU** • Positive DIF or Giemsa staining on BALF or transbronchial biopsy	Outcome and prognostic factors among PCP patients with acute respiratory failure admitted to an ICU.	44 patients • 14 HIV-positive • 30 HIV-negative	Overall in-hospital mortality: 63.3 % In-hospital mortality according to HIV-status: • HIV-positive: 57.1 % • HIV-negative: 66.7 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,2,3,4,5] among HIV-negative: • Prior corticosteroid therapy • Level of PEEP day 3 • Pneumothorax Longer duration before treatment Independent risk factors for *mortality*[4**] among HIV-negative: • Prior corticosteroid therapy • Level of PEEP day 3 • Pneumothorax

Enomoto et al. [133], 2009, Japan	Retrospective multicenter case review: 1997-2007 (~10 years)	• Adult **HIV** and **non-HIV patients** • New compatible finding on thoracic HRCT (e.g., bilateral GGO), morphologically confirmed PCP or positive PCR on sputum or BALF-specimens	Comparison of clinical characteristics and outcome of PCP between HIV and non-HIV patients.	35 patients • 18 HIV-positive • 17 HIV-negative	28-day-mortality: • HIV-positive: 0 % • HIV-negative: 35.3 % 90-day-mortality: • HIV-positive: 0 % • HIV-negative: 64.7 % PCP-related mortality: • HIV-positive: 0 % • HIV-negative: 52.9 %	Risk factors for *mortality*[4]: • Underlying pulmonary disease • HIV-negative status Other/focus of study: • Demographics and clinical presentation varied between HIV and non-HIV patients. • HIV-negative patients had significantly higher $CD4^+$ T-cell counts, and in six patients it was >300/μL.
Nakamura et al. [89], 2009, Japan	Retrospective monocenter case review: 1989-2006 (29 years)	• Adult **HIV** and **non-HIV patients** • Microscopic detection and/or positive PCR for *P. jirovecii* in BALF.	Evaluate the utility of β-D-glucan and KL-6 and non-HIV PCP.	35 patients • 19 HIV-positive • 16 HIV-negative	Overall in-hospital mortality: NA Mortality (non-specified): 25.7 %	Factors associated with *mortality* (survivors compared to non-survivors)[2,3]: • Lower PaO_2/FiO_2 • Lower serum-albumin • Mechanical ventilation • BALF-neutrophilia Other/focus of study: • Both β-D-glucan KL-6 levels were higher in HIV-positive patients, and the latter was related to duration of symptoms. • Neither β-D-glucan nor KL-6 were related to outcome.
Matsumura et al. [118], 2011, Japan	Retrospective multicenter case review: 2005-2010 (~6 years)	• Adult **non-HIV patients** • Positive PCR for *P. jiroveci*, new GGO on thoracic CT and clinical suspicion (presumptive treatment for PCP)	Clinical characteristics of PCP in non-HIV patients and their association with microbiological genotypes.	82 patients	30-day-mortality: 24 %	Risk factors for mortality (survivors compared to non-survivors)[1,2,4]: • Hypoxemia • Lower lymphocytes • Higher LDH • Pneumothorax • Invasive pulmonary aspergillosis • Lower serum-albumin Independent risk factors for *mortality*[4**]: • Lower serum-albumin • Mechanical ventilation

						Other/focus of study: • No association between genotype and clinical characteristics.
Moon et al. [149], 2011, Republic of Korea	Retrospective monocenter case review: 2007-2010 (4 years)	• Adult **non-HIV patients** with moderate to severe PCP • Morphologically confirmed PCP on BAL (direct immunofluorescence)	Demographics, clinical characteristics, and outcomes of PCP in non-HIV patients with and without CAT.	88 patients	Overall in-hospital mortality: NA Overall all-cause 30- and 90-day mortality: 31.8 % and 45.5 %, respectively 30- and 90-day all-cause mortality according to underlying disease: • SOT: 19.2 and 23.1 % • Hematological malignancy: 15.4 and 38.5 % • Interstitial lung disease: 55.6 and 77.8 % • Connective tissue disease: 57.1 and 57.1 % 30- and 90-day all-cause mortality according to treatment: • CAT (recent steroid use): 47.8 and 52.2 % • CAT (no recent steroid use): 19.4 and 44.4 % • No CAT (recent steroid use): 36.4 and 54.4 % • No CAT (no recent steroid use): 33.3 and 33.3 %	Factors associated with *survival*[1,3] (survivors compared to non-survivors): • Hematological malignancy (30-day mortality) • SOT (90-day mortality) Other/focus of study[7]: • No significant difference in 90-day survival between patients with/without CAT.

Wang et al. [105], 2011, Republic of China	Retrospective multicenter case review: 1959-2009 (10 years)	• Adult and pediatric **HIV** and **non-HIV patients** • Clinical with or without microbiological/morphological confirmation on sputum, BALF, biopsy, autopsy with PCR/microscopy	History of PCP in mainland China with focus on geographical and periodical distribution in relation to demographics, diagnostics, underlying disease, and prognosis.	2351 patients • 1646 HIV-positive (first patient reported in 1984) • 706 HIV-negative	Overall in-hospital mortality: NA Mortality according to anti-PCP specific treatment and HIV-status: • HIV-positive and no treatment: 100 % • HIV-positive and anti-PCP-treatment: 14.6 % • HIV-negative and no treatment: 86.2 % • HIV-negative and anti-PCP-treatment: 15.8 %	Focus of study: • Number of PCP-cases increased drastically with the advent of the HIV/AIDS-epidemic. • Underlying disease in PCP patients varied according to age group with infants and adults primarily affected by HIV/AIDS, and adolescents primarily affected by hematological malignancies. • Incidence of HIV-associated, non-HIV PCP cases in SOT and other non-HIV patients showed increasing trends during study period.
Asai et al. [120], 2012, Japan	Retrospective monocenter case review: 2001-2010 (10 years)	• Adult **non-HIV patients** • Community-acquired PCP, compatible symptoms, radiological findings, and microbiological detection (conventional staining and PCR on BALF or sputum)	Identify clinical factors contributing to survival of non-HIV patients with PCP and test whether the application of guidelines for CAP is suitable.	23 patients	Overall in-hospital mortality: 39.1 %	Factors associated with mortality[1,2,5] (survivors compared to non-survivors): • Interval from admission to diagnosis (days) • Interval from admission to treatment (days) Other/focus of study: • Guidelines for CAP underestimate mortality risk in non-HIV PCP patients and appear inadequate for PCP. • In non-survivors CAP severity increased significantly between admission and start of PCP-specific treatment.
Hardak et al. [121], 2012, Israel	Retrospective monocenter case review: 2005-2010 (6 years)	• Immunocompromised **non-HIV patients** (mean age 56±14 years) • Predisposing immunodeficiency, clinical and radiological signs, and positive PCR for *P. jiroveci* in BALF	Clinical manifestations, outcomes and factors associated with mortality due to PCP in non-HIV patients.	58 patients	Overall in-hospital mortality: 17.2 % According to management and complications: • Mechanical ventilation 59 % • Co-infections: 45 %	Factors associated with *mortality*[4]: • Co-infections • Higher LDH • Female gender • Higher pneumonia severity index at admission • Higher APACHE-III-scores

Li et al. [141], 2012, Taiwan	Retrospective monocenter case review: 2008-2011 (4 years)	• Adult **HIV** and **non-HIV patients** • Pulmonary symptoms, radiological manifestations (chest X-ray or thoracic CT), positive PCR, and anti-PCP-treatment during hospitalization	Clinical characteristics, management, and outcome of PCP in HIV and non-HIV patients and predictors of mortality in non-HIV patients.	43 patients • 23 HIV-positive • 20 HIV-negative	Overall in-hospital mortality: NA According to HIV-status: • HIV-negative: 9 % • HIV-positive: 60 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,4]: • HIV-negative status • Degree of lymphopenia • Shock during hospitalization Other/focus of study: • Treatment delay observed more frequently in HIV-negative patients. • Treatment delay was associated with mortality in HIV-negative patients.
Ainoda et al. [119], 2012, Japan	Retrospective monocenter case review: 2008-2011 (~4 years)	• Adult **non-HIV patients** • Presence of cellular immunodeficiency, compatible radiological manifestations, hypoxemia, positive PCR or DIF on BALF/sputum, and positive elevated β-D-glucan	Relationship between mechanical ventilation with intubation and treatment delay.	24 patients	Overall in-hospital mortality: NA 30-day mortality: 16.7 % 90-day mortality: 45.8 %	Factors associated with *survival* (survivors compared to non-survivors)[1,5,7:] • Underlying disease (renal SOT-recipients) Other/focus of study: • Treatment delay was significantly associated with invasive mechanical ventilation. • The difference between intubated and non-intubated patients' 90-day mortality was not significant.
Tamai et al. [124], 2013, Japan	Retrospective monocenter case review: 2006-2012 (~7 years)	• Adult **non-HIV patients** • Presence of clinical and radiological manifestations (on thoracic CT), positive PCR in BALF/morphological confirmation and elevated β-D-glucan	Prognostic factors for in-hospital mortality of PCP related to clinical factors, including BALF-parameters in non-HIV patients.	29 patients	Overall in-hospital mortality: 41 %	Predictive factors of *mortality*[4]: • Higher age • Renal transplantation • Lower PaO_2/FiO_2 • Mechanical ventilation • Lower serum-albumin • Higher LDH • Higher CRP • Higher BALF cellularity • Lower fraction of BALF lymphocytes Independent predictor of mortality[4]**:

						• Fraction of BALF neutrophils/BALF neutrophilia
Kim et al. [123], 2014, Republic of Korea	Retrospective multicenter case review: 2004 – 2011 (~7,5 years)	• Immunocompromised **non-HIV patients** (mean age 56±16 years) • Morphologically or molecularly detected *P. jiroveci* and clinical and radiological manifestations of PCP	Prognostic factors of PCP in non-HIV patients.	173 patients	Overall in-hospital mortality: 36 % • PCP-related: 32 % According to management: • ICU mortality rate 69.3 % • Mechanical ventilation 50.9 %	Factors associated with *mortality* (survivors compared to non-survivors)[3,5:] • Higher age • Diabetes mellitus • Chronic liver disease • Chronic lung disease • Dyspnea • Desaturation • Higher A-a-gradient • Higher CRP • Lower serum-albumin • Positive CMV antigenemia assay • Combined bacteremia • Lower fraction of BALF lymphocytes • Higher fraction of BALF neutrophils/ neutrophilia • Co-infection in BALF • Adjuvant corticosteroid • Mechanical ventilation Independent predictors of *mortality*[6]: • Higher Aa-gradient • Combined bacteremia • Increased BUN • Pre-existing chronic lung disease
Ko et al. [134], 2014, Republic of Korea	Retrospective monocenter case review: 2005 – 2011 (7 years)	• Adult **non-HIV patients** requiring mechanical ventilation in an **ICU** for PCP • Morphologically confirmed PCP and clinical and radiological signs of PCP	Outcome of non-HIV patients with PCP and acute respiratory failure requiring mechanical ventilation.	48 patients	Overall in-hospital mortality: 65 % ICU mortality: 52 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,2,3]: • Co-infection with CMV • Development of pneumothorax • Severity of illness on ICU-admission; SAPS-score • Failure of initial antimicrobial treatment for PCP • Newly developed shock during ICU-stay

						Factors independently associated with *mortality*[4**]: • Severity of illness on ICU-admission; SAPS-score • Failure of initial antimicrobial treatment for PCP • Newly developed shock during ICU-stay Factors associated with *reduced* 90-day *survival*[7]: • Failure of initial antimicrobial treatment for PCP
Guo et al. [122], 2016, Republic of China	Retrospective multicenter case review: 2008-2012 (5 years)	• Adult **HIV** and **non-HIV patients** • Host predisposition, clinical, laboratory and radiological evidence, microbiological confirmation, or response to anti-PCP treatment	Clinical characteristics of PCP in HIV *versus* non-HIV patients in mainland China and factors related to outcome.	151 patients • 105 HIV positive • 46 HIV-negative	Overall in-hospital mortality: NA All-cause mortality according to HIV-status: • HIV-positive 12.4 % • HIV-negative 15.2 Mechanical ventilation-associated mortality: • HIV-positive 60.0 % • HIV-negative 27.8 % According to primary underlying disease (non-HIV patients): • Transplant recipients 42.9 % • Chronic pulmonary disease 28.6 % • Connective tissue disease 14. 3 % • Solid tumor 14.3 %	Factors associated with *mortality*[6]: • Procalcitonin >0.5 ng/ml • Co-infection • Four or more symptoms: Presence of cough, dyspnea, fever, chest pain and/or weight loss • Admission to ICU Independent predictors of *mortality* of non-HIV patients[6**]: • Four or symptoms: Presence of cough, dyspnea, fever, chest pain and/or weight loss • Admission to ICU Other/focus of study: • Non-HIV patients were older, had longer duration of symptoms, but shorter interval to respiratory failure and required mechanical ventilation and ICU-admission more frequently. • All-cause mortality was similar across HIV-status.

Weng et al. [125], 2016, Republic of China	Retrospective multicenter case review: 2012-2015 (4 years)	• Adult immunocompromised **non-HIV patients** requiring admission to **ICU** due to respiratory insufficiency • Microbiologically confirmed PCP (by PCR or morphologically) in BALF, sputum, or aspirate.	Mortality predictors of PCP in non-HIV patients requiring ICU admission.	82 patients	Overall in-hospital mortality: 75.6 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,2,3,5]: • Higher age • Higher APACHE-II-score • Less fever • Hypotension • Pneumomediastinum Independent predictors of *mortality*[4**]: • Higher age • Lower leukocytes • Pneumomediastinum
Asai et al. [135], 2017, Japan	Retrospective monocenter case review: 2005-2012 (7.5 years)	• Adult **non-HIV patients** • Positive PCR, compatible radiological findings on HRCT and clinical symptoms.	Evaluate the clinical presentation and prognostic factors of non-HIV PCP.	38 patients	Overall 30-day mortality: 34 %	Factors associated with *mortality* (survivors compared to non-survivors)[4,5,7]: • ECOG PS 2-4 • Later anti-PCP therapy • Longer time to diagnosis • Higher KL-6 • Chronic heart disease • Cerebrovascular disease Independent predictors of *mortality*[4**]: • ECOG PS 2-4
Kotani et al. [146], 2017, Japan	Retrospective monocenter case review: 2008-2012 (~3 years)	• Adult **non-HIV** patients admitted to **ICU** with hypoxemic respiratory failure due to PCP.	Risk factors of mortality in PCP patients developing respiratory failure.	20 patients	Overall in-hospital mortality: 20.0 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,3,.5]: • Higher age • Lower serum-albumin at baseline • Higher KL6 • Higher SP-D Independent predictors of 90-day *mortality*[4**]: • Higher KL-6 • Interstitial lung disease

Inoue et al. [246], 2018, Japan	Retrospective nationwide database study using hospital discharge statistics: 2010-2016 (6 years)	• Adult **non-HIV patients** treated with TMS • Patients diagnosed with non-HIV PCP (criteria not specified)	Effectiveness of CAT in non-HIV patients with PCP with moderate (PaO_2 >60 mm Hg) and severe (PaO_2 <60 mm Hg) respiratory state, respectively.	1299 patients	Overall in-hospital mortality: NA 30- and 60-day all-cause mortality according to severity and therapy: • Moderate respiratory status and TMS only: 8.0 and 9.1 % • Moderate respiratory status and TMS with CAT: 9.4 and 10.9 % • Severe respiratory status and TMS only: 33.8 and 36.6 % • Severe respiratory status and TMS with CAT: 21.3 and 24.7 %	Factors independently associated with 30- and 60-day *survival* [6**]: • CAT in patients with severe respiratory status Other/focus of study: • CAT with TMS decreased 30- and 60-day all-cause mortality of patients with severe respiratory state. • CAT did not decrease 30- and 60-day all-cause mortality risk patients with moderate respiratory state.
Choi et al. [267], 2018, South Korea	Retrospective monocenter case review: 2013-2015 (3 years)	• Adult **non-HIV patients** admitted to an **ICU** for respiratory failure requiring ventilator or high-flow nasal oxygen treatment • Compatible symptoms and radiological findings, immunocompromised state, receival of anti-PCP treatment and positive PCR in induced sputum, BALF or tracheal aspirate.	Identify prognostic factors and examine PCP PCR negative conversion in non-HIV PCP patients with respiratory failure.	81 patients	Overall in-hospital mortality: NA Overall in-hospital survival: 35.8 %	Factors associated with *mortality* (survivors compared to non-survivors)[2,3,5:] • Higher APACHE-II score • Renal failure requiring renal replacement therapy • Absence of PCR negative conversion • Lower PaO_2/FiO_2 first 24 hours Factors independently associated with mortality [6**]: • Higher APACHE-II score • Absence of PCR negative conversion

Kumagai et al. [268], 2019, Japan	Retrospective case review: 2006-2015 (10 years)	• Adult immunocompromised **non-HIV patients** • Presence of PCP-associated immunodeficiency, clinical, laboratory (elevated β-D-glucan), radiological and microbiological (PCR + morphological confirmation) signs and evidence of PCP	Prognostic impact of crazy paving pattern on HRCT in non-HIV patients with PCP.	61 patients	Overall in-hospital mortality: 31.1 % According to findings on thoracic HRCT: • GGO without CPP: 6.2 % • GGO with CPP: 58.6 %	Prognostic factors associated with *mortality*[6]: • Lower PaO_2/FiO_2 • Lower serum-albumin • CPP • Consolidations • Bronchiectasis Independent prognostic factors associated with *mortality*[6**]: • Lower serum-albumin • CPP Prognostics for poor 100-day overall survival[7]: • GGO with CPP
Liu et al. [151], 2019, Taiwan	Retrospective monocenter case review: 2015-2016 (~1 year)	• Adult **HIV** and **non-HIV patients** • Clinical, radiological, and molecular evidence (positive qPCR, C_T-value <35) of PCP	Clinical characteristics, treatment, outcomes, and prognostic factors of PCP in non-HIV patients.	109 patients • 25 HIV-positive • 84 HIV-negative	Overall in-hospital mortality: NA Overall 60-day mortality: 39.4 % 60-day mortality according to HIV-status: • HIV-positive 16.0 % • HIV-negative 46.4 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,3:] • HIV-negative status • Respiratory failure Factors associated with *mortality* (survivors compared to non-survivors, HIV-negative patients)[1,3,4]: • C_T-value <24.8 • CAT • Pneumothorax Factors independently associated with increased 60-day mortality in HIV-negative patients[4**]: • Lymphopenia • CAT • Pneumothorax Prognostics for poor 60-day outcome in HIV-negative patients[7]: • C_T-value <24.8 • CAT

						Other/focus of study: • Non-HIV patients had longer duration between radiographic findings and treatment, higher rates of nosocomial PCP, hypoxia, respiratory failure, and mortality.
Ko et al. [237], 2019, Republic of Korea	Retrospective monocenter case review: 2005-2018 (~14 years)	• Adult **non-HIV patients** admitted to an **ICU** due to respiratory failure • Respiratory symptoms, radiological evidence of PCP, and positive microscopy on pulmonary specimen	Effects of early anti-PCP treatment on clinical outcomes in HIV-negative patients.	51 patients	Overall in-hospital mortality: 45.1 % ICU-mortality: 37.3 %	Factors associated with mortality (survivors compared to non-survivors)[2,3]: • Higher age • Mechanical ventilation day of ICU-admission • Higher SAPS-III scores • Co-infection with CMV • Failure to initial anti-PCP-treatment Independent predictors of *mortality*[4**]: • Increasing age • Failure to initial anti-PCP-treatment Focus of study: • Treatment delay not associated with mortality.
Kato et al. [219], 2019, Japan	Retrospective monocenter case review: 2008-2018 (~10.5 years)	• Adult **HIV** and **non-HIV patients** • Clinical suspicion, receival of preemptive anti-PCP treatment, and laboratory evidence (positive Grocott stain or PCR on BALF, and/or positive β-D-glucan-test)	Clinical characteristics of PCP in HIV vs. non-HIV patients.	96 patients • 31 HIV-positive • 44 HIV-negative	Overall in-hospital mortality: NA	Factors associated with mortality[7]: • HIV-negative status Focus of study: • Increasing incidence and PCR testing during study period. • HIV patients experienced more adverse effects. • Inferior survival and diagnostic accuracy in non-HIV patients who received more antibiotics possibly due to diagnostic difficulties.
Chen et al. [220], 2020,	Retrospective multicenter case review:	• Adult **HIV** and **non-HIV patients** mitted to an **ICU**	Determine key risk factors, informative	96 patients • 34 HIV-positive	Overall in-hospital mortality: 29.8 %	Focus of study: • Increase in total number of PCP-cases per year.

Republic of China	2015-2019 (4.5 years)	• Signs and symptoms of PCP, biochemical (elevated LDH), hypoxemia, radiological evidence, and positive microscopy for *P. jirovecii* in BALF, sputum, or lung biopsy	biochemical markers, and effective prophylaxis for PCP.	• 62 HIV-negative		• Increase in ratio of non-HIV vs. HIV patients during study period. • None of the patients had received prophylaxis. • 30 non-HIV patients had received immunosuppressant for less than 3 months prior to presentation.
Duan et al. [260], 2020, Republic of China	Retrospective monocenter case review: 2018-2022 (2 years)	• Adult **non-HIV patients** admitted to an ICU • Signs and symptoms of PCP, biochemical (elevated LDH and β-D-glucan), radiological evidence, and positive metagenomic next-generation sequencing test for *P. jirovecii* on BALF	Risk factors of the outcome of PCP in immunocompromised non-HIV patients diagnosed by metagenomic next-generation sequencing.	46 patients	Overall in-hospital mortality: 43.5 %	Factors associated with mortality (survivors compared to non-survivors)[2,3]: • Higher age • Higher LDH • Higher CRP • Lower serum-albumin Independent predictors of *mortality*[4**]: • Higher age • Higher LDH • Higher CRP • Lower platelet count
Kim et al. [238], 2021, Korea	Retrospective multicenter case review: 2013-2019 (~6.5 years)	• Adult **HIV, non-HIV,** and **non-immunocompromised patients** • Signs and symptoms of PCP, radiological evidence, positive PCR test for *P. jirovecii* on BALF or sputum, and receival of anti-PCP therapy	Compare clinical characteristics and prognoses between PCP patients with and without immunocompromised conditions.	121 patients • 26 HIV-positive • 147 non-HIV • 14 non-IC	Overall in-hospital mortality: NA In-hospital mortality according to predisposition: • HIV-positive: 15.4 % • HIV-negative: 49.0 % • Non-immunocompromised: 71.4 %	Factors associated with *mortality* (survivors compared to non-survivors)[4]: • Higher age • Non-immunocompromised status • Interstitial lung disease • Dyspnea • Higher A-a-gradient • Lower PaO_2/FiO_2 Independent predictors of *mortality*[4**]: • Higher age • Dyspnea • Higher alveolar-arterial oxygen pressure difference

						Factors associated with 90-day *mortality* (survivors compared to non-survivors)[6]: • Higher age • Non-immunocompromised status • Non-HIV • Interstitial lung disease • Dyspnea • Higher A-a-gradient • Higher LDH • Longer interval between admission and treatment • Higher PaO_2/FiO_2 Independent predictors of *mortality*[6**]: • PaO_2/FiO_2 • Higher age • Interstitial lung disease • Longer interval between admission and treatment Other/focus of study: • Non-immunocompromised patients were older, had higher mortality rates, and were more susceptible to treatment delay which in turn was associated with increased 90-day mortality.
Jin et al. [269], 2021, Republic of China	Retrospective monocenter case review: 2012-2018 (7 years)	• Adult **non-HIV patients** • Microbiological confirmation, either by microscopic examination or both a positive PCR test and increased level of serum 1,3-beta-D-glucan.	Relationships between the different types of lymphocytes and prognosis of patients.	88 patients	Overall in-hospital mortality: NA Overall 3-months mortality: 44 %	Factors associated with *mortality* (survivors compared to non-survivors)[2,3,4,6,7,8]: • Lower serum-albumin • Higher LDH levels • Higher demand for mechanical ventilation • Underlying lung disease • Lower NK-cell counts • Lower $CD8^+$ T-cell counts • Lower $CD4^+$ T-cell counts

						Factors independently associated with mortality [4**]: • Demand for mechanical ventilation • Lower $CD4^+$ T-cell counts
Hou et al. [261], 2022, Republic of China	Retrospective monocenter case review: 2018-2022 (4.5 years)	• Adult **non-HIV patients** • Signs and symptoms of PCP, biochemical (elevated LDH and beta-D-glucan), radiological evidence, and positive metagenomic next-generation sequencing test for *P. jirovecii* on BALF	Predictors of in-hospital mortality in PCP patients diagnosed by metagenomic next-generation sequencing.	154 patients	Overall in-hospital mortality: 36.4 %	Factors associated with mortality (survivors compared to non-survivors)[1,2,3,5]: • Higher APACHE-II • Higher Procalcitonin • Higher LDH • Higher neutrophil-lymphocyte ratio • Lower lymphocytes • $CD4^+$ T-cell counts • Lower PaO_2/FiO_2 Independent predictors of *mortality*[4**]: • Co-infection with other pathogens • Higher neutrophil-lymphocyte ratio
Zhang et al. [239], 2022, Republic of China	Retrospective monocenter case review: 2016-2020 (4.5 years)	• Adult **non-HIV patients** • Positive microscopy (methenamine silver stain) on BALF	Outcomes and associated risk factors of PCP patients after 2015.	30 patients	Overall in-hospital mortality: 30 %	Factors associated with mortality (survivors compared to non-survivors)[4]: • Higher LDH • Lower lymphocytes • Lower monocytes • Lower PaO_2
Jin et al. [262], 2022, Republic of China	Retrospective monocenter case review: 2012-2021 (10 years)	• Adult **non-HIV patients** • Microbiological confirmation, either by microscopic examination or a positive PCR test in combination with clinical manifestations, hypoxemia, increased serum beta-D-glucan, compatible radiological findings,	Develop and validate two clinical tools for predicting the risk of death and ICU-admission in non-HIV PCP patients.	508 patients • 423 in development set • 85 in validation set	Overall in-hospital mortality: 30 % 90-day mortality: • Development cohort: 39 % • Validation cohort: 26 %	Factors associated with mortality (survivors compared to non-survivors)[4]: • Higher age • Chronic lung disease • Shock • Invasive mechanical ventilation • Higher respiratory rate • Higher LDH • Higher CRP • Lower $CD8^+$ lymphocytes • Chronic kidney disease • Dyspnea • Lung moist rales

		and receival of anti-PCP therapy.				• Higher BUN • Lower PaO_2 • Second line therapy • CMV co-infection Independent predictors of *mortality*[4,6**] (included in death risk tool): • Higher age • Chronic lung disease • Shock • Invasive mechanical ventilation • Higher respiratory rate • Higher LDH • Higher BUN • CMV co-infection Focus of study: • External and internal validation demonstrated good discrimination of the tools. • The tool predicting ICU-admission including dyspnea, lung moist rales, respiratory rate, pleural effusion, BUN, CAR, and LDH was more informative and accurate than the CURB-65 score.
Arend et al. [106], 1993, Netherlands	Retrospective monocenter case review: 1980 – 1993 (14 years)	• Adult **non-HIV patients** • Definite PCP: Morphological confirmation • Probable PCP: Interstitial pulmonary disease, responding to anti-*pneumocystis* treatment in case of negative microscopic	Underlying disorder and previous immunosuppressive treatment in non-HIV patients with PCP.	78 patients	Overall mortality: 35 %	Factors associated with mortality (survivors compared to non-survivors)[3]: • Concomitant pulmonary infection • Mechanical ventilation • Predisposing condition other than renal transplant • Previous chemotherapy • Previous cyclophosphamide treatment Other/focus of study: • Increasing incidence of non-HIV cases.

		examination or unfeasible exam.				• Vast majority treated with corticosteroids, chemotherapy, or combinations in a variety of regimes prior to developing PCP.
Ewig et al. [106], 1995, Germany	Retrospective monocenter case review: 1985 – 1992 (8 years)	• Adult **HIV** and **non-HIV patients** • Morphological confirmation	Compare the first episode of PCP in HIV and immunosuppressed non-HIV patients.	74 patients • 58 HIV-positive • 16 HIV-negative	Overall mortality: NA According to HIV-status: • HIV-positive 17 % • HIV-negative 50 %	Factors associated with mortality (survivors compared to non-survivors)[1,2,3,4,5]: • HIV-negative status • Lower serum-albumin Other/focus of study: • Prognosis improved significantly for HIV PCP, but not non-HIV PCP. • Non-HIV patients were older, and outcome differed according to predisposition: patients with neoplastic disease and collagen vascular disease accounted for the high mortality. • Acute respiratory failure occurred more frequently in non-HIV patients.
Delclaux et al. [150], 1999, France	Retrospective multicenter case review: 1988 – 1996 (9 years)	• Adult **non-HIV patients** admitted to an **ICU** with severe PCP • Morphological confirmation and absence of co-pathogens in BALF, severe hypoxemia and receiving TMS as anti-PCP	Effect of CAT on survival in HIV negative patients with PCP admitted to an ICU.	31 patients	Overall in hospital mortality: NA According to therapy: • CAT: 39 % • No CAT: 50 %	Focus of study: • No significant difference between initial severity of illness, need for mechanical ventilation and mortality rate between patients who received or did not receive CAT.
Nüesch et al. [107], 1999, Switzerland	Retrospective monocenter case review: 1983-1998 (16 years)	• Immunocompromised **HIV** and **non-HIV patients** (mean age 39 and 48 years, respectively) • Microbiological detection (not specified) and clinical	Comparison of clinical characteristics and outcome of PCP over time in HIV and non-HIV patients.	121 patients • 89 HIV-positive • 32 HIV-negative	Overall in-hospital mortality: NA According to HIV-status: • HIV-positive 11 % • HIV-negative 19 %	Focus of study: • Non-HIV patients were significantly older, had shorter duration of symptoms, less thoracic pain, seating, weight loss, cachexia, and longer hospital stays. • The incidence of non-HIV PCP increased while the mortality from non-HIV decreased, respectively.

		or radiological signs of PCP				• The mortality from HIV PCP remained unchanged.
Roblot et al. [136], 2002, France	Retrospective multicenter case review: 1995-1999 (5 years)	• Adult immunocompromised **non-HIV patients** • Morphologically confirmed PCP and presence of clinical and/or radiological signs	Determine underlying disease associated with PCP in non-HIV patients and identify prognostic factors.	103 patients	Overall in-hospital mortality: NA Overall PCP-related mortality: 38 % According to immunocompromising condition: • Hematologic malignancy 33 % • Inflammatory disease 45 % • SOT 25 % • Solid tumors 53 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,2,4]: • Higher respiratory rate • Higher pulse rate • Higher CRP • Mechanical ventilation Other/focus of study: • Hematological malignancies predominated (58 %). • The vast majority had received corticosteroids in various regimes and/or chemotherapy.
Calderón et al. [143], 2004, Spain	Retrospective regionwide multicenter database study using hospital discharge data: 1998 – 1999 (2 years)	• Pediatric and adult **HIV** and **non-HIV patients** • ICD-codes for PCP	Impact of *P. jirovecii* in southern Spain following introduction of HAART.	498 patients • 434 HIV-positive • 64 HIV-negative	Overall in-hospital mortality: NA Overall PCP-related mortality:11.8 % • HIV-positive: 11.8 % • HIV-negative: 23.1 %	Factors associated with *mortality* (survivors compared to non-survivors)[1:] • HIV-negative status Other/focus of study: • Annual incidence in Andalusia: 3.4 per 100 000 population. • Higher age, costs, and longer stays in HIV-negative.
Mikaelsson et al. [126], 2005, Sweden	Retrospective monocenter case review: 1991 – 2001 (11 years)	• Pediatric and adult **HIV** and **non-HIV patients** • Proven (microbiological, radiological, and clinical evidence of PCP), probable (symptoms and	Incidence, symptoms, treatments, and risk factors for PCP in Gothenburg, Sweden over a decennium.	108 patients (118 episodes) • 84 proven PCP episodes • 24 HIV-positive	Overall hospital mortality: NA Overall mortality during follow up (to December 5, 2003): 63 % • HIV-positive 63 % • HIV-negative 41 %	Focus of study: • HIV-infection and SOT predominated, but non-HIV cases dominated (75 %). • Incidence remained stable during study period despite increase in cytotoxic regimens. • Five clusters of non-HIV PCP were identified, none among HIV patients. • Only 17 % were on prophylaxis.

		radiology suggestive of PCP in immunocompromised host without microbiological confirmation) or possible PCP (microbiological detection in immunosuppressed individual without clinical and radiological signs of PCP)		• 94 non-HIV	Death caused by PCP-associated complications: • 14/64 in proven non-HIV PCP-cases	
Overgaard et al. [108], 2007, Denmark	Retrospective monocenter case review: 2002-2004 (3 years)	• Pediatric and adult **non-HIV patients** • Microbiological detection of *P. jiroveci* (microscopy and/or PCR) and symptoms of PCP	Risk factors associated with PCP among HIV-negative patients.	50 patients • 11 pediatric patients • 39 adults	Overall hospital mortality: NA • PCP related mortality: 14 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,2]: • Admittance to ICU • Long admission to diagnosis interval • Long interval from admission to treatment • Lower mean lymphocyte count
Monnet et al. [137], 2008, France	Retrospective monocenter case review: 1993-2006 (14 years)	• Adult **HIV** and **non-HIV patients** admitted to an **ICU** • Microbiological detection of *P. jirovecii* (by microscopy; DIF and/or staining)	Compare critical care management and outcome according to HIV-status in PCP patients admitted to an ICU.	72 patients • 45 HIV-positive 46 (cases) • 27 HIV-negative	Overall hospital mortality: 29 % • HIV-positive: 17 % • HIV-negative: 48 % 28-day mortality: • HIV-positive: 26 % • HIV-negative: 52 % 90-day mortality: • HIV-positive: 30 % • HIV-negative: 59 %	Factors associated with *mortality*[4]: • HIV-negative status • Increasing age • Higher SAPS II-score at admission • Pneumothorax • NIV-failure Independent predictors of *mortality*[4**]: • HIV-negative status • Higher SAPS II-score at admission Other/focus of study: • Significant increase in HIV-negative PCP patients admitted to ICU. • Significant higher occurrence of NIV-failure in HIV-negative patients .

						• HIV-negative patients older, higher severity, occurrence of ARDS/ALI and need for renal replacement therapy.
Fily et al. [109], 2011, France	Retrospective monocenter case review: 2000-2007 (7 years)	• Adult **non-HIV patients** • Microbiological detection of *P. jiroveci* (microscopy and/or PCR) and symptoms of PCP	Describe PCP and colonization among non-HIV patients.	54 patients • 46 PCP patients • 8 colonized patients	Overall hospital mortality: 21 %	Focus of study: • The incidence increased during the study period. • Hematological malignancies predominated (54 %) • Long-term corticosteroid therapy was found in 65 % • The colonized patients had underlying immunodepression or chronic lung disease.
Magne et al. [110], 2011, France	Retrospective multicenter case review: 2000-2007 (8 years)	• Pediatric and adult **HIV** and **non-HIV patients** • Microscopic detection of *P. jirovecii* in BALF	Reevaluate the epidemiological characteristics of PCP in the Paris area and to analyze the relation between point mutation(s) in the DHPS gene and the breakthrough of prophylaxis.	805 patients • 541 HIV-positive • 264 HIV-negative	Overall hospital mortality: NA 14-day mortality: • HIV-positive: 13 % • HIV-negative: 26 %	Focus of study: • Total number of cases stable, but the proportion of HIV-negative cases increased from 25% to 41%.
Coyle et al., [111], 2012, Northern Ireland	Retrospective multicenter case review: 2008-2011 (3 years)	• Pediatric and adult **HIV** and **non-HIV patients** • Clinical and radiological signs of PCP in combination with microbiological confirmation PCR on any respiratory specimen	Observational review of PCP Northern Ireland.	51 patients • 13 HIV patients • 38 non-HIV patients	Overall hospital mortality: NA Overall mortality rate (not specified otherwise): 30 %	Focus of study: • Substantial increase in incidence, mainly due to non-HIV cases • Treatment resulted in rapid clearance (PCR conversion from repeated testing)

Lemiale et al. [147], 2013, France	Retrospective multicenter pooled analysis encompassing four study populations from 1988-2011	• Adult **non-HIV patients** admitted to an **ICU** • Morphologically confirmed PCP	Effect of steroids on survival in HIV negative patients with PCP admitted to an ICU.	139 patients	Pooled ICU-mortality: 26 % *Survival* according to CAT-category: • No steroids: 75 % • Low-dose: 80 % • High-dose: 71 %	Factors associated with *mortality*[4]: • Non-hematological disease • ICU-acquired infection • Higher SAPS-II • Lower PaO_2/FiO_2 at admission • Mechanical ventilation • Pneumothorax • Shock Independent predictors of *mortality*[4**]: • High-dose CAT • Higher SAPS-II • Non-hematological disease
Roembke et al. [144], 2013, Germany	Retrospective monocenter case review: 1999-2009 (11 years)	• Adult **HIV** and **non-HIV patients** • Clinical and radiological signs of PCP in combination with microbiological confirmation (PCR) on BALF.	PCP outcome in a tertiary referral center and evaluation of predictors of mortality on PCP patients with respect to potential risk factors.	51 patients • 21 HIV-positive • 30 HIV-negative	Overall in-hospital mortality: NA In-hospital mortality in patients requiring ventilatory support: 54 %	Negative predictors of *survival*[7]: • HIV-negative status • Age >50 year • Hb >10 g/dl • CRP >50 mg/L • LDH >500 U/L • >3 comorbidities Independent predictors of *mortality*[6**]: • HIV-negative status • ICU-necessity
Maini et al. [101], 2013, United Kingdom	Retrospective nationwide database study using hospital episode statistics, routine laboratory reporting, death certificates and HIV surveillance	• Adult **HIV** and **non-HIV patients** • ICD-codes for PCP and microbiological data not otherwise specified	Epidemiological trends of PCP in the United Kingdom based on national data sources.	Total: NA • 2258 HIV-negative (hospital admission data) • 779 HIV-positive (HIV surveillance data)	Overall in-hospital mortality: NA Death registrations: 779 cases	Focus of study: • Significant increase in incidence in all defined risk groups except for HIV-infection • PCP-attributed deaths increased significantly during study period. • Increasing cases in older patients towards study end.

	data: 2001-2011 (11 years)					
Bitar et al. [102], 2014, France	Retrospective nationwide database study using hospital discharge statistics: 2001-2010 (10 years)	• Pediatric and adult **HIV** and **non-HIV patients** • ICD-codes for PCP (mainly microscopy)	Epidemiology and trends of invasive fungal infections including PCP in France	9365 patients	Overall in-hospital mortality: NA Fatality rate overall 9.2 % Average fatality rate (er 100 000 person years) according to HIV-status: • HIV-positive 0.06 • HIV-negative 0.21	Epidemiological trends: • Overall incidence rate (1.5 per 100 1000 person years, 95 % CI 1.2-1.9) o Increasing in HIV-negative patients (+13.3 %), decreasing in HIV-positive patients (-14.3 %) o Decrease in total trend (-8.6 %) • Increasing fatality trend (+11.7 %), but only significant in HIV-associated Factors associated with mortality: • HIV-status: fatality rates 5.7 % vs. 21.5 % in HIV-positives vs. HIV- negative • Increasing age (significant for >20 years) • Hematological malignancy combined with neutropenia. • Solid tumors • Chronic renal failure • Acute renal failure • Intensive care
Kofteridis et al. [145], 2014, Greece	Retrospective monocenter case review: 2002 – 2014 (~10 years)	• Adult **non-HIV patients** • Morphologically confirmed PCP and clinical and radiological signs of PCP	Predisposing factors, clinical features, and outcome in HIV-negative patients.	62 patients	Overall death attributable to PCP: 29 % According to immunosuppressive condition: • Hematological malignancy: 26 % • Solid tumor: 50 % • Chronic inflammatory/autoimmune disease: 13 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,2,3,5]: • Solid tumor • Need for mechanical ventilation • Presence of SIRS-criteria on admission • Presence of pleural effusion • Respiratory failure Independent predictors of *mortality*[4**]: • Respiratory failure Focus of study: • Hematological malignancies predominated (50 %).

						• Chemotherapy alone predominated (42 %).
Roux et al. [127], 2014, France	Prospective multicenter cohort study: 2007-2010 (4 years)	• Adult **HIV** and **non-HIV patients** • Morphologically confirmed PCP	Comparison of PCP in HIV and non-HIV patients.	544 patients • 223 HIV-positive • 321 HIV-negative	In hospital-mortality according to HIV-status: • HIV-positive 4 % • HIV-negative 27 % According to immunosuppressive condition: • Kidney transplant patients: 4 % • Allogenic HSCT patients: 43 %	Factors associated with *mortality*[1]: • HIV-negative status Independent predictors of *mortality*[4**]: • Older age • Receipt of HSCT • Need for oxygen on admission • Need for invasive mechanical ventilation • Time to PCP treatment/additional day • HIV-negative status • Underlying disease other than SOT Prognostics for cumulative 90-day survival[7]: • Time to anti-PCP treatment • HIV-positive status Other/focus of study: • Treatment delay observed more frequently in HIV-negative patients. • Treatment delay was associated with mortality.
Bienvenu et al. [112], 2016, France	Retrospective monocenter case review: 2005-2013 (9 years)	• Pediatric and adult **HIV** and **non-HIV patients** • Clinical and radiological signs of PCP in combination with microbiological confirmation (microscopy and/or real-time PCR)	Describe clinical, diagnostic, and therapeutic characteristics of PCP patients with and without HIV-infections.	604 patients • 143 HIV-positive • 461 HIV-negative	Overall in-hospital mortality: NA 14-day mortality: 16 % 14-day mortality according to HIV-status: • HIV-positive: 1.4 % • HIV-negative: 2016 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,2,3,4,5]: • HIV-status Other/focus of study: • Non-HIV patients less symptomatic at presentation • Anti-PCP treatment initiated more frequently in HIV-positive patients • Increase in ratio of non-HIV vs. HIV patients during study period (from 1.7 to 5.6)

						• 39.5 % increase in total number of PCP-cases per year
Schoovaerts et al. [270], 2017, Belgium	Prospective monocenter case series: 2015-2016 (1 year) (1)) and retrospective monocenter analysis of positive BALFs: 2013-2016 (4 years) (2))	• Adult **non-HIV patients** • Positive PCR for *P. jiroveci* and clinical and radiological signs of PCP	Clinical risk factors for PCP in non-HIV patients	1) 7 patients 2) 59 patients	1) Overall in-hospital mortality: 0 %	Not addressed. Focus of study: • There was a predominance of positive PCR cases in malignancy patients compared to other departments (22 % vs. 7.3 %). • Breast cancer dominated among the seven patients. • Three patients had brain metastasis • Four patients were exposed to corticosteroids. • Five patients had lung diseases.
Ricciardi et al. [138], 2017, Italy	Retrospective monocenter case review: 2011-2015 (4 years)	• Adult **HIV** and **non-HIV** patients • Clinical and radiological signs of PCP in combination with microbiological confirmation (DIF and nested PCR)	Clinical and laboratory characteristics including genotype associated with worse outcome.	116 patients • 26 HIV-positive • 90 HIV-negative	Overall in-hospital mortality: 26 %	Factors associated with *mortality*: • Age >60 years • Chronic lungs disease • Admission to other ward other than Infectious Disease • Lack of prophylaxis Independent risk factors for *mortality*[4**]: • Admission to ward other than Infectious Disease Other/focus of study: • No association between genotype and outcome (50 patients with sufficient material for sequencing).
Schmidt et al. [244], 2018, Germany	Retrospective monocenter case review: 2000 – 2017 (17 years)	• **HIV** and **non-HIV patients** (mean age 45±15 years) • Morphologically confirmed PCP and typical clinical features	Clinical course, treatment and outcome of PCP and predictors of outcome.	240 patients • 125 HIV-positive • 115 non-HIV	Overall in-hospital mortality: 25.4 % According to primary underlying disease: • HIV 12.8 %	Factors associated with *mortality* (survivors compared to non-survivors)[1,4,6]: • HIV-negative status • Higher age • Higher BMI

					• SOT 38.5 % • Chemotherapy 44.7 % • Immunosuppression/rheumatic disease 35.7 % • (Miscellaneous 30 %) According to management: • Non-ICU 16 % • ICU 58.0 % • Ventilated 60.5 %	• Lower admission glomerular filtration rate • Higher initial LDH • Suboptimal TMS doses <15 mg/kg Independent risk factors for *mortality*[4**]: • LDH level
Gaborit et al. [240], 2019, France	Retrospective review of a prospective monocenter cohort study: 2012-2017 (4 years)	• Adult **HIV** and **non-HIV** patients • Typical clinical features and microbiological confirmation (morphological or positive real-time PCR)	Early risk factors for severe PCP and 90-day mortality, including BALF cytology profiles at diagnosis.	107 patients • 21 HIV-positive • 86 HIV-negative	Overall in-hospital mortality: NA 90-day mortality: 27.1 %	Factors associated with 90-day mortality (survivors compared to non-survivors)[1,2,4,8]: • Age >55 years • HIV-negative status • Absence of BAL fluid alveolitis • Viral co-infection • SAPS2 • SOFA-score • Severe ARDS • PaO_2/FiO_2 ratio >200 Independent risk factors for 90-day-*mortality*[4**]: • Worse SOFA-score • Absence of BALF alveolitis
Rego de Figueiredo et al. [243], 2019, Portugal	Retrospective monocenter case review: 2011-2016 (6 years)	• Adult **HIV** and **non-HIV patients** • ICD-9 code for PCP • Definite PCP: microbiological confirmation (RT-PCR) clinical, laboratory and radiological evidence supporting diagnosis	Comparison of PCP in HIV and non-HIV patients.	129 patients • 75 HIV-positive • 54 HIV-negative	Overall in-hospital mortality: 23 % According to HIV-status: • HIV-positive 13 % • HIV-negative 37 %	Independent risk factors for *mortality*[4**]: • HIV-negative status Focus of study: • Non-HIV patients were older, and there was a female preponderance. • HIV-negative patients had less respiratory complaints and lower LDH, but higher need for mechanical ventilation and mortality.

Pereira-Días et al. [216], 2019, Spain	Retrospective nationwide database study using hospital discharge statistics: 2008-2012 (5 years)	• Adult **HIV** and **non-HIV patients** • ICD-9 code for PCP	Epidemiological spectrum and risk factors for PCP	4550 patients • 3346 HIV-positive • 1204 HIV-negative	Overall in-hospital mortality: 25.5 % According to HIV-status: • HIV-positive 16.7 % • HIV-negative: 25.5 %	Risk factors for *mortality*[3]: • HIV-negative status Other/focus of study: • Increasing incidence in HIV-negative, decreasing in HIV-positive, total incidence stable during study period. • Higher age, clinical complexity, costs and longer stays in HIV-negative. • Increasing incidence in patients with chronic lung diseases.
Dunbar et al. [235], 2020, Belgium and Netherlands	Retrospective multicenter case review: 2012-2018 (6.5 years)	• Adult **HIV** and **non-HIV patients** • Positive real-time PCR for *P. jirovecii* on BALF with subsequent distinction between probable, possible, and colonized patients based on C_T-value, response to treatment, fatal outcome without receiving treatment.	Epidemiology of PCP in recent years and assess how many patients with PCP did or did not receive prophylaxis in the month preceding infection.	153 patients • 39 HIV-positive • 114 HIV-negative	Overall in-hospital mortality: 19 % Mortality according to indication for prophylaxis: • Not indicated/not received: 5/9 = 56 % • Indicated/not received: 19/133 = 14 % Mortality according to CAT: • No steroids: 16 % • Low-dose: 13 %	Focus of study: • 87 % diagnosed with PCP had not received prophylaxis despite indicated by local and international guidelines. • Majority of patients were HIV-negative, and a high proportion had a fatal outcome.
Bozzi et al. [263], 2022, Italy	Retrospective monocenter case review: 2019-2020 (2 years)	• Adult **non-HIV patients** • According to EORTC-criteria [195]; i.e., host factors, radioclinical criteria and microscopic demonstration (proven) and/or positive real-time PCR (probable)	Patient characteristics and evaluate overlooked risk factors and management issues.	20 patients • 11 proven • 9 probable	Overall in-hospital mortality: 20 %	Focus of study: • No patients had received prophylaxis despite main indication for prophylaxis according to guidelines in 9 patients and recognized host factor in the remaining patients. • $CD4^+$ T-cell counts >200 cells/mm^3 in all patients with available data.

Kolbrink et al. [222], 2022, Germany	Retrospective nationwide database study using hospital discharge statistics (1)) and retrospective multicenter case review (2)): 2014-2019 (6 years)	• Pediatric and adult **HIV** and **non-HIV patients** • ICD-codes for PCP	Recent epidemiology of PCP with focus on incidence trends in relation to underlying diseases, and course and outcomes of PCP.	1)12 455 patients • 2124 HIV-positive • 10331 HIV-negative 2) 68 patients • 19 HIV-positive • 49 HIV-negative	1) Overall in-hospital mortality: 27.4 % Overall in-hospital mortality according to HIV-status: • HIV-positive: 8.5 % • HIV-negative: 31.3 % 2) Overall in-hospital mortality: 22.1 % Overall in-hospital mortality according to HIV-status: • HIV-positive: 5.3 % • HIV-negative: 28.6 %	1) Focus of study: • Overall increase in PCP-incidence (+17.0 % overall) with opposing trends according to HIV-status: -4.3 % in HIV-positives and +21.8 % in HIV-positive • Overall increase in PCP-related deaths (+19.2 % overall) with opposing trends according to HIV-status: -34.2 % in HIV-positives and +23.4 % in HIV-negatives. • Incidence and deaths increased in patients with solid malignancy, autoimmune and pulmonary disease, whereas they decreased in patients with hematological malignancies and in SOT-recipients possibly due to international prophylaxis guidelines. 2) Focus of study: • Higher mortality in HIV-negative compared to HIV-positive patients. • Significantly later treatment initiation in HIV-negative patients and higher occurrence of detection failure.
Pates et al. [218], 2023, England	Retrospective nationwide database study using hospital episode statistics data: 12012-2022 (10 years)	• Pediatric and adult **HIV** and **non-HIV patients** • ICD-codes for PCP	Recent epidemiology of PCP in England.	NA	NA	Focus of study: • The incidence of PCP increased significantly from 2.2 to 4.5 per 100 000 population between 2012/2013 and 2019/2020. • The proportion of PCP patients aged 75 years and older increased from 14 % to 26 %.

Study, year, country	Design, patients, and period	Case inclusion	Focus and study objectives	Study population(s)	Main findings

Liu et al. [148], 2017, Republic of China	Meta-analysis of 13 studies including 867 **non-HIV patients** with PCP	• Microbiological identification and/or positive PCR from a sputum, BAL specimen or lung biopsy specimen	Clinical characteristics and factors associated with outcomes of PCP in non-HIV patients.	867 patients	Overall pooled mortality: 30.6 %
					
					Factors significantly associated with *higher mortality*[4]:
					• Demography, predisposition, and immunosuppression:
					  ○ Old age
					  ○ Female sex
					  ○ Solid tumor
					• Presentation, management, and complications:
					  ○ Symptomatology (dyspnea)
					  ○ Biochemistry:
					  ▪ Higher LDH
					  ▪ Lower serum-albumin
					  ○ Longer interval from symptom onset to treatment
					  ○ Respiratory failure
					  ○ ICU admission
					  ○ Co-infections (bacterial, CMV and aspergillus)
					  ○ Chemotherapy (hematological and oncological patients)
					  ○ Invasive ventilation (hematological and oncological patients)
					
					Factors significantly associated with *lower mortality*:
					• Hematological malignancy
					• Solid organ transplantation
					• Use of immunosuppressive agents
					
					Not significantly associated with increased mortality:
					• Autoimmune disease
					• Symptomatology (cough, fever)
					• Neutropenia
					• Lack of PCP-prophylaxis
					• CAT
					• Preceding corticosteroid therapy

Wang et al. [208], 2021, Republic of China	Meta-analysis of 19 studies including 1310 **non-HIV patients** with PCP	• NA	Risk factors of mortality from non-HIV related PCP and theoretical basis for disease management.	1310 patients	Overall pooled mortality: NA
					
					Factors significantly associated with *higher mortality*[4,11]:
					• Demography, predisposition, and immunosuppression:
					  ○ Old age
					  ○ Solid tumor
					  ○ Pulmonary comorbidity
					• Presentation, management, and complications:
					  ○ Biochemistry:
					  ▪ Higher LDH
					  ▪ Lymphopenia
					  ○ Co-infection with CMV
					  ○ Invasive ventilation
					
					Not significantly associated with increased mortality:
					• Sex
					• Lower serum-Albumin
					• Lack of PCP-prophylaxis
					• Corticosteroids exposure after admission
					• Time from onset of symptoms to treatment

[a]The papers are ordered according to continent (North America, Asia and Middle East, and Europe) and year of publication.
Statistical methods: [1]Fischer exact test; [2]Mann Whitney U-test, non-parametric Wilcoxon Rank sum test; [3]chi squared test; [4]linear or logistic regression (univariable and multivariable**); [5]Student T-test; [6]cox-proportional hazard regression; [7]log rank test for comparison of Kaplan Meier curves; [8]Kruskal Wallis test; [9]not specified/unclear,[10]Mantel-Haensszel test for trend, [11]propensity score matching, [12]mean difference, [13]one way analysis of variance.

7.2 Burden of PCP in selected countries

Author, year, country[a]	PCP definition	Material and methods	Estimate of PCP burden
Dufrense et al. [271], 2016, Canada	• NA	• Extrapolation from a single-center unpublished study	• Total burden per year: 252 cases • Estimated incidence: 0.71 per 100 000 people
Zhou et al. [272], 2020, Republic of China	• NA	• AIDS-register data (UNAIDS) • Cancer estimates (GLOBOCAN)	• Total burden per year: 27 723 cases o HIV: 13 o Cancer: 9241 • Estimated incidence: 1.93 per 100 000 people
Ben et al. [232], 2015, Israel	• NA	• Extrapolation of single center data (microbiology laboratory in Tel Aviv)	• Total burden per year: 305 cases o HIV: 97 o Cancer/SCT/SOT: 13 • Estimated incidence: 0.2 per 100 000 people
Rodriguez-Tudela et al. [223], 2014, Spain	• NA	• Epidemiological surveillance of AIDS • Extrapolation of data from peer-reviewed epidemiology papers	• Total burden per year: 305 cases o HIV: 97 o Cancer/SOT: 208 • Estimated incidence: 3.4 per 100 000 people
Ruhnke et al. [224], 2015, Germany	• NA	• AIDS-register data • ICD-codes from national statistics database	• Total burden per year: 1013 cases o HIV: 860 o Cancer/SOT: 153 • Estimated incidence: 1.3 per 100 000 people
Mortensen et al. [153], 2015, Denmark	• HIV-negative: positive microscopy and/or PCR, symptoms and response to anti-PCP treatment [108] • HIV-positive: NA.	• Extrapolation of data from the national hospital (Rigshospitalet) • AIDS-register data and previous publications on AIDS-defining illness in HIV-infected people	• Total burden per year: 82 cases o HIV: 15 o Respiratory condition: 67 • Estimated incidence: 1.5 per 100 000 people
Lagrou et al. [225], 2015, Belgium	• NA	• Extrapolation of data from unpublished epidemiology paper and national AIDS-registers	• Total burden per year: 120 cases o HIV: 15 o Respiratory disease: 105 • Estimated incidence: 1.1 per 100 000 people
Chrdle et al. [226], 2015, Czech Republic	• NA	• Extrapolation of data from Internal registry of the Department of tropical and infectious diseases in Prague	• Total burden per year: 72 o HIV: 12 o Cancer/transplantation: 60 • Estimated incidence: 0.7 per 100 000
Dorgan et al. [227], 2015, Ireland	• NA	• Extrapolation of national HIV/AIDS data and prior epidemiology paper	• Total burden per year: 50 o HIV: 13

			○ Cancer/chemotherapy: 37 • Estimated incidence: 0.8 per 100 000
Gamaletsou et al. [228], 2016, Greece	• NA	• Extrapolation of data from similar countries and national AIDS-registers	• Total burden per year: 112 ○ HIV: 28 ○ Cancer/chemotherapy: 84 • Estimated incidence: 1.0 per 100 000
Gangneux et al. [273], 2016, France	• Mainly microscopy	• Systematic literature search and extraction of data from published epidemiology papers	• Total burden per year: 658 cases ○ HIV: 449 ○ Respiratory condition: 4 ○ Cancer/SOT recipients: 144 ○ None/other: 61 • Estimated incidence: 1.0 per 100 000 people
Pegorie et al. [229], 2016, United Kingdom	• NA	• Extrapolation of data from published epidemiology papers and incidence estimates for known risk populations	• Total burden per year: 207-587 cases ○ SOT: 50 cases • Estimated incidence: 0.33-0.93 per 100 000 people
Sabino et al. [230], 2017, Portugal	• NA	• Previous study reporting HIV/AIDS data	• Total burden per year: 65 cases ○ HIV: 65 • Estimated incidence: 0.62 per 100 000 people
Nordøy et al. [155], 2018, Norway	• PCR, immunofluorescence	• Data collections from six medical microbiology laboratories in Norway	• Total burden per year: 262 cases ○ HIV: 4 ○ Respiratory disease: 258 • Estimated incidence: 5 per 100 000 people
Bassetti et al. [274], 2018, Italy	• NA	• AIDS-register data • Nationwide extrapolation from other countries for HIV-negatives	• Total burden per year: 82 cases ○ HIV: 300 ○ Non-HIV: 450 • Estimated incidence: 1.2 per 100 000 people
Özenci et al. [154], 2019, Sweden	• Positive PCR	• Nationwide extrapolation of data from the Karolinska University Hospital	• Total burden per year: 297 ○ HIV: 15 ○ Respiratory condition: 67 • Estimated incidence: 3 per 100 000 people
Buil et al. [231], 2020, Netherlands	• ECIL-guidelines for HIV-negative patients [80]	• AIDS-report data • Nationwide extrapolation of local data for HIV-negatives	• Total burden per year: 740 ○ HIV: 48 ○ Cancer/immunocompromised: 692 • Estimated incidence: 4.3 per 100 000 people

[a]The papers are ordered according to continent (North America, Asia and Middle East, and Europe) and year of publication.

7.3 Literature paper II

Author, year, country[a]	Reference method/gold standard and/or comparison	Population, design, and inclusion	Respiratory specimens	Gene target and assay	Sensitivity and specificity	Upper and lower cut-offs for PCP *versus* colonization
Larsen et al. [156], 2002, United States	• Conventional and immunofluorescence staining/microscopy • Conventional PCR	• 51 mixed patients • Retrospective • Monocenter	• BALF • Induced sputum • Oral wash	• MSG • In-house	• NA	• No
Arcenas et al. [275], United States, 2006	• Clinical/multimodal • Conventional staining/microscopy • In-house real-time PCR	• 214 mixed patients • Prospective • Monocenter	• BALF	• Cdc2 • In-house	• NA	• NA
Seah et al. [184], 2011, Canada and United States	• Conventional and immunofluorescence staining/microscopy • In-house real-time PCR	• 278 patients (HIV-status not specified) • Prospective? • Monocenter	• BALF • Bronchial washing • Induced sputum • Other	• Mt-LSU rRNA • Commercial	• 93.5 % • 95.1 %	• No
Hauser et al. [54], 2011, United States, Austria, and Switzerland	• Clinical/multimodal • Conventional and immunofluorescence staining/microscopy	• 110 mixed patients • Prospective • Multicenter	• BALF • Sputum • Other lower tract specimens	• Mt-LSU rRNA • Commercial	Compared to clinical diagnosis: • 93 % • 91 % Compared to microscopy: • 93 % • 90 %	• No
McTaggart et al. [176], 2011, Canada	• Conventional and immunofluorescence staining/microscopy • In-house real-time PCR • Conventional PCR with sequencing	• 105 mixed patients • Prospective • Monocenter	• BALF	• Mt-LSU rRNA • Commercial	• 100 % • 100 %	• NA

Church et al. [161], 2015, Canada	• Immunofluorescence staining/microscopy • In-house real-time PCR	• 127 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • In-house	• 65 % • 100 %	• Yes
Liu et al. [250], 2020, United States	• Clinical/multimodal • Immunofluorescence staining/microscopy • In-house real-time PCR	• 180 mixed patients • Retrospective • Monocenter	• BALF	• Mt-SSU rRNA • In-house	Compared to reference methods: • 96.9 % • 94.6 % Clinical discrimination: • 90.9 % • 99.3 %	• Yes
Kilic et al. [276], 2020, United States	• Immunofluorescence staining/microscopy	• 125 mixed patients • Inclusion: NA • Monocenter	• BALF	• Mt-LSU rRNA • Commercial	• 100 % • 87.2 %	• NA
Damhorst et al. [254], 2022, United States	• Pathology (not specified)	• 785 mixed patients • Retrospective • Multicenter	• BALF • Mini-BAL • Induced sputum • Sputum • Endotracheal aspirate • Tracheal aspirate • Bronchial wash • Bronchial brush	• Mt-LSU rRNA • Commercial	• 93 % • 94 %	• No
Fujisawa et al. [163], 2009, Japan	• Clinical/multimodal • Conventional staining/microscopy • Conventional PCR	• 86 non-HIV patients • Prospective • Monocenter	• Induced sputum	• ITS-2 • In-house	• 82.4 % • 98.6 %	• No
Matsumura et al. [172] 2012, Japan	• Clinical/multimodal • Conventional staining/microscopy	• 217 mixed patients • Prospective • Monocenter	• BALF • Induced sputum	• DHPS • In-house	Overall[5]: • 73.6 % • 73.3	• Yes

[5] Refers to discrimination between “definite” and “probable PCP” from “colonization”. See paper for more details.

Chien et al. [181], Taiwan, 2016	• Clinical/multimodal • Nested PCR	• 171 mixed patients • Retro- and prospective • Monocenter	• Induced sputum • BALF	• MSG • Commercial	• 92.6 % • 95.54 %	• No
Rudramurthy et al. [178], 2017, India	• Clinical/multimodal • Conventional staining/microscopy	• 104 mixed patients • Prospective • Multicenter	• BALF • Tracheal aspirate • Gastric aspirate • Induced sputum	• MSG • In-house	• 100 % • 97.8 %	• Yes
Sarasombath et al. [251], 2021, Thailand	• Clinical/multimodal • Conventional staining/microscopy	• 355 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • In-house	Non-HIV: • 100 % • 72.9 % HIV: • 100 % • 91.7 %	• Yes
Aguilar et al. [255], 2021, Colombia	• Clinical/multimodal and microscopy • Real-time PCR in validation	• 223 mixed patients • Prospective • Monocenter	• BALF • Oral washes	• Mt-LSU rRNA • In-house	BALF: • 100 % • 97.7 % Oral washes: • 76 % • 82 %	• No
Ruiz-Ruiz et al. [277], 2022, Chile	• Clinical/pathological • Immunofluorescence staining/microscopy • Nested PCR	• 73 patients (not specified) • Inclusion: NA • Monocenter	• BALF • Biopsies • Gargles • Nasal aspirates	• Multiplex: Mt-LSU rRNA and MSG • In-house	Compared to nested PCR: • 100 % • 100 %	• NA
Meliani et al. [278], 2003, France	• Conventional staining/microscopy	• 509 mixed patients • Inclusion: NA • Monocenter	• BALF	• Mt-LSU rRNA • In-house	• NA	• No
Brancart et al. [202], 2004, Belgium	• Conventional and immunofluorescence staining/microscopy	• 53 patients (not specified) • Prospective • Monocenter	• BALF	• β-tubulin • In-house	• NA	• NA
Flori et al. [175], 2004, France	• Conventional staining/microscopy • Conventional PCR	• 150 mixed patients • Prospective • Monocenter	• BALF	• MSG • In-house	• 100 % • 98.6 %	• Yes

Linssen et al. [168], 2006, Netherlands	• Conventional staining/microscopy	• 124 mixed patients • Retrospective • Multicenter	• BALF	• Mt-LSU rRNA (x 2) and DHPS • In-house	• NA	• Yes
Bandt et al. [166], 2007, Germany	• Clinical/multimodal • Conventional staining/microscopy	• 86 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA and DHFR2	Mt-LSU: • 100 % • 100 % DHFR2: • 100 % • 97 %	• Yes
Fillaux [174], 2008, France	• Immunofluorescence staining/microscopy	• 66 mixed patients • Retrospective • Multicenter	• BALF	• MSG • In-house	• 100 % and 90.5 %	• Yes
Rohner et al. [169], 2009, Switzerland	• Conventional staining/microscopy • In-house real-time PCR	• 186 mixed patients • Retrospective • Monocenter	• BALF	• Kex-1 • In-house	• 100 % • 92.4 %	• No
Chumpitazi et al. [177], 2011, France	• Clinical/multimodal • Conventional staining/microscopy	• 66 mixed patients • Prospective • Monocenter	• BALF	• MSG • In-house	• 100 % • 97.7 %	• Yes
Alanio et al. [173], 2011, France	• Clinical/multimodal • Conventional staining/microscopy	• 238 mixed patients • Prospective • Monocenter	• BALF • Induced sputum	• Mt-LSU rRNA • In-house	• 100 % • 85.7 %	• Yes
Botterel et al. [170], 2012, France	• Immunofluorescence staining/microscopy	• 287 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • In-house	• NA	• Yes
Mühlethaler et al. [160], 2012, Switzerland	• Clinical/multimodal • Immunofluorescence staining/microscopy	• 171 non-HIV patients • Retrospective • Multicenter	• BALF	• MSG • In-house	• 100 % • 87.5 %	• Yes
Orsi et al. [164], 2012, Italy	• Immunofluorescence staining/microscopy • Nested PCR	• 20 mixed patients • Retrospective • Monocenter	• BALF • Lung biopsy	• Mt-LSU rRNA • Commercial	• 88.9 % • 63.4 %	• No

	• Sequencing					
Damiani et al. [49], 2013, France	• Clinical/multimodal • Conventional and immunofluorescence staining/microscopy • Conventional PCR	• 46 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • In-house	• 100 %[6] • 100 %[6]	• Yes
Dalpke et al. [180], 2013, Germany	• Clinical/multimodal • In-house real-time PCR	• 242 mixed patients • Retro- and prospective • Monocenter	• BALF	• MSG • In-house	• NA	• No
Robert-Gangneux et al. [171], 2014, France	• Clinical/multimodal • Conventional and immunofluorescence staining/microscopy	• 137 mixed patients • Prospective • Monocenter	• BALF	• Mt-LSU rRNA • In-house	• NA[7]	• Yes
Maillet et al. [159], 2014, France	• Clinical/multimodal • Conventional staining/microscopy	• 35 mixed patients • Retrospective • Monocenter	• BALF • Broncho-aspirate	• MSG • In-house	• 80 % • 100 %	• Yes
Montesinos et al. [88], 2015, Belgium	• Clinical/multimodal • In-house real-time PCR	• 120 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • Commercial	• 72 % • 82 %	• No, but overlaps
Louis et al. [158], 2015, France	• Clinical/multimodal • Conventional and immunofluorescence staining/microscopy	• 1211 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • In-house	HIV: • 100% • 100 % Non-HIV: • 100 % • 88.1 %	• Yes (for non-HIV)

[6]Applying upper and lower cut-offs.
[7] Significant difference in fungal loads, but not cut-off proposed.

Orsi et al. [165], 2015, Italy	• Clinical/multimodal • Immunofluorescence staining/microscopy	• 41 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • Commercial	• 100 % • 94.4 %	• NA
Montesinos et al. [162], 2016, Belgium	• Clinical/multimodal • In-house real-time PCR and genotyping	• 120 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • DHPS (point mutations) • Commercial	• 70 % • 82 %	• No, but overlaps
Fauchier et al. [86], 2016, France	• Clinical/multimodal • Conventional staining/microscopy	• 225 mixed patients • Prospective • Monocenter	• BALF	• Mt-LSU rRNA • In-house	Overall • 72 % • 75 % HIV: • 74 % • 100 % Non-HIV: • 80 % • 60 %	• Yes
Unnewher et al. [157], 2016, Germany	• Clinical/multimodal • Immunofluorescence staining/microscopy	• 128 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • In-house	• 100 % • 80 %	• Yes
Guillaud-Saumur et al. [167], 2017, France	• Clinical/multimodal • Conventional and immunofluorescence staining/microscopy • In-house real-time PCR	• 34 mixed patients • Retrospective • Monocenter	• BALF • Biopsy • Induced sputum	• Mt-LSU rRNA • Commercial	• NA	• NA
Hoarau et al. [179], 2017, France	• Clinical/multimodal • Conventional staining/microscopy • In-house real-time PCR	• 133 mixed patients • Retrospective • Monocenter	• BALF • Induced sputum	• Mt-LSU rRNA • Commercial	• 100 % • 91.6 %	• Yes
Issa et al. [252], 2018, France	• Clinical/multimodal • Microscopy (staining not specified)	• 150 mixed patients • Retrospective • Monocenter	• BALF • Tracheal aspirate • Induced sputum	• MSG • Commercial	• 78 % • 86 %	• Yes

Perret et al. [253], 2020, Switzerland	• Clinical/multimodal • Conventional staining/microscopy	• 71 mixed patients • Retrospective • Monocenter	• BALF	• Mt-LSU rRNA • In-house	• 97 % • 82 %	• Yes

[a]The papers are ordered according to continent (North America, Asia and Middle East, and Europe) and year of publication.

7.4 Charlson comorbidity indices

Variable	Points[a]	Definition/comment
Age (years)		Only weighted in [197].
<50	0	
50-59	1	
60-69	2	
70-79	3	
>80	4	
Myocardial infarction	1	History of definite or probable myocardial infarction (changes on electro or echocardiogram and enzyme changes)
Congestive heart failure	1	Exertional or paroxysmal nocturnal dyspnea and has responded to digitalis, diuretics, or afterload reducing agents
Peripheral vascular disease	1	Intermittent claudication or past bypass for chronic arterial insufficiency, history of gangrene or acute arterial insufficiency, or untreated thoracic or abdominal aneurysm (≥6 cm)
Cerebrovascular accident or transient ischemic attack	1	
Dementia	1	Chronic cognitive deficit
Chronic obstructive pulmonary disease	1	
Connective tissue disease	1	
Peptic ulcer disease	1	Any history of treatment for ulcer disease or history of ulcer bleeding
Liver disease		
Mild	1	Severe = cirrhosis and portal hypertension with variceal bleeding history, moderate = cirrhosis and portal hypertension but no variceal bleeding history, mild = chronic hepatitis (or cirrhosis without portal hypertension)
Moderate to severe	2	
Diabetes mellitus		
Uncomplicated	1	
End-organ damage	2	
Hemiplegia	2	
Moderate to severe chronic kidney disease	2	Severe = on dialysis, status post kidney transplant, uremia, moderate = creatinine >3 mg/dL (0.27 mmol/L)
Solid tumor		
Localized	2	
Metastatic	6	
Leukemia	2	
Lymphoma	2	
AIDS	6	

[a] Refer to [196, 197] for the respective index validations.

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Paper I

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Epidemiological and clinical characteristics of immunocompromised patients infected with *Pneumocystis jirovecii* in a twelve-year retrospective study from Norway

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Abstract

Background: *Pneumocystis* pneumonia (PCP) severely menaces modern chemotherapy and immunosuppression. Detailed description of the epidemiology of *Pneumocystis jirovecii* today is needed to identify candidates for PCP-prophylaxis.

Methods: We performed a 12-year retrospective study of patients with *P. jirovecii* detected by polymerase chain reaction in Central Norway. In total, 297 patients were included. Comprehensive biological, clinical and epidemiological data were abstracted from patients' medical records. Regional incidence rates and testing trends were also assessed.

Results: From 2007 to 2017 we found a 3.3-fold increase in testing for *P. jirovecii* accompanied by a 1.8-fold increase in positive results. Simultaneously, regional incidence rates doubled from 5.0 cases per 100,000 person years to 10.8. A majority of the study population had predisposing conditions other than human immunodeficiency virus (HIV). Hematological (36.0%) and solid cancers (25.3%) dominated. Preceding corticosteroids were a common denominator for 72.1%. Most patients (74.4%) presented with at least two cardinal symptoms; cough, dyspnea or fever. Main clinical findings were hypoxia, cytopenias and radiological features consistent with PCP. A total of 88 (29.6%) patients required intensive care and 121 (40.7%) suffered at least one complication. In-hospital mortality was 21.5%. Three patients (1.0%) had received prophylaxis.

Conclusions: *P. jirovecii* is re-emerging; likely due to increasing immunosuppressants use. This opportunistic pathogen threatens the life of heterogenous non-HIV immunosuppressed populations currently at growth. Corticosteroids seem to be a major risk factor. A strategy to increase prophylaxis is called for.

Keywords: *Pneumocystis jirovecii*, PCP, Pneumonia, Immunosuppression, Immunocompromised

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Background

Iatrogenic immunosuppression represents a double-edged sword in the era of modern medicine. While improving the lives and life expectancy of individuals living with chronic autoimmune disorders, organ transplants or cancers, immunosuppressive drugs also increase the risk of opportunistic infections [1]. *Pneumocystis* pneumonia (PCP), primarily associated with the human immunodeficiency virus (HIV) epidemic in the 1980's, represents one of these [2]. PCP is often severe and mortality rates are high, especially in non-HIV immunocompromised patients [3].

Adequate identification of patients with PCP is challenging and relies on clinical suspicion. The manifestations of PCP are non-specific and include cough, dyspnea, fever and hypoxemia, in addition to constitutional symptoms like malaise and weight loss [4]. Therefore, PCP is often mistaken for pneumonia of another bacterial, viral or fungal etiology, malign processes, sarcoidosis and non-infectious interstitial pneumonitis [5], or in our experience pulmonary embolism. Polymerase chain reaction (PCR) for molecular detection of microorganisms and high-resolution CT-scans are of essential value in this context, particularly among immunocompromised hosts [5]. In the case of *Pneumocystis jirovecii*, distinction between colonization and PCP poses a particular challenge as the risk factors are overlapping [6]. Moreover, the high sensitivity of PCR for *P. jirovecii* detection compromises its specificity for PCP diagnosis [5]. In contrast, the sensitivity of microscopic visualization of cysts or trophic forms is limited, specifically among non-HIV individuals and on specimens from the upper respiratory tract due to low fungal inoculums and reduced chance of detection [3].

Knowledge and awareness about iatrogenic risk factors are required for considering PCP as a differential diagnosis and for prescribing prophylaxis to susceptible individuals. Immunosuppressants associated with PCP include corticosteroids, a wide spectrum of chemotherapeutic regimens, synthetic steroid-sparing drugs, and modern biological immunomodulators such as anti-tumor necrosis factor [2].

In Norway, PCP is not a notifiable disease unless it occurs in an HIV-infected individual as a manifestation of acquired immunodeficiency syndrome (AIDS) [7]. Therefore, the incidence in immunocompromised non-HIV patients, their host characteristics and the burden across HIV-status are unknown. Due to extensive use of immunosuppressants susceptible populations are currently at growth [2]. Herein we describe epidemiological and clinical characteristics among immunocompromised patients assessed for PCP in a 12-year retrospective study.

Methods

Setting

Our study was based on data from St. Olavs hospital, Trondheim University Hospital, which is the only tertiary referral hospital in Central Norway. The health region offers services to approximately 700,000 inhabitants representative of the national population [8, 9]. Until 2017, St. Olavs hospital had the only microbiology laboratory conducting *P. jirovecii* diagnostics in the region. All patients from central Norway with *P. jirovecii* detected in one or more respiratory samples by PCR in St. Olavs hospital between 2006 and 2017 were identified and linked to their respective medical records. Only primary episodes were included. All subjects 16 years or older at the time of testing were eligible. Alive patients were included on the basis of informed consent in 2018. There were no minors among these. The need for consent from next of kin or legal guardian of deceased patients was waived by the ethics committee.

Patient characteristics and data collection

We retrospectively reviewed the medical records of the study population and extracted comprehensive epidemiological, laboratory and clinical data. The software Epi Info™ (version 7.2.2.6; Centers for Disease Control and Prevention, Atlanta, GA, USA) was used to record patient data. The number and severity of combined comorbid conditions were assessed according to the Charlson weighted comorbidity index [10]. Corticosteroid exposure pattern 60 days preceding presentation was categorized as daily, intermittent or none. In case of ongoing corticosteroid intake on the date of *P. jirovecii* detection, the daily dose was converted into the equivalent in methylprednisolone expressed as milligrams per day and the median among users was calculated. Antimicrobial treatments administered after the detection of *P. jiroveci*, regardless of etiological indication, were also registered. Treatment and documented complications occurring in association with hospitalization were also recorded. Date of death was ascertained through linkage with the Norwegian Population Register through the end of June 2018 for sufficient follow-up.

Samples and definition of PCP

Diagnostic respiratory specimens included bronchoalveolar lavage fluid, lung biopsies, sputum samples, induced sputum, nasopharyngeal swabs and tracheal aspirates. In two patients, definitive detection of *P. jirovecii* was performed post-mortem upon autopsy. In cases where multiple respiratory samples were taken from a patient, those from the lower respiratory tract, primarily bronchoalveolar lavage fluid, were preferred due to their superior diagnostic yield in the setting of PCP.

The PCR analysis was performed as an in-house real-time PCR targeting the beta-tubulin gene of *P. jirovecii*, as previously described [11], with some modifications. PCR reagents and instruments used varied through the study period, but all changes were validated to ensure equal quality. The laboratory participated in a *Pneumocystis jirovecii* pneumonia (PCP) DNA EQA Programme (QCMD) from 2012. Semiquantitative estimation of fungal loads was performed on positive samples and results were reported with cycle threshold (C_T) values. C_T values are defined as the replicated number at which the fluorescence generated within a reaction crosses the fluorescens threshold line [12]. Accordingly, a low C_T value corresponds to a high fungal burden and vice versa. Microscopy (direct immunofluorescence (DIF) was performed with MONOFLUO *Pneumocystis jirovecii* IFA Test Kit #32515 (Bio-Rad). The assay was in use at the laboratory until 2017, mainly on samples resulting positive by PCR whenever positive controls were available. To discriminate cases of PCP (PCP^+) from colonization (PCP^-), we applied retrospective case-criteria in line with the European Conference on Infections in Leukaemia (ECIL) guidelines [13] with the available data. According to previous studies, we considered that C_T values above 35 corresponded to colonization and not overt PCP [12, 14] regardless of the host's HIV status. Thus, the criteria for PCP^+ among our PCR-positive cohort were i) positive DIF and/or ii) C_T value below 36. Patients with C_T values above 35 and negative or missing DIF result were considered colonized with *P. jirovecii* (i.e., PCP^-). Patients with missing C_T value and negative or missing DIF result were classified as "undetermined PCP-status".

Estimation of incidence rates

To estimate regional incidence rates, we accessed the online databank of Statistic Norway to retrieve the total number of people 16 years or older living in Central Norway during the study period [15]. These counts represented the denominators in our yearly incidence rate estimates. PCR detection of *P. jirovecii* was introduced at St. Olavs hospital, our referral laboratory, in late 2006. Thus, estimates were calculated for 2007 to 2017. In 2017, Molde hospital, a local hospital in the health region, established PCR-testing for *P. jirovecii* too. For completeness, individuals with a positive result at the laboratory in Molde hospital in 2017 were included in the regional incidence estimates for that year.

Statistics

Continuous quantitative variables are presented as medians with second (q_1) and third (q_3) quartiles or means with standard deviation ($\pm$ SD). Discrete variables are given as absolute numbers (percentages). Incidence rate estimates are given with 95% confidence intervals. All analyses were performed using Microsoft Excel (version 16.4; Microsoft Corporation, Redmond, WA, USA) or STATA/MP (version 15.1; College Station, TX, USA).

Results

Description of study population

A final 297 patients (117 F, 180 M) from Central Norway whose samples tested positive for *P. jirovecii* by PCR in the microbiology laboratory of St. Olavs hospital, were included in the study cohort (Fig. 1). The median patient age was 66 years and a majority (60.6%) of the patients were male. Each patient was classified with respect to their principal immunosuppressive condition associated with *P. jirovecii* and PCP development (Table 1). Hematological malignancies were the major predisposing conditions, present in more than one third of the patients (36.0%), with non-Hodgkin lymphomas appearing most frequently of these (51.4%). The second largest subpopulation was constituted by patients with solid tumors (25.3%). Therein, lungs including pleural membranes were the most common origin of the primary tumor (36.0%).

Behind malignancies, various non-HIV conditions appeared with decreasing frequencies, including immunological disorders (15.5%), solid organ transplantation (12.5%) and chronic lung disease (6.1%).

In our cohort, only a minority of seven patients (2.4%) presented with PCP in the context of HIV-infection. One patient from South-East Asia died pre-hospitally and resulted HIV positive in the referral hospital post-mortem. Four patients were unaware of their HIV-status and were naïve to anti-retroviral therapy, while the remaining two were not adherent to their anti-retroviral regimen.

Previous known comorbidities were present in 71.4% of patients, with hypertension being the most prevalent (Table 1). Moreover, the Charlson comorbidity index was skewed towards higher values, indicating an old study population and high degree of comorbidities overall.

Premorbid iatrogenic immunosuppression, chemotherapy and corticosteroid exposure

Nearly all of the non-HIV study subjects had received immunosuppressants or chemotherapy before assessment for PCP. Ongoing drug regimens prescribed in vicinity to presentation were registered and categorized (Table 2).

As expected, the regimens reflected the underlying conditions, namely the etiology for iatrogenic immunosuppression, with chemotherapy with adjuvant steroids

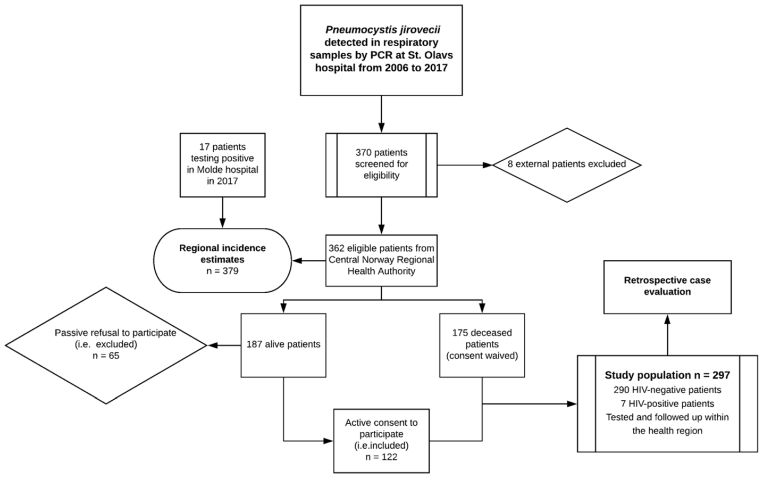

Fig. 1 Flowchart. Flowchart of the study population. Adult patients tested in the regional referral laboratory of St. Olavs hospital and followed up in hospitals comprised by the health region of Central Norway were included in the final study population. Recruitment of alive patients required active consent in accordance with the resolution of the regional ethical committee. Molde hospital, a local hospital in the health region, established PCR detection for *P. jirovecii* too in 2017. Individuals resulting positive there were included in the regional incidence estimates for 2017, but not in the study population. PCR, polymerase chain reaction

for hematological malignancy, being the most common category (22.6%).

Notably, 72.1% had been exposed to systemic corticosteroids in the 2 months preceding evaluation for PCP with a median dose in methylprednisolone of 8 (q_1-q_3 4–20) milligram per day among users the day of *P. jirovecii* detection ($n = 146$). Given the high prevalence of systemic corticosteroid usage, we went on to investigating the indications for prescription among the exposed ($n = 214$), though some patients had multiple indications. Here immunosuppression for immunological disorders and graft rejection prophylaxis had the highest occurrence (46.3%), followed by systemic corticosteroids as chemotherapeutic agents (35.0%), and other oncological indications combined (31.3%), indicating widespread application in treatment of cancer patients in general.

A subpopulation of 49 patients (16.5%), including the seven HIV-positive patients, were not being prescribed immunosuppressant or receiving chemotherapy at presentation. However, 29 of the non-HIV patients in this group (69.0%) had received iatrogenic immunosuppression, chemotherapy or both the last 5 years.

Only three patients (1.0%) were receiving primary PCP-prophylaxis at presentation.

Clinical, biological and radiological features

All but six patients presented with at least one cardinal symptom of pneumonia; cough, dyspnea, or fever prior to detection of *P. jirovecii* in a respiratory sample. The remaining patients reported reduced general condition, had abnormal findings on their physical or radiographic examinations. Clinical characteristics are summarized in Table 3.

A selection of objective manifestations and laboratory results are also presented though they were not retrievable for all patients. Decreased oxygen saturation (< 95%) was a common baseline finding, documented in 215 (72.4%) patients on presentation. Differential blood counts were incomplete overall, but therein lymphopenia ($< 1.0 \times 10^9$ cells/L) dominated (108 of 152 patients; 71.1%) whilst severe neutropenia ($< 0.5 \times 10^9$ cells/L) appeared infrequently (6 of 224 patients; 2.7%).

Plain chest radiography was performed in 254 cases, and abnormalities were noted in 219 (86.2%), with interstitial (nodular, linear or patchy) opacities being the most frequent features. Among 247 patients undergoing thoracic CT, 242 (98.0%) manifested abnormalities. Ground glass opacities were present in 73.7% of the cases, followed by thickening of interstitial septa (26.7%), both suggestive signs of PCP, but not pathognomonic.

Microbiological results and classification of PCP-status

A majority of the patients within our cohort underwent bronchoalveolar lavage for microbiological diagnostics ($n = 234$, 78.8%), followed by sputum ($n = 44$, 14.8%), induced sputum ($n = 9$, 3.0%), tracheal aspiration ($n = 5$, 1.7%), biopsy upon autopsy ($n = 2$, 0.7%), nasopharyngeal sampling ($n = 2$, 0.7%) and lastly transbronchial biopsy

Table 1 Characterization of the study population; 297 patients with positive PCR for *Pneumocystis jirovecii*

Male sex, n (%)	180 (60.6)
Ever smoking, n (%)	162 (54.5)
Age (years), median, (q_1-q_3)	66 (59–74)
Immunosuppressive conditions, n (%)	
Hematological malignancies	**107 (36.0)**
Non-Hodgkin's lymphoma	55 (18.5)
Chronic leukemia	17 (5.7)
Plasma cell disease	15 (5.1)
Acute leukemia	11 (3.7)
Hodgkin's lymphoma	9 (3.0)
Solid tumors	**75 (25.3)**
Lung including pleural membranes	27 (9.1)
Breast	14 (4.7)
Genitourinary tract	14 (4.7)
Gastrointestinal tract	9 (3.0)
Other primary tumor[a]	11 (3.7)
Immunological disorders	**46 (15.5)**
Rheumatoid arthritis	16 (5.4)
Connective tissue disorders and vasculitidies	15 (5.1)
Miscellaneous[b] disorders	15 (5.1)
Solid organ transplantations	**37 (12.5)**
Kidney	31 (10.4)
Heart, lung	6 (2.0)
Chronic lung diseases	**18 (6.1)**
Interstitial lung disease or sarcoidosis	11 (3.7)
Chronic obstructive pulmonary disease	7 (2.4)
HIV-infection	**7 (2.4)**
Other[c]	**7 (2.4)**
Comorbid conditions, n (%)	
Hypertension	92 (31.0)
Cardiovascular disease	83 (27.9)
Chronic pulmonary disease	52 (17.5)
Diabetes mellitus type 1 or 2	44 (14.8)
Solid tumor	30 (10.1)
Chronic kidney disease	38 (12.8)
Congestive heart failure	18 (6.1)
Rheumatic disease	12 (4.0)
Hematological malignancy[d]	13 (4.4)
Chronic liver disease	4 (1.4)
Charlson comorbidity index, n (%)	
< 4	47 (15.8)
4–6	132 (44.4)
> 6	118 (39.7)

Abbreviations: *AIHA* autoimmune hemolytic anemia, *ITP* immune thrombocytopenic purpura, *PCR* polymerase chain reaction

[a]Other primary tumors include brain tumors (i.e., astroglioma, meningioma), nasopharyngeal carcinoma, melanoma, adrenal gland tumor, sarcoma, and mesothelioma
[b]Miscellaneous immunological disorders include hematological disorders (ITP, AIHA), skin disorders, uveitis, inflammatory diseases of gastrointestinal tract and arthritidies other than rheumatoid arthritis
[c]Other immunosuppressive conditions include one patient with statin-induced myositis, one patient with common variable immunodeficiency and four patients with no established disorder at the time of presentation
[d]In 13 patients, hematological malignancy was not considered the primary immunosuppressive condition nor indication for immunosuppression but rather comorbidity

($n = 1$, 0.3%). DIF microscopy for *P. jirovecii* was performed on respiratory samples from 118 patients. The examinations resulted positive in 54 of these (45.8%). C_T values were retrievable for 243 patients irrespectively of patient characteristics, mainly from analysis of BAL-fluid samples ($n = 192$, 79.0%) Table S4 (Additional file 1). Based on the results of the microbiological analyses, 140 patients (47.1%), five of whom were HIV-positive, were diagnosed with PCP (PCP^+), whereas 116 patients (39.1%) were presumed colonized (PCP^-) (Figure S1 (Additional file 1)). Epidemiological and clinical characteristics and premorbid iatrogenic exposures of these are summarized in Table S1 and Table S2, respectively (Additional file 1). PCP^+-patients were generally comparable to the overall population in terms of demographics and predisposition. A tendency of more cardinal symptoms, hypoxia, low lymphocyte counts, elevated lactate dehydrogenase levels, and radiological remarks was noted. The yearly distribution of the three patient categories (PCP^+, PCP^- and "undetermined PCP-status") is depicted in Fig. 2. There was only one case of PCP in 2006, but the ensuing years saw an increase.

Complications, management and outcome

From examining the predisposition and clinical characteristics associated with *P. jirovecii*-detection, we went on to investigating the outcome (Table 3). Overall, anti-PCP treatment was instituted to 87.9% of the patients. Almost a third (29.6%) required treatment in an ICU and the same proportion received ventilation support (non-invasive and/or invasive). One hundred twenty-one patients (40.7%) experienced at least one complication, primarily respiratory failure or ARDS. Overall, in-hospital mortality was 21.5%, occurring in 64 patients. Cumulative all-cause 30-, 90- and 180-mortality rates for the study population were 20.2, 33.0 and 39.1% respectively. Accounting for surviving non-participants the rates were lowered resulting in an in-hospital mortality of 17.7% and 30-, 90- and 180-day mortality rates of 16.6, 26.8 and 32.0%, respectively. The clinical course of PCP^+ patients was broadly similar to the population overall. However, a greater proportion received anti-PCP treatment, intensive care, and ventilation support. Moreover,

Table 2 Premorbid immunosuppression, chemotherapy and corticosteroid exposure among 297 patients with positive PCR for *P. jirovecii*

Immunosuppression/chemotherapy regimens at presentation, n (%)	
Chemotherapy for hematological malignancy with adjuvant corticosteroids	67 (22.6)
Corticosteroids in monotherapy	44 (14.8)
Graft rejection prophylaxis after solid organ transplantation	36 (12.1)
Chemotherapy for solid malignancy with adjuvant corticosteroids	33 (11.1)
DMARDs with adjuvant corticosteroids	22 (7.4)
Chemotherapy for solid malignancy	16 (5.4)
Chemotherapy for hematological malignancy	12 (4.0)
Corticosteroids and other immunosuppressants[a]	8 (2.7)
DMARDs in monotherapy	5 (1.7)
Prophylaxis or treatment for GVHD after allogenic stem cell transplantation	3 (1.0)
Other combinations[b]	2 (0.7)
None	49 (16.5)
Systemic corticosteroid exposure last 60 days prior to presentation, n (%)	
Daily	125 (42.1)
Intermittent	91 (30.6)
No exposure to systemic corticosteroids	79 (26.6)
No information	2 (0.7)
Corticosteroid daily dosage in mg methylprednisolone at presentation, *n* = 292	
Median the day of *P. jirovecii* detection (q_1-q_3), *n* = 146	8 (4–20)
Minimum, maximum	0,120
Indications for corticosteroid administration among exposed[c], n (%)	
Immunosuppression for immunological disorders or graft rejection prophylaxis	99 (46.3)
Chemotherapy	75 (35.0)
Anti-emesis and other oncological indications[d]	51 (23.8)
Peritumoral oedema in primary and secondary intracranial tumors	16 (7.5)
Hematological and solid malignancies complicated by AIHA or ITP	9 (4.2)

Abbreviations: *AIHA* autoimmune hemolytic anemia, *DMARDs* disease-modifying anti-rheumatic drugs, *GVHD* graft-versus-host disease, *ITP* immune thrombocytopenic purpura

[a]Other immunosuppressants include mycophenolate, azathioprine, cyclophosphamide, calcineurin- and mTOR-inhibitors, cyclosporine and hydroxychloroquine

[b]Other combinations of immunosuppressive regimens include one patient receiving graft rejection prophylaxis for solid organ transplantation in combination with chemotherapy for hematological malignancy with adjuvant corticosteroids and one patient receiving azathioprine for vasculitis, respectively

[c]214 patients (72.1%) had known exposure to systemic corticosteroids last 60 days prior to presentation, and proportions are expressed with 214 as denominator. In some cases, corticosteroids were prescribed for more than one indication

[d]Other oncological indications include peritumoral oedema for patients with extracranial tumors, corticosteroids in combination with radiotherapy, vena cava superior syndrome, medulla compression etc.

complication and mortality rates were slightly higher (Table S3, Additional file 1).

Diagnostic and epidemiological trends

Our referral laboratory reported upward trends in molecular testing for *P. jirovecii* during the study period since the introduction of PCR in late 2006. A total of 1790 respiratory samples were referred for PCR analysis; 79 in 2007 compared to 259 in 2017. Accordingly, there was a 3.3-fold increase in analyses from 2007 to 2017. This was accompanied by a 1.8-fold increase in the incidence of samples with a positive PCR result; from 25 in 2007 to 46 in 2017 (Fig. 3). However, the proportion of positive samples remained more or less stable with a mean of 20.8% (SD 4.7) per year. All cases detected within Central Norway gave rise to regional incidence estimates. There were 5.0 cases per 100,000 person years in 2007 compared to 10.8 cases per 100,000 person years in 2017, with an increasing trend during this time interval (Fig. 4).

Discussion

In the first systematic evaluation of *P. jirovecii* in Norway we observed an apparent increasing incidence of PCP from 2007 to 2017. The vast majority were constituted by patients with other predispositions than HIV-

Table 3 Clinical characteristics, management and outcome among 297 patients with positive PCR for *Pneumocystis jirovecii*

Symptoms at baseline, n (%)	
Dyspnea	219 (73.7)
Fever	214 (72.1)
Cough	169 (56.9)
Two symptoms	221 (74.4)
Three symptoms	92 (31.0)
Objective baseline findings and biochemistry, median (q_1-q_3)	
Oxygen saturation, % ($n = 285$)[a]	89 (85–93)
Hemoglobin, g/dl ($n = 289$)	10.7 (9.7–11.7)
Leukocyte count, × 10^9/L ($n = 287$)	7.2 (4.3–10.1)
Neutrophil count, × 10^9/L ($n = 224$)	5.0 (3.0–7.7)
Lymphocyte count, × 10^9/L ($n = 152$)[b]	0.6 (0.4–1.1)
Albumin, g/L ($n = 207$)	32 (26–36)
Lactate dehydrogenase, U/L ($n = 165$)	293 (224–390)
Radiological findings, n (%)	
Remarks on chest X-ray ($n = 254$)	205 (80.7)
Nodular, linear and/or patchy opacities	219 (86.2)
Focal infiltrates	30 (11.8)
Consolidations	11 (4.33)
Remarks on thoracic CT ($n = 247$)	242 (98.0)
Ground glass opacities	188 (76.1)
Thickening of interstitial septa	69 (27.9)
Infiltrates	53 (21.5)
Consolidations	44 (17.8)
Lymphadenopathy	41 (16.6)
Bronchiectasis	18 (7.3)
Three-in-bud sign	16 (6.5)
Cysts	12 (4.9)
Management and complications, n (%)	
Receiving PCP-directed treatment	261 (87.9)
Antimicrobials for other pathogens[c]	176 (59.3)
Transferred to an ICU	88 (29.6)
Receiving ventilation support	88 (29.6)
Invasive and/or invasive and non-invasive	50 (16.8)
Non-invasive only	38 (12.8)
Developing complications	121 (40.7)
Respiratory failure/ARDS	83 (27.9)
Superinfection	50 (16.8)
Hemodynamic failure	37 (12.5)
Renal failure	33 (11.1)
Pneumothorax	7 (2.4)
Outcome, n (%)	
In-hospital mortality	64 (21.5)
Cumulative all-cause mortality	
30-days	60 (20.2)
90-days	97 (32.7)
180-days	116 (39.1)

Abbreviations: *ARDS* acute respiratory distress syndrome, *CT* computed tomography, *ICU* intensive care unit, *PCP Pneumocystis* pneumonia, *PCR* polymerase chain reaction

[a]68 patients received supplemental oxygen when oxygen saturation was measured

[b]Lymphopenia (< 1.0 lymphocytes × 10^9/L) was present among 108 patients (71.1%) with retrievable lymphocyte counts

[c]163 patients received antibiotics, 56 patients received antifungals, 25 patients received antivirals other than anti-retrovirals and four patients received anti-tuberculous drugs

infection, such as haematological and solid cancers, and immunosuppression in the form of corticosteroids in monotherapy or in combination with chemotherapy. Our research confirms the non-specific, thus challenging, clinical presentation of patients with suspected PCP and the association between *P. jirovecii* and high risk of in-hospital mortality.

Several studies have reported upward trends of PCP occurring in non-HIV patients [16–20], including one from Denmark [21]. In contrast, a study from Sweden did not register a rise, in spite of an increasing number of cytotoxic treatments, but that study ended in 2011 [22] which is before we saw a clear increase in our study. The authors proposed a more widespread administration of prophylaxis to patients at risk as the reason for this opposing trend [22]. In our study, only three patients were receiving primary prophylaxis at presentation. However, since only subjects who tested positive for *P. jirovecii* were included in our study population, a selection occurred. As a result, patients receiving prophylaxis without developing PCP, or without undergoing testing during the study period were not included. Nonetheless, our report reveals a gap between patients receiving adequate prophylaxis and those at risk. Concomitantly, a recent Cochrane review showed that trimethoprim/sulfamethoxazole was highly effective in preventing PCP in non-HIV immunocompromised patients with an 85% incidence reduction (95% CI 38 to 96%) and a number needed to treat of 19 patients for PCP prevention [23]. PCP-mortality was also reduced by 83% (95% CI 6 to 97%) without an increase in adverse events [23].

The apparent rise in the PCP-incidence in patients with secondary immunodeficiencies other than AIDS occurs with a concurrent escalation in the administration of immunosuppressants and chemotherapy regimens [1, 24]. Iatrogenic immunosuppression, as well as the impairing effects of the underlying disease itself, are probable explanations for patients developing PCP in

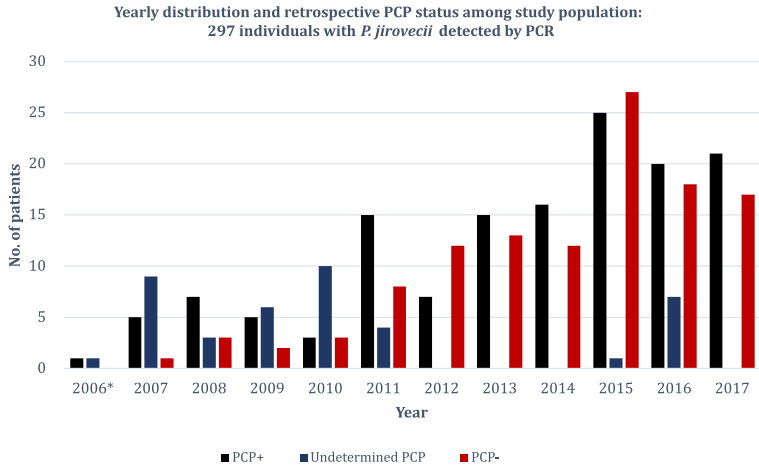

Fig. 2 *Pneumocystis* pneumonia (PCP)-status by study year. Yearly distribution of patients with **I)** PCP (PCP^+) based on i) positive direct immunofluorescence and/or ii) C_T value below 36 (black columns; $n = 140$, 47.1%), **II)** presumed colonization (PCP^-) not fulfilling the criteria for PCP (red columns; $n = 116$, 39.1) and **III**) "undetermined PCP-status"; patients without information about C_T value and negative or missing DIF result (dark blue columns; $n = 41$, 13.8). Criteria were applied in retrospect. *The study period was from 2006 to 2017, though PCR was introduced in late 2006. C_T, cycle threshold; DIF, direct immunofluorescence microscopy; PCP, *Pneumocystis* pneumonia; PCR, polymerase chain reaction

this context [25]. Moreover, we report a high prevalence of non-communicable comorbidities which may contribute to the "net state of immunosuppression" [26]. Altogether, this ageing population, cumulating endogenous and exogenous risk factors, may provide favorable conditions for *P. jirovecii* to re-emerge as an important opportunistic pathogen. Nevertheless, since the proportion of positive PCR results remained stable, it is uncertain whether the observations from our region reflects an actual increase in the number of people infected by the fungus, a changed clinical practice, or increased awareness of PCP, namely detection bias.

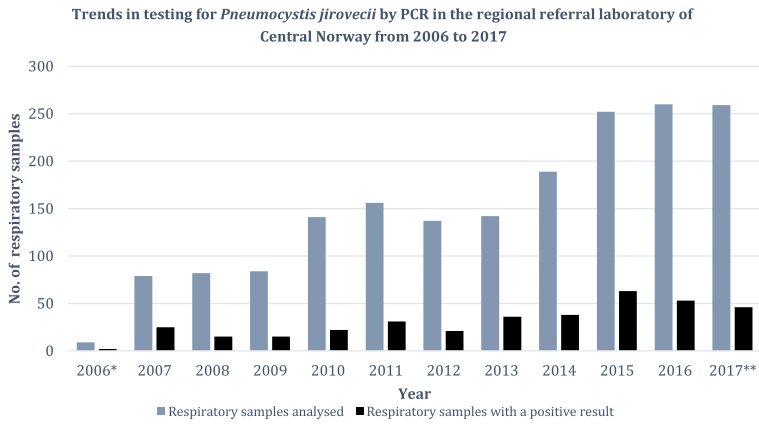

Fig. 3 Trends in testing for *Pneumocystis jirovecii* by PCR. Number of respiratory samples referred to the Department of Medical Microbiology of St. Olavs hospital for *P. jirovecii* detection by PCR during the study period (grey columns) and number of respiratory samples resulting positive (black columns). *PCR was introduced in late 2006, and there was a 3.3-fold increase in testing from 2007 to 2017 in our regional referral laboratory. The mean proportion of positive samples (not depicted) was 20.8% (SD 4.7). **In 2017 Molde hospital, a local hospital in the health region, established PCR detection for *P. jirovecii* too. That year an additional 70 respiratory samples were tested at their laboratory, and 20 (28.6%), representing 17 patients, resulted positive (not depicted). PCR, polymerase chain reaction

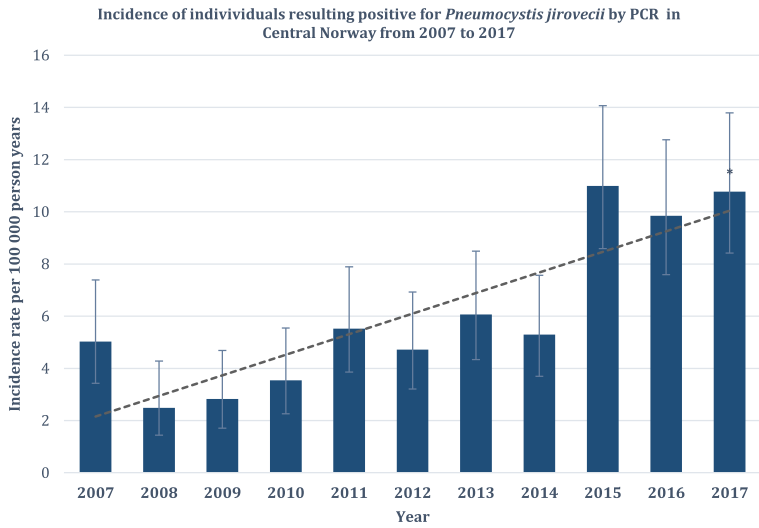

Fig. 4 Regional incidence of *Pneumocystis jirovecii* detected by PCR in Central Norway. Estimated incidence rates of individuals resulting positive for *P. jirovecii* by PCR in Central Norway health region (dark columns) with 95% confidence intervals and resulting linear trend (dotted line). PCR was introduced in late 2006 in our regional referral laboratory. Thus, estimates were calculated from 2007. Molde hospital, a local hospital in the health region, established PCR detection for *P. jirovecii* too in 2017. For completeness, individuals resulting positive there were included in the regional incidence estimates for 2017 (*). Regional population counts from Statistic Norway were used to compute the incidence rates. PCR, polymerase chain reaction

Reduction of $CD4^+$ T cells caused by iatrogenic immunosuppression is the most significant risk factor regarding developing PCP in non-HIV immunocompromised individuals [25]. Systemic corticosteroids are hazardous to lymphocyte proliferation and kinetics, especially in high doses [5]. For the grand majority of our study population, preceding exposure to systemic corticosteroids was a common denominator. We report a wide spectrum of indications, exposure patterns and doses at the time of presentation, as well as a diversity in the co-administered chemotherapy and immunosuppressants. Exposure to systemic corticosteroids preceding development of PCP in heterogeneous non-HIV populations has already been described in several studies [19–21, 27–38]. Even patients receiving systemic corticosteroids in tapering doses are at risk [21]. Moreover, patients with miscellaneous conditions not previously associated to PCP development per se, may develop PCP due to systemic corticosteroids exposure [39]. This was presumably the case for one of our PCP^+-patients receiving such treatment for statin-induced myositis.

In spite of the widespread use of corticosteroids and their lymphocytotoxic effects, lymphocyte counts were only documented in about half of the patient records (51.2%). Lymphopenia was present in the majority of these (72.1%), and even more prevalent among PCP^+-patients (93.2%). Neutrophil counts, on the other hand, were present in almost all the records. While neutropenic patients occasionally contract PCP, they do not appear to be unproportionally predisposed to PCP [3], though the risk may depend on the intensity and duration of neutropenic states [40]. Perhaps the missing data in our study, namely the incomplete lymphocyte counts, reflect a certain unawareness and unwariness regarding the impairing effects of immunosuppressants on other cell lines than neutrophils. Raised awareness regarding risk factors would probably lead to more patients receiving primary prophylaxis as well as prompter diagnosis in the case of infection. In fact, early treatment is crucial for the outcome since there appears to be a positive association between treatment delay and mortality [33, 41]. Non-HIV patients seem more susceptible to diagnostic delays in spite of more fulminant onset of symptoms [21, 33, 41].

Regarding outcomes, the in-hospital mortality observed in our study is in the lower range, also among the patients retrospectively classified as PCP^+. In comparison, it ranges from 15 to 49% for patients without HIV [17, 19, 27, 30, 32, 34–37, 42–45], and increases severely above 50% when ICU admission is required for respiratory failure [29, 46–48]. The differences in mortality may be due to heterogeneity in inclusion criteria in terms of underlying diseases, respiratory samples and diagnostic techniques. A recent meta-analysis reported a pooled overall in-hospital mortality of 30% for patients

without HIV [49]. The prognosis of patients with HIV-infection is reportedly better, with mortality ranging from 10 to 20% during the initial infection, but it increases considerably with the need for invasive respiratory support in this population too [3]. In our study, five out of seven patients diagnosed with PCP in the context of AIDS died, resulting in an in-hospital mortality of 71.4%. This sample is too small to draw any conclusions or comparisons but indicates that PCP in HIV-patients is still a serious and potential life-threatening diagnosis, even in an industrialized country like Norway. Indeed, all the HIV-positive individuals fell outside of UNAIDS' 90–90-90-treatment target for 2020 [50] in spite of high availability of anti-retroviral treatment.

With respect to the distribution of immunocompromising conditions, our cohort is broadly comparable to other reports [27, 30, 32]. In spite of the seemingly increasing incidence, PCP remains a relatively rare disease in non-HIV immunocompromised patients. This is confirmed by our regional incidence estimates for the study period. Importantly, they represent number of people with positive result for *P. jirovecii* by PCR. Hence, the incidence of clinical PCP was likely lower. Fillâtre et al. investigated incidence and risk furtherly; reporting incidence rates of PCP related to non-HIV predisposing conditions over two decades from France [39]. Their results demonstrate an apparent dissimilarity in the risk of contracting PCP within this heterogeneous population, presumably related to the underlying conditions and immunosuppressive treatment [39]. The prevalence of predisposing conditions influences how the risk translates into PCP occurrence. For instance, more patients with rheumatoid arthritis (RA) were assessed for PCP than patients with vasculitidies and connective tissue disorders combined in our cohort. This occurred in spite of RA patients' inferior risk of contracting PCP compared to the latter group [39]. In developed countries, it is estimated that between 0.5 and 1% of the population suffers from RA [51], whilst vasculitidies and connective tissue diseases are much rarer conditions [52]. This may explain our observations.

To diagnose PCP accurately remains a challenge, even with modern technologies. Herein, positive *P. jirovecii*-PCR was the primary inclusion criterion. To study the epidemiological trends of *P. jirovecii* in Norway, we believe it was important to describe this population as a whole since all the patients were tested on clinical indication and had a high pre-test probability of PCP. In addition, they represented potential candidates for prophylaxis, mostly unidentified at the time, an important aspect to shed light on per se.

DIF microscopy represents an alternative method for case inclusion and is the current gold standard for PCP diagnosis [3]. However, its sensitivity is known to be unacceptably low, especially in populations dominated by HIV-negative individuals [5]. This seemed to be the case in our population as well. Also, false positives may result due to morphologically interchangeable fluorescent material. Lastly, the validity relies on experienced examinators. In light of this, real-time PCR represents a rapid and objective detection tool, though extrapolation of results is confronted by heterogeneity in PCR-target, respiratory samples, quality of DNA-extraction, host-characteristics, quantification methods and so on [53].

Herein, C_T values from semiquantitative real-time PCR analysis and DIF results were collectively used to separate *probable* cases of PCP from those with presumed colonization. Of note, C_T values were not reported in the laboratory information system during the study period. Therefore, C_T values were collected retrospectively from the log of the PCR instruments. Unfortunately, some of the PCR-instruments had been replaced and discarded, and consequently C_T values for samples run on those instruments were lost. Since the retrievability of C_T values depended on which instrument the samples were analyzed, the missing pattern can be considered random and unrelated to patient characteristics. Analysis for beta-D-glucan was not available as a routine assay in our region; thus, such data were unavailable.

Retrospective PCP-classification was a secondary objective to see whether the general trends and characteristics in the overall population were representative. It was performed without considering heterogeneity in respiratory samples, which is a well-known issue [53]. Accordingly, a drawback of this approach is variability in microorganism gradients and volumes across respiratory samples, in addition to intra- and interindividual variability in host-pathogen biology. Collectively, these factors might have resulted in information bias. Yet, regardless of the exact number, the minority of patients with presumed colonization has important implications. Besides the possible role of colonization in chronic diseases, proposed interhuman transmission from individuals harboring *P. jirovecii* organisms is a concern [6].

To our knowledge, this is the largest study undertaken in a Nordic country regarding testing, epidemiology and clinical characteristics of patients assessed for PCP. However, the study design and methodology have several limitations and may provide grounds for biases. Firstly, the study population was sampled from only one region, thus, findings may not be generalizable to other areas. Secondly, the results of this report are based on retrospective case reviews of medical records, a method associated with certain limitations. Foremost, causal claims cannot be made, for instance regarding corticosteroid exposure and

risk of contracting PCP. Also, the case review is a qualitative method. Hind-sight bias is likely to affect all retrospective case record reviews in particular [54]. Further, this design does not allow us to examine unavailable patient characteristics, and we rely on the information provided by the health personnel who treated the patients. Thirdly, we were unable to include all alive patients, which might have resulted in selection bias. In spite of the stigma associated with HIV/AIDS, we have little reason to believe that the request for active consent influenced the recruitment of HIV-positive individuals. In fact, the number of HIV-related PCP cases in our cohort were comparable to the estimated incidence in the region according to the national HIV/AIDS surveillance and health reports [7, 55]. Finally, our approach to identify eligible candidates, using positive PCR might have introduced bias as discussed above.

Conclusions

In conclusion, PCP should always be suspected in susceptible patients manifesting consistent signs and symptoms. Systemic corticosteroid exposure and lymphopenia are dominating risk factors for PCP in non-HIV patients. These appeared to be frequent in our population. Awareness regarding predisposition and the spectrum of onsets, ranging from insidious to fulminant depending on the host's HIV status, is required to assure a high index of suspicion. Multimodal diagnostics across disciplines are often necessary for precise PCP diagnosing, though biological detection remains fundamental. Here we reveal that PCP is a rare disease in Norway, however the burden of *P. jirovecii* seems to be increasing, especially in non-HIV populations. In light of this, a strategy to increase administration of primary prophylaxis to individuals at risk seems called for.

Abbreviations

AIDS: Acquired immunodeficiency syndrome; AIHA: Autoimmune hemolytic anemia; ARDS: Acute respiratory distress syndrome; CI: Confidence interval; C_T : Cycle threshold; CT: Computed tomography; DIF: Direct immunofluorescence microscopy; DMARDs: Disease modifying anti-rheumatic drugs; ECIL: European conference on infections in leukaemia; GVHD: Graft versus host disease; HIV: Human immunodeficiency virus; ICU: Intensive care unit; ITP: Immune thrombocytopenic purpura; PCP: *Pneumocystis* pneumonia; PCR: Polymerase chain reaction; q_n: n^{th} quartile; RA: Rheumatoid arthritis; REC: Committee for Medical and Health Research Ethics; SD: Standard deviation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-021-06144-1.

Additional file 1.

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Authors' contributions

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

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Availability of data and materials

The dataset generated and analyzed during this study are not publicly available due to privacy concerns regarding individual study participants but are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Regional Committee for Medical and Health Research Ethics North (REC North); reference number 2017/2419. Patients still alive when the study was undertaken were included on the basis of active informed consent. No minors were eligible for inclusion and all consents were personal. All deceased patients were included since the need for consent from next of kin or legal guardian was waived by REC North. The Data Access Committee of Nord-Trøndelag Hospital Trust and the Data Protection Officer of Helse Møre og Romsdal Trust approved the project. All data were managed in accordance with the General Data Protection Regulation (GDPR), adapted by the European Union (EU) in 2016.
All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

All authors declare no competing interests.

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Publisher's Note

Supplementary material to: "Epidemiological and clinical characteristics of immunocompromised patients with positive *Pneumocystis jirovecii*-PCR in a 12-year retrospective study"

CONTENTS

Supplementary Tables:

Table S1 Characterization 140 of patients retrospectively diagnosed with *Pneumocystis* pneumonia (PCP$^+$)[a]	
Male sex, n (%)	89 (63.6)
Ever smoking, n (%) (n = 136)	76 (55.9)
Age (years), median, (q_1-q_3)	65 (58-73)
Immunosuppressive conditions, n (%)	
Hematological malignancies	**48 (34.3)**
Non-Hodgkin's lymphoma	24 (17.1)
Chronic leukemia	8 (5.7)
Acute leukemia	6 (4.3)
Plasma cell disease	7 (5.0)
Hodgkin's lymphoma	3 (2.1)
Solid tumors	**41 (29.3)**
Lung including pleural membranes	14 (10.0)
Breast	7 (5.0)
Genitourinary tract	6 (4.3)
Other primary tumor[b]	7 (5.0)
Gastrointestinal tract	7 (5.0)
Immunological disorders	**17 (12.1)**
Connective tissue disorders and vasculitidies	6 (4.3)
Rheumatoid arthritis	4 (2.9)
Miscellaneous[c] disorders	7 (5.0)
Solid organ transplantations	**22 (15.7)**
Kidney	18 (12.9)
Heart, lung	4 (2.9)
Chronic lung diseases	**5 (3.6)**
Interstitial lung disease or sarcoidosis	5 (3.6)
HIV-infection	**5 (3.6)**
Other[d]	**2 (1.4)**
Comorbid conditions, n (%)	
Hypertension	43 (30.7)
Cardiovascular disease	36 (25.7)
Chronic pulmonary disease	23 (16.4)
Diabetes mellitus type 1 or 2	20 (14.3)
Chronic kidney disease	19 (13.6)
Solid tumor	14 (10.0)
Congestive heart failure	8 (5.7)

Hematological malignancy	6 (4.3)
Rheumatic disease	4 (2.9)
Chronic liver disease	2 (1.4)
Charlson comorbidity index, n (%)	
< 4	21 (15.0)
4-6	59 (42.1)
> 6	60 (42.9)

[a]PCP$^+$ criteria: i) Positive direct immunofluorescence microscopy and/or ii) Cycle threshold value below 36 on semiquantitative PCR analysis. Patients not fulfilling the criteria were considered colonized (PCP$^-$). Patients missing microbiological data were classified with "undetermined PCP-status".
[b]Other primary tumors include brain tumors (i.e. astroglioma and meningioma), nasopharyngeal carcinoma, adrenal gland tumor, sarcoma, and mesothelioma.
[c]Miscellaneous immunological disorders include hematological disorders (ITP, AIHA), skin disorders, inflammatory diseases of gastrointestinal tract and arthritidies other than rheumatoid arthritis.
[d]Other/miscellaneous immunosuppressive conditions included one patient with no diagnosed condition who had received steroids for suspected autoimmune disorder and one patient with statin-induced myositis treated with corticosteroids.
Abbreviations: AIHA; autoimmune hemolytic anemia, ITP; immune thrombocytopenic purpura, PCR; polymerase chain reaction, PCP; *Pneumocystis* pneumonia

Table S2 Premorbid immunosuppression, chemotherapy and corticosteroid exposure among 140 patients retrospectively diagnosed with *Pneumocystis* pneumonia (PCP⁺)[a]	
Immunosuppression/chemotherapy regimens at presentation, n (%)	
Chemotherapy for hematological malignancy with adjuvant corticosteroids	31 (22.1)
Corticosteroids in monotherapy	24 (17.1)
Graft rejection prophylaxis after solid organ transplantation	22 (15.7)
Chemotherapy for solid malignancy with adjuvant corticosteroids	16 (11.4)
DMARDs with adjuvant corticosteroids	9 (6.4)
Chemotherapy for solid malignancy	6 (4.3)
Chemotherapy for hematological malignancy	6 (4.3)
Corticosteroids and other immunosuppressants[b]	4 (2.9)
DMARDs in monotherapy	2 (1.4)
Prophylaxis or treatment for GVHD after allogenic stem cell transplantation	1 (0.7)
None	19 (13.6)
Systemic corticosteroid exposure last 60 days prior to presentation, n (%)	
Daily	67 (47.9)
Intermittent	40 (28.6)
No exposure to systemic corticosteroids	31 (22.1)
No information	2 (1.4)
Corticosteroid daily dosage in mg methylprednisolone at presentation, n = 138	
Median the day of *P. jirovecii* detection (q_1-q_3), n = 82	10 (6-20)
Minimum, maximum	0,120
Indications for corticosteroid administration among exposed[c], n (%)	
Immunosuppression for immunological disorders or graft rejection prophylaxis	36 (33.6)
Chemotherapy	32 (29.9)
Anti-emesis and other oncological indications[d]	26 (24.3)
Peritumoral oedema in primary and secondary intracranial tumors	10 (9.3)
Hematological and solid malignancies complicated by AIHA or ITP	5 (4.7)

[a]PCP⁺ criteria: i) Positive direct immunofluorescence microscopy and/or ii) Cycle threshold value below 36 on semiquantitative PCR analysis. Patients not fulfilling the criteria were considered colonized (PCP⁻). Patients missing microbiological data were classified with "undetermined PCP-status".
[b]Other immunosuppressants include mycophenolate, azathioprine, cyclophosphamide, calcineurin- and mTOR-inhibitors, cyclosporine and hydroxychloroquine.
[c]107 patients (76.4 %) had known exposure to systemic corticosteroids last 60 days prior to presentation, and proportions are expressed with 107 as denominator. In some cases, corticosteroids were prescribed for more than one indication.
[d]Other oncological indications include peritumoral oedema for patients with extracranial tumors, corticosteroids in combination with radiotherapy, vena cava superior syndrome, medulla compression etc.
Abbreviations: AIHA, autoimmune hemolytic anemia; DMARDs, disease-modifying anti-rheumatic drugs; GVHD, graft-versus-host disease; ITP, immune thrombocytopenic purpura.

Table S3 Clinical characteristics, management and outcome among 140 patients retrospectively diagnosed with *Pneumocystis* pneumonia (PCP^+)[a]	
Symptoms at baseline, n (%)	
Dyspnea	104 (74.3)
Fever	107 (76.4)
Cough	85 (60.7)
Two symptoms	106 (75.7)
Three symptoms	52 (37.1)
Objective baseline findings and biochemistry, median (q_1-q_3)	
Oxygen saturation, %, (n = 125)[b]	87 (83.4-92.0)
Hemoglobin, g/dl, (n = 135)	10.7 (9.6-11.6)
Leukocyte count, x 10^9/L (n = 137)	7.6 (4.3-10.0)
Neutrophil count, x 10^9/L (n = 110)	5.02 (3.0-7.7)
Lymphocyte count, x 10^9/L (n = 73)[c]	0.6 (0.3-1.0)
Albumin, g/L (n = 106)	32 (27-36)
Lactate dehydrogenase, U/L (n = 86)	317 (243-439)
Radiological findings, n (%)	
Remarks on chest X-ray (n = 132)	116 (87.9)
Nodular, linear and/or patchy opacities	40 (30.3)
Focal infiltrates	12 (9.1)
Consolidations	7 (5.3)
Remarks on thoracic CT (n = 118)	116 (98.3)
Ground glass opacities	97 (82.2)
Thickening of interstitial septa	36 (30.5)
Infiltrates	27 (22.9)
Consolidations	22 (18.6)
Lymphadenopathy	18 (15.3)
Bronchiectasis	8 (6.8)
Three-in-bud sign	6 (5.1)
Cysts	6 (5.1)
Management and complications, n (%)	
Receiving PCP-directed treatment	135 (96.4)
Antimicrobials for other pathogens[d]	92 (65.7)
Transferred to an ICU	50 (35.7)
Receiving ventilation support	46 (33.8)
Invasive or invasive and non-invasive	26 (18.6)
Non-invasive only	20 (14.3)
Developing complications	59 (42.1)

Respiratory failure/ARDS	47 (33.6)
Superinfection	23 (16.4)
Hemodynamic failure	18 (12.9)
Renal failure	16 (11.4)
Pneumothorax	4 (2.9)
Outcome, n (%)	
In-hospital mortality	39 (27.9)
Cumulative all-cause mortality	
30-days	35 (25.0)
90-days	50 (35.7)
180-days	58 (41.4)

[a]PCP$^+$ criteria: i) Positive direct immunofluorescence microscopy and/or ii) Cycle threshold value below 36 on semiquantitative PCR analysis. Patients not fulfilling the criteria were considered colonized (PCP$^-$). Patients missing microbiological data were classified with "undetermined PCP-status".
[b]33 patients received supplemental oxygen when oxygen saturation was measured.
[c]Lymphopenia (<1.0 lymphocytes x 10^9/L) was present among 68 patients (93.2 %) with retrievable lymphocyte counts.
[d]86 patients received antibiotics, 35 patients received antifungals, 16 patients received antivirals other than anti-retrovirals and one patient received anti-tuberculous drugs.
Abbreviations: ARDS, acute respiratory distress syndrome; CT, computed tomography; ICU, intensive care unit; PCP, *Pneumocystis* pneumonia.

Table S4 Respiratory samples among study population and microbiological data as basis for *Pneumocystis* pneumonia-status[a]

Respiratory samples	Study population overall for reference, n (%)	Samples with retrievable results , n (%)[b]	
		Cycle threshold values semiquantitative PCR analysis	Direct immunofluorescence microscopy
Bronchoalveolar lavage fluid	234 (78.8)	192 (82.1)	97 (41.5)
Sputum	44 (14.8)	37 (84.1)	13 (29.5)
Induced sputum	9 (3.0)	7 (77.8)	4 (44.4)
Tracheal aspirate	5 (1.7)	4 (80.0)	3 (60.0)
Nasopharyngeal aspirate	2 (0.7)	2 (100.0)	0 (0)
Transbronchial biopsy	1 (0.3)	1 (100.0)	0 (0)
Biopsy upon autopsy	2 (0.7)	0 (0)	1 (50.0)
Total	**297 (100)**	**243 (81.8)**	**118 (39.7)**

[a] PCP^{+} criteria: i) Positive direct immunofluorescence microscopy and/or ii) Cycle threshold value below 36 on semiquantitative PCR analysis. Patients not fulfilling the criteria were considered colonized (PCP^{-}). Patients missing microbiological data were classified as "undetermined PCP-status".

[b]Cycle threshold values and DIF microscopy results were retrievable for 243 and 118 patients, respectively (%). The total number of the specific respiratory samples (e.g. BAL fluid) is the denominator of the proportions (%) in the two columns to the right.

Abbreviations: BAL, bronchoalveolar lavage; DIF, direct immunofluorescence; PCR, polymerase chain reaction

Paper II

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RESEARCH ARTICLE

Semiquantitative Real-Time PCR to Distinguish *Pneumocystis* Pneumonia from Colonization in a Heterogeneous Population of HIV-Negative Immunocompromised Patients

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ABSTRACT *Pneumocystis jirovecii* is a threat to iatrogenically immunosuppressed individuals, a heterogeneous population at rapid growth. We assessed the ability of an in-house semiquantitative real-time PCR assay to discriminate *Pneumocystis* pneumonia (PCP) from colonization and identified risk factors for infection in these patients. Retrospectively, 242 PCR-positive patients were compared according to PCP status, including strata by immunosuppressive conditions, human immunodeficiency virus (HIV) infection excluded. Associations between host characteristics and cycle threshold (C_T) values, semiquantitative real-time PCR correlates of fungal loads in lower respiratory tract specimens, were investigated. C_T values differed significantly according to PCP status. Overall, a C_T value of 36 allowed differentiation between PCP and colonization with sensitivity and specificity of 71.3% and 77.1%, respectively. A C_T value of less than 31 confirmed PCP, whereas no C_T value permitted exclusion. A considerable diversity was uncovered; solid organ transplant (SOT) recipients had significantly higher fungal loads than patients with hematological malignancies. In SOT recipients, a C_T cutoff value of 36 resulted in sensitivity and specificity of 95.0% and 83.3%, respectively. In patients with hematological malignancies, a higher C_T cutoff value of 37 improved sensitivity to 88.5% but reduced specificity to 66.7%. For other conditions, assay validity appeared inferior. Corticosteroid usage was an independent predictor of PCP in a multivariable analysis and was associated with higher fungal loads at PCP expression. Semiquantitative real-time PCR improves differentiation between PCP and colonization in immunocompromised HIV-negative individuals with acute respiratory syndromes. However, heterogeneity in disease evolution requires separate cutoff values across intrinsic and iatrogenic predisposition for predicting non-HIV PCP.

IMPORTANCE *Pneumocystis jirovecii* is potentially life threatening to an increasing number of individuals with compromised immune systems. This microorganism can cause severe pneumonia in susceptible hosts, including patients with cancer and autoimmune diseases and people undergoing solid organ transplantation. Together, these patients constitute an ever-diverse population. In this paper, we demonstrate that the heterogeneity herein has important implications for how we diagnose and assess the risk of *Pneumocystis* pneumonia (PCP). Specifically, low loads of microorganisms are sufficient

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Semiquantitative real-time PCR can improve differentiation between non-HIV PCP and colonization, but a significant heterogeneity in fungal loads at disease evolution requires separate cut-off values across non-HIV immunosuppressive predispositions.

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to cause infection in patients with blood cancer compared to those in solid organ recipients. With this new insight into host versus *P. jirovecii* biology, clinicians can manage patients at risk of PCP more accurately. As a result, we take a significant step toward offering precision medicine to a vulnerable patient population. One the one hand, these patients have propensity for adverse effects from antimicrobial treatment. On the other hand, this population is susceptible to life-threatening infections, including PCP.

KEYWORDS *Pneumocystis jirovecii*, PCP, colonization, immunosuppression, real-time PCR

Pneumocystis jirovecii is an atypical fungus and causative agent of *Pneumocystis* pneumonia (PCP) (1). Historically, PCP reemerged with the onset of the human immunodeficiency virus (HIV) epidemic as an opportunistic infection and hallmark of AIDS in the 1980s (2). Since the introduction of antiretroviral therapy and prompt administration of PCP prophylaxis, this disease burden is declining (3). Rather, it is becoming overshadowed by PCP in non-HIV immunocompromised populations, especially in resource-rich countries with universal health care (3). Nowadays, *P. jirovecii* represents a life threat to patients with malignancies, immunological disorders, chronic lung diseases, and those undergoing solid organ transplantation (SOT) (4). Their susceptibility to PCP is largely attributed to iatrogenic immunosuppression besides intrinsic host factors (5).

The clinical characteristics of PCP vary according to the degree of immunosuppression and, more markedly, with respect to the host's HIV status (3). First, non-HIV patients typically have a more fulminant onset, rapid progression of severe pneumonitis with respiratory failure, and higher mortality (4). Second, their respiratory samples contain fewer *P. jirovecii* organisms and more neutrophils, features of both diagnostic and prognostic importance (1). Although HIV status is the principal host distinction, HIV-negative patients represent a heterogeneous population with diverse risk profiles (3). Moreover, diagnosing non-HIV PCP is notoriously difficult due to absence of pathognomonic features and a broad differential (6).

Diagnostic guidelines for PCP recommend a multimodal algorithm including detection of *P. jirovecii* (7). Microscopic visualization has been the gold standard, since culturing of *P. jirovecii* is extremely difficult, but the sensitivity of microscopy is especially poor when applied to respiratory samples from non-HIV patients (1). Since the 1990s, highly sensitive PCR-based assays have become widely utilized (8). However, difficulties with differentiating between PCP and colonization, that is, presence of *P. jirovecii* in the absence of clinical pneumonia, has proven a drawback of this technology (4). In fact, this has repercussions for antimicrobial treatment guidance. Prompt initiation is vital for the prognosis of PCP, whereas management of colonization remains debated (1). Our objective was to assess the utility of an in-house semiquantitative real-time PCR-assay for diagnosing PCP in HIV-negative immunocompromised patients and identify predictors for infection.

RESULTS

Description of study population and comparisons according to PCP status. A total of 242 HIV-negative patients (100 female, 142 male) with positive *P. jirovecii* PCR were included, representing 84.0% of 288 presumed eligible patients (Fig. 1). Patient characteristics and univariate comparison according to PCP status are presented in Table 1.

With the present case definition, the condition was classified as PCP (PCP^+) in 196 patients and as colonization (PCP^-) in 46 patients. Demographics were comparable apart from cardiovascular comorbidity being more common among PCP^- patients. Chronic lung diseases were associated with colonization. Otherwise, PCP status seemed independent of immunosuppressive condition and regimen. However, the median corticosteroid dose (first quartile [q_1] to third quartile [q_3]) at presentation was higher among PCP^+ patients (10 [5 to 24] versus 4 [4 to 8] mg methylprednisolone/

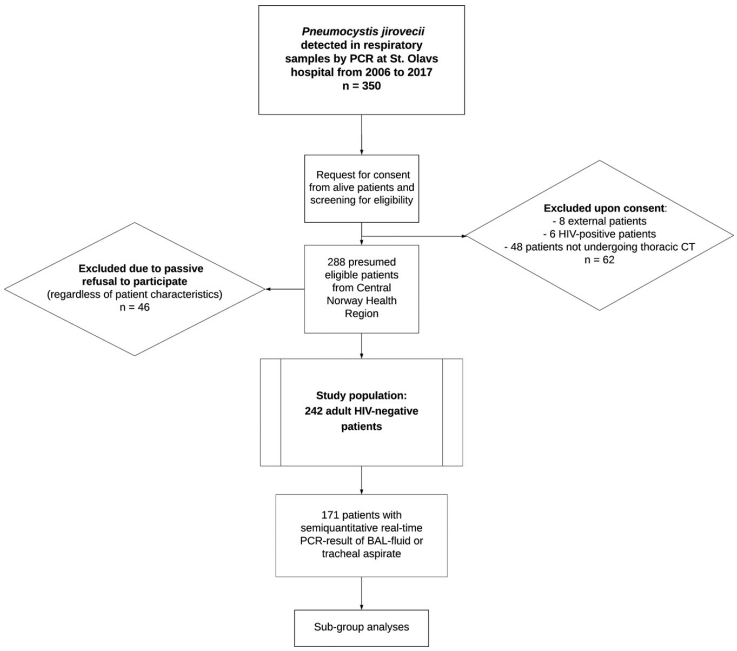

FIG 1 Flowchart of the study population. Adult patients tested in the regional referral laboratory and undergoing thoracic CT during diagnostic workup were eligible for inclusion. External referral and HIV seropositivity were exclusion criteria. All deceased patients were included, whereas recruitment of alive patients required active consent. BAL, bronchoalveolar lavage; CT, computed tomography; HIV, human immunodeficiency virus; PCP, *Pneumocystis* pneumonia.

day, $P < 0.001$). Besides, PCP$^+$ patients manifested more signs and symptoms of respiratory impairment and specific laboratory and radiological abnormalities (e.g., lymphopenia and crazy paving, respectively).

Sensitivity of microscopy and diagnostic discrimination by semiquantitative real-time PCR. Respiratory samples were mainly collected as bronchoalveolar lavage (BAL) fluid ($n = 203$, 83.9%), followed by sputum ($n = 25$, 10.3%), induced sputum ($n = 8$, 3.3%), tracheal aspirate ($n = 4$, 1.7%), respiratory biopsy sample ($n = 1$, 0.4%), and nasopharyngeal swab sample ($n = 1$, 0.4%) (see Fig. S1 in the supplemental material). Direct immunofluorescence (DIF) microscopy was performed on 99 samples, with 44 (44.4%) examinations resulting in positives. The sensitivity of DIF microscopy for *P. jirovecii* detection was positively associated with low cycle threshold (C_T) values, regardless of respiratory sample (adjusted odds ratio [OR], 0.77; 95% confidence interval [CI], 0.66 to 0.89) (Fig. S2).

C_T values from semiquantitative real-time PCR analysis of BAL fluid or tracheal aspirate were retrievable for 171 patients (Table S5). The median (q_1 to q_3) C_T value was lower among PCP$^+$ patients than among PCP$^-$ patients (35 [32 to 37] versus 38 [37 to 41], $P < 0.001$) (Fig. S3), confirming higher fungal loads in individuals with clinical infection. However, it was impossible to find an optimal C_T cutoff value for discrimination between PCP and colonization due to overlaps (Fig. S4). The receiver operating characteristic (ROC) curve analysis gave an area under the curve (AUC) of 0.80 (95% CI, 0.73 to 0.88) (Fig. 2A). A C_T value of 36 came closest to maximizing sensitivity and specificity simultaneously, being 71.3% (95% CI, 63.7% to 78.9%) and 77.1% (95% CI, 63.2% to 91.1%), respectively. This corresponded to a positive predictive value of 92.4% (95% CI, 87.3% to 97.5%) and a negative predictive value of 40.9% (95% CI, 29.0 to 52.8%). The validity and percentage of correctly classified patients varied according to C_T cutoff

TABLE 1 Characteristics of study population and comparison of patients with *Pneumocystis* pneumonia and colonization[a]

Characteristic	No. (%) in case of missing	Value			
		Study population (*n* = 242; 100%)	PCP$^+$ (*n* = 196; 81.0%)	PCP$^-$ (*n* = 46; 19.0%)	*P* value difference
Demographics and comorbidity					
Median age (yrs [q_1–q_3])	NA	66 (59–73)	65.5 (59–73)	68 (60–74)	0.39
Male sex (no. [%])	NA	142 (58.7)	119 (60.7)	23 (50.0)	0.18
History of smoking (no. [%])	235 (97.1)	131 (55.7)	106 (55.8)	25 (55.6)	0.98
Median Charlson comorbidity index (q_1–q_3)	NA	6 (4–8)	6 (4–8)	6 (4–8)	0.97
Comorbidities (no. [%])	NA				
Cardiovascular disease		66 (27.3)	45 (23.0)	21 (45.7)	0.002
Chronic kidney disease		32 (13.2)	26 (13.3)	6 (13.0)	0.97
Chronic liver disease		2 (0.83)	2 (1.0)	0 (0.0)	1.00
Chronic pulmonary disease		43 (17.8)	32 (16.3)	11 (23.9)	0.23
Congestive heart failure		13 (5.4)	10 (5.1)	3 (6.5)	0.72
Diabetes mellitus type 1 or 2		33 (13.6)	26 (13.3)	7 (15.2)	0.73
Hematological malignancy[b]		12 (5.0)	10 (5.1)	2 (4.3)	1.00
Hypertension		75 (31.0)	60 (32.1)	15 (27.3)	0.79
Rheumatic disease		7 (2.9)	6 (3.1)	1 (2.2)	1.00
Solid tumor		28 (11.6)	24 (12.2)	4 (8.7)	0.62
Any of the above		157 (64.9)	124 (63.3)	33 (71.7)	0.28
Primary PCP prophylaxis at presentation	NA	2 (0.8)	2 (1.0)	0 (0)	1.00
Microbiology					
C_T value of semiquantitative real-time PCR-analysis (median [q_1–q_3])					
Any respiratory sample[c]	202 (83.5)	36 (33 to 37)	35 (32–37)	38 (37–41)	<0.001
BAL fluid or tracheal aspirate[c]	171 (70.7)	36 (33–37)	35 (32–37)	38 (37–41)	<0.001
Immunosuppressive conditions					
Distribution across PCP groups	NA				0.19
Hematological malignancies		89 (37.6)	75 (38.3)	14 (30.4)	Ref.
Solid tumors		68 (28.7)	59 (30.1)	9 (19.6)	0.66
Immunological disorders		38 (16.0)	28 (14.3)	10 (21.7)	0.17
Solid organ transplantation		29 (12.2)	23 (11.7)	6 (13.0)	0.54
Chronic lung diseases		13 (5.5)	8 (4.1)	5 (10.9)	0.059
Other/miscellaneous[d]		5 (2.1)	3 (1.5)	2 (4.3)	Excluded
Pulmonary metastasis from solid tumor		12 (5.0)	9 (4.6)	3 (6.5)	0.70
Premorbid iatrogenic immunosuppression, chemotherapy and corticosteroid exposure					
Any immunosuppressive regimen (no. [%])	NA				
Last 5 yrs		230 (95.0)	187 (95.4)	43 (93.5)	0.70
At presentation		205 (84.7)	168 (85.7)	37 (80.4)	0.37
Regimen at presentation (no. [%])	NA				0.33
Chemotherapy for hematological malignancy and adjuvant steroids		54 (22.3)	47 (24.0)	7 (15.2)	
Chemotherapy for solid tumor and adjuvant steroids		31 (12.8)	26 (13.3)	5 (10.9)	
Chemotherapy for hematological malignancy		10 (4.1)	10 (5.1)	0 (0)	
Chemotherapy for solid tumor		14 (5.8)	11 (5.6)	3 (6.5)	
Corticosteroids in monotherapy		35 (14.5)	29 (14.8)	6 (13.0)	

(Continued on next page)

TABLE 1 (Continued)

Characteristic	No. (%) in case of missing	Value			*P* value difference
		Study population (*n* = 242; 100%)	PCP$^+$ (*n* = 196; 81.0%)	PCP$^-$ (*n* = 46; 19.0%)	
Graft rejection prophylaxis after SOT		28 (11.6)	23 (11.7)	5 (10.9)	
DMARDs with or without adjunctive steroids		22 (9.1)	15 (7.7)	7 (15.2)	
Other combinations[e]		11 (4.6)	7 (3.6)	4 (8.7)	
None		37 (15.3)	28 (14.3)	9 (19.6)	
Systemic corticosteroid exposure pattern 60 days preceding presentation (no. [%])	240 (99.2)				0.31
Daily		102 (42.5)	80 (41.2)	22 (47.8)	0.96
Intermittent		74 (30.8)	64 (33.0)	10 (21.7)	0.20
None		64 (26.7)	50 (25.8)	14 (30.4)	Ref.
Methylprednisolone equivalent dose (mg/day at presentation) (median [q_1–q_3])[f]	237 (97.9)	8 (4–20	10 (5–24)	4 (4–8)	<0.001
Symptomatology (no. [%])					
Cough	NA	140 (57.9)	117 (59.7)	23 (50.0))	0.23
Dyspnea	NA	184 (76.0)	156 (79.6)	37 (60.9)	0.007
Fever	NA	180 (74.4)	151 (77.0)	29 (63.0)	0.05
Minimum two cardinal symptoms	NA	184 (76.0)	154 (78.6)	30 (65.2)	0.056
All three cardinal symptoms	NA	81 (33.5)	74 (37.8)	7 (15.2)	0.004
No cardinal symptoms	NA	3 (1.2)	0 (0)	3 (6.5)	0.007
Objective findings and biochemistry					
Abnormal lung auscultation (no. [%])	NA	144 (59.5)	123 (62.8)	21 (45.7.)	0.033
Oxygen saturation (%) (median [q_1–q_3][g]	207 (85.5)	89 (84–93)	88 (84–93)	91.5 (88–95)	0.014
Leukocyte count × 10^9/liter (median [q_1–q_3]	235 (97.1)	7.0 (4.3–10)	6.9 (4.2–10.0)	7.7 (5.2–9.9)	0.36
Neutrophil count × 10^9/liter (median [q_1–q_3]	186 (76.9)	4.8 (2.8–7.3)	4.8 (2.8–7.3)	4.8 (3.1–7.0)	0.99
Neutropenia (<0.5 neutrophils 10^9/liter)	186 (76.9)	3 (1.6)	2 (1.3)	1 (3.6)	0.37
Lymphocyte count × 10^9/liter (median [q_1–q_3])[h]	122 (50.4)	0.63 (0.41–1.1)	0.6 (0.4–1.1)	1.0 (0.5–1.5)	0.047
Lymphopenia (<1.0 lymphocyte × 10^9/liter)	123 (50.8)	82 (66.7)	73 (70.2)	9 (47.4)	0.052
CD4$^+$ T cell count × 10^9/liter (median [q_1–q_3])	13 (5.4)	0.13 (0.07–0.25)	0.1 (0.05–0.25)	0.32 (0.22–0.41)	0.24
Lactate dehydrogenase (U/liter) (median [q_1–q_3]	142 (58.7)	293.5 (221–390)	308 (225–390)	224 (200–441)	0.082
Albumin (g/liter) (median [q_1–q_3]	174 (71.9)	33 (27–36)	32.5 (27–35.5)	33.5 (27–37.5)	0.95
C-reactive protein (mg/liter) (median [q_1–q_3]	235 (97.1)	76 (38–146)	81 (42–156)	53 (24.5–116.5)	0.019
Radiological features (no. [%])					
Any remarks on chest X-ray	204 (84.3)	160 (78.4)	133 (80.1)	27 (71.1)	0.22
Any remarks on thoracic CT	NA	237 (97.9)	196 (100)	41 (89.1)	<0.001
Findings on thoracic CT	NA				
Atelectasis		41 (16.9)	29 (14.8)	12 (26.1))	0.066
Bronchiectasis		18 (7.4)	11 (5.6)	7 (15.2)	0.025
Crazy paving pattern		55 (22.3)	53 (27.0)	4 8.7)	0.007
Consolidations		44 (18.2)	39 (19.9)	5 (10.9)	0.20
Cysts		9 (3.7)	6 (3.1)	3 (6.5)	0.38
Emphysema		26 (10.7)	20 (10.2)	6 (13.0)	0.58
Ground glass opacities[i]		180 (74.4)	171 (87.2)	12 (26.1)	<0.001
Infiltrates[i]		52 (21.5)	42 (21.4)	10 (21.7)	0.96
Lymphadenopathy		40 (16.5)	32 (16.3)	8 (17.4)	0.86

(Continued on next page)

TABLE 1 (Continued)

Characteristic	No. (%) in case of missing	Value			
		Study population (*n* = 242; 100%)	PCP$^+$ (*n* = 196; 81.0%)	PCP$^-$ (*n* = 46; 19.0%)	*P* value difference
Noduli		21 (8.7)	15 (7.7)	6 (13.0)	0.24
Pleural effusion		67 (27.7)	52 (26.5)	15 (32.6)	0.41
Pneumothorax		1 (0.41)	1 (0.5)	0 (0.0)	1.00
Reticular or septal thickening		63 (26.0)	55 (28.1)	8 (17.4)	0.14
"Tree-in-bud sign"		16 (6.6)	11 (5.3)	5 (10.9)	0.20

[a]Criteria for PCP were multimodal and based on available patient data (see Materials and Methods and Fig. S1 in the supplemental material). Patients not fulfilling the criteria for their respective groups were considered colonized with *P. jirovecii* (i.e., PCP$^-$). BAL, bronchoalveolar lavage; CT, computed tomography; C_T, cycle threshold; DMARDs, disease-modifying antirheumatic drugs; NA, not applicable; Ref., reference group in logistic regression analysis; SOT, solid organ transplantation.

[b]In 12 patients, hematological malignancy was not considered the primary immunosuppressive condition or an indication for immunosuppression but rather a comorbidity.

[c]Respiratory samples included bronchoalveolar lavage fluid (*n* = 203, 83.9%), sputum (*n* = 25, 10.3%), induced sputum (*n* = 8, 3.3%), tracheal aspirate (*n* = 4, 1.7%), respiratory biopsy specimen (*n* = 1, 0.4%) and nasopharyngeal swab sample (*n* = 1, 0.4%), in a total of 242 samples. C_T values were retrievable from analysis of 202 samples, including 171 BAL fluid samples and tracheal aspirates.

[d]Other/miscellaneous immunosuppressive conditions included two patients with no diagnosed condition, whereas two had received steroids for suspected autoimmune disorder and one patient with statin-induced myositis was treated with corticosteroids.

[e]Other combinations include exposure to other immunosuppressants (mycophenolate, azathioprine, cyclophosphamide, calcineurin and mTOR inhibitors, and cyclosporine and hydroxychloroquine with or without adjuvant steroids) and one patient receiving both graft rejection prophylaxis for solid organ transplantation and chemotherapy for hematological malignancy with adjuvant corticosteroids.

[f]Median methylprednisolone equivalent dose per day was calculated among 117 patients having an intake the day of *P. jirovecii* detection: 95 PCP$^+$ and 22 PCP$^-$ patients.

[g]Fifty-three patients were receiving supplemental oxygen when saturation was measured; 45 (23.0%) in the PCP$^+$ group and 8 (17.4%) in the PCP$^-$ group (*P* = 0.41 for difference).

[h]One patient with chronic lymphatic leukemia was excluded from the analysis due to an abnormally high lymphocyte count (i.e., 37.9 $\times$ 10^9/liter).

[i]Note: Ground glass opacities and infiltrates were among the criteria for PCP$^+$.

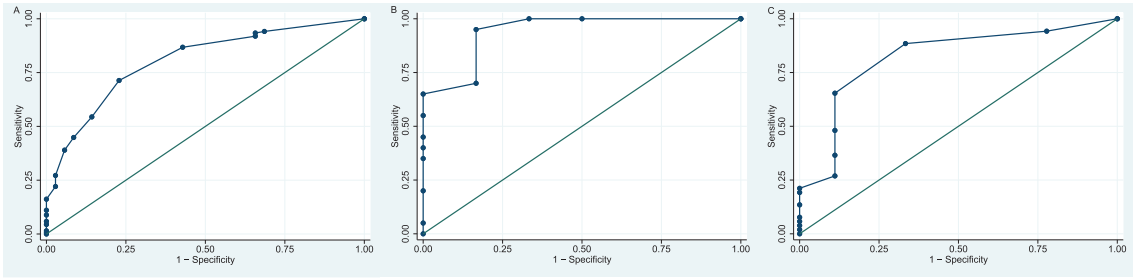

FIG 2 ROC curves of semiquantitative real-time PCR of BAL fluid or tracheal aspirate for discrimination between *Pneumocystis* pneumonia and colonization. (A) ROC curve for population overall, based on 171 samples. (B) ROC curve for SOT recipients, based on 26 samples. (C) ROC curve for patients with hematological malignancies, based on 61 samples.

value (Fig. S5). C_T values greater than 36 defined a gray zone without definitive discrimination, comprising 39 PCP$^+$ patients. Their characteristics are summarized in Table S2.

Subgroup analyses of PCP$^+$ patients. C_T values of <31 corresponded to 100% specificity. To identify characteristics of this subpopulation with higher fungal loads ($n = 22$), we compared it to PCP$^+$ patients with C_T values of 31 and higher ($n = 114$) (Table S3). Notably, fungal load appeared associated with immunosuppressive condition ($P = 0.05$). SOT recipients accounted for 36.4% of the high-fungal-load population, whereas patients with hematological malignancies dominated the low-fungal-load population, constituting 40.5%. Moreover, we noted an association between corticosteroid exposure and fungal burden, with more daily users and fewer unexposed subjects in the high-fungal-load population. Median doses were comparable.

Heterogeneity in fungal loads. Successively, we further analyzed the relationships to immunosuppressive predisposition, including corticosteroid exposure and fungal burden (Fig. 3; see also Fig. S6 and S7). A linear regression model was fitted comparing C_T values in BAL fluid or tracheal aspirate across immunosuppressive conditions, with patients with hematological malignancies as a reference group, ($F[4,162] = 3.03$, $P = 0.019$, $R^2 = 0.070$). Only SOT recipients had significantly lower C_T values (Table S4). Univariate analyses confirmed this difference in medians (q_1 to q_3) compared to patients with hematological malignancies overall (34.5 [28 to 36] versus 36 [34 to 37], $P = 0.072$), among PCP$^+$ patients (33 [28 to 36] versus 36 [33 to 37], $P < 0.01$), and to a lesser degree among PCP$^-$ patients (38 [37 to 38] versus 39.5 [37 to 41], $P = 0.54$).

Discrimination across immunosuppressive conditions. With caution regarding the number of patients and observations, we investigated the validity of semiquantitative real-time PCR across immunosuppressive conditions. Based on 26 samples from SOT recipients, the discrimination between PCP and colonization appeared outstanding and superior to the population overall (AUC, 0.94; 95% CI, 0.82 to 1.00) (Fig. 2B). A C_T value of 36 corresponded to a sensitivity of 95.0% (95% CI, 85.4% to 100.0%) and a specificity of 83.3% (95% CI, 53.5% to 100.0%). In spite of lower fungal loads, the validity was excellent for patients with hematological malignancies (AUC, 0.82; 95% CI, 0.66 to 0.98) based on 61 observations (Fig. 2C). Yet, a higher C_T cutoff value was needed to achieve a sensitivity of >75%. Here, a C_T value of 37 corresponded to a sensitivity of 88.5% (95% CI, 79.8% to 97.1%) and a specificity of 66.7% (95% CI, 35.9% to 97.5%). The validity of the PCR assay appeared inferior for the remaining conditions (Fig. S8A to C; Table S5).

Independent risk factors for PCP. Based on univariate comparisons, we performed multivariable analyses to identify independent risk factors for PCP (Table 2). Only chronic lung diseases were associated with markedly lower odds of PCP (OR, 0.30; 95% CI, 0.09 to 1.05). Presence of all three cardinal symptoms and abnormal lung auscultation were independent predictors for PCP. Moreover, corticosteroid dose at presentation was positively associated with PCP, while C_T value and oxygen saturation were negative predictors. The presence of crazy paving on computed tomography (CT) imaging was strongly associated with PCP.

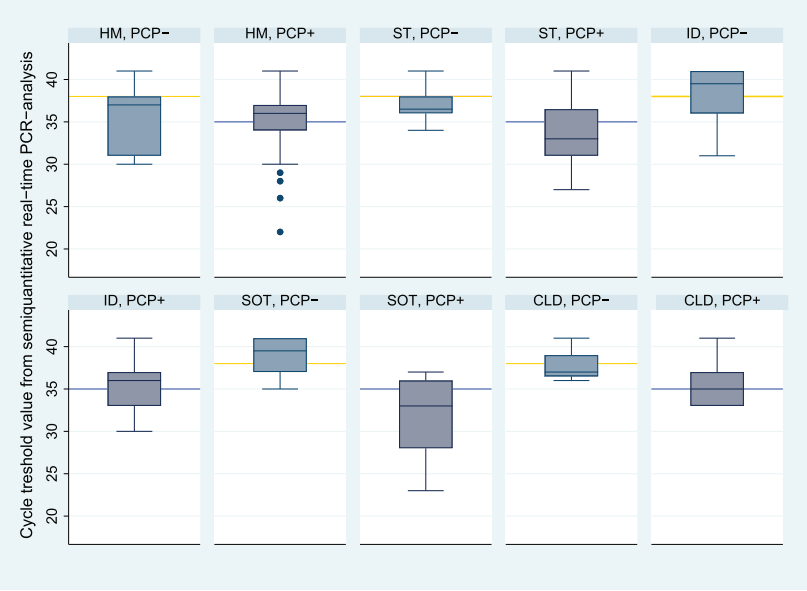

FIG 3 Relationship between semiquantitative real-time PCR-result, immunosuppressive conditions, and PCP status. C_T values from of BAL fluid or tracheal aspirate differed significantly according to PCP status ($P < 0.01$) with medians being 35 (blue line) and 38 (yellow line), respectively. Retrospectively, 196 patients were diagnosed with PCP (i.e., PCP^+) while 46 were presumed colonized (i.e., PCP^-). C_T, cycle threshold; CLD, chronic lung disease; HM, hematological malignancy; ID, immunological disorder; PCP, *Pneumocystis* pneumonia; PCR, polymerase chain reaction, SOT, solid organ transplant; ST, solid tumor.

DISCUSSION

This study demonstrates that semiquantitative real-time PCR can improve differentiation between PCP and colonization in immunocompromised HIV-negative patients. However, a significant heterogeneity in fungal loads across immunosuppressive predispositions implicates that universal cutoff values for predicting non-HIV PCP are inadequate.

Non-HIV populations at risk of opportunistic infections, including PCP, are growing rapidly because of prolonged survival and escalating use of immunosuppressants (3, 5). Diagnostic algorithms with high specificity are needed to avoid unnecessary treatment, especially among multimorbid patients with propensity for adverse effects and drug interactions (9). On the other hand, delayed diagnosis is associated with increased mortality risk, potentially exceeding 50% (1).

Semiquantitative real-time PCR gradually substituted microscopy for *P. jirovecii* detection in our regional referral laboratory during the last decades, but whether C_T values should be emphasized for treatment guidance remained unestablished. Here, the study subjects represented a selected population, and they had high pretest probability of PCP. Accordingly, the majority were classified as PCP^+ in retrospect. Although C_T values were significantly lower among PCP^+ patients, it was impossible to determine a cutoff with a 100% negative predictive value.

Several studies have assessed real-time PCR strategies to distinguish PCP from colonization. Extrapolation is limited by heterogeneity in PCR targets, PCP definitions, host characteristics, types of respiratory samples, sample volumes, DNA extraction, and quantification methods (C_T values or copies per milliliter) (10). Anyhow, the majority have found real-time PCR assays potentially useful (11–25), though gray zones are common and stratification by HIV status is of utmost importance. Inability to

TABLE 2 Uni- and multivariable analyses of risk factors for *Pneumocystis* pneumonia versus colonization[a]

Risk factor and covariate(s)[b]	No. of observations	OR[d]	95% CI	*P* value
Cardiovascular comorbidity	**NA**	**0.35**	**0.18–0.69**	**0.002**
Age and sex	NA	0.27	0.13–0.57	0.002
Any other comorbidity and sex	NA	0.29	0.14–0.60	0.001
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	0.47	0.17–1.26	0.13
C_T value of semiquantitative real-time PCR-analysis of BAL fluid or tracheal aspirate/unit increase	**171**	**0.68**	**0.58–0.80**	**<0.001**
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	82	0.54	0.38–0.80	< 0.001
Immunosuppressive condition[c]	**237**			
Hepatological malignancy	**89**	**1**	**Ref.**	**Ref.**
Solid tumor	**68**	**1.22**	**0.50–3.02**	**0.66**
Immunological disorder	**38**	**0.52**	**0.21–1.31**	**0.17**
Solid organ transplantation	**29**	**0.72**	**0.25–2.07**	**0.54**
Chronic lung disease	**13**	**0.30**	**0.09–1.05**	**0.059**
Daily methylprednisolone equivalent dose at presentation/mg increase	**237**	**1.05**	**1.00–1.10**	**0.035**
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	**117**	**1.11**	**1.02–1.20**	**0.011**
Dyspnea	**242**	**2.51**	**1.26–4.98**	**0.009**
Cardiovascular comorbidity	242	2.87	1.30–5.88	0.004
Immunosuppressive condition	237	2.83	1.36–5.89	0.005
Systemic corticosteroid exposure pattern 60 days preceding presentation	240	2.84	1.40–5.46	0.004
Fever	**242**	**1.97**	**0.99–3.90**	**0.053**
Daily methylprednisolone equivalent dose at presentation/mg increase	237	2.33	1.14–4.75	0.020
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	2.68	0.96–7.45	0.059
At least two cardinal symptoms (cough, dyspnea, fever)	**242**	**1.96**	**0.97–3.92**	**0.059**
Immunosuppressive condition	237	1.70	0.81–3.55	0.159
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	2.52	0.92–6.93	0.073
All three cardinal symptoms (cough, dyspnea, and fever)	**242**	**3.38**	**1.44–7.94**	**0.005**
Daily methylprednisolone equivalent dose at presentation/mg increase	237	4.28	1.71–10.7	0.002
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	6.23	1.30–29.7	0.022
Abnormal lung auscultation	**242**	**2.01**	**1.05–3.84**	**0.035**
Daily methylprednisolone equivalent dose at presentation/mg increase	237	1.81	0.93–3.51	0.080
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	3.35	1.22–9.21	0.019
Immunosuppressive regimen at presentation	242	2.17	1.10–4.28	0.026
Oxygen saturation in %/unit increase	**207**	**0.93**	**0.87–0.99**	**0.016**
Lymphocyte count × 10^9/liter/unit increase	**122**	**0.71**	**0.50–1.00**	**0.050**
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	59	1.13	0.55–2.32	0.745
Immunosuppressive condition[c]	119	0.64	0.43–0.94	0.024
Lymphopenia (<1.0 × 10^9/liter)	**123**	**2.62**	**0.97–7.07**	**0.058**
Charlson comorbidity index/unit increase	123	2.97	1.06–8.32	0.039
Daily methylprednisolone equivalent dose at presentation/mg increase	120	2.87	1.04–7.92	0.042

(Continued on next page)

TABLE 2 (Continued)

Risk factor and covariate(s)[b]	No. of observations	OR[d]	95% CI	*P* value
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	60	2.35	0.56–9.94	0.244
C-reactive protein in mg/liter/unit increase	**235**	**1.00**	**1.00–1.01**	**0.057**
Lactate dehydrogenase in U/liter/unit increase	**142**	**1.00**	**1.00–1.00**	**0.89**
Atelectasis	**242**	**0.49**	**0.23–1.06**	**0.070**
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	0.70	0.22–2.21	0.54
Lymphocyte count × 10^9/liter/unit increase	122	2.86	0.35–23.2	0.33
Immunosuppressive regimen at presentation	242	0.57	0.26–1.25	0.16
Bronchiectasis	**242**	**0.33**	**0.12–0.91**	**0.032**
Age, sex	242	0.37	0.13–1.05	0.063
Immunosuppressive condition[c]	237	0.43	0.15–1.27	0.13
Systemic corticosteroid exposure pattern 60 days preceding presentation	240	0.37	0.13–1.02	0.054
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	237	0.52	0.11–2.41	0.40
Immunosuppressive regimen at presentation	242	0.37	0.13–1.1	0.073
Crazy paving pattern on thoracic CT	**242**	**3.89**	**1.33–11.4**	**0.013**
Age and sex	242	4.28	1.45–12.7	0.009
C_T value of semiquantitative real-time PCR analysis of BAL fluid or tracheal aspirate	171	6.09	1.58–23.4	0.009
Immunosuppressive condition[c]	237	4.38	1.45–13.3	0.009
Immunosuppressive regimen at presentation	242	4.29	1.44–12.8	0.009
Daily methylprednisolone equivalent dose at presentation among exposed/mg increase	117	5.26	1.12–24.8	0.036
Lymphocyte count × 10^9/liter/unit increase	122	3.07	0.65–14.4	0.16

[a]Criteria for PCP were multimodal and based on available patient data (see Materials and Methods and Fig. S1). Patients not fulfilling the criteria for their respective groups were considered colonized with *P. jirovecii* (i.e., PCP^-). BAL, bronchoalveolar lavage; CT, computed tomography; C_T, cycle threshold; NA, not applicable; OR, odds ratio.

[b]Risk factors are in boldface. Plausible confounders were identified *a priori* and included in multivariable analyses. Covariates with ≥10% effect on OR are included in the table. For complete list of covariates, refer to Table S1.

[c]Five patients had immunosuppressive conditions classified as miscellaneous and were excluded from the comparative analysis. Adjustment for age and sex did not cause significant changes to odds ratios overall or *P* values and are not reported.

[d]Univariate analysis results are in boldface; adjusted ORs are in lightface.

discriminate the two entities has also been described (26, 27), perhaps due to a continuous progression from carriage to active infection (7).

Upon exposure, *P. jirovecii* adheres to type 1 pneumocytes, which in turn induces organism activation and multiplication (1). The passage from colonization to PCP and complications is ill defined in non-HIV patients (5), and CD4 counts fail in predicting disease (6). Paradoxically, the associated lung injury is proposed to result from an inappropriate inflammatory host response (5). Marked bronchoalveolar neutrophilia observed in HIV-negative patients likely reflects this reaction and aggravates the prognosis (1).

Since the fungus lives and thrives in the alveoli, an increasing density gradient from the upper to the lower respiratory tract is expected (7). In the attempt to avoid invasive sampling, researchers have assessed the validity of upper respiratory tract specimens compared to the gold standard of BAL fluid, with various results (10). Overall, the sensitivity appears too low to exclude PCP, while positive results support the diagnosis (7). Asymptomatic carriage in the upper respiratory tract due to recent exposure is a differential diagnosis (4), and a theoretical source of contamination unless protective invasive sampling is applied (28).

In light of the current knowledge gaps and diagnostic challenges, a major strength of this study is the large number of high-risk cases and high-yield respiratory specimens permitting subgroup analyses. Interestingly, SOT recipients and patients with hematological malignancies distinguished themselves at different ends of a spectrum, harboring high and low fungal loads, respectively. However, an R^2 of 7.0% suggests that endogenous host predisposition explains little of the diversity. Indeed, our results indicate that immunosuppression, including corticosteroid exposure, also influences the precise intersection of host response and *P. jirovecii* concentration that results in clinical infection.

Cancer patients are primarily subject to cycles of chemotherapy regimens, for instance, rituximab, cyclophosphamide, vincristine, and prednisolone (R-CHOP) and fludarabine, cyclosporine, and rituximab (FCR), both involving significant risk of PCP (5). Moreover, corticosteroids have vast supportive care indications in oncology, increasing exposure (2). In comparison, SOT recipients are prescribed daily multidrug regimens with explicit lymphocytotoxic effects to prevent allograft rejection (29). Although SOT regimens are pleiotropic and not CD4 specific, perhaps they come closest to mimicking the lymphocyte depletion occurring during the natural course of HIV infection considering their continuity and intensity (29).

Notably, Montesinos et al. found that *P. jirovecii* concentrations were markedly heterogeneous in samples from HIV-negative PCP patients (23). Relatedly, Robert-Gangneux et al. highlighted hematological malignancies particularly for the tendency of negative microscopy examinations, *per se*, to be associated with low fungal loads (26). Altogether, we hypothesize that intrinsic and iatrogenic host factors affect *P. jirovecii* multiplication and non-HIV PCP expression. Regardless of the pathogenesis, our findings have important implications. Foremost, the validity of real-time PCR strategies may vary across immunosuppressive predispositions, and optimal cutoff values for discrimination should be validated according to these strata.

Acknowledging the importance of the recent multicenter study from the Fungal PCR Initiative comparing the performance of several commercial and noncommercial *P. jirovecii* quantitative real-time PCR assays with emphasis on standardization, our in-house assay harbors certain shortcomings (30). Specifically, the protocol only tests the efficacy of the amplification step. Ideally, one should add a negative control prior to extraction to monitor the entire real-time process. Use of an alien negative control is preferable to avoid bias from human factors (e.g., unknown quantity of human DNA in eluate). Moreover, inherent variability of biologic systems is an important bottleneck in real-time PCR studies such as ours. To limit confounding from differences in sample volumes, relative quantification (e.g., the comparative [$\Delta\Delta$] C_T method) involving normalization of *P. jirovecii* to one or more reference genes with near constant expression should prevail over absolute quantification. Importantly, the genes must be amplified

with comparable efficacy for this method to be accurate (31). Owing to higher feasibility, easier clinical interpretation, and determination of cutoff values, diagnostic microbiology departments may still prefer absolute quantification.

The last concern regards the target gene for amplification. Beta-tubulin is a highly conserved single-copy nuclear gene (10). Single-copy genes are favorable to avoid bias in quantification and accurately reflect the quantity of organisms (30). This allows interstrain comparisons and direct determination of cutoff values, since varied copy numbers is a nonissue. However, compared to multicopy gene targets such as the major surface glycoprotein and mitochondrial genes, inferior analytical sensitivity is a drawback (10, 30). Extraction of whole nucleic acids demonstrates an even wider detection range for *P. jirovecii* compared to that with DNA only (30). In fact, to target the mitochondrial small subunit with whole nucleic acid as a starting material appears to yield the best sensitivity (30). The rationale for using assays with the highest sensitivity obtainable is vast. Principally, even low-amount *P. jirovecii* inoculums can be associated with non-HIV PCP. With the distinct exception of SOT patients, our study underscored this characteristic, particularly among patients with hematological malignancies. Hence, the nature of this disease strongly argues for high negative predictive values, including the lower spectrum of *P. jirovecii* inoculums. The growing implications of colonization are equally important. Molecular genotyping reports involving colonized patients in nosocomial transmission networks are worrisome and emphasize the urgency for strategies to reduce circulation of *P. jirovecii* (32). Furthermore, the possible risk of developing full-blown PCP from colonization in case of deteriorated immunity favors preemptive treatment (30).

Despite the above-described issues, we believe that the main findings of our study withstand. Considering the ever-diverse population susceptible to *P. jirovecii*, these indications warrant further investigations with emphasis on appropriate study design and stratified analyses.

Besides real-time PCR, this study underlines readily available clinical characteristics to emphasize for treatment guidance. In line with previous reports (12, 14, 16, 26, 33), the sensitivity of DIF microscopy appeared associated with *P. jirovecii* loads. Concerning noninvasive investigations, history of all three cardinal symptoms and decreased oxygen saturation were independent predictors of PCP in our PCR-positive cohort. Also, lymphopenia, an established risk factor for PCP (5), was associated with PCP, based on 123 observations. In our experience, a common pitfall is declaring patients immunocompetent if their neutrophil count is normal in spite of lymphopenia. In relation to this, cumulative corticosteroid dose is worth stressing due to lymphocytotoxic effects. Although we found a positive association, dose tapering, low doses, or no preceding intake does not exclude PCP (2). Lastly, both corticosteroids and lymphopenia are risk factors for colonization too, complicating clinical discrimination (8).

Cardiovascular comorbidity favored colonization in the univariate analysis. We hypothesize that shared clinical characteristics, particularly in cardiac patients, contributed to this. However, a multivariable analysis confirmed a positive confound by corticosteroids, moderating this relationship. A reluctance toward corticosteroid therapy to these patients because of adverse circulatory and metabolic effects may explain this finding.

This study has several limitations. First, we were unable to include all alive patients. Also, to strive for diagnostic homogeneity, validation of the semiquantitative real-time PCR was primarily performed on lower-respiratory-tract specimens. These limitations represent selection bias. Second, this was a retrospective analysis, challenging data collection and reliability. Third, the lack of a gold standard for diagnosing PCP might have resulted in information bias. Fourth, an increase in familywise error rate across reported statistical analyses was not controlled for. Finally, the comparison of fungal loads is challenged by variability in respiratory specimens, host pathogen biology, and procedural and analytical factors discussed above.

In conclusion, semiquantitative real-time PCR offers high objectivity and sensitivity

for *P. jirovecii* detection in HIV-negative immunocompromised individuals. However, heterogeneity across host predispositions requires multivariable models to optimize discrimination between life-threatening PCP and colonization. Prospective studies are needed to assess the external validity of our results while reducing the risk of bias and confounding.

MATERIALS AND METHODS

Setting and inclusion. St. Olavs hospital, Trondheim University Hospital, is the only tertiary referral hospital in the central Norway health region, covering approximately 700,000 inhabitants. Adult patients with respiratory samples testing positive for *P. jirovecii* by PCR at the Department of Medical Microbiology from 2006 to 2017 were identified. For inclusion, respiratory samples included BAL fluids, induced sputa, sputa, tracheal aspirates, respiratory biopsy specimens, and nasopharyngeal swab samples. Patients who were HIV negative, had been followed up regionally, and had undergone thoracic CT were eligible. Inclusion of alive patients required active consent, while all deceased patients were included.

Data collection. Comprehensive biological, clinical, and demographic data were collected retrospectively from patient records. Ongoing corticosteroid intake on the date of *P. jirovecii* detection was registered and converted into the equivalent in methylprednisolone expressed as milligrams per day. Degree of comorbidity was assessed according to the Charlson weighted comorbidity index (34). Cardiovascular comorbidities comprised coronary heart disease, stroke, and peripheral artery disease, whereas congestive heart failure and hypertension were registered separately. Epi Info (version 7.2.2.6; Centers for Disease Control and Prevention, Atlanta, GA, USA) was used for data recording.

Microbiological detection of *P. jirovecii*. DIF microscopy was performed with MONOFLUO *Pneumocystis jirovecii* IFA test kit number 32515 (Bio-Rad). Lack of positive controls from "definite" PCP patients was a challenge during the study period. For this reason and concerns regarding sensitivity and specificity, the laboratory used DIF as a complementary method in line with the guidelines (7), mainly on PCR-positive samples. In 2017, semiquantitative real-time PCR replaced DIF definitely. The in-house assay targeting the beta-tubulin gene of *P. jirovecii* was adapted from Brancart et al. (33) with some modifications as described in detail below (11, 33).

Semiquantitative real-time PCR-protocol. Respiratory tract samples that were viscous were pretreated with Sputolysin (dithiothreitol, volume 1:2) for 10 min 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 min. Thereafter, 500 μl of the supernatant was mixed with 50 μl proteinase K and incubated for 15 min at 65°C. If the sample volume was <10 μl, the centrifugation step was omitted, and 1 ml of sample was mixed with 100 ml proteinase K and incubated as described above. Then, the mixture was spun down, the supernatant was removed, and 500 μl of precipitate was used for DNA extraction on a NucliSENS easyMAG instrument (bioMérieux) with an eluate volume of 55 μl.

Reagents and PCR instruments used varied during the study period, but all changes were validated to ensure equal quality. During 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 qPCR supermix with uracil-*N*-glycosylase, 0.5 μl of each primer (12 μM) and probe (8 μM), and 3.5 μl molecular-grade water. BAL fluids, considered critical patient samples, were extracted and amplified in duplicates. Amplification reactions were carried out on either 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 min, 95°C for 3 min, and then 40 cycles of 95°C, 60°C, and 72°C for 10 s each. Results were reported to clinicians as negative/positive, with a comment about low concentration of *P. jirovecii* if the cycle threshold (C_T) value was high. 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 for inhibition, a separate real-time PCR targeting a human 237-bp intergenic region of chromosome 20 (position 104006 to 104242, sequence AL133466) was run, as previously described (35). All samples were positive, indicating absence of PCR inhibitors, and no samples were excluded due to nonamplification during the study period. The laboratory participated in a *Pneumocystis jirovecii* pneumonia (PCP) DNA EQA Program (QCMD) during the study period.

Retrieval of C_T values. C_T values were not reported in the laboratory information system during the study period. Therefore, C_T values were collected from the log of the PCR instruments in retrospect. Since some of the PCR instruments were replaced and discarded during the study period, C_T values for samples run on those instruments were lost. These were registered as "missing" during data collection. The retrievability of C_T values depended on which instrument the analyses were run, and the missing pattern was considered random and unrelated to patient characteristics.

Case definition. To separate infection from colonization in PCR-positive patients, multimodal criteria based on current clinical practice, previous reports (36–38), and existing diagnostic guidelines emphasizing biological detection were imposed *a posteriori* (7) (see Fig. S1 in the supplemental material). We identified three patient groups and applied the following criteria for PCP: group 1, (i) immunosuppressive state and (ii) positive DIF; group 2 (characterized by missing or negative DIF microscopy-result), (i) immunosuppressive state, (ii) at least one cardinal symptom of PCP (cough, dyspnea, and fever), (iii) typical findings on thoracic CT (ground glass opacities and/or infiltrates), and (iv) presumptive diagnosis at time of diagnosis, i.e., receiving anti-PCP treatment; group 3, patients who died in hospital within 30 days of detection without receiving anti-PCP treatment. We evaluated these patients individually with respect to cause of death and PCP status to exclude abrupt death from PCP without time to receive

anti-PCP treatment. The alternative diagnosis was colonization and PCP-unrelated death (i.e., terminal patients dying from underlying conditions). Patients not fulfilling the criteria for their respective groups were considered colonized with *P. jirovecii*. C_T values were compared to the retrospective PCP status, infection (PCP^+) or colonization (PCP^-).

Statistics. Continuous and categorical variables are presented as medians with second (q_1) and third (q_3) quartiles and proportions with percentages, respectively. Simple linear regression was used to compare C_T values across immunosuppressive conditions. Otherwise, univariate analyses were performed with the Wilcoxon rank sum, chi-square, or Fisher's exact test as appropriate, except for polychotomous independent variables, for which logistic regression was applied. Subsequently, multivariable logistic regression analyses were performed for variables having P values of <0.10 with covariates identified *a priori* (Table S1), with PCP versus colonization as outcomes. ROC curves were used to assess the validity of semiquantitative real-time PCR and determine sensitivity and specificity according to C_T cutoff values. Results are expressed as proportions, ORs, or AUC with 95% confidence intervals. All P values were two sided. Values of <0.05 were considered statistically significant.

Analyses were performed using Microsoft Excel (version 16.4; Microsoft Corporation, Redmond, WA, USA), STATA/MP (version 15.1; StataCorp, College Station, TX, USA), and IBM SPSS statistics for Macintosh (version 27.0; IBM Corp., Armonk NY, USA).

Ethics. This study was approved by the Regional Committee for Medical and Health Research Ethics (REC-North, reference number 2017/2419).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE S1, PDF file, 0.7 MB.

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We declare no conflicts of interest.

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Supplemental material to: "Semiquantitative real-time PCR to distinguish *Pneumocystis* pneumonia from colonization in a heterogenous population of HIV-negative immunocompromised patients"

CONTENTS

Supplementary Tables:

Supplementary Figures:

Other

Supplemental Table S1. Independent variables and covariates included in multivariable analyses reported in Table 3

Confounders identified *a priori* (Covariates)

Independent variables (Risk factors for PCP)	Age and sex	CCI with and without sex	Comorbidities other than cardiovascular with and without sex	Immunosuppressive condition	Immunosuppressive regimen at presentation	Systemic corticosteroid exposure 60 days preceding presentation	Immunosuppressive regimen last five years (any vs. none)	Daily methylprednisolone equivalent dose at presentation/mg increase	Daily methylprednisolone equivalent dose in mg at presentation among exposed[a]	Cardiovascular comorbidity	Cardiopulmonary comorbidity	Co-infections (antimicrobials other than anti-PCP as surrogate)	Smoking	Lymphocyte count in blood	C_T value of BAL-fluid or tracheal aspirate from semiquantitative real-time PCR-analysis for *P. jirovecii* (i.e., fungal load)
Cardiovascular comorbidity	**X**		**X**	X	X			X	**X**				X		
C_T value of BAL-fluid or tracheal aspirate from semiquantitative real-time PCR-analysis for P. jirovecii (i.e., fungal load)	X	X		X	X			X	**X**	X			X	X	
Immunosuppressive condition	X														
Daily methylprednisolone equivalent dose in mg per day at presentation among exposed[a]	X	X		X						X	X				
Methylprednisolone equivalent dose in mg per day at presentation	X	X		X						X	X				
Dyspnea	X	X		**X**	X	X	**X**			**X**	X	X			
Fever	X	X		X	X	X		**X**	**X**			X			
At least two cardinal symptoms	X	X		**X**	X	X	X	**X**	**X**	X	X	X	X		
All three symptoms	X	X		X	X	X	X	**X**	**X**	X	X	X	X		
Abnormal lung auscultation	X	X		X	**X**			**X**	**X**	X	X				
Oxygen saturation in %	X	X						X	X		X	X	X		
Lymphocyte count x 10^9/L	X	X		**X**	X	X	X	X	**X**						
Lymphopenia (< 1.0 x 10^9 cells/L)	X	**X**		X	X	X	X	**X**	**X**						
C-reactive protein in mg/L	X	X		X		X		X	X			X			
Lactase dehydrogenase level in U/L	X	X		X		X		X	X			X			
Atelectasis on thoracic CT	X	X		X	**X**	X	X	X	**X**	X	X	X	X	**X**	X
Bronchiectasis on thoracic CT	**X**	X		**X**	**X**	**X**	X	X	**X**	**X**	X	X	X	X	X
Crazy paving pattern on thoracic CT	**X**	X		**X**	**X**	X	X	X	**X**	X	X	X	X	**X**	X

[a]Methylprednisolone equivalent dose per day among 117 exposed patients having an intake the day of *P. jirovecii*-detection.

Abbreviations:

BAL, Bronchoalveolar lavage; CT, Computed tomography; C_T, cycle threshold; PCR, polymerase chain reaction.

Supplemental Table S2. Clinical data of 39 PCP^{+}-patients [a] with C_T values from semiquantitative real-time PCR-analysis of bronchoalveolar lavage fluid or tracheal aspirate in gray zone (> 36)

Patient ID	Age (years)	Sex	CCI	Smoking history	Respiratory sample and C_T value	Result DIF	Immunosuppressive condition	Immunosuppression at presentation	Corticosteroid exposure and dose at presentation (mg/day)[b]	Cardinal symptoms	Oxygen saturation (%)[c]	Neutrophil count (x 10^9/L)	Lymphocyte count (x 10^9/L)
2	64	F	8	Yes	BAL-fluid; 37	(.)	Solid tumor (genitourinary tract)	None	Intermittent; 0	Dyspnea, fever	94 (-)	2.4	(.)
7	72	F	4	No	BAL-fluid; > 40	Positive	Rheumatoid arthritis	sDMARDs in monotherapy	None	Dyspnea, fever	83 (-)	(.)	(.)
8	82	M	7	No	BAL-fluid; 37	Negative	Chronic lymphatic leukemia with AIHA/ITP	Chemotherapy and steroids	Intermittent; 0	Dyspnea	90 (-)	(.)	(.)
15	79	M	9	No	BAL-fluid; > 40	(.)	Solid tumor (genitourinary tract with pulmonary metastasis)	Chemotherapy	None	All three	93 (-)	3.5	(.)
22	69	F	6	Yes	BAL-fluid; 37	(.)	Non-Hodgkin lymphoma	Steroids in monotherapy	Intermittent; 8	Dyspnea, fever	75 (-)	17.9	(.)
25	67	M	5	Yes	BAL-fluid; 37	Positive	Non-Hodgkin lymphoma	Chemotherapy and steroids	(.); 16	Cough	(.)	17.0	2.8
30	83	M	5	No	BAL-fluid; 37	(.)	Rheumatoid arthritis	sDMARDs in monotherapy	None	Dyspnea, fever	90 (+)	16.3	0.9
36	79	F	6	No	BAL-fluid; > 40	Negative	Suspected autoimmune disease with AIHA and kidney involvement	None	None	Dyspnea	90 (-)	3.9	1.4
48	67	M	8	Yes	BAL-fluid; 38	(.)	Solid tumor (gastrointestinal tractus)	Chemotherapy	None	All three	93 (-)	0.79	(.)
49	83	M	14	Yes	BAL-fluid; 38	(.)	Solid tumor (lungs)	Chemotherapy and steroids	Intermittent; 0	All three	80 (-)	(.)	(.)
62	64	M	7	Yes	BAL-fluid; 37	(.)	Acute myeloblastic leukemia	GVHD prophylaxis or treatment	Daily; 4	Dyspnea	94 (-)	(.)	(.)
69	61	M	4	No	BAL-fluid; 39	Negative	Solid tumor (sarcoma neck region)	Chemotherapy	None	Cough, dyspnea	95 (-)	3.0	(.)
78	72	M	5	Yes	BAL-fluid; 37	Negative	Chronic lymphatic leukemia	None	None	Dyspnea, fever	92 (-)	4.0	0.45
85	55	F	3	No	BAL-fluid; > 40	(.)	Chronic myelogenous leukemia	GVHD prophylaxis or treatment	None	Dyspnea, fever	96 (-)	6.8	1.2
101	65	M	3	Yes	BAL-fluid; 37	(.)	Chronic obstructive pulmonary disease	None	None	All three	(.)	(.)	(.)
103	56	F	7	Yes	BAL-fluid; 39	(.)	Solid tumor (lungs)	Chemotherapy and steroids	Daily; 20	All three	96 (-)	8.3	0.66
109	72	M	5	No	BAL-fluid; 38	(.)	Non-Hodgkin lymphoma	Chemotherapy and steroids	Daily; 32	All three	(.)	9.4	(.)
117	82	F	6	No	BAL-fluid; 37	(.)	Non-Hodgkin lymphoma	Chemotherapy and steroids	Intermittent; 0	Dyspnea, fever	90 (-)	4.8	0.41
126	31	F	3	No	BAL-fluid; 37	Positive	Eosinophilic granulomatosis with polyangiitis	Steroids and azathioprine	Daily; 32	Dyspnea	(.)	12.8	1.0
132	33	M	2	No	BAL-fluid; 37	(.)	Hodgkin's lymphoma	Chemotherapy and steroids	Intermittent; 0	All three	98 (-)	11.2	(.)
135	67	M	6	Yes	Tracheal aspirate; > 40	(.)	Interstitial lung disease	None	None	Dyspnea, fever	74 (+)	(.)	(.)
136	64	M	5	Yes	BAL-fluid; 37	(.)	Solid organ transplant (kidney)	Graft rejection prophylaxis	Daily; 4	All three	96 (-)	(.)	(.)

143	66	M	4	No	BAL-fluid, 37	Positive	Non-Hodgkin lymphoma	Chemotherapy	None	All three	98 (-)	2.0	(.)
145	60	F	4	Yes	BAL-fluid; 37	(.)	Acute lymphoblastic leukemia	Chemotherapy and steroids	Intermittent; 0	Cough, fever	(.)	2.9	(.)
156	74	F	9	No	BAL-fluid; 37	(.)	Solid tumor (breast)	None	None	Dyspnea, fever	87 (-)	(.)	(.)
162	59	M	2	No	BAL-fluid; 37	(.)	Rheumatoid arthritis	sDMARDs and steroids	Intermittent; 0	All three	94 (-)	6.2	2.1
176	41	F	2	(.)	BAL-fluid; 37	Negative	Hodgkin's lymphoma	Chemotherapy	None	All three	93 (-)	1.5	(.)
194	76	M	7	Yes	BAL-fluid; 37	(.)	Chronic lymphatic leukemia	None	None	All three	81 (-)	2.4	1.6
203	77	M	6	Yes	BAL-fluid; 38	(.)	Autoimmune hemolytic anemia	None	Intermittent; 0	Dyspnea, fever	(.)	3.9	2.4
209	82	M	7	Yes	BAL-fluid; 37	(.)	Vasculitis	Steroids in monotherapy	Daily; 32	All three	75 (-)	9.8	0.50
211	62	M	8	Yes	BAL-fluid; 37	(.)	Solid tumor (genitourinary tract)	Chemotherapy and steroids	Intermittent; 0	Dyspnea, fever	92 (+)	1.9	0.60
220	64	M	9	Yes	BAL-fluid; > 40	Negative	Solid tumor (lungs)	Chemotherapy and steroids	Intermittent; 16	All three	78 (+)	9.5	0.20
236	62	F	5	Yes	BAL-fluid; > 40	(.)	Chronic myelogenous leukemia	Chemotherapy	None	Dyspnea, fever	92 (+)	5.7	0.99
245	25	F	2	No	BAL-fluid; 38	(.)	Non-Hodgkin lymphoma	Chemotherapy and steroids	None	Cough, dyspnea	100 (-)	2.3	1.1
253	29	F	2	No	BAL-fluid; 37	(.)	Hodgkin's lymphoma	Chemotherapy and steroids	Intermittent; 0	Cough, dyspnea	95 (-)	6.5	1.9
259	81	F	11	Yes	BAL-fluid; > 40	(.)	Multiple myeloma	Chemotherapy and steroids	Intermittent; 0	Dyspnea, fever	90 (-)	0.6	1.6
262	74	F	4	Yes	BAL-fluid; 40	(.)	Ulcerative colitis	None	None	Cough	(.)	8.2	1.4
282	76	F	5	No	BAL-fluid; 38	Negative	Non-Hodgkin lymphoma	Chemotherapy and steroids	Intermittent; 0	Cough, fever	(.)	2.3	0.30
284	73	F	5	Yes	BAL-fluid; 38	(.)	Anti-synthetase syndrome	Steroids in monotherapy	Intermittent; 0	Dyspnea, fever	85 (+)	15.6	1.1

[a]Criteria for PCP were multimodal and based on available patient data (See Methods and Supplemental Figure S1). Patients not fulfilling the criteria for their respective groups were considered colonized with *P. jirovecii* (i.e., PCP-)

[b]Systemic corticosteroid exposure 60 days preceding presentation and methylprednisolone equivalent dose in mg/day at presentation

[c]Oxygen saturation was measured with (+) or without (-) supplemental oxygen.

Abbreviations and notations:

AIHA, Autoimmune hemolytic anemia; BAL, bronchoalveolar lavage; CCI, Charlson Comorbidity index; DIF, direct immunofluorescence; F, female; GVHD, Graft *versus* host disease; ID, identification number; ITP, Immunologic thrombocytopenic purpura; M; male; PCP, *Pneumocystis* pneumonia; PCR, polymerase chain reaction; sDMARDs, synthetic disease modifying anti-rheumatic drugs; (.) = "missing".

Supplemental Table S3. Subgroup analyses of 136 PCP^{+}-patients based on semiquantitative real-time PCR-analysis of BAL-fluid or tracheal aspirate[a]				
	No. of observations in case of missing (%)	**C_T value < 31** No. (%) 22 (16.2)	**C_T value ≥ 31** No. (%) 114 (83.8)	**p-value difference**
Demographics				
Median age (q_1-q_3)	NA	65 (55-69)	65.5 (58-74)	0.31
Male sex, no. (%)	NA	15 (68.2)	64 (56.1)	0.29
History of smoking, no. (%)	131 (96.3)	12 (54.5)	63 (57.8)	0.78
Median Charlson comorbidity index (q_1-q_3)	NA	4.5 (4-7)	6 (4-8)	0.14
Immunosuppressive condition				
Distribution across PCP-groups no. (%)	133 (97.8)[b]			0.050
Hematological malignancies		7 (31.8)	45 (40.5)	
Solid tumors		6 (27.3)	31 (27.9)	
Immunological disorders		1 (4.5)	18 (16.2)	
Solid organ transplantation		8 (36.4)	12 (10.8)	
Chronic lung diseases		0 (0.0)	5 (4.5)	
Iatrogenic immunosuppression, chemotherapy and corticosteroid exposure at presentation				
Regimen at presentation, no. (%)	NA			0.059
Chemotherapy for hematological malignancy and adjuvant steroids		4 (18.2)	27 (23.7)	
Chemotherapy for solid tumor and adjuvant steroids		4 (18.2)	11 (9.6)	
Chemotherapy for hematological malignancy		2 (9.1)	5 (4.4)	
Chemotherapy for solid tumor		0 (0)	9 (7.9)	
Corticosteroids in monotherapy		2 (9.1)	14 (12.3)	
Graft rejection prophylaxis after SOT		8 (36.4)	12 (10.5)	
DMARDs with or without adjunctive steroids		1 (4.5)	11 (9.6)	
Other combinations[c]		0 (0)	5 (4.4)	
None		1 (4.5)	20 (17.5)	
Systemic corticosteroid exposure pattern 60 days preceding presentation, no. (%)	134 (98.5)			0.056
Daily		12 (57.1)	41 (36.3)	0.037
Intermittent		7 (33.3)	36 (31.9)	0.13
None		2 (9.5)	36 (31.9)	Ref.
Methylprednisolone equivalent dose in mg/day at presentation, median (q_1-q_3)[d]	134 (98.5)	10 (4-24)	10 (6-20)	0.57

[a]Criteria for PCP were multimodal and based on available patient data (See Methods and Supplemental Figure S1). Patients not fulfilling the criteria for their respective groups were considered colonized with *P. jirovecii* (i.e., PCP^{-})

[b]Three patients had immunosuppressive conditions classified as miscellaneous and were excluded from the comparative analysis.

[c]Other combinations include exposure to other immunosuppressants (mycophenolate, azathioprine, cyclophosphamide, calcineurin- and mTOR-inhibitors, cyclosporine and hydroxychloroquine with or without adjuvant steroids) and one patient receiving both graft rejection prophylaxis for solid organ transplantation and chemotherapy for hematological malignancy with adjuvant corticosteroids

[d]Median methylprednisolone equivalent dose was calculated among 63 patients having an intake the day of *P. jirovecii*-detection.

Abbreviations:

BAL, bronchoalveolar lavage; CT, computed tomography; C_T, cycle threshold; DMARDs, disease modifying anti-rheumatic drugs; NA, not applicable; PCR, polymerase chain reaction; Ref., reference group in logistic regression analysis; SOT, solid organ transplantation.

Supplemental Table S4. Summary of linear regression analysis for immunosuppressive conditions predicting C_T values from semiquantitative real-time PCR-analysis of BAL-fluid or tracheal aspirate for *Pneumocystis jirovecii*-detection[a]

Model (167 observations)	**Coefficient**	**Standard Error**	**t**	**P>|t|**	**95 % Confidence interval**
Immunosuppressive condition					
Hematological malignancy	NA	NA	NA	NA	NA
Solid tumor	-0.78	0.75	-1.04	0.30	(-2.26)-0.70
Immunological disorder	1.28	0.88	1.46	0.15	-0.45-3.01
Solid organ transplantation	-1.86	0.9	-2.09	0.038	(-3.60)-(-0.11)
Chronic lung disease	1.50	1.35	1.11	0.27	(-1.17)-4.17
Constant	35.2	0.48	72.5	<0.001	34.2-36.1

[a](F(4,162) = 3.03; p = 0.019, R^2 = 0.07), adjusted R^2 0.05, Root MSE 3.79.

Abbreviations:

BAL, bronchoalveolar lavage; C_T, cycle threshold; NA, PCR, polymerase chain reaction

Supplemental Table S5. Validity of semiquantitative real-time PCR-analysis of BAL-fluid or tracheal aspiration for discrimination between *Pneumocystis* pneumonia and colonization across immunosuppressive conditions in ROC analyses.

Immunosuppressive condition (no. of patients)	Observations, no. (%)[a]	AUC (95 % confidence interval)	p-value[c]
Hematological malignancy (89)	61 (68.5)	0.82 (0.66-0.98)	0.002
Solid tumor (68)	44 (64.7)	0.78 (0.63-0.92)	0.022
Immunological disorder (38)	27 71.1)	0.72 (0.48-0.97)	0.071
Solid organ transplantation (29)	26 (89.7)	0.94 (0.83-1.00)	0.001
Chronic lung disease (13)	9 (69.2)	0.73 (0.370-1.00)	0.27
Other/miscellaneous[b]	5 (2.1)	NA	NA
Population overall (N = 242)	171[a] (70.7)	0.80 (0.73-0.88)	< 0.001

[a]Missing data were independent of immunosuppressive condition (p = 0.25). Proportion (%) refers to the number of observations within the sub-group of immunosuppressive conditions.

[b]Other/miscellaneous immunosuppressive conditions included four patients with no diagnosed condition, whereas two had received steroids for suspected autoimmune disorder and one patient with statin-induced myositis treated with corticosteroids. Sub-group ROC-analysis was not performed for this group.

[c]The reported p-values corresponds to a null-hypothesis of AUC = 0.5.

Abbreviations:

AUC, area under curve; BAL, Bronchoalveolar lavage; NA, not applicable; PCR, polymerase chain reaction; ROC, receiver operating characteristics.

Supplemental figures

FIGURE LEGENDS

Figure S1. *Pneumocystis* pneumonia case definition for study population.

Based on available data three patient-groups were identified and the following criteria for PCP were applied: *Group 1* i) immunosuppressive state and ii) positive DIF, *Group 2* (characterized by missing or negative DIF microscopy-result) i) immunosuppressive state ii) at least one cardinal symptom of PCP (cough, dyspnea and fever) iii) typical findings on thoracic CT (ground glass opacities and/or infiltrates) and iv) presumptive diagnosis at time of diagnosis; i.e. receiving anti-PCP treatment, *Group 3*: Patients who died in-hospital within 30 days of detection without receiving anti-PCP treatment were evaluated individually with respect to cause of death and PCP-status. Patients not fulfilling the criteria for their respective groups were considered colonized with *P. jirovecii*.

Figure S2. Relationship between microscopic examination and semiquantitative real-time PCR-results. DIF microscopy was performed on 99 of 242 respiratory samples (BAL-fluid (n = 82), sputum (n = 10), induced sputum (n = 4), tracheal aspirate (n = 3). The presence of *Pneumocystis jirovecii* was confirmed in 44 (44.4%) samples. With PCR-analysis as a reference for *P. jirovecii*-detection, the sensitivity of DIF microscopy (i.e., positive examination) was positively associated with low C_T values (i.e., higher fungal loads), regardless of respiratory sample-type (adjusted OR 0.77 95 % CI 0.66-0.89, $p < 0.001$) (*). (**Supplemental Figure S2**).

BAL, bronchoalveolar lavage; C_T, cycle threshold; DIF, direct immunofluorescence; PCR, polymerase chain reaction.

Figure S3. Distribution of semiquantitative real-time PCR-results according to PCP-status. Retrospectively 196 patients were diagnosed with PCP (i.e., PCP^+) while 46 were presumed colonized (i.e., PCP^-). C_T values from semiquantitative real-time PCR-analysis of BAL-fluid or tracheal aspirate overlapped but were significantly lower (i.e., higher fungal loads) among PCP^+-patients ($p < 0.01$) (*). Median C_T value for the population overall was 36 (red horizontal line).

BAL, bronchoalveolar lavage; C_T, cycle threshold; PCP, *Pneumocystis* pneumonia; PCR, polymerase chain reaction.

Figure S4. Distribution of semiquantitative real-time PCR-results according to PCP-status. Retrospectively 196 patients were diagnosed with PCP (i.e., PCP^+) while 46 were presumed colonized (i.e., PCP^-). C_T values of BAL-fluid or tracheal aspirate overlapped though PCP^+-patients had a significantly lower median (i.e., higher fungal loads) (35 *vs.* 38, $p < 0.01$).

BAL, bronchoalveolar lavage; C_T, cycle threshold; PCP, *Pneumocystis* pneumonia; PCR, polymerase chain reaction.

Figure S5. Validity of semiquantitative real-time PCR for differentiation between *Pneumocystis* pneumonia and colonization. Sensitivity (blue line), specificity (orange line) and percentage correctly classified (green line) according to various C_T values as clinical cut-offs for differentiation between PCP and colonization based on 171 observations from semiquantitative real-time PCR-analysis of BAL-fluid or tracheal aspirate.

C_T, cycle threshold; PCP, *Pneumocystis* pneumonia; PCR, polymerase chain reaction.

Figure S6. Relationship between semiquantitative real-time PCR-results, corticosteroid dose and corticosteroid exposure pattern. Methylprednisolone equivalent dose (mg/day) at presentation and exposure pattern the preceding 60 days according to C_T values of BAL-fluid or tracheal aspirate based on 169 observations. The distribution of corticosteroid doses according to C_T values was non-linear. Median C_T value of BAL-fluid or tracheal aspirate was 36 (red horizontal line).

BAL, bronchoalveolar lavage; C_T, cycle threshold; *P. jirovecii*, *Pneumocystis jirovecii;* PCR, polymerase chain reaction.

Figure S7. Relationship between semiquantitative real-time PCR-results and immunosuppressive regimen. Immunosuppressive regimen at presentation according to C_T value of BAL-fluid or tracheal aspirate based on 171 observations.

BAL, bronchoalveolar lavage; Chemoth., chemotherapy; C_T, cycle threshold; hem, hematological; *P. jirovecii*, *Pneumocystis jirovecii;* PCR, polymerase chain reaction; SOT, solid organ transplantation.

Figure S8A-C. ROC-curves of semiquantitative real-time PCR-results of BAL-fluid or tracheal aspirate for discrimination between *Pneumocystis* pneumonia and colonization. **A)** ROC-curve for patients with solid tumors based on 44 samples; **B)** ROC-curve for patients with immunological disorders based on 27 samples; **C)** ROC-curve for patients with chronic lung diseases based on 9 samples.

BAL, bronchoalveolar lavage; C_T, cycle threshold; PCR, polymerase chain reaction, ROC, receiver operating characteristics.

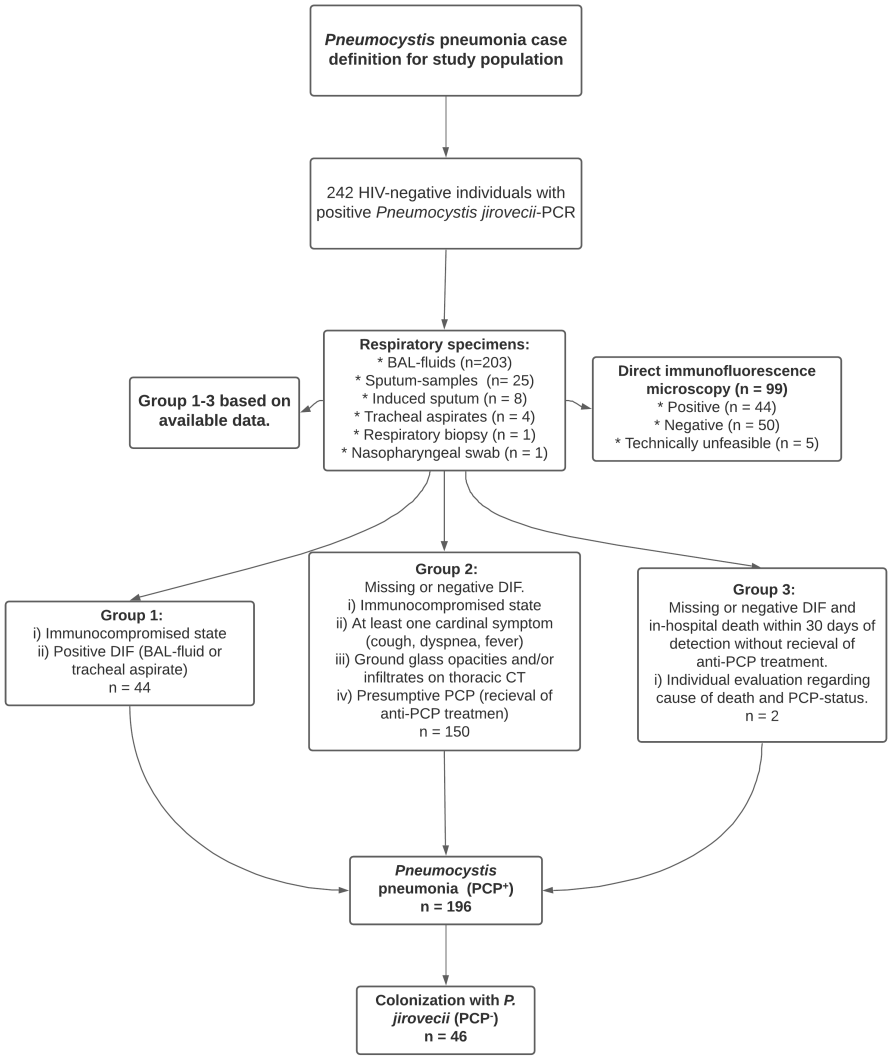

Pneumocystis pneumonia case definition for study population
242 HIV-negative individuals with positive *Pneumocystis jirovecii*-PCR
Respiratory specimens:
* BAL-fluids (n=203)
* Sputum-samples (n= 25)
* Induced sputum (n = 8)
* Tracheal aspirates (n = 4)
* Respiratory biopsy (n = 1)
* Nasopharyngeal swab (n = 1)
Group 1-3 based on available data.
Direct immunofluorescence microscopy (n = 99)
* Positive (n = 44)
* Negative (n = 50)
* Technically unfeasible (n = 5)
Group 1:
i) Immunocompromised state
ii) Positive DIF (BAL-fluid or tracheal aspirate)
n = 44
Group 2:
Missing or negative DIF.
i) Immunocompromised state
ii) At least one cardinal symptom (cough, dyspnea, fever)
iii) Ground glass opacities and/or infiltrates on thoracic CT
iv) Presumptive PCP (recieval of anti-PCP treatmen)
n = 150
Group 3:
Missing or negative DIF and in-hospital death within 30 days of detection without recieval of anti-PCP treatment.
i) Individual evaluation regarding cause of death and PCP-status.
n = 2
Pneumocystis pneumonia (PCP+)
n = 196
Colonization with *P. jirovecii* (PCP-)
n = 46

Figure S1

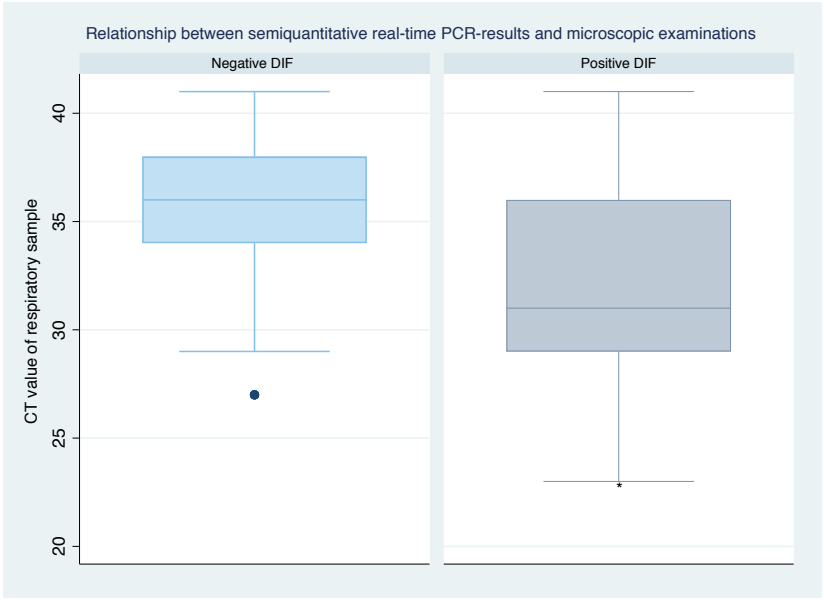


Figure S2

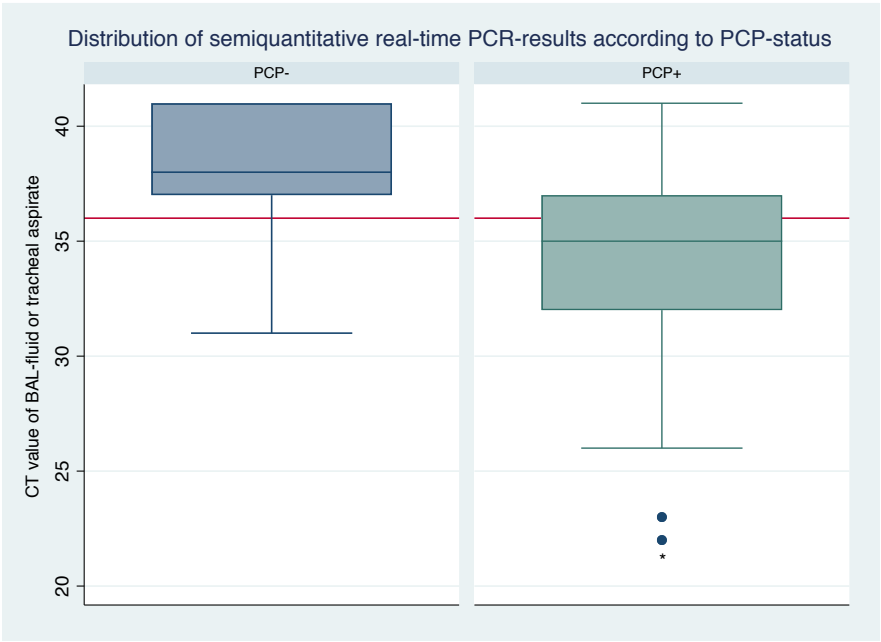


Figure S3

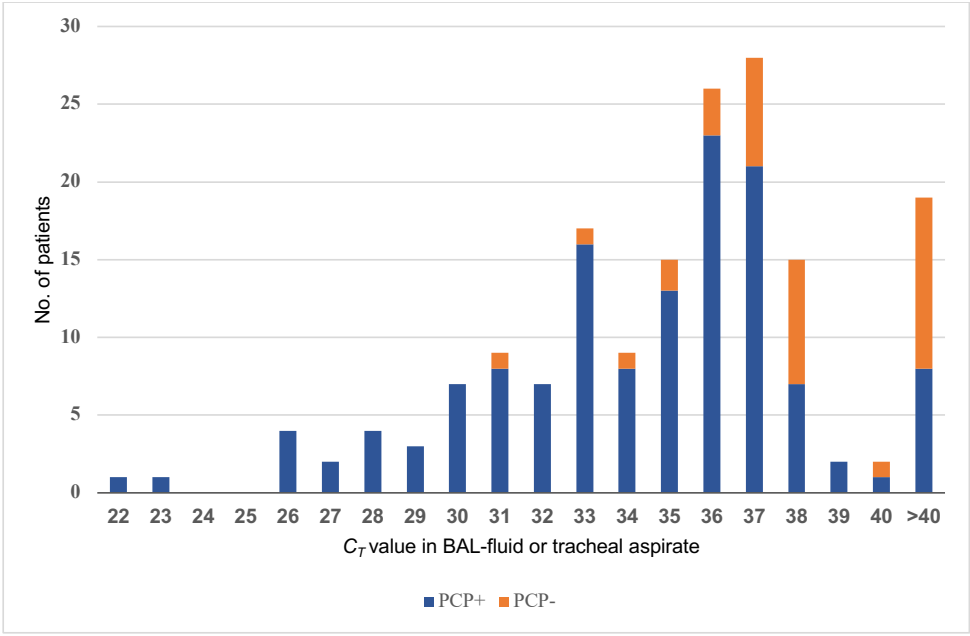


Figure S4

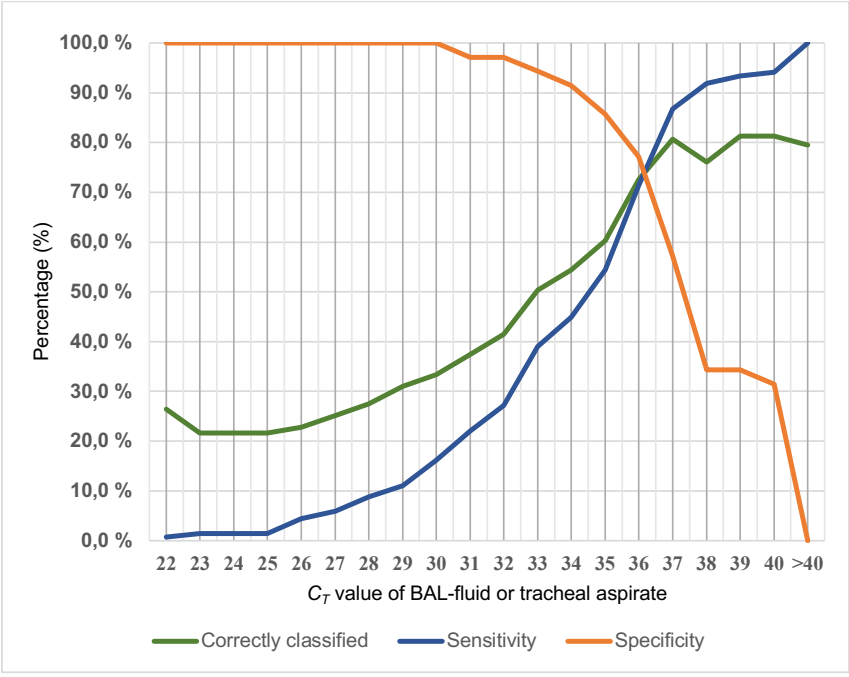
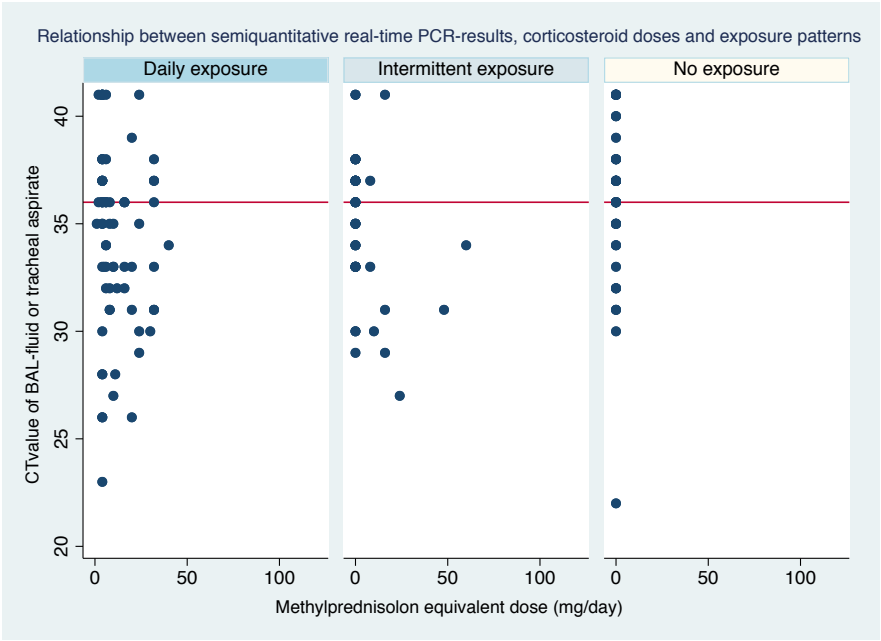


Figure S5



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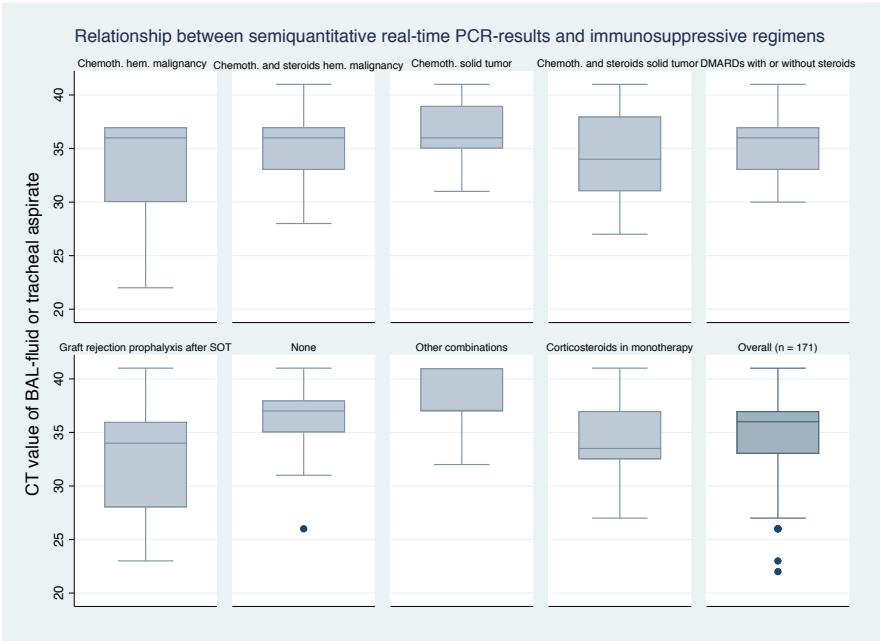

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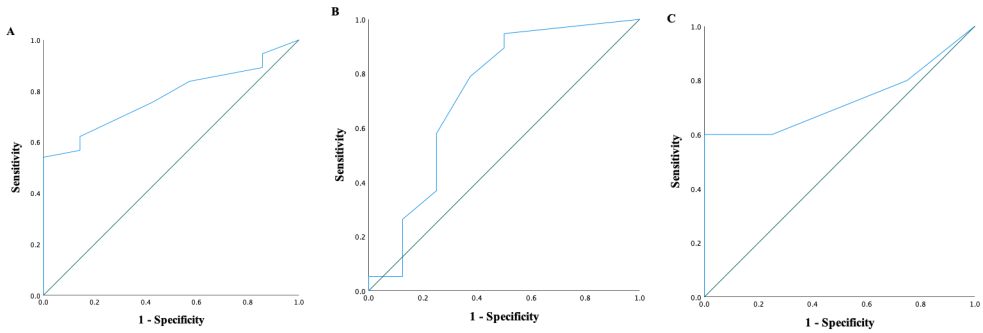
A
Sensitivity
1 - Specificity
B
Sensitivity
1 - Specificity
C
Sensitivity
1 - Specificity

Figure S8A-C

Paper III

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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 ≤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.

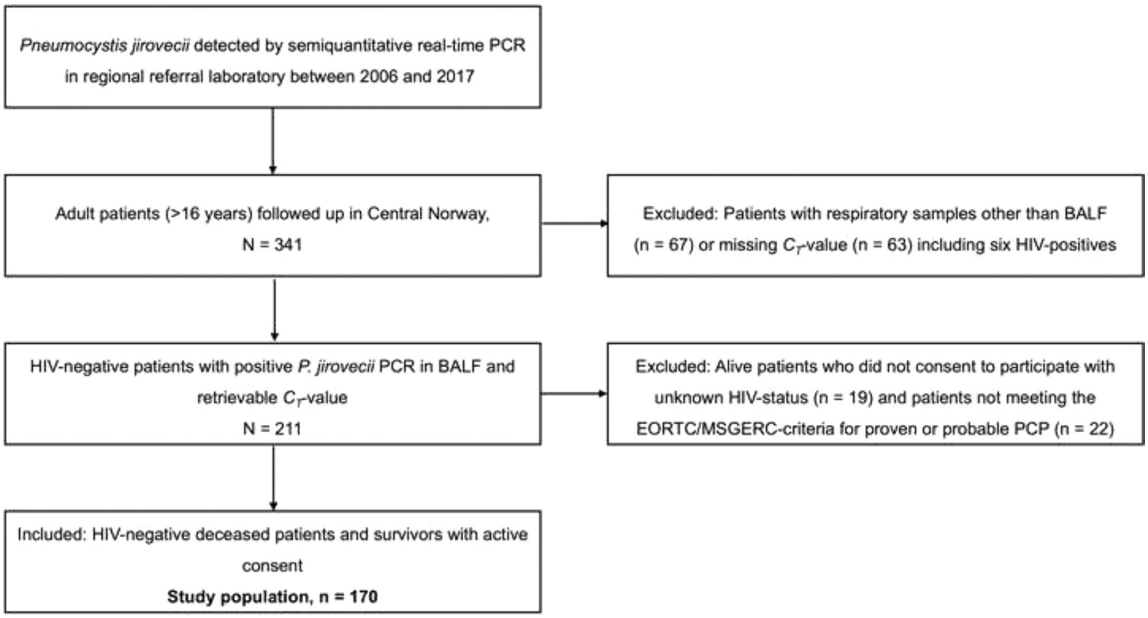

Figure 1. Flowchart of study design. Adult patients without HIV with positive *Pneumocystis jirovecii* semiquantitative real-time PCR in the regional referral laboratory from 2006 to 2017 were screened for eligibility. Patients with respiratory samples other than BALF, missing C_T value, or who did not 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.

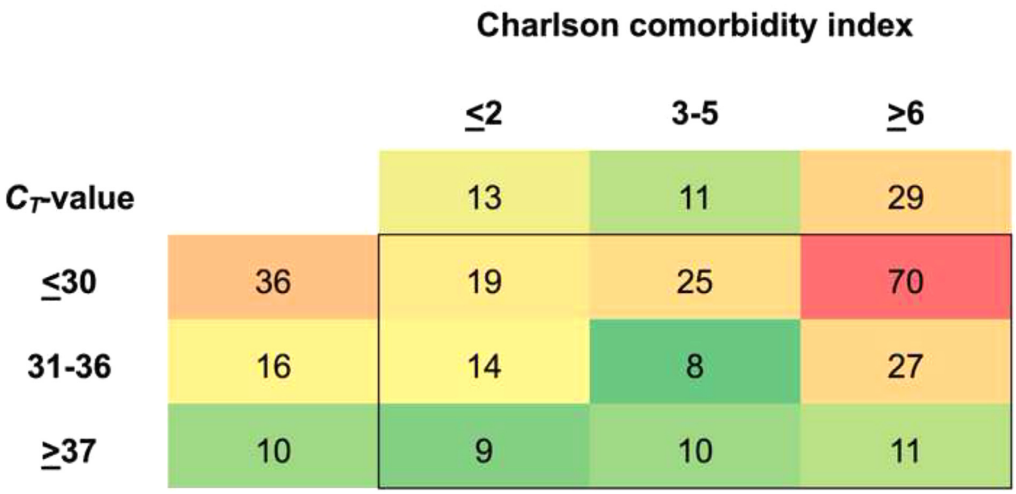

Figure 2. Mortality risk in patients with PCP. Heat map illustrating 30-day mortality (in %) in the study population of 170 patients without HIV within subgroups of Charlson comorbidity index, C_T value from semiquantitative real-time polymerase chain reaction for *P. jirovecii* detection in bronchoalveolar 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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Supplementary material to: "Role of fungal burden in risk stratification of non-HIV patients with *Pneumocystis* pneumonia: A 12-year retrospective observational multicenter cohort"

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Supplementary Table 1. Fulfillment of European Organization for Research and Treatment of Cancer/Mycoses Study Group Education and Research Consortium-criteria according to underlying disease.

Immunosuppressive condition, n (%)	n with data (%)	Hematological malignancy, n (%)	Solid tumor, n (%)	Solid organ transplantation, n (%)	Immunological disorder, n (%)	Chronic lung disease, n (%)	Miscellaneous conditions[a], n (%)	Study population
		65 (28.3)	43 (25.3)	28 (16.5)	25 (14.7)	7 (4.1)	2 (1.2)	170 (100.0)
Iatrogenic host criteria besides underlying disease								
Chemotherapy/iatrogenic immunosuppression at presentation, n (%)	170 (100)	53 (81.5)	38 (88.4)	28 (100)	23 (92.0)	7 (100)	2 (100)	150 (88.2)
Chemotherapy/iatrogenic immunosuppression preceding five years, n (%)	170 (100)	64 (98.5)	42 (97.7)[b]	28 (100)	24 (96.0)[c]	7 (100)	2 (100)	167 (98.2)
Median lymphocyte count	86 (50.6)	0.6 (0.3-1)	0.5 (0.2-1.1)	0.7 (0.6-1.1)	0.7 (0.5-1.1)	1.3 (0.8-1.7)	0.29 (0.29-0.29)	0.65 (0.4-1.1)
Clinical radiological criteria								
Compatible clinical presentation	170 (100)	65 (100)	43 (100)	28 (100)	25 (100)	7 (100)	2 (100)	170 (100)
Cardinal symptoms (cough, fever, dyspnea)	170 (100)	65 (100)	43 (100)	28 (100)	23 (92.0)	7 (100)	2 (100)	168 (98.8)
Documented hypoxemia by ABG or O_2-saturation[d]	151 (88.8)[e]	45/54 (83.3)	37/42 (88.1)	22/27 (81.5)	20/21 (95.2)	5/5 (100)	1/2 (50.0)	130/151 (86.1)
Compatible radiologic findings	170 (100)	65 (100)	43 (100)	28 (100)	25 (100)	7 (100)	2 (100)	170 (100)
On thoracic CT	153 (90.0)[e]	57/57 (100)	40/40 (100)	25/25 (100)	22/22 (100)	7/7 (100)	2/2 (100)	153/153 (100)
On CXR given missing CT	17 (10.0)[e]	8/8 (100)	3/3 (100)	3/3 (100)	3/3 (100)	0/0 (0)	0/0 (0)	17/17 (100)
Microbiological criteria								
Positive DIF	61 (35.9)[e]	17/27 (63.0)	4/14 (28.6)	11/13 (84.6)	2/6 (33.3)	0/1 (0)	NA	34/61 (55.7)
Positive *P. jirovecii* PCR	170 (100)	65 (100)	43 (100)	28 (100)	25 (100)	7 (100)	2 (100)	170 (100)
EORTC-classification	170 (100)							
Proven PCP		17 (26.2)	4 (9.3)	11 (39.3)	2 (8.7)	0 (0)	0 (0)	34 (20.0)
Probable PCP		48 (73.8)	39 (90.7)	17 (60.7)	23 (92.0)	7 (100)	2 (100)	136 (80.0)

[a]Miscellanoues conditions included statin-induced myositis treated with corticosteroids (n = 1) and no definite diagnosis at presentation (n = 1).
[b]One patient with solid tumor who suffered from pancreatic cancer with lung metastases had not received chemotherapy but radiotherapy. He was lymphopenic with a lymphocyte count of 0.5 cells/mm^3 and met the remaining criteria for "probable" PCP including positive *P. jirovecii* PCR (C_T-value = 35 in BALF).
[c]One patient with dermatopolymyositis had not received any immunosuppressants prior to presentation, but he was classified with "probable" PCP based on fulfilled radioloclinical criteria, lymphocyte count of 0.5 and positive *P. jirovecii* PCR (C_T-value = 35 in BALF).
[d]Hypoxemia was defined as partial oxygen pressure < 9.6 kPa on ABG or O_2-saturation < 95 %.
[e]Due to missing data, the row shows the fraction of "n with characteristic/n examined" (%).
Abbreviations: ABG, arterial blood gas; BALF, bronchoalveolar lavage-fluid; CXR, chest X-ray; CT, computed tomography; C_T, cycle threshold; DIF, direct immunofluorescence microscopy; NA, not assessed; PCP, *Pneumocystis* pneumonia; PCR, polymerase chain reaction.

Supplementary Table 2. Independent variables and respective covariates included in multivariable logistic regression analyses.										
		Confounders identified *a priori* (covariates)								
Exposure variables	**n (%) with available data**	Age	Sex	Cardiovascular and/or chronic heart failure	Comorbid chronic lung disease	Comorbid rheumatic disease	Underlying disease	Premorbid methylprednisolone-equivalent dose	Year/period (before or after 2011)[b]	Coinfection[c]
Age	170 (100)									
Sex	170 (100)									
Charlson comorbidity index	170 (100)									
Hypertension	170 (100)									
Cardiovascular disease/congestive heart failure	170 (100)									
Comorbid chronic lung disease	170 (100)									
Diabetes mellitus	170 (100)									
Malignancy	170 (100)									
Chronic kidney disease	170 (100)									
Any comorbidity	170 (100)									
Underlying disease	170 (100)									
Premorbid methylprednisolone-equivalent dose	168 (98.8)									
Cough	170 (100)									
Dyspnoea	170 (100)									
Fever	170 (100)									
Three cardinal symptoms	170 (100)									
O_2-saturation	146 (85.9)						X			
C_T-value from PCR in BALF	170 (100)						X			
Leukocytes x 10^9/L	168 (98.8)									
Neutrophils x 10^9/L	137 (80.6)									
Lymphocytes x 10^9/L	86 (50.6)							X		
Albumin, per g/L	127 (74.7)									
C-reactive protein mg/L	167 (98.2)						X			
Lactate dehydrogenase U/L	105 (61.8)									
GGO/infiltrates on thoracic CT	153 (90.0)									
Crazy paving pattern on thoracic CT	153 (90.0)									
Crazy paving pattern vs. GGO/infiltrates on thoracic CT	153 (90.0)									

[a]We draw direct acyclic graphs for each exposure variable of interest to identify confounders. Gray shading indicates inclusion of covariates in the multivariable analyses to adjust for confounding.

[b]Neither year nor period were associated with the outcomes and were not included as covariates in the multivariable analyses.

[c]Co-infections were not included as co-variates due to incomplete microbiological ascertainment in the records but based on available data four patients had probable CMV-infection and one patient was co-infected with *Legionella peneumophila.*

Abbreviations: BALF, bronchoalveolar lavage-fluid; CT, computed tomography, C_T value, cycle threshold-value; GGO, ground glass opacities; PCR, polymerase chain reaction; X, covariate was excluded from model due to multicollinearity.

Supplementary Table 3. Univariable comparison of patients with positive *Pneumocystis jirovecii* PCR in BALF and retrievable C_T-value in Central Norway between 2006 and 2017 by consent to participate.

	n with available data (%)	Passive refusal, n = 19	Active consent, n = 84	p	Presumed eligible population, N = 211
Age years, median (q_1-q_3)	211 (100.0)	56 (35-71)	65 (56-73)	0.13	66 (57-74)
Male sex, n (%)	211 (100.0)	7 (36.8)	50 (59.5)	0.08	118 (55.9)
Time period	211 (100.0)			0.08	
2006-2011		0 (0)	12 (14.3)		94 (27.6)
2012-2017		19 (100)	93 (80.2)		178 (84.4)
C_T-value in BALF, median (q_1-q_3)	211 (100.0)	36 (34-37)	36 (33-37)	0.73	36 (33-37)
Hospital	211 (100.0)			0.41	
University hospital		12 (63.2)	61 (72.6)		139 (65.9)
Local hospital		7 (36.8)	23 (27.4)		72 (34.1)

Abbreviations: BALF, bronchoalveolar lavage-fluid; C_T value, cycle threshold-value; PCR, polymerase chain reaction.

Supplementary Table 4. Sub-group analyses of fungal burden and risk of 30-day mortality in 158 patients receiving *anti*-pneumocystis treatment.

Risk factor	n with available data (%)	n category	Events, n (%)[a]	Crude and inverse probability weighted odds ratios[b]							
				Univariable				Multivariable			
Covariates[c]				Crude OR (95 % CI)	p	Weighted OR (95 % CI)	p	Crude OR (95 % CI)	p	Weighted OR (95 % CI)	p
***C*$_T$-value from PCR in BALF**	158 (100)				0.06		0.06				
≤30		28	10 (35.7)	3.61 (1.14-11.5)	0.03	3.52 (1.10-11.3)	0.03	4.21 (1.13-15.6)	0.03	4.19 (1.07-16.4)	0.04
31-36		85	14 (16.5)	1.28 (0.46-3.60)	0.64	1.28 (0.45-3.63)	0.64	1.09 (0.35-3.36)	0.88	1.09 (0.36-3.32)	0.88
≥37		45	6 (13.3)	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-
Age, per year								1.02 (0.98-1.06)	0.31	1.02 (0.98-1.07)	0.26
Male sex								1.42 (0.55-3.68)	0.47	1.47 (0.54-3.98)	0.45
Methylprednisolone-equivalent dose, mg/day											
0							-	1 (ref.)	-	1 (ref.)	-
1-7								0.76 (0.20-2.83)	0.68	0.75 (0.22-2.57)	0.64
8-19								4.07 (1.22-13.5)	0.02	4.22 (1.11-16.1)	0.04
≥20								2.51 (0.75-8.42)	0.14	2.62 (0.75-9.23)	0.13
Chronic lung disease								2.42 (0.81-7.19)	0.11	2.41 (0.73-7.99)	0.15

[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 non-participation 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) for comparisons.
[c]Covariates were included based on *a priori* knowledge and drawing of direct acyclic graphs.
Abbreviations: BALF, bronchoalveolar lavage-fluid; CI, confidence interval; C_T, cycle threshold; HR, hazard ratio; OR, odds ratio; PCR, polymerase chain reaction.

Supplementary Table 5. Sub-group analyses of risk of 30-day mortality in 143 patients with C_T-values ≤37.

Risk factor	n with available data (%)	n category	Events, n (%)[a]	Crude and inverse probability weighted odds ratios[b]							
				Univariable				Multivariable			
Covariates[c]				Crude OR (95 % CI)	p	Weighted OR (95 % CI)	p	Crude OR (95 % CI)	p	Weighted OR (95 % CI)	p
C_T-value from PCR in BALF	143 (100)				0.001		<0.01				
≤29		18	7 (38.9)	6.55 (1.92-22.3)	<0.01	6.42 (1.87-22.0)	<0.01	9.37 (2.23-39.3)	<0.01	9.53 (2.39-37.9)	0.001
30-33		46	14 (30.4)	4.50 (1.66-12.2)	<0.01	4.55 (1.67-12.4)	<0.01	3.87 (1.29-11.6)	0.02	3.94 (1.41-11.0)	<0.01
34-37		79	7 (8.9)	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-
Age, per year								1.03 (0.98-1.07)	0.23	1.03 (0.99-1.07)	0.17
Male sex								1.43 (0.52-3.91)	0.48	1.50 (0.53-4.24)	0.44
Methylprednisolone-equivalent dose, mg/day											
0								1 (ref.)	-	1 (ref.)	-
1-7								0.44 (0.09-2.06)	0.30	0.43 (0.12-1.50)	0.19
8-19								2.29 (0.65-8.07)	0.20	2.41 (0.67-8.64)	0.18
≥20								2.16 (0.61-7.64)	0.23	2.27 (0.57-9.05)	0.24
Chronic lung disease								1.44 (0.45-4.59)	0.54	1.45 (0.42-4.94)	0.56

[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 non-participation 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) for comparisons.
[c]Covariates were included based on *a priori* knowledge and drawing of direct acyclic graphs.
Abbreviations: BALF, bronchoalveolar lavage-fluid; CI, confidence interval; C_T, cycle threshold; HR, hazard ratio; OR, odds ratio; PCR, polymerase chain reaction.

Figure legends

Supplementary Figure 1. Flowchart of study design and statistical analyses. HIV-negative adult patients with positive *Pneumocystis jirovecii* semiquantitative real-time PCR in the regional referral laboratory from 2006-2017 were screened for eligibility. Patients with respiratory samples other than BALF, missing C_T-value, or who did not meet the EORTC/MSGERC 2021-criteria for "proven" or "probable" PCP were excluded [1]. Inclusion of survivors required active consent (i.e., returning signed information letter by postal mail) while all eligible deceased patients were recruited. HIV-status was available in consenting survivors and deceased patients, and six HIV-positives were excluded during the screening process. With the data available regardless of consent (age, sex, and hospital (university vs. local) we performed sensitivity analyses applying inverse probability weighted regression adjustment to address participation bias.

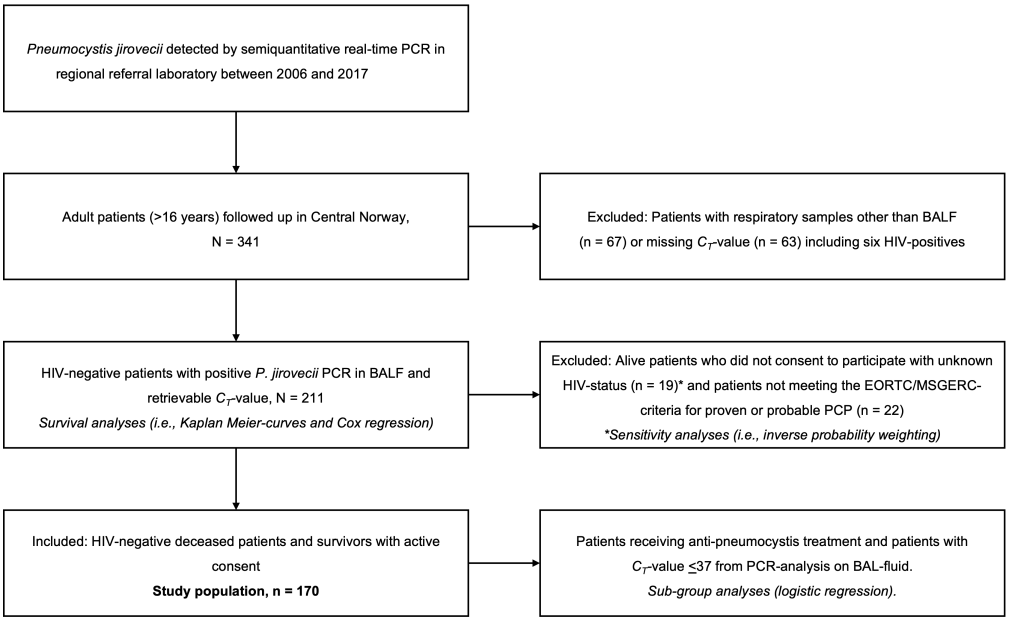
BALF, bronchoalveolar lavage-fluid; C_T, cycle threshold; EORTC/MSGERC, European Organization for Research and Treatment of Cancer/Mycoses Study Group Education and Research Consortium; HIV, human immunodeficiency virus; PCR, polymerase chain reaction.

Supplementary Figure 2. *Pneumocystis jirovecii* mortality in Central Norway between 2006 and 2017. Cumulative 30-day mortality by C_T-value among all patients with positive *P. jirovecii* PCR in BALF and retrievable C_T-value between 2006 and 2017 (N = 211).

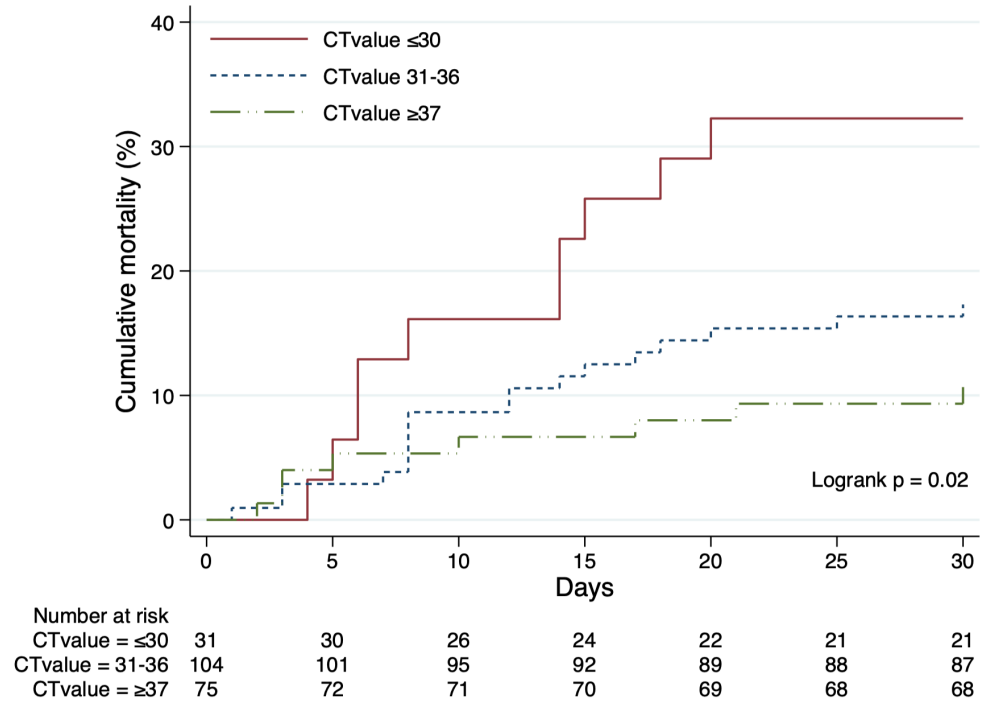
BALF, bronchoalveolar lavage-fluid; C_T, cycle threshold; HIV, human immunodeficiency virus; p, p-value; PCR, polymerase chain reaction.

Supplementary Figure 3. Mortality risk in sub-group of patients with positive *Pneumocystis jirovecii* PCR. Heat map of 143 patients with C_T-value $\leq$37 illustrating 30-day mortality (in %) within subgroups of Charlson comorbidity index, C_T-value from semiquantitative real-time PCR for *P. jirovecii* detection, and their interaction (framed in black). We adjusted for age, sex, and participation bias through inverse probability weighting.

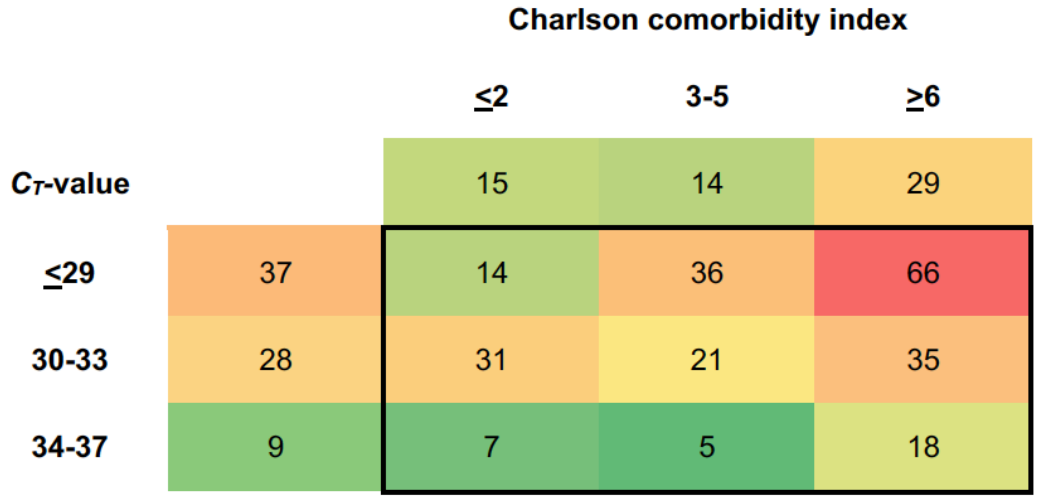
BALF, bronchoalveolar lavage-fluid; C_T, cycle threshold; HIV, human immunodeficiency virus; PCR, polymerase chain reaction.



Supplementary Figure 1.



Supplemental Figure 2.



Supplementary Figure 3.

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[1] K. Lagrou, S. Chen, H. Masur, C. Viscoli, C.F. Decker, L. Pagano, A.H. Groll, Pneumocystis jirovecii Disease: Basis for the Revised EORTC/MSGERC Invasive Fungal Disease Definitions in Individuals Without Human Immunodeficiency Virus, Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 72(Suppl 2) (2021) S114-s120. DOI:https://doi.org/10.1093/cid/ciaa1805

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