Invasive aspergillosis: burden of disease, management and surveillance: assessing clinical management challenges and exploring Aspergillus disease surveillance

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Abstract

Invasive aspergillosis (IA) is a global health threat with a rising number of at-risk patients. It is classically seen in immunocompromised patients, such as those with a haematological malignancy, or severe and prolonged neutropenia. However, recently IA has also been observed in patients without classical risk factors: patients treated with new therapies that affect the immune system, critically ill patients, and patients with a viral respiratory infection (influenza and more recently COVID-19). Diagnosis and treatment of IA is challenging. Current diagnostic tools have their limitations: a positive culture from a bronchoalveolar lavage (BAL) does not necessarily reflect disease, yet [...]



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Invasive aspergillosis: burden of disease, management and surveillance

Assessing clinical management challenges and exploring *Aspergillus* disease surveillance

Rebecca van Grootveld

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Invasive aspergillosis: burden of disease, management and surveillance

Assessing clinical management challenges
and exploring *Aspergillus* disease surveillance

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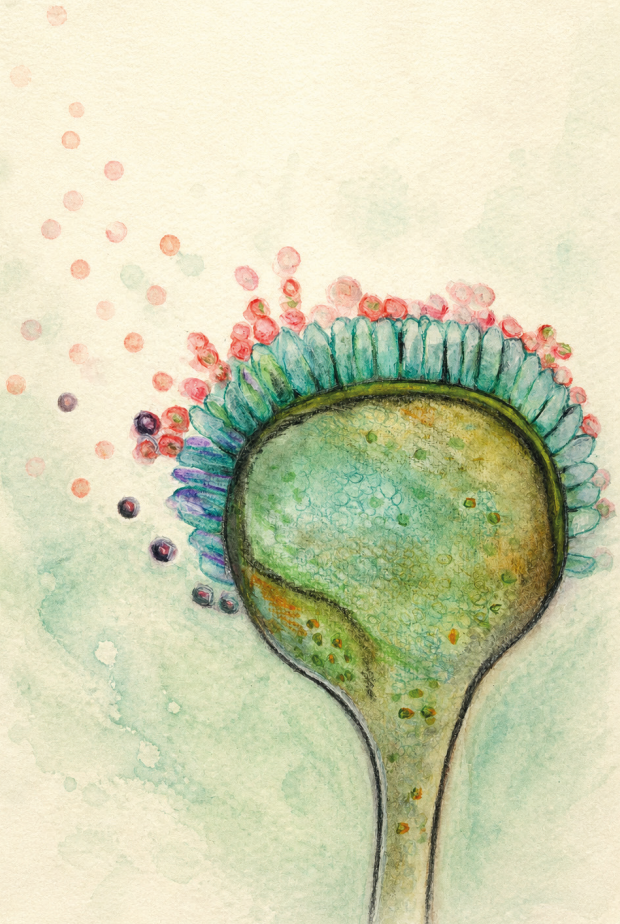
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1

Introduction

PATHOGEN

Aspergillus is a saprophytic fungus that plays an important ecological role in recycling environmental carbon and nitrogen.[1, 2] The fungus is common and globally widespread in nature, and can be found in soil, decaying vegetation, food, water and air.[3]

Aspergillus was first described in 1729 by Pier Antonio Micheli, an Italian priest and biologist. The microscopic spore-bearing structure (conidiophore) of *Aspergillus* reminded him of an aspergillum, a devise used by the Roman Catholic clergy to sprinkle holy water (from the Latin aspergere, "to sprinkle") (figure 1).[4] Since the first description of the genus *Aspergillus* by Micheli, numerous species within the genus have been described. Currently, the genus *Aspergillus* (kingdom Fungi, subkingdom Dikarya, phylum Ascomycota) is known to consist of 446 species, subdivided in 6 subgenera and 27 sections.[5] However, only a few number of species are associated with infections in humans. *Aspergillus fumigatus* is the most common species to cause disease,[6] and other relevant species for human disease include: *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger* and *Aspergillus terreus.*[3, 6, 7]



Figure 1. Conidial head of *Aspergillus fumigatus*.
Photo by A. Sidorencu.

Aspergillus fumigatus has multiple characteristics that contribute to its efficacy as an opportunistic pathogen.[1, 3, 8] First, this fungus can adapt well to a broad range of environmental conditions such as variable temperatures or pH values. Second, *Aspergillus* sporulates abundantly and thousands of conidia are produced, and dispersed through the air efficiently due to their hydrophobicity.[3, 8] Third, their physical features allow conidia to reach the alveoli, for example small particle size (2–3 µm) and the melanin containing cell wall that offers protection from reactive oxygen species (ROS) and lysis by host cells.[3, 8] Finally, in immunocompromised patients conidia can efficiently adapt their physiology to the host environment, by regulating their gene expression, and germinate into hyphae.[8]

DISEASE

Humans inhale > 100 *A. fumigatus* airborne conidia every day (based on an estimated indoor or outdoor conidial concentration 1-100 conidia/m3).[1, 8] In most people this will not cause disease, because airborne conidia that reach the alveoli will be killed by the innate immune system. Especially the presence of functional neutrophils plays an important role in the host defence against *Aspergillus.*[9] However, immunocompromised patients or patients with a history of bronchopulmonary disease (COPD or asthma for example) can develop pulmonary aspergillosis.

Aspergillosis includes a wide spectrum of clinical syndromes depending on the immune status of the host, and can be divided into three groups (figure 2).[10-12] Firstly, invasive aspergillosis (IA) which mostly affects immunocompromised patients,[13] e.g. those with classical host risk factors for IA: severe and prolonged neutropenia, haematological malignancy, recipients of an allogeneic hematopoi-

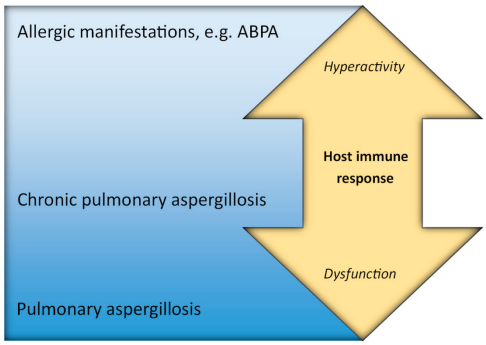


Figure 2. The spectrum of aspergillosis depending on the host immune response.
APBA: allergic bronchopulmonary aspergillosis

etic stem cell transplantation (HSCT), recipients of a solid organ transplantation, or patients treated with corticosteroids. In these patients infection is predominantly located in the respiratory tract (pulmonary aspergillosis, sinus aspergillosis and tracheobronchial aspergillosis), but the infection may disseminate to other organs in up to approximately 10% of cases.[14-16] In particular patients receiving intensive chemotherapy for acute myeloid leukaemia (AML) or myelodysplastic syndrome (MDS) or recipients of an allogeneic HSCT have a high risk of developing IA.[17-20] A meta-analysis reported a frequency of IA ranging from 4% (with antifungal prophylaxis) to 11% (without antifungal prophylaxis) in patients with haematological malignancies receiving intensive chemotherapy, and a frequency of IA ranging from 7% to 9% after HSCT.[21] Secondly, chronic pulmonary aspergillosis (CPA) which mostly affects patients with underlying lung disease with no or mild immune compromise.[11, 22] Clinical presentations of CPA are diverse, varying from a single aspergilloma to subacute invasive aspergillosis or chronic necrotising pulmonary aspergillosis, requiring different treatments.[12, 22] Finally, a very different group of patients presents with allergic manifestations caused by hypersensitivity to *A. fumigatus.* Allergic bronchopulmonary aspergillosis (ABPA) or severe asthma with fungal sensitisation are allergic responses to *Aspergillus* in patients with asthma or cystic fibrosis.[11] The main focus of this thesis is IA.

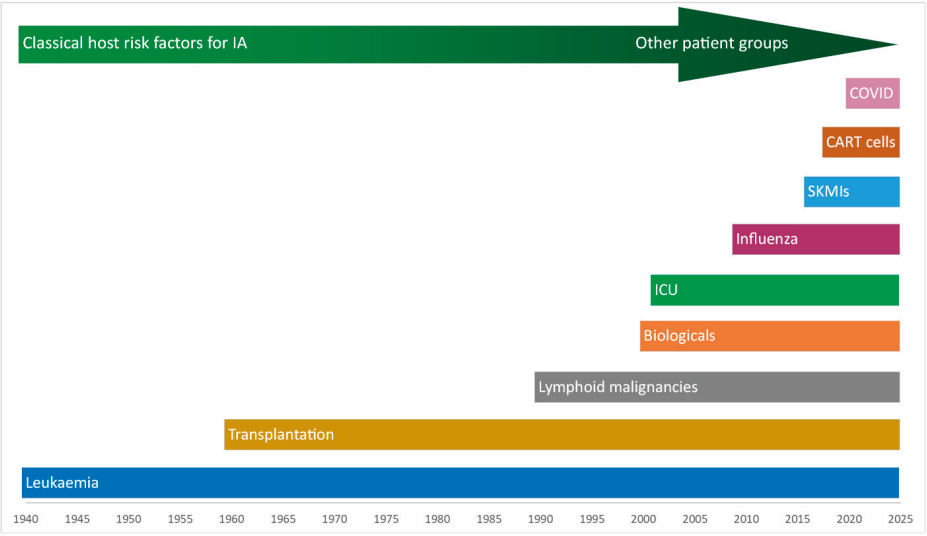


Figure 3. Patients at risk for invasive aspergillosis over time.
Based on figure 5 of Latgé and Chamilos.[1] Classical host risk factors for IA include severe and prolonged neutropenia, haematological malignancy, recipients of an allogeneic hematopoietic stem cell transplantation, recipients of a solid organ transplantation, or patients treated with corticosteroids. Other patient groups include patients treated with new immunomodulators, critically ill patients, or patients with a respiratory viral infection. CAR T cells: chimeric antigen receptor T cells, COVID: COVID-19, IA: invasive aspergillosis, ICU: intensive care unit, SMKI: small-molecule kinase inhibitor.

In recent years IA has also been described in patients without classical host risk factors for IA. This group includes patients treated with new therapies affecting the immune system, such as ibrutinib, a Brutons's tyrosine kinase inhibitor used for the treatment of chronic lymphocytic leukaemia,[13, 23, 24] critically ill patients[25] and patients with a viral respiratory infection (influenza and COVID-19) admitted to the ICU.[26-30] Since this at-risk population is increasing and variable, so is the number of patients with IA. In figure 3 the patients at risk for IA over time are depicted.

DIAGNOSIS

Diagnosis of IA is challenging, and should be based on a combination of clinical, radiological and mycological data. Invasive aspergillosis must be considered in patients at risk for IA with fever of unknown origin, with or without symptoms of lower respiratory tract infection, who remain febrile despite broad-spectrum antibiotic treatment.[17, 20] In these patients thin-section chest computed tomography (CT) imaging is recommended to identify the site of infection and guide diagnostic procedures like bronchoalveolar lavage (BAL) or CT-guided biopsies.[17, 20] Typical CT findings of IA include nodules, that may be surrounded by a halo of ground-glass opacity (halo sign) which reflects haemorrhage and is the result of invasion of *Aspergillus* into the pulmonary vessels, consolidations or wedge-shaped haemorrhagic infarcts.[17, 20] Besides imaging, bronchoscopy with BAL is recommended in patient with suspicion of IA[17, 20] so that the airways can be inspected, and lower respiratory tract samples can be acquired for microbiological tests. In patients with peripheral nodular lesions the yield of BAL is low and lung biopsies should be considered.[20]

There are several tools for diagnosis of aspergillosis, i.e., microscopy, culture and fungal biomarkers for antigen detection (galactomannan, (1-3)-β-d-glucan) or DNA detection by *Aspergillus* polymerase chain reaction (PCR). The sensitivity and specificity of these tests can vary in different patient populations.

Histopathology

Diagnosis of IA is confirmed if a biopsy/needle aspiration of a sterile site has been obtained and there is histopathological or direct microscopic evidence of disease, or *Aspergillus* is isolated from culture, or *Aspergillus* DNA is detected.[13] Histological examination can be performed if biopsies/needle aspirations have been obtained. After tissues have been stained with Gomori's methenamine silver stain or periodic acid-Schiff stain, they can be examined under the microscope to detect invasion by fungal hyphae in tissues and vessels.

Confirmation by culture or *Aspergillus* PCR is necessary to identify the fungus, since microscopic examination cannot differentiate between *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., *Trichoderma* spp., *Paecilomyces* spp., *Scopulariopsis* spp., *Acremonium* spp., *Schizophyllum* spp., *Phaeoacremonium* spp., *Trichosporon* spp., or other non-pigmented (hyaline) fungi with septated hyphae with acute-angle (45˚) branching.[31] Also, differentiation from *Mucorales* spp. (non-pigmented, pauciseptate ribbon-like hyphae with right angle (90˚) branching) can sometimes be difficult.[31] Positive diagnostic tests from a sterile site are valuable as diagnosis of IA can be confirmed, but these procedures are infrequently performed due to contra-indications such as thrombocytopenia, coagulation disorders or respiratory/hemodynamic instability of patients.

Microscopy and culture

Direct microscopic examination of samples, mostly lower respiratory tract samples or less frequently biopsies/aspirates, allows for rapid analysis of the presence of fungal elements. However, specificity is low, as fungi such as *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., or even *Mucorales* spp. can have a similar appearance in direct microscopy.[32] Also, identification up to *Aspergillus* species level is not possible. Sensitivity is 50% at best[17] and variable depending on factors such as the use of fluorescent dyes (to increase sensitivity) and the experience of the laboratory technician.

In most laboratories fungal cultures can be performed. It is an easy low-cost method and if *Aspergillus* is cultured identification and susceptibility testing is possible. Though, sensitivity is low (< 50%) in patients with host risk factors for IA.[33, 34] *Aspergillus* can usually be identified by microscopic examination of a positive culture due the typical conidiophore structure: a foot cell and non-septate stipe ending in a vesicle covered with either a single layer of phialides (uniseriate) or a layer of metulae from which the phialides radiate (biseriate). The phialides produce conidia, and some species (e.g. *A. nidulans*) produce Hülle cells. The conidial head (vesicle, phialides, metulae) can have a columnar or radial appearance. For example, *A. fumigatus* is uniseriate and the conidial head has a columnar shape and *A. niger* is biseriate and the conidial head has a radial shape. Colonies also have different colours depending on the species, e.g. *A. fumigatus* green/grey, *A. niger* dark brown/black, or *A. terreus* cinnamon brown. Besides microscopy, matrix assisted laser desorption/ionisation is increasingly used to identify fungal colonies.[35, 36] Molecular methods can also be used, for example to distinguish rare or cryptic species.[37]

Galactomannan

Galactomannan (GM) is a polysaccharide that is an important component of the cell wall of all *Aspergillus* spp. and is released during fungal growth. Testing for GM has been validated in serum and BAL samples, but GM can be detected in urine, cerebrospinal fluid or pleural fluid as well.[38]

The sensitivity and specificity of the GM test depend on the cutoff, the patient population and treatment with mould-active antifungals at time of sample collection (decreased sensitivity). False-positive results have been described in patients in whom GM was introduced into the body as part of another product, e.g. many organic molecules are produced on an industrial scale by fermentation through *A. niger* or *A. terreus*: beta-lactam antibiotics (piperacillin/tazobactam or amoxicillin/clavulanate), or gluconate used in plasma expanders.[39] However, the amount of GM in these products has been reduced and currently cross-reactivity is rarely seen.[39] Also, in patients receiving immunoglobulins false-positive GM results have been described.[40] Galactomannan in food can cause false-positive results if the gastrointestinal permeability is increased in patients with mucositis or graft versus host disease.[39] Galactomannan or GM cross-reacting epitopes have been described in other fungi such as *Histoplasma capsulatum*, *Cryptococcus* spp., *Geotrichum* spp., *Talaromyces* spp., *Acremonium* spp., *Alternaria* spp., *Fusarium* spp., or *Penicillium* spp.[39] and bacteria, e.g. *Bifidobacterium* spp..[41]

Sensitivity of serum GM is highest in haematology patients who are neutropenic, whereas in non-neutropenic patients, such as critically ill patients or recipients of a solid organ transplant, sensitivity is low.[42] A meta-analysis showed that in patients with neutropenia with proven or probable IA (vs possible/no IA) sensitivity of serum GM (optical density (OD) cutoff 0.5) was 0.78 (95% confidence interval (CI), 0.70–0.85) and specificity was 0.85 (95% CI, 0.78–0.91).[43] Another meta-analysis showed that in recipients of a solid organ transplant sensitivity of serum GM was 0.22 (95% CI, 0.03–0.6) and specificity was 0.84 (95% CI 0.78–0.88).[42]

In contrast to serum GM, sensitivity of BAL GM is similar in neutropenic and non-neutropenic patients. In a study that included three meta-analyses to calculate sensitivity and specificity, sensitivity of BAL GM (OD cutoff 1) was 0.75–0.86 and specificity was 0.94–0.95.[39]

(1-3)-β-d-glucan

(1-3)-β-d-glucan (BDG) is a component of the cell wall of many species and genera of fungi and can be detected in serum of patients with IA.[17] It has a limited

role in diagnosis of IA, because of reduced sensitivity and specificity compared to the GM assay,[44] and plays no role in classification of IA.[13, 25, 26, 30]

Aspergillus PCR

Aspergillus PCR assays can detect and identify *Aspergillus* to species level, and can be applied to different sample types. Sensitivity and specificity of a commercial real-time PCR assay were 84% and 80% respectively in BAL samples from patients with hematological disease suspected for IA.[34] An additional advantage of PCR is the possibility to detect resistance mutations in a sample without the need for culture and susceptibility testing.

CLASSIFICATION

Based on the underlying disease of the patient, there are different criteria for classifying IA[13, 25, 26, 30] for the purpose of scientific studies.

In patients with classical host risk factors for IA the EORTC/MSGERC criteria are used[13] and patients are classified as proven, probable or possible. Criteria for classification include a host risk factor for IA, clinical criteria and mycological criteria.[13] Diagnosis of IA is considered proven if there is histopathological or direct microscopic evidence of disease in a biopsy or aspirate, or if *Aspergillus* is isolated from culture of a sterile site, or *Aspergillus* DNA is detected in a sterile site, and can apply to any patient regardless of their immune status.[13] Diagnosis of IA is considered probable if there is at least one host factor, a clinical criterion and mycological evidence of disease.[13] Patients with a host factor and clinical criteria, but without mycological evidence (negative or not performed) are considered possible IA.[13] The EORTC/MSGERC criteria were originally developed for research purposes, but are used in clinical practice as well. The percentage of proven cases of IA was around 25% versus 75% probable IA in several studies.[14, 33, 45] Therefore diagnosis of IA mostly relies on culture of respiratory samples or non-culture based methods, galactomannan for example.

In critically ill patients other criteria than the EORTC/MSGERC criteria are necessary, because host risk factors for IA are often absent and this is a requirement for classification. Furthermore, typical radiological features of IA are non-specific in the majority of patients and fungal biomarkers may have lower diagnostic accuracy than in 'classical at-risk populations'.[25, 46] For patients admitted to the ICU different classification criteria have been developed: AspICU[25] for critically ill patients, IAPA[26] for patients with influenza admitted to the ICU and CAPA[30] for patients with COVID-19 admitted to the ICU. Classification of IA in

critically ill patients requires the presence of a host factor, at least one clinical criterion and mycological evidence.

TREATMENT

Currently, triazoles are the first line treatment in aspergillosis.[17, 20, 47] Before the introduction of the triazoles IA was associated with high morbidity and mortality.[48] In the pre-triazole era overall mortality in patients with IA was 58%, and increased to 87% in bone marrow transplant recipients and 88% in patients with central nervous system or disseminated IA.[16] The class of triazole drug includes itraconazole (first registered 1997), voriconazole (2002), posaconazole (2006), and isavuconazole (2015), and is used for treatment of acute IA or CPA, and as prophylaxis to prevent fungal infection in patients with prolonged neutropenia following intensive chemotherapy for AML, MDS or recipients of an allogeneic HSCT.[17-20, 48] Unfortunately, triazole resistance in *A. fumigatus* has emerged globally, limiting antifungal treatment options.[48, 49] Triazole resistance selection occurs mostly in the environment by exposure to azole fungicides used for both plant protection in agriculture and material protection.[50, 51] In the Netherlands, hotspots for resistance selection include flower bulb waste, green waste material and wood chippings.[52] Mutations associated with environmental resistance selection include a 34 base pair tandem repeat (TR) with an L98H mutation (TR_{34}/L98H) and TR_{46}/Y121F/T289A combination in the Cyp51A gene, that both lead to pan triazole resistance.[50] Furthermore, triazole resistance is associated with an increased mortality in patients with IA (49% vs 28%).[53] In 2022 the WHO published the WHO fungal priority pathogens list (WHO FPPL)[54] that aims to help research and policy interventions to strengthen the global response to fungal infections and increasing antifungal resistance. In the list, *A. fumigatus* was included in the critical priority group together with *Candida auris*, *Candida albicans* and *Cryptococcus neoformans*. One of the key action points of the WHO FPPL is to improve surveillance, to gain more insight into the burden of disease and antifungal resistance.

SURVEILLANCE

In the Netherlands, *A. fumigatus* resistance surveillance is currently performed by the mycology reference laboratory (Radboudumc-CWZ Center of Expertise for Mycology and National Public Health Institute, RIVM) by using data from ten sentinel hospitals (five academic medical centres and five teaching hospitals).[55, 56] The results are published in a yearly report on antimicrobial use and

antimicrobial resistance in the Netherlands (Nethmap-Maran) by The Dutch Working Party on Antimicrobial Policies (Dutch acronym SWAB) and the National Institute for Public Health and the Environment (RIVM).[55]

Clinical *A. fumigatus* isolates are usually screened for triazole resistance using a four-well agar plate containing voriconazole, itraconazole and posaconazole and a growth control (VIPcheck™, MediaProducts, Groningen, the Netherlands). Resistant isolates are sent to the reference laboratory for in vitro susceptibility testing and sequence analysis of the Cyp51A gene. With this programme resistance is monitored as well as the effect of intervention strategies to manage antifungal resistance. The national invasive mycosis guideline was revised in 2017 due to increasing triazole resistance frequencies exceeding 10%.[47] Presently, the national guideline from 2017 is being revised.

The clinical implications of triazole resistance in the Netherlands are unknown, because clinical information is not collected for the current resistance surveillance. To gain more insight into changes in the spectrum of disease, the spread of antifungal resistance, and to estimate the impact on health in different populations, disease oriented surveillance is imperative. Moreover, in this era of globalisation, modern medicine, and challenges such as global warming and decreasing biodiversity new forms of the disease or known forms of aspergillosis in new or previously unaffected patient populations may occur.

However, *Aspergillus* disease surveillance is difficult to conduct, due to the complexity of defining inclusion criteria. Positive mycology cultures from airway samples do not necessarily reflect disease, as a patient may be colonised with *Aspergillus*. Besides in patients with IA mycology cultures are often negative, and mycological evidence of aspergillosis mostly depends on fungal biomarkers, for instance GM or *Aspergillus* PCR. In addition, host factors and clinical factors are equally important for classification of invasive fungal disease.[13, 22, 25, 26, 30, 46] However, the different classification systems for the diverse groups of patients at risk for IA have different definitions for host, clinical and mycological criteria, which complicates case selection as well.

OUTLINE OF THE THESIS

The aim and scope of this thesis was to explore the possibilities of surveillance of IA in the Netherlands and to obtain more insight into the burden of disease and management of IA. The start of the PhD programme for this thesis unexpectedly coincided with the global COVID-19 pandemic, which influenced the scope of this thesis. Soon after the start of the pandemic, secondary infections with aspergillosis were described in patients with COVID-19 admitted to the intensive care unit (ICU) requiring ventilatory support, 'COVID-19 associated pulmonary aspergillosis (CAPA)'.[57-59] For a better comprehension of the new clinical entity CAPA, patients with COVID-19 admitted to the ICU of the Leiden University Medical Center (LUMC) were routinely screened for *Aspergillus*. The local CAPA surveillance programme was assessed in two studies, and in a multicentre study burden and management of CAPA in the Netherlands were determined.

The first part consists of three chapters about the burden of disease and challenges in the management of IA. In **chapter 2** the local treatment guideline for IA was evaluated 5 years after its implementation. The local guideline was implemented in 2015 because of the high local frequency of triazole resistance. Instead of combination therapy with voriconazole and liposomal amphotericin B, to avoid toxicity, a risk based empirical treatment strategy with voriconazole or liposomal amphotericin B was initiated depending on clinical parameters. In **chapter 3** the burden of IA was evaluated in a prospectively collected cohort of patients with acute myeloid leukaemia or high-risk myelodysplastic syndrome. A model was developed to determine the effect of IA on risk for different causes of death. In **chapter 4** frequency, risk factors and management of CAPA were evaluated in a multi-centre study in the Netherlands.

The second part of the thesis consists of three chapters about surveillance of IA. In **chapter 5 and 6** local surveillance of CAPA is described during the first COVID-19 peak in 2020 (chapter 5) and over a longer period 2020-2022 (chapter 6). In **chapter 7** a pilot study for national *Aspergillus* disease surveillance is described.

In **chapter 8** the results of the thesis are summarised and discussed. Additionally, recommendations are made for future research based on important findings in this thesis, and connections are identified in acquired knowledge from the different studies.

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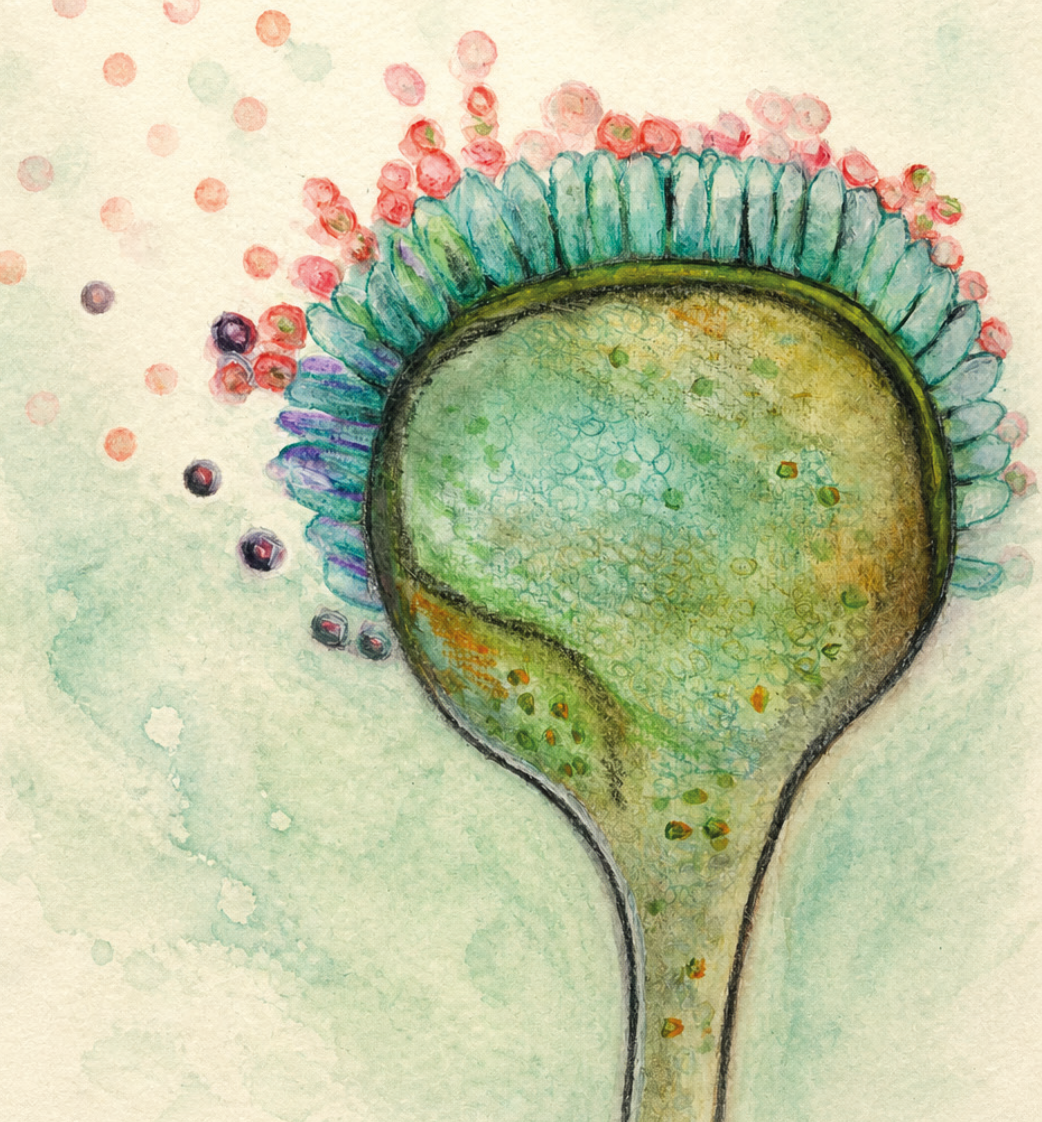
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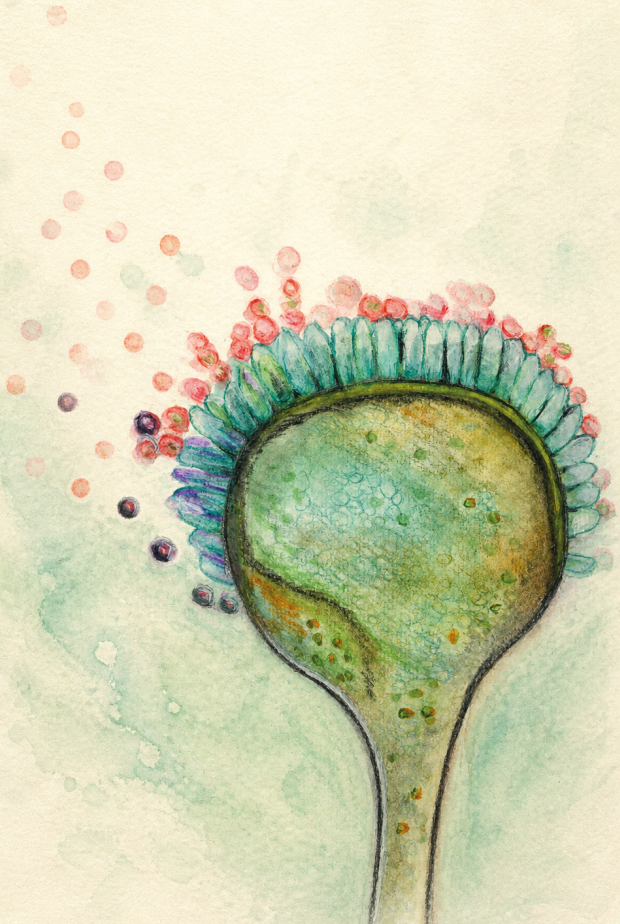
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Part I

Clinical management and burden of disease





2

Implementation of a clinical decision rule for selecting empiric treatment for invasive aspergillosis in a setting with high triazole resistance

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ABSTRACT

World-wide, emerging triazole resistance increasingly complicates treatment of invasive aspergillosis (IA). In settings with substantial (>10%) prevalence of triazole resistance, empiric combination therapy with both a triazole and liposomal amphotericin B (LAmB) can be considered because of the low yields of susceptibility testing. To avoid toxicity while optimizing outcome, a strategy with monotherapy would be preferable. A newly designed treatment algorithm based on literature and expert consensus provided guidance for empiric monotherapy with either voriconazole or LAmB. Over a four and a half year period, all adult patients in our hospital treated for IA were included and patient data were collected. An independent committee reviewed the attributability of death to IA for each patient. Primary outcomes were 30- and 100-day crude mortality and attributable mortality. In total, 110 patients were treated according to the treatment algorithm. Fifty-six patients (51%) were initially treated with voriconazole and 54 patients (49%) with LAmB. Combined attributable and contributable mortality was 13% within 30 days and 20% within 100 days. Treatment switch to LAmB was made in 24/56 (43%) of patients who were initially treated with voriconazole. Combined contributable and attributable 100-day mortality in this subgroup was 21% and was not increased when compared with patients initially treated with LAmB (P = 0.38). By applying a comprehensive clinical decision algorithm, an antifungal-sparing regime was successfully introduced. Further research is warranted to explore antifungal treatment strategies that account for triazole-resistance.

INTRODUCTION

Since the introduction of the antifungal drug voriconazole, mold-active triazole formulations are the primary treatment of choice for invasive aspergillosis (IA) in leading European and American guidelines.[1-3] However, over the past decades, emerging triazole resistance has developed as a new important threat to effective prevention and treatment of IA.[4-7] This development has given rise to an increase in the application of liposomal amphotericin B (LAmB), which is the drug of second choice for this indication.[8, 9] This is concerning due to decreased efficacy and increased toxicity of Amphotericin B formulations when compared to voriconazole.[1, 10-17] The highest incidence rates of IA can be found in patients with a hematological malignancy who receive intensive chemotherapeutic treatment and/or undergo hematopoietic stem cell transplantation (HSCT).[18-21] Despite the use of antifungal chemoprophylaxis, the residual incidences observed in this population often remain 5–10% or more.[21-23] Furthermore, due to the increasing number of patients that survive with temporary or chronic diseases of the immune system and by introduction of new treatment modalities e.g., tyrosine kinase inhibitors like ibrutinib, the population at risk of IA is expanding.[15, 24, 25]

Triazole-resistance mutations in the fungal DNA, specifically the CYP51A-gene and its promotor-region, are most often associated with pan-triazole resistance and therefore necessitate the use of LAmB. Use of LAmB can be associated with lower rates of treatment success and high rates of toxicity when compared to voriconazole.[10, 12, 13] Demonstrated resistance against all triazoles is a clear indication for the use of LAmB.[9] Deciding between voriconazole and LAmB as empiric therapy is difficult when the clinician suspects the presence of triazole resistant IA, but definite proof is missing. The background resistance rate, which varies among different populations at risk and different regions, needs to be taken into account when making this decision.

The first clinical isolates with triazole-resistance mechanisms have been recognized in Northern Western Europe.[7, 26] At present, resistance against triazoles in environmental as well as clinical isolates has developed to a global problem.[27-32] Results of a nationwide surveillance study in 2014 showed a triazole resistance rate of 5% in the USA, and presence of resistant isolates in the environment has been demonstrated as well.[29, 31] In the Netherlands, average resistance rates of *Aspergillus fumigatus* were 14.7% of 764 isolates screened in academic hospitals and 7.8% of 784 isolates screened in non-academic teaching hospitals in 2018.[33] It is complicated to measure the impact of triazole resistance in the clinical setting. Due to the fact that cultures and antifungal susceptibility testing fails in the majority of clinical specimens, the clinician often has to resort to an em-

pirical treatment strategy. Although our ability to determine susceptibility has recently been improved due to the introduction of PCR, the combined results of both culture and PCR are conclusive only in 30–60% of patients with probable invasive aspergillosis.[34-36] The resulting uncertainty about susceptibility easily gives rise to overtreatment with LAmB. When there is a high background resistance rate, any clue that raises the suspicion of resistance could motivate the clinician to opt for the use of LAmB instead of voriconazole. To ensure both the addition of the survival benefit of treatment with voriconazole and covering the risk of triazole resistance, Dutch national guidelines advise to empirically treat IA with combination therapy of both voriconazole and LAmB in case of unknown susceptibility.[37] However, the expected benefits of this strategy need to be weighed against a higher rate of serious adverse events associated with combination therapy, as well as higher costs,[16, 38, 39] but no randomized study data are available on this topic. Furthermore, no randomized head-to-head comparison between LAmB and voriconazole has been published, leaving some uncertainty about the superiority of voriconazole.[10-13]

To evade unnecessary toxicity while optimizing outcome, a clinical decision rule guiding to monotherapy with either voriconazole or LAmB was designed and validated in our hospital in a region with resistance rates between 16 and 24% reported in the last 10 years.[33]

METHODS

Development of a clinical treatment strategy

In 2014, all medical specialties in the Leiden University Medical Center involved in the diagnosis and treatment of patients with IA participated in constructing a treatment algorithm that provided guidance for empiric monotherapy with either voriconazole or LAmB (Figure 1). The algorithm aimed to optimize the balance between the risk of treating triazole-resistant IA with voriconazole and the risk of unnecessarily treating triazole-susceptible IA with LAmB. The treatment protocol was based on literature and guidelines[1, 21, 23, 38, 40-42] and information about local resistance rates.[33]

Criteria were formulated that either predispose for a worse outcome (e.g., presence of respiratory insufficiency) or were thought to predispose for infection with a triazole-resistant isolate (development of IA during chemoprophylaxis with a triazole). On the basis of these criteria, a decision about empiric (i.e., awaiting susceptibility testing) treatment was made. Both predicted worse outcome and breakthrough infections were considered indications for empiric treatment with

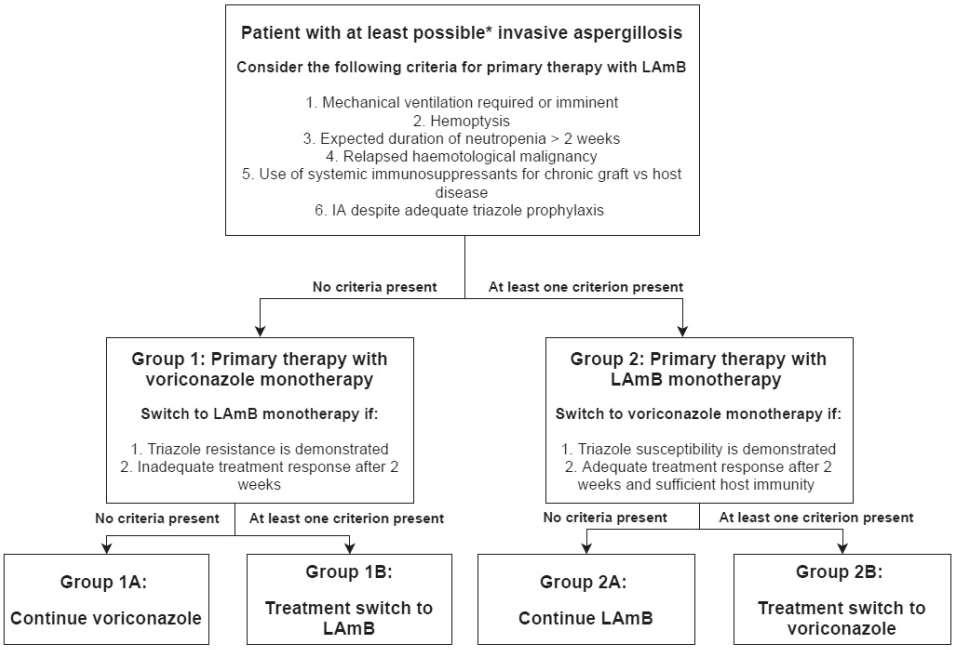


Figure 1. Flowchart of the treatment protocol.
Legend: the treatment protocol was implemented in a setting with high >10% of triazole resistance. IA denotes invasive aspergillosis; LAmB Liposomal amphotericin B. *as defined by the 2008 EORTC/MSG criteria for the diagnosis of invasive fungal infections[40]

LAmB. Although it is currently not supported by the literature that prophylaxis using a triazole predisposes for infection with a triazole-resistant isolate, the committee that constructed the algorithm decided to apply this criterion based on its rational concept. The reason to initially treat patients with a predicted worse outcome with LAmB was to mitigate the risk of applying ineffective treatment to a severely ill patient with potentially triazole-resistant IA. As treatment failure in the first phase of disease is associated with higher mortality, avoiding this risk was considered a high priority.[20, 41, 43] Combination therapy was not applied to avoid possible toxicity or other complications.

During treatment, results of susceptibility testing were used directly to switch treatment if appropriate. After two weeks of treatment without information on susceptibility, an evaluation of treatment outcome was made. In case of clinical non-response in a patient initially treated with voriconazole, the risk of triazole-resistance was considered high and treatment was switched to LAmB. In case of clinical non-response in a patient initially treated with LAmB, the risk of an (intrinsically) polyene-resistant infectious agent was considered to still be very low. Because host factors were considered to be the most likely cause of non-

response in this case, no benefit was expected to switch treatment to voriconazole. In case of response to treatment in patients treated with LAmB, treatment was de-escalated to voriconazole monotherapy under close monitoring.

The designed algorithm was approved by the institution's antimicrobial steering committee and was implemented from January 2015 onwards.

Study protocols and definitions

All patients who were considered to have a high risk of IA routinely received prophylaxis with a triazole with activity against *Aspergillus*. This included patients receiving remission-induction therapy prior to allogeneic HSCT, patient who underwent allogeneic HSCT, patients with prolonged neutropenia (at least two weeks), or other patients receiving high risk chemotherapeutics that induce neutropenia or impaired granulocyte function. Therapeutic drug monitoring was applied in this population.[44]

Protocolized CT-scanning and bronchoalveolar lavage (BAL) were performed upon suspicion of IA. BAL samples were examined by direct microscopy, culture, galactomannan assay (cut off at 0.5 optical density) and from 2017 onwards also by PCR. All available BAL samples from patients initially not tested by PCR were retrospectively tested. The AsperGenius® PCR assay (Pathonostics, Maastricht, the Netherlands) was used. Triazole resistance was routinely tested by four well agar plate screening (VIP check, Groningen, The Netherlands).

Data collection

All adult patients were retrospectively included if they had been treated according to our treatment protocol between January 2015 and September 2019. Patients who received either LAmB, voriconazole, isavuconazole or posaconazole in our center were identified through the hospital pharmacy database. Of these patients, the electronic medical records were examined to ensure eligibility for inclusion in our study. Extracted patient characteristics included age, diagnosis of immunocompromising disease, diagnosis of IA, comorbidity and immune status, as well as information about performed diagnostics, triazole susceptibility, dosage of therapy, duration of therapy, treatment history, switch of antifungal therapy, renal function and outcome of IA. IA was classified according to the 2008 revised European Organisation for Research and Treatment of Cancer – Mycoses Study Group criteria for the classification of invasive fungal infections.[40] After retrieval of all relevant information, the data of all participants was pseudonymized.

Definition of attributable mortality

The role of IA with regard to the cause of death was classified as either 'non-attributable to IA', 'contributable', 'attributable', or 'unknown' (see criteria in the textbox 1). The last category was introduced because the clinical data at time of death were insufficient for a few cases. Attributable and contributable mortality were the primary outcome measures for this study. The classification was constructed by the investigator group prior to analysis of the data and was based on modification of definitions from literature.[41, 43] A committee was instructed to determine attributability in all patients who died within 100 days. The commit-

Textbox 1

Attributable
The immediate cause of death was defined as the disease process, injury, or complication immediately preceding death. Invasive aspergillosis (IA) was considered the cause of death when the immediate cause of death was due to this infection. Examples are neurological complications of an *Aspergillus* infection that disseminated to the brain, lung bleeding or respiratory insufficiency in a patient with pulmonary aspergillosis.

or

IA was judged to have played a major role if death would not have occurred had the patient not had IA, even though another condition was present that also contributed to death. This includes toxicity, interactions and other side effects of antifungal treatment that played a major role in the cause of death. Another example is a *Pseudomonas* bacteremia in a patient with a cavitating pulmonary aspergillosis in which the lungs are considered the most likely source of the bacteremia.

Contributable
IA or treatment of IA was defined as playing a minor role if it was probably not essential in explaining the patient's death but arguably did play some role in the event. Example is a patient with an *Aspergillus* infection as well as severe uncontrolled gastrointestinal graft versus host disease at the time of death.

Non-attributable
Mortality was classified as not related to IA if there was a clear other cause of death not related to IA.

Unknown
If insufficient data were present about the circumstances in which death occurred.

tee consisted of three reviewers who were not directly involved with the study, with experience in the fields of infectious diseases, clinical microbiology and haematology. The reviewers received written instructions to use the medical correspondence, post-mortem reports and laboratory findings as reported in the patient files to categorize the deceased patients according to the above definitions. In case of disagreement between the reviewers, the case was discussed between all three reviewers until consensus was reached.

Statistics

Survival proportions were calculated and comparative analyses were performed using SPSS version 25 (IBM Corp., Armonk, NY).

Ethics

The study was reviewed by the institutional review board, which deemed that the Dutch law on research on human subjects was not applicable to our study. Data were processed in accordance with the national Personal Data Protection Act.

RESULTS

Protocol adherence and study population characteristics

A total number of 115 patients were treated for IA at our institution from January 2015 to September 2019. Of this total, 110 patients (96%) were treated for IA with either voriconazole or LAmB monotherapy according to the designed algorithm. The remaining five patients received off-protocol treatment regimens (Figure 2). These patients received either combination therapy, or violated the criteria mentioned in the decision tool otherwise. For all results, the remaining 110 patients were used to calculate percentages, with the exception of the triazole resistance rates.

Study population characteristics

Out of 110 included patients, 76 (69%) were male; the median age was 63 (range 20–83), and 100 (90%) patients were treated for an underlying hematologic malignancy or had undergone allogeneic stem cell transplantation (Table 1). Of patients with hematologic malignancy, 14 suffered from a relapsed malignancy and eight from a secondary (treatment related) malignancy. A small subgroup of patients (10%) did not suffer from hematologic malignancy but were recipients of a solid organ transplantation, received chemotherapy for other malignancies, or were immunocompromised for other reasons.

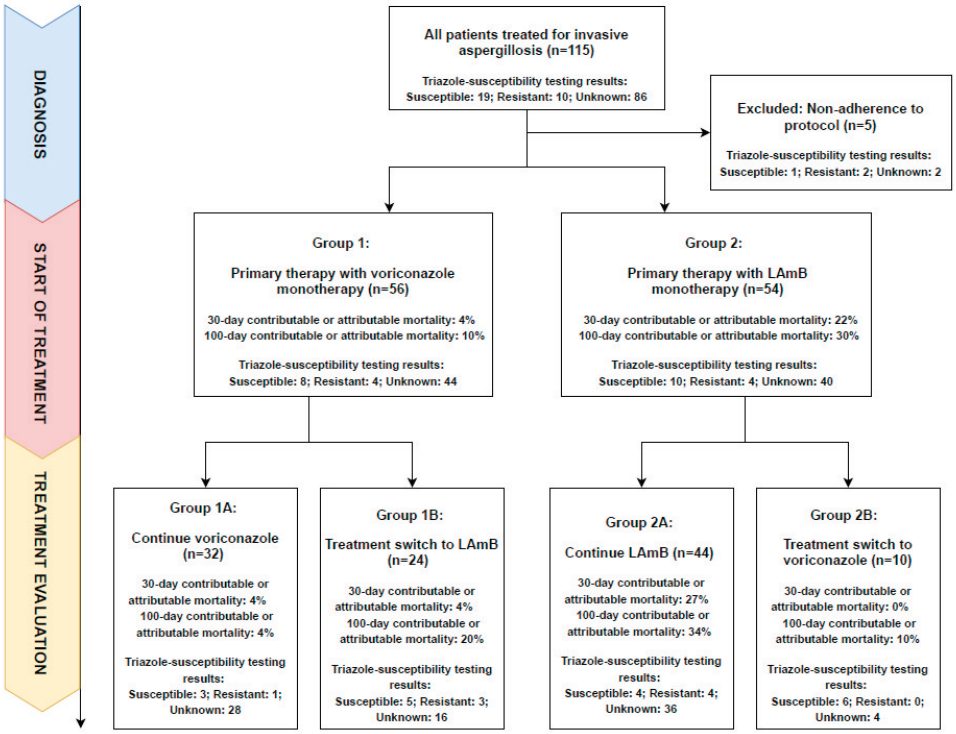


Figure 2. The treatment protocol with numbers of patients in each arm as well as resistance and mortality rates.
Legend: LAmB, Liposomal amphotericin B. Susceptibility testing was done by applying both PCR and culture techniques. Resistance data were not yet known at the moment of start of therapy, but were known at the moment of treatment evaluation. Contributable and attributable mortality rates were defined in the process as described in the methods section and can also be found in Table 3. *possible IA was defined according to the 2008 revised definitions for the diagnosis of invasive fungal infections[40]

Treatment and outcomes

Fifty-six patients (51%) were initially treated with voriconazole and 54 (49%) were initially treated with LAmB. Cultures were positive in 16/115 (14%) patients and phenotypical voriconazole resistance was detected in 5/16 (31%). Overall, susceptibility testing was successful in 29 cases, yielding 10 (34%) triazole-resistant and 19 (66%) triazole-susceptible isolates. Susceptibility was determined phenotypically by culture in 13 clinical isolates, and genetically with PCR in 16 clinical isolates. In one case, triazole resistance was detected by both PCR and culture. Five non-fumigatus *Aspergillus* fungi were identified with PCR or culture, none of which with intrinsic LAmB-resistance (two *Aspergillus flavus*, two *Aspergillus niger*, one *Aspergillus nidulans*).

Table 1.Characteristics of patients treated for invasive aspergillosis.

Total number of patients	110
Sex, male (%)	76 (69)
Age, median (range)	63 (20-83)
Underlying predisposing disease, number of pts. (%)	
Total with hematologic malignancy or after HSCT	100 (91)
Total who underwent HSCT	54 (49)
Patients with hematologic malignancy:	
AML/MDS-RAEB2	45 (41)
ALL	29 (26)
Multiple Myeloma	12 (11)
MDS	4 (4)
Aplastic Anemia	4 (4)
CLL	2 (2)
Myelofibrosis	2 (2)
CML	1 (1)
Received HSCT for Sickle Cell Disease	1 (1)
Receiving chemotherapy for solid tumor	2 (2)
Kidney transplantation	3 (3)
Liver transplantation	2 (2)
Other* (not malignant, not transplant-related)	3 (3)

Legend: IA denotes invasive aspergillosis, HSCT hematopoietic Stem Cell Transplantation, LAmB Liposomal Amphotericin B, ALL Acute Lymphoid Leukaemia, AML Acute Myeloid Leukemia, CML Chronic Myeloid leukemia, CLL Chronic Lymphoid leukemia, MDS Myelodysplastic Syndrome, MDS-RAEB2 Myelodysplastic Syndrome - Refractory Anaemia with Excess Blasts grade 2. *Other underlying diseases: severe anorexia nervosa, badly regulated diabetes type 1, and influenza.

Upon clinical evaluation of empiric therapy and of resistance data, a switch was made to LAmB in 24/56 (43%) of patients who were initially treated with voriconazole. Table 2 displays the reasons for treatment switch. In the group that started treatment with LAmB, 9 out of 54 (17%) patients switched to voriconazole. Treatment was completed with voriconazole in 42 cases and with LAmB in 68 patients. The flowchart (Figure 2) shows the number of patients in each treatment group.

Therapy-related adverse events occurred in both treatment arms. Hepatotoxicity was a reason to stop treatment with voriconazole in seven patients (13%) and nephrotoxicity was a reason to stop treatment with LAmB in 11 patients (20%). Allergic reaction was a reason to stop treatment with voriconazole in one case. Serious alteration of mental state and/or visual hallucinations was a reason to stop treatment with voriconazole in two cases. Reasons to start treatment with LAmB are displayed in Table 2. The most common reason to initially treat with LAmB was adequate prophylaxis with voriconazole before establishing the

Table 2. Motivation of treatment decisions.

Number of patients initially treated with voriconazole (group 1)	**56**
Number of patients initially treated with LAmB (group 2)	**54**
*Reason to initially treat with LAmB**, number of patients, (% of patients treated with LAmB)	
Mechanical ventilation required or imminent	9 (17)
Hemoptysis	0
Expected duration of neutropenia >2 weeks	2 (4)
Relapsed hematologic disease	14 (26)
Use of systemic immunosuppression for chronic graft versus host disease	0
IA occurred despite adequate prophylaxis with a triazole	28 (52)
Broader antifungal spectrum deemed necessary (e.g. suspicion of mucormycosis)	5 (9)
Intolerance or significant drug interaction with voriconazole	4 (7)
Number of patients who switched from voriconazole to LAmB (group 1B)	**24 (23)**
Reasons to switch, number of patients, (% of patients who switched)	
Resistance to azoles demonstrated	3 (13)
Progression of IA	15 (63)
Intolerance to voriconazole	6 (25)
Number of patients who switched from LAmB to voriconazole (group 2B)	**10 (9)**
Reasons to switch, number of patients (% of patients who switched)*:*	
Susceptibility to azoles demonstrated	6 (60)
Adequate treatment response and sufficient recovery of host immunity	2 (20)
Intolerance to LAmB	2 (20)

Legend: LAmB denotes liposomal amphotericin B, IA invasive aspergillosis. *More than one reason could be present for one patient.

diagnosis of IA (28 patients). Of these 28 patients, susceptibility testing was successful in four cases, of which only one was triazole resistant.

Crude and attributable mortality rates per treatment stratum are listed in Table 3. The flowchart (Figure 2) shows a combination of attributable and contributable rates only. Contributable or attributable mortality was lower in patients initially treated with voriconazole (30-day contributable or attributable mortality was 6%) than in patients initially treated with LAmB (30-day contributable or attributable mortality was 22%). Mortality was lowest in patients who were only treated with voriconazole monotherapy. Mortality was highest in patients who were only treated with LAmB monotherapy and in patients initially treated with voriconazole and later switched to treatment with LAmB as per the rules of the decision tree. The mortality rates differed only slightly (61 vs 54% crude 100-day mortality, 34 vs 21% combined attributable and contributable 100-day mortality). Attributable mortality was highest in the first period after diagnosis of IA. After 40 days, most mortality was either non-attributable to IA or of unknown cause (Figure 3). Combined contributable and attributable mortality for

Table 3. Outcomes of patients treated according to the protocol.

	Total	Group 1 (VOR)	Group 2 (LAmB)	Group 1A (VOR)	Group 1B (VOR, LAmB)	Group 2A (LAmB)	Group 2B (LAmB, VOR)
Number of patients	**110**	**56**	**54**	**32**	**24**	**44**	**10**
30-day mortality # patients (%)							
total	**26 (24)**	**7 (13)**	**19 (35)**	**4 (13)**	**3 (13)**	**19 (43)**	**0**
attributable	3 (3)	2 (4)	1 (2)	1 (4)	1 (4)	1 (2)	0
contributable	11 (10)	0	11 (20)	0	0	11 (25)	0
unrelated	11 (10)	5 (9)	6 (11)	3 (9)	2 (8)	6 (11)	0
unknown	1 (1)	0	1 (2)	0	0	1 (2)	0
100-day mortality # patients (%)							
total	**51 (46)**	**20 (34)**	**31 (57)**	**9 (28)**	**11 (54)**	**27 (61)**	**4 (40)**
attributable	6 (5)	3 (5)	3 (6)	1 (4)	2 (8)	2 (5)	1 (10)
contributable	16 (15)	3 (5)	13 (24)	0	3 (13)	13 (29)	0
unrelated	24 (22)	12 (21)	12 (22)	6 (18)	6 (28)	9 (20)	3 (30)
unknown	5 (9)	2 (4)	3 (6)	2 (6)	0	3 (7)	0
Azole-resistance *# of patients (%)*	8 (5)	4 (7)	4 (7)	1 (3)	3 (13)	4 (10)	0
Azole-susceptibility *# of patients (%)*	18 (15)	8 (14)	10 (19)	3 (9)	5 (21)	4 (9)	6 (60)
Diagnostic certainty # of patients (%)							
Possible	36 (33)	16 (29)	20 (37)	13 (41)	3 (13)	19 (43)	1 (10)
Probable	72 (65)	39 (70)	33 (61	19 (59)	20 (83)	25 (57)	8 (80)
Proven	2 (2)	1 (2)	1 (2)	0	1 (4)	0	1 (10)

Legend: LAmB denotes liposomal amphotericin B, VOR voriconazole. Diagnostic certainty was defined according to the revised 2008 EORTC/MSG criteria for the diagnosis of Invasive Fungal Infection.[40] Group 1 consists of patient initially treated with voriconazole. Subgroup 1A continued treatment with voriconazole, while subgroup 1B switched to LAmB eventually. Group 2 consists of patients initially treated with LAmB. Subgroup 2A continued treatment with LAmB, while subgroup 2B switched to voriconazole eventually. The treatment rules for the different groups can be found in Figure 1.

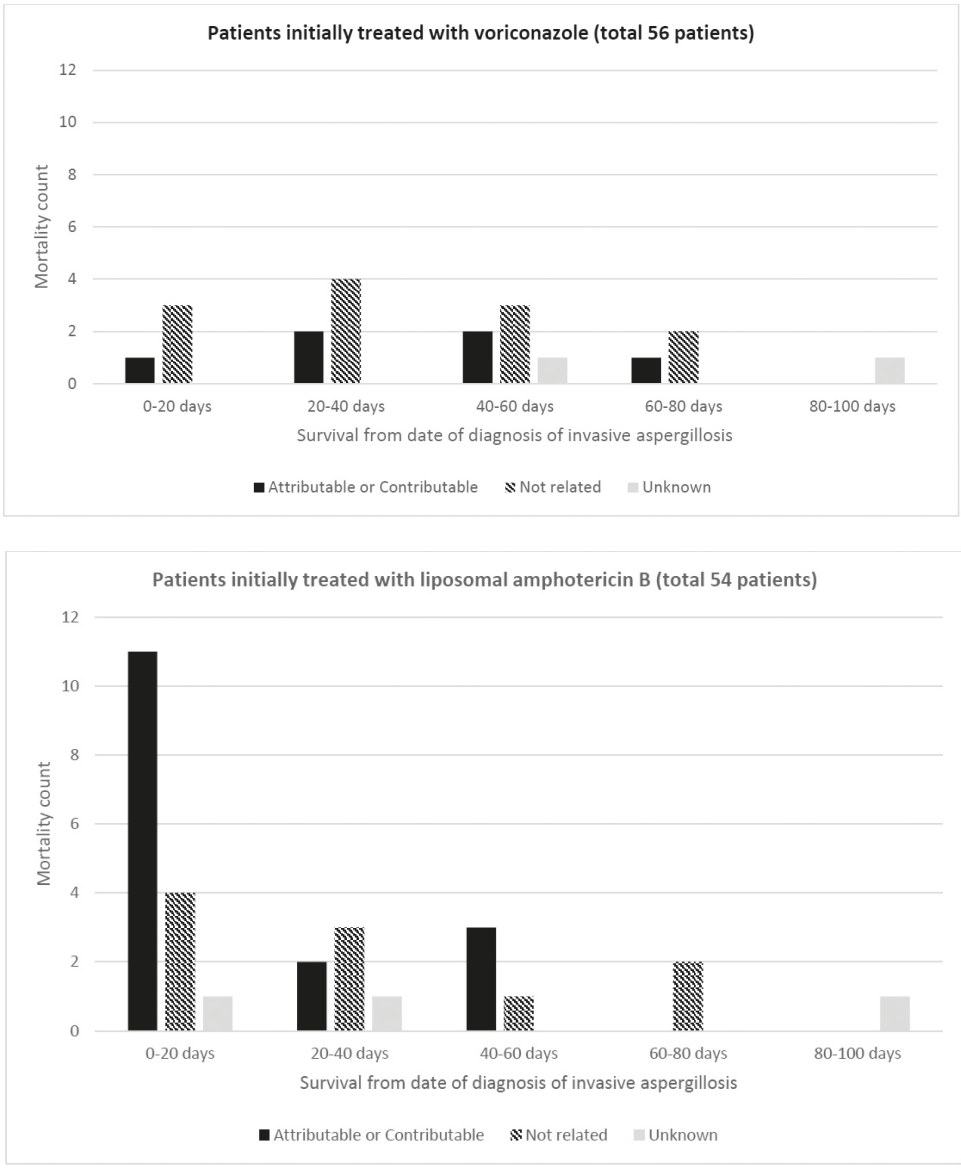


Figure 3. Mortality counts of patients treated for invasive aspergillosis.
Legend: IA denotes invasive aspergillosis. Attributable or contributable mortality was determined in the procedure as described in the methods section.

all groups was 12% after 30 days and 20% after 100 days. Mortality unrelated to IA within 30 days was lower in patients with proven or probable IA only when compared to all patients (4 versus 10% overall).

The calculation of survival rates was repeated for proven and probable cases of IA (defined according to the 2008 revised EORTC criteria[40]) and detailed in supplement A. The results were similar to the results of the overall analysis.

DISCUSSION

By applying a comprehensive clinical decision algorithm in our area with high (>10%) triazole-resistance rates, 51% of patients were empirically treated with monotherapy voriconazole, without indications for excess crude mortality even if a later switch to LAmB was needed. In 29% of patients, therapy with LAmB could be avoided during the entire course of treatment. Our study provides a rationale to effectively account for possible triazole resistance while preventing the negative effects associated with combination therapy. However, due to the way the study has been designed, it is not possible to make direct comparisons between treatment groups; the basis on which the choices for therapy were made were dependent on clinical factors which are also associated with outcomes, thought to be helpful in identifying the patients who would benefit the most from the relevant treatment strategy. This distinction causes factors that are correlated with a worse outcome to not be equally distributed between the treatment groups. Mortality rates of patients initially directed to voriconazole monotherapy were lower when compared to people who were treated with LAmB. However, the group of patients that were initially treated with LAmB were expected to have a worse outcome at baseline.

The treatment decision tree was constructed in accordance with our local antibiotics steering committee, with knowledge about local epidemiology and triazole-resistance rates, current literature and the relevant guidelines. National and international guidelines that describe the optimal management for IA need to rely on low evidence levels, in part due to the relative rarity of IA. Additionally, because of improvements in the management of patients with hemato-oncological disease, the population at risk is becoming harder to define and is ever-changing. Emerging triazole resistance makes it even more difficult to formulate an unambiguous treatment advice.

No data are available that support an increased risk of triazole-resistant IA in patients that develop IA despite receiving adequate prophylaxis with a tri-

azole. We did choose to include this as a criterion to initially treat with LAmB. Nonetheless, no remarkable additional risk of triazole-resistance was found in this subgroup (of four successful susceptibility tests in 28 patients, one isolate showed triazole resistance). A limitation of this study is that detection of resistance was not as successful as reported in literature despite the use of both PCR and conventional culture.[34-36, 45] Reported rates of triazole resistant IA in our region are amongst the highest in the Netherlands, and in the world.[32, 33] The high local resistance rates have been linked to the extensive use of fungicides in agriculture.[7] All-cause mortality in our cohort was high with a 46% mortality rate within 100 days. In patients with proven and probable invasive aspergillosis only, this rate was similar (41%). In literature, the mortality rates differed greatly between different subpopulations, but were on average lower than in our population.[15, 22, 23] However, within the total mortality count, a minority of cases was attributable or contributable to IA. The nature of the subpopulation plays a large role in the expected case fatality rate. On average, our population consisted of patients with both a high risk of developing IA and a high risk of mortality due to haematological malignancy. Additionally, T-cell depleted HSCT is standard practice in our center. This method of transplantation is associated with a substantially lower risk of graft versus host disease, but at the cost of more difficulty in the treatment of opportunistic infections due to an initially less effective T-cell mediated immune response.

In conclusion, the results of our study can provide new insight in the application of an antifungal-sparing clinical decision tool while minimizing the risks of the consequences of undertreatment. The non-randomized approach and heterogeneous population make it difficult to make generalized statements about treatment effectiveness, and future research could further expand on the hypothesis that LAmB can have an important role in the treatment of IA in areas with high triazole resistance without the necessity of combination therapy.

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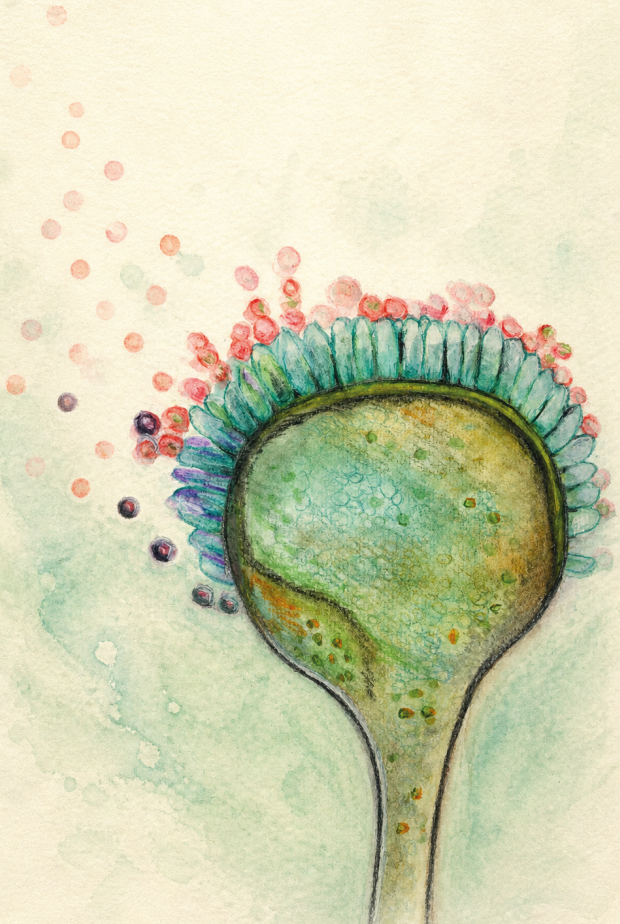
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Supplementary Table A. Outcomes for patients with proven and probable invasive aspergillosis only.

	Total	Group 1 (VOR)	Group 2 (LAmB)	Group 1A (VOR)	Group 1B (VOR, LAmB)	Group 2A (LAmB)	Group 2B (LAmB, VOR)
Number of patients	**74**	**40**	**34**	**19**	**21**	**25**	**9**
30-day mortality # patients (%)							
total	**12 (16)**	**3 (8)**	**9 (27)**	**1 (5)**	**2 (10)**	**9 (36)**	**0**
attributable	1 (1)	1 (3)	0	0	1 (5)	0	0
contributable	8 (11)	0	8 (24)	0	0	8 (32)	0
unrelated	3 (4)	2 (5)	1 (3)	1 (5)	1 (5)	1 (4)	0
unknown	0	0	0	0	0	0	0
100-day mortality # patients (%)							
total	**30 (41)**	**13 (33)**	**17 (50)**	**5 (26)**	**8 (38)**	**14 (56)**	**3 (33)**
attributable	4 (5)	2 (5)	2 (6)	0	2 (10)	1 (4)	1 (11)
contributable	11 (15)	2 (5)	9 (26)	0	2 (10)	9 (36)	0
unrelated	13 (18)	8 (20)	5 (15)	4 (21)	4 (19)	3 (12)	2 (22)
unknown	2 (3)	1 (3)	1 (3)	1 (5)	0	1 (4)	0
Azole-resistance *# of patients (%)*	8 (11)	4 (10)	4 (12)	1 (5)	3 (14)	4 (16)	0
Azole-susceptibility *# of patients (%)*	16 (22)	8 (20)	10 (29)	3 (16)	5 (23)	4 (16)	6 (66)
Diagnostic certainty # of patients (%)							
Probable	72 (97)	39 (98)	33 (97)	19 (100)	20 (95)	25 (100)	8 (89)
Proven	2 (3)	1 (2)	1 (3)	0	1 (5)	0	1 (11)

Legend: LAmB denotes liposomal amphotericin B, VOR voriconazole. Diagnostic certainty was defined according to the revised 2008 EORTC/MSG criteria for the diagnosis of Invasive Fungal Infection.[40] Group 1 consists of patient initially treated with voriconazole. Subgroup 1A continued treatment with voriconazole, while subgroup 1B switched to LAmB eventually. Group 2 consists of patients initially treated with LAmB. Subgroup 2A continued treatment with LAmB, while subgroup 2B switched to voriconazole eventually. The treatment rules for the different groups can be found in Figure 1.



3

Effect of invasive aspergillosis on risk for different causes of death in older patients with acute myeloid leukaemia or high-risk myelodysplastic syndrome

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ABSTRACT

Purpose: Study objectives were to estimate the cumulative incidence of death due to different causes of death (CODs) and investigate the effect of invasive aspergillosis (IA) on each separate COD in a cohort of older patients with acute myeloid leukaemia (AML) or high-risk myelodysplastic syndrome (MDS) included in the Haemato-Oncology Foundation for Adults in the Netherlands (HOVON) 43 randomized controlled trial.

Methods: Pre-collected data from the trial was obtained from the HOVON data center and relevant clinical information was extracted. The cumulative incidence of death due to different CODs was estimated with a competing risk model and the association between each COD and prognostic factors, including IA, were investigated with a cause-specific hazard Cox regression model.

Results: In total 806 patients were included, mean age of 70 years and 55% were male. The cumulative incidences of death due to leukaemia or infection at 3, 6, 12 and 36 months were 0.06, 0.11, 0.23, 0.42 and 0.17, 0.19, 0.22, 0.25 respectively. Incidence of IA was 21% and diagnosis of IA up until the final chemotherapy cycle was associated with an increased risk of dying from leukaemia (cause-specific hazard ratio ($_{CS}$HR): 1.75, 95% CI 1.34–2.28) and a trend was seen for infection ($_{CS}$HR: 1.36, 95% CI 0.96–1.91).

Conclusion: Leukaemia was the most likely cause of death over time, however in the first year after diagnosis of AML or high-risk MDS infection was the most likely cause of death. Patients with IA had a relatively increased risk of dying from leukaemia or infection.

INTRODUCTION

The incidence of invasive aspergillosis (IA) in patients with haematological malignancies ranges from 4 (with prophylaxis) to 11% (without prophylaxis) during remission induction chemotherapy and is approximately 8% after allogeneic stem cell transplantation (SCT).[1] In patients with acute myeloid leukaemia (AML) the most common risk factor for development of IA is prolonged and severe neutropenia which is induced by AML or by intensive chemotherapy.[2] In addition, lack of response to chemotherapy and age have been reported to be a risk factors for development of IA.[3-5]

AML is mostly seen in older patients with a median age of around 70 years.[6, 7] The 5-year survival for patients older than 15 years diagnosed with AML in Europe in 2000–2007 was 17% but survival rates decreased steeply with age.[8] Most patients with AML have active leukaemia at time of death. Other reported causes of death (CODs) include transplant related complications, infection (pneumonia in particular), bleeding, or other causes.[9-14] The contribution of IA to COD in patients with AML is difficult to quantify, because IA has multiple risk factors and these risk factors also directly contribute to AML treatment failure, complications and mortality.[15, 16]

In the past years overall survival has improved for younger AML patients, mostly due to improved supportive care and improvements of allogeneic-SCT.[17-19] Conversely, for older patients with AML limited progress has been made.[17-19] This can be explained by patient and disease specific factors. Older patients with AML are more likely to have comorbidities, poor performance status, less tolerance to intensive chemotherapy and a higher incidence of poor prognostic factors, such as unfavourable cytogenetics and secondary leukaemia after myelodysplastic syndrome (MDS).[6, 20, 21] Moreover, older patients are more susceptible for infections due to immunosenescence, a gradual decrease in immune system function associated with physiological aging that affects both the innate and the adaptive immune system, malnutrition and age related physiological changes.[22, 23]

Study objectives were to estimate the cumulative incidence of death due to different CODs and to investigate the effect of IA on each separate COD in a cohort of older patients with AML (≥ 61 years) or high-risk MDS included in the Haemato-Oncology Foundation for Adults in the Netherlands (HOVON) 43 randomized controlled trial. The HOVON focuses on improving treatment methods for adult patients with malignant haematological disorders by implementing randomized controlled trials.

MATERIALS AND METHODS

Study design

Pre-collected data from the HOVON-43 randomized controlled trial[13] was requested from the HOVON data center. This trial included patients with AML or high-risk MDS. From all patients clinical information was extracted about diagnosis, treatment, response to treatment, survival and COD. The trial data contained an infection form in which all infections were registered during each chemotherapy treatment cycle. From the infection form, all patients with an occurrence of IA were identified. A competing risk model was applied to estimate the cumulative incidence of death with different competing events (different CODs). A cause specific hazard Cox regression model was used to investigate the association between survival time and multiple covariates.

All ethical considerations were within the intended use of the HOVON-43 randomized controlled trial and in accordance to the ethical principles as posed in the declaration of Helsinki as well as current national legislation.

HOVON-43 trial data

Data was previously collected during the HOVON-43 randomized controlled trial that was performed from 2000–2006.[13] This trial was performed to determine the optimal dose of daunorubicin in older patients (≥ 61 years old) treated for AML or high-risk MDS. In short, after diagnosis eligible patients were randomly assigned to receive a first cycle of conventional or escalated dose of daunorubicin in combination with cytarabine. Next, a second cycle of treatment was administered in both groups consisting of high dose cytarabine. After each cycle of remission induction chemotherapy response was assessed. Patients in complete remission after the second cycle could undergo a reduced intensity allogeneic-SCT if an HLA-matched donor had been identified. Otherwise, patients were randomly assigned to receive post remission chemotherapy with gemtuzumab ozogacimin (GO) or no treatment. Patients who were not in complete remission or who experienced a serious adverse event were not further randomized. Follow-up was performed until five years after the second chemotherapy cycle. Patients did not receive antifungal prophylaxis. At the time, chemoprophylaxis to prevent filamentous fungal infections was not standard practice.

Patient population and data collection

A selection of baseline patient characteristics was made to investigate the effect of these prognostic factors on different CODs before start of chemotherapy. We obtained the following baseline variables: date of birth, gender, WHO

performance status, AML or MDS, white blood cell (WBC) count at diagnosis, prior haematological or oncological disease, previous chemotherapy or radiotherapy, bone marrow cellularity, bone marrow fibrosis. Time dependent variables included outcome (death/alive), COD and occurrence of IA.

From the infection form the variable 'occurrence of IA' was created by identification of all patients with an occurrence of IA at any time point in the treatment schedule. Cases in which the diagnosis of IA was uncertain (n = 72) were discussed by two investigators (MdB and RvG) and final decision was based on consensus (17 IA; 55 no IA). In case consensus would not be reached, a third expert was consulted (MvdB). In most cases that were discussed only treatment with an antifungal agent was registered without mention of IA. That was not considered enough evidence for diagnosis IA. The uncertain cases that were classified as IA had a lung abnormality on imaging and were treated with an antifungal agent. The timepoint of IA was estimated from the chemotherapy cycle date in which the occurrence of IA was registered. Diagnosis of IA was made by the treating physician at the time. The data did not contain information about clinical characteristics, which diagnostic test was performed for the diagnosis of IA, or certainty of diagnosis according to EORTC/MSGERC guidelines.[24]

In the dataset the COD was grouped into the following categories: leukaemia (including high-risk MDS), pneumonitis, infection, haemorrhage, veno-occlusive disease, secondary malignancy, other COD or unknown. Cause of death was recorded according to the clinical judgement of the treating physician at the time of death, as the rate of autopsy in this patient population was very low. In most patients the COD was described in a free text field as well. By checking the description of the COD, a few misclassified COD were reclassified.

For modelling purposes different CODs were grouped together. The variables 'infection' and 'other COD' were grouped together ('infection and other COD'), because 'other COD' included 12/70 patients with infection as one of the documented CODs as well. In total, 158/216 (73%) in the group 'infection and other COD' had an infection. The variables 'pneumonitis', 'haemorrhage', 'veno-occlusive disease', 'secondary malignancy' and 'unknown' were grouped together as 'miscellaneous'. In haematology patients pneumonitis is mostly defined as lung inflammation induced by chemotherapy. Therefore pneumonitis was not considered an infection.

Statistical methods

In this study, a competing risk model[25] was applied to estimate the cumulative incidence of death with the competing events 'leukaemia', 'infection and other COD', and 'miscellaneous'.

To study the association between each competing event ('leukaemia', 'infection and other COD' and 'miscellaneous') and prognostic factors a Cox proportional-hazard model[26] was estimated. Time fixed prognostic factors included in the model were AML or high-risk MDS, prior haematological or oncological disease, previous chemo- or radiotherapy, WBC count at diagnosis and age. Invasive aspergillosis was included in the model as time dependent. The last documented cycle of chemotherapy was selected for the diagnosis of IA. If a patient did not have IA from the time of inclusion until the end of the final chemotherapy treatment cycle, the patient was registered as not having IA. Hazard ratios (HR) along with their 95% confidence intervals (CI) were reported. A p-value of < 0.05 was considered statistically significant. Bone marrow cellularity and bone marrow fibrosis were not included as variable in the Cox proportional hazard model due to a high number of missing data, as was WHO performance status, because values were similar for all patients.

All analyses were performed in R software environment (https://www.R-project.org/) with the mstate library.[27]

RESULTS

The HOVON-43 trial included a total of 866 patients. Overall, 60 patients were a priori excluded from the analysis (Table 1).

Table 1 summarises the patient characteristics at baseline for the included 806 patients. The mean age at the time of diagnosis was 70 years (range: 61–88). In total, 710 (88.1%) patients died during the study period. Table 2 displays the number of patients and percentage for each COD. A total of 171 (21.2%) patients developed IA during follow-up, of whom 40 recovered. The remaining 131 patients had IA up until in their final chemotherapy cycle and of these patients 121 (92.4%) died. Most patients developed IA during remission induction chemotherapy in cycle I and II (Table 3). Table 2 shows the number and percentage of patients who had IA up until their final chemotherapy treatment cycle for the different CODs.

Table 1. Descriptive statistics of the selected relevant variables at time of diagnosis of AML or high-risk MDS.

Characteristics	N (%)
Total	806
Age (mean)	70
Age	
≤ 70 years	427 (53)
> 70 years	379 (47)
Sex	
Male	441 (55)
Female	365 (45)
AML-MDS	
AML	677 (84)
MDS	129 (16)
WHO performance status	
WHO 0	262 (33)
WHO 1	448 (56)
WHO 2	83 (10)
WHO 3	2 (0.2)
Bone marrow cellularity	
Low	60 (7)
Medium	123 (15)
High	430 (53)
WBC count (x10^9 cells/L)	
1st quantile	2
Median	5.5
3rd quantile	27
Prior haematological or oncological disease	
No	747 (93)
Yes	59 (7)
Previous chemo- or radiotherapy	
No	596 (74)
Yes	210 (26)
Bone marrow fibrosis	
No	497 (62)
Yes	112 (14)

AML: acute myeloid leukaemia; MDS: myelodysplastic syndrome; WBC: white blood cell. Unknown entry is not displayed.
The HOVON trial included 866 patients of whom 60 were excluded from the analysis. Patients were excluded from the analysis for the following reasons: incomplete records (n=33), time of diagnosis and or time of follow-up not documented correctly (n=8), registered twice (n=4), missing informed consent (n=1), diagnosis of acute promyelocytic leukaemia (n=6), no AML (n=4), diagnosis of refractory anaemia with an international prognostic score of less than 1.5 (low-risk MDS) (n=1), previously treated AML (n=1), medical history of malignant lymphoma (n=1) or concurrent liver cancer (n=1).

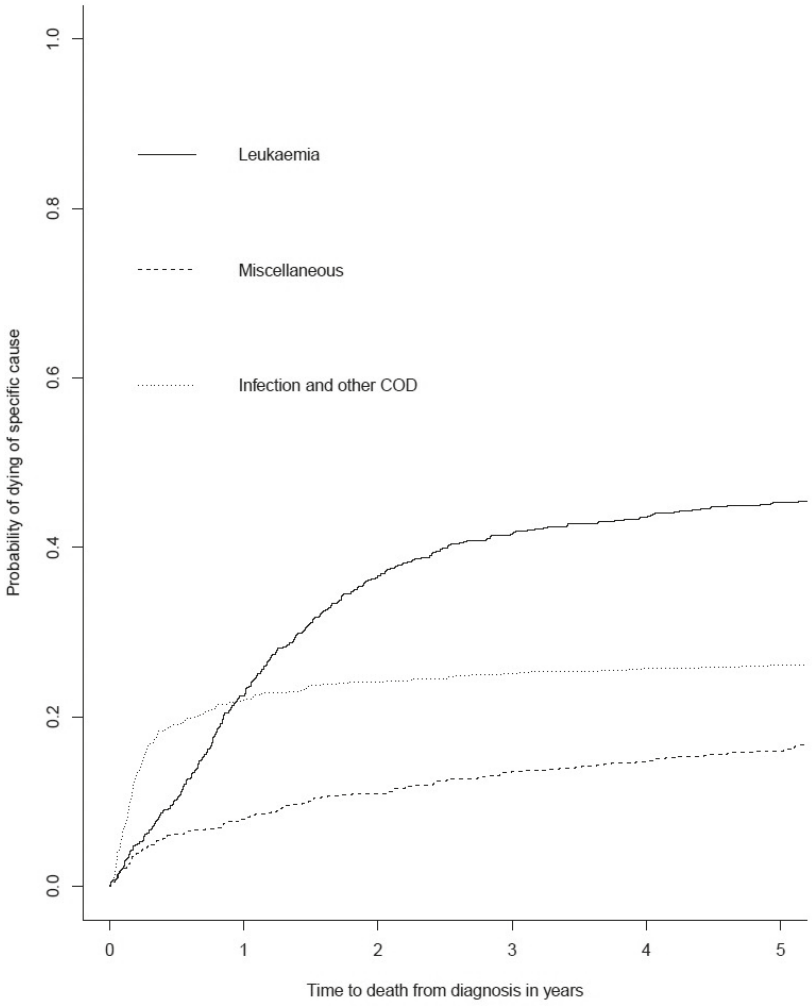


Figure 1. Cumulative incidence of different causes of death.
The cumulative incidence of an event is displayed graphically as the plot of the cumulative incidence function. It displays the probability that a specific event (death due to leukaemia, infection and other cause of death (COD) or miscellaneous) has occurred by a specific time. Other COD included the CODs: leukaemia in combination with: infection (n=8), infection and haemorrhage (n=2), infection and multi organ failure (n=1), haemorrhage (n=2), pneumonitis (n=2), cardiac disease (n=2), respiratory insufficiency and pneumonitis (n=1), respiratory and cardiac insufficiency (n=1), pulmonary embolism (n=1) or ileus (n=1); and infection, renal failure and haemorrhage (n=1), multiorgan failure (n=8), liver failure (n=1), renal failure (n=1), cardiac disease (n=14), respiratory insufficiency (n=4), cardiac disease and haemorrhage (n=1), acute respiratory distress syndrome (n=2), graft versus host disease (n=2), encephalopathy (n=2), cerebrovascular accident (n=3), pulmonary embolism (n=2), ileitis (n=1), hypoxic brain damage (n=1), progressive supranuclear paralysis (n=1), subdural bleeding (n=1), progressive degeneration of general state (n=1), euthanasia (n=2) and suicide (n=1). Miscellaneous included the CODs: pneumonitis, haemorrhage, veno-occlusive disease, secondary malignancy and unknown.

Figure 1 displays the cumulative incidence of death for the different CODs. As expected from the summary presented in Table 2, leukaemia was the most likely COD over time. However, the probability of dying from an infection or other COD in the first year after diagnosis of AML or high-risk MDS was higher than the probability of dying from another cause.

Table 2. Cause of death/alive grouped by patients with or without invasive aspergillosis up until their final chemotherapy cycle.

Cause of death / Alive	With IA N (%)	Without IA N (%)	Total N (%)
Total (known)	131 (100)	675 (100)	806 (100)
Leukaemia	59 (45)	321 (47.6)	380 (47.1)
Pneumonitis	10 (7.6)	22 (3.3)	32 (4)
Infection	31 (23.7)	115 (17)	146 (18.1)
Haemorrhage	5 (3.8)	30 (4.4)	35 (4.3)
Veno-occlusive disease	1 (0.8)	1 (0.1)	2 (0.2)
Secondary malignancy	1 (0.8)	9 (1.3)	10 (1.2)
Other	9 (6.9)	61 (9)	70 (8.7)
Unknown	5 (3.8)	30 (4.4)	35 (4.3)
Alive	10 (7.6)	86 (12.7)	96 (11.9)

The variables 'infection' and 'other cause of death (COD)' were grouped together ('infection and other COD'), because 'other COD' included 12/70 patients with infection as one of the CODs as well. The variables 'pneumonitis', 'haemorrhage', 'veno-occlusive disease', 'secondary malignancy' and 'unknown' were group together as 'miscellaneous', because of the relatively small number of patients in all categories.

Table 3. Chemotherapy treatment cycle in which invasive aspergillosis was first diagnosed.

Chemotherapy treatment cycle	Number of patients that underwent chemotherapy treatment cycle N	Cases of IA not recovered by the final chemotherapy cycle N (%)	All IA cases N (%)
Total	795	131 (16)	171 (22)
Cycle I	795	46 (6)	83 (10)
Cycle II	577	84 (15)	87 (15)
GO cycle I	114	0 (0)	0 (0)
GO cycle II	92	1 (1)	1 (1)
GO cycle III	65	0 (0)	0 (0)

Cycle I and II remission induction chemotherapy cycle I and II; GO cycle I, II and III gemtuzumab ozogacimin, post remission chemotherapy cycle I, II and III, IA invasive aspergillosis.
Of note: Post-remission, 49 patients received reduced intensity allogeneic-stem cell transplantation (SCT) consolidation therapy. There were no data available about infections, or IA in particular, that occurred during or after SCT.

The cause specific HRs ($_{CS}$HR) estimated from the Cox model are provided in Table 4. Diagnosis of IA in the final cycle of treatment was associated with an increased risk of dying due to 'leukaemia' ($_{CS}$HR 1.750 (95% CI 1.341-2.282)) and less markedly due to 'infection and other COD' ($_{CS}$HR 1.359 (0.958-1.909)). Age was included as a categorical variable in the model. Older age (>70 years) seemed to have a protective effect for death due to leukaemia ($_{CS}$HR 0.745 (95% CI 0.614-0.904)). Higher WBC count at diagnosis was significantly associated with an increased risk of dying from leukaemia. Note that WBC count was included as a linear term in the model, implying that the HR indicates the increase in risk for every unit of WBC count.

DISCUSSION

Cumulative incidence of death and effect of IA on death

We found that from 12 months onwards after diagnosis of AML or high-risk MDS leukaemia was the most likely cause of death. However, in the first 12 months after diagnosis of AML or high-risk MDS the probability of dying from an infection or other COD was higher than the probability of dying from another cause. By estimating the cumulative incidence with a competing risk model we were able to estimate the cumulative incidence for each COD and study the effect of prognostic factors on these CODs ('leukaemia', 'infection and other COD', and 'miscellaneous'). With regard to IA, patients with IA up until their final chemotherapy cycle had an increased risk of dying from 'leukaemia' or 'infection and other COD'. This is in concordance with a study that reported that IA was associated with a higher risk of death in patients with AML as compared to patients without IA.[10] Another study showed that no development of IA during first remission induction chemotherapy was associated with complete remission and complete remission at three years.[28]

The predisposing host risk factor for IA in patients with AML or MDS is prolonged and severe neutropenia.[2, 29] Neutropenia can be caused by intensive cytotoxic treatment or by the leukaemia itself. Patients with refractory leukaemia are at highest risk of developing IA, because they might be treated with multiple chemotherapy cycles and they might be neutropenic due to the underlying leukaemia.[29] These patients also have a higher risk of dying, because the presence of underlying disease, cytotoxic treatment and immunocompromised status can all cause severe complications that lead to death. Invasive aspergillosis may often not be the direct COD. If death occurs in a patient suffering from multiple diseases, each disease can be either the direct COD (attributable), can have contributed to the events that lead to death (contributable), or be unrelated to

Table 4. Estimated cause-specific hazard ratio along with their 95% confidence interval for the different causes of death 'leukaemia', 'infection and other cause of death' and 'miscellaneous'.

Cause of death	$_{CS}$HR	95% CI	p-value
Leukaemia			
IA up until final chemotherapy cycle	1.750	1.341 - 2.282	<0.001*
AML/MDS (MDS)	0.988	0.771 - 1.266	0.923
WBC count	1.002	1.001 - 1.004	0.054*
No prior haematological or oncological disease	0.938	0.751 - 1.169	0.567
No previous chemo- or radiotherapy	0.849	0.560 - 1.288	0.443
Age > 70	0.745	0.614 - 0.904	0.003*
Infection and other COD			
IA up until final chemotherapy cycle	1.359	0.958 - 1.909	0.085
AML/MDS (MDS)	0.827	0.623 - 1.210	0.403
WBC count	1.002	0.996 - 1.005	0.106
No prior haematological or oncological disease	1.318	0.978 - 1.775	0.068
No previous chemo- or radiotherapy	1.320	0.870 - 2.003	0.191
Age > 70	0.961	0.757 - 1.210	0.747
Miscellaneous			
IA up until final chemotherapy cycle	1.085	0.621 - 1.895	0.773
AML/MDS (MDS)	0.942	0.603 - 1.453	0.792
WBC count	0.999	0.997 - 1.005	0.733
No prior haematological or oncological disease	0.934	0.626 - 1.393	0.738
No previous chemo- or radiotherapy	0.538	0.231 - 1.258	0.153
Age > 70	0.855	0.607 - 1.206	0.373

*Statistically significant, AML acute myeloid leukaemia, COD cause of death, $_{CS}$HR cause specific hazard ratio, IA invasive aspergillosis, MDS myelodysplastic syndrome, WBC white blood cell count, 95% CI 95% confidence interval

death.[30] Despite, assessing the COD in patients with IA remains difficult and is further complicated because there is no universal approach to discriminate between association and causation with regard to IA and death.[30]

Incidence of invasive aspergillosis in older patients with AML or high-risk MDS

The incidence of IA in this cohort was 21% and IA occurred mostly during remission induction chemotherapy. This is similar to findings in other studies described in a recent meta-analysis, although incidences of IA ranged from 3.2%-43.1% during remission induction chemotherapy in patients without prophylaxis.[1] In this meta-analysis a crude incidence of 11.1% was reported during remission induction chemotherapy in patients without prophylaxis.[1]

Influence of age and other co-variables

The results from this study suggest that older age (>70 years) had a slight protective effect with regard to the risk of dying due to leukaemia itself ($_{CS}$HR: 0.745 (95% CI 0.641–0.904)). This was an unexpected finding since a higher incidence of poor prognostic factors is mostly found in older patients.[6, 20, 21] In an assessment of the outcomes of patients with secondary AML included in 13 EORTC trials conducted between 1986 and 2008, patients >60 years did not have better outcomes than patients >70 years. On the other hand, in this study WHO performance status was strongly associated with overall survival with a median of 0.8 years in patients with WHO performance status 0–1 and 0.4 years in patients with WHO performance status 2–4.[19] It is unclear how these results should be explained. In our study, WHO performance status was comparable in patients ≤70 years and >70 years. Thus, WHO performance status does not explain the slight protective effect of older age with regard to the risk of dying to leukaemia itself. The finding suggests that there might be a confounder, for example, differences in AML or MDS cytogenetics. Alternatively, the generalizability of the study might be limited by characteristics of the studied patient population. The finding that older age is protective may be biased by the phenomenon that less fit older patients may not have wanted to participate in the trial.

Antifungal prophylaxis

At the time the HOVON-43 trial was performed antifungal prophylaxis was not prescribed to patients. Nowadays, *Aspergillus*-effective antifungal prophylaxis is standard of care in patients with AML or high-risk MDS undergoing intensive chemotherapy. Current national and international guidelines advise antifungal prophylaxis during prolonged neutropenia following chemotherapy for patients who are at high risk for IA.[31-34] In general, antifungal prophylaxis is not recommended beyond remission induction chemotherapy.[32]

From the observed overall incidence of IA of 21% it becomes clear that antifungal prophylaxis would have been indicated in this group, in particular during remission induction chemotherapy. Only one patient developed IA during post remission chemotherapy. The reported incidence of IA during consolidation chemotherapy is low, around 2–3%.[35, 36] A retrospective multicentre study showed that antifungal prophylaxis during consolidation chemotherapy decreased the incidence of IA.[35] In this study, IA was diagnosed in 34/1137 (2.9%) who did not receive antifungal prophylaxis and in 22/1451 (1.5%) who did receive prophylaxis ($p < 0.01$).[35] The number needed to treat to prevent IA was 71. The authors concluded that antifungal prophylaxis during consolidation chemotherapy may be required in older patients receiving high dose cytarabine, as age ≥60 years and

treatment with high dose cytarabine were both independently associated with mortality. This only partly applied to our cohort. Although antifungal prophylaxis decreases the incidence of IA, IA cannot be fully prevented.[1] As an alternative to standard prophylaxis, screening strategies using a galactomannan serum assay can be deployed in high risk groups such as this study cohort.[37]

Study strengths and limitations

Strengths of the study included the size of the study population and the quality of the data which was prospectively collected during a randomized controlled trial. However, it should be considered that the cohort dates from 2006 and since then advances in diagnostics and treatment of IA as well as improvements in prophylactic strategies have been made. Still though, the competing risk analysis enabled us to study the effect of IA on mortality for each separate COD ('leukaemia', 'infection and other COD' and 'miscellaneous'). Furthermore, we focused on IA as COD in older patients with AML or high-risk MDS. Though there are some limitations. First, IA was described in the infection form, but in a small number of patients the diagnosis was uncertain. These cases were discussed and final decision was based on consensus. Secondly, no information was available about clinical characteristics, diagnostic tests that were performed, or the certainty of diagnosis. We were dependent on the diagnosis of IA that was made by the treating physician at the time and registered in the infection form. However, even though exact certainty of the diagnosis of IA could not be established in this cohort, as is more often the case in real-life clinical practice, the diagnosis (and treatment) of IA are usually not made and reported without consideration of host characteristics, imaging and clinical signs and symptoms. Thirdly, the COD was based on clinical diagnosis and not autopsy. In some cases, IA itself was the COD, but numbers are uncertain as COD infection was not always further specified. Fourthly, for modelling purposes different COD needed to be grouped together.

Conclusions

In this cohort of older patients with AML or high-risk MDS, leukaemia was the most likely cause of death over time. However, in the first year after diagnosis of AML or high-risk MDS infection was the most likely cause of death. The high incidence of IA that was found underscores the indication for antifungal prophylaxis in this older patient population, in particular during remission induction chemotherapy. Since diagnosis of IA is not made and reported lightly in this population, the absence of the currently used EORTC classification system at the time, may only have had limited influence on the findings of this study. Patients with IA up until their final chemotherapy cycle had an increased risk of dying from leukaemia or infection, although the exact role IA played in the COD

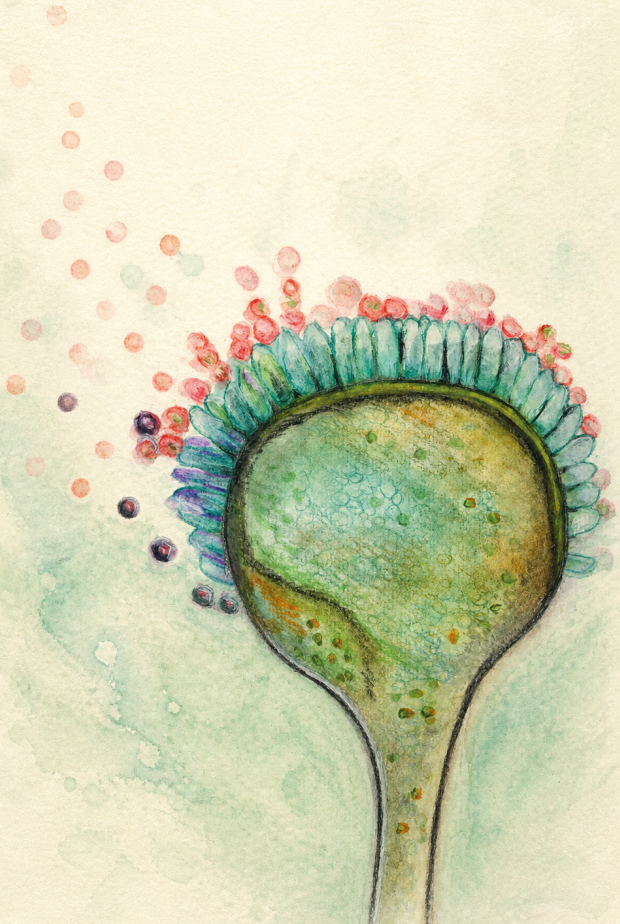
is unknown. Our results are suggestive of a contributable effect of IA, but further research is necessary that focusses on a universal classification to determine which role IA plays in the COD and the implementation of such a classification. Determining whether death is attributable, contributable or unrelated to IA will enhance our understanding of IA, and can lead to improved management of patients with leukaemia and IA.

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4

Incidence, risk factors and pre-emptive screening for COVID-19 associated pulmonary aspergillosis in an era of immunomodulant therapy

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ABSTRACT

Purpose: COVID-19 associated pulmonary aspergillosis (CAPA) is associated with increased morbidity and mortality in ICU patients. We investigated the incidence of, risk factors for and potential benefit of a pre-emptive screening strategy for CAPA in ICUs in the Netherlands/Belgium during immunosuppressive COVID-19 treatment.

Materials and methods: A retrospective, observational, multicentre study was performed from September 2020–April 2021 including patients admitted to the ICU who had undergone diagnostics for CAPA. Patients were classified based on 2020 ECMM/ISHAM consensus criteria.

Results: CAPA was diagnosed in 295/1977 (14.9%) patients. Corticosteroids were administered to 97.1% of patients and interleukin-6 inhibitors (anti-IL-6) to 23.5%. EORTC/MSGERC host factors or treatment with anti-IL-6 with or without corticosteroids were not risk factors for CAPA. Ninety-day mortality was 65.3% (145/222) in patients with CAPA compared to 53.7% (176/328) without CAPA ($p = 0.008$). Median time from ICU admission to CAPA diagnosis was 12 days. Pre-emptive screening for CAPA was not associated with earlier diagnosis or reduced mortality compared to a reactive diagnostic strategy.

Conclusions: CAPA is an indicator of a protracted course of a COVID-19 infection. No benefit of pre-emptive screening was observed, but prospective studies comparing pre-defined strategies would be required to confirm this observation.

BACKGROUND

Invasive aspergillosis (IA) has been reported in patients with COVID-19 admitted to the intensive care unit (ICU) requiring ventilatory support since the beginning of the COVID-19 pandemic.[1–3] During the first COVID-19 period (March 2020–August 2020) patients were not routinely treated with immunosuppressive medication. After the RECOVERY trial and a meta-analysis showed that patients with COVID-19 receiving either invasive mechanical ventilation or oxygen alone benefited from treatment with systemic corticosteroids this became the mainstay of treatment.[4, 5] Subsequently, interleukin-6 inhibitors (anti-IL-6), were introduced in clinical practice after two randomized controlled trials showed lower mortality in patients with respiratory failure treated with anti-IL-6 compared to the control group. Most patients in both studies were treated with corticosteroids, so the beneficial effect could be explained by a combination of anti-IL-6 on top of corticosteroids.[6, 7] From September 2020 patients with COVID-19 receiving oxygen or invasive mechanical ventilation were routinely treated with dexamethasone and from January 2021 anti-IL-6 was added to the treatment guideline in critically ill ventilated patients in the Netherlands. Anti-IL-6 was not routinely given in Belgium. Immunosuppressive treatment is classically a risk factor for IA.[8] Therefore, it was anticipated that the incidence of IA would increase. Previous studies have shown that patients with COVID-19 admitted to the ICU were at risk of developing COVID-19 associated pulmonary aspergillosis (CAPA) after approximately 7.28 days of mechanical ventilation[9] and CAPA mortality rates exceed 50%.[10, 11] Any form of diagnostic screening for *Aspergillus* might lead to earlier detection of CAPA and thus earlier initiation of antifungal treatment and possibly reduce mortality. The current study investigated the incidence and characteristics of CAPA in patients receiving immunosuppressive treatment for COVID-19. Furthermore, the potential benefits of a pre-emptive screening strategy for CAPA were studied.

MATERIALS & METHODS

Study design

A retrospective, observational, multicentre study was performed from September 2020–April 2021 in patients with COVID-19 admitted to the ICU who had undergone diagnostic procedures for CAPA. Clinical data were collected and patients were classified based on 2020 ECMM/ISHAM consensus criteria[12] with one minor modification: all positive *Aspergillus* BAL PCR results were considered relevant irrespective of cycle threshold-value. These criteria are further referred to as CAPA criteria. Patients with positive *Aspergillus* test results who did

not fulfil CAPA criteria were classified as colonised and included in the group of patients without CAPA. Patients with CAPA were compared to patients without CAPA.

Study population

All patients with PCR confirmed SARS-CoV-2 infection admitted to the ICU who had undergone diagnostic procedures including broncho-alveolar lavage (BAL) sampling, non-bronchoscopic lavage (NBL) sampling, serum galactomannan (GM) testing and/or blood *Aspergillus* PCR testing.

Participating ICUs

ICUs in the Netherlands were invited to take part in this retrospective study via a newsletter issued regularly during the COVID-19 pandemic by the Dutch Intensive Care Society (NVIC). Centres that expressed interest were invited to participate and received financial support for data collection. One large Belgian centre was invited to take part as well. Funding was provided by the Dutch National Institute for Public Health and the Environment (RIVM) and the ZonMw COVID-19 Programme.

Data collection

Clinical data were collected by the participating medical centres and captured in an electronic case report form (eCRF) created in Castor EDC, and included demographics, comorbidities, EORTC/MSGERC host factors, severity of illness scores at ICU admission (APACHE IV and SOFA score), COVID-19 treatment, CAPA diagnosis, CAPA treatment, confirmed CAPA tracheobronchitis, length of stay and mortality. The cumulative dose of corticosteroids (prednisone equivalent) was calculated without collecting data about corticosteroid type, duration or dose. Participating centres were requested to report all GM results and only positive results of the other tests. The eCRF included questions about the timing of the clinical CAPA suspicion and clinical CAPA diagnosis that was documented in the electronic health record.

Ethics

All ethical considerations were in accordance with the ethical principles as posed in the declaration of Helsinki, the principles of good clinical practice, as well as current national legislation. The study was initiated by Radboudumc, Nijmegen, The Netherlands and ethical approval was granted by the medical ethical committee Arnhem-Nijmegen (CMO 2020–6339). All other participating centres received approval from their medical ethical committee before participating in the study. An opt-out system was employed, ensuring that medical data from patients who objected against use of their clinical data were not

included in the study. Data were pseudonymised and data entry was performed by local physicians or research nurses.

Statistical analyses

CAPA incidence was the primary study outcome. Secondary outcomes included 30- and 90-day mortality, time to event (first positive mycological test, clinical CAPA suspicion and clinical CAPA diagnosis during ICU stay) and incidence of triazole resistance. Further analyses were performed to determine risk factors for CAPA, and to establish the effect of pre-emptive diagnostic screening for CAPA. Categorical variables were reported as numbers and percentages. Continuous variables were reported as median and interquartile range (IQR). Groups were compared with the Mann Whitney U test for continuous data and Chi-square test or Fisher's exact test for categorical data depending on sample size. Proportion confidence intervals were calculated with the Wilson score interval. Survival curves were constructed with Kaplan-Meier and compared with the log-rank test. A generalized estimating equation (GEE) logistic regression model was applied to the data to identify baseline variables at time of admission to the ICU associated with CAPA and 30-day mortality. Treatment for COVID-19 or pre-emptive screening for CAPA were considered baseline variables. A GEE model was chosen because each centre had their own protocol for patients with CAPA (centres were included as subject variables and patients as within-subject variables). Statistically significant variables from GEE univariable logistic regression analysis that were considered clinically relevant were included in the GEE multivariable logistic regression analysis. Odds ratios (OR) with their 95%-CI were reported. Variables were included in GEE logistic regression model if they were reported in >5% of patients and all EORTC/MSGERC criteria were grouped together. Two sided tests were used and a p-value of <0.05 was considered statistically significant. Statistical analyses were performed in SPSS version 25.0 and Microsoft Excel Version 2180.

RESULTS

CAPA incidence

In the ten participating centres (5 university medical centres (UMCs) and 5 non-UMCs) 1977 patients with COVID-19 were admitted to the ICU in the research period (total cohort). Of these, 793 patients had undergone diagnostic procedures for CAPA and were included in our study (diagnostic cohort). CAPA incidence in the total cohort was 14.9% (295/1977) (95%-CI: 13.4–16.6%) (Fig. 1), with variable incidences in the centres (range: 5.4–27.3%). In the diagnostic cohort the incidence of CAPA was 37.2% (295/793) (95%-CI: 33.9–40.6%) (7 proven

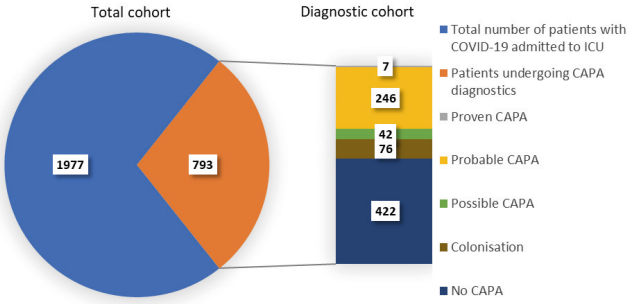


Figure 1. Total number of patients with COVID-19 admitted to the ICU in the study period (total cohort) and patients undergoing CAPA diagnostics (diagnostic cohort). CAPA classification according to 2020 ECMM/ISHAM consensus criteria (proven, probable or possible CAPA).[12] Patients with positive *Aspergillus* test results who did not fulfil CAPA criteria were classified as colonised. Patients who did not have any positive *Aspergillus* test results were classified as no CAPA. CAPA: COVID-19 associated pulmonary aspergillosis, ICU: intensive care unit

including 5 with histologically-confirmed tracheobronchitis, 246 probable, 42 possible). In three patients (1 probable and 2 possible) post-mortem autopsy reported evidence of IA. A clinical diagnosis of tracheobronchitis was made in 15/673 (2.2%) patients that could be classified as proven (6), probable (7) or no CAPA (2).

Baseline characteristics and risk factors for CAPA of the study population (table 1 and supplemental table 1)

The diagnostic cohort consisted of mostly male patients with a median age of 66 years. Patients with CAPA were slightly older. No significant differences were observed in comorbidities. Presence of EORTC/MSGERC risk factors was similar (CAPA 12.2% vs no CAPA 12.9%) in both groups. The majority of patients were treated with corticosteroids (97.1%). Treatment with anti-IL-6 with or without corticosteroids was comparable in patients with and without CAPA (CAPA: 24.8%, no CAPA: 22.7%). There was no difference in the cumulative dose of corticosteroids per kg bodyweight in patients with and without CAPA from ICU admission to first diagnosis of CAPA, preadmission to hospital or preadmission to the ICU (in hospital), but the median cumulative dose of corticosteroids per kg bodyweight during total ICU stay was higher for patients with CAPA 8.33 vs 5.73 mg prednisone/kg bodyweight ($p = 0.015$).

Patients with CAPA had a slightly higher APACHE IV score on admission to the ICU and were treated with renal replacement therapy (RRT) and vasopressors and/or inotropes more frequently (Table 1).

Table 1. Baseline characteristics of patients included in the diagnostic cohort with and without CAPA.

		No CAPA (n=498)		CAPA (n=295)		Total (n=793)		Significance level
Age	Median (IQR)	65	(56-72)	67	(61-72)	66	(58-72)	<0.001
BMI (787)	Median (IQR)	29	(25.4-33)	27.8	(25.5-31.5)	28.4	(25.4-32.4)	0.115
Sex (male)	n, %	356	71.5	239	81.0	595	75.0	0.003
Current or former smoker (493)	n, %	151	49.5	119	63.3	270	54.8	0.003
Comorbidities								
Any comorbidities	n, %	406	81.5	243	82.4	649	81.8	0.765
Acute leukaemia	n, %	3	0.6	2	0.7	5	0.6	1
Stem cell transplantation	n, %	4	0.8	5	1.7	9	1.1	0.305
Other haematological malignancy	n, %	18	3.6	13	4.4	31	3.9	0.578
Solid organ transplant	n, %	31	6.2	13	4.4	44	5.5	0.28
Cardiovascular disease	n, %	275	55.2	165	55.9	440	55.5	0.846
Diabetes mellitus	n, %	136	27.3	99	33.6	235	29.6	0.062
Asthma	n, %	59	11.8	28	9.5	87	11.0	0.305
COPD	n, %	48	9.6	32	10.8	80	10.1	0.585
Cystic fibrosis	n, %	2	0.4	0	0.0	2	0.3	0.532
Pulmonary tuberculosis	n, %	2	0.4	1	0.3	3	0.4	1
Multiple sclerosis	n, %	1	0.2	1	0.3	2	0.3	1
Liver cirrhosis	n, %	1	0.2	1	0.3	2	0.3	1
Inflammatory bowel disease	n, %	7	1.4	2	0.7	9	1.1	0.497
Rheumatological disease	n, %	30	6.0	19	6.4	49	6.2	0.814
Psoriasis	n, %	4	0.8	0	0.0	4	0.5	0.303
HIV/AIDS	n, %	0	0.0	1	0.3	1	0.1	0.372
Solid organ malignancy	n, %	39	7.8	27	9.2	66	8.3	0.515
Other malignancy	n, %	0	0.0	1	0.3	1	0.1	0.372
Chronic kidney disease	n, %	32	6.4	17	5.8	49	6.2	0.708
Thyroid disease	n, %	36	7.2	18	6.1	54	6.8	0.542

Table 1. Baseline characteristics of patients included in the diagnostic cohort with and without CAPA. (continued)

		No CAPA (n=498)		CAPA (n=295)		Total (n=793)		Significance level
Other	n, %	132	26.5	84	28.5	216	27.2	0.547
EORTC/MSGERC criteria[8]*								
Recent history of neutropenia (748)	n, %	1	0.2	1	0.4	2	0.3	1
Active haematological malignancy (790)	n, %	9	1.8	7	2.4	16	2.0	0.577
Allogeneic stem cell transplantation	n, %	4	0.8	3	1.0	7	0.9	0.715
Prolonged use of corticosteroids (756)	n, %	28	5.9	17	6.0	45	6.0	0.946
T-cell immunosuppressants (789)	n, %	27	5.5	16	5.4	43	5.4	0.994
B-cell immunosuppressants (790)	n, %	11	2.2	6	2.0	17	2.2	0.86
Inherited severe immunodeficiency	n, %	0	0.0	0	0.0	0	0.0	1
Any EORTC criterium (791)	n, %	64	12.9	36	12.2	100	12.6	0.775
APACHE IV and SOFA scores on ICU admission								
APACHE IV (624)	Median (IQR)	53	(28-69)	59	(38-73)	57	(31-71)	0.005
SOFA (635)	Median (IQR)	6	(4-8)	7	(4-9)	6	(4-8)	0.016
Supportive care on ICU								
SDD (738)	n, %	335	73.1	216	77.1	551	74.7	0.226
Renal replacement therapy (786)	n, %	85	17.2	80	27.5	165	21.0	0.001
Vasopressors and/or inotropes (789)	n, %	441	88.9	277	94.5	718	91.0	0.008
Most invasive ventilatory support during ICU stay								
ECMO	n, %	39	7.8	16	5.4	55	6.9	0.197
Invasive	n, %	437	87.8	274	92.9	711	89.7	0.022
Non-invasive	n, %	20	4.0	4	1.4	24	3.0	0.035
None	n, %	2	0.4	1	0.3	3	0.4	1
Treatment for COVID-19								
Remdesivir (792)	n, %	71	14.3	31	10.5	102	12.9	0.132
Corticosteroids (792)	n, %	479	96.2	290	98.6	769	97.1	0.047

Table 1. Baseline characteristics of patients included in the diagnostic cohort with and without CAPA. (continued)

		No CAPA (n=498)		CAPA (n=295)		Total (n=793)		Significance level
Tocilizumab (792)	n, %	113	22.7	73	24.8	186	23.5	0.493
Corticosteroids and tocilizumab (792)	n, %	111	22.3	73	24.8	184	23.2	0.413
Time to event from ICU admission in days								
Performance of BAL or NBL[†] (647)	Median (IQR)	6	(3-11)	7	(4-12)	7	(3-11)	0.033
First positive BAL or NBL GM[‡] (193)	Median (IQR)			10	(5-15)	10	(5-15)	NA
First positive mycological test[§] (318)	Median (IQR)	9	(3-15)	8	(4-13)	8	(4-13)	0.95
Clinical suspicion CAPA[§] (444)	Median (IQR)	7	(4-11)	8	(5-12)	8	(4-12)	0.162
Clinical diagnosis CAPA[§] (216)	Median (IQR)	12	(9-16)	12	(7-16)	12	(7-16)	0.729
Length of ICU stay in days[¶] (729)	Median (IQR)	21	(13-36)	28	(18-42)	23	(14-38)	<0.001
Mortality at 30 days after ICU admission (658)	n, %	134	33.8	94	36.0	228	34.7	0.551
Mortality at 90 days after ICU admission (550)	n, %	176	53.7	145	65.3	321	58.4	0.008
Mortality during ICU stay (789)	n, %	170	34.2	138	47.3	308	39.0	<0.001

*Solid organ transplant can be found under comorbidities, [†]Date of first GM, date of first BAL or NBL not available, [‡]If NBL GM was positive based on a GM index > 1.2 twice or more, the date of the first positive NBL GM result was selected, [§]According to medical centres, [¶]In patients in whom the total ICU stay was known

BAL: bronchoscopic alveolar lavage, BMI: body mass index, GM: galactomannan, ICU: intensive care unit, IQR: interquartile range, NBL: non-bronchoscopic lavage, SDD: selective digestive decontamination

Table 2. Uni- and multivariable generalized estimating equations (GEE) logistic regression model for CAPA.

Univariable analysis	**OR (95%-CI)**
Age	1.033 (1.024-1.042)
BMI	0.981 (0.954-1.008)
Sex (male)	1.702 (1.190-2.435)
Any comorbidities	1.059 (0.636-1.765)
Cardiovascular disease	1.029 (0.873-1.214)
Diabetes mellitus	1.344 (0.954-1.895)
Asthma	0.780 (0.545-1.117)
COPD	1.141 (0.795-1.637)
Rheumatological disease	1.074 (0.640-1.802)
Solid organ malignancy	1.185 (0.756-1.859)
Chronic kidney disease	0.890 (0.586-1.354)
Thyroid disease	0.834 (0.514-1.353)
Any EORTC/MSGERC criterium[8]	0.938 (0.574-1.534)
APACHE IV at ICU admission	1.008 (1.003-1.013)
SOFA at ICU admission	1.073 (1.002-1.149)
Remdesivir*	0.709 (0.511-0.984)
Corticosteroids*	2.875 (0.795-10.402)
Tocilizumab*	1.125 (0.797-1.589)
Corticosteroids and tocilizumab*	1.151 (0.804-1.650)
Pre-emptive screening†	0.742 (0.507-1.085)
Multivariable analysis	**OR (95%-CI)**
Age	1.025 (1.017-1.034)
Sex (male)	1.730 (1.257-2.380)
APACHE IV at ICU admission	1.005 (1.001-1.010)
Remdesivir*	0.668 (0.456-0.976)

*Treatment for COVID-19, †Medical centres that pre-emptively screened for CAPA: *Aspergillus* diagnostics performed in all patients with COVID-19 admitted to the ICU irrespective if there was a suspicion of CAPA. OR: odds ratio, 95%-CI: 95% confidence interval

Older age, male sex, higher APACHE IV and SOFA score on ICU admission and treatment with remdesivir (decreased risk) were associated with CAPA diagnosis in univariable analysis, and all remained associated with CAPA in multivariable analysis (SOFA score not included in analysis) (Table 2).

Time to event

Median time from ICU admission until the first positive mycological test, clinical CAPA suspicion and clinical CAPA diagnosis by the treating physician was eight, eight and twelve days respectively (Table 1). Fig. 2 depicts on which day the first NBL/BAL GM test became positive from ICU admission.

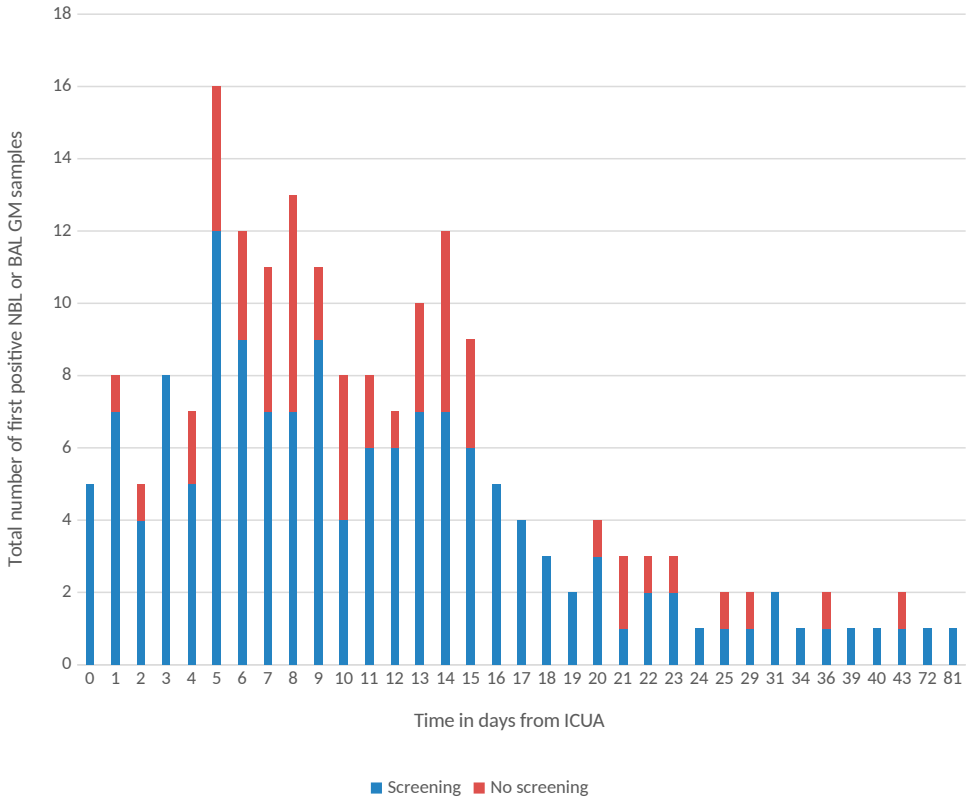
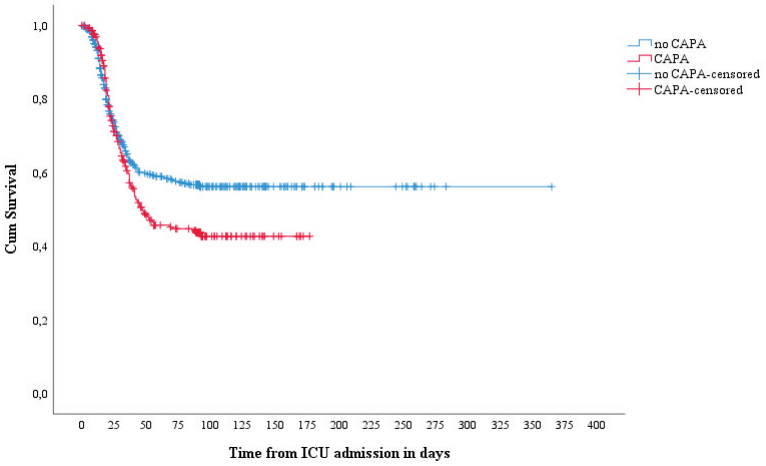


Figure 2. First positive NBL or BAL GM sample after ICU admission in days.
Screening: medical centres that screened for CAPA systematically (pre-emptive screening strategy). No screening: medical centres that performed diagnostics for *Aspergillus* spp. on clinical suspicion of CAPA (reactive diagnostic strategy). BAL: bronchoscopic alveolar lavage, CAPA: COVID-19 associated pulmonary aspergillosis, GM: galactomannan, ICU: intensive care unit, ICUA: ICU admission, NBL: non-bronchoscopic lavage

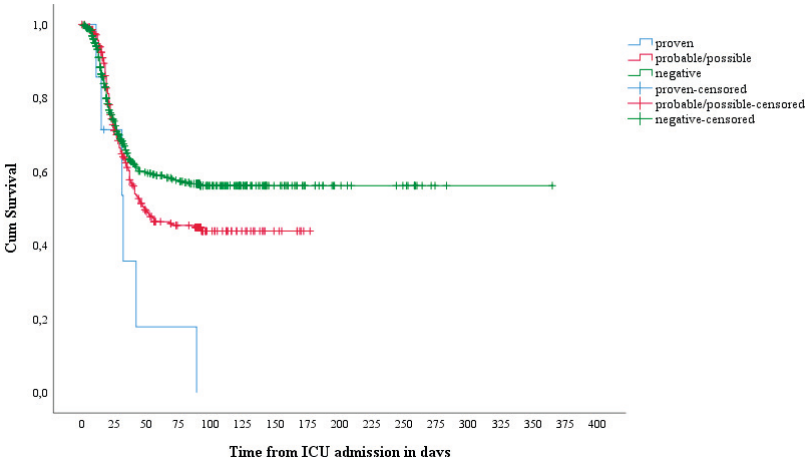
Mortality

ICU mortality in the total cohort was 500/1977 (25.3%). In the diagnostic cohort mortality during ICU stay was 39%, and was significantly higher for patients with CAPA 47.3% vs 34.2% (p <0.001). There was no statistically significant difference in 30-day mortality between patients with and without CAPA, whereas 90-day mortality was increased in patients with CAPA (CAPA: 145/222 (65.3%) vs non-CAPA: 176/328 (53.7%), p = 0.008) (Table 1). Mortality in patients with probable and possible CAPA was similar and was lower than patients with proven CAPA (Fig. 3). Ninety-day mortality in patients with tracheobronchitis was 11/14.

Of the 295 patients with CAPA, 192 (65%, 7 proven, 153 probable and 32 possible) were treated with an antifungal agent for ≥3 days. There was no difference in 30- or 90-day mortality between patients treated or not treated with an anti-



	Timepoint								
Numbers at risk	0	25	50	75	100	125	150	175	200
CAPA	295	185	109	89	32	18	8	1	0
No CAPA	498	292	201	177	88	60	38	24	16



	Timepoint				
Numbers at risk	0	25	50	75	100
CAPA proven	7	4	1	1	0
CAPA probable/possible	288	181	108	88	32
No CAPA	498	292	201	177	88

Figure 3a. Kaplan-Meier survival curve of patients with and without CAPA. Log-rank test: p=0.020. Figure 3b. Kaplan-Meier survival curve of patients classified as proven, probable/possible or no CAPA. Log-rank test: 0.006
CAPA: COVID-19 associated pulmonary aspergillosis

fungal agent (30-day mortality: not treated 34/85 (40%), treated 60/176 (34.1%), p = 0.351; 90-day mortality: not treated 46/68 (67.6%), treated 99/154 (64.3%), p = 0.628). Also, when excluding patients with possible CAPA (data not shown).

Older age, any comorbidity, diabetes mellitus, chronic kidney disease, thyroid disease, any EORTC/MSGERC criterium and treatment with remdesivir (all increased risk), and corticosteroids or tocilizumab (both decreased risk) were associated with 30-day mortality in univariable analysis (Table 3). In multivari-

Table 3. Uni- and multivariable generalized estimating equations (GEE) logistic regression model for 30-day mortality.

Univariable analysis	**OR (95%-CI)**
Age	1.071 (1.063-1.080)
BMI	0.976 (0.946-1.008)
Sex (male)	0.780 (0.554-1.097)
Any comorbidities	1.797 (1.133-2.846)
Cardiovascular disease	1.310 (0.583-1.811)
Diabetes mellitus	1.749 (1.218-2.512)
Asthma	1.001 (0.441-2.270)
COPD	1.564 (0.806-3.031)
Rheumatological disease	1.305 (0.710-2.401)
Solid organ malignancy	0.995 (0.650-1.523)
Chronic kidney disease	2.568 (1.132-5.824)
Thyroid disease	1.795 (1.021-3.155)
Any EORTC/MSGERC criterium[8]	2.273 (1.454-3.550)
APACHE IV at ICU admission	1.011 (0.999-1.022)
SOFA at ICU admission	1.034 (0.953-1.122)
Remdesivir*	1.531 (1.034-2.268)
Corticosteroids*	0.519 (0.411-0.656)
Tocilizumab*	0.614 (0.405-0.931)
Corticosteroids and tocilizumab*	0.628 (0.419-0.944)
Pre-emptive screening[†]	1.578 (0.999- 2.489)
Multivariable analysis	**OR (95%-CI)**
Age	1.073 (1.060-1.085)
Diabetes mellitus	1.415 (0.953-2.100)
Chronic kidney disease	1.7288 (0.756-3.955)
Any EORTC/MSGERC criterium[8]	2.509 (1.468-4.284)
Remdesivir*	1.365 (0.876-2.125)
Corticosteroids*	0.492 (0.319-0.757)
Tocilizumab*	0.802 (0.495-1.298)

*Treatment for COVID-19, [†]Medical centres that pre-emptively screened for CAPA: *Aspergillus* diagnostics performed in all patients with COVID-19 admitted to the ICU irrespective if there was a suspicion of CAPA. OR: odds ratio, 95%-CI: 95% confidence interval

able analysis older age, any EORTC/MSGERC criterium and treatment with corticosteroids remained associated with 30-day mortality (Table 3).

Pre-emptive screening strategy for CAPA

All ICUs had their own protocol for suspected CAPA. Of the ten participating ICUs five screened for CAPA systematically (3 BAL or NBL on admission to ICU, of which 2 UMCs; 2 centres screened weekly on a tracheal or bronchial aspirate, both in UMCs) whilst the other ICUs performed diagnostics for *Aspergillus* spp. when CAPA was suspected (Fig. 4). Centres were considered screening

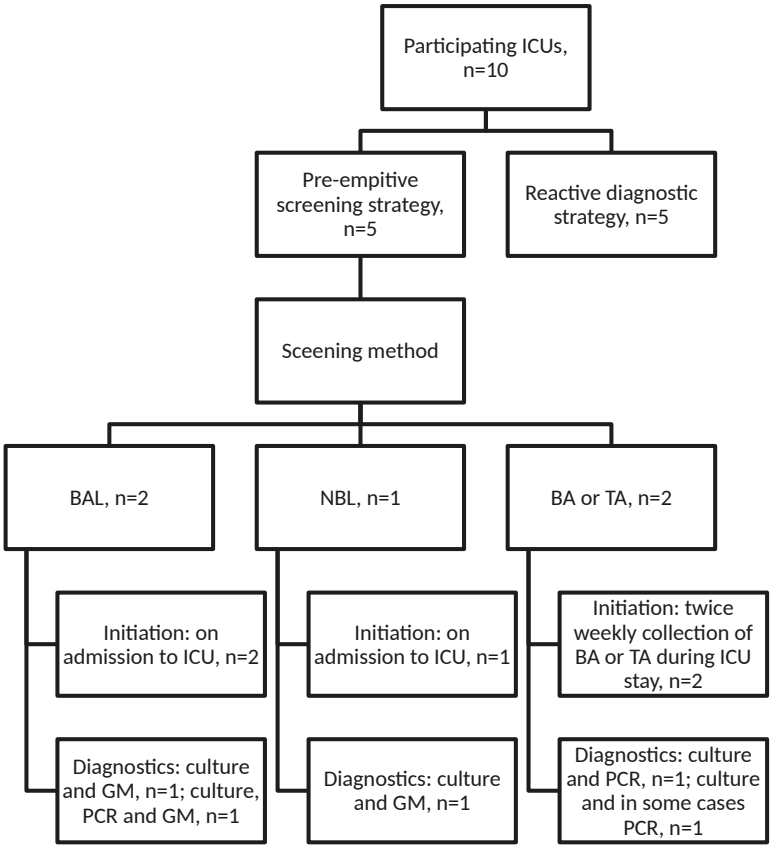


Figure 4. Flow chart depicting the different CAPA protocols of centres that screened for CAPA pre-emptively.

Pre-emptive screening strategy: centres in which *Aspergillus* diagnostics were routinely performed in all patients with COVID-19 admitted to the ICU. Reactive diagnostic strategy: centres in which *Aspergillus* diagnostics were only performed when there was suspicion of CAPA.

BA: bronchial aspirate, BAL: bronchoalveolar lavage, CAPA: COVID-19 associated pulmonary aspergillosis, GM: galactomannan, ICU: intensive care unit, NBL: non-bronchoscopic lavage, TA: tracheal aspirate

centres if *Aspergillus* diagnostics were routinely performed in all patients with COVID-19 admitted to the ICU irrespective if there was a suspicion of CAPA (pre-emptive screening strategy). Centres were considered non-screening centres if *Aspergillus* diagnostics were only performed when there was suspicion of CAPA (reactive diagnostic strategy). The diagnostic tests that were routinely performed differed between the centres, although most often culture and BAL/NBL GM were performed. Two centres prophylactically treated patients with an antifungal agent: one systemically in patients receiving high-dose methylprednisolone and the other by inhalation therapy from initiation of invasive mechanical ventilation[13] (neither of them during the entire study period).

Patients in whom screening was performed were slightly younger and more often had a classical host risk factor for IA (supplemental table 2). The average time frame between ICU admission and collection of the first NBL or BAL GM was 6 days (IQR: 2–10) for centres with a pre-emptive screening strategy and 10 days (IQR: 6–14) for centres with a reactive diagnostic strategy, $p < 0.001$. The first positive BAL or NBL GM result was found after 9 days (IQR: 5–15) in centres that screened and 10 days (IQR: 7–14) in centres that did not screen, $p = 0.362$. Treatment initiation from ICU admission was 10 days in centres that screened vs 12 days in those that did not. There was no difference in time from clinical CAPA suspicion/diagnosis until treatment initiation (supplemental table 2).

CAPA incidences were similar in the centres with a pre-emptive screening strategy or a reactive diagnostic strategy (210/593 (35.4%) vs 85/200 (42.5%), $p = 0.073$). Both 30- and 90-day mortality were higher in hospitals that screened for CAPA (supplemental table 2). The Kaplan-Meier survival curve can be found in supplemental Fig. 1. A pre-emptive screening strategy was not associated with CAPA incidence or 30-day mortality in univariable analysis (Table 2, Table 3).

CAPA classification and mortality

Different positive test combinations of patients with probable or possible CAPA are depicted in supplemental table 3 and 4. An evaluation was performed of positive test results for CAPA classification and mortality. Test results of patients with probable and possible CAPA were ranked on the basis of the positive test results theoretically most indicative of *Aspergillus* invasiveness or load: 9 serum GM, 1 PCR blood, 90 BAL microscopy, 16 NBL microscopy, 51 BAL culture, 12 NBL culture, 40 BAL PCR, 55 BAL GM and 14 NBL GM. Mortality was highest in patients with at least a positive serum GM, followed by at least positive BAL/NBL microscopy (Fig. 5 and supplemental tables 5 and 6). Patients with BAL/NBL GM as maximum positive test had lower or similar mortality compared to patients with negative test results.

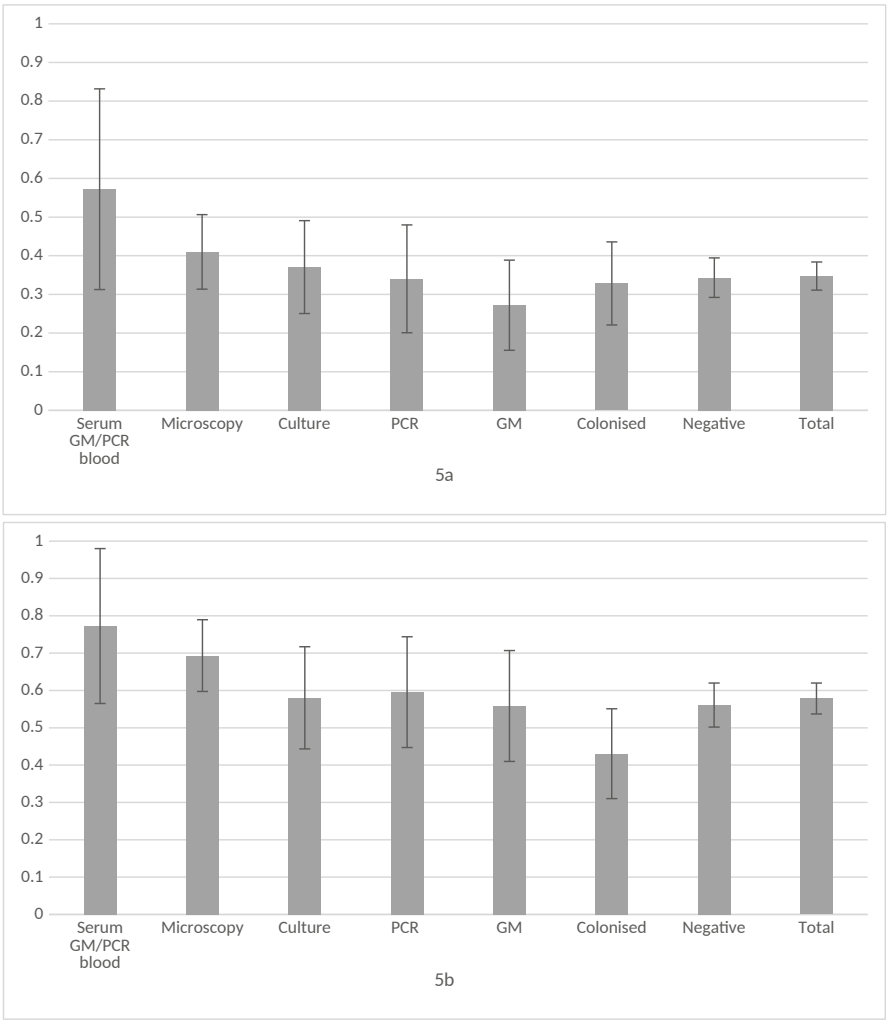


Figure 5. 30- and 90-day mortality in patients with positive test results ranked on the basis of positive test results theoretically indicative of *Aspergillus* invasiveness or load.

The estimated probability of death at day 30 (5a) or 90 (5b) with corresponding 95% confidence interval (95%-CI), calculated with the Wilson score interval.

Patients with probable or possible CAPA according to 2020 ECMM/ISHAM consensus criteria,[12] colonised (patients with positive *Aspergillus* test results who did not fulfil CAPA criteria) or no CAPA.

Ranking: serum GM/PCR blood > microscopy: BAL/NBL microscopy > culture: BAL/NBL culture > PCR: BAL PCR > GM: BAL/NBL GM*†‡ > colonised > negative test results

BAL: bronchoscopic alveolar lavage, GM: galactomannan, NBL: non-bronchoscopic lavage

* NBL GM positivity according to 2020 ECMM/ISHAM consensus criteria[12].† 30- and 90-day mortality and estimated probability of death with 95%-CI of patients with a positive BAL GM: day 30: 29.7% (31.6%; 95%-CI: 17.5-45.9); day 90: 56.7% (55.9%; 95%-CI: 39.2-72.6). ‡ 30- and 90-day mortality and estimated probability of death with 95%-CI of patients with a positive NBL GM: day 30: 14.3% (22%; 95%-CI: 4-39.9); day 90: 55.6% (53.9%; 95%-CI: 26.7-81.1)

Triazole resistance

Aspergillus species were cultured from samples of 188 patients of which 175 contained *A. fumigatus* (supplemental table 7) and phenotypical tests to detect triazole resistance were performed in 80/188 (42.6%) patients. Triazole resistance was detected in 11/80 (13.8%) patients. In total, 125 patients had one or more samples in which *Aspergillus* DNA was detected by PCR. Resistance PCR yielded results in 35/125 (28%) patients, of which 4/35 (11.4%) had resistance associated mutations. Combined culture and PCR results indicated triazole resistance in 12/93 (12.9%) patients. 30- and 90-day mortality were similar in patients with a triazole-resistant or triazole-susceptible isolate.

DISCUSSION

In this large observational multicentre study incidence of CAPA, characteristics of and risk factors for CAPA and a pre-emptive screening strategy for CAPA were investigated.

In the total cohort a CAPA incidence of 14.9% was found. This finding is comparable to a previous multicentre study (Dutch CAPA1.0 study) that observed an incidence of 15%[14] with the ECMM/ISHAM consensus definition[12] although different inclusion criteria were used. The Dutch CAPA1.0 study was performed from February–May 2020, when treatment with corticosteroids and anti-IL-6 was not standard of care for patients with COVID-19 admitted to the ICU with respiratory insufficiency. We expected to find a higher incidence of CAPA because these immunomodulating drugs are now being used routinely, but CAPA incidences remained similar. This might be explained by the relatively short duration or dose of corticosteroids so that the immunomodulating effects for becoming a host for IA are minor, or possibly by the improved care for COVID-19 and prevention of SARS-CoV-2 infection by vaccination. Variable CAPA incidences have been reported, which can be explained by the use of different classifications at the start of the COVID-19 pandemic before the ECMM/ISHAM consensus definition had been published.[10] A recently published meta-analysis reported a pooled reported CAPA incidence of 10% (95%-CI: 7–14%) and showed that the incidence was lower when patients were reclassified and also differed depending on the classification used.[11]

Treatment with anti-IL-6 alone or in combination with corticosteroids was not a risk factor for CAPA. Neither was there a difference in cumulative dose of corticosteroids before CAPA was suspected in patients with and without CAPA. Patients with CAPA were treated with a higher cumulative dose of corticoste-

roids during their total ICU stay, but this may be explained by the longer ICU stay and is therefore not a certain CAPA risk factor. An association of anti-IL-6 with occurrence of CAPA has been described before,[15, 16] but the number of patients treated with anti-IL-6 were small in both previous studies (39/581 (6.7%)[15] and 38/506 (7.5%)[16]) as compared to our series. Looking into other possible risk factors for CAPA, we found that EORTC/MSGERC host criteria were not associated with CAPA. This is in concordance with other reports.[11] Risk factors we observed were older age, male sex and higher APACHE IV or SOFA score on ICU admission. Treatment with remdesivir decreased the risk for CAPA, but conclusions cannot be drawn from our data because its administration may not have been random and confounding by indication may have occurred.

In the diagnostic cohort, 90-day mortality was higher in patients with CAPA compared to patients without CAPA (65.3% vs 53.7%, p = 0.008). Other reports have found similar mortality rates in patients with CAPA.[10, 11, 15] Interestingly, we did not find a difference in mortality at 30 days which is in line with another study that did not find any difference in early mortality at 28 days, whereas a difference was seen at 84 days.[15] ICU mortality in the total cohort (including the diagnostic cohort) was considerably lower with 25%. The higher mortality in the diagnostic cohort might be attributable to the selection of patients in whom invasive diagnostics like BAL or NBL were performed, likely because of clinical deterioration or lack of clinical improvement during ICU stay. The increase in ICU mortality in confirmed CAPA patients was relatively modest (13.1%) in comparison to the increase in mortality of those found to have had an indication for BAL or NBL diagnostics compared to the total cohort (ICU mortality in the diagnostic cohort: 39%). Possibly, CAPA occurs mainly in patients who already have a worse prognosis because of a protracted course of the COVID-19 infection and mortality is mainly determined by the course of their COVID-19 rather than by CAPA. This hypothesis is supported by the fact that antifungal treatment did not improve outcome, regardless of the strength of CAPA diagnosis. Also, it is striking that other opportunistic pathogens like CMV and HSV seem to re-activate in COVID-19 patients with a protracted course in ICU.[17, 18] Whether higher mortality as observed in patients with CAPA can be attributed to IA is thus unknown. In the diagnostic cohort, autopsies were only performed in a few patients, but did confirm CAPA in some. Autopsy reports seldom reveal proven CAPA.[19, 20] Van de Veerdonk et al. postulate that CAPA should be seen as a disease with a continuous spectrum, from colonisation, to tissue invasion and eventually angioinvasion and dissemination, in which multiple factors contribute to *Aspergillus* becoming invasive.[21] Hard criteria, such as a cut-off value for GM or PCR as suggested in the 2020 ECMM/ISHAM definition[12] would do this no justice. The hypothesis that CAPA is a disease with a continuous spectrum

was underlined in a study evaluating *Aspergillus* test results and mortality.[22] In this study mortality was incremental in the presence and classification of CAPA. Positive serum GM and serum (1,3)-β-D-glucan were associated with increased mortality in comparison to CAPA patients who were blood biomarker negative. Of the nine patients in our study who were serum GM positive, suggestive of angio-invasive disease, one was lost to follow-up at day 90 but of the remaining eight patients seven had died. Also, mortality appeared higher in patients with diagnostic tests indicative of a higher fungal load, despite being classified as possible CAPA. The 2020 ECMM/ISHAM definition[12] may therefore not be optimal to classify patients according to the strength of diagnostic evidence, as was also shown by the similar mortality in patients with possible and probable CAPA. Furthermore, not all patients with CAPA according to the definition[12] were treated with antifungal agents and one of the reasons for this was that there was no clinical CAPA suspicion. As was recently suggested, in order to correctly identify patients with CAPA, local epidemiology and clinical details need to be taken into account[23] as well as strong diagnostic evidence demonstrated by a combination of different positive markers for *Aspergillus* spp..[24]

The optimal diagnostic work-flow for CAPA is still unknown, which was reflected by the variable protocols retrieved from all participating ICUs. Some ICUs performed diagnostics when CAPA was suspected, whilst others pre-emptively screened for CAPA from the start of ICU admission. The diagnostic tests that were routinely performed differed per centre as did the method of screening. In our opinion the benefit of pre-emptive screening and its optimal form remains uncertain as we found no clear benefit of screening. However, due to the retrospective observational nature of this study results should be interpreted with caution. Prospective studies comparing pre-defined strategies are required to establish an optimal form of screening. The incidence of CAPA was similar in centres that screened and did not screen for CAPA. In addition, there was no difference in time from ICU admission until first positive GM between those hospitals. Higher mortality was observed in the hospitals that screened pre-emptively, possibly because screening was mostly performed in the UMCs, where more complex patients are treated. For example, patients in the pre-emptive screening group more often had an EORTC/MSGERC host risk factor for IA.

Our study has a number of limitations. Firstly, the variable CAPA protocols in the participating ICUs may have caused inclusion bias and may have influenced outcome. Secondly, negative test results and clinical parameters were not taken into account when classifying patients according to the CAPA classification whereas these may be relevant for the final diagnosis. Thirdly, sample size

was not calculated as it was considered challenging to predict the number of patients eligible to participate in the study, because the characteristics and epidemiology of the pandemic were rapidly changing. Lastly, a comparison of the total cohort with the diagnostic cohort was not possible as clinical information was not collected of patients in the total cohort.

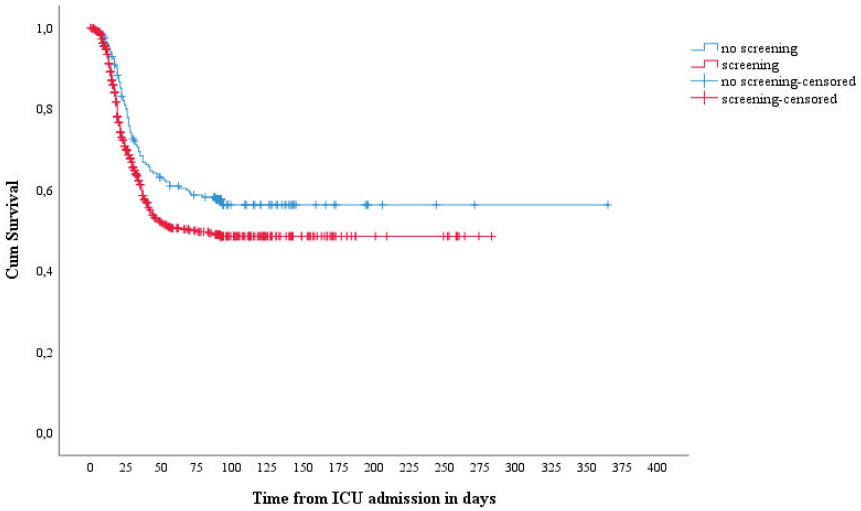
In conclusion, treatment with anti-IL-6 with or without corticosteroids was not a risk factor for CAPA and CAPA incidence was similar to the first COVID-19 period when treatment with immunosuppressive agents was not standard of care. In general, our observations support the hypothesis that CAPA is an indicator of a protracted course of the COVID-19 infection. We did not observe a benefit of pre-emptive screening for CAPA, but prospective studies comparing predefined strategies would be required to confirm this observation.

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SUPPLEMENTAL MATERIAL



	Timepoint										
Numbers at risk	0	25	50	75	100	125	150	175	200	250	275
Screening	593	324	193	160	88	53	35	18	12	9	1
No screening	200	153	117	106	32	25	11	7	4	2	0

Supplemental figure 1. Kaplan-Meier survival curves of medical centres with a pre-emptive screening strategy (screening) or a reactive diagnostic strategy (no screening) for CAPA. Log-rank test: p=0.023. CAPA: COVID-19 associated pulmonary aspergillosis.

Supplemental Table 1. Baseline characteristics of patients included in the diagnostic cohort with and without CAPA (additional information besides table 1).

			No CAPA (n = 498)		CAPA (n = 295)		Total (n = 793)		Significance level
Number of days on ventilatory support	ECMO (55)	Median (IQR)	13	*(9-30)*	22	*(11-31)*	14	*(10-30)*	0.349
	Invasive (699)	Median (IQR)	16	*(8-30)*	23	*(12-34)*	18	*(9-32)*	<0.001
	Non-invasive (499)	Median (IQR)	4	*(2-8)*	5	*(2-9)*	4	*(2-8)*	0.152
Treatment for COVID-19	Landelumab (792)	n (%)	0	*0.0*	0	*0.0*	0	*0.0*	1
	Eculizumab (792)	n (%)	0	*0.0*	0	*0.0*	0	*0.0*	1
	Anakinra (792)	n (%)	9	*1.8*	2	*0.7*	11	*1.4*	0.227
	Lcatibant (792)	n (%)	0	*0.0*	0	*0.0*	0	*0.0*	1
	Convalescent serum (792)	n (%)	5	*1.0*	5	*1.7*	10	*1.3*	0.512
	Cholorquine (792)	n (%)	0	*0.0*	0	*0.0*	0	*0.0*	1
	Hydroxycholorquine (792)	n (%)	0	*0.0*	0	*0.0*	0	*0.0*	1
	Lopinavir/ritonavir (792)	n (%)	2	*0.4*	0	*0.0*	2	*0.3*	0.533
	Ivermectin (792)	n (%)	0	*0.0*	1	*0.3*	1	*0.1*	0.371
	Other (792)	n (%)	97	*19.5*	51	*17.3*	148	*18.7*	0.457
Cumulative dose of corticosteroids	ICU admission until first CAPA suspicion* (380)	Median (IQR)	320	*(200-400)*	320	*(240-400)*	320	*(200-400)*	0.512
	ICU admission until first CAPA suspicion† (373)	Median (IQR)	3.38	*(2.22-5)*	3.62	*(2.46-4.82)*	3.56	*(2.31-4.95)*	0.478
	Total ICU stay* (600)	Median (IQR)	475	*(360-1535)*	700	*(400-1785)*	537	*(400-1599)*	0.011
	Total ICU stay† (594)	Median (IQR)	5.73	*(3.85-17.27)*	8.33	*(4.43-20)*	6.67	*(4-18.34)*	0.015
	Pre admission to hospital* (56)	Median (IQR)	400	*(300-600)*	300	*(180-450)*	300	*(300-461)*	0.301
	Pre admission to hospital† (56)	Median (IQR)	4.55	*(3.12-6.65)*	4	*(2.17-6.52)*	4.41	*(3.12-6.6)*	0.758
	Pre admission to ICU (from hospital admission)* (295)	Median (IQR)	120	*(40-200)*	100	*(40-190)*	120	*(40-200)*	0.341
	Pre admission to ICU (from hospital admission) † (292)	Median (IQR)	1.34	*(0.63-2.32)*	1.18	*(0.54-2.19)*	1.27	*(0.59-2.29)*	0.556

Supplemental Table 1. Baseline characteristics of patients included in the diagnostic cohort with and without CAPA (additional information besides table 1). (continued)

			No CAPA (n = 498)		CAPA (n = 295)		Total (n = 793)		Significance level
Antifungal treatment	Treatment with an antifungal agent - including for candidemia	n (%)	100	*20.1*	230	*78.0*	330	*41.6*	<0.001
	Treatment duration - including for candidemia (330)	Median (IQR)	4	*(2-11)*	12	*(5-27)*	10	*(3-22)*	<0.001
	Treatment with an antifungal agent - active against Aspergillus spp.	n (%)	88	*17.7*	228	*77.3*	316	*39.8*	<0.001
	Treatment duration - active against Aspergillus spp. (316)	Median (IQR)	4	*(2-11)*	12	*(5-27)*	10	*(3-23)*	<0.001
	Treatment with an antifungal agent - active against Aspergillus spp.‡ (419)	n (%)	50	*17.2*	93	*72.7*	143	*34.1*	<0.001
	Treatment duration - active against *Aspergillus* spp.‡ (143)	Median (IQR)	5	*(3-12)*	22	*(9-41)*	14	*(5-35)*	<0.001

*Cumulative dose of corticosteroids (prednisone equivalent in mg), †Cumulative dose of corticosteroids per kg bodyweight (prednisone equivalent in mg/kg bodyweight), ‡Exclusion of deaths/transfers. IQR: interquartile range.

Supplemental Table 2. Baseline characteristics of patients in diagnostic cohort in whom screening was or was not performed.

			No screening (n = 200)		Screening (n = 593)		Total (n = 793)		Significance level
Demographics	Age	Median (IQR)	67	*(59-73)*	65	*(58-72)*	66	*(58-72)*	0.043
	BMI	Median (IQR)	28.4	*(25.6-32.2)*	28.4	*(25.4-32.4)*	28.4	*(25.4-32.4)*	0.828
	Sex (male)	n (%)	154	*77.0*	441	*74.4*	595	*75.0*	0.457
Smoking	Current or former smoker (493)	n (%)	83	*61.0*	187	*52.4*	270	*54.8*	0.085
Comorbidities	Any comorbidities	n (%)	164	*82.0*	485	*81.8*	649	*81.8*	0.946
	Acute leukaemia	n (%)	0	*0.0*	5	*0.8*	5	*0.6*	0.338
	Stem cell transplantation	n (%)	0	*0.0*	9	*1.5*	9	*1.1*	0.122
	Other haematological malignancy	n (%)	6	*3.0*	25	*4.2*	31	*3.9*	0.443
	Solid organ transplantation	n (%)	4	*2.0*	40	*6.7*	44	*5.5*	0.011
	Cardiovascular disease	n (%)	117	*58.5*	323	*54.5*	440	*55.5*	0.321
	Diabetes mellitus	n (%)	52	*26.0*	183	*30.9*	235	*29.6*	0.193
	Asthma	n (%)	24	*12.0*	63	*10.6*	87	*11.0*	0.59
	COPD	n (%)	24	*12.0*	56	*9.4*	80	*10.1*	0.299
	Cystic fibrosis	n (%)	0	*0.0*	2	*0.3*	2	*0.3*	0.622
	Pulmonary tuberculosis	n (%)	0	*0.0*	3	*0.5*	3	*0.4*	0.576
	Multiple sclerosis	n (%)	0	*0.0*	2	*0.3*	2	*0.3*	0.622
	Liver cirrhosis	n (%)	0	*0.0*	2	*0.3*	2	*0.3*	0.622
	Inflammatory bowel disease	n (%)	4	*2.0*	5	*0.8*	9	*1.1*	0.241
	Rheumatological disease	n (%)	11	*5.5*	38	*6.4*	49	*6.2*	0.645
	Psoriasis	n (%)	0	*0.0*	4	*0.7*	4	*0.5*	0.363
	HIV/AIDS	n (%)	0	*0.0*	1	*0.2*	1	*0.1*	1
	Solid organ malignancy	n (%)	16	*8.0*	50	*8.4*	66	*8.3*	0.848
	Other malignancy	n (%)	0	*0.0*	1	*0.2*	1	*0.1*	1
	Chronic kidney disease	n (%)	8	*4.0*	41	*6.9*	49	*6.2*	0.139
	Thyroid disease	n (%)	11	*5.5*	43	*7.3*	54	*6.8*	0.395

Supplemental Table 2. Baseline characteristics of patients in diagnostic cohort in whom screening was or was not performed. (continued)

			No screening (n = 200)		Screening (n = 593)		Total (n = 793)		Significance level
	Other	n (%)	36	*18.0*	180	*30.4*	216	*27.2*	0.001
EORTC/MSGERC criteria* [8]	Recent history of neutropenia (748)	n (%)	0	*0.0*	2	*0.3*	2	*0.3*	1
	Active haematological malignancy (790)	n (%)	0	*0.0*	16	*2.7*	16	*2.0*	0.016
	Allogeneic stem cell transplantation	n (%)	0	*0.0*	7	*1.2*	7	*0.9*	0.201
	Prolonged use of corticosteroids (756)	n (%)	7	*4.0*	38	*6.5*	45	*6.0*	0.213
	T-cell immunosuppressants (789)	n (%)	6	*3.0*	37	*6.3*	43	*5.4*	0.08
	B-cell immunosuppressants (790)	n (%)	4	*2.0*	13	*2.2*	17	*2.2*	1
	Inherited severe immunodeficiency	n (%)	0	*0.0*	0	*0.0*	0	*0.0*	1
	Any EORTC/MSGERC criterium (791)	n (%)	14	*7.1*	86	*14.5*	100	*12.6*	0.006
APACHE IV and SOFA scores on ICU admission	APACHE IV (624)	Median (IQR)	58	*(39-71)*	56	*(29-71)*	57	*(31-71)*	0.133
	SOFA (635)	Median (IQR)	6	*(4-8)*	6	*(4-8)*	6	*(4-8)*	0.079
Supportive care on ICU	SDD (738)	n (%)	153	*78.1*	398	*73.4*	551	*74.7*	0.202
	Renal replacement therapy (786)	n (%)	37	*18.5*	128	*21.8*	165	*21.0*	0.316
	Vasopressors and/or inotropes (789)	n (%)	184	*92.0*	534	*90.7*	718	*91.0*	0.568
Most invasive ventilatory support during ICU stay	ECMO	n (%)	0	*0.0*	55	*9.3*	55	*6.9*	<0.001
	Invasive total	n (%)	199	*99.5*	512	*86.3*	711	*89.7*	<0.001
	Non-invasive	n (%)	0	*0.0*	24	*4.0*	24	*3.0*	0.004
	None	n (%)	1	*0.5*	2	*0.3*	3	*0.4*	1
Treatment for COVID-19	Corticosteroids (792)	n (%)	199	*99.5*	570	*96.3*	769	*97.1*	0.019
	Tocilizumab (792)	n (%)	76	*38.0*	110	*18.6*	186	*23.5*	<0.001
	Corticosteroids and tocilizumab (792)	n (%)	76	*38.0*	108	*18.2*	184	*23.2*	<0.001

Supplemental Table 2. Baseline characteristics of patients in diagnostic cohort in whom screening was or was not performed. (continued)

			No screening (n = 200)		Screening (n = 593)		Total (n = 793)		Significance level
Time to event from ICU admission in days	Performance of BAL or NBL GM† (647)	Median (IQR)	10	*(6-14)*	6	*(2-10)*	7	*(3-11)*	<0.001
	First positive BAL GM or NBL GM‡ (193)	Median (IQR)	10	*(7-14)*	9	*(5-15)*	10	*(5-15)*	0.362
	First positive mycological test§ (318)	Median (IQR)	9	*(4-15)*	8	*(4-13)*	8	*(4-13)*	0.27
	Clinical suspicion CAPA§ (444)	Median (IQR)	9	*(5-12)*	7	*(4-11)*	8	*(4-12)*	0.018
	Clinical diagnosis CAPA§ (216)	Median (IQR)	13	*(8-16)*	11	*(6-16)*	12	*(7-16)*	0.17
Length of ICU stay in days	Length of ICU stay¶ (729)	Median (IQR)	29	*(19-44)*	21	*(13-36)*	23	*(14-38)*	<0.001
Mortality	Mortality at 30 days after ICU admission (658)	n (%)	53	*27.6*	175	*37.6*	228	*34.7*	0.015
	Mortality at 90 days after ICU admission (550)	n (%)	81	*47.9*	240	*63.0*	321	*58.4*	0.001
Time to treatment initiation in days	Clinical suspicion CAPA until treatment initiation (293)	Median (IQR)	1	*(0-4)*	2	*(0-4)*	2	*(0-4)*	0.39
	Clinical diagnosis CAPA until treatment initiation (207)	Median (IQR)	0	*(0-0)*	0	*(0-1)*	0	*(0-1)*	0.698
	First positive GM until treatment initiation (167)	Median (IQR)	0	*(-1-1)*	1	*(0-2)*	1	*(0-2)*	0.002
	First collected GM until treatment initiation (278)	Median (IQR)	0	*(0-2)*	2	*(0-7)*	2	*(0-5)*	<0.001
	ICU admission until treatment initiation (313)	Median (IQR)	12	*(7-16)*	10	*(6-14)*	11	*(6-15)*	0.056

*Solid organ transplant can be found under comorbidities, †Date of first GM, date of first BAL or NBL not available, ‡If NBL GM was positive based on a GM index >1.2 twice or more, the date of the first positive NBL GM result was selected, §According to medical centres, ¶In patients in whom the total ICU stay was known.

BAL: bronchoscopic alveolar lavage, BMI: body mass index, GM: galactomannan, ICU: intensive care unit, IQR: interquartile range, NBL: non-bronchoscopic lavage.

Supplemental Table 3. Different test combinations of patients with probable CAPA.

	n	%	% of group
Serum GM	6	2.4	4.1
Serum GM and BAL GM	1	0.4	
Serum GM, BAL culture and BAL GM	1	0.4	
Serum GM, BAL microscopy, culture, PCR and BAL GM	1	0.4	
PCR blood	1	0.4	
BAL microscopy	14	5.7	36.6
BAL microscopy and culture	10	4.1	
BAL microscopy and PCR	9	3.7	
BAL microscopy, culture and PCR	8	3.3	
BAL microscopy and BAL GM	17	6.9	
BAL microscopy, culture and BAL GM	8	3.3	
BAL microscopy, PCR and BAL GM	7	2.8	
BAL microscopy, culture, PCR and BAL GM	17	6.9	
BAL culture	14	5.7	20.7
BAL culture and PCR	6	2.4	
BAL culture and BAL GM	20	8.1	
BAL culture, PCR and BAL GM	11	4.5	
BAL PCR	20	8.1	16.3
BAL PCR and BAL GM	20	8.1	
BAL GM	55	22.4	22.4
Total	246	100	100

Different test combinations of patients with probable CAPA (according to 2020 ECMM/ISHAM consensus criteria[12] ranked on the basis of positive test results theoretically most indicative of *Aspergillus* invasiveness or load: serum GM or PCR on blood > BAL microscopy > BAL culture > BAL PCR > BAL GM. BAL: bronchoscopic alveolar lavage, GM: galactomannan.

Supplemental Table 4. Different test combinations of patients with possible CAPA.

	n	%	% of group
NBL microscopy	5	11.9	38.1
NBL microscopy and culture	2	4.8	
NBL microscopy, culture and GM index >4.5	3	7.1	
NBL microscopy and GM index at least 2x >1.2	1	2.4	
NBL microscopy, culture and GM index at least 2x >1.2	1	2.4	
NBL microscopy, culture, GM index > 4.5 and GM index at least 2x >1.2	3	7.1	
NBL microscopy, culture and GM index at least 2x >1.2 + PCR	1	2.4	
NBL culture	5	11.9	28.6
NBL culture and GM index >4.5	1	2.4	
NBL culture, GM index >4.5 and GM index at least 2x >1.2	6	14.3	
NBL GM index >4.5	2	4.8	33.3
NBL GM index at least 2x >1.2	7	16.7	
NBL GM index >4.5 and GM index at least 2x >1.2	5	11.9	
Total	42	100	100

Different test combinations of patients with possible CAPA (according to 2020 ECMM/ISHAM consensus criteria[12] ranked on the basis of positive test results theoretically most indicative of *Aspergillus* invasiveness or load: NBL microscopy > NBL culture > NBL GM (NBL GM positivity according to 2020 ECMM/ISHAM consensus criteria.[12] GM: galactomannan, NBL: non-bronchoscopic lavage.

Supplemental Table 5. 30-day mortality in patients with positive test results ranked on the basis of the positive test results theoretically indicative of Aspergillus invasiveness or load.

	Positive test result (at least)															
	Serum GM/PCR	%	Micros-copy	%	Culture	%	PCR	%	BAL/NBL GM	%	Colo-nised	%	Nega-tive	%	Total	%
Alive	4	40	57	59.4	37	63.8	27	67.5	38	74.5	47	68.1	216	65.9	426	65.3
Death	6	60	39	40.6	21	36.2	13	32.5	13	25.5	22	31.9	112	34.1	226	34.7
Total	10	100	96	100	58	100	40	100	51	100	69	100	328	100	652	100

Patients with probable or possible CAPA according to 2020 ECMM/ISHAM consensus criteria,[12] colonised (patients with positive *Aspergillus* test results who did not fulfil CAPA criteria) or no CAPA.
Ranking: serum GM/PCR blood > microscopy: BAL/NBL microscopy > culture: BAL/NBL culture > PCR: BAL PCR > GM: BAL/NBL GM (NBL GM positivity according to 2020 ECMM/ISHAM consensus criteria[12] > colonised > negative test results.
BAL: bronchoscopic alveolar lavage, GM: galactomannan, NBL: non-bronchoscopic lavage.

Supplemental Table 6. 90-day mortality in patients with positive test results ranked on the basis of the positive test results theoretically indicative of Aspergillus invasiveness or load.

	Positive test result (at least)															
	Serum GM/PCR	%	Microscopy	%	Culture	%	PCR	%	BAL/NBL GM	%	Colonised	%	Negative	%	Total	%
Alive	1	11.1	25	29.8	19	41.3	15	39.5	17	43.6	35	57.4	117	43.8	229	42.1
Death	8	88.9	59	70.2	27	58.7	23	60.5	22	56.4	26	42.6	150	56.2	315	57.9
Total	9	100	84	100	46	100	38	100	39	100	61	100	267	100	544	100

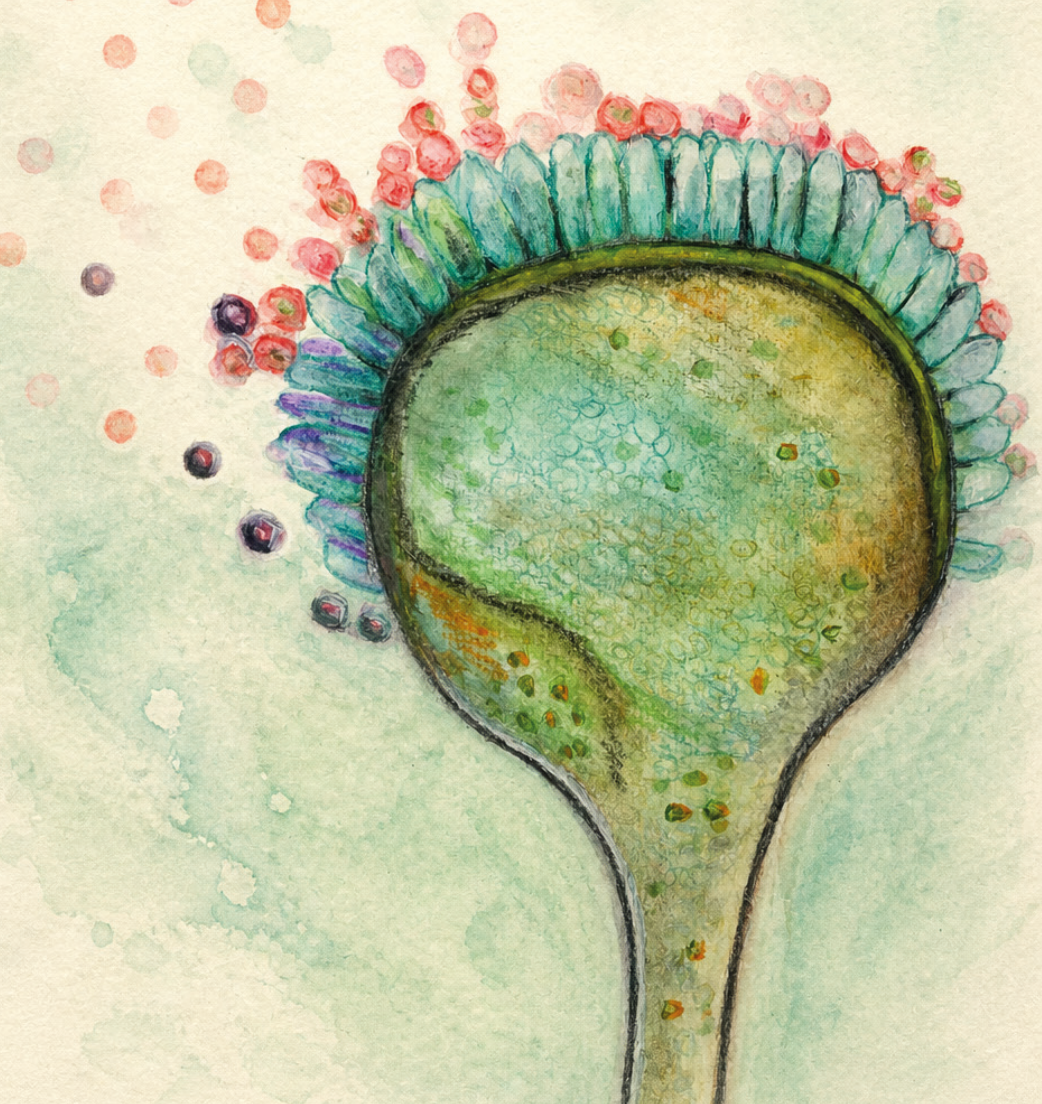
Patients with probable or possible CAPA according to 2020 ECMM/ISHAM consensus criteria,[12] colonised (patients with positive *Aspergillus* test results who did not fulfil CAPA criteria) or no CAPA.
Ranking: serum GM/PCR blood > microscopy: BAL/NBL microscopy > culture: BAL/NBL culture > PCR: BAL PCR > GM: BAL/NBL GM (NBL GM positivity according to 2020 ECMM/ISHAM consensus criteria[12] > colonised > negative test results.
BAL: bronchoscopic alveolar lavage, GM: galactomannan, NBL: non-bronchoscopic lavage.

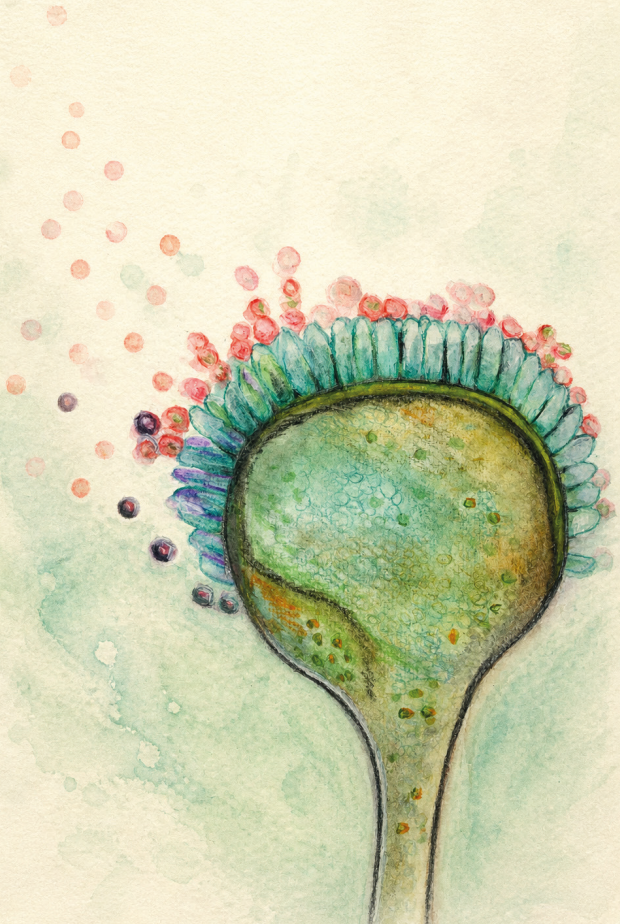
Supplemental Table 7. Different *Aspergillus* spp. cultured from respiratory samples.

	n	%
Aspergillus fumigatus	169	90
A. niger	3	2
A. flavus	2	1
A. fumigatus + *A. flavus*	2	1
A. terreus	1	1
A. fumigatus + *A. nidulans*	1	1
A. fumigatus + *A. terreus*	1	1
A. fumigatus + *A. flavus* + *A. terreus*	1	1
A. fumigatus + *Aspergillus* spp.	1	1
A. niger + *Aspergillus* spp.	1	1
Aspergillus spp.	6	3
Total	188	100

Part II

Screening and surveillance





5

Systematic screening for COVID-19 associated invasive aspergillosis in ICU patients by culture and PCR on tracheal aspirate



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ABSTRACT

Background: A high prevalence of COVID-19 associated pulmonary aspergillosis (CAPA) has been reported, though histopathological evidence is frequently lacking. To assess the clinical significance of *Aspergillus* species in respiratory samples of mechanically ventilated COVID-19 patients, we implemented routine screening for *Aspergillus* in tracheal aspirate (TA).

Patients/methods: From all adult COVID-19 patients admitted to the intensive care unit (ICU), TA samples were collected twice a week for *Aspergillus* screening by PCR and or culture. Bronchoalveolar lavage (BAL) sampling was performed in patients with a positive screening result if possible. Clinical information was obtained from the electronic patient record and patients were categorised according to the recently published consensus case definition for CAPA.

Results: Our study population consisted of 63 predominantly (73%) male patients, with a median age of 62 years and total median ICU stay of 18 days. *Aspergillus* species were present in TA screening samples from 15 patients (24%), and probable CAPA was diagnosed in 11 (17%) patients. Triazole resistance was detected in one patient (14%). Concordance between TA and BAL was 86%, and all TA culture positives were confirmed in BAL. We were able to withhold treatment in three of fifteen patients with positive screening (20%) but negative BAL results.

Conclusions: Positive culture, molecular detection and or antigen detection of *Aspergillus* species do not equal infection. Until we understand the clinical relevance of *Aspergillus* species detected in respiratory samples of COVID-19 patients, minimal-invasive screening by TA is a feasible method to monitor patients. Positive screening results should be an indication to perform a BAL to rule out upper airway colonisation.

INTRODUCTION

Soon after the COVID-19 pandemic emerged worldwide a high prevalence of COVID-19 associated pulmonary aspergillosis (CAPA) was reported in patients admitted to the intensive care unit (ICU).[1-4] Histopathological evidence for CAPA has been described in a number of cases.[5-7] Furthermore, in recent years, severe influenza requiring mechanical ventilation has been recognised as an independent risk factor for invasive pulmonary aspergillosis (IPA) in immunocompetent patients.[8] However, histopathological evidence for CAPA is missing in most reports.[9] Furthermore, a recent summary of available autopsy findings in a series of seven cases did not show signs of invasive aspergillosis (IA) in patients with positive *Aspergillus* cultures and or positive bronchoalveolar lavage (BAL) galactomannan (GM).[10] Neither was IA reported in a meta-analysis of pulmonary histopathology reports of 129 COVID-19 patients,[11] but standard histological stains for fungi were not always performed. Hence, the true prevalence of invasive infection in COVID-19 patients remains to be elucidated. Also, neither the benefit of (pre-emptive) antifungal treatment nor the consequences of withholding treatment with antifungals are known yet.[4, 12]

Given that at the moment dexamethasone is the cornerstone of treatment for patients with COVID-19 admitted to hospital requiring oxygen,[13, 14] more COVID-19 patients may be put at risk for IA. Diagnosing CAPA is difficult, because most patients do not fulfil EORTC/MSGERC or AspICU criteria[1-4, 15, 16] and CT-scans usually reveal severely damaged lungs because of COVID-19.[2, 4] Hence, in clinical practice, *Aspergillus* positive respiratory cultures from COVID-19 patients cause the following clinical dilemma: are we dealing with colonisation or infection? Although, excess mortality has been observed in patients with COVID-19 with suspicion of *Aspergillus* in the lower respiratory tract.[7, 17, 18] During the COVID-19 pandemic diagnosing CAPA was further complicated by concerns about the risk of SARS-CoV-2 infection in healthcare workers by aerosol forming diagnostic procedures such as BAL sampling.[19]

Currently the optimal diagnostic workflow for CAPA is unknown, but recently a CAPA consensus definition has been proposed.[20] To fully comprehend the diagnostic value and dilemmas of *Aspergillus* screening we initiated systematic screening for *Aspergillus* in COVID-19 patients admitted to the ICU of our academic medical centre. In this study, we evaluated our CAPA screening programme after one month of screening for *Aspergillus* species by tracheal aspirate (TA). Our goals were to assess the value of TA as a screening sample, to compare different diagnostic tests and to characterise patients with CAPA. By doing so, we wanted to optimise the diagnostic workflow.

MATERIALS AND METHODS

Study setting and patient population

From all adult patients with PCR confirmed COVID-19, admitted to the ICU of the Leiden University Medical Center (LUMC), TA samples were routinely collected twice a week for bacterial culture, SARS-CoV2 PCR and *Aspergillus* screening (culture and *Aspergillus* PCR). Few BAL procedures were performed at first, but BAL samples were obtained more frequently from mid-April. In comparison to BAL sampling, TA sampling is a minimal-invasive method to collect a specimen from the lower respiratory tract. Because TA samples can be contaminated with micro-organisms from the upper respiratory tract, confirmation by BAL sampling was performed in patients with a positive screening result if deemed possible and clinically indicated. Treatment was initiated in patients with a positive culture or PCR of BAL and in patients with a positive culture and PCR of TA if no BAL was performed. The diagnostic workflow is depicted in Figure 1.

Study definitions

Categorisation according to CAPA consensus definition

All patients were categorised according to the recently published case definition for CAPA.[20] Tracheobronchitis was not observed. Non-broncoscopic alveolar lavage was not performed, so patients could not be classified as possible CAPA. Patients not meeting criteria for probable CAPA with negative BAL diagnostics were considered colonised with *Aspergillus*. If no BAL was performed patients were considered unclassifiable.

Data collection

We obtained data from the laboratory information system about bacterial culture, SARS-CoV-2 PCR, *Aspergillus* culture and resistance, *Aspergillus* PCR and GM results between April 1st 2020 and May 11th 2020 (first COVID-19 peak). These data included TA samples collected for *Aspergillus* screening as well as other samples collected in this period. From patients with *Aspergillus* positive culture and or PCR and or GM relevant clinical data about age, sex, underlying illness, diagnosis and follow-up (dates of first day of illness, hospital admission, ICU admission, discharge and end of follow-up), treatment (antifungal therapy, antibacterial therapy, treatment with chloroquine or remdesivir, supportive care on ICU), and outcome (death or alive) was obtained from the electronic patient records (EPR). From patients with negative results, we obtained clinical data from the LUMC-COVID-19 registration database, as well as all smoking data. Follow-up was until discharge from hospital, transfer to another hospital or death.

The study was approved by the hospitals' Institutional Review Board (Medical Ethical Committee Leiden-Delft-Den Haag, The Netherlands). Patients were only included if they had consented via an approved opt-out procedure active in our institution. If patients weren't able to consent because they were intubated, the opt-out procedure for clinical data for the National Intensive Care Evaluation (NICE) was applied. The screening programme was implemented as temporary routine care.

Aspergillus culture, PCR techniques and galactomannan

Both TA and BAL samples were inoculated on sheep blood agar, chocolate agar, sheep blood agar with colistin and nalidixic acid and cystine-lactose-electrolyte-deficient (CLED) agar and incubated in an atmosphere enriched with 5% CO2 at 37°C for two days according to standard protocol. In addition, BAL samples were inoculated on Sabouraud dextrose agar with gentamicin (0.04 g/L) and chloramphenicol (0.5 g/L) and incubated at 28°C and 35°C for 10 days. Tracheal aspirate samples were inoculated on Sabouraud agar and were incubated at 35°C for 10 days if they were part of the *Aspergillus* screening programme.

Triazole resistance screening was performed on *Aspergillus fumigatus* isolates by a four-well agar plate with voriconazole (2 mg/L), itraconazole (4 mg/L), posaconazole (0.5 mg/L) and a growth control (VIPcheck™, Groningen, The Netherlands). Up to four colonies of an isolate were inoculated on Sabouraud agar and after growth of the isolate a suspension of 0.5 - 2.0 McFarland was prepared which was added to the four-well agar plate. MIC testing was performed on isolates by microbroth dilution (MD) method according to EUCAST by a reference laboratory (Radboudumc, Nijmegen, The Netherlands).

The PathoNostics AsperGenius® PCR assay (PathoNostics, Maastricht, The Netherlands) was performed according to the manufacturer's instructions for detection of *Aspergillus fumigatus* complex, *Aspergillus terreus* and *Aspergillus* species, and TR34/L98H and Y121F/T289A resistance mutations. Samples were diluted in TE buffer and bead beating was carried out. DNA extraction was performed with the MagNA Pure 96, DNA and viral NA small volume kit. Galactomannan testing was performed using the Platelia™ *Aspergillus* Ag (Bio-rad laboratories, Marnes-la-Coquette, France). A GM optical density (OD) cut-off of ≥ 0.5 was deemed positive.

Statistical analysis

Categorial variables were described as numbers and percentages. Continuous variables were described as median and interquartile range (IQR) or range. Pa-

tients with positive and negative *Aspergillus* culture, PCR or GM results were compared with the Mann Whitney U-test for numerical data and Chi-square test or Fisher's exact test for categorical data depending on sample size. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS statistics (IBM SPSS Statistics for Windows, version 25.0. Armonk, NY: IBM Corp).

RESULTS

Clinical characteristics

Patient characteristics

From April 1st to May 11th 2020 63 COVID-19 patients were admitted to the ICU of the LUMC. Median age of the patients was 62 years, 73% were male and median ICU stay was 18 days (range 3-55). Additional patient characteristics are depicted in Table 1. Classical risk factors for invasive fungal infection[15] were present in three (5%) patients (1 allogeneic stem cell transplantation, 2 organ transplant).

During the study period, *Aspergillus* species were identified in TA screening by culture and or PCR in 15 of 63 (24%) patients. Probable CAPA was diagnosed in 11 of 63 (17%) patients and included four patients who were only BAL GM positive and negative in screening. Patient categorisation is depicted in Figure 1. The clinical characteristics of the patients with positive *Aspergillus* diagnostics are described in more detail in Table S1. At the time of diagnosis of CAPA CT-scanning revealed COVID-19 associated radiographic abnormalities in nine of these patients and cavitation in one patient, who was also diagnosed with a bacterial lung abscess due to *Streptococcus anginosus*. Six patients with probable CAPA received antifungal treatment. Of the six patients who were treated with antifungals four died, three within one week after treatment initiation and one 23 days after treatment initiation without any signs of antifungal treatment failure as follow-up samples had become negative. The five untreated patients included three patients with only a positive GM in BAL without other positive *Aspergillus* diagnostics, one patient positive in screening and only GM positive in BAL and one patient who was screening and BAL PCR positive. No treatment was given because of clinical improvement and or negative follow-up diagnostics in three patients of whom 2 survived, treatment restrictions in one patient who died shortly thereafter and because of death on the day of BAL results in another patient. One autopsy report was available from a patient with probable CAPA who was only BAL GM positive, but no histological evidence of IA was de-

Table 1. Patient characteristics of COVID-19 patients with positive and negative *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples.

	***Aspergillus* positive (n=19)**	***Aspergillus* negative (n=44)**	**Total (n=63)**	**Significance level, p-value***
Baseline				
Age, years (IQR)	65 (59 – 72)	61 (55 – 68)	62 (57 – 71)	0.076[I]
Sex, male (%)	14 (73.7)	32 (72.7)	46 (73.0)	0.937[II]
Underlying illness				
Chronic pulmonary disease or asthma (physician diagnosed), n (%)[‡]	7 (36.8)	10 (22.7)[†]	17 (27)	0.247[II]
Chronic liver disease, n (%)	0	0	0	N/A
Diabetes, n (%)[‡]	5 (26.3)	10 (22.7)[†]	15 (23.8)	1.000[III]
HIV/AIDS, n (%)	0	0[†]	0	N/A
Malignant neoplasm, n (%)[‡§]	2 (10.5)	3 (6.8)[†]	5 (7.9)	0.643[III]
Organ transplant, n (%)	1 (5.3)	1 (2.3)	2 (3.2)	0.516[III]
Smoking				
Current smoking, n (%)[‡]	0	3 (6.8)	3 (4.8)	0.537[III]
Stopped smoking, n (%)[‡]	7 (36.8)	9 (20.5)	16 (25.4)	0.263[III]
Smoking unknown, n (%)	7 (36.8)	22 (50)	29 (46)	0.336[II]
Supportive care on ICU				
Invasive ventilation, n (%)	19 (100)	44 (100)	63 (100)	1.000
Prone position, n (%)	19 (100)	37 (81.4)	56 (88.9)	0.091[III]
Renal replacement therapy or dialysis, n (%)	6 (31.6)	7 (15.9)	13 (20.6)	0.186[III]
Extracorporeal support, n (%)	1 (5.3)	0	1 (1.6)	0.302[III]
Vasoactive drugs, n (%)	15 (100)	48 (100)	63 (100)	1.000
SARS-CoV-2 PCR¶				
SARS-CoV-2 PCR conducted during ICU stay, n	19/19 (100)	40/44 (90.9)	59/63 (93.7)	0.306[III]
Positive SARS-CoV-2 PCR result, n	18/19 (94.7)	28/40 (70)	46/59 (78)	0.44[III]
Ct-value peak load, median (IQR)	24.3 (22.6 – 28.3)	27.7 (23.6 – 30)	25.7 (23.2 – 29.5)	0.105[I]
SARS-CoV-2 PCR became negative during ICU stay, n	8/18 (44.4)	11/28 (39.3)	19/46 (41.3)	0.729[II]
Outcome				
Mortality, n (%)	10 (52.6)	9 (20.5)	19 (30.2)	0.011[II]*

Abbreviations: ct-value, cycle threshold value; ICU, intensive care unit; IQR, interquartile range

* Statistically significant (p-value < 0.05); I Mann Whitney U-test; II Chi-square test; III Fischer's exact test; † In the *Aspergillus* negative group the medical history was unknown for asthma (n=1), diabetes (n=1), HIV/AIDS (n=1)) and malignant neoplasm (n=2); ‡ Unknowns were left out for the statistical test; § Of the patients with a malignant neoplasm, four patients had a solid organ malignancy ≤ 5 years ago and one patient had a haematological malignancy and was recently treated by undergoing an allogenic stem cell transplantation. At presentation in hospital, the patient did not have leukopenia and was not treated with immunosuppressive drugs; ¶ SARS-CoV-2 PCR results of tests performed during stay in our hospital.

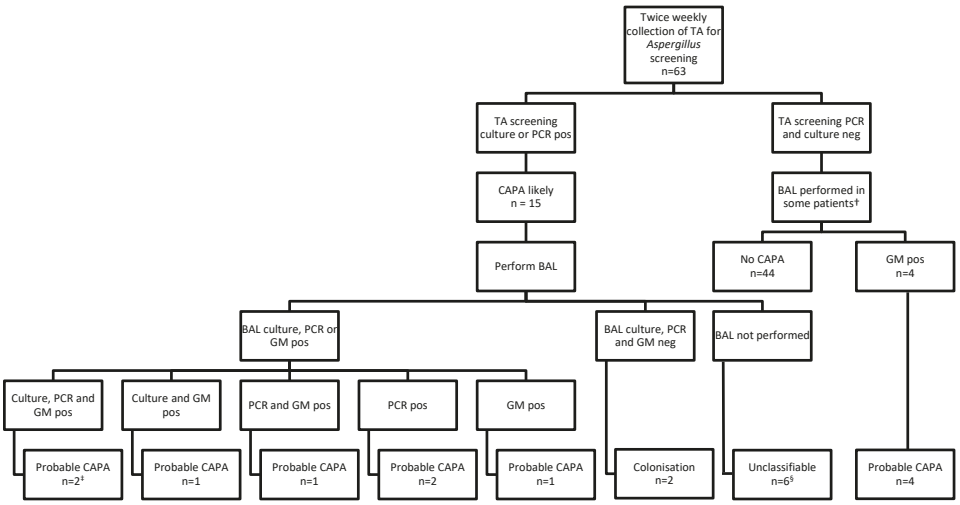


Figure 1. Screening for and classification of COVID-19 associated pulmonary aspergillosis.
TA, tracheal aspirate; BAL, bronchoalveolar lavage; CAPA, COVID-19 associated pulmonary aspergillosis; GM, galactomannan; pos: positive; neg, negative. Patients were classified according to the CAPA consensus definition.[20] †BAL sampling was performed in patients with negative screening results if there was a clinical indication, respiratory deterioration for example. ‡One of the patients with probable CAPA had a positive cytological smear of BAL showing branching hyphae. §In one of the patients branching hyphae were seen in a TA Gram stain.

scribed. Mortality was significantly higher in patients with probable CAPA (7/11, 63.6%) compared to patients not classified as having an infection (12/52, 23.1%) (p-value: 0.013). Triazole resistance was detected in one patient (1/7 culture-positive patients, 14.3%) by VIPcheck™ and MD in a TA screening sample and a T289A/Y12F mutation was detected in two other TA samples from the same patient, but *A. fumigatus* was not isolated from these samples. Bronchoalveolar lavage was not performed in this patient.

Time to positivity

Screening for IA was initiated at a median of 16 days from first day of illness (FDI) of COVID-19 (range: 7 - 21). The median duration from FDI and ICU admission to first positive screening result was respectively 18 days (range: 11 - 27) and 8 days (range: 2 - 23), Figure 2. Twelve patients were positive in the first screening sample. Two patients were first positive in the second screening sample and one patient was first positive in the seventh screening sample. Overall 13/15 (87%) patients were found positive in screening within the first 14 days after ICU admission, including 6/7 patients with probable CAPA who were positive in screening. Two patients became positive later, a patient with probable CAPA (positive screening on day 20) who was admitted to the ICU eight days before

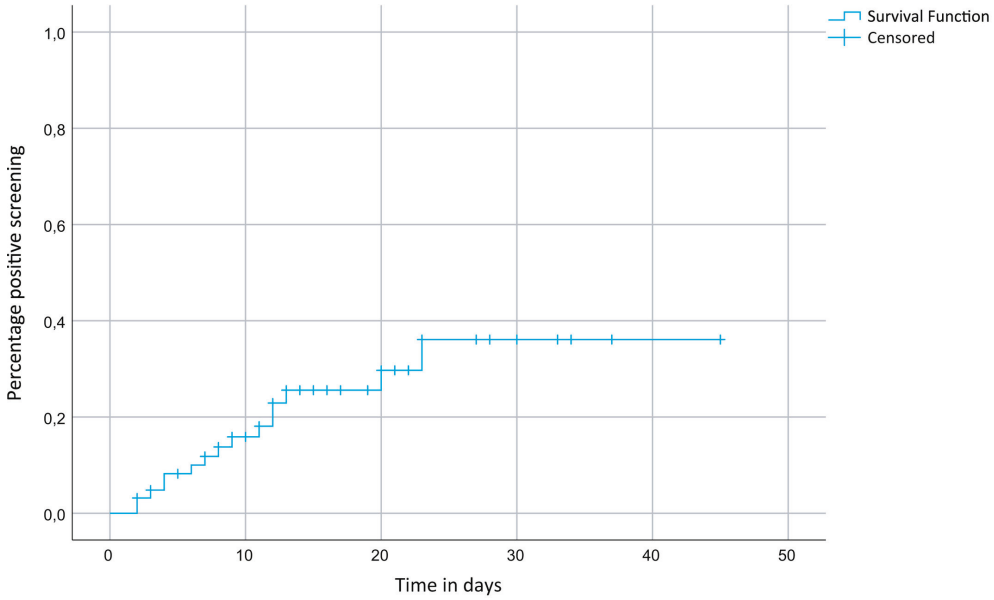


Figure 2. Cumulative incidence of positive screening results from ICU admission.
Positive screening result: tracheal aspirate PCR or culture positive. Follow-up time for patients with negative screening was until last negative culture and or PCR.

the screening programme was initiated and a patient who was not classifiable (positive screening on day 23) who was admitted to the ICU in the screening period. Of the seven patients with probable CAPA who were positive in screening, two remained culture and PCR positive and three remained PCR positive in all subsequent TA samples collected during their ICU stay. Of the six patients who were unclassifiable two remained culture and PCR positive. Both colonised patients remained positive in screening. The time to positivity for the different groups is presented in Figure S1.

Diagnostics

Samples

In total, 421 samples were collected for culture and or PCR which included 366 (86.9%) TA and 55 (13.1%) BAL samples. The median number of samples collected per patient was five (range: 1-20). A total of 412 (97.9%) samples were cultured, of which 26/412 (6.3%) were positive. *Aspergillus* PCR was performed on 267/421 (63.4%) samples, of which 49/267 (18.4%) were positive. PCR detected *A. fumigatus* complex (n = 45), *A. terreus* (n = 3) and *Aspergillus* species (n = 1). Of the remaining samples 215 were negative and three were not interpretable.

Fungal typing and resistance

Aspergillus species were cultured from 26 samples (21 TA and 5 BAL). *A. fumigatus* was cultured most often (n = 26). *A. niger* (n = 1) and *A. flavus* (n = 1) were cultured as well. The VIPcheck™ was performed in 23/26 (88%) and 22 *A. fumigatus* isolates were susceptible in this screening. One isolate was resistant to triazoles and this was confirmed by MD. Microbroth dilution was performed 17 times for 15 *A. fumigatus* isolates, 1 *A. niger* isolate and 1 *A. flavus* isolate, and results for first cultured TA and BAL isolates from culture-positive patients can be found in Table S1. Cyp-51 PCR was performed on 28 samples (21 TA and 7 BAL), was successful in 18/28 (64%) samples and could not be interpreted in 10 samples (5 TA and 5 BAL) due to low loads. A T289A/Y121F mutation was detected in 2/18 samples, both from the same patient, but *A. fumigatus* was not cultured from these samples.

Galactomannan

Galactomannan was performed on 36 BAL samples from 16 patients and was positive (OD cut-off ≥ 0.5) in 9/19 samples from patients with positive *Aspergillus* screening in previous TA samples. Positive results were also seen in 7/17 samples from *Aspergillus* screening negative patients. Using a GM OD cut-off of ≥ 1 these numbers would have been 8/19 samples and 5/17 samples respectively. PCR was positive in two of these sixteen GM-positive BAL samples, culture was positive in one sample and both PCR and culture were positive in four samples. The four patients with positive GM as the only positive finding in BAL had negative *Aspergillus* screening in TA. Details can be found in Table S1. Serum was not routinely collected for GM testing. In the beginning of April, at the start of the screening programme, serum samples from all COVID-19 patients were tested once, and all were negative (GM OD < 0.5) in spite of positive cultures and or PCR of TA in nine patients (data not shown). Galactomannan was performed on a random subset of 10 TA samples but was weakly positive in three samples (GM OD values: 0.51, 0.66 and 1.36) despite negative PCR and culture, possibly due to the bloody aspect and mucus in the samples and was not performed on this type of sample any further.

TA-BAL concordance

Concordance of TA and BAL samples could only be analysed in a select group, because mostly BAL sampling was performed if TA screening samples were positive. Bronchoalveolar lavage samples were collected within three days after TA samples for culture and *Aspergillus* PCR 33 times from 19 different patients for confirmation of a positive TA result, or because there was another clinical indication to perform a BAL. In 22 instances both PCR and culture had been performed on both TA and BAL. Overall concordance between BAL and

Table 2. Concordance of sequentially collected tracheal aspirate and bronchoalveolar lavage samples.

	BAL PCR/culture			
TA PCR/culture	**+/+**	**+/-**	**-/-**	**% TA finding confirmed in BAL**
+/+	3[†]	0	0	100
+/-	0	3[‡]	2[§]	60
-/-	0	1[¶]	13[&]	93

Abbreviations: −/−, PCR and culture negative; +/−, PCR positive and culture negative; +/+, PCR and culture positive; BAL, bronchoalveolar lavage; CAPA, COVID-19 associated pulmonary aspergillosis; GM, galactomannan; TA, tracheal aspirate.
[†] Samples from three patients with probable CAPA. [‡] Samples from three patients with probable CAPA; one patient was culture-positive in a later collected BAL sample. § Samples from two patients; 1 colonisationand 1 sample from a patient later diagnosed with probable CAPA based on positive GM in a later collected BAL sample. ¶ Follow up sample from a patient with probable CAPA who was TA PCR positive in an earlier sample. & Samples from eight patients; 3 negative and 5 patients with probable CAPA. Of the patients with probable CAPA, three patients were only BAL GM positive but negative in all TA screening sample, one patient was TA PCR positive in an earlier sample and only BAL GM positive and one patient was PCR positive in earlier TA and BAL samples.

TA was 19/22 (86%). One additional positive was detected by BAL PCR from a patient with probable CAPA who had been positive in TA screening in earlier samples. No additional positives were detected by BAL culture compared to TA. BAL confirmed all culture-positive TA samples, but confirmed three of five TA samples that were only PCR positive. Overall TA-BAL concordance for both PCR and culture is depicted in Table 2 and in Table S2.

PCR-culture concordance

For 255 samples results were available for both PCR and culture on the same sample, and overall concordance was 225/255 (88.2%). Altogether, PCR detected 28/255 (11.0%) additional positive samples (22 TA and 6 BAL) and missed two culture positives (1 TA and 1 BAL). The additional PCR-positive samples were from patients who were classified as probable CAPA (n = 6), colonisation (n = 2 and unclassifiable CAPA (n = 3). The samples positive in culture only were a TA sample from a patient who was not classifiable and a BAL sample from a patient with probable CAPA. From this last patient three BAL samples were collected simultaneously and *Aspergillus* was detected in 2/3 samples, one by culture and one by PCR.

Time to diagnostic result

Aspergillus was detected by PCR and culture in TA screening samples of six patients. All were PCR and culture positive in the same screening sample, except for one patient with probable CAPA. In this patient the first positive screening result by culture was in a sample collected 17 days after the sample of the first

positive screening by PCR and after five sequential PCR-positive and culture-negative TA samples.

DISCUSSION

Whether CAPA is a clinical entity that requires routine screening, invasive diagnostics and treatment is yet unknown. One month of systematic screening yielded positive findings for *Aspergillus* in 24% of COVID-19 patients admitted to the ICU. Most patients (87%) were found positive in screening within 14 days after ICU admission. Probable CAPA was diagnosed in 17% of patients, all of whom did not have EORTC/MSGERC host factors for IPA. A large number of samples, 421 in total, were collected for culture and or PCR with a median of five samples per patient. Most isolates were susceptible to triazoles. Concordance between culture and PCR was high (88.2%). We were able to withhold treatment in three of fifteen patients with positive screening (20%) because of negative BAL results, of whom two improved clinically and one died, which demonstrates the added value of BAL sampling. Overall mortality was 30% and mortality was significantly higher in patients with probable aspergillosis (64%) compared to patients not classified as having an infection (23%). Establishing the cause of death was not possible, because mostly autopsies were not performed. These numbers are comparable to other studies in which excess mortality is seen in patients with COVID-19 with suspicion of *Aspergillus* in the lower respiratory tract as well, ranging from 44% (30-day mortality) to 71%.[7, 17, 18] To what extent *Aspergillus* infections contribute to mortality in ICU patients with COVID-19 and whether antifungal treatment can reduce mortality remains uncertain.

The observed CAPA prevalence in this cohort is within the range reported by other clinical centres from different parts of the world who have reported CAPA prevalence percentages in ICU patients ranging from 3.8 - 35%,[21, 22] but prevalence numbers should be interpreted with caution as clinical and mycological evidence of CAPA varied per study. Currently, the optimal diagnostic workflow for CAPA is unknown and still under investigation as described in recent literature.[9, 17, 22-24] Optimal diagnostics depend both on feasibility for the treating physician and laboratory as well as the diagnostic value. Regarding feasibility, TA samples can be safely and relatively easily obtained from intubated COVID-19 ICU patients. Fungal culture is a simple and cheap method and prolonged incubation did not seem necessary in our cohort, with all cultures becoming positive within three days (data not shown). With regard to the diagnostic value, TA screening is not sufficient based on the current CAPA consensus definition for which bronchoscopy for inspection of the trachea and BAL sampling or non-

bronchoscopic lavage is required,[20] but may be a screening step to reduce the number of patients in whom invasive BAL sampling is required.

Before the CAPA consensus definition was published, an expert opinion on IAPA in ICU patients proposed a CAPA definition, awaiting further histopathological studies, in which the entry criterion was pulmonary infiltrates with at least one of the following: positive GM in serum > 0.5 or BAL ≥ 1, positive *Aspergillus* culture of BAL (or if BAL is not performed sputum or TA), or cavitation in an area of pulmonary consolidation patients.[23] Bartoletti and colleagues categorised intubated COVID-19 patients according to this CAPA definition.[7] Probable CAPA was diagnosed in 27.7% patients, whilst by applying the AspICU criteria 17.6% were diagnosed with putative IPA.[7] They concluded that the use of the newly proposed CAPA criteria may allow earlier diagnosis than AspICU,[7] but it may also lead to overtreatment. One of the difficulties is that most COVID-19 ICU patients have extensive pulmonary infiltrates. Also, *Aspergillus* is considered a core component of the basal oral microbiome,[9] and false-positive BAL GM results occur in patients without pulmonary aspergillosis due to various causes.[25] By applying a GM cut-off value of one, still 32% false-positive results were seen even in a population including patients with classical host risk factors for IA.[26] By applying the CAPA consensus definition[20] four cases of probable CAPA were found that were only BAL GM positive and had been missed in screening. These patients were not (or only shortly) treated, because IA was not suspected because of spontaneous clinical improvement in one patient who survived, because of treatment restrictions in another patient who died with no evidence of IA in autopsy reports and because of negative follow-up diagnostics in 2 patients who died. It is our suspicion that the BAL GM was false-positive in these cases, but CAPA cannot be completely ruled out in the two patients that died without autopsies.

False-positive GM results can occur in patients colonised with *Aspergillus*, patients infected or colonised with other fungi such as penicillium, contamination of food and in the past false-positive results were seen in patients treated with co-amoxicillin/clavulanic acid and piperacillin-tazobactam or gluconate-containing solutions containing GM antigens.[26-28] However, these reasons do not explain the positive results in our cohort. In our validation, TA GM resulted in false positives as well possibly due to the bloody aspect and mucus in the samples. For this reason, we would not advise to perform TA GM. Besides, TA is not validated for the GM assay. The role of BAL GM testing in this setting needs further evaluation.

Concordance between culture and PCR was high (88.2%) for both TA and BAL, and most discordant samples were samples that were PCR positive and culture negative. PCR has the advantage of a rapid time to results and possibly a higher sensitivity compared to culture. However, culture was positive within two days in the majority of patients and hence the increased speed of diagnostics compared to culture is highly dependent on PCR logistics. The added value of PCR for *Aspergillus* screening on TA remains uncertain. Of the eight patients found positive only by PCR, four were shown to be probable CAPA in BAL samples and two received antifungal treatment, whereas six were left untreated of whom four survived without antifungal treatment suggesting positive *Aspergillus* PCR on TA may reflect colonisation or contamination of the upper airways. On the other hand, two patients with probable CAPA who were treated with antifungals were only PCR positive in screening and in subsequent BAL samples as well and one patient with probable CAPA had several PCR-positive samples before becoming culture positive suggesting it may precede CAPA diagnosis by other tests. PCR of BAL is included in the new CAPA consensus definition and a single-positive *Aspergillus* PCR in BAL (ct-value < 36 cycles) is considered mycological evidence for probable CAPA.[20] Before the CAPA consensus definition was available, we also considered it likely for patients with a positive BAL PCR to have CAPA and treated patients accordingly. PCR is included in the revised EORTC/MSGERC,[15] but not in the AspICU criteria.[16] Gangneux and colleagues have proposed two different adaptions of the AspICU criteria by adding i) PCR on TA or ii) PCR TA in combination with blood biomarkers (GM or PCR).[29] By incorporating PCR on TA into the algorithm the amount of patients with putative CAPA increased from 9/45 to 15/45. By incorporating PCR on TA and blood biomarkers into the algorithm the percentage of putative patients subsequently decreased from 9/45 to 7/45. The authors were in favour of the latter algorithm, because it allowed further categorisation of the patients with a better relevance than the regular AspICU classification and had better specificity than PCR on TA alone and this is in accordance with our cohort in which several patients with only a positive PCR on TA were considered colonised, were not treated and had a favourable outcome. In our cohort blood GM was not of added value because it lacked sensitivity, but it was tested in a limited population.

Previous studies on diagnosis of CAPA used various definitions for CAPA and thus sensitivities and specificities of the various diagnostic tests for IA in COVID-19 patients cannot be reliably compared between studies and cannot be extrapolated from studies on IA in other settings. In general, sensitivity of culture is limited, around 50%[30] and a positive culture cannot discriminate between infection and colonisation,[31] but specificity does increase in immunocompromised patients.[32] In a prospective pilot study including 44 haematological and

non-haematological ICU patients, categorised according to AspICU criteria, BAL culture, PCR and GM (OD cut-off 1) and serum GM (OD cut-off 0.5) were compared for putative IPA.[33] Sensitivity (range) for BAL PCR, BAL GM and serum GM was 44% (13.7 - 78.8), 55.6% (21.2 - 86.3) and 33.3% (7.5-70.1) respectively. Specificity (range) for BAL PCR, BAL GM and serum GM was 94.3% (80.8 - 99.3), 94.3% (80.8 - 99.3) and 97.1% (85.1-99.9) respectively.

In our centre patients were screened for *Aspergillus* twice weekly and a BAL was performed if screening was positive, or if there was a clinical indication for a BAL. Although a BAL is a high risk procedure with formation of aerosols which some guidelines advise against,[19] we consider it necessary in the management of COVID-19 patients with positive *Aspergillus* screening results. By applying our diagnostic workflow we were able to withhold treatment in three of fifteen patients with positive screening (20%). These patients were only PCR positive in screening. Of these patients two improved clinically and were discharged from the ICU and one patient died. Although TA is considered to be a sample from the lower respiratory tract there is more chance of contamination by tube colonisation with micro-organisms from the oropharyngeal cavity. A recently published paper proposed a screening and diagnostic algorithm for CAPA that is similar to our proposed workflow.[24] In the algorithm-positive *Aspergillus* sputum or TA culture and or clinical deterioration should be an indication to perform a BAL.

In severely ill COVID-19 patients with positive *Aspergillus* screening results, withholding antifungal treatment based on only moderately validated algorithms is difficult in clinical practice. In our cohort treatment was withheld in five of six patients (2 probable CAPA, 2 colonisation and 1 unclassifiable patient) with positive screening because no clinical signs of CAPA were present of whom four (1 probable, 2 colonisation and 1 unclassifiable patient) had a favourable outcome.

Until there is more clarity about CAPA we will continue screening COVID-19 patients admitted to the intensive care of the LUMC by culture and PCR on TA. Especially now patients admitted to the intensive care for mechanical ventilation are treated with six mg of dexamethasone (equivalent of 37.5 mg prednisone) for a maximum duration of 10 days.[13, 14] Prolonged use of corticosteroids is a classical risk factor for IA.[15] If this puts COVID-19 patients on the ICU at an additional risk for IA remains to be studied. However, our screening in its current extensive form may be simplified for a number of reasons. The large amount of samples pose an extra workload on top of the regular and COVID-19 associated workload of the laboratory. Since 87% of patients had positive screening results in the first 14 days after ICU admission by PCR or culture, screening might only

be necessary in the first two weeks after ICU admission. Finally, screening was done with a combination of both PCR and culture, but screening by culture alone is a possibility. This would cut costs and spare equipment necessary for the PCR. The added value of blood and BAL GM testing seems limited but requires further study.

Due to limited clinical experience with CAPA and new insights during the COVID-19 pandemic, few BAL procedures were performed in the first months of the pandemic. Because of this clinical decision making could only be based on TA results. We argued that if both TA PCR and culture were positive there was a relevant possibility of actual infection with *Aspergillus* species and therefore patients were treated as CAPA in case of compatible signs and symptoms, but colonisation cannot be ruled out. Finally, for clinical information about the patients with positive screening and motivation to start antifungal treatment for example we had to rely on information registered in the EPR.

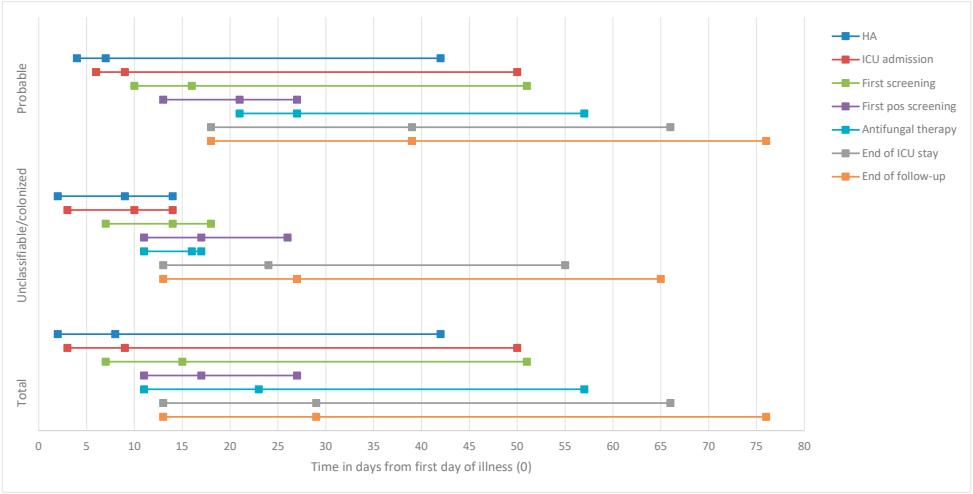
By screening for *Aspergillus* twice weekly by PCR and culture *Aspergillus* was detected in respiratory samples of nearly 25% of patients with COVID-19 admitted to the ICU. Probable CAPA was diagnosed in 17%. Clearly, positive culture, molecular detection (PCR) and/or antigen detection (GM) as evidence of presence of *Aspergillus* species, do not equal infection. Our findings emphasise that robust and validated definitions and histopathological studies are urgently needed as well as more information on the precise diagnostic performance of serum and BAL GM in this setting. Until we can better comprehend the clinical relevance of *Aspergillus* species detected in respiratory samples of COVID-19 patients, minimal-invasive methods like screening by TA can be performed to monitor patients. Positive screening results should be a trigger to perform a BAL for culture and, if possible, PCR.

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SUPPLEMENTAL MATERIAL



Supplemental Figure 1. Time to event (median and range) from first day of illness for different patient groups.
Time to event (median and range) in days from first day of illness for patients with probable CAPA (n=11) and unclassifiable/colonized patients (n=8). The outer squares represent the range and the inner square represents the median.
Abbreviations: CAPA, COVID-19 associated pulmonary aspergillosis; HA, hospital admission; ICU, intensive care unit; pos, positive.
First screening: first day of *Aspergillus* screening, day of sample collection; first positive finding: first positive finding of *Aspergillus* in screening: day that PCR or culture was positive; antifungal therapy: initiation of treatment with an antifungal agent; follow-up: end of follow-up until discharge from hospital, transfer to another hospital or death.
For one of the patients with probable CAPA the first day of illness was unknown, so we calculated results from hospital admission for the variables first screening, first positive finding, antifungal therapy and end of follow-up from first day of illness.

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20]

CAPA classification	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Age, years	59	74	66	70	62	70	59	52	59	51	74
Sex	Male	Female	Male	Male	Male	Female	Male	Male	Male	Male	Male
Host risk factors for IPA according to EORTC/MSGERC[15] or AspICU[16] criteria	None	None	None	None	None	None	None	None	None	None	None
Other relevant underlying illnesses	None	Thrombo-embolic event	Asthma	None	None	None	Chronic cardiac disease, diabetes mellitus, hypertension	Diabetes mellitus, hypertension,	Asthma, chronic pulmonary disease, diabetes mellitus	Asthma, diabetes mellitus	Chronic pulmonary disease
Time from FDI to HA, days	10	15	≥4[†]	9	7	FDI not mentioned	7	5	5	7	42
Time from FDI to ICU admission, days	11	18	≥8[†]	9	7	FDI not mentioned	7	7	6	10	50
1st day of screening from FDI (day of sample collection)	Day 20	Day 20	Day 14	Day 20	Day 21	Day 12 (from HA)	Day 16	Day 10	Day 16	Day 11	Day 51
1st day of pos culture and or PCR in screening from FDI (not day of collection, but of first pos result)	Day 22	Day 21	Day 15	Day 21	Day 27	Day 13 (from HA)	Day 16	NA	NA	NA	NA
Length ICU stay, days	55	27	17	16	44	18	22	38	20	29	12
End of ICU stay from FDI	66	45	25	25	51	18 (from HA)	29	45	26	39	62
End of follow-up from FDI[‡]	76	52	25	25	51	18 (from HA)	29	54	26	39	62
Day start mechanical ventilation from FDI	12	18	8	12	14	0 (from HA)	7	8	6	10	51

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Probable	Probable	probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
P/f ratio on day of intubation	19	20	13	13	17	Unknown	15	15	Unknown	Unknown	12
P/f ratio	19[§]	17[§]	18[§]	10[§]	18[§]	27[§§]	16[§§§]	20[§§§§]	17[§§§§]	20[§§§§]	12[§§§§]
Position	Supine, status after prone position[§]	Supine, status after prone position[§]	Prone[§]	Prone[§]	Prone[§]	Prone[§§]	Prone[§§§]	Prone[§§§§]	Prone[§§§§]	Prone[§§§§]	Supine, status after prone position[§§§§]
IS therapy	No	No	No	Methyl prednisone	No	No	Methyl prednisone	No	Prednisone	No	No
CRP mg/L	331.1[§]	345.7[§]	103.8[§]	283.7[§]	199.7[§]	51.8[§§]	124.7[§§§]	252[§§§§]	192[§§§§]	322.4[§§§§]	251[§§§§]
Leukocytes 10^9/L	26.34[§]	11.5[§]	41.33[§]	10.28[§]	26.77[§]	14.42[§§]	32.32[§§§]	15[§§§§]	8.59[§§§§]	24.74[§§§§]	9.95[§§§§]
Lymphocytes 10^9/L	2.19[§]	Not tested	0.99[§]	0.42[§]	Not tested	1.62[§§]	Not tested	1.5[§§§§]	0.67[§§§§]	0.77[§§§§]	0.78[§§§§]
CT-imaging[¶]	Ground glass, consolidations, crazy paving, PE, progression	Not available	Ground glass, consolidations, PE, progression	Consolidations, PE, 1st CT	Consolidations, tree-in-bud, cavity, progression	Ground glass, consolidations, PE, progression	Consolidations, PE, progression	Consolidations, nodules	Consolidations	Ground glass, consolidations, PE, progression	Ground glass, consolidations, progression
Antifungal therapy	Yes (day 25)	Yes (day 23)	Yes (day 20)	Yes (day 21)	Yes (day 28)	No	No	No	No	No	Yes (day 57)
Antifungal therapy spec	LAmB > voriconazole > LAmB	LAmB > voriconazole	LAmB	LAmB	LAmB	NA	NA	NA	NA	NA	LAmB

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Probable	Probable	probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Motivation for initiating or not initiating antifungal therapy	No/minimal clinical improvement and persistently high CRP levels. Pos cytological smear of BAL and BAL PCR pos. Restart LAmB because of pos PCR and strongly pos GM on BAL	Fever and rise in CRP levels. 2 of 3 BAL pos: 1x PCR and 1x GM	Prone position and mechanical ventilation with high pressure necessary for adequate gas exchange. Multiple TA PCR pos and BAL PCR and GM pos	Difficulty in maintaining adequate gas exchange with mechanical ventilation. TA and BAL PCR pos and BAL GM pos	Respiratory deterioration not responding to antibiotics. New findings on CT-scan, although not indicative of a fungal infection. TA PCR pos and BAL GM weakly pos	No clinical suspicion of IPA (respiratory stable/improvement and drop in CRP levels), differential diagnosis: colonisation	Mechanical ventilation with high pressure necessary for adequate gas exchange. TA PCR pos, but BAL negative. BAL GM result available on day of death of patient	No fever, decreasing CRP levels. Respiratory stable	Respiratory instable, CRP rise and fever after stopping flucloxacillin for VAP with *S. aureus*. Flucloxacillin was restarted. Expectant policy regarding pos GM due to restricted treatment policy because of severity of pulmonary damage	Respiratory deterioration with lots of sputum production, suspicion of a VAP. Clinical response to antibiotics was observed Expectant policy regarding pos GM. Follow-up diagnostics were performed, GM OD declined becoming negative in 10 days without antifungal treatment	Treatment with LAmB was initiated after positive GM, but was stopped after two days because culture and PCR of repeat diagnostics remained negative. GM was not repeated[&&&&]
Chloroquine	Yes	Yes	No	Yes	Yes	Yes	Yes	No	No	Yes	Yes
Remdesivir	No	No	No	No	No	No	No	Yes	No	No	No

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Probable	Probable	probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Antibacterial therapy for pulmonary infection (at the same time *Aspergillus* was detected)	No	No	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes
Antibacterial therapy spec			Cefuroxime		Penicillin/ metronidazole > meropenem > penicillin/ metronidazole > meropenem/vancomycin > clindamycin		Flucloxacillin > cefuroxime > flucloxacillin/ceftazidime	Cefuroxime	Flucloxacillin	Cefuroxime / gentamicin	Ceftazidime
Pulmonary bacterial infection (at the same time *Aspergillus* was detected)	No	No	HAP/VAP, culture: *K. pneumoniae* (previous not in BAL)	No	Lung abscess, culture: *S. constellatus*, throat flora	No	HAP, cultures: *S.aureus, H. influenzae, C. kosteri, E. coli* and *P. aeruginosa*	VAP, culture: *S. aureus*	VAP, culture: *S. aureus*	Suspicion of VAP, cultures: negative	HAP/VAP, culture: *P. aeruginosa*
SARS-CoV-2 PCR, ct-value	31.93[§]	22.64[§]	26.43[§]	23.14[§]	30.21[§]	29.3[§§]	24.06[§§§]	Neg[§§§§]	31.47[§§§§]	30.7[§§§§]	Neg[§§§§]
BAL culture	Pos (day 42); pos cytological smear of BAL (day 25)	Pos (day 26)	Pos (day 21)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Probable	Probable	probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Culture spec	*A. fumigatus*, VIPcheck™ S, MD: AMB 0.5, ISA 0.5, ITR 0.5, POZ 0.063, VOR 0.5	*A. fumigatus*, VIPcheck™ S, MD: AMB 0.5, ISA 0.5, ITR 0.5, POZ 0.063, VOR 0.25	*A. fumigatus*, VIPcheck™ S, MD: AMB 0.5, ISA 0.5, ITR 0.5, POZ 0.125, VOR 0.5								
BAL PCR	Pos (day 25)	Pos (day 23)	Pos (day 19)	Pos (day 21)	Pos (day 32)	Pos (day 15 from HA)	Neg	Neg	Neg	Neg	Neg
PCR spec	*A. fumigatus* complex, resistance PCR not successful due to low load	*A. fumigatus* complex, resistance PCR not performed due to low load	*A. fumigatus* complex, no mutations	*A. fumigatus* complex, resistance PCR not performed due to low load	*A. fumigatus* complex, resistance PCR not successful due to low load	*A. fumigatus* complex, resistance PCR not performed due to low load					
BAL PCR lowest ct-value	33.32	38.06	31	35.26	34.08	34.47	Neg	Neg	Neg	Neg	Neg
BAL GM	Pos	Pos	Pos	Pos	Pos	Not performed	Pos	Pos	Pos	Pos	Pos
BAL GM peak value	7.234 (day 41, performed once)	1.65 (day 23, performed once)	4.69 (day 19, performed once)	4.2 (day 21, performed once)	0.76 (day 21)	Not performed	5.66 (day 28)	3.8 (day 26)[&]	6.87 (day 20, performed once)[&&]	1.41 (day 24)[&&&]	6.15 (day 56, performed once)[&&&&]
TA culture	Pos (day 41)	Pos (day 21)	Pos (day 17)	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Probable	Probable	probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Culture spec	*A. fumigatus*, VIPcheck™ S, MD: AMB 0.5, ISA 0.5, ITR 0.5, POZ 0.063, VOR 0.5	*A. fumigatus*, VIPcheck™ S, MD: AMB 0.5, ISA 0.5, ITR 0.5, POZ 0.063, VOR 0.25	*A. fumigatus*, VIPcheck™ S, MD: AMB 0.5, ISA 0.5, ITR 0.5, POZ 0.063, VOR 0.5								
TA PCR	Pos (day 22)	Pos (day 22)	Pos (day 15)	Pos (day 21)	Pos (day 27)	Pos (day 13 from HA)	Pos (day 16)	Neg	Neg	Neg	Neg
PCR spec	*A. fumigatus* complex, resistance PCR not performed	*A. fumigatus* complex, no mutations	*A. fumigatus* complex, no mutations	*A. fumigatus* complex, resistance PCR not performed due to low load	*A. fumigatus* complex, resistance PCR not performed due to low load	*A. fumigatus* complex, resistance PCR not successful due to low load	*A. fumigatus* complex, resistance PCR not successful due to low load				
Total number of samples	20	8	7	5	20	3	13	20	5	19	9
Total number of BAL samples	4	3	2	2	8	1	5	3	2	7	3
Total number of TA screening samples (culture and PCR performed)	9	4	4	2	6	2	3	6	2	8	1
First positive screening sample	2nd sample	1st sample	1st sample	1st sample	1st sample	1st sample	1st sample	NA	NA	NA	NA
Number of follow-up screening samples that were positive	7	3	3	1	1	1	0	NA	NA	NA	NA

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Probable	Probable	probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable	Probable
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Number of BAL samples that were collected after positive screening/after positive BAL GM	4	3	2	2	6	1	5	2 BAL performed after pos BAL GM	0 (no BAL performed after pos BAL GM)	6 BAL performed after pos BAL GM	2 BAL performed after pos BAL GM
Number of BAL samples that were positive	3 (1: PCR pos, 2: culture, PCR and GM pos)	2 (1: PCR pos, 1: culture and GM pos)	2 (culture, PCR and GM pos)	2 (PCR and GM pos)	2 (PCR pos)	1 (PCR pos)	1 (GM pos)	1; none of the BAL follow-up samples were positive (BAL GM was only performed on one sample)	1; no BAL performed after pos BAL GM	4; 3 BAL follow-up samples were BAL GM pos	1; none of the BAL follow-up samples were positive (BAL GM was not performed)[&&&&]
Outcome	Alive	Alive	Death (day 25)	Death (day 25)	Death (day 51)	Alive	Death (day 29)	Alive	Death (day 26)[^]	Death (day 39)	Death (day 62)

CAPA classification	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Colonisation	Colonisation
Patient	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Age, years	65	72	78	77	63	60	64	71
Sex	Male	Female	Male	Male	Male	Female	Male	Female
Host risk factors for IPA according to EORTC/MSGERC[15] or AspICU[16] criteria	None	None	None	None	Recent lung transplantation. Treatment with mycophenolate mofetil, tacrolimus and prednisone	None	None	None
Other relevant underlying illnesses	Chronic cardiac disease	Asthma, malignant neoplasm, thromboembolic event	Chronic pulmonary disease, hypertension, thromboembolic event	Chronic cardiac disease, hypertension	Chronic pulmonary disease	Hypertension	Diabetes mellitus, hypertension	Coagulopathy, malignant neoplasm
Time from FDI to HA, days	9	8	9	2	10	14	7	7

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Colonisation	Colonisation
Patient	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Time from FDI to ICU admission, days	10	10	9	3	10	14	7	9
1st day of screening from FDI (day of sample collection)	Day 10	Day 15	Day 16	Day 7	Day 12	Day 18	Day 15	Day 10
1st day of pos culture and or PCR in screening from FDI (not day of collection, but of first pos result)	Day 12	Day 16	Day 17	Day 26	Day 14	Day 18	Day 19	Day 11
Length ICU stay, days	45	8	16	40	3	4	30	13
End of ICU stay from FDI	55	18	25	43	13	18	37	22
End of follow-up from FDI‡	65	18	25	55	13	18	53	28
Day start mechanical ventilation from FDI	10	10	12	3	13	14	7	9
P/f ratio on day of intubation	9	18	Unknown	Unknown	15	13	Unknown	16
P/f ratio	30§	17§	15§§§	27§§§	15§§§	7§§§	15§§§	22§§§
Position	Prone§	Prone§	Supine, status after prone position§§§	Prone§§§	Prone§§§	Prone§§§	Supine, status after prone position§§§	Prone§§§
IS therapy	No	No	No	No	No	No	No	No
CRP mg/L	111.9§	226.5§	83.3§§§	141.9§§§	99.6 (one day before culture was pos, transferred) §§§	> 20 (highly sensitive CRP) §§§	286.5§§§	424.9§§§
Leukocytes 10^9/L	11.17§	9.47§	10.18§§§	9.32§§§	1.85 (one day before culture was pos, transferred) §§§	11.42§§§	12.01§§§	16.67§§§
Lymphocytes 10^9/L	2.01§	0.62§	Not tested	Not tested	Not tested	Not tested	Not tested	1.16§§§

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Colonisation	Colonisation
Patient	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
CT-imaging[1]	Ground glass, consolidations, crazy paving, 1st CT	Ground glass, consolidations, PE, 1st CT	Ground glass, consolidations, 1st CT	X-ray lungs: consolidation. NB previous CT, but > 7 days ago	Ground glass, crazy paving, 1st CT	Ground glass, consolidations, crazy paving, 1st CT	Ground glass, consolidations, PE, 1st CT	Ground glass, consolidations, crazy paving, 1st CT
Antifungal therapy	Yes (day 11)	Yes (day 16)	Yes (day 17)	No	No	No	No	No
Antifungal therapy spec	LAmB > voriconazole + echinocandine > LAmB	LAmB	LAmB	NA	NA	NA	NA	NA
Motivation for initiating or not initiating antifungal therapy	Fever and rise in CRP levels. Gram stain TA with branching hyphae	Respiratory deterioration which could not only be explained by PE. TA culture and PCR pos	TA PCR pos. No suspicion of a fungal infection (respiratory stable/improvement and drop in CRP levels), but because of unfamiliarity of *Aspergillus* in COVID-19 patients antifungal treatment was initiated	No clinical suspicion of IPA (respiratory improvement)	Respiratory instability, no fever, rise in CRP levels. TA culture became pos after patient was transferred to another hospital	Poor oxygenation, fever, high CRP levels. TA PCR became pos after treatment was stopped	Fever and elevated inflammation parameters due to HAP with clinical improvement on antibiotics	Prone position and mechanical ventilation with high pressure necessary for adequate gas exchange. Respiratory deterioration in supine position. Rise in CRP levels. TA PCR pos, but BAL negative
Chloroquine	No	Yes	Yes	Yes	No	Yes	Yes	Yes
Remdesivir	Yes	No	No	No	No	No	No	No
Antibacterial therapy for pulmonary infection (at the same time *Aspergillus* was detected)	No	No	No	No	No	No	Yes	No
Antibacterial therapy spec							Vancomycin > cotrimoxazole	

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Colonisation	Colonisation
Patient	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Pulmonary bacterial infection (at the same time *Aspergillus* was detected)	No	No	No	No	No	No	HAP, culture: MRSA	No
SARS-CoV-2 PCR, ct-value	25.46[$]	24.8[$]	29.95[§§§]	Neg[§§§]	21.96[§§§]	14.34[§§§]	34.41[§§§]	27.01[§§§]
BAL culture	No BAL[$]	No BAL	No BAL	No BAL	No BAL	No BAL	Neg	Neg
BAL PCR	No BAL[$]	No BAL	No BAL	No BAL	No BAL	No BAL	Not performed	Neg
BAL GM	No BAL[$]	No BAL	No BAL	No BAL	No BAL	No BAL	Not performed	Neg
BAL GM peak value	No BAL[$]	No BAL	No BAL	No BAL	No BAL	No BAL	Not performed	0.07 (day 12, performed once)
TA culture	Pos (day 12)	Pos (day 17)	Pos (day 18)	Neg	Pos (day 14)	Neg	Neg	Neg
Culture spec	Two *A. fumigatus* isolates, VIPcheck™ S and R, MD 1: AMB 0.5, ISA >16, ITR 8, POZ 0.5, VOR >16, MD2 MD: AMB 0.5, ISA 0.5, ITR 0.25, POZ 0.063, VOR 0.5 AND *A. flavus*, AMB 1, ITR 0.25	*A. fumigatus*, VIPcheck™ S, MD not performed	*A. fumigatus*, VIPcheck™ S, MD: AMB 0.5, ISA 0.5, ITR 0.25, POZ 0.063, VOR 0.5		*A. fumigatus*, VIPcheck™ S, MD not performed			
TA PCR	Pos (day 12)	Pos (day 16)	Pos (day 17)	Pos (day 26)	Neg	Pos (day 18)	Pos (day 19)	Pos (day 11)
PCR spec	*A. fumigatus* complex, resistance PCR not performed due to low load[#]	*A. fumigatus* complex, no mutations	*A. fumigatus* complex, no mutations	*Aspergillus* species		*A. fumigatus* complex, resistance PCR not performed due to low load	*A. terreus*	*A. terreus*

Supplementary Table 1. Clinical information of patients with positive *Aspergillus* culture, PCR or galactomannan results on tracheal aspirate or bronchoalveolar lavage samples categorised according to the CAPA consensus definition.[20] (continued)

CAPA classification	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Not classifiable	Colonisation	Colonisation
Patient	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Total number of samples	15	2	5	11	1	2	10	4
Total number of BAL samples	0	0	0	0	0	0	1	1
Total number of TA screening samples (culture and PCR performed)	9	2	3	9	1	1	6	3
First positive screening sample	1st sample	1st sample	1st sample	7th sample	1st sample	1st sample	2nd sample	1st sample
Number of follow-up screening samples that were positive	4	1	2	0	0	0	1	0
Number of BAL samples that were collected after positive screening	0	0	0	0	0	0	1	1
Number of BAL samples that were positive	NA	NA	NA	NA	NA	NA	0	0
Outcome	Alive	Death (day 18)	Death (day 25)	Alive	Alive	Death (day 18)	Alive	Alive

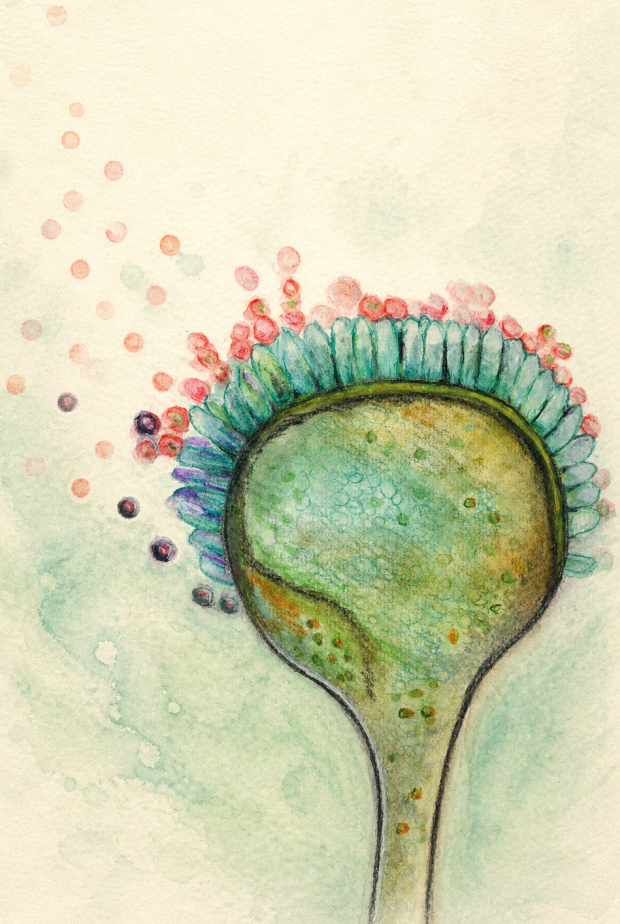
Abbreviations: 1st CT, 1st CT-scan; AMB, amphotericin B; BAL, bronchoalveolar lavage; CAPA, COVID-19 associated pulmonary aspergillosis; CRP, c-reactive protein; ct-value, cycle threshold value; FDI, first day of illness; GM, galactomannan; HA, hospital admission; HAP, hospital acquired pneumoniae; ICU, intensive care unit; IPA, invasive pulmonary aspergillosis; IS therapy, immunosuppressive therapy; ISA, isavuconazole; ITR, itraconazole; LAmB, liposomal amphotericin B; MRSA, methicillin-resistant *Staphylococcus aureus*; NA, not applicable; neg, negative; PE, pulmonary embolism; p/f ratio, Pa02/fi02 ratio in mmHg; POZ, posaconazole; pos, positive; R, resistant; S, sensitive; spec, specified; TA, tracheal aspirate; VAP, ventilator associated pneumoniae; VOR, voriconazole

† From 1st day of presentation, not hospital admission, FDI not mentioned. ‡ Follow-up until discharge from hospital, transfer to another hospital or death. § On day antifungal treatment was initiated, because of positive culture and or PCR of *Aspergillus* species. §§ On day PCR of BAL was positive, but invasive aspergillosis was deemed unlikely so treatment was not initiated. §§§ On day PCR OR culture of tracheal aspirate was positive (followed by negative culture/PCR of BAL OR no BAL if invasive aspergillosis was deemed unlikely OR no BAL because patient was lost to follow-up). §§§§ On day BAL GM was positive. ¶ CT-imaging: up to one week before positive culture and or PCR of *Aspergillus* species. & BAL GM was negative in a follow-up BAL sample collected two days later (GM OD 3.8 > 0.11). && Last available BAL sample. &&& BAL GM became negative within 10 days (GM OD 1.41 > 1.19 > 0.9 > 0.55 > 0.03). &&&& BAL GM of the follow-up sample collected two days later was tested in retrospect and was negative (GM OD 0.138). Serum GM performed on the same day was negative. ^ No histological evidence of IA was described in the autopsy report. Cause of death: severe COVID-19 pneumonia, complicated by a superinfection with *S. aureus*. $ BAL sampling was performed after multiple positive TA screening samples and after treatment was initiated, so the BAL was not considered reliable for classification. # A T289A/Y121F mutation was detected in two TA screening samples that were PCR positive on day 17 and 21.

Supplementary Table 2. Concordance of sequentially collected tracheal aspirate and bronchoalveolar lavage samples.

	BAL		
TA	**+**	**-**	**Concordance**
Culture			
+	3[†]	0	
-	0	28[‡]	
			31/31 (100%)
PCR			
+	6[§]	3[¶]	
-	2[&]	13[^]	
			19/24 (79%)

Abbreviations: +, positive; -, negative; BAL, bronchoalveolar lavage; CAPA, COVID-19 associated pulmonary aspergillosis; GM, galactomannan; TA, tracheal aspirate
[†] Samples from three patients with probable CAPA. [‡] Samples from 17 patients; 5 negative, 1 unclassifiable, 2 colonisation and 9 probable CAPA. Of the patients with probable CAPA, four were only BAL GM positive, three were only BAL PCR positive, one was TA PCR positive and BAL GM positive and one patient was culture-positive in a later collected BAL sample. [§] Samples from five patients; 4 patients with probable CAPA and 1 colonisation. [¶] Samples from three patients; 1 colonisation and 2 probable CAPA. Of the patients with probable CAPA, one patient was BAL GM positive in a later collected BAL sample and one patient was culture-positive in a later collected BAL sample. [&] Follow up samples from a patient with probable CAPA who was TA PCR positive in an earlier sample. [^] Samples from eight patients; 3 negative and 5 probable CAPA. Of the patients with probable CAPA, three were only BAL GM positive, one was TA PCR positive and BAL GM positive and one was BAL PCR positive in an earlier sample.



6

Prospective and systematic screening for invasive *aspergillosis* in the ICU during the COVID-19 pandemic, a proof of principle for future pandemics



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ABSTRACT

The diagnostic performance of a prospective, systematic screening strategy for COVID-19 associated pulmonary aspergillosis (CAPA) during the COVID-19 pandemic was investigated. Patients with COVID-19 admitted to the ICU were screened for CAPA twice weekly by collection of tracheal aspirate (TA) for *Aspergillus* culture and PCR. Subsequently, bronchoalveolar lavage (BAL) sampling was performed in patients with positive screening results and clinical suspicion of infection. Patient data were collected from April 2020–February 2022. Patients were classified according to 2020 ECMM/ISHAM consensus criteria. In total, 126/370 (34%) patients were positive in screening and CAPA frequency was 52/370 (14%) (including 13 patients negative in screening). CAPA was confirmed in 32/43 (74%) screening positive patients who underwent BAL sampling. ICU mortality was 62% in patients with positive screening and confirmed CAPA, and 31% in CAPA cases who were screening negative. The sensitivity, specificity, positive and negative predictive value (PPV & NPV) of screening for CAPA were 0.71, 0.73, 0.27, and 0.95, respectively. The PPV was higher if screening was culture positive compared to PCR positive only, 0.42 and 0.12 respectively. CAPA was confirmed in 74% of screening positive patients, and culture of TA had a better diagnostic performance than PCR. Positive screening along with clinical manifestations appeared to be a good indication for BAL sampling since diagnosis of CAPA was confirmed in most of these patients. Prospective, systematic screening allowed to quickly gain insight into the epidemiology of fungal superinfections during the pandemic and could be applicable for future pandemics.

INTRODUCTION

COVID-19 associated pulmonary aspergillosis (CAPA) has been described as a complication of COVID-19 in critically ill patients admitted to the intensive care unit (ICU). Despite uncertainties regarding CAPA classification and pathogenesis,[1] most reports show that COVID-19 patients with CAPA have high mortality rates compared to patients without CAPA.[2-4] Since the start of the COVD-19 pandemic a CAPA screening programme was implemented in the ICU of our hospital consisting of twice weekly culture and PCR on tracheal aspirate (TA) as a routine procedure. The screening programme for CAPA was evaluated after the first COVID-19 peak (April 2020–May 2020) and was found to be a feasible and simple method to monitor patients.[5] Since the first peak many changes have taken place in both viral virulence and therapeutic management of COVID-19 in ICU patients.

Immunosuppressive agents now play an important role in the treatment of patients with COVID-19 including corticosteroids and interleukin-6 Inhibitors (anti-IL-6). Treatment with immunosuppressants is a known risk factor for invasive aspergillosis.[6] Therefore, an increased frequency of CAPA could be expected in COVID-19 periods when ICU patients were treated with immunosuppressive agents. At the same time SARS-CoV-2 variants have appeared with different virulence profiles.[7-9]

Therefore, the purpose of this study was to investigate the yield of screening for CAPA and the frequency of CAPA in the ICU over a 2-year period in which medical management of COVID-19 changed and new SARS-CoV-2 variants appeared. Also, the diagnostic performance of various diagnostic tests for CAPA screening and diagnosis in relation to mortality were investigated. This evaluation may aid in designing fungal infection monitoring programmes during future pandemics.

METHODS

Study population

All adult patients with COVID-19 admitted to the ICU in the Leiden University Medical Center (LUMC, Leiden, The Netherlands) were screened for CAPA twice weekly by collecting TA for *Aspergillus* culture and PCR (Figure 1). Confirmation bronchoalveolar lavage (BAL) sampling was performed in patients with positive screening results if clinically indicated (respiratory deterioration not caused by a pulmonary embolism) and feasible (depending on oxygen saturation levels). *As-*

pergillus culture, PCR and in some cases galactomannan (GM) were performed on BAL samples. The screening programme was implemented as temporary routine care.

From September 2020 patients with COVID-19 were treated with dexamethasone and from January 2021 with dexamethasone and tocilizumab.[10] In patients who deteriorated with an unknown cause, hyperinflammation was suspected and these patients were mostly treated with methylprednisolone.

Culture, susceptibility testing, PCR, resistance PCR and GM

Methods of culturing, susceptibility testing, (resistance) PCR testing, and GM testing have been described in a previous manuscript.[5] In short, TA and BAL samples were inoculated on various agar plates and incubated for 2–10 days. Undiluted samples were inoculated onto culture media, using a one µl sterile inoculation needle. For TA samples, this was done directly, for BAL samples this was performed after sedimentation of undiluted sample. Triazole resistance screening was performed with VIPcheck™ and MIC testing was performed with the microbroth dilution method according to EUCAST. The PCR (AsperGenius®, PathoNostics®, Maastricht, The Netherlands) was performed for detection of *Aspergillus fumigatus* complex, *Aspergillus terreus* and *Aspergillus* species, and TR34/L98H and Y121F/T289A resistance mutations. Galactomannan testing was performed using the Platelia™ *Aspergillus* Ag (Bio-rad laboratories, Marnes-la-Coquette, France).

Classification of patients (Figure 1)

CAPA classified according to the 2020 ECMM/ISHAM consensus criteria[11] was considered the gold standard. Patients were classified according to the 2020 ECMM/ISHAM consensus criteria,[11] with one minor modification: all positive BAL PCR results were considered positive irrespective of cycle threshold value. Patients were considered colonised with *Aspergillus* if TA samples were *Aspergillus* positive, but confirmatory BAL samples remained negative. Patients were considered unclassifiable if screening was positive but no BAL was performed. A BAL was not performed in patients in whom CAPA was not suspected at all or in patients who were too ill for bronchoscopy. When comparing patients with CAPA to patients without CAPA, the group of unclassifiable and colonised patients were included in the no CAPA group. According to our local screening strategy, BAL sampling was performed in patients with a clinical suspicion of CAPA, and therefore unclassifiable patients were included in the group of patients with no CAPA. A BAL performed within 3 days after positive screening was considered a confirmatory BAL. In our centre non-bronchoscopic lavages were not performed, thus patients could not be classified as possible CAPA.

Data collection

From the laboratory information system (LIMS) data were extracted about culture, susceptibility, PCR and GM results from April 2020 until February 2022. Clinical data about age, sex, admission to the ICU of the Leiden University Medical Center, and mortality were extracted from electronic hospital databases.

SARS-CoV-2 variants

To describe the dominant variant at different time points, data from national[7] and local pathogen surveillance, and SNP typing were investigated. Structural pathogen surveillance has been performed since June 2021. Before then, SNP typing was performed for research purposes.[12]

Ethics

The study was approved by the hospitals' Institutional Review Board, the COVID-committee (CoCo): CoCo 2020-044. Patients were only included if they had consented via an approved opt-out procedure active in our institution. If patients were not able to consent because they were intubated, the opt-out procedure for clinical data for the National Intensive Care Evaluation (NICE) was applied.

Statistical analyses

Screening outcome, CAPA frequency and concordance between screening outcome and CAPA classification were the primary study outcome parameters. This was assessed by sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of screening for CAPA. Secondary outcomes included time to event (ICU admission until collection of TA and until first positive TA or BAL sample), ICU mortality, and 60-day mortality.

Categorical variables were described as numbers and percentages per category, numerical continuous variables were described as medians and interquartile ranges. Categorical data were compared with the Fisher's exact test or Chi-square test depending on sample size and numerical data were compared with the Mann Whitney U-test (two groups) or Kruskal-Wallis test (> two groups). Proportion confidence intervals were calculated with the Wilson score interval. Kaplan-Meier survival curves for 60-day mortality were constructed and groups were compared using the log-rank test. ICU mortality was defined as in-hospital ICU mortality (31 patients, of whom 3 were classified as CAPA, were transferred to another ICU and censored at discharge). Statistical analyses were performed with IBM SPSS Statistics for Windows version 25.0.

RESULTS

Screening outcome and CAPA frequency

From April 2020 until February 2022 370 patients were routinely screened for CAPA from a total number of 424 patients with COVID-19 admitted to the ICU (Figure 1). The cohort consisted out of 270 (73%) males and median age was 62.5 years (IQR: 54–70). Of the patients who were screened 126/370 (34.1%) were positive in screening. Patients positive in screening were older than patients negative in screening (64.5 vs 60 years, P = 0.037). The median time from ICU admission at our medical centre until collection of first TA sample was one day and until collection of the first positive TA was 3 days. A BAL was performed within 3 days of positive screening in 43/126 (34.1%) of these patients and CAPA was confirmed in 32/43 (74.4%) (Figure 2).

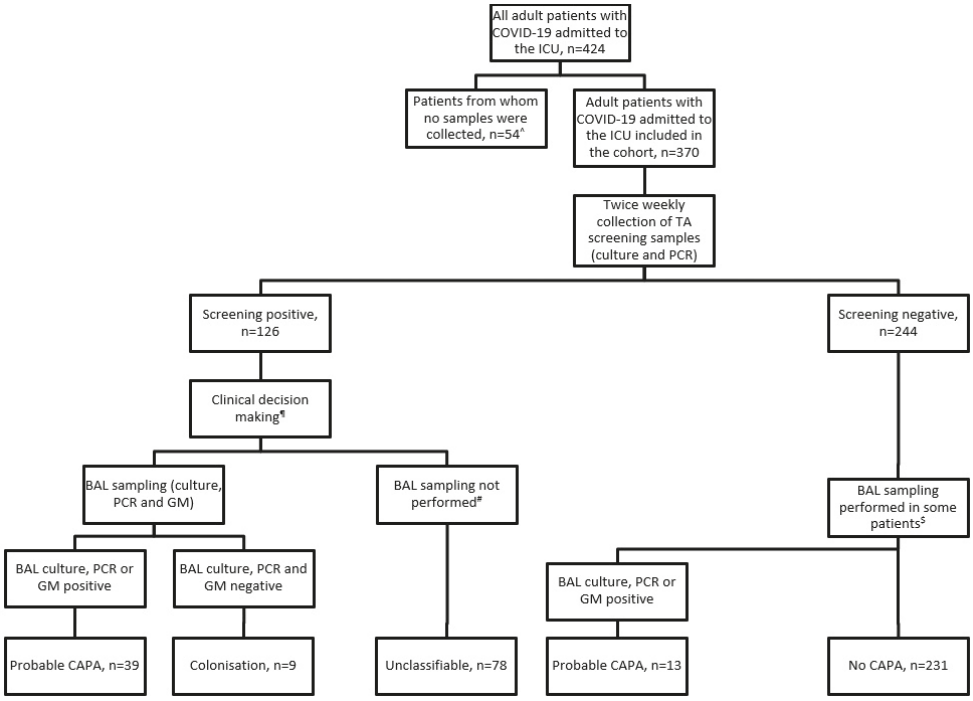


Figure 1. Screening method for detection of CAPA*.

*According to 2020 ECMM/ISHAM consensus criteria.[11] ¶ Clinical decision-making was based on a combination of screening results and clinical manifestations (respiratory deterioration or clinical/radiological signs of infection). # BAL sampling was not performed if patients improved clinically, if there was another explanation for respiratory deterioration (pulmonary embolism for example), or if patients died. $ BAL sampling was performed in patients with negative screening if there was a clinical indication to perform a BAL (respiratory deterioration). ^ Of the 54 patients not included in the cohort median ICU stay was 3 days (IQR 2-5) and reason for discharge was transfer to another hospital n=13, discharge to nursing department n=33 or home n=1, or death n=7. BAL: bronchoscopic alveolar lavage; CAPA: COVID-19 associated pulmonary aspergillosis; GM: galactomannan; TA: tracheal aspirate

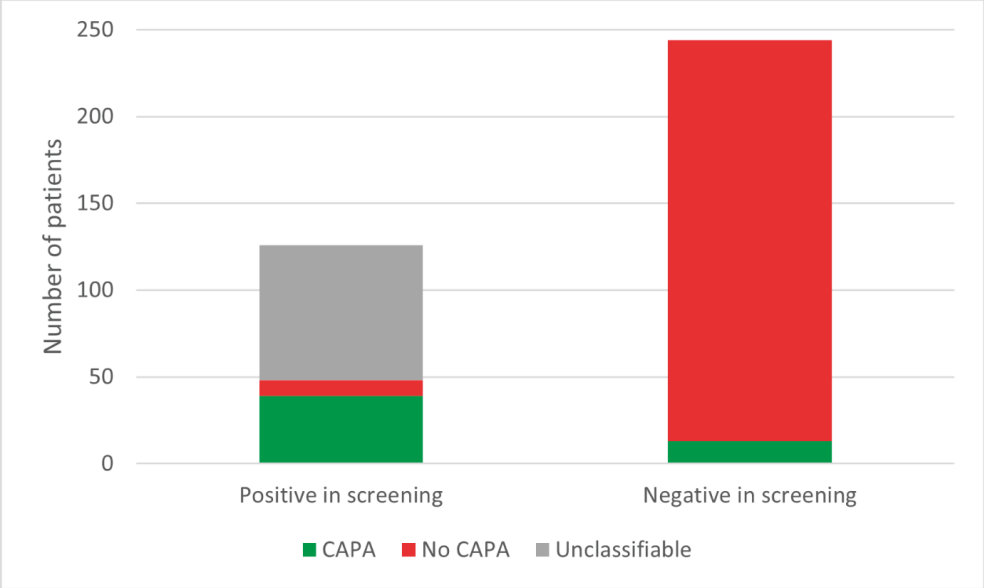


Figure 2. Screening outcome and CAPA* classification.
* According to 2020 ECMM/ISHAM consensus criteria.[11] CAPA: COVID-19 associated pulmonary aspergillosis, screening: screening by collection of tracheal aspirate for culture and PCR. Patients were considered unclassifiable if screening was positive but no BAL was performed. Of note: CAPA was diagnosed in 32/43 (74.4%) of patients in whom BAL was performed within three days, in 32/126 (25.4%) of screening positive cohort, and in 32/370 (8.6%) of total cohort.

Overall CAPA frequency was 52/370 (14.1%), and 32/52 (62%) cases were diagnosed through the screening programme. CAPA cases not detected by screening had a positive BAL sample obtained more than 3 days after a positive TA sample (seven patients) or were negative in screening (13 patients).

Influence of CAPA treatment regimen and viral subtype on screening outcome

Figure 3 depicts screening outcome and CAPA classification[11] by COVID-19 period based on viral subtype dominance and standard treatment regimen (see also Supplemental Table S1). The frequency of screening positive patients was higher in COVID-19 periods when patients were treated with immunosuppressive agents (from June 2020 onwards), but not statistically significant (P = 0.451). Frequency of CAPA varied from 9.7–18.6% in the different COVID-19 periods without a statistically significant difference between the groups (proportion CAPA vs no CAPA/unclassifiable patients, P = 0.385).

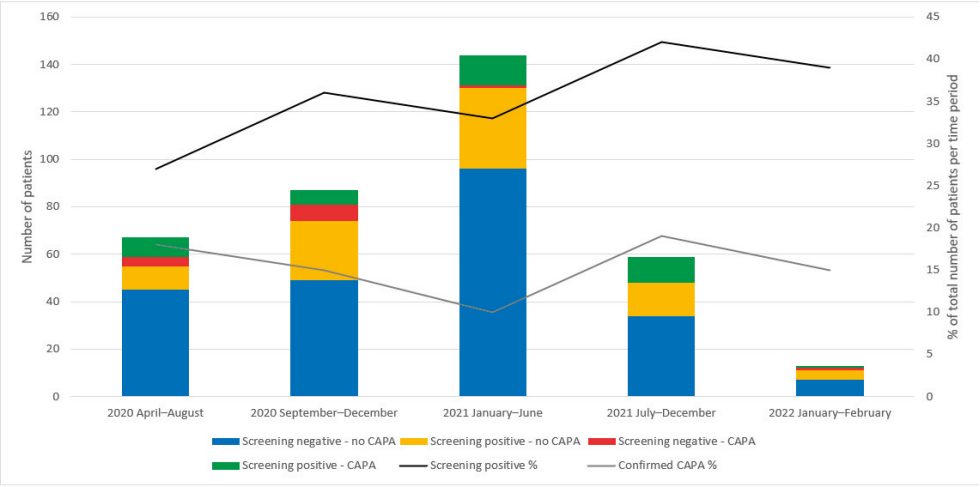


Figure 3. Screening outcome and CAPA* classification per COVID-19 period¶.
* According to 2020 ECMM/ISHAM consensus criteria.[11] ¶ Based on viral subtype dominance and standard treatment regimen: 2020 April-August [baseline], 2020 September-December [corticosteroids], 2021 January-June [viral subtype Alpha, corticosteroids + anti-IL-6], 2021 July–December [viral subtype Delta, corticosteroids + anti-IL-6], 2022 January–February [viral subtype Omicron BA1, corticosteroids + anti-IL-6]. Anti-IL-6: interleukin-6 Inhibitors, CAPA: COVID-19 associated pulmonary aspergillosis, screening: screening by collection of tracheal aspirate for culture and PCR. The group 'screening positive - no CAPA' consisted out of patients in whom CAPA was ruled out by negative BAL results n = 9 and patients who were unclassifiable n = 78.

Diagnostic value of screening

In the patients with a positive TA sample, PCR was positive more often than culture (91% and 51%, respectively) (Supplemental Table S2). The PPV and NPV of screening can be found in Table 1. The PPV and NPV of screening for different hypothetical attack rates of CAPA can be found in Supplemental Table S3. CAPA cases negative in screening were most often only GM positive in BAL (11 of 13 cases).

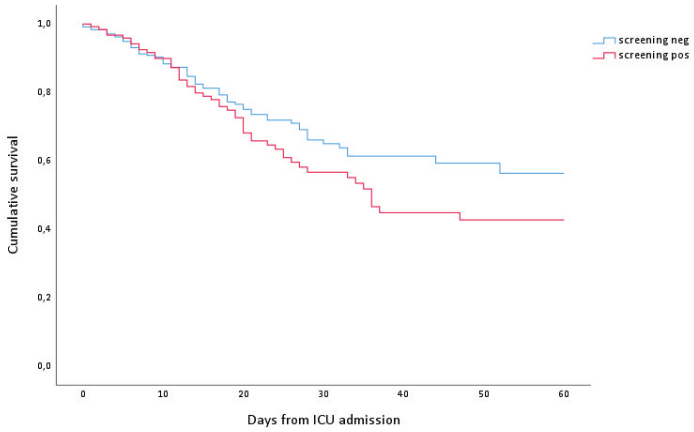
Screening and mortality

Overall ICU mortality was 119/423 (28.1%) and mortality in patients who were screening positive was 49/126 (38.9%). ICU mortality in patients with confirmed CAPA was 28/52 (53.8%) and was higher than patients with no CAPA/unclassifiable patients 84/318 (26.4%), $P < 0.001$. ICU mortality in the CAPA population missed by TA screening was 4/13 and was lower than mortality in patients with CAPA detected by screening (31% vs 62% $P = 0.054$, Supplemental Table S4). Kaplan-Meier survival curves of patients positive or negative in screening and patients with or without CAPA are depicted in Figure 4. ICU mortality in patients with no CAPA and unclassifiable patients was similar (61/240 (25.4%) vs 23/78

Table 1. Sensitivity, specificity, positive and negative predictive value of screening for CAPA* with TA.

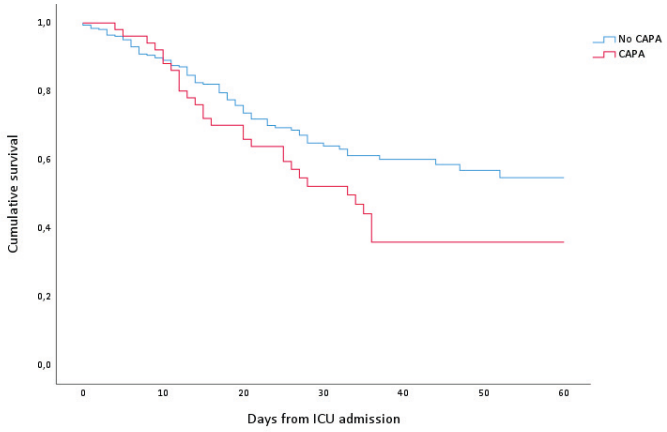
	CAPA, n (%)	No CAPA, n (%)	Total, n (%)	Sensitivity	Specificity	PPV	NPV
Screening total							
Screening positive	32 (71)	87 (27)	119 (33)				
Screening negative	13 (29)	231 (73)	244 (67)				
Total	45 (100)	318 (100)	363 (100)	32/45, 0.71	231/318, 0.73	32/119, 0.27	231/244, 0.95
Screening culture positive							
Screening positive	24 (65)	33 (13)	57 (19)				
Screening negative	13 (35)	231 (88)	244 (81)				
Total	37 (100)	264 (100)	301 (100)	24/37, 0.65	231/264, 0.88	24/57, 0.42	231/244, 0.95
Screening only PCR positive							
Screening positive	8¶ (38)	54 (18)	62 (20)				
Screening negative	13 (62)	231 (81)	244 (80)				
Total	21 (100)	285 (100)	306 (100)	8/21, 0.38	231/285, 0.81	8/62, 0.12	231/244, 0.95

* According to 2020 ECMM/ISHAM consensus criteria.[11] ¶Of the eight patients who were only PCR screening positive, three had a culture positive TA sample at another moment and in the remaining five TA were never culture positive. CAPA: COVID-19 associated pulmonary aspergillosis, NPV: negative predictive value, PPV: positive predictive value, TA: tracheal aspirate.
A BAL performed within three days after positive screening was considered a confirmatory BAL for CAPA diagnosis. Patients classified as CAPA with a positive BAL sample collected at another moment were excluded from the analysis (screening positive with a negative confirmatory BAL sample (n=2) or no BAL collected within three days after positive screening (n=5)).



	Day 0-10	Day 10-20	Day 20-30	Day 30-40	Day 40-50	Day 50-60	Day 60
Screening negative (number/ death/censored)	244/22/38	184/24/55	105/12/33	60/4/19	37/1/14	22/1/7	14/0/14
Screening positive (number/ death/censored)	126/12/11	103/18/20	65/13/15	37/7/4	26/1/7	18/0/5	13/0/13

Figure 4a. Life table and Kaplan-Meier survival curves of patients who were positive and negative in screening (60-day mortality from ICU admission).
Neg: negative, pos: positive.



	Day 0-10	Day 10-20	Day 20-30	Day 30-40	Day 40-50	Day 50-60	Day 60
No CAPA (number/death/ censored)	318/30/47	241/31/74	136/17/43	76/5/21	50/2/18	30/1/9	20/0/20
CAPA (number/death/ censored)	52/4/2	46/11/1	34/8/5	21/6/2	13/0/3	10/0/3	7/0/7

Figure 4b. Life table and Kaplan-Meier survival curves of patients classified as CAPA* and patients without CAPA or unclassifiable patients (60-day mortality from ICU admission). * According to 2020 ECMM/ISHAM consensus criteria.[11]

(29.5%), P = 0.479), whilst a difference was found in patients with CAPA and unclassifiable patients (28/52 (53.8%) vs 23/78 (29.5%), P = 0.005). Kaplan-Meier survival curves of patients with or without CAPA or unclassifiable patients are depicted in Supplemental Figure S1.

DISCUSSION

From April 2020 until February 2022 34% of COVID-19 ICU patients in our hospital were culture or PCR positive in CAPA screening. In approximately 1/3 of patients a BAL was performed within 3 days after positive screening results and CAPA was confirmed in most of these patients (32/43 (74%)). Overall CAPA frequency was 14%. COVID-19 treatment regimen or viral subtype had no effect on screening outcome or CAPA frequency.

Screening for CAPA had a high NPV of 0.95. The overall PPV was 0.27 and was higher for culture-positive TA screening samples (with or without positive PCR) than for PCR positive only TA screening samples (0.42 and 0.12, respectively). Based on our findings, screening by culture alone seems acceptable. The PPV of a positive TA culture was 0.58 in another study,[13] but definitions for CAPA included possible cases based on TA GM as well, complicating a comparison. Also, predictive values are by definition dependent on disease prevalence in a population. The observed CAPA attack rate of 14% was similar to previous (Dutch) studies,[2, 14-16] but higher than the prevalence reported in a systemic review and meta-analysis (10% (95%-CI: 8–13%) with wide variation among studies (0–34%)).[4] Hence, local validation of a screening strategy is mandatory. Screening by culture is a cheaper alternative than screening by PCR, but the time-to-result will be reduced by PCR.

Given the fact that *Aspergillus* and other invasive fungal infections can complicate various viral respiratory diseases, we calculated NPV and PPV of our screening strategy for different hypothetical frequencies of IA following viral infection (see Supplemental Table S3). However, our screening strategy included clinical decision making regarding BAL follow up diagnostics, which was influenced by increasing knowledge about the disease. We acknowledge that this factor can be variable between hospitals, even within a country and also over time during a pandemic.[2] Therefore, our strategy cannot simply be adopted to screen for IA in the context of viral pandemic preparedness. The high percentage of confirmed CAPA (74% of patients undergoing confirmatory testing) demonstrates that in most patients the indication for a BAL was correct. Screening might reduce the

indications for BAL sampling. However, the effect on reduction of BAL sampling is unknown since there was no comparator arm.

Most CAPA patients missed by screening were only BAL GM positive (11/13). Diagnosis of CAPA in this group remains uncertain given that mortality was low compared to patients with BAL culture and/or PCR confirmed CAPA (33%, 61%, and 64%, respectively). Nevertheless, diagnosis of CAPA even remains uncertain in patients with culture and/or PCR confirmed CAPA as histopathological evidence of CAPA is limited. Lower mortality in patients with only BAL GM confirmed CAPA has been observed before[2] questioning this diagnostic marker for CAPA if it is the only positive test result. No association was found between BAL GM concentration and 30-day ICU mortality in a study that compared *Aspergillus* test profiles of CAPA patients and controls.[17] Another study showed that BAL GM was often found as an isolated marker in patients with CAPA and poorly repeatable in sequential samples.[18] Whether a GM only result is a consequence of test aspecificity or a marker of low fungal burden is unknown.

Whether screening results in lower mortality due to earlier diagnosis of CAPA cannot be concluded given the observational nature of our study. In a multicentre study, that compared hospitals with a pre-emptive screening strategy for CAPA to those with a reactive diagnostic strategy, no benefit of screening was observed.[2] However, as it was a retrospective study screening protocols varied per hospital and because it was observational, bias or confounding is likely. Furthermore, mortality is high in patients with COVID-19 with CAPA, but determining the attributability of IA in the cause of death remains difficult.[19]

Moreover, in the case of CAPA, there is debate about the clinical entity itself.[20] This is due to the variably reported attack rate of CAPA,[1, 4, 20] the limited histopathological evidence of IA[21, 22] and the observation that treatment with an antifungal agent did not improve the outcome in CAPA cases.[2, 23] A number of studies have suggested that CAPA should probably be seen as a disease with a continuous spectrum from colonisation to tissue invasion and angioinvasion in which multiple factors contribute to *Aspergillus* becoming invasive.[17, 24] However, distinguishing CAPA patients with IPA from those without IPA is challenging.[1] Besides knowledge on local epidemiology and clinical manifestations, combination testing might improve diagnosis of CAPA especially if tests show concordant results.[1, 18] For the purpose of research standardisation, the 2020 ECMM/ISHAM consensus criteria[11] were urgently needed, but an improvement based on recent findings may be warranted given the limited specificity of BAL GM. Interestingly, after large scale COVID-19 vaccination, patients with CAPA are more likely to have an EORTC/MSGERC host factor for IA.[25]

The strength of our study is that our screening and confirmation strategy was unchanged over time, after some reluctance to perform BAL sampling in the first period. Hence, we present a reliable overview of frequency of positive screening and confirmed CAPA over time. Our study has a number of limitations. Firstly, our cohort includes patients who were unclassifiable because no BAL sampling was performed. We assumed that this wasn't done because there was no clinical CAPA suspicion and these patients were included in the group not classified as having CAPA but this may not be true in all cases. Secondly, the COVID-19 periods were based on viral subtype dominance and SARS-CoV-2 treatment regimens in that period. Clinical information was limited so on patient level exact treatment for COVID-19 and SARS-CoV-2 variant were unknown.

In conclusion, screening for CAPA by regular and systematic collection of TA is a feasible and simple bedside method to monitor COVID-19 patients admitted to the ICU and can be performed by culture alone. A pre-emptive screening strategy for CAPA or other fungal superinfections of severe viral respiratory tract infections should include screening results in combination with clinical decision making, and should ideally be standardised. Further research is necessary to improve accuracy of CAPA diagnosis and to evaluate whether screening improves survival.

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Supplemental table 1. Screening positive and CAPA confirmed cases by COVID-19 period, viral subtype dominance and standard treatment regimen.

Period	2020 April–August	2020 September–December	2021 January–June	2021 July–December	2022 January–February	TOTAL
Viral subtype dominance			Alpha	Delta	Omicron BA1	NA
Standard treatment regimen [10]	None	Corticosteroids	Corticosteroids + anti-IL-6	Corticosteroids + anti-IL-6	Corticosteroids + anti-IL-6	NA
Number of patients who were screened	67	87	144	59	13	370
Number of patients who were screening positive	18	31	47	25	5	126
% screening positive of patients screened	26.9	35.6	32.6	42.4	38.5	34.1
Estimated probability of % screening positive patients with 95%-CI[¶]	28.1 (17.7 – 38.5)	36.2 (26.4 – 46.1)	33.1 (25.5 – 40.7)	42.8 (30.6 – 55.1)	41.1 (17.7 – 64.5)	34.2 (29.4 – 39.0)
Number of patients with BAL performed within 3 days of positive screening	8	6	15	12	2	43
% BAL performed of screening positive patients	44.4	19.4	31.9	48.0	40.0	34.1
Number of patients with confirmed CAPA* from screening positive patients[#]	5	4	12	10	1	32
% confirmed CAPA* of patients with positive screening	27.8	12.9	25.5	40.0	20.0	25.4
Number of patients with confirmed CAPA* total	12	13	14	11	2	52
% confirmed CAPA* total of patients screened	17.9	14.9	9.7	18.6	15.4	14.1
Estimated probability of % confirmed CAPA* total of patients screened with 95%-CI[¶]	19.7 (10.6 – 28.8)	16.4 (8.9 – 23.9)	10.8 (5.9 – 15.7)	20.6 (10.7 – 30.3)	23.3 (4.3 – 42.2)	14.4 (10.9 – 17.8)
Number of deaths in ICU cohort (%)	18/67 (26.9)	29/87 (33.3)	41/144 (28.5)	18/59 (30.5)	6/13 (46.2)	112/370 (30.3)
Number of deaths in ICU in confirmed CAPA* patients (%)	7/12 (58.3)	4/13 (30.8)	9/14 (64.3)	7/11 (63.6)	½ (50)	28/52 (53.8)

* According to 2020 ECMM/ISHAM consensus criteria.[11] [¶] 95%-CI calculated with Wilson score interval. [#] Of whom the BAL was performed within 3 days of positive screening. Anti-IL-6: interleukin-6 inhibitors, BAL: broncho-alveolar lavage, CAPA: COVID-19 associated pulmonary aspergillosis, 95%-CI: 95% confidence interval, ICU: intensive care unit, screening: screening by collection of tracheal aspirate for culture and PCR.

Supplemental table 2. Patients with positive TA or BAL samples.

	Patients (n, %)
Number of patients who were TA screening positive	126
Screening PCR positive (% of screening positive)	115 (91)
Screening culture positive (% of screening positive)	64 (51)
BAL total positive	52
BAL GM positive (% of BAL total positive)	38 (73)
BAL PCR positive (% of BAL total positive)	34 (65)
BAL culture positive (% of BAL total positive)	23 (44)

Anti-IL-6: interleukin-6 inhibitors, BAL: broncho-alveolar lavage, GM: galactomannan, ICU: intensive care unit, TA: tracheal aspirate.
Of note: resistance associated mutations (TR46/T289A/Y121F) were detected in two TA samples, both from the same patient in whom the resistant *A. fumigatus* isolate was also found by culture. This patient was classified as CAPA based on a positive BAL GM. In this cohort, overall triazole resistance was one in 63 patients (1.6%) who were TA or BAL culture positive (patients with and without CAPA) and one in 32 patients (3.1%) with CAPA who were TA or BAL culture positive. From two patients with CAPA two other fungi were cultured besides *Aspergillus*: *Paecilomyces* species (considered colonisation) and *Lichtheimia ramosa* (considered infection).

Supplemental table 3. Sensitivity, specificity, positive and negative predictive value of screening for CAPA* with TA for different attack rates of CAPA.

	CAPA	No CAPA	Total	Sensitivity	Specificity	PPV	NPV
CAPA frequency 1%							
Screening total							
Screening positive	2.58	97.03	99.61				
Screening negative	1.05	262.34	263.39				
Total	3.63	359.37	363	0.71	0.73	0.03	1.00
Screening culture positive							
Screening positive	1.96	35.76	37.72				
Screening negative	1.05	262.23	263.28				
Total	3.01	297.99	301	0.65	0.88	0.05	1.00
Screening only PCR positive							
Screening positive	1.16	57.56	58.72				
Screening negative	1.90	245.38	247.28				
Total	3.06	302.94	306	0.38	0.81	0.02	0.99
CAPA frequency 5%							
Screening total							
Screening positive	12.89	93.11	106				
Screening negative	5.26	251.74	257				
Total	18.15	344.85	363	0.71	0.73	0.12	0.98
Screening culture positive							
Screening positive	9.78	34.31	44.10				
Screening negative	5.27	251.64	256.90				
Total	15.05	285.95	301	0.65	0.88	0.22	0.98
Screening only PCR positive							
Screening positive	5.81	55.23	61.05				

Supplemental table 3. Sensitivity, specificity, positive and negative predictive value of screening for CAPA* with TA for different attack rates of CAPA. (continued)

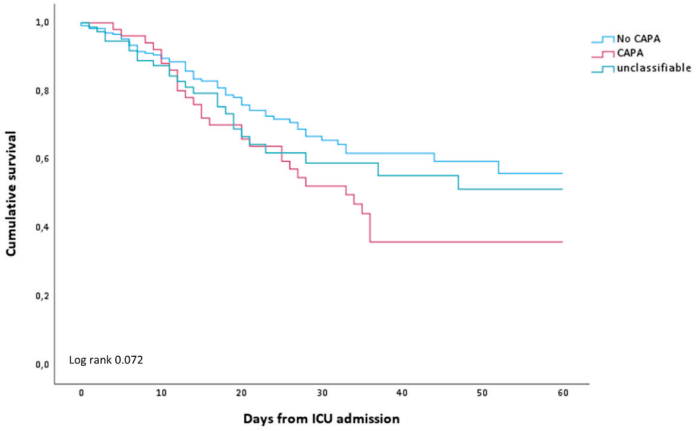
	CAPA	No CAPA	Total	Sensitivity	Specificity	PPV	NPV
Screening negative	9.49	235.47	244.95				
Total	15.30	290.70	306	0.38	0.81	0.10	0.96
CAPA frequency 20%							
Screening total							
Screening positive	51.55	78.41	129.95				
Screening negative	21.05	211.99	233.05				
Total	72.60	290.40	363	0.71	0.73	0.40	0.91
Screening culture positive							
Screening positive	39.13	28.90	68.03				
Screening negative	21.07	211.90	232.97				
Total	60.20	240.80	301	0.65	0.88	0.58	0.91
Screening only PCR positive							
Screening positive	23.26	46.51	69.77				
Screening negative	37.94	198.29	236.23				
Total	61.20	244.80	306	0.38	0.81	0.33	0.84

* According to 2020 ECMM/ISHAM consensus criteria.[11] CAPA: COVID-19 associated pulmonary aspergillosis, NPV: negative predictive value, PPV: positive predictive value, TA: tracheal aspirate.

Supplemental table 4. ICU mortality per screening outcome and CAPA* classification.

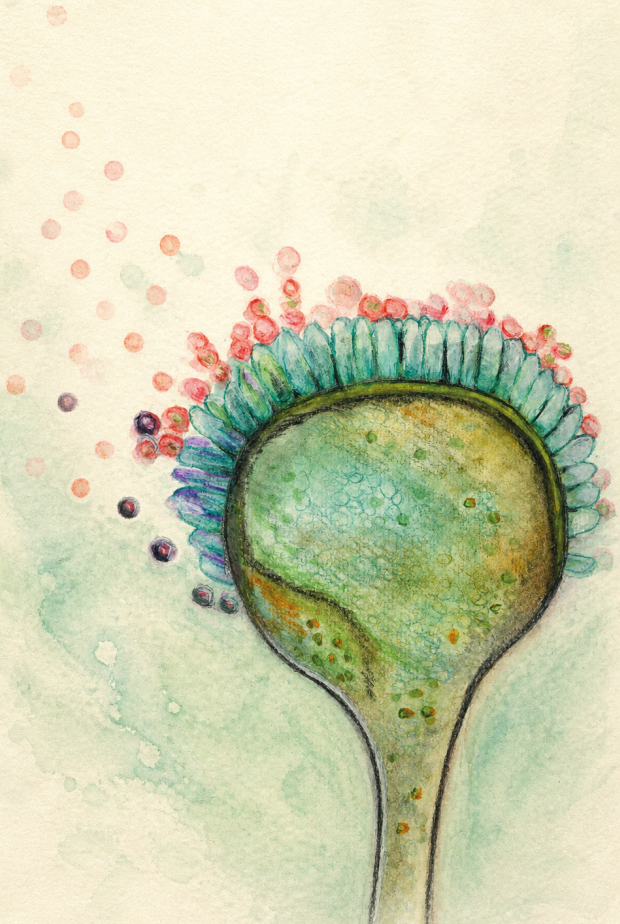
	Death during ICU stay		
Screening outcome and CAPA classification	**Yes**	**No**	**Total**
Screening positive/confirmed CAPA, n (%)	24 (62)	15 (38)	39 (100)
Screening negative/confirmed CAPA, n (%)	4 (31)	9 (69)	13 (100)
Screening positive/no CAPA, n (%)	25 (29)	62 (71)	87 (100)
Screening negative/no CAPA, n (%)	59 (26)	172 (74)	231 (100)
Total, n (%)	112 (30)	258 (70)	370 (100)

* According to 2020 ECMM/ISHAM consensus criteria.[11] CAPA: COVID-19 associated pulmonary aspergillosis, ICU: intensive care unit, screening: screening by collection of tracheal aspirate for culture and PCR. ICU mortality: mortality during ICU stay.
Screening positive/CAPA vs screening positive/no CAPA p< 0.001, screening positive/CAPA vs screening negative/CAPA p=0.054, screening positive/CAPA vs screening negative/no CAPA p<0.001, screening positive/no CAPA vs screening negative/no CAPA p=0.565, screening positive/no CAPA vs screening negative/CAPA p=1, screening negative/CAPA vs screening negative/no CAPA p=0.746



	Day 0-10	Day 10-20	Day 20-30	Day 30-40	Day 40-50	Day 50-60	Day 60
No CAPA (number/death/censored)	240/21/37	182/21/56	105/13/34	58/4/19	35/1/15	19/1/5	13/0/13
CAPA (number/death/censored)	52/4/2	46/11/1	34/8/5	21/6/2	13/0/3	10/0/3	7/0/7
Unclassifiable (number/death/censored)	78/9/10	59/10/18	31/4/9	18/1/2	15/1/3	11/0/4	7/0/7

Supplemental figure 1. Life table and Kaplan-Meier survival curves of patients with CAPA*, patients without CAPA and unclassifiable patients (60-day mortality from ICU admission).
* According to 2020 ECMM/ISHAM consensus criteria.[11]



7

From *Aspergillus fumigatus* pathogen surveillance to *Aspergillus* disease surveillance

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STRUCTURED SUMMARY

Objective: The aim of this study was to determine which mycological diagnostic test(s) and clinical data are required for detecting and classifying *Aspergillus* disease cases for surveillance purposes.

Methods: This pilot study for an *Aspergillus* disease surveillance programme was performed in two academic medical centres in the Netherlands in collaboration with the National Institute for Public Health with data from 2019. Patients with a positive bronchoalveolar lavage (BAL) *Aspergillus fumigatus* culture, *Aspergillus* polymerase chain reaction (PCR), or galactomannan (GM) immune assay were included in the study. Data were retrospectively collected from the laboratory information system and supplemented with clinical and treatment data from electronic patient records (EPRs). Using mycology and clinical data, cases were classified using international definitions for disease classification (considered gold standard).

Results: In total, 89 patients with a positive diagnostic test for *Aspergillus* were included in the cohort (53 culture positive; 36 PCR/GM positive only). Of these by-pathogen-identified patients, 38 (43%) had classifiable *Aspergillus* disease (24 culture positive). 32 of 38 (84%) *Aspergillus* disease cases were detected if a positive diagnostic test in combination with antifungal treatment was considered indicative of disease. The most complete detection and classification was obtained when EPR data were extracted.

Conclusions: Positive *Aspergillus* BAL results and additional antifungal treatment data resulted in a good estimate of *Aspergillus* disease cases. However, EPR data provided the most accurate case identification but – at present – require manual chart review which is laborious and less feasible for surveillance. Future research should focus on systems for automated data extraction from the EPR to allow reliable case classification with minimal workload.

INTRODUCTION

Reliable infectious disease surveillance is essential for guiding public health and diagnostic/therapeutic interventions. The importance of surveillance of fungal infections has been stressed by the World Health Organization[1] as fungal infections and emerging antifungal resistance are a growing global public health concern. The number of patients with invasive aspergillosis (IA) is expected to increase due to an expanding at-risk population. Besides patients with classic host risk factors for IA,[2] new at-risk groups include patients admitted to the intensive care (ICU), patients treated with novel biological therapies targeting immune-signalling pathways, patients with COPD, and patients with viral respiratory tract infections such as influenza or COVID-19.[3-7] In *Aspergillus fumigatus* triazole resistance has emerged globally, severely limiting antifungal treatment options. Furthermore, triazole resistance selection occurs predominantly in the environment through exposure to triazole fungicides used to prevent fungal diseases in agriculture (protection of crops).[8, 9] Environmental exposure of *A. fumigatus* to triazole fungicides can lead to tandem repeat (TR)–mediated mutations in the cyp51A gene (such as the 34 base pair TR with L98H mutation [TR34/L98H] and TR46/Y121F/T289A), resulting in pan-triazole resistance not only to agricultural fungicides but also to those used in medical treatments and thereby limiting treatment options.[8, 9] This underscores the need for One Health surveillance programmes.

Unlike bacterial and yeast bloodstream infections, where recovery of micro-organisms in blood culture commonly represents infection, disease surveillance of fungal infections is difficult to conduct. Mycological culture from bronchoalveolar lavage (BAL) may not represent IA as host factors and clinical factors, e.g. imaging, are required to classify the presence of invasive fungal disease. Furthermore, fungal culture is commonly negative, and mycological evidence then relies on the detection of *Aspergillus* biomarkers, such as galactomannan (GM), *Aspergillus* IgG/IgE, and *Aspergillus* polymerase chain reaction (PCR). Detection of triazole resistance in *A. fumigatus* may be challenging due to negative cultures (precluding minimum inhibitory concentration (MIC) testing) and limited sensitivity of triazole resistance PCR assays. In addition, consensus criteria exist, but they are disease specific, meaning that there is no single case definition that covers all diverse patient groups at risk for acute or chronic *Aspergillus* disease (i.e. IA, chronic pulmonary aspergillosis [CPA] or allergic bronchopulmonary aspergillosis [ABPA]).[2, 5, 10-14] This complicates case selection and comparison between studies. Also, previous studies focussing on epidemiology or disease burden used manual chart review to classify *Aspergillus* diseases which is labour-intensive.[15-18]

In the Netherlands, *A. fumigatus* resistance pathogen-based sentinel surveillance (culture only) is currently coordinated by the mycology reference laboratory (Radboudumc-CWZ Center of Expertise for Mycology and National Public Health Institute, RIVM) aimed to monitor the frequency of triazole-resistant *A. fumigatus* and trends in cyp51A-resistance genotypes.[19] All *A. fumigatus* isolates cultured in 10 participating sentinel surveillance laboratories are screened for triazole resistance using an agar-based method (VIPcheck™, Mediaproducts, Groningen), and resistant isolates are sent to the mycology reference laboratory for in-vitro susceptibility testing and cyp51A-sequencing. Of these patients, limited clinical data are collected from the laboratory information system (LIMS; age, sex, and aggregated clinical data). This programme serves as the basis for intervention strategies to manage and decrease antifungal resistance. Triazole-resistance frequencies exceeding 10% led to revision of the national invasive mycoses guideline in 2017[20] in which combination antifungal therapy was recommended in patients with suspected IA at least until additional information on triazole resistance was available.

However, this current pathogen-based surveillance has evident shortcomings. First, all positive cultures are screened, including those considered clinically irrelevant. Second, limited clinical data are collected, and third, culture-negative cases of IA are not included. Consequently, it is not possible to assess the clinical implications of triazole resistance and to monitor the effect of interventions, for instance, if the initial combination therapy reduces excess mortality associated with triazole-resistant IA.

The aim of the current pilot study was to investigate which mycological diagnostic test(s) and clinical data are most useful for detecting patients with *Aspergillus* disease and to assess if reliable *Aspergillus* disease classification, using international definitions, was possible and feasible with data from both the LIMS and the electronic patient record (EPR).

Several approaches were followed to determine how *Aspergillus* disease cases with at least one positive mycological diagnostic finding could best be detected and classified according to the subsequent research questions:

1. Can *Aspergillus* disease cases be detected by culture alone or should *Aspergillus* PCR or GM results be considered as well?
2. Can *Aspergillus* disease cases be detected by combining a positive diagnostic test with a limited clinical data set from the EPR, such as the medical speciality of the attending physician or initiation of antifungal treatment?
3. Can *Aspergillus* disease cases be detected and classified based on data obtained from the LIMS alone or is a full EPR review necessary?

METHODS

The pilot study, with a retrospective design, was performed at the Leiden University Medical Center and Radboud University Medical Center using clinical data from 2019. The year 2019 was chosen because it was prior to the start of the COVID-19 pandemic in the Netherlands.

Study population

Patients of all ages with a positive BAL culture (*A. fumigatus*), BAL PCR (*Aspergillus* species) (AsperGenius®, PathoNostics®, Maastricht, the Netherlands, or an in-house PCR targeting the 28S rRNA gene[21]), or BAL GM (GM, Platelia™ *Aspergillus* Ag Bio-Rad laboratories, Marnes-la-Coquette, France) (optical density [OD] index cut-off ≥ 1) were selected via the LIMS. After selection of patients with positive mycology test results (potential *Aspergillus* disease cases) in the LIMS, clinical data from these patients were collected in the EPR to confirm the diagnosis (Figure 1) and to assess whether information about the medical speciality of the attending physician and administration of antifungal treatment was sufficient for detecting *Aspergillus* disease cases.

Data collection

Data were extracted from the LIMS by using a query and included year of birth, sex, clinical data added to the mycology diagnostic test orders (minimal information, mostly one word or sentence), BAL culture results (including results from susceptibility testing), PCR results (including results of resistance PCR) or GM results, results of follow-up BAL samples, detection of resistant *Aspergillus* isolates from any location, and detection of other fungi.

After patients with a positive BAL culture, PCR or GM had been identified, additional data from these patients were collected from the EPR. Data extracted from the EPR included underlying disease, host risk factors for IA, medical speciality of the attending physician, diagnosis of IA (date and location), the initial empirical antifungal treatment for IA, relevant data for classification of *Aspergillus* disease, six weeks and six months mortality, and autopsy reports.

Data were collected by two clinical microbiologists (one from each medical centre: M.vdB. and J.B.) who decided which *Aspergillus* disease classification criteria were relevant to the patient and classified patients accordingly. The data were captured in an electronic case report form (eCRF) that consisted of two parts: the first part was for data collected from the LIMS, and the second part was for data collected from the EPR. This was done to evaluate whether the LIMS would be an adequate source for identifying patients with *Aspergillus*

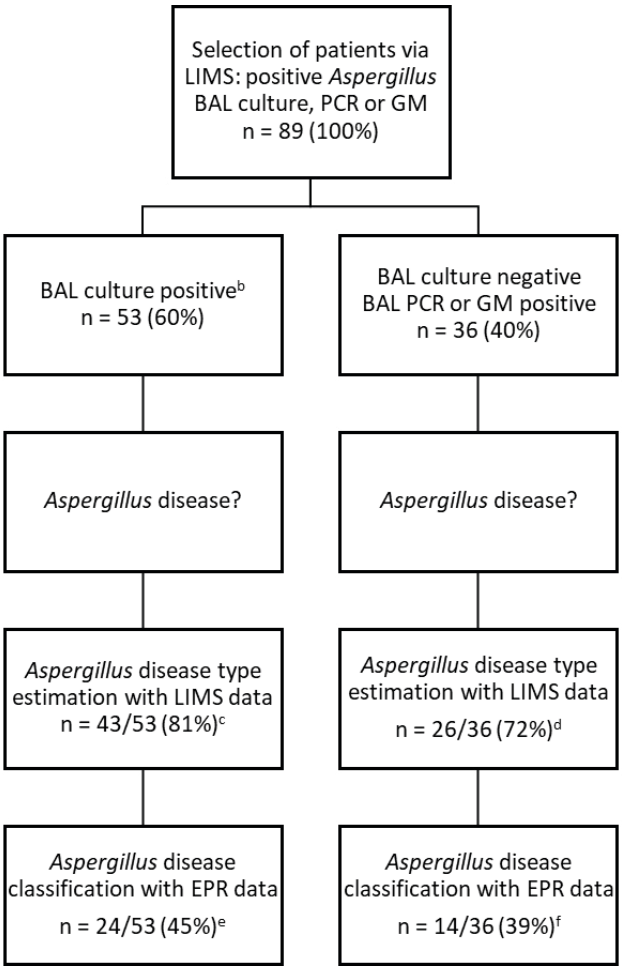


Figure 1. Detection and classification of *Aspergillus* disease[a] cases.
BAL: bronchoalveolar lavage; EORTC/MSGERC: European Organization for Research and Treatment of Cancer-Mycoses Study Group Education and Research Consortium; EPR: electronic patient record; GM: galactomannan; IAPA: influenza-associated pulmonary aspergillosis; LIMS: laboratory information system; PCR: polymerase chain reaction. [a] *Aspergillus* disease includes patients with invasive aspergillosis (IA) (according to EORTC/MSGERC,[2] AspICU,[10] or IAPA criteria[11]), chronic pulmonary aspergillosis (CPA) or allergic bronchopulmonary aspergillosis (ABPA). [b] BAL culture positive, regardless of PCR and GM results: 11 culture, PCR and GM positive; six culture and PCR positive (GM negative or not done); four culture and GM positive (PCR negative or not done); 32 culture positive (PCR and GM negative or not done). [c] *Aspergillus* disease type estimation: 19 IA and 24 CPA/ABPA; 21/43 (49%) confirmed *Aspergillus* disease after formal classification. [d] *Aspergillus* disease type estimation: 22 IA and four CPA/ABPA; 12/26 (46%) confirmed *Aspergillus* disease after formal classification. [e] *Aspergillus* disease: 16 IA and eight CPA/ABPA. [f] *Aspergillus* disease: 11 IA and three CPA/ABPA.

disease without manual chart review in the EPR. The approximate time spent on entering the data for both parts of the eCRF was recorded by the clinical microbiologists collecting the data.

Classification of patients

With the data extracted from the LIMS, a rough estimation of *Aspergillus* disease type was performed by the clinical microbiologists collecting the data, based on (limited) clinical information provided in the mycological diagnostic test request, or other cultures such as surveillance cultures for haematology or ICU patients. The categories in the eCRF included 'LIMS haematology', 'LIMS intensive care (ICU)', 'LIMS influenza', 'LIMS lung', 'LIMS other', 'LIMS none', or 'LIMS unknown' and could be further specified in a free-text field.

Subsequently, patients were formally classified based on data acquired through full EPR review with international definitions: the European Organization for Research and Treatment of Cancer-Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) consensus criteria,[2] the AspICU 2012 clinical algorithm for ICU patients[10] (at the time of the study, the 2024 consensus definitions for invasive fungal disease in adult patients in the ICU[14] were not yet available), or the influenza-associated pulmonary aspergillosis (IAPA) consensus criteria[11] (further referred to as IA if the case fulfilled the criteria of one or more of these definitions). Patients with another type of aspergillosis (CPA or ABPA) were labelled according to the diagnosis of the attending physician (further referred to as 'other type of aspergillosis').

All types of classifiable aspergillosis were considered *Aspergillus* disease, i.e. IA, CPA, or ABPA. Patients who could not be classified were considered unclassifiable. Patients were not classified as possible IA because a positive mycological test was an entry criterion – thus most were not included in the study – and if mycological evidence was not met (only one BAL PCR positive), then patients were considered unclassifiable because in all cases, either host or radiological criteria were lacking as well.

To calculate the accuracy of *Aspergillus* disease type estimation based on LIMS data, a comparison was made between LIMS-based *Aspergillus* disease categories and EPR-based *Aspergillus* disease classification. The following combinations were considered concordant: 'LIMS haematology' – formal EORTC/MSGERC, 'LIMS other' (only solid organ transplant or congenital immunodeficiency) – formal EORTC/MSGERC, 'LIMS ICU' – formal AspICU, 'LIMS influenza' – formal IAPA, 'LIMS lung' – formal other type of aspergillosis, 'LIMS other or none' – formal not classifiable.

Statistical methods

Categorical variables were described as numbers and percentages per category. Continuous variables were described as medians and interquartile ranges. Venn diagrams were constructed to show test concordance between BAL culture, GM, and PCR results. Life tables and Kaplan–Meier survival curves were constructed. All statistical analyses were performed using IBM SPSS Statistics for Windows version 29.0 (IBM Corp., Armonk, New York, USA).

RESULTS

In total, 89 patients had a positive BAL culture or PCR or GM test result and were included in this retrospective pilot study. Patient characteristics are shown in Table I.

Aspergillus disease was classifiable in 38 of 89 (43%) patients of which 27 (30%) were patients with IA and 11 (13%) were patients with another type of aspergillosis (five ABPA and six CPA). *Aspergillus* disease was unclassifiable in 51 of 89 (57%). Patients with unclassifiable disease exhibited a range of underlying diseases, with 25% having chronic pulmonary disease and 84% were untreated (Table I). Overall mortality rates at six weeks and six months were 22 of 82 (27%) and 29 of 80 (36%), respectively (Table I, Supplementary Figure A1).

Three different approaches for detection of *Aspergillus* disease cases:

1. Culture, PCR or GM

Of the patients with classifiable *Aspergillus* disease in the cohort, 24 of 38 (63%) had a positive BAL culture, whereas 12 of 38 (37%) would have been missed if *Aspergillus* biomarkers had not been included as entry criteria (Figure 1).

No test combination was unique for classifiable cases of *Aspergillus* disease as compared with unclassifiable cases, but not all three tests had been performed in BAL samples of all patients. BAL cultures had been performed in 88 of 89 (99%) patients, GM in 66 of 89 (74%), and PCR in 53 of 89 (60%) (all irrespective of test result). All three tests had been performed in 51 (57%) patients.

The various test combinations that were positive in the first collected BAL sample in the different patient groups are depicted in Figure 2 and Supplementary Table A2. Information about triazole resistance can be found in Supplementary Table A3.

Table I. Baseline characteristics, treatment and mortality of patients grouped according to classification.[a]

	Invasive aspergillosis, n	%	Other type of aspergillosis,[b] n	%	Unclassifiable,[c] n	%	Total, n	%
	27	30	11	13	51	57	89	%
Age, median (IQR)	59	(38-68)	66	(41-74)	58	(45-71	60	(46-70
Male	19	70	7	64	27	53	53	60
Underlying disease (86)	25	93	11	100	44	92	80	93
Acute leukaemia	5	19			3	6	8	9
Other haematological malignancy	8	30			3	6	11	12
Allogeneic stem cell transplantation	8	30			5	10	13	15
Autologous stem cell transplantation	1	4					1	1
Solid organ transplantation	2	7			5	10	7	8
Congenital immunodeficiency syndrome	4	15			2	4	6	7
Cardiovascular disease					3	6	3	3
Diabetes mellitus	2	7			2	4	4	4
Chronic pulmonary disease	2	7	5	45	12	25	19	21
Asthma	1	4	4	36	4	8	9	10
Cystic fibrosis					3	6	3	3
Solid organ malignancy			3	27	4	8	7	8
Chronic kidney disease					2	4	2	2
Chronic liver disease					1	2	1	1
Thromboembolic event	1	4			1	2	2	2
Rheumatological disease	1	4	1	9	1	2	3	3
Thyroid disease					1	2	1	1
Other	1	4			3	6	4	4
Host risk factors for IA (87)	18	67			16	33	34	39

Table I. Baseline characteristics, treatment and mortality of patients grouped according to classification.[a] (continued)

	Invasive aspergillosis, n	%	Other type of aspergillosis,[b] n	%	Unclassifiable,[c] n	%	Total, n	%
Recent history of neutropenia (86)	4	15			1	2	5	6
Haematological malignancy (86)	12	44			6	13	18	20
Allogeneic stem cell transplantation (87)	8	30			5	10	13	15
Solid organ transplantation (87)	2	7			5	10	7	8
Prolonged use of corticosteroids (86)	7	26			8	17	15	17
T-cell immunosuppressants (84)	6	23			6	13	12	14
B-cell immunosuppressants (82)	3	13			1	2	4	5
Inherited severe immunodeficiency (84)	2	8			2	4	4	5
Hospital admission	24	89	4	36	26	51	54	59
Treatment with an antifungal agent (87)	23	85	9	82	8	16	40	46
Voriconazole	3	13	3	33	6	75	12	30
Amphotericin B	10	43					10	25
Voriconazole + echinocandin	7	30			2	25	9	23
Voriconazole + amphotericin B	1	4					1	3
Isavuconazole	1	4					1	3
Posaconazole	1	4	3	33			4	10
Other			3	33			3	8
Mortality								
Six weeks (82)	10	38	2	18	10	22	22	27
Six months (80)	14	54	2	20	13	30	29	36

N: number; IA: invasive aspergillosis; IQR: interquartile range; EORTC/MSGERC: European Organization for Research and Treatment of Cancer-Mycoses Study Group Education and Research Consortium; IAPA: influenza-associated pulmonary aspergillosis. [a] Patients were classified according to EORTC/MSGERC,[2] AspICU,[10] or IAPA criteria.[11] [b] Patients with chronic pulmonary aspergillosis or allergic bronchopulmonary aspergillosis. [c] Patients were classified as unclassifiable if they did not meet the criteria for invasive aspergillosis or other aspergillosis.

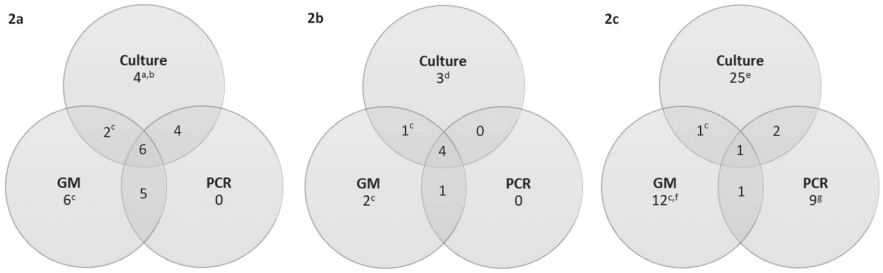


Figure 2. Venn diagrams showing positive culture, GM and PCR results of the first collected BAL sample of patients classified according to EORTC/MSGERC,[2] AspICU,[10] or IAPA criteria[11] with (a) invasive aspergillosis, n=27; (b) other type of aspergillosis (ABPA or CPA), n=11; or (c) unclassifiable patients, n=51.

ABPA: allergic bronchopulmonary aspergillosis; BAL: bronchoalveolar lavage; CPA: chronic pulmonary aspergillosis; EORTC/MGSERC: European Organization for Research and Treatment of Cancer-Mycoses Study Group Education and Research Consortium; GM: galactomannan; IAPA: influenza-associated pulmonary aspergillosis; PCR: polymerase chain reaction. [a] For three patients, PCR was not done (nd); [b] for one patient, PCR and GM were nd; [c] for one patient, PCR was nd; [d] for three patients, PCR and GM were nd; [e] for five patients, PCR was nd, and for 18 patients, PCR and GM were nd; [f] for one patient, culture was nd; [g] for one patient, GM was nd.

Of note: of the nine unclassifiable patients with only a positive PCR, two had underlying haematological disease and were classified according to EORTC/MSGERC criteria, but were deemed unclassifiable. Of these two patients, one patient only had a single positive BAL PCR result.

2. Positive diagnostic test with limited clinical data

The different classifications of *Aspergillus* disease per medical speciality of the attending physician are depicted in Figure 3. Medical speciality alone was insufficient to recognize *Aspergillus* disease in this cohort.

Overall, treatment with an antifungal agent was initiated in 40 of 89 (45%) patients. If antifungal treatment in combination with positive BAL diagnostics was considered a proxy for *Aspergillus* disease, 32 of 38 (84%) patients with *Aspergillus* disease were detected (Table I and Supplementary Figure A4).

3. Detection and classification with LIMS or EPR data

Although data could be collected from the LIMS very rapidly (approximately five minutes vs approximately 15–20 min for the EPR per patient), diagnosis and precise classification of *Aspergillus* disease was only possible after manual chart review in the EPR (Table II). A rough classification of *Aspergillus* disease was performed with LIMS data, and overall concordance between the LIMS and EPR was found in 37 of 80 (46%) patients (LIMS category was unknown in nine patients). Concordance in patients with *Aspergillus* disease was observed in 31 of 38 cases (82%).

Table II. *Aspergillus* disease[a] surveillance with data collected from LIMS or EPR.

	LIMS	Manual chart review in EPR
Selection of patients	+	-
Demographic data (age, sex)	+	+
Underlying disease	± (only rough classification of disease type)	+
Host risk factor for IA	-	+
Classification of *Aspergillus* disease	-	+
Treatment	-	+
Mortality	+	+
Microbiological data	+	± (more work to retrieve)
Feasibility	+ (approximately five minutes per patient)	- (approximately 15-20 minutes per patient)
Pathogen based surveillance	+	- (might be possible, but more work to retrieve data)
Disease based surveillance	-	+

EPR: electronic patient record, IA: invasive aspergillosis, LIMS: laboratory information system; EORTC/MSGERC: European Organization for Research and Treatment of Cancer-Mycoses Study Group Education and Research Consortium; IAPA: influenza-associated pulmonary aspergillosis. [a]*Aspergillus* disease includes patients with invasive aspergillosis (according to EORTC/MSGERC,[2] AspICU,[10] or IAPA criteria[11]), chronic pulmonary aspergillosis or allergic bronchopulmonary aspergillosis.

DISCUSSION

Aspergillus disease surveillance should aid in decision-making regarding preventative and therapeutic efforts for the patient populations affected. Essentially, sensitivity for disease detection is not required to reach 100% because individual medical decision-making is not based on surveillance. Formal aims for the sensitivity of this type of surveillance have not been set. Nevertheless, representative surveillance, for attaining reliable estimates of disease type and of the affected patient population (without missing certain patient groups), is essential to tailor interventions. This pilot study aimed to gain information on the optimal entry criteria and clinical data needed for this purpose.

Three different approaches for detection of *Aspergillus* disease cases:

1. Culture, PCR or GM

We conclude that both BAL cultures and *Aspergillus* PCR or GM results should be considered for detection of *Aspergillus* disease cases. The sensitivity and specificity of culture are known to be low.[22, 23] Hence, inclusion of positive *Aspergillus* BAL cultures, PCR or GM results leads to better detection of *Aspergillus*

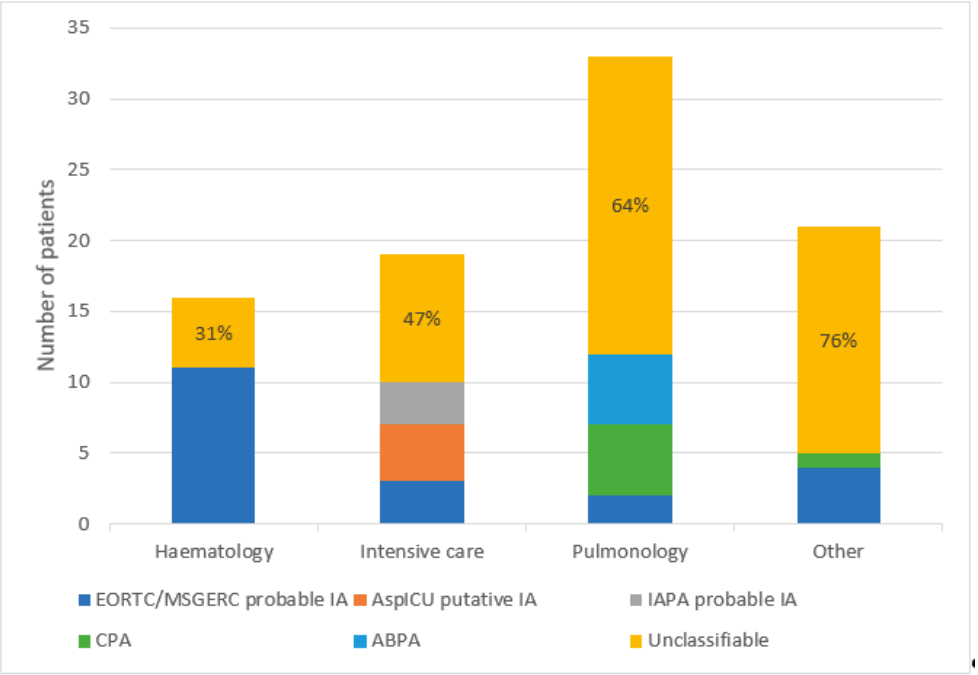


Figure 3. *Aspergillus* disease classification[a] per medical specialty of the attending physician.
ABPA: allergic bronchopulmonary aspergillosis; CPA: chronic pulmonary aspergillosis; EORTC/MSGERC: European Organization for Research and Treatment of Cancer-Mycoses Study Group Education and Research Consortium; IA: invasive aspergillosis; IAPA: influenza-associated pulmonary aspergillosis**.** [a] Classification according to EORTC/MGSERC,[2] AspICU,[10] or IAPA criteria.[11]

disease cases. In the cohort, an additional 12 of 38 (37%) patients with *Aspergillus* disease were detected if *Aspergillus* PCR or GM was considered. The BAL test result on itself (culture, PCR, or GM positivity) or BAL test combinations did not aid in distinguishing *Aspergillus* disease cases from unclassifiable cases. However, definitive conclusions cannot be drawn as not all three tests were consequently performed in BAL samples of all patients. Probably only cultures, without PCR or GM, are performed in patients in whom IA is not suspected. Furthermore, the role of PCR and antigen testing in CPA has not been well established.

Other epidemiological studies on *Aspergillus* disease have selected patients based on one or more combinations of the following: positive culture, positive serum or BAL GM, histological evidence of fungal disease, antifungal treatment via pharmacy records, or coding data for fungal diseases.[15-18] Including pharmacy records as an inclusion criterion can aid in refining inclusion criteria (and

patients with possible IA could be included as well) but will unlikely improve feasibility if results have to be combined to microbiological test results manually.

2. Positive diagnostic test with limited clinical data

Detection of patients was improved by including clinical information from the EPR about antifungal treatment. Considering positive BAL findings with antifungal treatment as a proxy for disease detected 84% patients.

Additional clinical data from the EPR about the medical speciality of the attending physician also led to a more accurate detection of *Aspergillus* disease cases, especially if patients were treated by a haematologist or intensivist. However, of the patients treated by other medical specialities, only a minority of cases with positive BAL findings had classifiable *Aspergillus* disease. Inclusion of *Aspergillus*-specific IgG antibodies results might improve specificity for detection of patients with CPA,[12] but were not included in the current study.

One of the difficulties of *Aspergillus* disease classification is that there are different case definitions for different patient groups. Furthermore, the current case definitions were developed for research purposes, i.e. clinical trials, using higher GM cut-off values or certain radiological findings not typically applied as rigidly in clinical practice. These criteria mostly rely on a single positive test at a certain moment in time, but in clinical practice, diagnosing *Aspergillus* disease is more complex and might be considered when other aspects are taken into account as well, such as comorbidities, clinical response, or multiple test results.

While classification of patients with the various types of IA was performed with current consensus definitions, CPA diagnosis was based on the diagnosis made by the attending physician. There are consensus definitions for CPA,[12] but these are too complicated to include in surveillance criteria. CPA consists of different clinical forms; these forms can overlap with one another, and each has its own clinical definition based on clinical, serological, mycological, and radiological findings. Furthermore, radiological progression over time is an important parameter in certain forms, which is difficult to capture at a single timepoint in surveillance. Adjustment of these definitions for surveillance purposes might be needed to include patients who were diagnosed and treated at the bedside.

3. Detection and classification with LIMS or EPR data

With data from the LIMS, potential *Aspergillus* disease cases could be detected (positive BAL culture, PCR, or GM). Furthermore, collection of demographic data (age and sex) and a rough classification of disease type were possible with a moderate concordance with formal EPR-based classification. LIMS data

were poor at excluding non-*Aspergillus* disease cases but moderately good at confirming classification type among true disease cases. However, detailed information about underlying disease or host risk factors for IA was not available; thus, classification was not reliably possible. With additional data from the EPR, patient classification was possible, but it is labour-intensive to manually review the chart to collect necessary data. In addition, European and national legislation restricts the use of medical data for research purposes.[24-26] The Dutch Public Health Act obligates physicians or laboratories to notify the municipal health service if certain infectious diseases are discovered (e.g. tuberculosis) and is intended to protect and promote the public health.[27] One of the goals of reporting certain infectious diseases includes monitoring and surveillance of disease for prevention and control strategies. However, this is not applicable for patients with IA as it is not a notifiable disease. This clearly poses a barrier for designing an EPR-based surveillance programme because of lengthy procedures for approval in the participating hospitals and of the possible bias introduced if only patients who have consented for inclusion can be included.

Study strengths and limitations

Strengths of the study included that data were collected from the LIMS and the EPR so that a comparison between the two could be made and the possibilities of surveillance via both datasets could be evaluated. By performing the study in two centres with different LIMSs and EPRs, we were able to evaluate whether surveillance would be possible in different centres. Classification of patients with international definitions for disease classification was done by one clinical microbiologist per medical centre and was not based on the diagnosis of the attending physician but could have been further improved by classifying patients in a multi-disciplinary team.

Limitations of the study included that not all diagnostics were performed on BAL so that a comparison could not be made between culture, PCR, and GM for the purpose of detecting *Aspergillus* disease cases. ABPA and CPA were not classified but based on the diagnosis of the attending physician. However, most unclassifiable patients were not treated with an antifungal agent, so probably the majority of these patients were included in the group with *Aspergillus* disease. Finally, most possible cases of IA were not included as a positive BAL was an entry criterion, and in patients where mycological evidence was insufficient (only one BAL PCR positive), host or clinical features of IA according to classification criteria were not met either. Thus patients with unclassifiable disease in our cohort were not classified as possible IA.

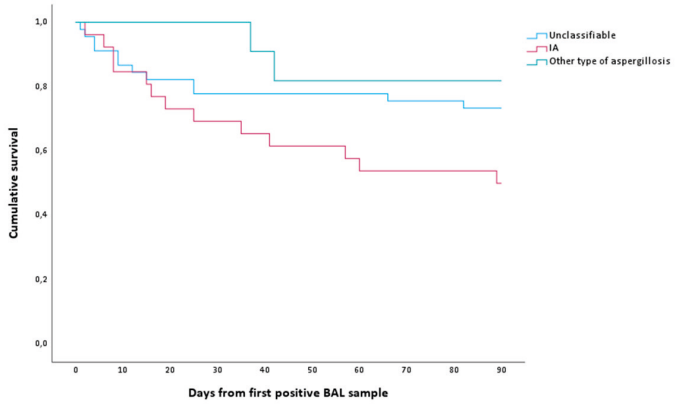
In conclusion, *Aspergillus* disease surveillance should include patients with positive BAL culture and/or PCR and/or GM results (37% of *Aspergillus* disease cases were only biomarker positive) and limited clinical data from the EPR including initiation of antifungal treatment or the medical speciality of the attending physician. This combination provides a representative overview of *Aspergillus* disease cases. For more precise patient classification, manual chart review is necessary, but the additional time needed greatly reduces the feasibility of this approach. Future research should focus on refining current definitions of IA for surveillance purposes and on advancing systems that enable automated extraction of anonymized data from EPRs, thereby facilitating precise disease classification with minimal time expenditure. Machine learning algorithms and/or artificial intelligence for detecting patients with IA provide opportunities for efficient data collection,[28] safeguarding privacy, and facilitating the refinement and standardization of inclusion criteria for surveillance and reporting of results.

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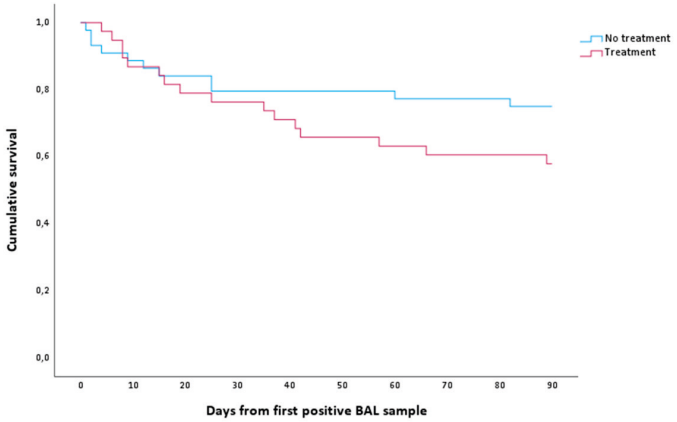
SUPPLEMENTARY MATERIAL



	Day 0-15	Day 15-30	Day 30-45	Day 45-60	Day 60-75	Day 75-90	Day 90
IA (number/death)	26/4	22/4	18/2	16/1	15/1	14/1	13/0
Other type of aspergillosis (number/death)	11/0	11/0	11/2	9/0	9/0	9/0	9/0
Unclassifiable (number/death)	45/7	38/3	35/0	35/0	35/1	34/1	33/0

Supplementary Figure A1a. Life table and Kaplan-Meier survival curves of patients classified[a] as IA, other type of aspergilossis (ABPA or CPA) or unclassifiable patients (90-day mortality from first positive BAL sample). Follow-up until day 90 was available for all 82 patients included in the analysis.

ABPA: allergic bronchopulmonary aspergillosis, CPA: chronic pulmonary aspergillosis, IA: invasive aspergillosis, IAPA: influenza associated pulmonary aspergillosis. [a] Classification according to EORTC/MGSERC,[2] AspICU,[10] or IAPA criteria.[11]



	Day 0-15	Day 15-30	Day 30-45	Day 45-60	Day 60-75	Day 75-90	Day 90
No treatment (number/death)	44/6	38/3	35/0	35/0	35/1	34/1	33/0
Treatment (number/death)	38/5	33/4	29/4	25/1	24/1	23/1	22/0

Supplementary Figure A1b. Kaplan-Meier survival curves of patients treated and not treated with an antifungal agent (90-day mortality from first positive BAL sample). Follow-up until day 90 was available for all 82 patients included in the analysis.

Supplementary Table A2. Culture, PCR and GM results of the first collected BAL sample of different patients groups according to classification[a].

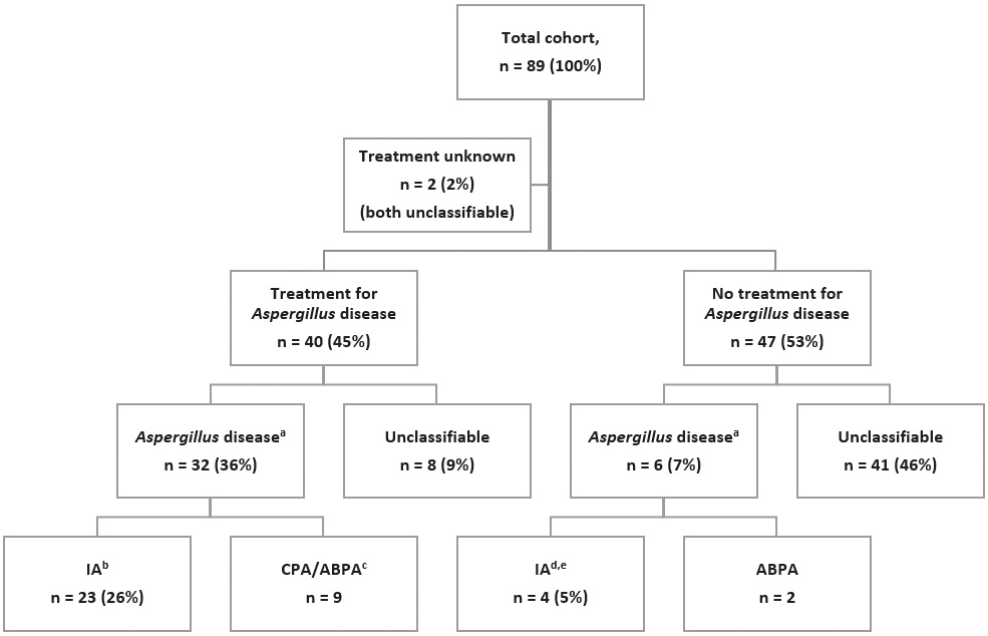
Different combinations of BAL culture, PCR and GM results			IA	%	Other (ABPA / CPA)	%	Unclassifiable	%	Total	%
Culture pos,	PCR pos,	GM pos	6	22	4	36	1	2	11	12
Culture pos,	PCR pos,	GM neg	4	15			2	4	6	7
Culture pos,	PCR neg,	GM pos	1	4					1	1
Culture pos,	PCR neg,	GM neg					2	4	2	2
Culture pos,	PCR nd,	GM pos	1	4	1	9	1	2	3	3
Culture pos,	PCR nd,	GM neg	3	11			5	10	8	9
Culture pos,	PCR nd,	GM nd	1	4	3	27	18	35	22	25
Culture neg,	PCR pos,	GM pos	5	19	1	9	1	2	7	8
Culture neg,	PCR pos,	GM neg					8	16	8	9
Culture neg,	PCR pos,	GM nd					1	2	1	1
Culture neg,	PCR neg,	GM pos	5	19	1	9	10	20	16	18
Culture neg,	PCR nd,	GM pos	1	4	1	9	1	2	3	3
Culture nd,	PCR neg,	GM pos					1	2	1	1
Total			27	100	11	100	51	100	89	100

ABPA: allergic bronchopulmonary aspergillosis, BAL: bronchoalveolar lavage, CPA: chronic pulmonary aspergillosis, GM: galactomannan, IA: invasive aspergillosis, IAPA: influenza associated pulmonary aspergillosis, nd: not done, neg: negative, pos: positive. [a] Patients were classified according to EORTC/MSGERC,[2] AspICU,[10] or IAPA criteria.[11]

Supplementary Table A3. Triazole resistance test results in relation to treatment and mortality in the different patient groups according to classification[a].

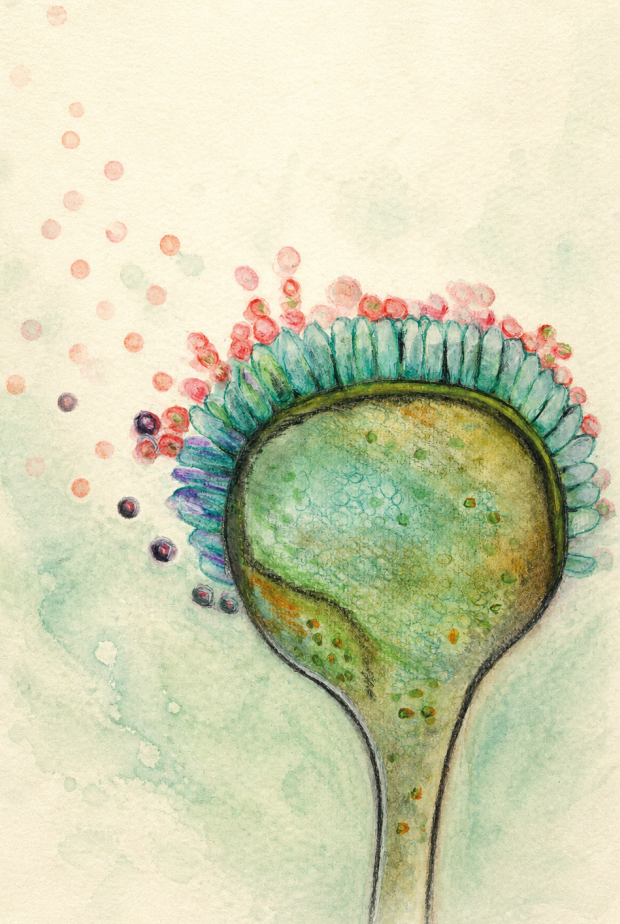
	IA, n=27 (%)	Unclassifiable, n=51 (%)	Other (ABPA/CPA), n=11 (%)	Total, n=89 (100)
Triazole resistance testing performed	18/27 (67)	30/51 (59)	8/11 (73)	56/89 (63)
Triazole resistance testing successful	18/18 (100)	29/30 (97)	8/8 (100)	55/56 (98)
Triazole resistance detected[b]	5/18 (28)	5/29 (17)	0	10/55 (18)
Number of patients with a resistant isolate treated with an antifungal agent	3/5 (60)	0		3/10 (30)
Antifungal treatment	2 liposomal amphotericin B 1 voriconazole + echinocandin			
Mortality (in patients with a triazole resistant isolate)	3/5 (60)	1/5 (20)		4/10 (40)
Within 6 weeks	2 (both patients were treated with liposomal amphotericin B)			2/10 (20)
Within 6 months	1 (not treated)	1 (not treated)		2/10 (20)

ABPA: allergic bronchopulmonary aspergillosis, CPA: chronic pulmonary aspergillosis, IA: invasive aspergillosis. [a] Patients were classified according to EORTC/MSGERC,[2] AspICU,[10] or IAPA criteria.[11] [b] Compared to phenotypical testing no additional cases with azole-resistance were found by PCR (TR34/L98H resistance mutations were found in 2 patients).



Supplementary Figure A4. Total cohort grouped according to treatment with an antifungal agent and *Aspergillus* disease[a].

ABPA: allergic bronchopulmonary aspergillosis, CPA: chronic pulmonary aspergillosis, IA: invasive aspergillosis. [a] *Aspergillus* disease includes patients with invasive aspergillosis (according to EORTC/MSGERC,[2] AspICU,[10] or IAPA criteria[11]), CPA or ABPA. [b] IA: 16 EORTC/MSGERC, four AspICU, three IAPA. [c] six CPA and three ABPA. [d] IA: four EORTC/MSGERC. [e] Of the four patients classified as probable IA who were not treated two died: two and 60 days after performance of BAL with positive test result for *Aspergillus*.



8

Summary and discussion

The growing number of patients at risk for invasive aspergillosis (IA) is due to an increase in diverse populations of immunocompromised patients. Besides patients with classical host risk factors for IA – such as patients with underlying haematological disease or recipients of a solid organ transplantation – critically ill patients, patients with influenza or SARS-CoV-2 infection, or patients treated with certain new immunomodulatory therapies affecting both the cellular and humoral immunity (for example small-molecule kinase inhibitors, e.g. ibrutinib (Bruton's tyrosine kinase inhibitor), eculizumab (monoclonal complement C5 inhibitor), or monoclonal TNF-α inhibitors)[1, 2] are also at risk for IA. In addition to this development, triazole resistance has emerged globally, reducing – the already limited – antifungal treatment options. Recent estimates of the global burden of invasive fungal infections have shown that IA is the leading fungal infection in terms of incidence and mortality.[3] However, these estimates were based on extrapolations due to unavailability of *Aspergillus* disease surveillance data as compared to pathogen based surveillance. Besides the burden of disease (e.g. incidence rate, morbidity, mortality) other burdens can be considered in IA, such as the burden of invasive diagnostics (e.g. biopsy, bronchoalveolar lavage (BAL)), the burden of therapy (e.g. toxicity), or the burden of antifungal resistance (figure 1). For more reliable estimates of all *Aspergillus* burdens in at-risk populations, disease surveillance is essential. In addition, disease surveillance will improve our understanding of the epidemiology of IA and antifungal resistance, potentially leading to improved diagnostic and therapeutic approaches. In this thesis the burden and management of IA were investigated and the possibilities to improve the national surveillance of IA were explored. In this chapter key study outcomes are summarised and discussed.

BURDEN AND MANAGEMENT

In **chapter 2 to 4** the burden and management of *Aspergillus* disease were studied, focussing on incidence rate, morbidity, mortality and (burden of) fungal diseases, antifungal therapy and prophylaxis.

Currently, there are three classes of antifungal drugs available for the treatment of *Aspergillus*: triazoles (e.g. itraconazole, voriconazole, isavuconazole, posaconazole; all targeting lanosterol 14α-demethylase, and thereby inhibiting ergosterol synthesis), polyenes (e.g. liposomal amphotericin B (LAmB), which binds to ergosterol in the fungal cell membrane), and echinocandins (e.g. caspofungin, micafungin, anidulafungin; all inhibiting β-(1,3)-D-glucan-synthase in the cell wall). Within these classes only a few drugs are active against infections with *Aspergillus*. For infections with triazole susceptible *Aspergillus* voricon-

	Clinical case	Burden
	60 year old woman with AML for whom intensive remission-induction chemotherapy is initiated, day=0	Incidence rate of aspergillosis during remission-induction without prophylaxis: 11%[15]
	Prophylaxis with voriconazole is indicated because prolonged and profound neutropenia is expected during/after remission-induction therapy, day=0	Incidence rate of aspergillosis during remission-induction with prophylaxis: 4%[15]
	Patient is nauseous and mild elevation of liver enzymes is observed, both most likely related to treatment with voriconazole. As symptoms are mild therapy is continued under observation, day=3	Toxicity of medication
	Patient is neutropenic and develops a fever, day=7 Blood cultures and a urine culture are obtained, and treatment with piperacillin-tazobactam iv is initiated	Diagnostic test, possible toxicity of medication
	Persistent fever and patient has developed a cough and is dyspnoeic. Blood cultures remain negative, day=10	Morbidity
	HR-CT is performed, day=11 Ground glass opacities and nodules are observed	Radiation exposure
	A BAL is performed because of lesions seen on HR-CT, and serum is obtained for serum GM, day=11	(Invasive) diagnostic test
	After the BAL has been performed the patient develops severe hypoxemia and is admitted to the ICU for intubation and mechanical ventilation	Complications of BAL; ICU admission
	After the BAL has been performed antifungal treatment with voriconazole + anidulafungin is initiated	Possible toxicity of medication
	Microbiology results, day=12 Microscopy: negative / culture: no growth after 1 day / PCR: Aspergillus fumigatus, TR34/L98H mutation detected	Triazole resistant A. fumigatus
	Treatment with liposomal amphotericin B is initiated. Voriconazole + anidulafungin are stopped	Possible toxicity of medication
	Clinical deterioration and death, day=20	Mortality

Figure 1. The burden of *Aspergillus* disease, illustrated by a case-vignette.
AML: acute myeloid leukaemia; BAL: bronchoalveolar lavage; GM: galactomannan; HR-CT: high-resolution computed tomography; ICU: intensive care unit

azole or isavuconazole are first line therapy, and in case of contraindications LAmB or posaconazole are considered suitable alternatives. For infections with triazole resistant *Aspergillus* LAmB is the first choice. The echinocandins have fungistatic activity against *Aspergillus* species, and are used for salvage treatment of IA.

Thus, there are limited antifungal therapeutic options. In particular for patients with triazole resistant *Aspergillus fumigatus* infection, patients with underlying organ dysfunction or patients treated with medication interacting with antifungal drugs. Drug-drug interactions often occur during treatment with triazoles (inhibitors of cytochrome P450). These include interactions with commonly used immunosuppressive agents (e.g. tacrolimus or cyclosporine), targeted haematological therapies or anticoagulants.[4] Promising antifungal agents, with a different mechanism of action and beneficial pharmacokinetic properties, are in development – mostly phase III trials – for prophylaxis or treatment of fungal infections.[4-7] Antifungal drug development is slow, because it is challenging to develop new antifungal agents with desired traits (broad spectrum, good bioavailability, minimal toxicity and side-effects) due to similarities between fungal and human eukaryotic cells.[8] However, given the increasing number of fungal infections and limited treatment options, there is a growing interest in the development of new antifungal drugs.

Future options for treatment of IA include olorofim (orotomide – reversible inhibitor of the enzyme dihyroorotate dehydrogenase (DHODH); oral; phase III trials), fosmanogepix (glycosylphosphatidylinositol-anchoredwalltransferprotein-1 inhibitor; oral or intravenous; phase III), ibrexafungerp (triterpenoid – β-(1,3)-D-glucan-synthase inhibitor; oral or intravenous; phase III), opelconazole (triazole; inhalation; phase III), thin film freezing (TFF) voriconazole (triazole; inhalation; phase II) or rezafungin (echinocandin; intravenous; phase III).[4-7] Olorofim and fosmanogepix are promising options for monotherapy of IA, including infections with triazole resistant isolates.[4-7] Ibrexafungerp has synergistic effects *in vitro* when combined with voriconazole, isavuconazole or LAmB, and this drug might be of added value in combination therapy for (triazole resistant) IA.[6] Future applications of the inhalation triazoles, opelconazole and TFF voriconazole, might include prophylaxis or combination therapy for IA.[4-7] Finally, rezafungin, a second-generation echinocandin with a mean half-life of 150 hours after two dosages, is an interesting option for outpatient therapy in combination with other antifungals.[4-7] The activity of rezafungin is comparable to that of other echinocandins.[5]

How promising these new drugs may be, the dual use of antifungals in medicine and agriculture is concerning. It has been found that in triazole resistant *A. fumigatus* infection in humans resistance associated mutations are mostly derived from the environment.[9] In other words, most mutations causing resistance to first-line triazole therapy do not occur in a patient during therapy with voriconazole or isavuconazole. Instead, patients are infected with *Aspergillus*

that already harbour resistant associated mutations that were acquired in the environment as a result of exposure to triazole fungicides.

Development of triazole resistance during therapy is rare, but has been shown to occur in patients with lung cavities, such as chronic cavitary pulmonary aspergillosis or aspergilloma.[10, 11] In these patients the fungus is able to reproduce asexually, and sporulation under antifungal therapy may be important in promoting the expression of azole-resistant *Aspergillus.*[11] In contrast, in IA the infection develops through hyphal growth. During hyphal growth, spontaneous mutations are likely to occur in the hyphal nuclei, but since the hyphae contain millions of nuclei, these mutations will have little effect on the phenotype of the fungus.[12] The mutated nuclei are outnumbered by wild-type nuclei, making the development of phenotypic resistance during treatment highly unlikely in patients with IA.[12] When in-patient triazole resistance occurs in patients with lung cavities, it develops rapidly and is characterised by point mutations in the cyp51A gene or unknown (non-cyp51A) resistance mechanisms. In contrast, environmental acquired resistance is characterised by mutations in the cyp51A gene combined with tandem repeats (TR) in the gene promoter (e.g. TR34/L98H or TR46/Y121F/T289A).[10, 11]

A. fumigatus is widespread in nature and is exposed to triazole fungicides used for crop protection, e.g. vegetables, fruit, flowers. This environmental exposure to triazoles can lead to TR-mediated mutations in the cyp51A gene of *A. fumigatus* which results in resistance to agricultural triazole fungicides, and consequently cross-resistance to medicinal triazoles. Of the new antifungal drugs in development, olorofim has the same mode of action as the newly developed fungicide ipflufenoquin. Ipflufenoquin belongs to a new class of fungicides called quinolines and has been recommended to prevent fungal infections in certain crops. Ipflufenoquin targets the same enzyme (DHODH) as olorofim developed for clinical use in humans, with a potential risk for cross resistance and thus a possible human health concern.[13] Therefore, a one health approach is urgently needed, involving a thorough risk assessment to prevent the emergence of environmental resistance selection to these new drugs. Triazole resistance has also been detected in clinical *A. fumigatus* isolates from animals in the Netherlands, and – similar to human infections – is predominantly caused by cyp51A TR-mediated mutations, thereby indicating an environmental origin.[14]

Chapter 2 showed that with a risk-stratified approach empirical combination antifungal treatment with voriconazole and LAmB could be safely avoided in an area with high local resistance rates. Monotherapy is generally preferred over combination therapy due to less toxicity and fewer drug-drug interactions.

However, with the global emergence of environmental resistance to triazoles and possibly to newer antifungal drugs, empirical combination therapy might become unavoidable in the future. Current antifungal agents might have to be combined with one of the newly developed antifungal agents for effective empirical therapy, e.g. voriconazole combined with ibrexafungerp. Nonetheless, local resistance rates are critical in guiding the choice of antifungal empirical therapy. A balanced approach is necessary to achieve optimal therapy while minimising side effects.

In **chapter 3**, a high incidence rate of IA of 21% was observed in a cohort of older patients with acute myeloid leukaemia (AML) or high-risk myelodysplastic syndrome (MDS), primarily during remission induction therapy in the absence of antifungal prophylaxis. The overall mortality rate was 88%. While the diagnosis of IA up to the final chemotherapy cycle was associated with an increased risk of death due to leukaemia or infection, the exact contribution of IA to the cause of death remained unclear.

A universal classification system to determine the role of IA in the cause of death (COD) could provide valuable insights into the number of deaths related to IA. In **chapter 2** such a classification method was developed and tested. Remarkably, among the total number of deaths, only a minority were attributable or contributable to IA. However, is it clinically relevant to determine whether death is attributable, contributable or unrelated to IA? Patients with AML or high risk MDS have a high risk of developing IA due to prolonged and severe neutropenia caused by intensive cytotoxic treatment or the haematological malignancy. These patients also face a high overall risk of mortality, as the malignancy itself, chemotherapy, and resulting immunosuppression can each lead to severe, potentially fatal complications. In a patient with multiple diseases, death may result directly from each disease, from a sequence of events triggered by each disease, or may occur independently of the diseases. The direct COD may often not be IA, i.e. patients are more likely to die with IA than because of IA, but determining the exact role IA played in the COD is complex and even impossible in some cases.

While understanding the role of IA in mortality is valuable, preventing IA itself remains a key priority, as mortality is not the only burden it imposes. A meta-analysis showed that the incidence of IA during remission-induction therapy for a haematological malignancy was higher in patients not receiving antifungal prophylaxis (prophylaxis 4%; no prophylaxis 11%).[15] Currently, antifungal prophylaxis is standard of care in patients with a high risk of IA, such as patients with AML or high risk MDS undergoing intensive chemotherapy, with the intention to

lower the incidence of IA. Current guidelines recommend triazoles, in particular posaconazole.[16-18] By lowering the incidence of IA, the morbidity of IA can be reduced/prevented in this at-risk population. This could have a beneficial effect on the prevention of direct clinical complications of IA (e.g. haemoptysis, or disseminated infection such as brain abscesses), as well as on the decrease of other indirect burdens such as the need for invasive procedures to diagnose IA (e.g. BAL), long-term therapies that in potential could be toxic or interact with other drugs, and a longer hospital stay or admission to the intensive care unit. Besides antifungal prophylaxis other preventative measures to minimise fungal exposure are important, and are recommended by guidelines: protective isolation in HEPA-filtered rooms, avoidance of construction, renovation or building sites, and avoidance of potted plants or flowers in the patients rooms and in the ward.[16-18]

In **chapter 4**, an incidence rate of 15% of COVID-19 associated pulmonary aspergillosis (CAPA) was observed in patients admitted to the ICU in the Netherlands in the pre-vaccination era when treatment with immunosuppressive agents was standard therapy, which was comparable to the preceding COVID-19 period when patients were not treated with immunosuppressive therapy. The EORTC/MSGERC host risk factors for IA or treatment with corticosteroids or anti-IL-6 were not found to be risk factors for CAPA. The 90-day overall mortality was 65% in patients with CAPA compared to 54% without CAPA (p = 0.008). Whether mortality was attributable, contributable or unrelated to CAPA was not analysed and was unknown, again demonstrating the complexity of determining COD in patients with IA.

A meta-analysis, that included 28 studies of which 17 from the pre COVID-vaccination era, detected nine risk factors for CAPA: chronic liver disease, haematological malignancy, chronic obstructive pulmonary disease, cerebrovascular disease, diabetes, invasive mechanical ventilation, use of renal replacement therapy, treatment with anti-IL-6 and treatment with corticosteroids.[19] Interestingly, besides haematological malignancy other EORTC/MSGERC host factors were not associated with CAPA.[19] Currently, in the COVID-vaccination era, hospital admission because of COVID-19 mostly happens in vulnerable patients such as older patients or patients with underlying comorbidities, in particular immunocompromised patients with EORTC/MSGERC host factors for IA.[20] This has changed the CAPA epidemiology: a retrospective study in mechanically ventilated adult patients with COVID-19 observed a significantly higher incidence of CAPA in the COVID-vaccination era as compared to the pre-vaccination era (59% vs 24%), mainly due to an increased proportion of patients with EORTC/MSGERC host risk factors.[21]

SCREENING

When screening tests for disease (development) are considered in routine practise, potential benefits and risks have to be weighed, and overall benefits of screening should outweigh the risks. International criteria for population screening were developed by Wilson and Junger[22] and the WHO[23], and are centred around the following principle: "*The central idea of early disease detection and treatment is essentially simple. However, the path to its successful achievement (on the one hand, bringing to treatment those with previously undetected disease and, on the other, avoiding harm to those persons not in need of treatment) is far from simple though sometimes it may appear deceptively easy.*" These principles are the basis for national cancer and prenatal/neonatal screening programmes in the Netherlands. Participation in the Dutch national screening programmes is voluntary, and eligible individuals are informed about the advantages and disadvantage of the screening test.

Prospective systematic screening for the presence of *Aspergillus* in patients with COVID-19 was initiated in the ICU of the LUMC from April 2020 onwards in an attempt to rapidly assess the epidemiology of fungal superinfections during the COVID-19 pandemic. The results of screening for the presence of *Aspergillus* and development of CAPA after the first COVID-19 peak (**chapter 5**) and over a 2-year period (**chapter 6**) showed that minimal-invasive screening by tracheal aspirate (TA) by culture and PCR was a feasible and rapid method to longitudinally monitor patients, and that positive screening together with a clinical suspicion of a superinfection appeared to be a good indication for BAL sampling. As described in **chapter 4**, screening for CAPA was performed in a number of other clinical centres as well but was not associated with earlier CAPA diagnosis or reduced mortality compared to a reactive strategy. However, it is hard to draw definitive conclusions as this was an observational study and the participating clinical centres had different methods of screening for CAPA.

Collection of TA, for culture and *Aspergillus* PCR, was the starting point of the diagnostic algorithm in the LUMC in COVID-19 patients. Positive TA results combined with clinical signs/symptoms prompted further diagnostic testing for *Aspergillus* by performing a BAL. In the ICU, samples could easily be collected because patients were intubated. For COVID-19 patients admitted to other non-ICU departments, collection of TA is more complicated because patients are not intubated. Another non-invasive method of screening for IA – in all patients at risk for *Aspergillus* disease – could be by collection of serum for GM testing. In neutropenic patients, however, international guidelines recommend against serial screening with serum GM in patients receiving antifungal prophylaxis be-

cause of the low prevalence of IA in this setting and the consequently low positive predictive value. [17, 18] Yet, in most high-risk patients, antifungal prophylaxis is standard of care, limiting the utility of routine serum GM testing in this population. In immunocompetent patients with influenza or COVID-19 serum GM has limited to poor sensitivity,[24] thus a negative test cannot rule out disease, which makes it an unsuitable tool for screening. It can serve as a prognostic marker though: a positive test is indicative of angio-invasive *Aspergillus* disease, and is associated with high mortality.[24]

An alternative approach could be routine BAL GM testing, which has a good performance for diagnosing IA.[17] If routine BAL GM was to be considered in patients at risk for *Aspergillus* disease, what would the number needed to undergo a bronchoscopy be to detect one *Aspergillus* disease case? Would this outweigh the invasiveness of the procedure itself, or the possible complications following bronchoscopy, such as respiratory or haemodynamic instability? For routine BAL GM the benefits of screening would probably not outweigh the harm, and would also carry additional financial costs. Currently, BAL sampling is performed in a select group of at-risk patients: those with clinical signs/symptoms, not responding to broad spectrum antibiotics, or positive screening results (in COVID-19 patients). In this group the incidence rate of *Aspergillus* will be higher, and the benefits of performing a BAL for diagnosis and treatment of IA will outweigh the harm. Conversely, given the high incidence rate of IA (~20%) in patients with influenza admitted to the ICU in the Netherlands, BAL sampling is recommended in the first 48 hours and treatment with an antifungal should be initiated immediately on admission.[25] In patients with COVID-19 either antifungal prophylaxis or systematic screening for CAPA might be beneficial in the current post-vaccination era. Which strategy is preferential needs to be determined.

Thus, the chosen screening method and consequential diagnostic algorithm depend on multiple factors, for example patient characteristics, incidence rate of *Aspergillus*, antifungal prophylaxis, feasibility of sample collection, or interpretation of test results. In the end, minimizing the overall burden is the primary objective. A suggested algorithm to decide upon screening for IA is shown in figure 2.

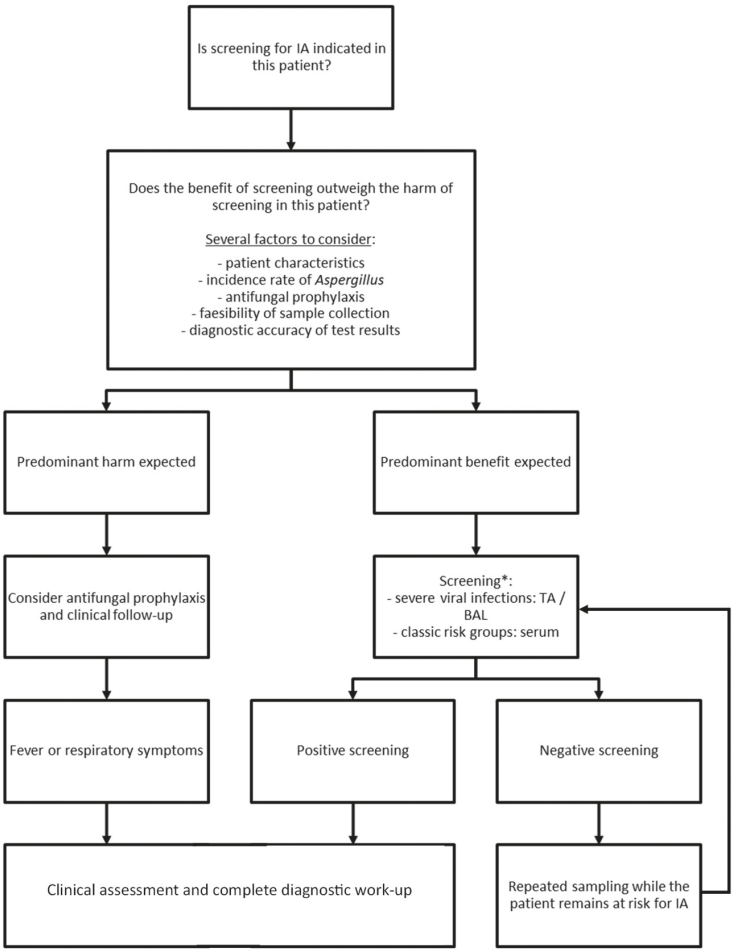


Figure 2. Algorithm for screening for invasive aspergillosis in a patient at risk for invasive aspergillosis. IA: invasive aspergillosis; BAL: bronchoalveolar lavage; TA: tracheal aspirate. *: Also consider prophylaxis in patients with a high risk of IA.

SURVEILLANCE

The terms "screening" and "surveillance" are often used as a synonym. However, Wilson & Jungner[22] made an important distinction between the two: "screening" is considered to be a cross-sectional, short-term operation of a population at risk, whilst "surveillance" is considered to be a long-term process in which screening examinations are repeated at intervals over time. The U.S. Centers for Disease Control and Prevention (CDC) defines surveillance as: "*The ongoing, systematic collection, analysis, and interpretation of health-related data essential to planning, implementation, and evaluation of public health practice,*

closely integrated with the timely dissemination of these data to those responsible for prevention and control".[26]

In **chapter 7**, a pilot study, performed in two academic medical centres, for future national surveillance of *Aspergillus* disease was described. One of the challenges was defining inclusion criteria, because unlike bacterial or yeast bloodstream infections where a positive blood culture reflects infection, a positive BAL culture does not necessarily indicate the presence of an infection. Defining IA is complex, and depends on a combination of host factors, clinical factors and mycological evidence of disease. For immunocompromised patients the EORTC/MSGERC criteria[27] are used for classification. For ICU patients, the FUNDICU criteria[28] were recently published with the aim of developing a standard definition for *Aspergillus* disease in critically ill patients with or without influenza or COVID-19 for research purposes.

Classification criteria for IA have mainly been developed for use in clinical studies, but are currently used in clinical routine practice as well. For clinical studies these criteria are required to determine whether a patient can be included in a study, and are mostly based on a single positive microbiological test result at a certain moment. Classification criteria create uniformity and allow studies to be compared with one another. In clinical practice, determining whether a patient has IA is more nuanced, and diagnosis of IA might be considered when besides the certainty of diagnosis several other aspects are taken into account such as comorbidities, clinical response, multiple diagnostic test results over time, treatment options, etc. Patients who do not fulfil the classification criteria might be diagnosed with IA, and patients who fulfil the classification criteria might not be diagnosed with IA. For example, a patient with haematological disease with persistent fever under broad-spectrum antibiotics with atypical lesions on imaging not described in EORTC/MSGERC criteria,[27] with mycological evidence of disease is unclassifiable according to EORTC/MSGERC criteria,[27] but IA will most likely be considered in a clinical context. In contrast, a patient with COVID-19 admitted to the ICU for respiratory support with a positive BAL GM is classifiable according to FUNDICU[28] criteria, but might be improving clinically without antifungal therapy, thus IA might be deemed unlikely as was shown in **chapter 5 and 6**.

For disease surveillance purposes, defining IA by using antifungal treatment as a criterion may provide a more accurate estimate of the presence of disease than a single positive diagnostic test result or complying to a set of other non-treatment scientific criteria, as it likely better represents the patients who were actually suspected of having *Aspergillus* disease. Though, while this approach

may reduce the diagnostic certainty of infection, this is not necessarily problematic, as the objective differs from that of a clinical study. This was underscored in **chapter 7**: most patients with *Aspergillus* disease according to EORTC/MSGERC,[27] AspICU,[29] or IAPA[30] criteria were detected by including those with positive mycological test results who were treated with an antifungal agent.

FUTURE DIRECTIONS

Invasive aspergillosis is a global health threat with a rising number of at-risk patients. Diagnosis and management of IA is challenging because of limitations in current diagnostic tools and the emergence of antifungal resistance. It has become clear from our work and that of others that at the moment there are limited antifungal treatment options, and that these options are further reduced by the global spread of environmentally acquired triazole resistance in *A. fumigatus*. Therefore, a one health interdisciplinary collaboration to improve the health of humans, animals and ecosystems by integrating these fields is necessary. Risk-assessments and policy interventions should be developed to prevent environmental resistance selection for novel antifungal drugs.

Climate change is an important part of a one health approach as well. Due to global warming areas where fungi can survive may expand, extreme weather could boost the growth and spread of fungi, and higher temperatures may cause fungi to adapt to survive in warmer conditions such as the human body.[31] This will unavoidably lead to a further increase in exposure of patients at-risk for (new) fungal infections as described in **chapter 1**. *Cryptococcus gattii* has been proposed as an example of a fungal pathogen whose emergence may be facilitated by climate change. *C. gattii* was previously confined to (sub)tropical areas, but has established itself and has become endemic in temperate zones such as Vancouver Island in Canada and the Pacific Northwest region of the United States.[32] The first cases were identified in wild and domestic animals after which human cases were detected.[32, 33] In response to the growing concern of invasive fungal infections the WHO fungal priority pathogens list[34, 35] was published, and one of the key areas of action is surveillance of fungal infections.

However, one of the difficulties that were encountered when setting up a pilot surveillance for *Aspergillus* disease, was how to detect patients and how the disease itself should be defined for this purpose. The inclusion criterion for the study was a positive BAL test (culture, GM or PCR), but most patients included in the study were formally unclassifiable. This emphasises the need for new (non-invasive) sensitive and specific biomarkers for IA. Also, in patients with

a high-risk of IA receiving antifungal prophylaxis, a non-invasive test – not affected by antifungal prophylaxis – would be ideal.

New diagnostic approaches for diagnosis of IA include molecular techniques such as metagenomic next-generation sequencing (mNGS) to detect microbial cell-free DNA in plasma;[36, 37] mNGS of BAL fluid;[38] a mold plasma cell-free DNA (cfDNA) PCR.[39] Also host metabolites have been studied as diagnostic markers such as pentraxin 3 – an acute phase protein that possibly contributes to the antifungal innate immune response – in plasma or BAL,[40] and analysis of cytokine profiles.[41, 42] Fungal metabolites such as siderophores – iron chelators secreted by *Aspergillus* to obtain iron for growth, e.g. triacetylfusarinine C (TAFC), the principal secreted siderophore of *A. fumigatus* which is produced only by actively growing cells and is absent in conidia – have been detected by mass spectrometry in blood, urine or BAL fluid;[43, 44] the use of siderophores in functional imaging with positron emission tomography (PET) has been described as well.[44] Of these new tests/biomarkers mNGS of microbial cell free DNA in plasma and the siderophores seem most promising. However, the effect of prophylaxis on these tests has to be further explored.

The current classification methods for IA – EORTC/MGSERC,[27] FUNDICU,[28] AspICU,[29] IAPA,[30] 2020 ECMM/ISHAM consensus criteria for CAPA[45] – are not practical for surveillance purposes, because of incongruent criteria for classification. An attempt to restructure the classification methods for ICU patients has been done by combining the AspICU,[29] IAPA[30], and 2020 ECMM/ISHAM consensus criteria for CAPA,[45] in the FUNDICU[28] for classification of proven or probable IA. However, the feasibility of these criteria for surveillance purposes is still questionable. The classification methods have all been developed for use in clinical studies, for uniformity so that studies can be compared, and are therefore less applicable for use in a clinical setting or for surveillance purposes. To allow comparison with older studies these criteria will probably still be used in the future. Nevertheless, further work is necessary to refine and harmonise definitions of IA to make them more practical for surveillance purposes. Maybe additional clinical information about consecutive test results or antifungal treatment for example could support diagnosis of IA, and aid in detecting patients. The role machine learning or artificial intelligence can play in detecting and classifying patients, for clinical studies and surveillance purposes, should be further explored.[46] Currently, the FUNGISENS study is investigating if artificial intelligence can be used to monitor *Aspergillus* disease.[47]

In conclusion, in the future the number and diversity of patients at risk for IA will most certainly increase. Disease surveillance will be crucial for following this

development, for identifying new patient populations at risk and to be able to assess the burden, prevention and management of *Aspergillus* disease. New technologies, including artificial intelligence, might enhance identification and classification of patients, and their application in this context warrants further investigation. Ultimately, adopting a one health approach is essential to effectively address IA and prepare for future challenges.

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NEDERLANDSE SAMENVATTING

Aspergillus is een schimmel die veelvuldig voorkomt in de natuur. Het is een saprotrofe schimmel, wat inhoudt dat *Aspergillus* voedingsstoffen uit dood organisch materiaal haalt, zoals bijvoorbeeld afgevallen bladeren, compost of rottend voedsel. De schimmel vermeerdert zich door de vorming van sporen. Deze sporen kunnen zich verspreiden in de omgeving en in de lucht, en uitgroeien tot een nieuwe schimmel. De meeste mensen ademen dagelijks meerdere *Aspergillus* sporen in zonder hiervan ziek te worden. Bij sommige mensen kan het inademen van sporen echter wel leiden tot een infectie. Hoe het ziektebeeld zich uit hangt samen met het afweersysteem van de persoon die de sporen heeft ingeademd.

De ziektebeelden veroorzaakt door *Aspergillus* kunnen grofweg ingedeeld worden in drie verschillende groepen: invasieve aspergillose, chronische pulmonale aspergillose en allergische bronchopulmonale aspergillose, een aparte groep patiënten met allergische manifestaties veroorzaakt door overgevoeligheid voor *Aspergillus*. Invasieve aspergillose wordt gezien in patiënten met een verminderde afweer, o.a. ernstig en langdurig tekort aan witte bloedcellen (neutropenie), kwaadaardige aandoeningen van bloed, beenmerg of lymfklieren zoals leukemie, of een stamceltransplantatie. Dit zijn zogenoemde klassieke risicofactoren voor het ontwikkelen van invasieve aspergillose. Neutrofielen zijn witte bloedcellen die een belangrijke rol spelen in de afweer tegen bacteriële en schimmelinfecties, en bij een laag aantal werkt de afweer onvoldoende, waardoor er een verhoogde kans is op infecties. Neutropenie kan worden veroorzaakt door leukemie of door behandelingen hiervoor.

Bij invasieve aspergillose bevindt de infectie zich voornamelijk in de longen/luchtwegen – het wordt ook wel invasieve pulmonale aspergillose genoemd – maar het kan zich ook uitbreiden naar andere locaties zoals bijvoorbeeld het brein. Chronische pulmonale aspergillose wordt gezien in patiënten met onderliggend longlijden die een normale of mild verminderde afweer hebben, met aandoeningen waarbij de longen zijn aangetast zoals bijvoorbeeld COPD. In de afgelopen jaren is invasieve aspergillose ook beschreven in patiënten met onder andere een auto-immuunziekte of vormen van kanker die worden behandeld met nieuwe therapieën die het immuunsysteem kunnen beïnvloeden, zoals monoklonale antilichamen, small-molecule-kinaseremmers, of cellulaire behandelingen. Daarnaast wordt invasieve aspergillose ook gezien in patiënten met influenza of COVID-19, die opgenomen zijn op de intensive care voor invasieve beademing. Deze patiëntengroepen, met een verhoogd risico op invasieve aspergillose, zijn heterogeen en nemen in aantal toe, door vergrijzing en doordat

meer patiënten met nieuwe therapieën worden behandeld. Hierdoor zal naar verwachting het aantal patiënten met deze infectie ook toenemen. Dit promotieonderzoek richtte zich voornamelijk op patiënten met invasieve aspergillose.

Het stellen van de diagnose invasieve aspergillose is ingewikkeld en hangt af van de aan- of afwezigheid van klinische kenmerken (de risicofactoren van de patiënt), radiologische bevindingen (typische afwijkingen op CT-thorax) en mycologische bevindingen. Mycologische diagnostiek bestaat uit microscopie, kweek, *Aspergillus* DNA onderzoek zoals de polymerase ketting reactie (PCR), en *Aspergillus* antigeentest. Invasieve aspergillose is zeker als histopathologische schimmelkleuringen van een biopt positief zijn. Biopten worden echter zelden afgenomen vanwege mogelijke complicaties zoals een bloeding. Veelal wordt materiaal uit de luchtwegen, zoals sputum of een broncho-alveolaire lavage monster (spoelsel uit de longen afgenomen tijdens een bronchoscopie), afgenomen voor diagnostiek. Met deze materialen kan een diagnose heel aannemelijk worden gemaakt, maar niet met zekerheid bevestigd zoals bij een biopt wel kan. Negatieve mycologische diagnostiek sluit ziekte namelijk niet uit en een positieve test kan ook op kolonisatie van de luchtwegen duiden zonder dat sprake is van een infectie. Op deze materialen kunnen bovendien verschillende mycologische testen worden gedaan die niet altijd met elkaar in overeenstemming zijn; een longspoelsel monster kan bijv. microscopie en kweeknegatief zijn, maar PCR en antigeen positief. Kweken zijn veelal negatief bij patiënten met een verminderde afweer met invasieve aspergillose, waardoor de diagnostiek vooral afhangt van PCR en *Aspergillus* antigeentest. Bij het stellen van de diagnose is het daarom van belang om risicofactoren, radiologische bevindingen en mycologische testresultaten mee te nemen. Daarnaast bestaan voor de verschillende patiëntengroepen verschillende classificatiecriteria, die ontwikkeld zijn voor wetenschappelijke studies, maar die ook in de medische praktijk worden toegepast voor het stellen van de diagnose. Er zijn richtlijnen voor patiënten met een verminderde afweer (EORTC/MSGERC richtlijn waarin risicofactoren worden beschreven voor invasieve aspergillose), voor patiënten met influenza, voor patienten met COVID-19 en voor patiënten opgenomen op de intensive care.

Op dit moment zijn de triazolen, een bepaalde groep antischimmelmiddelen zoals voriconazol en posaconazol, de eerste keuze behandeling van invasieve aspergillose. Helaas worden wereldwijd triazoolresistente *Aspergillus* stammen gevonden, wat de – toch al gelimiteerde – behandelopties van ziekte veroorzaakt door *Aspergillus* beperkt. Antifungale resistentie ontstaat voornamelijk in het milieu doordat *Aspergillus*, die veelvuldig voorkomt in de omgeving, wordt blootgesteld aan triazool fungiciden. Dit zijn schimmelbestrijdingsmiddelen die worden gebruikt in de landbouwsector om gewassen te beschermen tegen

schimmelinfecties. Doordat deze middelen lijken op de medische azolen die worden gebruikt voor de behandeling van patiënten, verliezen de medische azolen ook hun werkzaamheid. Patiënten moeten dan behandeld worden met andere middelen, meestal liposomaal amfotericine B, wat per infuus moet worden toegediend, veel bijwerkingen heeft en duurder is dan de triazolen.

In Nederland wordt al een aantal jaar *Aspergillus fumigatus* (*A. fumigatus*) pathogeensurveillance uitgevoerd waar tien ziekenhuizen aan deelnemen. Surveillance betekent toezichthouden door middel van het verzamelen, analyseren en interpreteren van gegevens, om trends te ontdekken, uitbraken vroeg te herkennen, en beleid en preventieve maatregelen te ondersteunen of maatregelen te treffen. Bij de *A. fumigatus* pathogeensurveillance worden *A. fumigatus* stammen, gekweekt uit patiëntmaterialen die in screening triazoolresistent zijn, opgestuurd naar het referentielaboratorium voor verder onderzoek. Deze vorm van surveillance geeft een goed beeld van de mate van triazoolresistentie. Het biedt echter geen inzicht in de medische gevolgen van triazoolresistentie omdat er geen patiëntgegevens worden verzameld. Daarnaast worden kweek-negatieve gevallen niet meegenomen. Om meer inzicht te krijgen in de impact van en risicofactoren voor ziekte veroorzaakt door (triazoolresistente) *Aspergillus* in verschillende patiëntenpopulaties is *Aspergillus* ziektesurveillance noodzakelijk. De zogenoemde ziektelast van *Aspergillus* kan inzichtelijk worden gemaakt door gegevens te verzamelen over de incidentie (hoeveel nieuwe gevallen van invasieve aspergillose in een bepaalde populatie in een specifieke periode), de morbiditeit (complicaties of beperkingen door invasieve aspergillose), en mortaliteit (sterfte). Daarnaast kunnen andere factoren die de ziektelast bepalen worden meegenomen, zoals de belastende diagnostiek die nodig is om een diagnose te stellen (bijv. het ondergaan van een longspoeling), bijwerkingen van therapie, of antifungale resistentie.

De doelen van dit promotieonderzoek waren om meer inzicht krijgen in ziektelast veroorzaakt door invasieve aspergillose, de doeltreffendheid van diagnostiek en behandeling van invasieve aspergillose te onderzoeken, en om de mogelijkheden van *Aspergillus* ziektesurveillance goed in kaart te brengen.

Deel I: ziektelast, diagnostiek en behandeling van invasieve aspergillose

In **hoofdstuk 2** wordt de lokale behandelrichtlijn voor invasieve aspergillose beschreven, vijf jaar nadat deze in gebruik was genomen. Deze lokale richtlijn werd geïmplementeerd in 2015 omdat in de regio Leiden een hoge mate van triazoolresistentie was vastgesteld in de *A. fumigatus* pathogeensurveillance. Er werd een behandelalgoritme opgesteld op basis van patiëntkenmerken,

waarbij patiënten empirisch werden behandeld met voriconazol of liposomaal amfotericine B, in plaats van een combinatie van deze middelen. Empirische behandeling betekent dat met antibiotica of een antifungaal middel wordt gestart gericht op de te verwachten meest voorkomende bacteriën of schimmels, in afwachting van een identificatie van de bacterie of schimmel en gevoeligheidsbepaling. Behandeling met één middel heeft de voorkeur boven dubbeltherapie doordat het leidt tot minder bijwerkingen of interacties met andere medicatie. Ziekere of kwetsbare patiënten of patiënten die ziekte ontwikkelden onder antifungale profylaxe (behandeling om ziekte te voorkomen) werden behandeld met liposomaal amfotericine B, andere patiënten kregen voriconazol ondanks de kans op resistentie. De vraagstelling was of het veilig was om niet alle patienten liposomaal amfotericine B te geven in afwachting van diagnostiek. Door een panel werd beoordeeld of sterfte op 30- en 100-dagen te maken had met de invasieve aspergillose, waarbij de verschillende opties waren: toerekenbaar, mogelijk toerekenbaar, niet toerekenbaar, of onbekend.

Empirische behandeling met voriconazol werd bij 51% (56/110) van de patiënten gestart, en bij 43% (24) van deze patiënten werd alsnog een switch gemaakt naar liposomaal amfotericine B. Behandeling met liposomaal amfotericine B kon bij 29% van de patiënten worden vermeden. De toerekenbare of mogelijk toerekenbare sterfte aan invasieve aspergillose op 100-dagen was 21% in de patiëntgroep die een switch had gemaakt van voriconazol naar liposomaal amfotericine B, maar was niet hoger dan de patiëntgroep die direct met liposomaal amfotericine B was gestart. Deze studie toonde aan dat dubbeltherapie met het behandelalgoritme veilig kon worden vermeden. Gezien de wereldwijde opkomst van triazoolresistentie en mogelijk ook resistentie tegen nieuwe antifungale middelen, is empirische dubbeltherapie in de toekomst wellicht noodzakelijk. Het blijft echter van belang om lokale resistentiecijfers mee te nemen in het behandelbeleid, om te komen tot een gebalanceerde afweging tussen optimale therapie met minimale bijwerkingen.

In **hoofdstuk 3** wordt de ziektelast van invasieve aspergillose beschreven in een prospectief verzameld cohort, een patiëntgroep die in de tijd wordt gevolgd, van oudere patiënten met acute leukemie. Er werd een rekenkundig model ontwikkeld om een schatting te maken van de cumulatieve incidentie van sterfte door leukemie, infectie of overige oorzaken, en om het effect van invasieve aspergillose op de verschillende doodsoorzaken vast te stellen. Cumulatieve incidentie geeft de kans weer dat een specifieke gebeurtenis (sterfte door leukemie, infectie of overige oorzaken) zich heeft voorgedaan op een bepaald moment in de tijd.

Leukemie was de meest waarschijnlijke doodsoorzaak in de hele periode dat patiënten werden gevolgd, maar in het eerste jaar na de leukemie diagnose was infectie de meest waarschijnlijke doodsoorzaak. Incidentie van invasieve aspergillose was 21% en patiënten met invasieve aspergillose hadden een relatief verhoogd risico op overlijden door leukemie of infectie, hoewel het onduidelijk was welke rol de schimmelinfectie had gespeeld.

Het is ingewikkeld om te bepalen of sterfte toerekenbaar is aan invasieve aspergillose. Patiënten met acute leukemie hebben een hoog risico op het ontwikkelen van invasieve aspergillose door een diepe en langdurige neutropenie die wordt veroorzaakt door de hematologische ziekte en door de intensieve chemotherapie. Deze patiënten hebben ook een grote kans om te overlijden doordat de hematologische ziekte, chemotherapie, en neutropenie kunnen leiden tot ernstige of dodelijke complicaties. Als een patiënt meerdere ziektes heeft en komt te overlijden, dan kan elke ziekte toerekenbaar, mogelijk toerekenbaar, of niet toerekenbaar zijn aan sterfte. De directe doodsoorzaak is veelal waarschijnlijk niet invasieve aspergillose, wat betekent dat patiënten overlijden met, maar niet ten gevolge van de infectie. Dit werd bevestigd in **hoofdstuk 2**: sterfte was meestal niet toerekenbaar aan invasieve aspergillose.

Hoewel, het bepalen van de rol van invasieve aspergillose in sterfte belangrijk is, is het vooral van belang om deze infectie te voorkomen in patiënten met een hoog risico. De incidentie van deze infectie in patiënten met een hematologische ziekte zoals leukemie, tijdens behandeling met chemotherapie, die geen antifungale profylaxe krijgen is ~11%. Daarom wordt aan deze patiëntgroep antifungale profylaxe gegeven, om invasieve aspergillose te voorkomen. Doordat de incidentie van invasieve aspergillose wordt verlaagd, wordt de ziektelast ook verlaagd (o.a. minder complicaties zoals verspreiding van de schimmel naar andere organen), of minder noodzaak voor belastende diagnostiek om een infectie vast te stellen). Andere preventiemaatregelen om blootstelling aan schimmels te verminderen zijn high-efficiency particulate air (HEPA)-gefilterde kamers, dat zijn filters die heel kleine deeltjes uit het lucht kunnen filteren, waaronder *Aspergillus* sporen; het voorkomen van bouwwerkzaamheden; en planten/bloemen weren in de patiëntkamers en op de afdelingen.

In **hoofdstuk 4** worden de incidentie, risicofactoren, mortaliteit en diagnostiek bij patiënten met COVID-19 geassocieerde pulmonale aspergillose beschreven in meerdere ziekenhuizen in Nederland tussen september 2020 en april 2021. De studie was retrospectief, wat bekent dat gegevens van een patiëntgroep in het verleden reeds zijn verzameld voor een ander doel en achteraf geanalyseerd worden. In deze periode waren COVID-19 vaccins nog niet beschikbaar in Ne-

derland en werden patiënten met COVID-19 opgenomen op de intensive care voor invasieve beademing behandeld met afweer onderdrukkende middelen zoals dexamethason en interleukine-6 remmers.

De incidentie van COVID-19 geassocieerde pulmonale aspergillose was 15% en was vergelijkbaar met eerdere perioden waarin behandeling met afweer onderdrukkende middelen nog geen vast onderdeel was van de behandeling van COVID-19 patiënten. De klassieke EORTC/MSGERC risicofactoren voor invasieve aspergillose en behandeling met immunosuppressieve middelen waren geen risicofactor voor COVID-19 geassocieerde pulmonale aspergillose. De 90-dagen mortaliteit was 65% in patiënten met COVID-19 geassocieerde pulmonale aspergillose en 54% in patiënten zonder infectie. De rol van invasieve aspergillose op het overlijden werd niet geanalyseerd. De diagnostische aanpak verschilde per centrum: in een aantal centra werd screening naar COVID-19 geassocieerde pulmonale aspergillose verricht vanaf opname op de intensive care, terwijl in andere centra pas op indicatie diagnostiek werd ingezet. De wijze van screening verschilde ook per centrum. Er werd geen duidelijk voordeel van screening gevonden, maar gezien de studieopzet – retrospectief, observationeel – kon hier geen eenduidige conclusie getrokken worden. In de huidige COVID-19 vaccinatie tijdperk worden vooral kwetsbare patiënten, zoals ouderen, of patienten met een verminderde afweer waaronder patiënten met EORTC/MSGERC risicofactoren voor invasieve aspergillose opgenomen met COVID-19. Door een groter aandeel van COVID-19 patiënten met EORTC/MSGERC risicofactoren, wordt momenteel een hogere COVID-19 geassocieerde pulmonale aspergillose incidentie gezien onder patiënten met COVID-19 opgenomen op de intensive care voor invasieve beademing.

Deel II: screening en surveillance

Tijdens de start van de COVID-19 pandemie werd screening naar COVID-19 geassocieerde pulmonale aspergillose opgestart met als doel om inzicht te krijgen in het voorkomen van schimmelinfecties bij patiënten met COVID-19. Bij patiënten met COVID-19 opgenomen op de intensive care voor invasieve beademing werd twee keer per week een tracheaal aspiraat afgenomen voor *Aspergillus* kweek en PCR. Tracheaal aspiraat is luchtwegmateriaal uit de onderste luchtwegen dat bij geïntubeerde patiënten via de tube kan worden afgenomen. Bij patiënten met een positieve tracheaal aspiraat kweek of PCR in combinatie met aanwijzingen voor een infectie werd, indien mogelijk, een longspoeling verricht om COVID-19 geassocieerde pulmonale aspergillose te bevestigen. De screeningresultaten na de eerste COVID-19 piek (april 2020 – mei 2020) zijn beschreven in **hoofdstuk 5** en de screeningsresultaten na twee jaar (april 2020 – februari 2022) zijn beschreven in **hoofdstuk 6**. Deze hoofdstukken laten

zien dat screening, d.m.v. kweek en PCR van makkelijk en minimaal belastend te verkrijgen tracheaal aspiraat monsters, een haalbare en snelle manier was om patiënten te monitoren. Bovendien was positieve screening in combinatie met een klinische COVID-19 geassocieerde pulmonale aspergillose verdenking een goede manier om patiënten te selecteren voor een – invasieve – longspoeling; de infectie werd bevestigd in 74% van patiënten waarbij een longspoeling werd verricht.

Voordat een screeningtest wordt geïmplementeerd in routine diagnostiek dienen de voor- en nadelen van een test te worden afgewogen, en moeten de voordelen groter zijn dan de nadelen. Een ideale screeningtest is een test die op materiaal uitgevoerd kan worden wat op niet belastende wijze verkregen kan worden, en waarbij de test zowel een hoge sensitiviteit en specificiteit heeft. Sensitiviteit is de kans dat een testuitslag positief is als iemand de ziekte heeft (terecht positieve uitslag). Specificiteit is de kans dat een testuitslag negatief is als iemand de ziekte niet heeft (terecht negatieve uitslag). Een test met een lage sensitiviteit zal ziekte missen bij mensen die wel de ziekte hebben, en een test met een lage specificiteit zal ten onrechte positief zijn bij mensen die de ziekte niet hebben.

De wijze van screenen naar *Aspergillus* beschreven in **hoofdstuk 5 en 6** is goed uitvoerbaar op de intensive care omdat patiënten zijn geïntubeerd, maar is ingewikkeld op verpleegafdelingen waar patiënten niet zijn geïntubeerd. Een niet-belastende screening methode voor invasieve aspergillose is bijvoorbeeld serum antigeentest. Deze test heeft een goede sensitiviteit in patiënten met neutropenie, maar de sensitiviteit is beperkt in patiënten of COVID-19 die een normale afweer hebben. Een negatieve serum antigeentest bij een patiënt met COVID-19 sluit ziekte dus niet uit en is daardoor ongeschikt als screeningstest. Een antigeentest op longspoelsel heeft een goede sensitiviteit voor het diagnosticeren van invasieve aspergillose, maar een longspoeling is belastend en de procedure kan leiden tot complicaties. Op dit moment wordt een longspoeling – terecht – alleen verricht in een selecte groep patiënten: risicogroep voor invasieve aspergillose, klachten/symptomen, niet reagerend op breedspectrum antibiotica, of positieve screening resultaten (COVID-19 patiënten). In deze groep zal de incidentie van invasieve aspergillose groter zijn en wegen de voordelen van een longspoeling op tegen de nadelen. De screeningmethode en daaropvolgend diagnostisch algoritme zijn dus afhankelijk van verschillende factoren, o.a. incidentie van invasieve aspergillose in een specifieke patiëntengroep, hoe makkelijk het materiaal te verkrijgen is, en testprestatie (sensitiviteit en specificiteit).

De termen screening en surveillance worden vaak door elkaar gebruikt, maar ze hebben een andere betekenis. Screening beschrijft een dwarsdoorsnede onderzoek op één moment in een populatie met risicofactoren, terwijl surveillance een lange termijn proces is waarbij screeningonderzoek wordt herhaald op vaste tijdsintervallen.

In **hoofdstuk 7** wordt een pilotstudie voor nationale *Aspergillus* ziektesurveillance beschreven, waarin werd geëvalueerd welke mycologische testen en patiëntgegevens nodig zijn voor het detecteren en classificeren van ziekten veroorzaakt door *Aspergillus* (invasieve aspergillose, chronische pulmonale aspergillose en allergische bronchopulmonale aspergillose). De gouden standaard was invasieve aspergillose volgens de verschillende classificatiecriteria voor patiënten met EORTC/MSGERC risicofactoren, patiënten met influenza of patiënten opgenomen op de intensive care. Het definiëren van inclusiecriteria was een uitdaging gezien de complexiteit van diagnostiek en classificatiecriteria. Classificatiecriteria voor invasieve aspergillose zijn met name ontwikkeld voor wetenschappelijke studies om te beoordelen of een patiënt deel kan nemen aan een studie en is meestal afhankelijk van één positieve mycologische test. Deze criteria zorgen voor uniformiteit, zodat studies met elkaar vergeleken kunnen worden. In de medische praktijk is het stellen van de diagnose meer genuanceerd en worden, naast een mycologische testuitslag, andere aspecten meegenomen in de beoordeling, zoals de ziektes die een patiënt heeft, respons op behandeling met antifungale middelen en de uitslagen van opeenvolgende mycologische testuitslagen. Hierdoor kan een patiënt die niet voldoet aan de classificatiecriteria in de dagelijkse medische praktijk toch worden gediagnostiseerd met invasieve aspergillose, en kan de diagnose worden verworpen bij een patiënt die wel voldoet aan de classificatiecriteria. Het classificeren van patiënten was te tijdrovend en complex voor surveillance doeleinden. Antifungale behandeling geeft mogelijk een beter beeld van patiënten met een verdenking op invasieve aspergillose, dan een mycologische testuitslag of classificatiecriteria alleen, want behandeling wordt over het algemeen alleen gestart bij patiënten die verdacht worden van een infectie. Dit werd bevestigd in **hoofdstuk 7**: een goede schatting van ziektegevallen veroorzaakt door *Aspergillus* werd verkregen door patiënten te includeren met positieve longspoelsel kweek, PCR of antigeentest in combinatie met antifungale behandeling.

Conclusies

In de toekomst zal naar verwachting het aantal patiënten met een verhoogd risico op invasieve aspergillose toenemen. Diagnostiek en behandeling van deze infectie blijven uitdagend door de beperkingen van de huidige diagnostische testen en de opkomst van triazoolresistentie. Een one-health benadering – een

integrale benadering van gezondheid van mens, dier en milieu – is essentieel, onder andere om resistentieselectie in de omgeving te voorkomen. Klimaatverandering speelt hierin ook een belangrijke rol. Stijgende temperaturen kunnen leiden tot een toename van schimmels, die zich mogelijk beter kunnen aanpassen aan hogere temperaturen, zoals die van het menselijk lichaam. Dit zal leiden tot een verdere toename van patiënten met een verhoogd risico op invasieve aspergillose. Ziektesurveillance is essentieel om patiënten te herkennen die risico lopen en om meer inzicht te krijgen in ziektelast, preventie, diagnostiek en behandeling van ziekten veroorzaakt door *Aspergillus*. De ontwikkeling van nieuwe (niet-invasieve) diagnostische testen met een hoge sensitiviteit en specificiteit is noodzakelijk. Evenals eenduidige definities voor ziekten veroorzaakt door *Aspergillus* voor surveillance doeleinden, waarbij kunstmatige intelligentie een rol kan spelen in het identificeren en classificeren van patiënten. Uiteindelijk is het belangrijk om vanuit een one-health perspectief vooruit te kijken en ons voor te bereiden op toekomstige uitdagingen.

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CURRICULUM VITAE

Rebecca werd geboren op 12 december 1986 in Bristol in het Verenigd Koninkrijk, als eerste kind van Anne van Grootveld en Robert van Grootveld. Zij groeide op in Bristol, Kaya (Burkina Faso), Hillegom, Noordwijkerhout, Oegstgeest, Nuwara Eliya (Sri Lanka) en Wieringerwerf, samen met haar zussen Eleanor en Jessica. In 2005 behaalde ze haar VWO diploma aan de Regionale Scholengemeenschap Wiringherlant in Wieringerwerf. Daarna begon ze met haar studie geneeskunde in het Leids Universitair Medisch Centrum (LUMC) in Leiden. Ze deed een keuzestage tropengeneeskunde in Sengerema in Tanzania, een semi-arts stage interne geneeskunde in het Reinier de Graaf ziekenhuis in Delft, en een wetenschappelijke stage op de afdeling Parasitologie van het LUMC. In 2012 behaalde zij haar artsexamen. Daarna werkte zij als arts-assistent interne geneeskunde in het Reiner de Graaf ziekenhuis, als arts-assistent psychiatrie bij GGZ Rivierduinen locatie Voorhout en als arts-onderzoeker bij de afdeling Parasitologie van het LUMC (supervisor dr. L. van Lieshout).

In 2015 startte ze met de opleiding tot arts-microbioloog in het LUMC (opleiders prof. dr. A.C.M. Kroes en dr. M.T. van der Beek). Tijdens de opleiding ontstond de interesse in schimmelinfecties en startte zij met een promotieonderzoek onder begeleiding van prof. dr. E.J. Kuijper, prof. dr. M.G.J. de Boer en dr. M. T. van der Beek. Sinds de afronding van haar opleiding in 2023 is ze werkzaam als arts-microbioloog bij het Regionaal Laboratorium Medische Microbiologie in het Albert Schweitzer ziekenhuis in Dordrecht en het Beatrixziekenhuis in Gorinchem.

Rebecca woont samen met Maarten Onderwater in Rijswijk, en samen hebben zij twee dochters Livia (2021) en Valerie (2022).

LIST OF PUBLICATIONS

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