Thesis

Pulmonary Aspergillosis in Critically Ill COVID-19 Patients in Intensive Care Units

submitted by

Jonas Gregor Frost

in partial fulfillment of the requirements for the degree of

Doktor der gesamten Heilkunde

(Dr. med. univ.)

at the

Medical University of Graz

executed at the **University department of**
Internal Medicine, Division of Infectious Diseases

under the supervision of

Priv.-Doz. Dr. med. univ. Dr. scient. med. Jürgen Prattes

Dr. med. univ. Peter Zechner

Santa Elena, 18.10.2022

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Santa Elena, 18.10.2022 *Jonas Gregor Frost m.p.*

Acknowledgements

First, I sincerely thank my supervisor Jürgen Prattes for giving me the opportunity to conduct my diploma thesis on this current topic. It was a challenging, but also very rewarding time in which I was able to build up knowledge and skills in the field of clinical research. Without his guidance, support and numerous consultations, I would not have been able to carry out the work in this form.

I would also like to thank Alexander Avian for his support during several video calls in questions about the appropriate use of statistic methods in this study. Furthermore, I would like to express my gratitude to my godmother, who helped to answer my questions about the English language and proofread this work.

Special thanks go to my friends who made my time in Graz an unforgettable experience. Finally, I dedicate this thesis to my parents. Besides the luxury of being able to study without financial worries, I am infinitely grateful for their emotional support, trust and kindness.

Table of Contents

List of Abbreviations

% = per cent

°C = degree Celsius

+ssRNA = positive-sense single-stranded ribonucleic acid

Abs = antibodies

ACE2 = angiotensin-converting enzyme 2

ARAF = azole-resistant A. *fumigatus*

ARDS = acute respiratory distress syndrome

ARF = acute respiratory failure

BALF = bronchoalveolar lavage fluid

BDG = (1,3)-β-D-glucan

CAPA = COVID-19-associated pulmonary aspergillosis

CAR = community-acquired respiratory

CD = cluster of differentiation

CFR = case fatality rate

CI = confidence interval

COPD = chronic obstructive pulmonary disease

CoV = coronavirus

COVID-19 = coronavirus disease 2019

CPA = chronic pulmonary aspergillosis

CRP = C-reactive protein

CT = computed tomography

DC = dendritic cell

ECMM = European Confederation for Medical Mycology

ECMO = extracorporeal membrane oxygenation

eCRF = electronic case report form

EIA = enzyme immunoassay

EORTC MSGERC = European Organization for Research and Treatment of Cancer/Mycosis Study Group Education and Research Consortium

GM = galactomannan

HCoV = human coronavirus

HFNC = high-flow nasal cannula

HSCT = hematopoietic stem cell transplant

IA = invasive aspergillosis

IAPA = influenza-associated pulmonary aspergillosis

IATB = invasive *Aspergillus* tracheobronchitis

IC = informed consent

ICU = intensive care unit

IFD = invasive fungal disease

IFN = interferon

IFR = infection fatality rate

IL = interleukin

ILI = influenza-like illness

IMV = invasive mechanical ventilation

IPA = invasive pulmonary aspergillosis

ISG = IFN-stimulated gene

ISHAM = International Society for Human and Animal Mycology

JAK = Janus kinase

L = liter

LFA = lateral flow assay

LFD = lateral flow device

LRT = lower respiratory tract

MERS = Middle East respiratory syndrome

mg = milligram

NIV = non-invasive ventilation

NSP = non-structural protein

ODI = optical density index

ORF = open reading frame

PCR = polymerase chain reaction

POC = point-of-care

R_0 = basic reproduction number

R_E = effective reproduction number

RBD = receptor-binding domain

RCT = randomized-controlled trial

SARS = severe acute respiratory syndrome

SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

SOT = solid organ transplant

TA = tracheal aspirate

TLR = Toll-like receptor

VOC = variant of concern

WHO = World Health Organization

List of Figures

List of Tables

Zusammenfassung

Hintergrund: Die invasive pulmonale Aspergillose (IPA) ist eine lebensbedrohliche Pilzinfektion, die in erster Linie Personen mit Immundefizienz betrifft. Neben dieser Hauptrisikogruppe wurde in erheblichem Umfang auch von IPA auf der Intensivstation, insbesondere bei schwerer Influenza, berichtet. Nach Ausbruch der coronavirus disease 2019 (COVID-19)-Pandemie deuteten erste Studien auf einen ähnlichen Zusammenhang zwischen einem schweren Verlauf von COVID-19 und IPA hin, wie er auch bei Influenza beobachtet wurde. Die Bestimmung der Häufigkeit der COVID-19-assoziierten pulmonalen Aspergillose (CAPA) war jedoch aufgrund fehlender einheitlicher Diagnosekriterien schwierig. Mit dieser Studie wollten wir daher die Prävalenz der CAPA auf der Intensivstation anhand der kürzlich veröffentlichten Konsenskriterien bestimmen. Außerdem wollten wir Erkenntnisse über den diagnostischen Stellenwert von Biomarkern wie z.B. Galactomannan (GM) und Risikofaktoren im Zusammenhang mit der CAPA gewinnen.

Methoden: PatientInnen, die wegen einer COVID-19-assoziierten akuten respiratorischen Insuffizienz auf die Intensivstation des Universitätsklinikums Graz eingewiesen wurden, wurden von März 2020 bis Mai 2021 in diese Studie eingeschlossen. Die relevanten klinischen Daten der PatientInnen wurden aus dem internen Dateninformationssystem des Klinikums extrahiert und in ein pseudonymisiertes elektronisches Formular übertragen. Die Entscheidung über die Durchführung von diagnostischen Verfahren oder Therapien bei den eingeschlossenen PatientInnen lag allein in der Verantwortung der behandelnden ÄrztInnen. Die PatientInnen wurden auf Grundlage der Diagnosekriterien der European Confederation for Medical Mycology und International Society for Human and Animal Mycology entweder als CAPA (gesichert/wahrscheinlich/möglich) oder als kein Nachweis für eine CAPA klassifiziert. Es wurde eine deskriptive Analyse der Variablen und eine Überlebensanalyse mit Kaplan-Meier-Schätzern durchgeführt.

Ergebnisse: Auf der Grundlage von insgesamt 119 eingeschlossenen PatientInnen wurden elf wahrscheinliche CAPA-Fälle und ein möglicher CAPA-Fall diagnostiziert. Daraus ergibt sich eine CAPA-Prävalenz von 10,1% [95% Konfidenzintervall (CI) 5.9–14.3] in der gesamten Kohorte, während bei invasiv beatmeten PatientInnen eine CAPA-Prävalenz von 20% (95% CI 12,7–29,1) berechnet wurde. CAPA-PatientInnen hatten eine signifikant längere Aufenthaltsdauer auf der Intensivstation im Vergleich zu PatientInnen ohne

Nachweis für eine CAPA. Die Diagnose einer CAPA wurde im Median 7 Tage (25%–75% Quartil: 3,5–12,75) nach Aufnahme auf die Intensivstation gestellt. In jedem CAPA-Fall wurde eine bronchoalveoläre Lavage (BAL) durchgeführt. Der GM-Wert in der BAL war bei 54,5% der CAPA-Fälle positiv [GM>1,0 optical density index (ODI)] mit einem Median GM-Wert von 6,81 ODI (25%–75% Quartil: 2,64–6,96). Bei 41,7% der CAPA-Fälle fiel der GM-Wert im Serum positiv aus (GM>0,5 ODI). Bei allen CAPA-PatientInnen wurde eine antimykotische Behandlung eingeleitet. Die Überlebensrate der CAPA-PatientInnen betrug 30 Tage nach der Aufnahme auf die Intensivstation, bei der Entlassung aus der Intensivstation und am Ende der Nachbeobachtung 75%, 50% bzw. 33,3%. Die Überlebensrate der PatientInnen ohne CAPA betrug 30 Tage nach der Aufnahme auf die Intensivstation, bei der Entlassung aus der Intensivstation und am Ende der Nachbeobachtung 60,7%, 57,9% bzw. 53,3%.

Konklusion: Die CAPA ist eine schwerwiegende Komplikation bei invasiv beatmeten COVID-19 PatientInnen. Zur frühzeitigen Diagnose der CAPA ist in den meisten Fällen eine Bronchoskopie zur Entnahme von respiratorischen Proben erforderlich. Allerdings hat ein erheblicher Anteil der CAPA-PatientInnen auch ein positives Serum-GM-Resultat und erreicht damit das angio-invasive Stadium der Erkrankung, das mit niedrigen Überlebensraten verbunden ist. Die Diagnose der meisten CAPA-Fälle konnte durch die Bestimmung von GM gestellt werden. Die Gesamtmortalität der CAPA-Fälle war nicht signifikant höher als bei PatientInnen ohne Nachweis einer CAPA.

Abstract

Background: Invasive pulmonary aspergillosis (IPA) is a life-threatening fungal infection that primarily affects individuals with immunodeficiencies. In addition to this main risk group, cases of IPA in the intensive care unit (ICU), especially in severe influenza, have also been reported. After the onset of the coronavirus disease 2019 (COVID-19)-pandemic, initial studies suggested a similar association between a severe course of COVID-19 and IPA as was observed with influenza. However, determining the frequency of COVID-19-associated pulmonary aspergillosis (CAPA) has been difficult because of the lack of standardized diagnostic criteria. Therefore, with this study, we aimed to determine the prevalence of CAPA in the ICU using the recently published consensus criteria. Furthermore, we sought to gain insight into the diagnostic value of biomarkers, such as galactomannan (GM), and risk factors associated with CAPA.

Methods: Patients admitted to the ICU of the University Hospital Graz due to COVID-19-associated acute respiratory failure were enrolled in this study from March 2020 to May 2021. Relevant patient clinical data were extracted from the hospital's internal data information system and transferred into a pseudonymized electronic case report form. The decision to perform diagnostic procedures or therapy for the included patients was the sole responsibility of the treating physicians. Patients were classified as either CAPA (proven/probable/possible) or no evidence for CAPA based on the European Confederation for Medical Mycology and the International Society for Human and Animal Mycology diagnostic criteria. Descriptive analysis of variables and survival analysis with Kaplan-Meier estimators were performed.

Results: Based on a total of 119 enrolled patients, eleven probable CAPA cases and one possible CAPA case were diagnosed. This results in a CAPA prevalence of 10.1% [95% confidence interval (CI) 5.9–14.3] in the entire cohort, whereas among invasively ventilated patients a CAPA prevalence of 20% (95% CI 12.7–29.1) was calculated. CAPA patients had a significantly longer ICU stay compared to those without evidence for CAPA. Patients were diagnosed with CAPA after a median of 7 days (25^{th}–75^{th} quartile: 3.5–12.75) after ICU admission. Bronchoalveolar lavage fluid (BALF) sampling was performed in every CAPA case. BALF GM turned out positive [GM>1.0 optical density index (ODI)] in 54.5% of CAPA cases with a median GM of 6.81 ODI (25^{th}–75^{th} quartile: 2.64–6.96). Serum GM turned out positive (GM>0.5 ODI) in 41.7% of CAPA cases. In all CAPA patients antifungal

treatment was initiated. Survival of CAPA patients at 30 days after ICU admission, at ICU discharge and at the end of follow-up was 75%, 50% and 33.3%, respectively. Survival of non-CAPA patients at 30 days after ICU admission, at ICU discharge and at the end of follow-up was 60.7%, 57.9% and 53.3%, respectively.

Conclusion: CAPA is a serious complication in invasively ventilated COVID-19 patients. To diagnose CAPA early, bronchoscopy is required in most cases to obtain respiratory samples. However, a substantial proportion of CAPA patients also have a positive serum GM result, reaching the angio-invasive stage of the disease, which is associated with poor survival rates. The diagnosis of the majority of CAPA cases could be made based on the detection of GM. Overall mortality in CAPA cases was not significantly higher than in patients without evidence of CAPA.

Publications

Data of this study already have been published as part of a conducted multinational study:

Prattes J, Wauters J, Giacobbe DR, Salmanton-García J, Maertens J, Bourgeois M, et al. **Risk factors and outcome of pulmonary aspergillosis in critically ill coronavirus disease 2019 patients—a multinational observational study by the European Confederation of Medical Mycology**. Clin Microbiol Infect. 2021;28(4):580–7.

1 Introduction

1.1 Invasive Pulmonary Aspergillosis

1.1.1 *Aspergillus* Species

Fungi, in general are eukaryotic organisms and one of their primary purposes is to degrade organic matter. Within the taxonomic domain of eukaryotes fungi build their own kingdom besides plants and animals. The differentiation to other eukaryotes can be made by their cell structure. This includes a rigid cell wall built by chitin and glucan (1). Instead of cholesterol, the cell membrane is mostly composed of ergosterol, which can be targeted by antifungal agents (2). The fungus kingdom comprises a huge class of different species. Based on their morphology and type of spore production fungi can be divided into a simplified way in yeasts, molds or dimorphic fungi. Dimorphic fungi can grow either as a yeast or as a mold. Molds are multicellular fungi, consisting of filamentous tubular structures, called hyphae (1). The entirety of colonies formed from the hyphae is called mycelium. In the mycelium an extracellular matrix can promote adherence between the hyphae and under static growth condition a biofilm may be formed (3).

Aspergillus spp. are hyaline molds. Their septate hyphae [width=3–6 micrometers (µm)] have a homogenous formation and are growing dichotomous branches in approximately 45-degree angles. Reproduction in this species of fungi is performed most often asexually (=anamorph), which means that the reproductive elements only perform mitosis. Hyphae may grow on or below certain surfaces or reach above the surface up to the air. The then so-called aerial hyphae are producing conidiophores (1). Conidiophores are highly organized stalks and at their end conidial heads are borne. Conidial heads are composed of a terminal vesicle and on the top of the vesicle phialides emerge. Multiple layers of phialides finally produce the asexual spores, called conidia (1). The conidia are the elements responsible for infection and are readily airborne, leading to the dissemination and spreading of the fungus via the air. While hyphae as the vegetative structure can be found in tissue of infected patients, conidial heads are usually absent in tissue (3). An exception to this are pre-existing body cavities, which can arise, for example, from healed tuberculosis lesions in the lungs (4). In such cavities existence of conidia can be found and an aspergilloma, may be formed. An aspergilloma is per definition a rounded conglomerate of fungal material as a late manifestation of chronic pulmonary aspergillosis (CPA) (5).

The different species complexes of *Aspergillus* spp. can be distinguished by the morphology of their conidial heads in the different stains (1). The most important human pathogenic species complexes are A. *fumigatus*, A. *flavus*, A. *niger*, A. *tereus*, A. *nidulans* and A. *clavatus*. A. *fumigatus* is the most common causative pathogen of invasive aspergillosis (IA). This species complex possesses growth from 25 degrees Celsius (°C) to over 37°C, which is one important requirement for fungal pathogens to cause invasive fungal disease (IFD) in humans (3). Each species complex, in turn, comprises many different sometimes cryptic species. However, to identify the specific species within the complex, modern molecular and biochemical methods like rapid deoxyribonucleic acid (DNA) sequencing are required (1). This might be important to identify those species, which developed resistance against antifungal agents (6). *Aspergilli* are saprotrophic species and A. *fumigatus* has one of the fastest growth rates in the fungal kingdom. Saprotrophic fungi acquire their nutrition by decomposing vegetation and *Aspergillus* is present ubiquitously in the environment worldwide (3). Environmental niches include soil, water, air and decaying organic material, such as dead plants or animals. Due to this ubiquitous existence, humans are constantly exposed to conidia of *Aspergillus* (7).

1.1.2 *Aspergillus*-related Diseases

Aspergillus spp. are the causative pathogens of various cutaneous, subcutaneous and opportunistic infections. All of them may be summarized in the collective term aspergillosis. The spectrum of diseases can be grouped into invasive, chronic, saprophytic (=heterotrophic nutrition of dead or decaying matter) and allergic types of disease (1). The underlying immune status of the host determines deeply the clinical presentation of the *Aspergillus* species, which results in a broad variety of diseases in humans (Fig. 1) (8). The most important entities of aspergillosis are IA, CPA, chronic necrotizing pulmonary aspergillosis (CNPA), aspergilloma, and fungal asthma including allergic bronchopulmonary aspergillosis (ABPA) and severe asthma with fungal sensitization (SAFS) (3). While immunodeficiency is the most important factor of invasive disease, chronic diseases mostly occur in patients with chronic lung and cavitary lung disease (8). In allergic diseases, such as ABPA, an exuberant type 2 helper T cell-mediated immune response to hyphae of *Aspergillus* causes a dysbalanced inflammation, especially in cystic fibrosis patients (9). Among the types of disease caused by *Aspergilli*, the global incidence of allergic disease is the highest, followed by chronic and invasive disease. Conversely, mortality is the highest

in invasive disease, followed by chronic disease, and significantly lower in allergic disease (10).

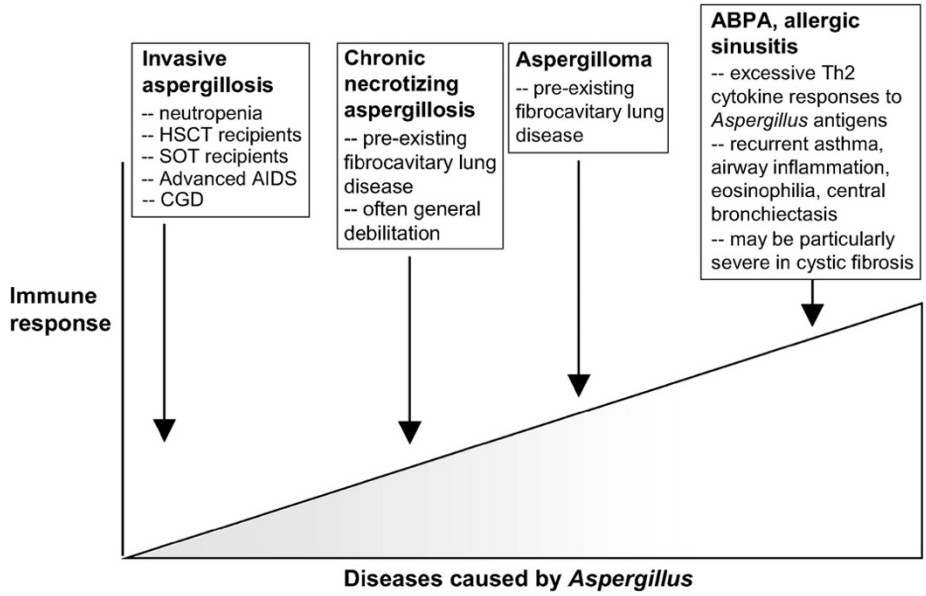

Figure 1: ***Spectrum of diseases caused by Aspergillus infection***

Estimates of worldwide incidence is difficult, but IA is thought to affect over 200 000 patients annually (11). Mortality of IA is ranging from 30 per cent (%) to over 90%, which depends on the host immune status, site of infection and type of treatment administration (4). Without treatment IA almost always leads to death in immunocompromised patients, as well as in non-neutropenic patients who are critically ill (12). One of the first case reports of IA was published in 1953 (13). Since then, there has been a steady increase in the incidence of IFDs in the following decades of the last century. As a result of advances in modern medicine, allogeneic hematopoietic stem cell transplants (HSCTs) and solid organ transplants (SOTs) could be performed. These have been made possible by the administration of immunosuppressive drugs, which have also found increased use in the treatment of other diseases such as autoimmune disorders (3). In the 1990s IA has become the predominant IFD and surpassed invasive candidiasis as the most common finding of fungal infection in hematological patients. IA is still an underdiagnosed disease in the clinical setting, which was noted through the ascertainment of IA in autopsy studies (14). In the vast majority of IA, the disease manifests in the lungs, called invasive pulmonary aspergillosis (IPA). Besides affecting directly lung parenchyma, the airways can also be invaded in form of invasive *Aspergillus* tracheobronchitis (IATB), which is a common

manifestation in lung transplant recipients (15). Less common are primary manifestations of IA in other organs (e.g., cerebral aspergillosis, endocarditis). Furthermore, in its severest course, IA can spread to multiple different organs (e.g., brain, heart, kidney, liver) by hematogenous dissemination (16).

1.1.3 Pathogenesis – from Inhalation to Invasive Disease

The main entry port of airborne conidia to the human body is through inhalation. Due to its small spore size, A. *fumigatus* can reach into the smallest airways, the distal alveoli (17). Therefore, *Aspergillus* infections primarily affect the lungs and paranasal sinus (9). Although the respiratory tract is exposed to approximately 100–1000 conidia daily, invasive disease in healthy individuals is scarce (3). This is accomplished through several pulmonary defense mechanisms of the host. Firstly, respiratory epithelial cells serve as a physiologic barrier. Inhaled conidia are rapidly evacuated from the lungs by the mucociliary clearance. Secondly, alveolar macrophages survey the lumen of the respiratory tract and ingest inhaled spores as the first line of defense (4). Finally, other phagocytes like neutrophil granulocytes and monocytes from the peripheral blood are recruited to the site of infected epithelial cells. When inhaled conidia germinate to hyphae neutrophils become the most important cell type in the fungal host defense (9). Innate immune cells are stimulated through the interaction of their pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), with pathogen-associated molecular patterns (PAMPs) of *Aspergillus* (4). Subsequently, downstream production of proinflammatory cytokines and chemokines is initiated. Certain cytokines as well as dendritic cells (DCs) provide the link to the adaptive immune system by activation of T cell responses (9).

The capacity of the host to limit the impact of fungal infection is determined by the combination of resistance and tolerance. Resistance means the capability to restrict fungal burden and the effective clearance of the pathogen. Tolerance represents the avoidance of the host to take excess damage of its own dysbalanced immune response to the pathogen (4). As an opportunistic infection, development of IA requires a dysfunction of the host defense. Most studies on the pathogenesis of IA refer to neutrophil granulocyte defects, but the increasing incidence of IA in non-neutropenic patients suggests the importance of other immunopathological mechanisms (4). Predisposing factors for IA are numerous and will be presented in the following chapter. Whether *Aspergillus* spp. cause invasive disease also depends on fungal and environmental factors. These factors are complex and not fully

understood yet, but in combination with a dysfunctional immune response they may facilitate the survival and growth of the mold in the host (3).

Pathogenesis of IA is different in neutropenic and non-neutropenic hosts, which has an impact on the clinical presentation, radiologic patterns, diagnostic value of microbiologic samples and therapy of IA patients (5,18). The histopathology of IPA in neutropenic patients is characterized by angio-invasion of the hyphae preferably into the pulmonary arteries. Thus, hemorrhagic infarct in the lung may occur. As a further consequence, other organs may be affected by hematogenous spread. In the vessels of all affected organs the mycelium may be found, which leads to thrombotic events and then again causes hemorrhagic infarct and necrosis in the particular organ (19). Different histopathological patterns occur in non-neutropenic patients. On the one hand, less hyphal angio-invasion is observed and the *Aspergillus* burden in general is less abundant. On the other hand, more tissue-invasion with signs of severe inflammation is observed in the lungs (14). In addition, the lower vascular invasion by *Aspergillus* leads to lower release of fungal elements into the bloodstream that may be detected through biomarker testing in serum (20). This is an important diagnostic obstacle and emphasizes the importance of obtaining respiratory specimen in non-neutropenic patients, where in culture presence of *Aspergillus* is more frequent than in neutropenic patients (18). However, when IPA is not treated adequately, rates of dissemination appear to be similar in neutropenic as well as in non-neutropenic hosts in autopsies (14). This is due to the fact, that IPA can also progress in non-neutropenic hosts from the tissue-invasive (=bronchoalveolar) stadium to the angio-invasive stadium. However, this evolution takes more time to develop in contrast to neutropenic hosts (21).

1.1.4 Patients at Risk for Invasive Pulmonary Aspergillosis

Patients with profound and long neutropenia carry the highest risk of developing IFD (22). This neutropenia can be caused either by certain diseases like aplastic anemia or by immunosuppressive therapy like cytotoxic chemotherapy for acute leukemia (8). Further predisposing conditions leading to development of IFD are summarized by The European Organization for Research and Treatment of Cancer and the Mycosis Study Group Education and Research Consortium (EORTC/MSGERC) in their host factors. They are used for clinical trials in scientific research as well as in their diagnostic algorithm for IPA, which will be explained later.

To illustrate which individuals, carry the highest risk of developing an invasive pulmonary mold disease the EORTC/MSGERC host factors are listed below (22):

- Recent history of neutropenia (<0.5 × 10^9 neutrophils/L [<500 neutrophils/mm^3] for >10 days) temporally related to the onset of invasive fungal disease
- Hematologic malignancy
- Receipt of an allogeneic stem cell transplant
- Receipt of a solid organ transplant
- Prolonged use of corticosteroids (excluding among patients with allergic bronchopulmonary aspergillosis) at a therapeutic dose of ≥0.3 mg/kg corticosteroids for ≥3 weeks in the past 60 days
- Treatment with other recognized T-cell immunosuppressants, such as calcineurin inhibitors, tumor necrosis factor-α blockers, lymphocyte-specific monoclonal antibodies, immunosuppressive nucleoside analogues during the past 90 days
- Treatment with recognized B-cell immunosuppressants, such as Bruton's tyrosine kinase inhibitors (eg., ibrutinib)
- Inherited severe immunodeficiency (such as chronic granulomatous disease, STAT 3 deficiency, or severe combined immunodeficiency)
- Acute graft-versus-host disease grade III or IV involving the gut, lungs, or liver that is refractory to first-line treatment with steroids

Several risk groups for developing IPA beyond the traditional host factors have emerged. These include patients with liver disease like severe alcoholic hepatitis and liver cirrhosis (23,24), and with pulmonary diseases like chronic obstructive pulmonary disease (COPD) (25). Furthermore, critically ill patients in the intensive care unit (ICU) have been increasingly reported for being at risk for developing IPA (24,26). The population represents large heterogeneity regarding their individual risk profiles. Nonetheless, most important are the use of high-dose corticosteroids, which promotes active fungal growth and tissue invasion in already *Aspergillus* colonized patients (27). Diabetes mellitus, malnutrition and burns also contribute to the individual risk profile of ICU patients (28,29).

Moreover, pulmonary epithelial damage induced by acute respiratory distress syndrome (ARDS) and its causative agents in pneumonia (e.g., bacterial, viral) have been identified as a main risk factor for IPA in the ICU setting (30). The course of critically ill influenza patients with acute respiratory failure (ARF) and consecutively ARDS can be complicated

by developing IFD, especially IPA. Recently, influenza was found to be an independent risk factor to the development of IPA. Influenza-associated pulmonary aspergillosis (IAPA) affects up to 32% of critically ill influenza patients and is associated with a 51% 90-day mortality rate (27). In addition, a significant amount of influenza patients in the ICU presents with signs of IATB, which may occur alone or in combination with lung tissue infection (31).

Since the worldwide spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the coronavirus disease 2019 (COVID-19)-pandemic has challenged health care systems worldwide. Although the vast majority of COVID-19 cases are mild, a significant proportion of patients require hospitalization. Deterioration in certain COVID-19 patients can lead to development of ARF, ARDS and ICU admission (32). Shortly after COVID-19 was declared a pandemic in March 2020 (33), the first case reports of IPA complicating the course of COVID-19 patients were published (34,35). Subsequently, retrospective studies were conducted and suggested, that COVID-19-associated pulmonary aspergillosis (CAPA) appear to have a high incidence and mortality (36,37). Together with the knowledge of the high mortality in IAPA patients, concerns were raised about the additional burden of CAPA on patients with already poor prognoses (38). Therefore, the following chapters presents COVID-19, its severe course and the association with development of invasive mold diseases.

1.2 Coronavirus Disease 2019

1.2.1 Severe Acute Respiratory Syndrome Coronavirus 2

COVID-19 is caused by SARS-CoV-2, previously called 2019-nCoV. Coronaviruses (CoVs) are phylogenetically part of the family *Coronaviridae* (39). *Coronaviridae* in general are characterized as positive-sense single-stranded ribonucleic acid (+ssRNA) viruses with a lipid envelope built up by outer-membrane lipoproteins and a helical shape capsid structure. The virus family received the prefix corona due to their morphology. Crown shaped spike proteins on the virion surface can be observed in the electron microscopy (7).

Among other human pathogenic +ssRNA virus families (e.g., *Picornaviridae*, *Caliciviridae*, *Togaviridae*, *Flaviridae*) *Coronaviridae* are the largest with a genome size of 26–32 kilo base pairs (kbp) (40). The family and its subfamily *Orthocoronavirinae* are divided into four genera, two of which, *Alphacoronavirus* and *Betacoronavirus*, contain viruses which are known to be pathogenic for humans. CoVs can cause a wide variety of diseases in mammals and birds, whereas in humans they predominantly cause respiratory diseases. The first human coronaviruses (HCoVs) were recovered in the 1960s. Nowadays seven different HCoVs are known (7).

On the one hand, HCoVs can be sub-divided into the group of community-acquired respiratory (CAR)-HCoVs. CAR-HCoVs usually cause mild diseases like the common cold in the cold time of the year (7). For example, influenza-like illness (ILI) is actually caused by CAR-HCoVs in up to 18% of cases and not by seasonal influenza viruses (41). ILI is an acute respiratory infection caused by influenza virus or other respiratory pathogens without requirement of hospitalization (42).

On the other hand, there are the remaining HCoVs, which are part of the *Betacoronavirus* clade: severe acute respiratory syndrome (SARS)-CoV, Middle East respiratory syndrome (MERS)-CoV and SARS-CoV-2. All aforementioned may cause severer diseases, led to significant outbreaks and have the potential to break the species barrier (43,44). The diseases caused by SARS-CoV and MERS-CoV are called SARS and MERS, respectively. Mortality from SARS and MERS is significantly higher compared to COVID-19 (45). Contrarily, SARS-CoV-2 is more contagious than SARS-CoV and MERS-CoV (46). Timely ascertainment and isolation of cases resulted in rapid containment of the newly emerged SARS-CoV and MERS-CoV. Thus, neither SARS-CoV with its outbreak and extinction in

2002–2003, nor MERS-CoV which has been circulating since 2012, reached the status of a global pandemic as was observed with SARS-CoV-2 (7).

Within the taxonomy SARS-CoV-2 and SARS-CoV are both part of the species *Severe acute respiratory syndrome-related coronavirus* and have therefore substantial similarities in common (39). They share up to 80% homology in their genome sequence, including the spike protein (S-protein). The S-proteins in SARS-CoV and SARS-CoV-2 have a similar structure including the receptor-binding domain (RBD) (Fig. 2). The SARS-CoV-2 genome has 14 open reading frames (ORFs), which encode for a total of 29 viral proteins, consisting of structural, accessory and non-structural proteins (NSPs). Two polyproteins are digested by viral proteases resulting in 16 NSPs, that are responsible for the viral replication and transcription (45). Other ORFs generate the structural proteins nucleocapsid (N-protein), membrane (M-protein), envelope (E-protein) and S-protein with its subunits S1 and S2. The structural proteins are essential for the virion assembly and are also playing a role in suppression of the host immune response. The accessory proteins are involved in regulation of the viral infection (45).

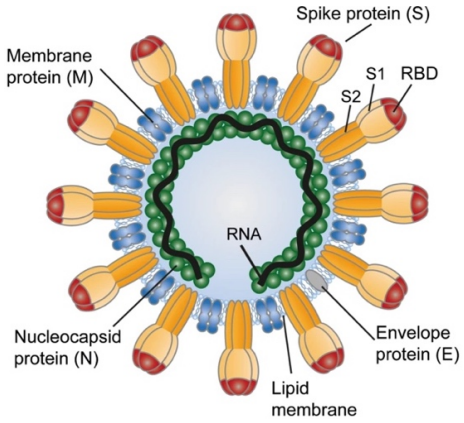

Figure 2: ***Schematic of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virion and its structural proteins***

Like CoVs in general SARS-CoV-2 has the possibility to frequent genetic recombination, resulting in a large number of mutations in the SARS-CoV-2 genome (45). Continuing evolution of the wild-type strain (e.g., Wuhan-Hu-1) led to multiple variants. Identifying new variants is important to recognize mutations (especially of the S-protein) that are causing higher virulence and escape from vaccine induced immunity and immunity induced due to previous SARS-CoV-2 infection (47). Several different variants have emerged up to

date, of which five are considered a variant of concern (VOC) by the World Health Organization (WHO) (including several sub-lineages) (48):

1. B.1.1.7 (Alpha)
2. B.1.351 (Beta)
3. P.1 (Gamma)
4. B.1.617.2 (Delta)
5. B.1.1.529 (Omicron)

SARS-CoV-2 is transmitted primarily over short distance through a continuum of respiratory particles in the form of smaller aerosol particles up to larger droplets. To a smaller extent direct physical contact and indirect contact via fomites are responsible for viral transmission. Transmission over longer distances by aerosol particles is happening mostly by prolonged exposure in poorly ventilated enclosed spaces (49). The first outbreak of COVID-19 in Wuhan showed the high transmissibility of SARS-CoV-2 with an estimated basic reproduction number (R_0) of 3.54 (46). R_0 is defined as the average number of new infections, that are caused by one infected individual in a population where everyone is susceptible (50). Susceptible means that a totally immune-naive population is exposed to an infectious agent without any non-pharmaceutical interventions (48). In comparison, the R_0 of seasonal influenza strains is estimated with a mean of 1.3 (50). Subsequent VOCs of SARS-CoV-2 showed even much higher transmissibility. For instance, for the currently dominant Omicron variant an average R_0 of 9.5 was estimated, which exceeds all previous variants (48). This infectivity almost approaches that of the most contagious diseases such as measles, which has a R_0 ranging between 12 and 18 (51).

A key role for the rapid spread of SARS-CoV-2 is played by its pre-symptomatic infectiousness, which leads to the delayed ascertainment of infections. Even asymptomatic SARS-CoV-2 infected people may transmit the virus to other people, not aware that they are infected. These circumstances resulted to the introduction of multipronged public health measures all over the globe to decrease the effective reproduction number (R_E) and flatten the exponential growth (46). R_E is defined as the average number of secondary infections that are caused by a primary infected individual in a population, where already a background immunity or non-pharmaceutical interventions are existent (48).
The differentiation between asymptomatic (=individuals who never develop symptoms) and pre-symptomatic (=individuals before symptom onset) SARS-CoV-2-positive individuals is

important. Both groups can transmit the virus, but transmissibility of SARS-CoV-2 seems to be the highest directly before and in the first days after symptom onset and viral shedding is decreasing in the following days (52,53). Thus, pre-symptomatic SARS-CoV-2 infected people have a greater influence on the dynamic of infections as asymptomatic individuals (49).

1.2.2 Pathogenesis – from Infection to Severe Disease

The angiotensin-converting enzyme 2 (ACE2) is the main cellular receptor of SARS-CoV-2 for entry into host cells. The S-protein of SARS-CoV-2 has a strong binding affinity to human ACE2 (45). After binding to ACE2 via the RBD of subunit S1, the S-protein is primed by transmembrane protease, serine 2 (TMPRSS2) through proteolytic cleavage. This results in membrane fusion, viral entry and subsequently viral replication in the host cells (54). Although COVID-19 is primarily considered a respiratory disease with the lungs as the most affected organ, it may cause multi-organ dysfunction, especially in critically ill patients (55). The alveolar epithelial type II cells (AECII) in the lungs are the main cells for expression of ACE2 (56). Nevertheless, ACE2 expression is distributed over many different tissues like heart, kidney, intestine and endothelium, which may explain the manifestation of COVID-19 in multiple organs (57).

The clinical severity of SARS-CoV-2 infection ranges from asymptomatic cases to critical illness and death. A significant proportion of individuals who get infected never develop symptoms and remain asymptomatic. A review stated that at least one third of SARS-CoV-2 infections may be asymptomatic (58). The median incubation period of COVID-19 accounts for four to five days, however, there were cases in which people showed symptoms not earlier than 14 days after infection (59,60).
COVID-19 patients can undergo a course of three stages with increasing severity of disease over the time (Fig. 3). While the viral response is high at the beginning and declining over the time, the host inflammatory response is increasing, and the two phases are overlapping. To consider in which stage the patient currently is plays a crucial role for choosing a targeted therapy at the right time (61).

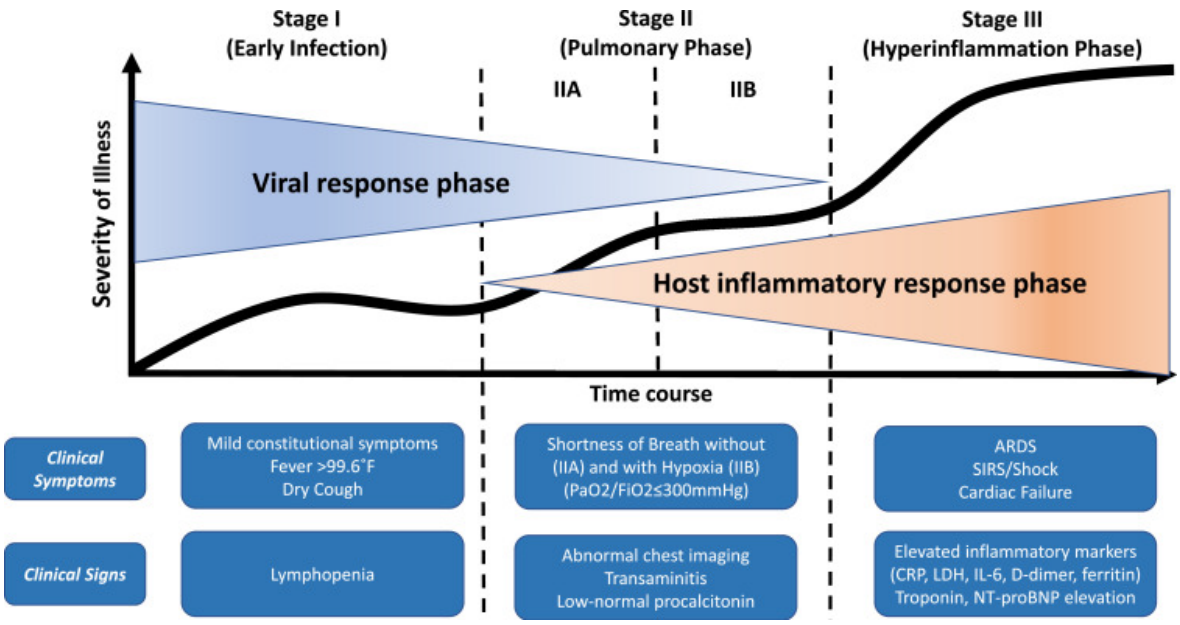

Figure 3: ***Classification of COVID-19 stages with associated signs and symptoms***

Reprinted and modified from COVID-19 illness in native and immunosuppressed states: A clinical-therapeutic staging proposal, 39/5, Siddiqi HK, Mehra MR, 2020, Copyright (2020), with permission from Elsevier.

The first stage is characterized by only mild symptoms and a high virus replication (61). Initial presentation and occurrence of specific symptoms in the course of time in COVID-19 may be different for each patient. Most common symptoms, however, are cough, fever, myalgia/fatigue, headache, sore throat, nasal congestion and rhinorrhea. Less common are gastrointestinal symptoms like diarrhea, nausea or vomiting (62–65). In addition, loss of smell (anosmia) and taste (ageusia) have been reported as hallmark symptoms of COVID-19 (66).

In the course of approximately one week after disease onset the patient may develop the second stage (67). It is characterized by signs of pneumonia and dyspnea as the most common symptom. Normally this is the moment when hospitalization is required. Further deterioration leads to severe disease with the need of oxygen supplementation due to hypoxemia (61).

After a median of eight to twelve days after disease onset the clinical progression may lead to critical illness. The third stage is usually composed of worsening hypoxemia, ARF and admission to the ICU (62,65,68,69). In most of the cases, COVID-19 patients must be admitted to the ICU due to ARF, and ARDS is the most commonly reported organ dysfunction (62–65,69). ARDS is a certain form of ARF with various pulmonary and extrapulmonary triggers, but the majority is caused by sepsis or pneumonia (bacterial, fungal, viral, aspiration or opportunistic). It is a severe inflammatory reaction of the lungs to

pulmonary damage (70). Diagnosis of this condition can be stated when the Berlin criteria of 2012 are met. The criteria depend on the timing, chest imaging, origin of edema and oxygenation of the patient (71). Besides the severe lung injury, acute cardiac, hepatobiliary and renal dysfunction can contribute to multiple organ failure (MOF) in critical COVID-19 (47,62,68). Remarkable complications in critically ill COVID-19 patients also include thrombosis and thromboembolic events (72) with pathological correlates of endothelial damage and alveolar capillary microthrombi (73). Moreover, secondary infections caused by bacteria or fungi were reported since the start of the pandemic in severe COVID-19 cases (62,63,69). Bacterial superinfections in COVID-19 were observed in studies from the beginning of the year 2020 with an approximate frequency of 14% in the ICU (74).

The third stage may also be characterized by a dysregulated inflammatory response. Lymphopenia as the most common laboratory finding in COVID-19 can be accompanied by elevated inflammatory markers [e.g., C-reactive protein (CRP), ferritin, lactate dehydrogenase (LDH)] (62,64,75). In addition, elevated serum concentrations of proinflammatory cytokines like interleukins (ILs) (e.g., IL-1β, IL-6, IL-8) and tumor necrosis factor-α (TNFα) may occur, which was described as "cytokine storm" in the literature (76,77). However, the term may be misleading, because in comparison to other syndromes of critical illness (i.e., non-COVID-19 ARDS, sepsis, cytokine release syndrome) the levels are significantly lower (78).

1.2.3 Patients at Risk for Severe COVID-19

Although deterioration of COVID-19 can also happen in young individuals without chronic health conditions, it is a rare phenomenon. There are several risk factors, which may have an impact on the course of the disease with a higher probability of developing severe or even fatal disease. Age has emerged as the most important risk factor for increased disease severity and mortality (75). The case fatality rate (CFR) of COVID-19 is significantly higher in older cohorts and it increases substantially in every additional decade. The CFR (lethality) is defined as the percentage of patients with a specific disease, who die because of the disease within a specific time period (79). For instance, the reported mortality of the initial epidemic in China showed a CFR of 8% in the 70–79 year-old patients and a CFR of 15% in the individuals who were older than 80 years (32). Considering the large percentage of asymptomatic infections, infection fatality rates (IFRs) elucidate an exponential relation between age and mortality. The IFR is defined as the ratio of deaths to total infections within a specific time interval. Estimated age-specific IFR rises to 0.4% at the age of 55 and

increases significantly afterwards to 1.4% at the age of 65, 4.6% at the age of 75 and 15% at the age of 85 (79). Moreover, underlying health conditions have a great impact on severity and mortality of COVID-19. These include specifically cardiovascular disease, diabetes mellitus, chronic respiratory disease, chronic kidney disease, immunosuppression, cancer, hypertension and obesity (32,47,67). Lastly, male gender is also an important risk factor contributing to worse outcome in COVID-19 (75). It is hypothesized, that an impaired immune system with enhanced innate immunity and hampered T cell activation are more frequent in men (47).

It is important to recognize whether a COVID-19 patient is still in a severe course of disease or is already critically ill. This is essential for the decision whether the patient needs to be transferred to the ICU. The WHO uses the following diagnostic features to distinguish between the two severity stages of COVID-19. (80):

- Severe COVID-19: Clinical signs of pneumonia (fever, cough, dyspnea) and one of the following:
    - Respiratory rate > 30 breaths/min
    - Severe respiratory distress (accessory muscle use, inability to complete full sentences)
    - Oxygen saturation ($Sp0_2$) < 90% on room air
- Critical COVID-19: requirement of life-sustaining treatment, ARDS or respiratory failure, sepsis, septic shock

1.2.4 Diagnostic Procedures

Evidence of SARS-CoV-2 infection through detection of viral ribonucleic acid (RNA) or direct specific antigens (protein fragments) in respiratory samples is required to confirm COVID-19. Quantitative reverse transcription real-time polymerase chain reaction (RT-qPCR) is a nucleic acid amplification test (NAAT) and considered as the “gold standard” with the highest sensitivity of all diagnostic tests. Specimen can be obtained from the upper respiratory tract (e.g., oro- or nasopharyngeal swab) and the lower respiratory tract (LRT). In severe cases specimen of the LRT normally contain a higher viral load, and the diagnostic yield is increased (59,81). As a point-of-care (POC) test, antigen tests are broadly available, which deliver quick results with similar specificity but reduced sensitivity (82).

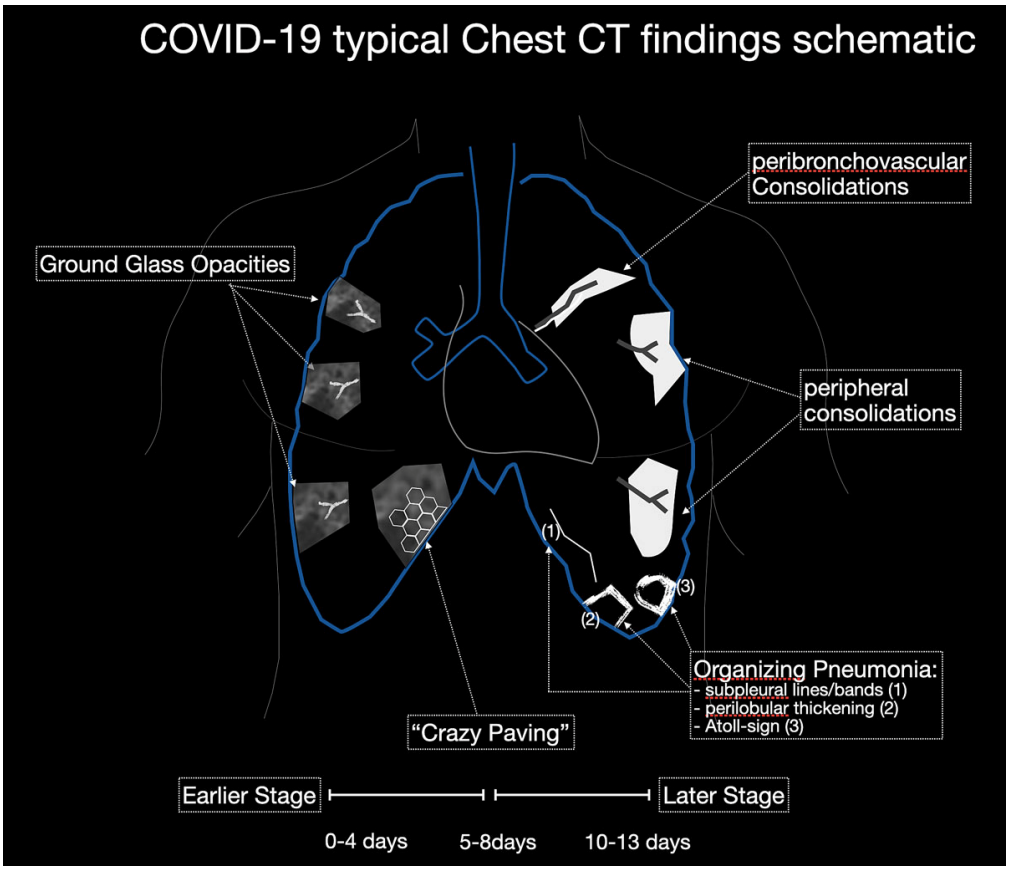

Figure 4: ***Schematic depiction of typical chest computed tomography (CT) findings for COVID-19***

Imaging findings on standard chest x-ray of COVID-19 in early stages include mostly bilateral ground-glass opacities, which can progress into consolidations in later stages of the disease (83). The chest computed tomography (CT) has a higher sensitivity in the detection of typical COVID-19 imaging features (Fig. 4). It may be repeated because of clinical deterioration to monitor disease progression and in case bacterial or fungal superinfection is suspected (83). The distribution of bilateral opacities is normally subpleural or peribronchovascular and as a sign of disease progression they may transform into consolidations. In addition, signs of organizing pneumonia or fibrosis may occur, especially in the late stage of the disease (62–64,83). The so-called "crazy paving" pattern appears less often but is also suggesting a progressive stage of COVID-19 (83). It stands for ground-glass attenuation with superimposed reticular thickening of intra- and interlobular septa (84). All aforementioned patterns are characteristic of COVID-19 but may also be observed in a wide variety of other diseases, including IPA (83,84).

1.2.5 Treatment in the Intensive Care Unit

Treatment of COVID-19 in the ICU includes airway management and oxygen administration according to severity of hypoxemia. It ranges from conventional low-flow oxygen therapy, high-flow nasal cannula (HFNC), non-invasive ventilation (NIV) up to invasive mechanical ventilation (IMV) (i.e., endotracheal tube, tracheostomy) and extracorporeal membrane oxygenation (ECMO). In intubated patients with ARDS, a "lung-protective" IMV with low tidal volumes (V_t) and plateau pressures (P_{plat}) should be established to prevent ventilator-induced lung injury. In addition, prone positioning over 12 to 16 hours and neuromuscular blockade in patients with refractory hypoxemia can be considered. Moreover, the initiation of pharmacologic venous thromboembolism (VTE) prophylaxis should be performed (67,80).

As mentioned earlier, the immune response in COVID-19 patients proceeds in two main phases. Early after infection, high viral replication dominates, which may be followed later by a dysregulated immune response with hyperinflammation (76). Based on this knowledge antiviral agents (i.e., nirmatrelvir/ritonavir, remdesivir) that target SARS-CoV-2 should be applied early on (85). Remdesivir shortens time to recovery and may improve survival in patients who require supplemental oxygen (86,87). However, when patients have already developed severe hypoxemia and are in need for ICU care, the benefit of antiviral drugs has diminished. Instead, it is more likely that immunomodulatory drugs have an impact on the dysregulated inflammatory response that is now predominant (85).
The use of systemic corticosteroids in severe and critically ill COVID-19 patients is strongly recommended in the guidelines (85,88). As shown in the RECOVERY trial, a daily application of 6 milligrams (mg) dexamethasone for ten days resulted in a significant reduction of mortality among intubated patients and those with respiratory support (89). The use of other corticosteroids like methylprednisolone seems to have similar effects on the outcome (90,91). Recently conducted studies suggest, that the application of dexamethasone in higher doses (12 mg versus 6 mg) might have a benefit in patients with severe hypoxemia (92,93).

Several studies indicated that anti-IL-6 pathway inhibitors are improving outcomes in certain COVID-19 patients (81,94). A meta-analysis pointed out that administration of tocilizumab with concomitant corticosteroids has the potential to lower the mortality in severe COVID-19 (95), which especially applies to patients with high CRP levels as sign of an immune

response dysregulation (96). These results were therefore also considered in the international guidelines of COVID-19 treatment. The WHO recommends strongly the usage of IL-6 receptor blockers (e.g., tocilizumab, sarilumab) together with corticosteroids for patients with severe or critical COVID-19 (88). The National Institutes of Health (NIH) recommends the combination of dexamethasone and tocilizumab in patients receiving mechanical ventilation or ECMO treatment within 24 hours after ICU admission. The recommendation of this treatment combination also applies to hospitalized patients with rapidly increasing oxygen requirement (high-flow device or NIV) and elevated markers of systemic inflammation (85).

Likewise, the Janus kinase (JAK) inhibitor baricitinib is an immunomodulatory drug that can be used as an alternative to IL-6 receptor blockers. As an additional immunosuppressive drug besides corticosteroids, baricitinib has the potential to lower the mortality in severe and critical COVID-19 (97). In the absence of comparative studies between IL-6 receptor blockers and baricitinib, they are considered equivalent. Therefore, the decision whether to use baricitinib or an IL-6 receptor blocker should be based on drug availability, local recommendations and patient comorbidities. Baricitinib should not be given together with an IL-6 receptor blocker, because it is associated with increased susceptibility to secondary infection (bacterial or fungal) (85,88).

The international guidelines for the therapy of COVID-19 patients acknowledge that the risk for developing a secondary infection may be elevated under the treatment of corticosteroids, IL-6 receptor blockers and JAK inhibitors. This risk is particularly increased when corticosteroids are combined with an additional immunosuppressant (85,88). With suppression of the nuclear factor κB (NF-κB)-dependent production of proinflammatory cytokines and chemokines, corticosteroid use can facilitate the germination of conidia by impairment of alveolar macrophages and other innate immune cells (74). IL-6 seems to have a protective proinflammatory and immunomodulatory effect on the pulmonary host defense. Therefore, excessive suppression by IL-6 receptor blockers might explain the favoring of secondary infections (4). However, the authors state that the risks outweigh the benefits of such a treatment in critically ill COVID-19 patients (85,88).

1.3 Association between COVID-19 and Invasive Pulmonary Aspergillosis

Most patients admitted to the ICU due to viral-induced ARF and ARDS don't present with EORTC/MSGERC host factors (27). Instead, numerous risk factors are contributing to the development of IPA in non-immunocompromised patients. Risk factors can be divided into extrinsic factors such as the above-mentioned treatment with corticosteroids, and intrinsic factors such as underlying disorders (98). Extrinsic or clinical factors are not specific for COVID-19 and present in other individuals in the ICU such as COPD patients. These factors do include IMV and ECMO, prolonged ICU stay, intravenous catheters and broad-spectrum antibiotics (5,15,99,100). Although angio-invasion is the trademark feature of IPA in neutropenic hosts, it is also reported in severe influenza patients affected by IATB (31). Similarly, the spectrum of pathological findings caused by *Aspergillus* spp. in COVID-19 patients seems to have a wide span of colonization, tissue-invasion and angio-invasion. However, the frequency of angio-invasion in CAPA is unclear due to the limited data of performed biopsies and autopsies (101). Van de Veerdonk et al. (102) proposed an angio-invasion threshold model (Fig. 5), which displays the cumulating influence of factors that contribute to the progression of IATB into the stage of angio-invasion.

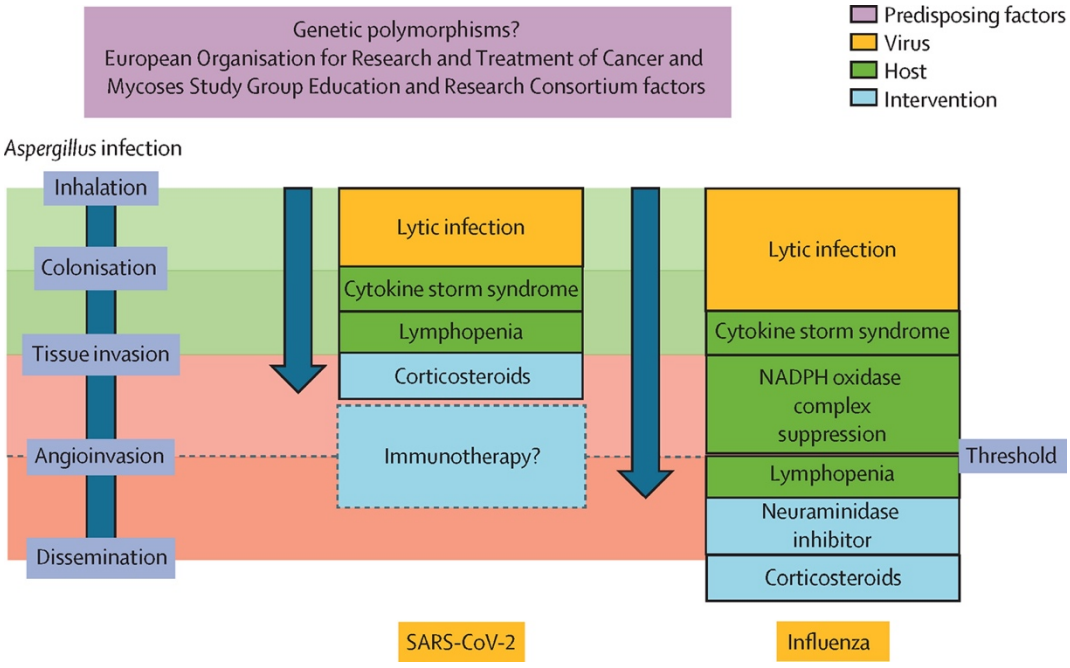

Figure 5: ***The angio-invasion threshold model***

Reprinted from The Lancet Respiratory Medicine, 9, van de Veerdonk FL et al., COVID-19-associated *Aspergillus* tracheobronchitis: the interplay between viral tropism, host defence, and fungal invasion, 795-802, Copyright 2021, with permission from Elsevier.

In addition to the extrinsic risk to secondary IFD, intrinsic effects of SARS-CoV-2 and genetic polymorphisms may explain why critically ill COVID-19 patients are affected by IPA (102). Underlying causes that facilitate the development of CAPA and possible links between severe COVID-19 and the pathophysiology of IPA will be discussed here in further detail.

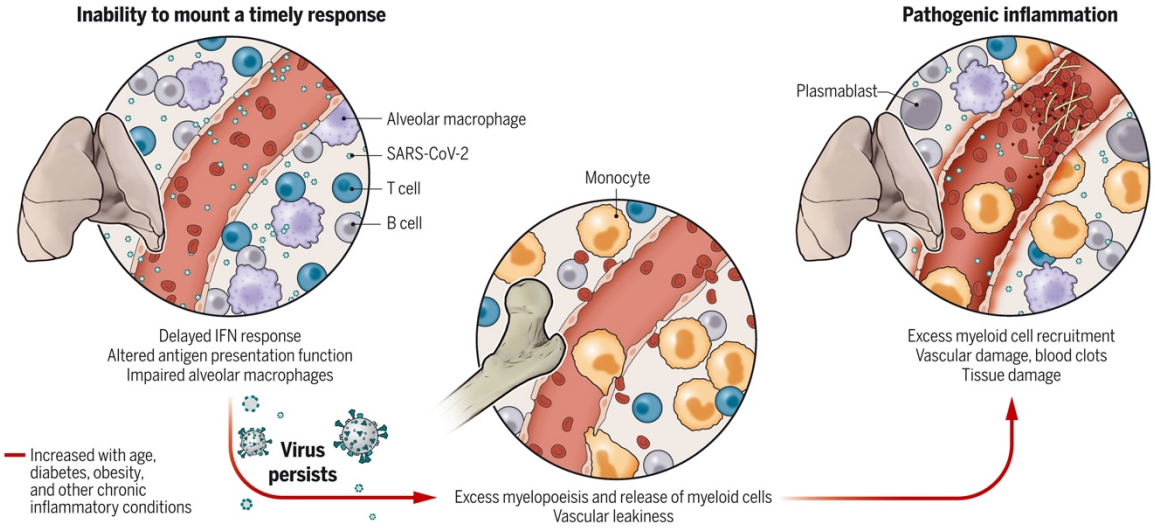

Figure 6: ***COVID-19 pathogenesis***

From Merad M, Blish CA, Sallusto F, Iwasaki A. The immunology and immunopathology of COVID-19. Science. 2022;375(6585):1122–7. Reprinted with permission from AAAS. ILLUSTRATION: V. ALTOUNIAN/*SCIENCE*

Although not fully understood yet, the immunopathological mechanisms of severe COVID-19 appear to be similar to SARS and divided into two main phases (Fig. 6) (103). It starts with the viral replication phase, where the virus is causing direct cytopathic damage in the infected tissue, which may also facilitate the invasion of the tissue by *Aspergillus* (104). The duration and dimension of this first phase has a direct impact on the progress of the secondary phase. In individuals where the immune system is not able to rapidly stop SARS-CoV-2 replication, prolonged viral replication may trigger further steps resulting in critical disease (47). Suspected reasons for the delayed viral clearance of SARS-CoV-2 are an impaired interferon (IFN) response and a dysbalanced adaptive immune response. The IFN induction and the generation of IFN-stimulated genes (ISGs) are important parts of the innate immune response to viral and fungal infections (4,59). Viral proteins of SARS-CoV-2 are interfering with the expression of ISGs and blocking the IFN-I and IFN-III immune response (45). Furthermore, adaptive autoimmunity in form of neutralizing immunoglobulin G (IgG) autoantibodies (auto-Abs) against IFNs-I was detected in critically ill patients (Fig. 7) (105).

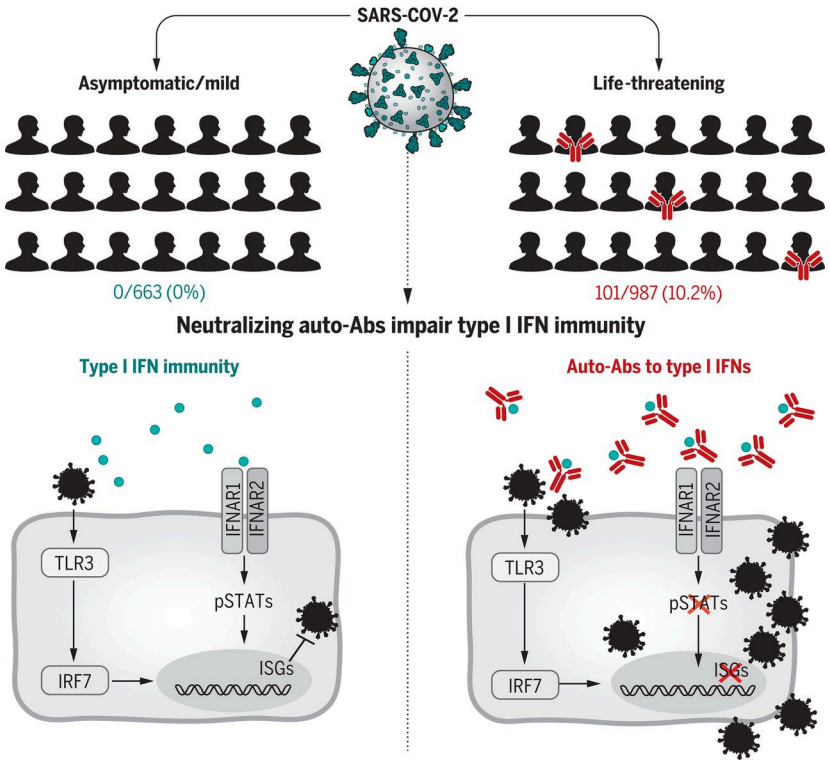

Figure 7: ***Neutralizing autoantibodies (auto-Abs) to type I interferons (IFNs) underlie life-threatening COVID-19 pneumonia.*** In a significant number of COVID-19 patients with critical illness neutralizing auto-Abs against type I IFNs were found. An impaired IFN response and interference with the downstream production of IFN-stimulated genes (ISGs) can hamper the antiviral and antifungal immune response.

In addition, inborn errors of TLR3- and interferon regulatory factor 7 (IRF7)-dependent IFN-I secretion were found in a small number of patients with fatal disease (106). Therefore, IFN-I (IFN-α, IFN-β) and IFN-III (IFN-λ) levels in the early phase of COVID-19 are detected to be lower than in other respiratory virus diseases (107). Both IFN-I and IFN-III play a role in antifungal immune defense. IFN-β can prime DCs to enhance the cluster of differentiation (CD)4+ T cell response against *Aspergillus* (108). IFNs-λ are stimulated by IFNs-I and directly regulating neutrophils in their antifungal defense response (109). In contrast to this, in later stages of the disease prolonged and elevated proinflammatory cytokine (e.g., IFNs) and chemokine release are observed in severely ill COVID-19 patients. This is accompanied by a profound reduction of sentinel cells like DCs and alveolar macrophages (47). Exhaustion, depletion and dysfunction of natural killer (NK) cells, $CD4^+$ and $CD8^+$ T cells are probably promoted by elevated inflammatory cytokine levels and have a great impact on the delayed viral clearance (76). If the virus persists, the tissue damage continues and triggers the second phase (Fig. 6), which is the dysbalanced recruitment of effector immune cells like immature monocytes, neutrophils and myeloid progenitors to the affected site.

Consequences of this excess and prolonged myeloid cell recruitment are a dysregulated immune response. Increased vascular permeability through enhanced proinflammatory molecules and emergency myelopoiesis result in further tissue damage, vascular lesions and development of microthrombi (47). The pathophysiology of IPA is complex and mainly understood in neutropenic patients. Non-neutrophil defense-related factors leading to development of IPA need to be investigated further (4). However, the aforementioned impaired innate immune response that persists later and causes the state of hyperinflammation could be a major predisposing factor of CAPA. (104). Together with the initial direct damage to the lung epithelium by SARS-CoV-2 leading to development of ARDS, this immunological environment may favor the development of fungal infections (98).

1.4 Diagnosis of Invasive Pulmonary Aspergillosis

For a long period of time, IPA was primarily observed in immunocompromised hosts. Therefore, existing diagnostic criteria applied specifically to patients with cancer, recipients of HSCT or SOT (8). The EORTC/MSGERC published their consensus definition of IFD for the patient groups mentioned before the first time in 2002 and revised and updated it the last time in 2019 (22). Patients are categorized into proven, probable or possible IFD. Proven IFD requires presence of fungi in histopathology or culture obtained by specimen from usually sterile body sites (e.g., blood, tissue). Presence of fungal elements in sterile body sites, is always considered as a proven IFD, regardless of the underlying diseases and conditions of the host (22). In contrast to proven IFD, diagnosis of probable and possible IPA predominantly rely on the presence of the earlier mentioned EORTC/MSGERC host factors and specific radiological signs. The definitions of probable and possible IPA are only applicable for immunocompromised hosts and cannot be used for patients lacking EORTC/MSGERC host factors (22).

With rising evidence that patients in the ICU may also be at risk for developing IPA it was urgent to develop a suitable diagnostic algorithm, which helps clinicians to reliably diagnose IPA in ICU patients. Diagnosing IPA in the population of ICU patients is difficult because of several reasons. First, necessary tissue sampling for proven IPA is not feasible in many patients due to the associated risk of complications in instable patients (29). Second, the vast majority of ICU patients don't present with underlying diseases qualifying as a EORTC/MSGERC host factor (30), making these criteria not suitable for IPA categorization in the majority of ICU patients. Third, thoracic imaging is complicated in ICU patients as CT scans may not always be performed easily and chest x-ray comes along with limited sensitivity, especially in highly damaged lungs as in ARDS patients (24). Lastly, invasively ventilated patients may present with *Aspergillus* colonization rather than invasive disease and discrimination between the two is not always possible (25).

Blot and colleagues (24) addressed this issue in 2012 with their AspICU algorithm. The proven IPA category remained idem to the EORTC/MSGERC criteria with ensuring the greatest certainty in diagnosis, but a newly proposed putative IPA category replaced the probable IPA category. The major difference was the introduction of an *Aspergillus* positive LRT specimen culture as the entry criterion (24). Additionally, clinical signs and symptoms were adapted to the presentation of ICU patients, and it didn't rely anymore to specific

imaging signs. However, mycological evidence through non-culture based biomarkers [i.e., galactomannan (GM)] was still not implemented into the diagnostic scheme (24). Subsequently, the AspICU algorithm was adapted for different specific risk groups, for instance, diagnostic criteria for IAPA (27,30). The individual diagnostic components of IPA relevant to the treating physician in the ICU are presented below.

1.4.1 Mycological Work-up

The most important mycologic samples in the ICU can be divided into blood (e.g., serum, plasma) and LRT specimens [e.g., bronchoalveolar lavage fluid (BALF), tracheal aspirate (TA)]. While blood samples are readily available, BALF samples are more difficult to obtain. A bronchoscope must be inserted into the deepest airways and therefore a sufficiently stable patient is required (110). Most diagnostic tests can be performed in blood as well as in respiratory samples. However, in non-neutropenic patients respiratory samples are in general better for the performance of diagnostic tests (5).

Culture:

The cultures from obtained respiratory specimen are an important cornerstone in the diagnosis of IPA, especially in ICU patients. However, the significance of *Aspergillus* isolates in cultures differs depending on the site where the samples were taken and the underlying immune status of the patient. For instance, a positive culture out of sputum samples in immunocompetent patients is most probably suggesting colonization than actual IFD (15). BALF specimens on the other hand have better performance in non-neutropenic patients. The prolonged tissue-invasive phase in these patients results in the expulsion of fungal material at the site of infection (21). However, the higher specificity of a fungal culture from BALF samples is still opposed by a sensitivity of approximately 50%. Therefore, the culture should be supplemented with additional tests if possible, also because a single positive culture from the BALF may still represent colonization (5). Positive *Aspergillus* culture out of blood samples representing fungemia is only reported in few cases, even in immunocompromised patients (15). Thus, it is not performed for the regular diagnosis of IPA in ICU patients.

Fungal biomarkers:

Detection of biomarkers like GM and (1,3)-β-D-glucan (BDG) as indirect fungal tests has become an important part in the diagnosis of IFDs. BDG is a fungal cell wall component, which can be elevated in serum by many different IFDs (e.g., candidiasis, aspergillosis, fusariosis). As a nearly pan-fungal marker the value of BDG in the diagnosis of IPA is limited due to its low specificity (111). In contrast, GM is a universal cell wall molecule of *Aspergillus*, which is present in conidia as well as in hyphae. These molecules are polysaccharides, composing together with BDG, α-glucan and chitin the cell wall structure of *Aspergillus* (3). Although highly specific for *Aspergillus* species GM can also be detected in other fungi species such as *Fusarium*, *Penicillium* and *Histoplasma* (16). The conventional test method of GM is based on a double sandwich enzyme immunoassay (EIA). The EIA uses a monoclonal antibody to detect the antigen of GM, specifically several epitopes on galactofuranose (16). Repeated measurement of GM in serum has become an important screening tool of IPA in high-risk immunocompromised patients. However, circulating GM in the blood as the sign of angio-invasive disease is often not met in non-neutropenic patients (110). Instead, the GM measurement in BALF became the "gold standard" in these patients. Detection of GM in a BALF sample represents active fungal growth, corresponding to the airway-invasive pattern in the earlier stages of IPA (21). The value of GM is specified by the optical density index (ODI). A GM ODI ≥0.5 in serum and GM ODI >1.0 in BALF have become established as the thresholds for a positive result (110). Detection of GM in BALF has a high sensitivity and specificity in diagnosing IPA, especially in the ICU setting (26,112).

Polymerase chain reaction:

Aspergillus-specific polymerase chain reaction (PCR) was implemented as a mycological criterion for probable IA the first time in the last revision of the EORTC/MSGERC criteria. Its value has been investigated the most for patients with hematologic malignancies and HSCT (22). Testing of *Aspergillus* spp. by using PCR in serum has a low sensitivity but a high specificity in non-neutropenic patients (113). *Aspergillus*-specific PCR from BALF samples on the other hand represents a good diagnostic possibility in non-neutropenic patients and is considered superior to fungal culture (5). The unique ability of PCR is the possibility to specify genus and species of *Aspergillus*. The identification of azole resistant mutations due to PCR, may be faster available in comparison to antifungal resistance testing in culture (2,110).

Point-of-care diagnostic tests:

There are two relatively new POC tests available, namely the *Aspergillus*-specific lateral-flow device (LFD) test and the *Aspergillus* GM lateral-flow assay (LFA). The *Aspergillus*-specific LFD uses a monoclonal antibody for binding to an extracellular glycoprotein antigen of *Aspergillus* spp., which is only secreted during active fungal growth (114). The *Aspergillus* GM LFA is using another method than EIA for GM measurement and results can be quantified either by the naked eye or through a digital read-out (Fig. 8) (115). These tests might be helpful in the early diagnosis of IPA, due to significantly lower turnaround times in comparison to established GM measuring and fungal culture. Both tests are now CE marked in the European Union and deliver results within 15–30 minutes (116). In addition, POC tests could fill the gap in the diagnostic work-up of IPA in regions such as Asia, where conventional GM detection is often unavailable in laboratories (117). The tests can theoretically also be performed in blood samples. However, this has currently no diagnostic significance in the ICU, as the sparse data available indicates that the sensitivity is either too low or only investigated for hematological patients (110).

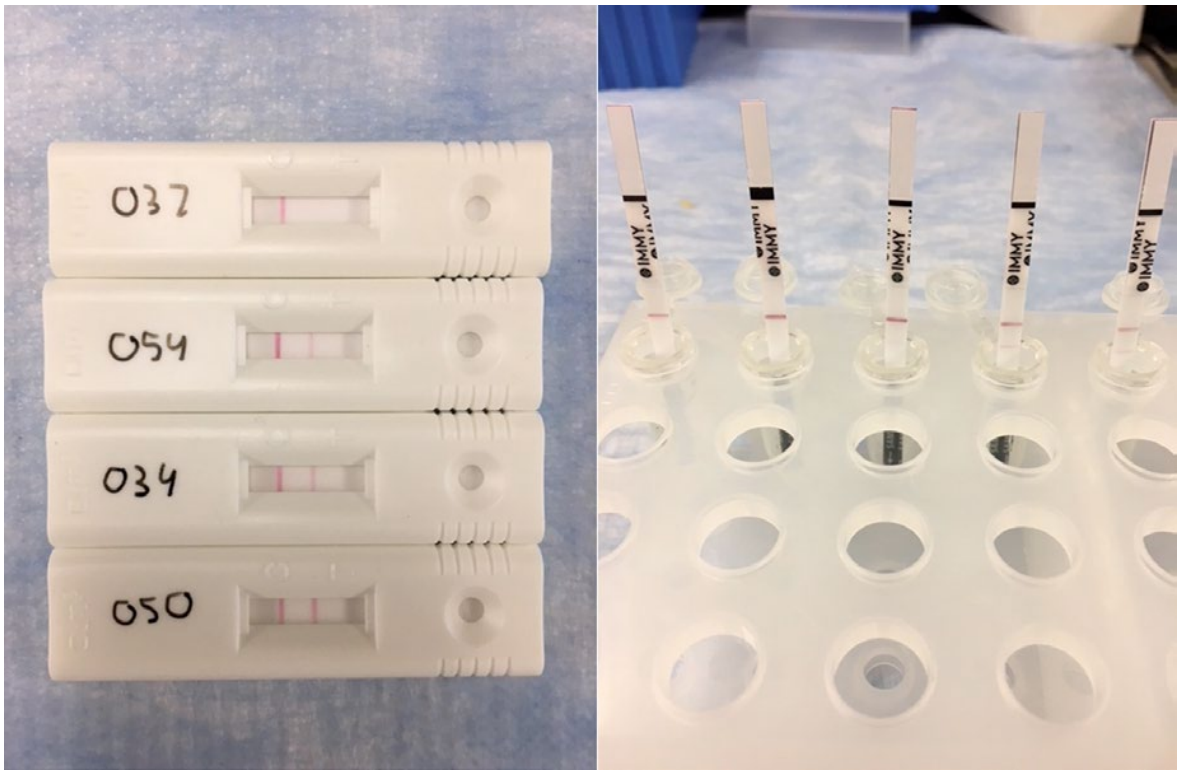

Figure 8: ***Point-of-care (POC) tests in the diagnosis of invasive pulmonary aspergillosis (IPA).*** Left: *Aspergillus*-specific Lateral Flow Device test. Right: *Aspergillus* Galactomannan Lateral Flow Assay.

Reprinted from Jenks JD, Mehta SR, Taplitz R, Aslam S, Reed SL, Hoenigl M. Point-of-care diagnosis of invasive aspergillosis in non-neutropenic patients: *Aspergillus* Galactomannan Lateral Flow Assay versus *Aspergillus*-specific Lateral Flow Device test in bronchoalveolar lavage. Mycoses. 2019;62(3):230–6., with permission of John Wiley and Sons.

1.4.2 Imaging

The grade of immunosuppression influences the radiologic presentation of IPA. Chest CT signs of angio-invasive disease are more frequent in patients with acute leukemia and in those, who are profoundly neutropenic in general. Most typical finding is the nodule with halo and air-crescent sign in the neutropenic patient, which is highly suspicious of IPA (18). Signs of airway-invasive disease, on the other hand, are more frequent among other hematological patients than acute leukemia and non-neutropenic patients. Airway-invasive disease may present in the chest CT with signs like centrilobular micronodules and tree-in-bud opacities (18). However, in patients without EORTC/MSGERC host factor these typical findings are missing in most IPA cases and ICU patients present mostly with non-specific radiographic features (12). These include unspecific infiltrates and consolidations that resemble the findings in severe COVID-19, making them indistinguishable in most cases (110).

1.4.3 Diagnostic Approach in COVID-19 patients

Onset of clinical signs and symptoms presented by CAPA patients is variable (113). Like the pathogenesis of IPA, a differentiation between the clinical presentation of neutropenic and non-neutropenic patients must be made. Fever, cough and chest pain are less frequent in non-neutropenic patients (110). Moreover, the clinical findings in general are unspecific and overlap with symptoms caused by COVID-19 (98). Nonetheless, clinical symptoms in non-neutropenic IPA patients can include worsening of the respiratory situation, refractory or recrudescent fever despite appropriate antibiotic therapy, pleural friction rub and chest pain, hemoptysis and dyspnea (24).

Clinical deterioration in COVID-19 patients with therapy-resistant respiratory failure can also be caused by venous thromboembolic events and cardiac disease (62,72). To rule out other factors for a worsening course, repetition of diagnostic procedures like CT is indicated (113). Imaging results should be accompanied with specimen acquisition of the LRT. This should be followed by intensive microbiological investigations consisting of microscopy, culture, *Aspergillus*-specific PCR, *Aspergillus*-specific LFD and GM measurement by EIA or LFA, respectively (113). To distinguish between *Aspergillus* colonization and invasive disease is important, because it affects the diagnosis of IPA in the ICU as the largest confounder (74). Respiratory tract specimen, like sputum or TA may only represent

colonization instead of true infection (113). Furthermore, validated thresholds of GM in respiratory samples like tracheal aspirate and sputum are not available (118). Therefore, performing bronchoscopy and consecutively obtaining a BALF sample is the most important investigation in the ICU setting to diagnose IPA (113).

1.4.4 Diagnostic Criteria for COVID-19-associated Pulmonary Aspergillosis

The European Confederation for Medical Mycology (ECMM) and the International Society for Human and Animal Mycology (ISHAM) proposed the following case definition of CAPA for clinical research in 2020 (113):

- SARS-CoV-2 infection confirmed by a positive RT-PCR result, either during two weeks between hospital and ICU admission or within 72–96 hours after ICU admission (entry criterion)
- IPA in temporal relationship to COVID-19-associated acute respiratory failure and requirement for treatment in the ICU

The diagnostic criteria for CAPA proposed by the ECMM/ISHAM are displayed in table 1. Within the probable CAPA definition the criteria distinguish between a diagnostic approach for IATB and one for other pulmonary manifestations (113). For the diagnosis of IATB in COVID-19 patients, direct visualization and consecutive biopsy and histopathology of tracheobronchial ulcers is considered as diagnostic "gold standard" (113). IATB may present as ulcerations, nodules, pseudomembranes, plaques or eschar on bronchoscopy. This again emphasizes the importance of performing bronchoscopy in the diagnosis of CAPA. Apart from the tracheobronchial form, a combination of an imaging feature [preferably by high resolution computed tomography (HRCT)] and mycological evidence through microbiological investigations is required to diagnose probable CAPA (113).

Table 1: ***Diagnostic criteria for proven, probable or possible COVID-19-associated pulmonary aspergillosis (CAPA) by the European Confederation for Medical Mycology/International Society for Human and Animal Mycology*** *(113)*

Entry criterion: Patient with COVID-19 needing intensive care and a temporal relationship

Proven tracheobronchitis or other pulmonary form

Mycological evidence (at least one of the following):

- histopathological or direct microscopic detection of fungal hyphae, showing invasive growth with associated tissue damage
- aspergillus recovered by culture or microscopy or histology or PCR obtained by a sterile aspiration or biopsy from a pulmonary site, showing an infectious disease process

Probable tracheobronchitits

Clinical factors:

- Tracheobronchitis, indicated by tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis

Mycological evidence (at least one of the following):

- microscopic detection of fungal elements in bronchoalveolar lavage, indicating a mould
- positive bronchoalveolar lavage culture or PCR*
- serum galactomannan index >0.5 or serum LFA index >0.5**
- bronchoalveolar lavage galactomannan index ≥1.0 or bronchoalveolar lavage LFA index ≥1.0**

Probable pulmonary form

Clinical factors:

- Pulmonary infiltrate, preferably documented by chest CT, or cavitating infiltrate (not attributed to another cause)

Mycological evidence (at least one of the following):

- microscopic detection of fungal elements in bronchoalveolar lavage, indicating a mould
- positive bronchoalveolar lavage culture*
- serum galactomannan index >0.5 or serum LFA index >0.5**

- bronchoalveolar lavage galactomannan index ≥1.0 or bronchoalveolar lavage LFA index ≥1.0**
- two or more positive aspergillus PCR tests in plasma, serum, or whole blood*
- a single positive aspergillus PCR in bronchoalveolar lavage fluid (<36 cycles)*
- a single positive aspergillus PCR in plasma, serum, or whole blood, and a single positive in bronchoalveolar lavage fluid (any threshold cycle permitted)*

Possible pulmonary form***

Clinical factors:

- Pulmonary infiltrate, preferably documented by chest CT, or cavitating infiltrate (not attributed to another cause)

Mycological evidence (at least one of the following):

- microscopic detection of fungal elements in non-bronchoscopic lavage indicating a mould
- positive non-bronchoscopic lavage culture*
- single non-bronchoscopic lavage galactomannan index >4.5
- non-bronchoscopic lavage galactomannan index >1.2 twice or more
- non-bronchoscopic lavage galactomannan index >1.2 plus another non-bronchoscopic lavage mycology test positive (non-bronchoscopic lavage PCR or LFA)

Abbreviations: COVID-19=coronavirus disease 2019. ICU=intensive care unit. PCR=polymerase chain reaction. LFA=lateral flow assay.

* In case of patients with chronic obstructive pulmonary disease or chronic respiratory disease, the PCR or culture results should be confirmed by galactomannan testing to rule out colonization or chronic aspergillosis. Galactomannan index should be available; galactomannan-index threshold applies to both enzyme immunoassay and LFA.

** Visual reader should be used for a primary result and confirmatory galactomannan testing should be sought.

*** Classification of possible CAPA will most likely be sufficient to initiate antifungal therapy in the clinic but, in line with other consensus statements, it is not recommended for enrolling patients into clinical trials. Possible CAPA could serve as a secondary endpoint in a randomized prophylaxis study. Additional studies are needed to support the specificity of non-bronchoscopic lavage testing. Non-bronchoscopic lavage is considered a blind application of 10–20 mL saline recovered by aspiration via the closed suction system in an intubated patient. Bronchoalveolar lavage and non-bronchoscopic lavage are currently not considered equal for diagnosing CAPA.

1.5 Treatment of Invasive Pulmonary Aspergillosis

1.5.1 Classification of Antifungal Agents

The antifungal armamentarium against IPA consists of the major antimycotic classes of azoles, polyenes and echinocandins, which will be explained here. Treatment guidelines of IPA are published by the Infectious Diseases Society of America (IDSA) (119) and by the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) in cooperation with the ECMM and the European Respiratory Society (ERS) (28).

Azoles:

Azoles have a fungicide mechanism of action against *Aspergillus* spp. (16). They ligate to the heme-iron center of the cytochrome P450 (CYP450) enzyme 14-α-demethylase. This leads to the inhibition of the demethylation of lanosterol, thereby preventing the synthesis of ergosterol in the endoplasmic reticulum. The result is an interference in the function of the fungal cell membrane with an increase of permeability (120). Azoles currently in use against IPA are the second-generation azoles voriconazole, isavuconazole and posaconazole (16).

Azoles can be administered intravenously and are the only class where oral formulations are available as treatment and prophylaxis options against IPA. Voriconazole and isavuconazole (administration as a prodrug) are two first-line treatment options against IPA (16). Posaconazole is currently used as antifungal prophylaxis, especially in hematological high-risk patients (28,119). However, recently published results from a randomized controlled trial highlighted that posaconazole is not inferior to voriconazole when used as first-line treatment for IPA. As posaconazole is causing less treatment-related adverse events, it may be recommended as a first-line option in guidelines in the future (121). An important disadvantage of azoles is the many drug-drug interactions that can be a problem in both hematologic and ICU patients (16).

Polyenes:

Polyenes have a fungicide mechanism of action. By ligating to ergosterol in a hydrophobically manner the lipophile interaction between the polyene and ergosterol results in the integration of channels into the lipid bilayer membrane. The outflow through the channel of small molecules and electrolytes, especially potassium, leads to cell death (120). Currently the only polyene used for treatment of IPA is liposomal amphotericin B (16).

Administration is usually intravenous, although there are also nebulized formulations that are applied by inhalation (122). Liposomal amphotericin B can be established as the first alternative in case azoles are contraindicated, not tolerated or in case of infection due to azole-resistant *Aspergillus*. In contrast to most of the azoles, liposomal amphotericin B is way less hepatotoxic and therefore a potential alternative for first-line treatment of IPA in patients with liver disease. However, liposomal amphotericin B may be nephrotoxic, thus renal markers need to be checked on a regular basis (28,119).

Echinocandins:

In contrast to azoles and polyenes, echinocandins have a fungistatic (=inhibition of fungal cell growth) mode of action against *Aspergillus*. The cellular target of this class is the fungal cell wall. The inhibition of the synthesis of BDG disrupts the construction of the cell wall, which results in cell death (120). The three currently available echinocandins are caspofungin, anidulafungin and micafungin (16).

Echinocandins can only be administered intravenously. Echinocandins are not recommended as a first-line regiment of IPA but can be established alone or in combination with a mold-active azole in case of salvage therapy and in case of infection due to azole-resistant *Aspergillus*. Additionally, echinocandins may be used as prophylaxis in SOT recipients such as lung transplant recipients and hematologic patients at high risk for IFD (28,119). One of the main advantages of echinocandins are their low number of drug-drug interactions and good tolerability (16).

1.5.2 Treatment Approach in COVID-19-associated Pulmonary Aspergillosis

In general, the treatment of CAPA does not differ significantly from that of other IPA in the ICU. One of the main challenges in the treatment of ICU patients is reaching an appropriate drug exposure (30). In critically ill COVID-19 patients hampered renal or hepatic function, renal replacement therapy and ECMO are frequent, which have an impact on the pharmacokinetic variability. For assurance of sufficient efficacy and patient safety during antifungal treatment, therapeutic drug monitoring at least once a week is recommended (101,113). However, recommendations are currently only established for voriconazole and posaconazole. For voriconazole a plasma concentration range of 2–6 mg per liter (L) is

recommended, whereas for posaconazole the lower threshold for treatment of IPA is 1 mg/L (28).

The current treatment recommendations of the ECMM/ISHAM CAPA guideline (113) are displayed in figure 9. The efficacy of azoles is threatened by the emergence of azole-resistant A. *fumigatus* (ARAF) (2). In sectors such as agriculture, azoles are widely used to control molds on plants. Though they are not primarily used against A. *fumigatus*, this can create a selection pressure in the environment that favors the occurrence of ARAF (16). Cases of CAPA caused by ARAF have already been reported (98). Therefore, treatment recommendations were provided against CAPA caused by suspected or proven (testing of antifungal susceptibility) azole-resistant *Aspergillus* spp. (113).

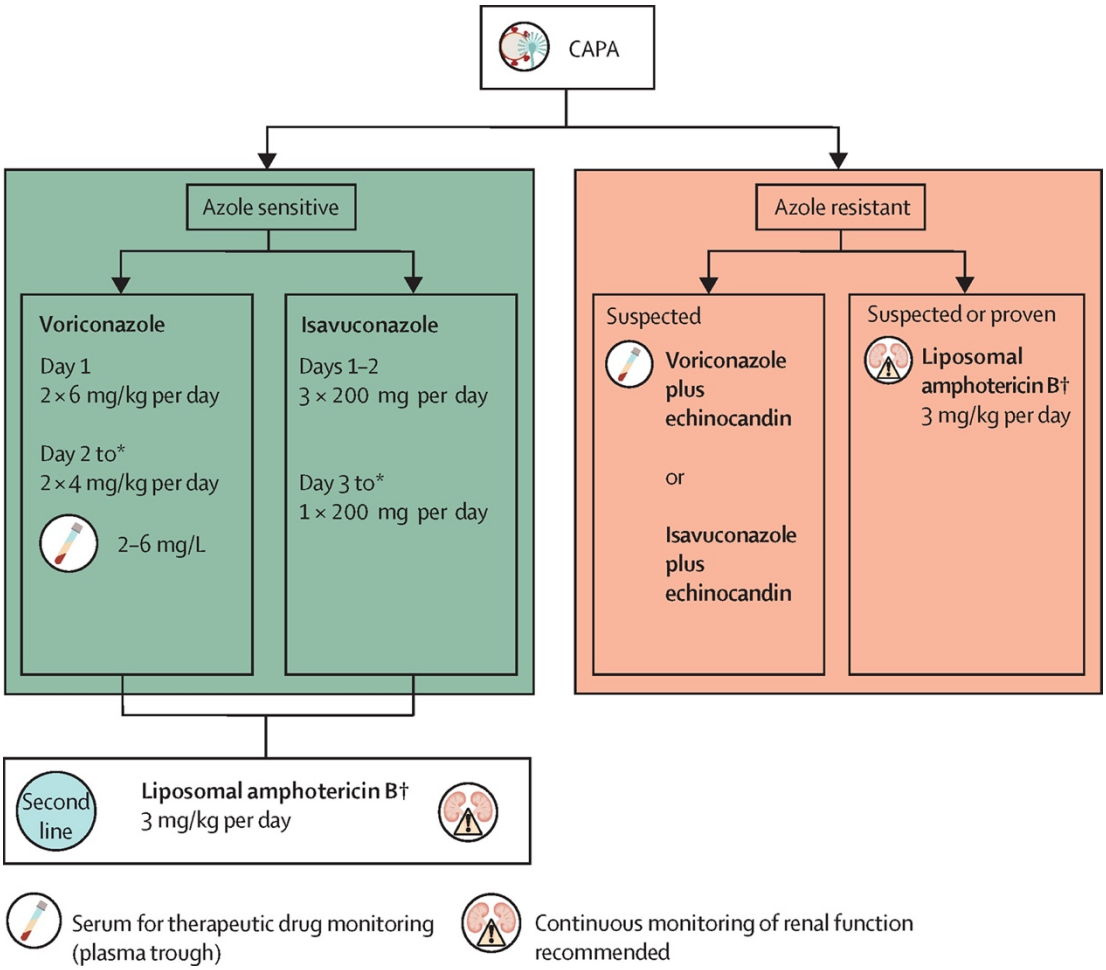

Figure 9: ***Recommended treatment for COVID-19-associated pulmonary aspergillosis (CAPA)***

*The optimal duration is unknown, but the expert panel suggests 6–12 weeks as a treatment course. In immunocompromised patients (eg, with haematological malignancy or receiving immunosuppressive therapy), longer treatment might be necessary. †Salvage therapy: caspofungin 70 mg loading dose on the first day followed by 50 mg/day. If body weight is more than 80 kg, then 70 mg loading dose on the first day followed by 70 mg/day.

1.6 Importance of Evaluating the Prevalence of COVID-19-associated Pulmonary Aspergillosis

IPA is a life-threatening disease. In order to improve the outcome of patients affected by IPA, rapid initiation of antifungal therapy is essential (21). The identification of specific patient collectives affected by this disease therefore assumes an important role. Awareness of a disease can only be raised among treating physicians if solid epidemiological data on the disease burden are available. However, especially the early diagnosis of IPA has always been challenging (15). Hence, the estimation of the actual prevalence of CAPA was uncertain at the relative beginning of the pandemic due to several reasons. One of them was the restrictive use of aerosol-generating procedures (AGPs) like bronchoscopy due to the related danger to health care workers or nosocomial infections (74). Therefore, diagnosis of CAPA was often driven by obtainment of TA samples. Detected GM out of TA samples is lacking specificity in comparison to BALF samples and is rather representing colonization than invasive disease (123). Second, diagnostic tests out of blood samples lack sensitivity in non-neutropenic patients (116). Third, both the clinical picture and radiologic signs of CAPA resemble those already seen in critically ill COVID-19 patients (37,124). Furthermore, in multiple case series the published prevalence of CAPA had a wide range from 5% up to 30% (74). In the absence of consensus criteria at this date, these studies used multiple different diagnostic definitions of CAPA, making it difficult to compare epidemiological data (24,118,124).

Therefore, the primary aim of this study was to estimate the prevalence of CAPA in patients admitted to the ICU due to COVID-19-associated ARF by uniformly applying the ECMM/ISHAM consensus criteria (113). Secondly, we wanted to evaluate the diagnostic value of fungal biomarkers and elucidate possible risk factors associated with the development and outcome of CAPA.

2 Materials and Methods

2.1 Aim of the Study

The primary objective of this study was to elucidate the prevalence of CAPA and its epidemiology in COVID-19 patients who required admission to ICU due to COVID-19-associated ARF.

Secondary objectives were:

1. To assess the diagnostic and prognostic performance of routinely performed fungal biomarkers for diagnosing CAPA in critically ill COVID-19 patients.
2. To evaluate the risk factors linked with diagnosis of CAPA and the outcomes of patients with CAPA in the ICU.

2.2 Study Design and Setting

This is a prospective monocentric cohort study at the University Hospital of Graz, Austria. Case entry started in March 2020 and was open until May 2021. After admission to ICU patients underwent checking for study inclusion criteria. If included the patients were followed and provided data were collected. Since September 1, 2020, antifungal prophylaxis has been recommended in the local standard operating procedure (SOP) for management of critically ill COVID-19 patients. The recommendation included the systemic application of intravenous posaconazole for the duration of HFNC oxygen supplementation, NIV or IMV in every patient admitted to the ICU due to COVID-19-associated ARF. However, whether posaconazole was implemented as a prophylaxis was solely the decision of the treating physicians.

2.3 Participants

All consecutive COVID-19 patients who were admitted to ICU due to COVID-19-associated ARF were included in this study.

Inclusion criteria:

- PCR confirmed SARS-CoV-2 infection
- ICU admission because of COVID-19-associated ARF
- Written informed consent (IC) given by the subject (in case patients were not able to give IC due to the underlying conditions, IC was obtained after clinical improvement. In case patients died and were not able to give IC before death, need for IC was waived by local ethic committee)

Exclusion criteria:

- < 18 years of age
- ICU admission because of other reasons than COVID-19-associated ARF

2.4 Specimen Sampling and Data Acquisition

The quantity and type of specimen which could be sampled depended on the necessary diagnostic procedures during the ICU stay and clinical course of each patient. All collected serum and respiratory tract (BALF/TA) samples were consequence of investigations performed in a routinely manner indicated by the treating physician only. The screening parameters for the study purpose included GM EIA in serum and BALF, *Aspergillus* spp. culture in BALF and TA, *Aspergillus* spp. specific LFD in BALF and *Aspergillus* specific spp. PCR in BALF. Left-over samples after routine work-up were collected and aliquoted, labelled in an anonymized way and stored at minus 80°C.

The sample storage was documented using a Microsoft Excel (Microsoft Corporation, Redmond, WA, United States) file. To identify patients anonymously they received a short identification document (ID) number. All data was acquired through browsing the hospital own medical data information system (openMEDOCS), which provides the patient records electronically for the medical staff in charge. Relevant details were transferred into an electronic case report form (eCRF), which included demographic data, baseline characteristics like underlying diseases and other risk factors leading to IFD, performed diagnostic procedures (microbiological and radiological investigations), antifungal treatment and outcome data. The anonymized eCRF was provided by FungiScope® (NCT 01731353) and accessible through (113) (EFS Fall 2018 Questback, Cologne, Germany) (125). The registry is in conformity with all applicable laws and regulations including the International Conference on Harmonization (ICH) Guideline for Good Clinical Practice (GCP) the ethical principles that are based on the Declaration of Helsinki (current official version: Fortaleza, Brazil 2013), and applicable privacy laws (Regulation European Union 2016/679). In addition, all Good Epidemiological Practice (GEP) requirements are fulfilled by the registry.

2.5 Diagnostic Criteria

To define CAPA cases, the 2020 ECMM/ISHAM consensus criteria (113) were utilized (Fig. 10). In relation to the proposed case definition the patients were classified as either proven CAPA (pulmonary and/or tracheobronchial form), probable CAPA (pulmonary and/or tracheobronchial form), possible CAPA (pulmonary form) or no evidence for CAPA. All patients fulfilling the definition for proven, probable or possible CAPA were considered as CAPA cases for this study.

2.6 Statistics

In terms of creating the statistics IBM SPSS 27 (SPSS Inc., Chicago, IL) was used for all analyses. For the descriptive analysis categorical variables are shown as absolute and relative frequencies with counts and percentages. Quantitative variables are presented as medians and quartiles or as mean plus 95% confidence interval (95% CI), as appropriate. To compare the group of patients who developed CAPA versus those with no evidence for CAPA (independent samples) and test for statistical significance, categorical variables were tested with chi-squared test and Fisher's exact test, respectively. All quantitative variables were analyzed graphically with quantile-quantile plots, checked for skewness and kurtosis and tested with Shapiro-Wilk-test and Kolmogorov-Smirnov-test if there are any normally distributed variables. Quantitative variables were then tested for statistical significance between the two groups with Mann-whitney-u-test or unpaired t-test, as appropriate. A p value below 0.05 was declared as statistically significant. For survival analysis between the patients who developed CAPA and those who did not a Kaplan-Meier estimator and plots were used for overall survival. After admission to the ICU, CAPA is diagnosed in each patient after different periods of time. Thus, for comparability between the two groups, a point in time of 14 days after ICU admission was defined as the starting point for the survival analysis. Patients who died or were discharged from the ICU before this time point were excluded from survival analysis. The remaining patients diagnosed with CAPA before this date were included in the CAPA group, while those not diagnosed with CAPA by this date were included in the non-CAPA group. The study protocol was approved by the local ethics committee of the Medical University of Graz, Austria (EC #32–296 ex 19/20).

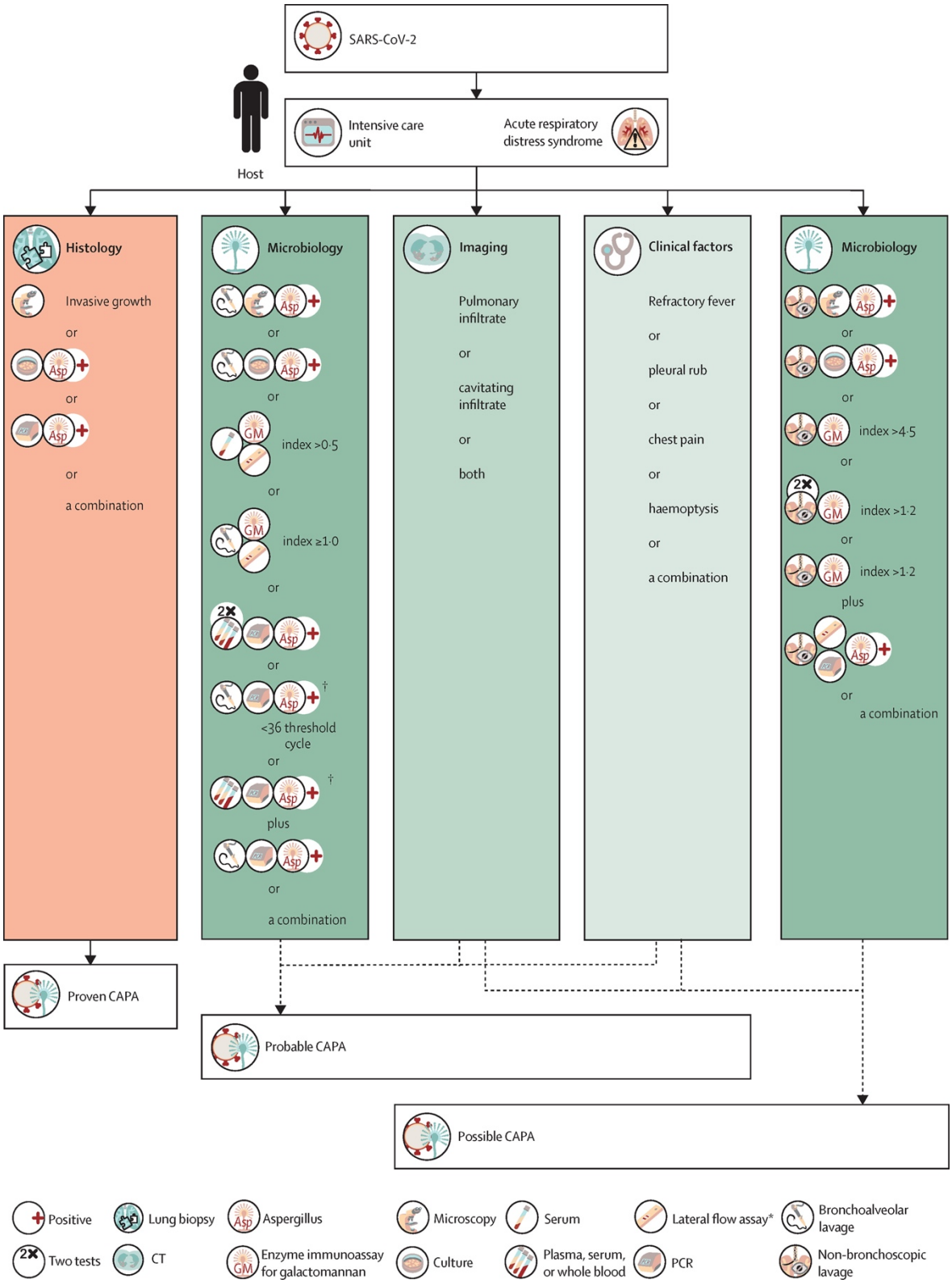

Figure 10: ***Defining and diagnosing COVID-19-associated pulmonary aspergillosis (CAPA) (pulmonary form)***

*Visual reader must be used for primary result and confirmatory galactomannan testing should be sought. †In case of patients with chronic obstructive pulmonary disease or chronic respiratory disease, the PCR or culture results should be confirmed by galactomannan testing to rule out colonization or chronic aspergillosis. Galactomannan index must be available; galactomannan index threshold applies to both enzyme immunoassay and lateral flow assay.

Reprinted from The Lancet Infectious Diseases, 21, Koehler P et al., Defining and managing COVID-19-associated pulmonary aspergillosis: the 2020 ECMM/ISHAM consensus criteria for research and clinical guidance, E149–E162, Copyright 2021, with permission from Elsevier.

3 Results

A total of 119 patients from the University Hospital of Graz were enrolled in this study. Out of 119 patients, 11 (9.2%) had probable CAPA, one (0.8%) had possible CAPA and 107 (89.9%) had no evidence for CAPA. This results in a CAPA prevalence (proven, probable and possible) of 10.1% (95% CI 5.9–14.3) with 12 CAPA cases [n=12 (eleven probable, one possible)] observed in the given case entry period. Out of 119 patients, 55 (46.2%) received IMV. Out of these 55 patients, 11 patients developed CAPA resulting in a prevalence of 20% (95% CI 12.7–29.1) among intubated patients. Out of 119 patients, eight (6.7%) were enrolled before local recommendation for antifungal prophylaxis, whereas 111 (93.3%) were enrolled after local recommendation for antifungal prophylaxis.

3.1 Baseline Characteristics of Patient Cohort

In table 1 the demographic characteristics are displayed to point out the major differences between the CAPA cases and the remaining patients without CAPA. Patients were diagnosed with CAPA after a median of 7 days (25^{th}–75^{th} quartile: 3.5–12.75) after ICU admission. The overall median follow-up time was 30 days (25^{th}–75^{th} quartile: 15–36) after admission to the ICU and 26 days (25^{th}–75^{th} quartile: 16.75–32.5) after patients had been diagnosed with CAPA. Patients diagnosed with CAPA received IMV significantly (p=0.001) more often compared to those who were not diagnosed with CAPA [11/12 patients (91.7%) versus 44/107 patients (41.1%)]. In addition, median ICU stay was significantly (p<0.001) longer in patients who were diagnosed with CAPA compared to non-CAPA patients [33 days (25^{th}–75^{th} quartile: 28.5–38) versus 10 days (25^{th}–75^{th} quartile: 5–20)].

Both groups received systemic corticosteroids in a high percentage as the primary COVID-19 therapy [10/12 CAPA patients (83.3%) versus 93/107 non-CAPA patients (86.9%)]. Tocilizumab was initiated as COVID-19 therapy in only a few patients [1/12 CAPA patient (8.3%) versus 5/107 non-CAPA patients (4.7%)].

Table 2: ***Baselines characteristics of patient cohort***

	Total (n=119)	CAPA group (n=12)	Non-CAPA group (n=107)	P value
Age (years), median (25^{th}–75^{th} quartile)	66 (55–76)	69 (55.5–77.5)	66 (55–75)	n.s.
Male sex, n (%)	76 (63.9)	10 (83.3)	66 (61.7)	n.s.
Baseline characteristics, n (%)				
Cardiovascular disease	76 (63.9)	7 (58.3)	69 (64.5)	n.s.
Diabetes mellitus	33 (27.7)	3 (25)	30 (28)	n.s.
History of smoking	16 (13.4)	2 (16.7)	14 (13.1)	n.s.
Malignant disease	17 (14.3)	1 (8.3)	16 (15)	n.s.
Obesity (BMI >30 kg/m^2)	35 (29.4)	5 (41.7)	30 (28.0)	n.s.
Pulmonary disease	31 (26.1)	2 (16.7)	29 (27.1)	n.s.
Solid organ transplantation	6 (5)	1 (8.3)	5 (4.7)	n.s.
Oxygen supplementation on ICU, n (%)				
Non-invasive ventilation	32 (26.9)	0 (0)	32 (29.9)	0.035
Invasive mechanical ventilation	55 (46.2)	11 (91.7)	44 (41.1)	0.001
ECMO	6 (5)	1 (8.3)	5 (4.7)	n.s.

COVID-19 therapy, n (%)				
Systemic corticosteroids	103 (86.6)	10 (83.3)	93 (86.9)	n.s.
Tocilizumab	6 (5)	1 (8.3)	5 (4.7)	n.s.
Survival day 30, n (%)	74 (62.2)	9 (75)	65 (60.7)	n.s.
Survival at ICU discharge, n (%)	68 (57.1)	6 (50)	62 (57.9)	n.s.
Survival end of follow-up, n (%)	61 (51.3)	4 (33.3)	57 (53.3)	n.s.
ICU stay (days), median (25^{th}–75^{th} quartile)	11 (5–23)	33 (28.5–38)	10 (5–20)	<0.001

Abbreviations: BMI=body mass index CAPA=COVID-19-associated pulmonary aspergillosis. COVID-19=coronavirus disease 2019. ECMO=extracorporeal membrane oxygenation. ICU=intensive care unit. n.s.=not significant (p>0.05).

3.2 Diagnostic Approaches

In table 3 the diagnostic strategies among CAPA cases are displayed. Bronchoscopy and BALF sampling were performed in 59 out of 119 patients (49.6%). BALF GM was measured in 41 out of these BALF samples (69.5%). Out of these 41, 11 patients were diagnosed with CAPA. Out of these 11, BALF GM turned out positive (GM>1.0 ODI) in six patients with CAPA (54.5%). BALF GM ODI in positive results was detected with a median of 6.81 (25^{th}–75^{th} quartile: 2.64–6.96). *Aspergillus*-specific LFD was performed in 19 out of these BALF samples (32.2%). Out of these 19, two patients were diagnosed with CAPA. Out of these two, *Aspergillus*-specific LFD turned out positive in one patient (50%). Out of these 19, three patients without CAPA had a positive *Aspergillus* specific LFD result. The *Aspergillus*-specific PCR was performed in 33 out of these BALF samples (55.9%). Out of these 33, six patients were diagnosed with CAPA. Out of these six, *Aspergillus*-specific PCR turned out positive in four patients (66.7%). BALF *Aspergillus* spp. culture was performed in 54 out of these BALF samples (91.5%). Out of these 54, 12 patients were diagnosed with CAPA. Out of these 12, *Aspergillus* spp. culture turned out positive in three patients with CAPA (25%). GM testing in serum was performed in 85 out of 119 patients (71.4%). Out of these 85, 12 patients were diagnosed with CAPA. Out of these 12, serum GM turned out positive (GM>0.5 ODI) in five patients (41.7%). Out of these 85, one patient without CAPA had a positive serum GM.

TA sampling was performed in 16 out of 119 patients (13.4%). Out of these 16, six patients were diagnosed with CAPA. Out of these six, *Aspergillus* spp. culture turned out positive in three patients (50%).

In table 4 the performed diagnostic procedures in each individual CAPA case are displayed. Among 11 probable CAPA cases, 5 out of 11 patients (45.5%) had a combination of two or more positive diagnostic procedures, whereas 6 out of 11 patients (54.5%) had only one positive diagnostic procedure (excluding TA *Aspergillus* spp. culture).

Table 3: ***Performed diagnostic procedures in COVID-19-associated pulmonary aspergillosis (CAPA) cases***

Diagnostic procedures	Positivity in CAPA cases (n=12)
BALF Galactomannan >1.0 ODI	6/11 (54.5%)
BALF Galactomannan ODI (if >1.0), median (25^{th}–75^{th} quartile)	6.81 (2.64–6.96)
Serum Galactomannan >0.5 ODI	5/12 (41.7%)
BALF positive *Aspergillus* spp. culture	3/12 (25%)
TA positive *Aspergillus* spp. culture	3/6 (50%)
BALF positive *Aspergillus* spp. PCR	4/6 (66.7%)
BALF positive *Aspergillus* spp. LFD	1/2 (50%)

Abbreviations: BALF=bronchoalveolar lavage fluid. CAPA=COVID-19-associated pulmonary aspergillosis. LFD=lateral flow device. ODI=optical density index. TA=tracheal aspiration. PCR=polymerase chain reaction.

Table 4: ***Overview of performed diagnostic procedures in individual COVID-19-associated pulmonary aspergillosis (CAPA) cases.*** Numbering of CAPA cases represents the same as in Table 5.

Cases	BALF GM >1.0 ODI	Serum GM >0.5 ODI	BALF Asp culture	TA Asp culture	BALF Asp PCR	BALF Asp LFD	Days from ICU admission to CAPA diagnosis
1	P	P	P	NA	P	P	3
2	P	P	N	NA	NA	NA	12
3	N	P	N	N	NA	NA	5
4	N	P	N	NA	NA	N	19
5	NA	P	N	N	N	NA	13
6	P	N	N	P	NA	NA	18
7	P	N	N	NA	P	NA	3
8	P	N	N	NA	P	NA	8
9	P	N	P	N	NA	NA	3
10	N	N	N	NA	P	NA	9
11	N	N	P	P	NA	NA	6
12*	N	N	N	P	N	NA	5

Abbreviations: P=positive. N=negative. NA=not available. Asp=*Aspergillus* spp. BALF=bronchoalveolar lavage fluid. CAPA=COVID-19-associated pulmonary aspergillosis. GM=Galactomannan. ICU=intensive care unit. LFD=lateral flow device. ODI=optical density index. TA=tracheal aspiration. PCR=polymerase chain reaction. *Possible CAPA.

3.3 Antifungal Treatment

Systemic antifungal treatment was initiated in all CAPA cases. In table 5 the different antifungal agents that had been used for treatment of CAPA cases are highlighted. Most cases (10/12; 83.3%) received a treatment with isavuconazole, followed by liposomal amphotericin B (4/12; 33.3%), voriconazole (2/12; 16.7%) and echinocandin (1/12; 8.3%). Five cases (5/12; 41.7%) were treated with antifungal combination of isavuconazole or voriconazole combined with liposomal amphotericin B or echinocandin. In addition, posaconazole was added in three cases (3/12; 25%) as the primary antifungal prophylaxis. Data of antifungal prophylaxis administration in the group of patients not diagnosed with CAPA was not available.

Table 5: ***Antifungal therapy initiated in COVID-19-associated pulmonary aspergillosis (CAPA) cases***. Numbering of CAPA cases represents the same as in Table 4.

Cases	Isavuconazole	Voriconazole	Liposomal Amphotericin B	Echinocandin
1*	X			
2	X		X	
3	X			
4	X			
5	X			
6		X		
7	X		X	
8*	X			
9		X	X	
10	X			
11*	X			X
12	X		X	

* Patient additionally received posaconazole as primary antifungal prophylaxis.

3.4 Survival Analysis

Overall, 58 deaths occurred in the study cohort during the observation period. 30 days after ICU admission nine out of 12 patients (75%) who developed CAPA were still alive, whereas 65 out of 107 patients (60.7%) without CAPA survived. At ICU discharge six out of 12 patients (50%) who developed CAPA were still alive, whereas 62 out of 107 patients (57.9%) without CAPA survived. At the end of follow-up 4 out of 12 patients (33.3%) who developed CAPA were still alive, whereas 57 out 107 patients (53.3%) without CAPA survived.

Of those CAPA patients who had a positive serum GM (ODI>0.5) result, four out of five patients (80%) survived at day 30 after ICU admission. Out of these five, two patients survived at ICU discharge (40%), whereas one out of five patients (20%) was still alive at the end of follow-up.

The cumulative survival after 70 days observation period between individuals with versus without CAPA is shown in figure 11 as Kaplan-Meier curve. Median survival time was estimated with 22 days (95% CI 15.02–28.98) in the CAPA group and 25 days (95% CI 4.91–45.09) in the group of patients without developing CAPA (p>0.05).

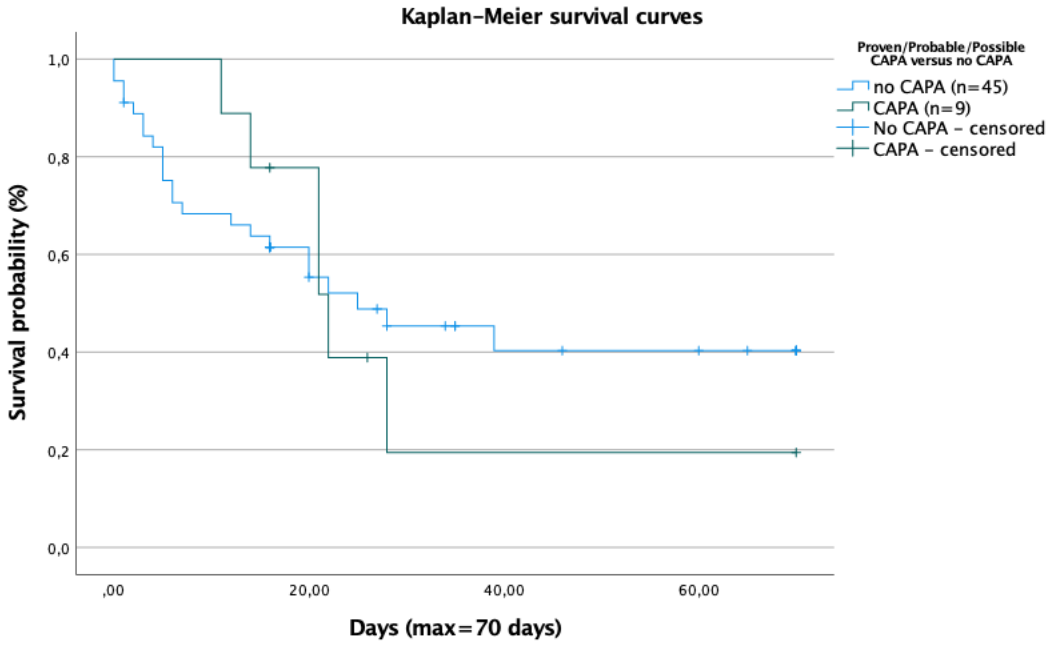

Figure 11: ***Kaplan-Meier curves between patients diagnosed with COVID-19-associated aspergillosis (CAPA) until 14 days after ICU admission and patients not diagnosed with CAPA until 14 days after ICU admission.*** Patients who died or were discharged before this date were excluded from analysis. Day 0=day 14 after ICU admission. Maximal observation period=70 days. The p-value was calculated by the log-rank test.

4 Discussion

In this study, involving patients who were admitted to the ICU because of COVID-19-associated ARF, we investigated the prevalence of CAPA. Occurrence of CAPA was found as a frequent complication with a prevalence of 10% in the overall cohort and 20% among patients receiving IMV, respectively. For classifying patients, the ECMM/ISHAM criteria (113) were used, and we found 11 patients with probable and 1 patient with possible CAPA.

A major factor influencing the wide range of reported CAPA incidence at the beginning of the pandemic was the use of numerous different diagnostic criteria. Retrospective application of ECMM/ISHAM criteria to previously conducted case series and prospective studies resulted in a notable reduction in the incidence of CAPA (126). This suggests that the incidence of CAPA was overestimated in many initial studies, especially in those reporting incidences over 30% (36,127). Our CAPA frequency results are close to data published in the largest ICU cohort studies to date using the ECMM/ISHAM criteria. In a multinational study by Janssen et. al (128) including two cohorts, frequency of patients receiving IMV was 82% and 76%, while CAPA incidence was estimated with 15% and 10%, respectively (128). In another large multicenter study by Gangneux et. al (129) only patients requiring IMV were included and systematically screened for IFD, which resulted in a CAPA prevalence of 15% (129). In our study the proportion of patients receiving IMV in the overall cohort was below 50%, the observation period has been over one year, and no systemic mycological testing was implemented in clinical care. Patients with CAPA were significantly more often treated with IMV than patients without evidence of CAPA and prevalence of CAPA among patients receiving IMV was twice as high as in the overall cohort. In addition, the group of patients diagnosed with CAPA faced a significantly longer stay in the ICU with a median of 33 days in comparison to 10 days in the non-CAPA group. These findings are in line with observations in other studies, reporting that CAPA predominantly occurs in COVID-19 patients with a worsening oxygenation course, need for intubation and prolonged ventilation (118,129,130). Possible explanation for this is that the development of CAPA is more likely to be favored in lungs severely damaged by ARDS, which requires IMV. IMV in general is known to be a clinical risk factor for development of IPA. As an entry route, colonization of the airways with *Aspergillus* spores is facilitated. Although colonization with fungal species does not necessarily cause infection it represents an antecedent for local invasive disease (17). Underlying immunopathological processes of COVID-19, alone or in combination with other factors, may favor that colonization develops

into IFD. However, as the pathophysiology of COVID-19 itself is not fully understood yet, further investigations are required to elucidate this association.

Since proven CAPA is indisputably the safest diagnosis and biopsies are rarely performed ante-mortem it is important to look at autopsy studies to validate our prevalence results. In a large systematic review of autopsy studies performed in 2020, invasive mold disease was only found in 2% of 677 deceased COVID-19 cases (131). Most of the included studies did not focus on identification of IFDs, hence fungal stains were only used in 38% of autopsies. Moreover, IMV was only present in 58% of the analyzed cohort and individual clinical data of each patient (e.g., administration of immunomodulatory treatment) was not available in several studies (131). However, the review suggests that when using the consensus criteria, the incidence of CAPA is still overestimated due to overdiagnosis in many studies. This is supported by the observation in a case series of deceased invasively ventilated COVID-19 patients. In six patients diagnosed as probable CAPA based on positive BALF specimens, postmortem needle core lung biopsies showed no histologic evidence of IA (132). Larger studies in which deceased probable CAPA cases are subsequently verified in autopsies for correctness of diagnosis would be of interest. On the other hand, an autopsy study by Fortarezza et al. (133) found a high frequency of proven CAPA, which supports our study results. Proven CAPA was diagnosed in nine out of 45 (20%) deceased severely ill COVID-19 patients. Patients were consecutively added into the study and included patients without ICU admission and requirement of IMV. In all cases foci of fungal pulmonary infection were found and in five cases signs of angio-invasive disease were present (133). Another single center study reported a high rate of four autopsy proven CAPA cases in eight ICU patients receiving long-term period of treatment, including ECMO. In all patients ante-mortem secondary IFD was not suspected (134). This observation is consistent with that in another large-scale study, which showed that IA in the ICU in general is only diagnosed in 40% of cases ante-mortem (135).

Considering that COVID-19 is a recently emerged disease, therapeutic approaches have been constantly changing over the past two years. This also has an impact on the epidemiology of the conducted studies during this time. After the first publication of the RECOVERY trial in summer 2020 (89), systemic corticosteroids have received the strongest recommendation in the therapeutic guidelines of COVID-19 and are broadly used in the ICU since then (85,88). Most of the patients in our study were included since autumn 2020 and only several patients

from the first wave in early 2020 were included. Hence, almost 90% of the overall patient cohort received systemic corticosteroids as standard therapy for COVID-19. This overall high percentage might be the reason why the difference in the administration of corticosteroids between CAPA and non-CAPA patients was not significant. In contrast to this, in the above-mentioned studies by Janssen et. al (128) and Gangneux et. al (129) less than 50% of the total populations received systemic corticosteroids as COVID-19 treatment, because patients in these studies were enrolled in the first half of 2020. The more frequent administration of systemic corticosteroids could be an explanation why the prevalence of CAPA in invasively ventilated patients was found higher in our study than in the other two studies. An increased likelihood of CAPA development due to application of high-dose corticosteroids, before or during ICU stay, was reported in several studies (118,130,136). Furthermore, corticosteroid use is already known as one of the main risk factors in ICU patients for developing IPA, which also depends of the applied cumulative dose (28). A recently formed CAPA task force recommends in their report, that tapering or discontinuation of corticosteroid therapy in CAPA patients without therapy response could be considered (101). However, because the benefits of corticosteroid therapy in COVID-19 are clearly established, most treating physicians are unlikely to discontinue therapy, especially if the CAPA diagnosis is uncertain.

Only a small number of six patients in the overall patient cohort received tocilizumab as a second immunomodulatory drug. Therefore, the impact on the prevalence of CAPA in our study is likely to be small. However, in other studies tocilizumab was reported as an independent risk factor for CAPA development (136). In addition, the combination therapy of tocilizumab and concomitant corticosteroids was found independently associated with development of CAPA (129). Therefore, especially COVID-19 patients receiving both corticosteroids and an anti-IL-6 pathway inhibitor should be monitored for signs and symptoms of a concomitant IFD (88).

The frequency of underlying diseases and conditions was equally distributed between CAPA patients and non-CAPA patients in our study. However, patients diagnosed with CAPA presented significantly more often with several risk factors in the published data, including history of pulmonary vascular disease, liver disease, coagulopathy, solid tumor, chronic renal failure and active smoking (130,136). Moreover, older age (>62 years), COPD, human immunodeficiency virus (HIV) infection and administration of immunosuppressive drugs other than corticosteroids before ICU admission, were independently associated with CAPA

development (128,129). In summary, even when the same diagnostic criteria are used, the prevalence of CAPA may vary in different studies depending on diagnostic monitoring, environmental factors (e.g., construction-related increase of fungal spores in the air), underlying treatment, risk factors and heterogeneity of the study population (137).

Clinical (e.g., refractory fever) and radiological signs (e.g., pulmonary infiltrates) are required in addition to mycological evidence to complete the probable and possible CAPA diagnosis according to the ECMM/ISHAM criteria (113). These signs are unspecific and often indistinguishable from those already present in severe COVID-19 (124). Specific radiologic findings of IPA are rare in non-immunocompromised patients, but typical signs such as nodules or cavitation were also reported in CAPA patients (118). Suspicion of IPA by radiologic signs should be also high in COVID-19, because these signs can occur before angio-invasion is reached, hence improving the survival of patients by earlier initiation of antifungal treatment (21). Nevertheless, mycologic evidence plays the greatest role in the diagnosis of CAPA. Bronchoscopy and subsequently obtained respiratory specimens have become the cornerstone in the early diagnosis of IPA in non-neutropenic patients (21). In our study patients were not systematically screened for the development of CAPA. This represents a realistic scenario of how patients could be diagnosed with CAPA in the ICU. Although more than half of the overall patient cohort did not receive IMV, bronchoscopy was performed in nearly 50% of the included patients. Hence, we were able to have access to a high number of sampled BALFs. In all patients diagnosed with CAPA at least one BALF sample was obtained. Out of these BALF samples, a fungal culture and GM detection were performed in almost all CAPA patients.

Detection of positive BALF GM in CAPA cases was relatively low with 54.5% out of 11 samples and only in 25% of the BALF samples in CAPA cases the fungal culture turned out positive for *Aspergillus*. In a study of patients at-risk for IPA, a BALF GM ODI ≥3 corresponded to a specificity of 100%, allowing IPA to be diagnosed independent of pretest probability (112). In our study positive BALF GM results were high with a median ODI of 6.81, suggesting a high fungal burden in these patients. In a study was shown that BALF GM concentration correlated with mortality in CAPA patients (138). Conversely, in another study BALF GM concentration was non-significant to ICU mortality of CAPA patients (139). Detection of GM is influenced by several factors. For one, cases of false-positive serum and BALF GM results due to administration of certain antibiotics (e.g., piperacillin-

tazobactam) were reported in the past (110). However, this does not seem to be a major problem with new solutions anymore these days (140). The exact location where the sample is taken and the amount of saline used also influences the BALF GM concentration (139). Furthermore, contamination of the BALF sample or culture with ubiquitous *Aspergillus* spores can lead to false-positive culture results (141). On the other hand, the fungal burden is by antifungal treatment and mold-active prophylaxis decreased, which may result in false-negative GM and culture results (142). However, only a minority of three patients diagnosed with CAPA received an antifungal prophylaxis with posaconazole in our study. Isolated positive GM and culture results from BALF samples may represent rather weak evidence for CAPA, which was particularly investigated in a recent study. BALF culture and BALF GM was often seen as the only positive mycologic evidence for CAPA and wasn't reproducible in many cases (141). However, in our study only in three out of eleven probable CAPA cases the diagnosis depended solely on either a positive BALF culture or a positive BALF GM result.

Interestingly, we observed detection of positive GM in serum in 41.7% of CAPA patients, which is higher than in other studies (138). Sensitivity of serum GM in CAPA is approximately 25% (139), which is the same as in other non-neutropenic patients in the ICU (26). Furthermore, serum GM was reported to remain negative in several proven CAPA cases (98). Only one out of five patients with a positive serum GM result did survive at the end of follow-up in our study. Although, we were not able to validate these differences statistically due to small sample size, this suggest that we had a relatively high number of CAPA cases in our cohort that reached the stage of angio-invasion. In recent investigations serum GM positivity in CAPA patients was associated with mortality rates reaching up to 100% (139,141). In addition, elevated serum BDG showed an association to higher mortality in CAPA patients. Although not suitable as a diagnostic criterium due to its non-specificity, serum BDG could serve together with serum GM as a prognostic marker indicative for angio-invasion in the management of CAPA patients (139). This reflects the clinical evolution that CAPA can progress to the stage of angio-invasion in non-neutropenic patients, albeit less frequently and significantly later than in neutropenic patients (21).
However, when we compare CAPA to IAPA, angio-invasion is observed more frequently in IAPA with a serum GM positivity of 65% (27). In addition, IAPA is diagnosed earlier after a median of three days after ICU admission. In contrast to this, we observed the diagnosis of CAPA after a median of seven days after ICU admission as was observed similarly in

other studies (128,129). Although both viruses cause epithelial damage, only influenza infects monocytes and macrophages, and thereby has a direct immunomodulatory effect by suppression of the NADPH-oxidase in these cells. This probably enables *Aspergillus* to reach the angio-invasion threshold earlier and more frequent in IAPA (102). The reported CAPA prevalence of 10–20% appears to be in a similar range to that of IAPA, which was calculated with 19% in the overall cohort and 14% in exclusively non-immunocompromised patients (27). However, in a large comparative study performed at the beginning of the COVID-19-pandemic, the incidence of IPA in invasively ventilated patients was found significantly higher in influenza than in COVID-19 patients (143). Furthermore, IAPA patients have been reported to have signs of IATB from 29% to over 50% (31,144). In comparison to that, manifestation as IATB was reported with a frequency of 10–20% among CAPA patients in some cohort studies (127,138).

An interesting approach to increase the diagnostic confidence of CAPA and to rule out more cases of colonization could be the combination of multiple diagnostic procedures for mycological evidence (118). In a recently conducted study mortality was significantly higher in patients who had three or more positive mycologic criteria results (141). This suggests that multiple positive tests better portray, if actually invasive disease developed. For instance, a positive BALF culture could be supplemented subsequently with a non-culture-based test (e.g., GM by EIA or LFA, PCR) and vice versa. POC tests (e.g., GM LFA) in particular could fill this role in the future, as they provide rapid results and do not require extensive laboratory testing (145). In the ECMM/ISHAM criteria a GM test in addition to a positive BALF culture or PCR is only required in patients with COPD or chronic respiratory disease to rule out colonization (113). BALF *Aspergillus* spp. PCR could be a suitable addition in the future to a single positive BALF culture or GM result, because quantifying the fungal burden is possible at the same time (146). In our study BALF PCR had the highest positivity rate with 66.7% among all diagnostic procedures but was only performed in half of the CAPA cases. However, thresholds must be evaluated further for different specimen and for each available PCR assay. The current recommended threshold of <36 cycles in the ECMM/ISHAM criteria may be not appropriate. Instead, in a recent study a threshold of <32 cycles showed the best sensitivity and specificity and was significantly associated with mortality (141).

Patients diagnosed with CAPA died in a larger percentage at ICU discharge and at the end of follow-up when compared to non-CAPA patients in our study. However, these findings were not statistically significant, including overall survival in Kaplan-Meier survival analysis. Nevertheless, evidence for excess mortality in CAPA was delivered by several other cohort studies (118,130,138,139). In addition, development of CAPA was proofed to be an independent predictor for mortality in the ICU (129). The significantly higher mortality of CAPA patients tends to argue against overdiagnosis of CAPA, which has been suggested in autopsy studies (131). The CAPA task force strongly recommends initiation of antifungal therapy in CAPA patients (101). According to the ECMM/ISHAM criteria also diagnosis of possible CAPA is likely to be sufficient to initiate antifungal treatment (113). All CAPA patients in our study cohort received antifungal treatment. In 40% of cases a combination of two antifungal agents were applied, which might have been indicated due to deterioration of the patients despite adequate monotherapy or due to antifungal resistance of the causative pathogen. Swift initiation of antifungal therapeutic regimes resulted to a significant decrease of the mortality rates in CAPA cases (118,138). As in other studies not all patients received antifungal treatment, this may explain why mortality in our study between CAPA and non-CAPA patients was not statistically different (129). However, there were also several cases reported, where patients classified as CAPA survived without receiving antifungal treatment (36,118,130). Moreover, in some studies mortality between CAPA patients, who received antifungal treatment and those who did not, was not significantly different (129,139,141). This again could reflect, similarly to the discrepancy between clinical CAPA diagnosis and autopsy findings, that several colonized patients are still misclassified as CAPA cases. Even positive BALF samples may represent colonization rather than invasive disease in some cases (141). In these patients, antifungal treatment cannot have a beneficial influence on the outcome. Colonization, in turn, may be a surrogate marker for more severe disease in COVID-19 itself, thus explaining the higher mortality rate of clinically diagnosed CAPA patients (131). However, it must be considered that cohort studies were not specifically designed to proof significance of antifungal treatment in CAPA cases. The impact of early and effective antifungal treatment needs to be investigated in randomized-controlled trial (RCTs) (129).

High mortality rates in CAPA cases also raises the question whether antifungal prophylaxis is appropriate and should be established. In a conducted RCT for posaconazole prophylaxis in influenza patients no conclusions could be drawn, because of the early development of many IAPA patients within the first 48 hours after ICU admission (120). A recently

published observational study showed, that antifungal prophylaxis in COVID-19 patients admitted to the ICU can lower the CAPA incidence significantly, but did not have an impact on lowering the mortality (148). Currently, an antifungal prophylaxis in general is not recommended in the guidelines for the treatment of critically ill COVID-19 patients (85,88).

Despite the great strengths (e.g., large patient cohort, sufficient respiratory samples, uniform application of ECMM/ISHAM criteria) of our study, some limitations should be mentioned. Even though the overall patient cohort was large, further statistical analyses such as regression analysis were not feasible because of the relatively small group of CAPA patients. Therefore, comparisons between the diagnostics and therapy performed within the group of CAPA patients have limited statistical validity. Although not an objective of this study, the impact of antifungal prophylaxis on the prevalence of CAPA would have been of interest. Unfortunately, data of posaconazole use in the non-CAPA group was not available. Furthermore, other information of interest such as the applied cumulative doses of glucocorticoids, antifungal treatment duration and specific dates of performed diagnostic procedures was not available.

In conclusion, CAPA is a serious complication in mainly invasively ventilated COVID-19 patients with prolonged stay in the ICU. Surveillance for CAPA should be high in COVID-19 patients receiving IMV with clinical deterioration despite appropriate therapy. Utilization of bronchoscopy in compliance with the personal protective measures should not be hesitated in case of suspected secondary infection. Combination of different mycologic criteria could increase diagnostic certainty of CAPA further. While some cases of colonization may still be misdiagnosed as CAPA by current consensus criteria, the poor outcome of CAPA when it progresses to the angio-invasive stage warrants initiation of antifungal therapy. Whether COVID-19 is like influenza an independent risk factor for developing IPA remains unclear and studies with head-to-head comparison with other control groups in the ICU are required. Further investigations with RCTs regarding the role of antifungal prophylaxis and therapy in lowering mortality of CAPA are needed.

4.1 A Brief Outlook

Since CAPA occurs primarily in critically ill COVID-19 patients, its incidence directly depends on the further course of the pandemic. The relative likelihood of developing severe disease by infection with Omicron is lower than in previous VOCs (149,150). Omicron causes more often only mild or moderate disease due to alterations in virological features and the high background immunity against COVID-19 based on vaccination and infection (151). Nevertheless, Omicron can also lead to an increase in critically ill patients due to very high case numbers. However, the impact in the general population is limited, and immunocompromised and elderly patients are now primarily affected by severe COVID-19 (152). Adequate protection by vaccination often cannot be achieved in this population due to the lack of immune response. This group of patients have conditions that overlap with those described in the EORTC/MSGERC host factors. An increase of critically ill COVID-19 patients presenting with EORTC/MSGERC host factors has a direct impact of the risk of developing CAPA. Hence, overall CAPA cases are expected to decrease due to lower incidence of severe COVID-19 cases, but the relative likelihood of a COVID-19 patient admitted to the ICU being affected by CAPA may increase. On the other hand, the evolution of SARS-CoV-2 is unpredictable. Mutations of epitopes in the S-protein targeted by neutralizing Abs can result in escape from innate or adaptive host immune response (45). Therefore, the occurrence of an immune-escape variant with higher pathogenicity cannot be excluded. This would then again lead to severe courses in broader sections of the population and thus also favor the occurrence of CAPA.

References

1. Murray PR, Rosenthal KS, Pfaller MA. Medical microbiology. 8th ed. Philadelphia: Elsevier; 2016. 836 p.
2. Fisher MC, Alastruey-Izquierdo A, Berman J, Bicanic T, Bignell EM, Bowyer P, et al. Tackling the emerging threat of antifungal resistance to human health. Nat Rev Microbiol [Internet]. 2022 [cited 2022 Apr 8]; Available from: https://www.nature.com/articles/s41579-022-00720-1
3. van de Veerdonk FL, Gresnigt MS, Romani L, Netea MG, Latgé JP. Aspergillus fumigatus morphology and dynamic host interactions. Nat Rev Microbiol. 2017;15(11):661–74.
4. Lass-Flörl C, Roilides E, Löffler J, Wilflingseder D, Romani L. Minireview: host defence in invasive aspergillosis: Host defence in invasive aspergillosis. Mycoses. 2013;56(4):403–13.
5. Kosmidis C, Denning DW. The clinical spectrum of pulmonary aspergillosis. Thorax. 2015;70(3):270–7.
6. Hoenigl M, Sprute R, Egger M, Arastehfar A, Cornely OA, Krause R, et al. The Antifungal Pipeline: Fosmanogepix, Ibrexafungerp, Olorofim, Opelconazole, and Rezafungin. Drugs [Internet]. 2021 [cited 2021 Oct 24]; Available from: https://link.springer.com/10.1007/s40265-021-01611-0
7. Bennett JE, Dolin R, Blaser MJ. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 9th ed. Philadelphia: Elsevier; 2019.
8. Segal BH, Walsh TJ. Current Approaches to Diagnosis and Treatment of Invasive Aspergillosis. Am J Respir Crit Care Med. 2006;173(7):707–17.
9. Segal BH. Aspergillosis. N Engl J Med. 2009;360(18):1870–84.
10. Roadmap - Gaffi | Gaffi - Global Action For Fungal Infections [Internet]. 2015 [cited 2022 Jun 15]. Available from: https://gaffi.org/roadmap/
11. Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden Killers: Human Fungal Infections. Sci Transl Med [Internet]. 2012 [cited 2022 Jul 19];4(165). Available from: https://www.science.org/doi/10.1126/scitranslmed.3004404
12. Vandewoude K, Blot S, Depuydt P, Benoit D, Temmerman W, Colardyn F, et al. Clinical relevance of Aspergillus isolation from respiratory tract samples in critically ill patients. Crit Care. 2006;10(1):R31.
13. Rankin NE. Disseminated aspergillosis and moniliasis associated with agranulocytosis and antibiotic therapy. Br Med J. 1953;1:918–9.

14. Chamilos G, Luna M, Lewis RE, Bodey GP, Chemaly R, Tarrand JJ, et al. Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989-2003). Haematologica. 2006;91(7):986–9.
15. Zmeili OS, Soubani AO. Pulmonary aspergillosis: a clinical update. QJM. 2007;100(6):317–34.
16. Arastehfar A, Carvalho A, Houbraken J, Lombardi L, Garcia-Rubio R, Jenks JD, et al. *Aspergillus fumigatus* and aspergillosis: From basics to clinics. Stud Mycol. 2021;100(1):100115–100115.
17. Gago S, Denning DW, Bowyer P. Pathophysiological aspects of *Aspergillus* colonization in disease. Med Mycol. 2019;57(Supplement_2):S219–27.
18. Bergeron A, Porcher R, Sulahian A, de Bazelaire C, Chagnon K, Raffoux E, et al. The strategy for the diagnosis of invasive pulmonary aspergillosis should depend on both the underlying condition and the leukocyte count of patients with hematologic malignancies. Blood. 2012;119(8):1831–7.
19. Böcker W, Denk H, Heitz PU, Höfler G, Kreipe H, Moch H, editors. Pathologie. 5th ed. München: Elsevier, Urban & Fischer; 2012. 1064 p.
20. Cordonnier C, Botterel F, Ben Amor R, Pautas C, Maury S, Kuentz M, et al. Correlation between galactomannan antigen levels in serum and neutrophil counts in haematological patients with invasive aspergillosis. Clin Microbiol Infect. 2009;15(1):81–6.
21. Nucci M, Nouer SA, Cappone D, Anaissie E. Early diagnosis of invasive pulmonary aspergillosis in hematologic patients: an opportunity to improve the outcome. Haematologica. 2013;98(11):1657–60.
22. Donnelly JP, Chen SC, Kauffman CA, Steinbach WJ, Baddley JW, Verweij PE, et al. Revision and Update of the Consensus Definitions of Invasive Fungal Disease From the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium. Clin Infect Dis. 2019;71(6):1367–76.
23. Gustot T, Maillart E, Bocci M, Surin R, Trépo E, Degré D, et al. Invasive aspergillosis in patients with severe alcoholic hepatitis. J Hepatol. 2014;60(2):267–74.
24. Blot SI, Taccone FS, Van den Abeele AM, Bulpa P, Meersseman W, Brusselaers N, et al. A Clinical Algorithm to Diagnose Invasive Pulmonary Aspergillosis in Critically Ill Patients. Am J Respir Crit Care Med. 2012;186(1):56–64.
25. Prattes J, Flick H, Prüller F, Koidl C, Raggam RB, Palfner M, et al. Novel Tests for Diagnosis of Invasive Aspergillosis in Patients with Underlying Respiratory Diseases. Am

J Respir Crit Care Med. 2014;190(8):922–9.
26. Meersseman W, Lagrou K, Maertens J, Wilmer A, Hermans G, Vanderschueren S, et al. Galactomannan in Bronchoalveolar Lavage Fluid: A Tool for Diagnosing Aspergillosis in Intensive Care Unit Patients. Am J Respir Crit Care Med. 2008;177(1):27–34.
27. Schauwvlieghe AFAD, Rijnders BJA, Philips N, Verwijs R, Vanderbeke L, Van Tienen C, et al. Invasive aspergillosis in patients admitted to the intensive care unit with severe influenza: a retrospective cohort study. Lancet Respir Med. 2018;6(10):782–92.
28. Ullmann AJ, Aguado JM, Arikan-Akdagli S, Denning DW, Groll AH, Lagrou K, et al. Diagnosis and management of Aspergillus diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline. Clin Microbiol Infect. 2018;24:e1–38.
29. Bassetti M, Azoulay E, Kullberg BJ, Ruhnke M, Shoham S, Vazquez J, et al. EORTC/MSGERC Definitions of Invasive Fungal Diseases: Summary of Activities of the Intensive Care Unit Working Group. Clin Infect Dis. 2021;72(Supplement_2):S121–7.
30. Verweij PE, Rijnders BJA, Brüggemann RJM, Azoulay E, Bassetti M, Blot S, et al. Review of influenza-associated pulmonary aspergillosis in ICU patients and proposal for a case definition: an expert opinion. Intensive Care Med. 2020;46(8):1524–35.
31. Nyga R, Maizel J, Nseir S, Chouaki T, Milic I, Roger PA, et al. Invasive Tracheobronchial Aspergillosis in Critically Ill Patients with Severe Influenza. A Clinical Trial. Am J Respir Crit Care Med. 2020;202(5):708–16.
32. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. JAMA. 2020;323(13):1239–42.
33. WHO Director-General's opening remarks at the media briefing on COVID-19 - 11 March 2020 [Internet]. [cited 2021 Feb 14]. Available from: https://www.who.int/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020
34. Blaize M, Mayaux J, Nabet C, Lampros A, Marcelin AG, Thellier M, et al. Fatal Invasive Aspergillosis and Coronavirus Disease in an Immunocompetent Patient. Emerg Infect Dis. 2020;26(7):1636–7.
35. Prattes J, Valentin T, Hoenigl M, Talakic E, Reisinger AC, Eller P. Invasive pulmonary aspergillosis complicating COVID-19 in the ICU - A case report. Med Mycol Case Rep. 2021;31:2–5.

36. Alanio A, Dellière S, Fodil S, Bretagne S, Mégarbane B. Prevalence of putative invasive pulmonary aspergillosis in critically ill patients with COVID-19. Lancet Respir Med. 2020;8(6):e48–9.
37. Koehler P, Cornely OA, Böttiger BW, Dusse F, Eichenauer DA, Fuchs F, et al. COVID-19 associated pulmonary aspergillosis. Mycoses. 2020;63(6):528–34.
38. Wauters J, Lamoth F, Rijnders BJA, Calandra T. Invasive Pulmonary Aspergillosis Goes Viral Again? Am J Respir Crit Care Med. 2021;203(3):275–7.
39. Gorbalenya AE, Baker SC, Baric RS, de Groot RJ, Drosten C, Gulyaeva AA, et al. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nat Microbiol. 2020;5(4):536–44.
40. Jameson JL, editor. Harrison's principles of internal medicine. Twentieth edition. New York: McGraw-Hill Education; 2018. 1 p.
41. van Beek J, Veenhoven RH, Bruin JP, van Boxtel RAJ, de Lange MMA, Meijer A, et al. Influenza-like Illness Incidence Is Not Reduced by Influenza Vaccination in a Cohort of Older Adults, Despite Effectively Reducing Laboratory-Confirmed Influenza Virus Infections. J Infect Dis. 2017;216(4):415–24.
42. WHO | WHO surveillance case definitions for ILI and SARI [Internet]. WHO. World Health Organization; [cited 2021 May 17]. Available from: http://www.who.int/influenza/surveillance_monitoring/ili_sari_surveillance_case_definition/en/
43. Drosten C, Günther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome. N Engl J Med. 2003;348(20):1967–76.
44. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. N Engl J Med. 2012;367(19):1814–20.
45. Yang H, Rao Z. Structural biology of SARS-CoV-2 and implications for therapeutic development. Nat Rev Microbiol. 2021;19(11):685–700.
46. Hao X, Cheng S, Wu D, Wu T, Lin X, Wang C. Reconstruction of the full transmission dynamics of COVID-19 in Wuhan. Nature. 2020;584(7821):420–4.
47. Merad M, Blish CA, Sallusto F, Iwasaki A. The immunology and immunopathology of COVID-19. Science. 2022;375(6585):1122–7.
48. Liu Y, Rocklöv J. The effective reproductive number of the Omicron variant of SARS-CoV-2 is several times relative to Delta. J Travel Med. 2022;29(3):taac037.

49. Escandón K, Rasmussen AL, Bogoch II, Murray EJ, Escandón K, Popescu SV, et al. COVID-19 false dichotomies and a comprehensive review of the evidence regarding public health, COVID-19 symptomatology, SARS-CoV-2 transmission, mask wearing, and reinfection. BMC Infect Dis. 2021;21(1):710.
50. Coburn BJ, Wagner BG, Blower S. Modeling influenza epidemics and pandemics: insights into the future of swine flu (H1N1). BMC Med. 2009;7(1):30.
51. Guerra FM, Bolotin S, Lim G, Heffernan J, Deeks SL, Li Y, et al. The basic reproduction number (R 0) of measles: a systematic review. Lancet Infect Dis. 2017;17(12):e420–8.
52. Cheng HY, Jian SW, Liu DP, Ng TC, Huang WT, Lin HH, et al. Contact Tracing Assessment of COVID-19 Transmission Dynamics in Taiwan and Risk at Different Exposure Periods Before and After Symptom Onset. JAMA Intern Med. 2020;180(9):1156.
53. Pan Y, Zhang D, Yang P, Poon LLM, Wang Q. Viral load of SARS-CoV-2 in clinical samples. Lancet Infect Dis. 2020;20(4):411–2.
54. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell. 2020;181(2):271-280.e8.
55. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. Lancet Respir Med. 2020;8(4):420–2.
56. Zhang H, Penninger JM, Li Y, Zhong N, Slutsky AS. Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. Intensive Care Med. 2020;46(4):586–90.
57. Liu J, Li Y, Liu Q, Yao Q, Wang X, Zhang H, et al. SARS-CoV-2 cell tropism and multiorgan infection. Cell Discov. 2021;7(1):17.
58. Oran DP, Topol EJ. The Proportion of SARS-CoV-2 Infections That Are Asymptomatic: A Systematic Review. Ann Intern Med. 2021;174(5):655–62.
59. Hu B, Guo H, Zhou P, Shi ZL. Characteristics of SARS-CoV-2 and COVID-19. Nat Rev Microbiol. 2020;19(3):141–54.
60. McAloon C, Collins Á, Hunt K, Barber A, Byrne AW, Butler F, et al. Incubation period of COVID-19: a rapid systematic review and meta-analysis of observational research. BMJ Open. 2020;10(8):e039652.
61. Siddiqi HK, Mehra MR. COVID-19 illness in native and immunosuppressed states:

A clinical–therapeutic staging proposal. J Heart Lung Transplant. 2020;39(5):405–7.
62. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. The Lancet. 2020;395(10223):497–506.
63. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. The Lancet. 2020;395(10223):507–13.
64. Guan W jie, Ni Z yi, Hu Y, Liang W hua, Ou C quan, He J xing, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med [Internet]. 2020 [cited 2021 Feb 2]; Available from: https://www.nejm.org/doi/10.1056/NEJMoa2002032
65. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus–Infected Pneumonia in Wuhan, China. JAMA. 2020;323(11):1061.
66. Lechien JR, Chiesa-Estomba CM, De Siati DR, Horoi M, Le Bon SD, Rodriguez A, et al. Olfactory and gustatory dysfunctions as a clinical presentation of mild-to-moderate forms of the coronavirus disease (COVID-19): a multicenter European study. Eur Arch Otorhinolaryngol. 2020;277(8):2251–61.
67. Berlin DA, Gulick RM, Martinez FJ. Severe Covid-19. Solomon CG, editor. N Engl J Med. 2020;383(25):2451–60.
68. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. The Lancet. 2020;395(10229):1054–62.
69. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020;8(5):475–81.
70. Thompson BT, Chambers RC, Liu KD. Acute Respiratory Distress Syndrome. Drazen JM, editor. N Engl J Med. 2017;377(6):562–72.
71. Acute Respiratory Distress Syndrome: The Berlin Definition. JAMA [Internet]. 2012 [cited 2022 Mar 19];307(23). Available from: http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2012.5669
72. CRICS TRIGGERSEP Group (Clinical Research in Intensive Care and Sepsis Trial Group for Global Evaluation and Research in Sepsis), Helms J, Tacquard C, Severac F, Leonard-Lorant I, Ohana M, et al. High risk of thrombosis in patients with severe SARS-CoV-2 infection: a multicenter prospective cohort study. Intensive Care Med.

2020;46(6):1089–98.
73. Ackermann M, Verleden SE, Kuehnel M, Haverich A, Welte T, Laenger F, et al. Pulmonary Vascular Endothelialitis, Thrombosis, and Angiogenesis in Covid-19. N Engl J Med. 2020;383(2):120–8.
74. Apostolopoulou A, Esquer Garrigos Z, Vijayvargiya P, Lerner AH, Farmakiotis D. Invasive Pulmonary Aspergillosis in Patients with SARS-CoV-2 Infection: A Systematic Review of the Literature. Diagnostics. 2020;10(10):807.
75. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, et al. Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. JAMA. 2020;323(20):2052.
76. Yang L, Liu S, Liu J, Zhang Z, Wan X, Huang B, et al. COVID-19: immunopathogenesis and Immunotherapeutics. Signal Transduct Target Ther. 2020;5(1):128.
77. Hu B, Huang S, Yin L. The cytokine storm and COVID-19. J Med Virol. 2021;93(1):250–6.
78. Leisman DE, Ronner L, Pinotti R, Taylor MD, Sinha P, Calfee CS, et al. Cytokine elevation in severe and critical COVID-19: a rapid systematic review, meta-analysis, and comparison with other inflammatory syndromes. Lancet Respir Med. 2020;8(12):1233–44.
79. Levin AT, Hanage WP, Owusu-Boaitey N, Cochran KB, Walsh SP, Meyerowitz-Katz G. Assessing the age specificity of infection fatality rates for COVID-19: systematic review, meta-analysis, and public policy implications. Eur J Epidemiol. 2020;35(12):1123–38.
80. Alhazzani W, Evans L, Alshamsi F, Møller MH, Ostermann M, Prescott HC, et al. Surviving Sepsis Campaign Guidelines on the Management of Adults With Coronavirus Disease 2019 (COVID-19) in the ICU: First Update. Crit Care Med. 2021;49(3):e219–34.
81. Alhazzani W, Møller MH, Arabi YM, Loeb M, Gong MN, Fan E, et al. Surviving Sepsis Campaign: Guidelines on the Management of Critically Ill Adults with Coronavirus Disease 2019 (COVID-19). 2020;48(6):30.
82. CDC. Healthcare Workers [Internet]. Centers for Disease Control and Prevention. 2020 [cited 2022 Apr 11]. Available from: https://www.cdc.gov/coronavirus/2019-ncov/hcp/testing-overview.html
83. Fechner C, Strobel K, Treumann T, Sonderegger B, Azzola A, Fornaro J, et al. COVID-19 and the role of imaging: early experiences in Central Switzerland. Swiss Med Wkly [Internet]. 2020 [cited 2022 Mar 19]; Available from:

https://doi.emh.ch/smw.2020.20304
84. Chiarenza A, Esposto Ultimo L, Falsaperla D, Travali M, Foti PV, Torrisi SE, et al. Chest imaging using signs, symbols, and naturalistic images: a practical guide for radiologists and non-radiologists. Insights Imaging. 2019;10(1):114.
85. COVID-19 Treatment Guidelines Panel [Internet]. Coronavirus Disease 2019 (COVID-19) Treatment Guidelines. National Institutes of Health; [cited 2022 Jul 15]. Available from: https://www.covid19treatmentguidelines.nih.gov/
86. Remdesivir and three other drugs for hospitalised patients with COVID-19: final results of the WHO Solidarity randomised trial and updated meta-analyses. The Lancet. 2022;399(10339):1941–53.
87. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the Treatment of Covid-19 — Final Report. N Engl J Med. 2020;383(19):1813–26.
88. Therapeutics and COVID-19: living guideline, 14 July 2022 [Internet]. Geneva: World Health Organization; 2022 (WHO/ 2019-nCoV/therapeutics/2022.4). [cited 2022 Jul 16]. Available from: https://www.who.int/publications-detail-redirect/WHO-2019-nCoV-therapeutics-2022.4
89. The RECOVERY Collaborative Group. Dexamethasone in Hospitalized Patients with Covid-19. N Engl J Med. 2021;384(8):693–704.
90. The WHO Rapid Evidence Appraisal for COVID-19 Therapies (REACT) Working Group, Sterne JAC, Murthy S, Diaz JV, Slutsky AS, Villar J, et al. Association Between Administration of Systemic Corticosteroids and Mortality Among Critically Ill Patients With COVID-19: A Meta-analysis. JAMA. 2020;324(13):1330.
91. Ranjbar K, Moghadami M, Mirahmadizadeh A, Fallahi MJ, Khaloo V, Shahriarirad R, et al. Methylprednisolone or dexamethasone, which one is superior corticosteroid in the treatment of hospitalized COVID-19 patients: a triple-blinded randomized controlled trial. BMC Infect Dis. 2021;21(1):337.
92. Granholm A, Munch MW, Myatra SN, Vijayaraghavan BKT, Cronhjort M, Wahlin RR, et al. Dexamethasone 12 mg versus 6 mg for patients with COVID-19 and severe hypoxaemia: a pre-planned, secondary Bayesian analysis of the COVID STEROID 2 trial. Intensive Care Med. 2022;48(1):45–55.
93. The COVID STEROID 2 Trial Group, Russell L, Uhre KR, Lindgaard ALS, Degn JF, Wetterslev M, et al. Effect of 12 mg vs 6 mg of Dexamethasone on the Number of Days Alive Without Life Support in Adults With COVID-19 and Severe Hypoxemia: The

COVID STEROID 2 Randomized Trial. JAMA. 2021;326(18):1807.
94. The REMAP-CAP Investigators. Interleukin-6 Receptor Antagonists in Critically Ill Patients with Covid-19. N Engl J Med. 2021;384(16):1491–502.
95. The WHO Rapid Evidence Appraisal for COVID-19 Therapies (REACT) Working Group, Domingo P, Mur I, Mateo GM, Gutierrez M del M, Pomar V, et al. Association Between Administration of IL-6 Antagonists and Mortality Among Patients Hospitalized for COVID-19: A Meta-analysis. JAMA. 2021;326(6):499.
96. Mariette X, Hermine O, Tharaux PL, Resche-Rigon M, Steg PG, Porcher R, et al. Effectiveness of Tocilizumab in Patients Hospitalized With COVID-19: A Follow-up of the CORIMUNO-TOCI-1 Randomized Clinical Trial. 2021;3.
97. Marconi VC, Ramanan AV, de Bono S, Kartman CE, Krishnan V, Liao R, et al. Efficacy and safety of baricitinib for the treatment of hospitalised adults with COVID-19 (COV-BARRIER): a randomised, double-blind, parallel-group, placebo-controlled phase 3 trial. Lancet Respir Med. 2021;9(12):1407–18.
98. Arastehfar A, Carvalho A, van de Veerdonk FL, Jenks JD, Koehler P, Krause R, et al. COVID-19 Associated Pulmonary Aspergillosis (CAPA)—From Immunology to Treatment. J Fungi. 2020;6(2):91.
99. Guinea J, Torres-Narbona M, Gijón P, Muñoz P, Pozo F, Peláez T, et al. Pulmonary aspergillosis in patients with chronic obstructive pulmonary disease: incidence, risk factors, and outcome. Clin Microbiol Infect. 2010;16(7):870–7.
100. Delsuc C, Cottereau A, Frealle E, Bienvenu AL, Dessein R, Jarraud S, et al. Putative invasive pulmonary aspergillosis in critically ill patients with chronic obstructive pulmonary disease: a matched cohort study. Crit Care. 2015;19(1):421.
101. Verweij PE, Brüggemann RJM, Azoulay E, Bassetti M, Blot S, Buil JB, et al. Taskforce report on the diagnosis and clinical management of COVID-19 associated pulmonary aspergillosis. Intensive Care Med. 2021;47(8):819–34.
102. van de Veerdonk FL, Brüggemann RJM, Vos S, De Hertogh G, Wauters J, Reijers MHE, et al. COVID-19-associated Aspergillus tracheobronchitis: the interplay between viral tropism, host defence, and fungal invasion. Lancet Respir Med. 2021;9(7):795–802.
103. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. Semin Immunopathol. 2017;39(5):529–39.
104. Hoenigl M, Seidel D, Sprute R, Cunha C, Oliverio M, Goldman GH, et al. COVID-19-associated fungal infections. Nat Microbiol. 2022;7(8):1127–40.

105. Bastard P, Rosen LB, Zhang Q, Michailidis E, Hoffmann HH, Zhang Y, et al. Autoantibodies against type I IFNs in patients with life-threatening COVID-19. Science. 2020;370(6515):eabd4585.
106. Zhang Q, Bastard P, Liu Z, Le Pen J, Moncada-Velez M, Chen J, et al. Inborn errors of type I IFN immunity in patients with life-threatening COVID-19. Science. 2020;370(6515):eabd4570.
107. Merad M, Blish CA, Sallusto F, Iwasaki A. The immunology and immunopathology of COVID-19. Science. 2022;375(6585):1122–7.
108. Gafa V, Remoli ME, Giacomini E, Severa M, Grillot R, Coccia EM. Enhancement of anti-Aspergillus T helper type 1 response by interferon-β-conditioned dendritic cells: IFN-β and anti-Aspergillus Th1 response. Immunology. 2010;131(2):282–8.
109. Espinosa V, Dutta O, McElrath C, Du P, Chang YJ, Cicciarelli B, et al. Type III interferon is a critical regulator of innate antifungal immunity. Sci Immunol. 2017;2(16):eaan5357.
110. Jenks JD, Nam HH, Hoenigl M. Invasive aspergillosis in critically ill patients: Review of definitions and diagnostic approaches. Mycoses. 2021;64(9):1002–14.
111. Hoenigl M. Invasive Fungal Disease Complicating Coronavirus Disease 2019: When It Rains, It Spores. Clin Infect Dis. 2021;73(7):e1645–8.
112. D'Haese J, Theunissen K, Vermeulen E, Schoemans H, De Vlieger G, Lammertijn L, et al. Detection of Galactomannan in Bronchoalveolar Lavage Fluid Samples of Patients at Risk for Invasive Pulmonary Aspergillosis: Analytical and Clinical Validity. J Clin Microbiol. 2012;50(4):1258–63.
113. Koehler P, Bassetti M, Chakrabarti A, Chen SCA, Colombo AL, Hoenigl M, et al. Defining and managing COVID-19-associated pulmonary aspergillosis: the 2020 ECMM/ISHAM consensus criteria for research and clinical guidance. Lancet Infect Dis. 2020;S1473309920308471.
114. Heldt S, Hoenigl M. Lateral Flow Assays for the Diagnosis of Invasive Aspergillosis: Current Status. Curr Fungal Infect Rep. 2017;11(2):45–51.
115. Jenks JD, Prattes J, Frank J, Spiess B, Mehta SR, Boch T, et al. Performance of the Bronchoalveolar Lavage Fluid *Aspergillus* Galactomannan Lateral Flow Assay With Cube Reader for Diagnosis of Invasive Pulmonary Aspergillosis: A Multicenter Cohort Study. Clin Infect Dis. 2021;73(7):e1737–44.
116. Jenks JD, Mehta SR, Taplitz R, Aslam S, Reed SL, Hoenigl M. Point-of-care diagnosis of invasive aspergillosis in non-neutropenic patients: *Aspergillus* Galactomannan

Lateral Flow Assay versus *Aspergillus* -specific Lateral Flow Device test in bronchoalveolar lavage. Mycoses. 2019;62(3):230–6.
117. Chindamporn A, Chakrabarti A, Li R, Sun PL, Tan BH, Chua M, et al. Survey of laboratory practices for diagnosis of fungal infection in seven Asian countries: An Asia Fungal Working Group (AFWG) initiative. Med Mycol. 2018;56(4):416–25.
118. White PL, Dhillon R, Cordey A, Hughes H, Faggian F, Soni S, et al. A National Strategy to Diagnose Coronavirus Disease 2019–Associated Invasive Fungal Disease in the Intensive Care Unit. Clin Infect Dis. 2021;73(7):e1634–44.
119. Patterson TF, Thompson GR, Denning DW, Fishman JA, Hadley S, Herbrecht R, et al. Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America. Clin Infect Dis. 2016;63(4):e1–60.
120. Whalen K, editor. Pharmacology. 6th ed., international ed. Philadelphia [etc.]: Wolters Kluwer; 2015. 664 p. (Lippincott's illustrated reviews).
121. Maertens JA, Rahav G, Lee DG, Ponce-de-León A, Ramírez Sánchez IC, Klimko N, et al. Posaconazole versus voriconazole for primary treatment of invasive aspergillosis: a phase 3, randomised, controlled, non-inferiority trial. The Lancet. 2021;397(10273):499–509.
122. Melchers M, van Zanten ARH, Heusinkveld M, Leeuwis JW, Schellaars R, Lammers HJW, et al. Nebulized Amphotericin B in Mechanically Ventilated COVID-19 Patients to Prevent Invasive Pulmonary Aspergillosis: A Retrospective Cohort Study. Crit Care Explor. 2022;4(5):e0696.
123. Roman-Montes CM, Martinez-Gamboa A, Diaz-Lomelí P, Cervantes-Sanchez A, Rangel-Cordero A, Sifuentes-Osornio J, et al. Accuracy of galactomannan testing on tracheal aspirates in COVID-19-associated pulmonary aspergillosis. Mycoses. 2021;64(4):364–71.
124. Verweij PE, Gangneux JP, Bassetti M, Brüggemann RJM, Cornely OA, Koehler P, et al. Diagnosing COVID-19-associated pulmonary aspergillosis. Lancet Microbe. 2020;1(2):e53–5.
125. Seidel D, Durán Graeff LA, Vehreschild MJGT, Wisplinghoff H, Ziegler M, Vehreschild JJ, et al. FungiScope ^{TM} -Global Emerging Fungal Infection Registry. Mycoses. 2017 Aug;60(8):508–16.
126. Fekkar A, Neofytos D, Nguyen MH, Clancy CJ, Kontoyiannis DP, Lamoth F. COVID-19-associated pulmonary aspergillosis (CAPA): how big a problem is it? Clin Microbiol Infect. 2021;27(9):1376–8.

127. Rutsaert L, Steinfort N, Van Hunsel T, Bomans P, Naesens R, Mertes H, et al. COVID-19-associated invasive pulmonary aspergillosis. Ann Intensive Care. 2020;10(1):71.
128. Janssen NAF, Nyga R, Vanderbeke L, Jacobs C, Ergün M, Buil JB, et al. Multinational Observational Cohort Study of COVID-19–Associated Pulmonary Aspergillosis. Emerg Infect Dis. 2021;27(11):2892–8.
129. Gangneux JP, Dannaoui E, Fekkar A, Luyt CE, Botterel F, De Prost N, et al. Fungal infections in mechanically ventilated patients with COVID-19 during the first wave: the French multicentre MYCOVID study. Lancet Respir Med. 2022;10(2):180–90.
130. Permpalung N, Chiang TPY, Massie AB, Zhang SX, Avery RK, Nematollahi S, et al. Coronavirus Disease 2019–Associated Pulmonary Aspergillosis in Mechanically Ventilated Patients. Clin Infect Dis. 2022;74(1):83–91.
131. Kula BE, Clancy CJ, Hong Nguyen M, Schwartz IS. Invasive mould disease in fatal COVID-19: a systematic review of autopsies. Lancet Microbe. 2021;2(8):e405–14.
132. Flikweert AW, Grootenboers MJJH, Yick DCY, du Mée AWF, van der Meer NJM, Rettig TCD, et al. Late histopathologic characteristics of critically ill COVID-19 patients: Different phenotypes without evidence of invasive aspergillosis, a case series. J Crit Care. 2020;59:149–55.
133. Fortarezza F, Boscolo A, Pezzuto F, Lunardi F, Jesús Acosta M, Giraudo C, et al. Proven COVID-19—associated pulmonary aspergillosis in patients with severe respiratory failure. Mycoses. 2021;64(10):1223–9.
134. Evert K, Dienemann T, Brochhausen C, Lunz D, Lubnow M, Ritzka M, et al. Autopsy findings after long-term treatment of COVID-19 patients with microbiological correlation. Virchows Arch. 2021;479(1):97–108.
135. Tejerina EE, Abril E, Padilla R, Rodríguez Ruíz C, Ballen A, Frutos-Vivar F, et al. Invasive aspergillosis in critically ill patients: An autopsy study. Mycoses. 2019;62(8):673–9.
136. Calderón-Parra J, Mills-Sanchez P, Moreno-Torres V, Tejado-Bravo S, Romero-Sánchez I, Balandin-Moreno B, et al. COVID-19-associated pulmonary aspergillosis (CAPA): Risk factors and development of a predictive score for critically ill COVID-19 patients. Mycoses. 2022;65(5):541–50.
137. Thompson III GR, Cornely OA, Pappas PG, Patterson TF, Hoenigl M, Jenks JD, et al. Invasive Aspergillosis as an Under-recognized Superinfection in COVID-19. Open Forum Infect Dis. 2020;7(7):ofaa242.

138. Bartoletti M, Pascale R, Cricca M, Rinaldi M, Maccaro A, Bussini L, et al. Epidemiology of Invasive Pulmonary Aspergillosis Among Intubated Patients With COVID-19: A Prospective Study. Clin Infect Dis. 2020;ciaa1065.
139. Ergün M, Brüggemann RJM, Alanio A, Dellière S, van Arkel A, Bentvelsen RG, et al. Aspergillus Test Profiles and Mortality in Critically Ill COVID-19 Patients. Hanson KE, editor. J Clin Microbiol. 2021;59(12):e01229-21.
140. Farmakiotis D, Le A, Weiss Z, Ismail N, Kubiak DW, Koo S. False positive bronchoalveolar lavage galactomannan: Effect of host and cut-off value. Mycoses. 2019;62(3):204–13.
141. Dellière S, Dudoignon E, Voicu S, Collet M, Fodil S, Plaud B, et al. Combination of Mycological Criteria: a Better Surrogate to Identify COVID-19-Associated Pulmonary Aspergillosis Patients and Evaluate Prognosis? Hanson KE, editor. J Clin Microbiol. 2022;60(3):e02169-21.
142. Eigl S, Prattes J, Reinwald M, Thornton CR, Reischies F, Spiess B, et al. Influence of mould-active antifungal treatment on the performance of the Aspergillus-specific bronchoalveolar lavage fluid lateral-flow device test. Int J Antimicrob Agents. 2015;46(4):401–5.
143. Rouzé A, Lemaitre E, Martin-Loeches I, Povoa P, Diaz E, Nyga R, et al. Invasive pulmonary aspergillosis among intubated patients with SARS-CoV-2 or influenza pneumonia: a European multicenter comparative cohort study. Crit Care. 2022;26(1):11.
144. Wauters J, Baar I, Meersseman P, Meersseman W, Dams K, De Paep R, et al. Invasive pulmonary aspergillosis is a frequent complication of critically ill H1N1 patients: a retrospective study. Intensive Care Med. 2012;38(11):1761–8.
145. Autier B, Prattes J, White PL, Valerio M, Machado M, Price J, et al. Aspergillus Lateral Flow Assay with Digital Reader for the Diagnosis of COVID-19-Associated Pulmonary Aspergillosis (CAPA): a Multicenter Study. Hanson KE, editor. J Clin Microbiol. 2022;60(1):e01689-21.
146. White PL, Bretagne S, Caliendo AM, Loeffler J, Patterson TF, Slavin M, et al. *Aspergillus* Polymerase Chain Reaction—An Update on Technical Recommendations, Clinical Applications, and Justification for Inclusion in the Second Revision of the EORTC/MSGERC Definitions of Invasive Fungal Disease. Clin Infect Dis. 2021;72(Supplement_2):S95–101.
147. Vanderbeke L, Janssen NAF, Bergmans DCJJ, Bourgeois M, Buil JB, Debaveye Y, et al. Posaconazole for prevention of invasive pulmonary aspergillosis in critically ill

influenza patients (POSA-FLU): a randomised, open-label, proof-of-concept trial. Intensive Care Med. 2021;47(6):674–86.
148. Hatzl S, Reisinger AC, Posch F, Prattes J, Stradner M, Pilz S, et al. Antifungal prophylaxis for prevention of COVID-19-associated pulmonary aspergillosis in critically ill patients: an observational study. Crit Care. 2021;25(1):335.
149. Abdullah F, Myers J, Basu D, Tintinger G, Ueckermann V, Mathebula M, et al. Decreased severity of disease during the first global omicron variant covid-19 outbreak in a large hospital in tshwane, south africa. Int J Infect Dis. 2022;116:38–42.
150. Menni C, Valdes AM, Polidori L, Antonelli M, Penamakuri S, Nogal A, et al. Symptom prevalence, duration, and risk of hospital admission in individuals infected with SARS-CoV-2 during periods of omicron and delta variant dominance: a prospective observational study from the ZOE COVID Study. The Lancet. 2022;399(10335):1618–24.
151. Reynolds CJ, Pade C, Gibbons JM, Otter AD, Lin KM, Muñoz Sandoval D, et al. Immune boosting by B.1.1.529 **(** Omicron) depends on previous SARS-CoV-2 exposure. Science. 2022;eabq1841.
152. Pilz S, Theiler-Schwetz V, Trummer C, Krause R, Ioannidis JPA. SARS-CoV-2 reinfections: Overview of efficacy and duration of natural and hybrid immunity. Environ Res. 2022;209:112911.