Microbiological Diagnosis of Pulmonary *Aspergillus* Infections

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Abstract

As microbiological tests play an important role in our diagnostic algorithms and clinical approach towards patients at-risk for pulmonary aspergillosis, a good knowledge of the diagnostic possibilities and especially their limitations is extremely important. In this review, we aim to reflect critically on the available microbiological diagnostic modalities for diagnosis of pulmonary aspergillosis and formulate some future prospects. Timely start of adequate antifungal treatment leads to a better patient outcome, but overuse of antifungals should be avoided. Current diagnostic possibilities are expanding, and are mainly driven by enzyme immunoassays and lateral flow device tests for the detection of *Aspergillus* antigens. Most of these tests are directed towards similar antigens, but new antibodies towards different targets are under development. For chronic forms of pulmonary aspergillosis, anti-*Aspergillus* IgG antibodies and precipitins remain the cornerstone. More studies on the possibilities and limitations of molecular testing including targeting resistance markers are ongoing. Also, metagenomic next-generation sequencing is expanding our future possibilities. It remains important to combine different test results and interpret them in the appropriate clinical context to improve performance. Test performances may differ according to the patient population and test results may be influenced by timing, the tested matrix, and prophylactic and empiric antifungal therapy. Despite the increasing armamentarium, a simple blood or urine test for the diagnosis of aspergillosis in all patient populations at-risk is still lacking. Research on diagnostic tools is broadening from a pathogen focus on biomarkers related to the patient and its immune system.

Keywords
- invasive *aspergillosis*
- microbiological diagnosis
- galactomannan
- *Aspergillus* antigen
- *Aspergillus* PCR
- lateral flow assay

Unless we demonstrate the fungus in a normal sterile sample, we have a degree of uncertainty regarding the diagnosis of invasive pulmonary aspergillosis (IPA) as reflected in the revised classification by the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSGERC).[1] Although these criteria are mainly for study purposes, a similar reasoning is used in clinical practice in which we combine the patient's risk profile, the presence of clinical or radiological arguments for fungal disease, and the results of microbiological tests to, when

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combined, support a possible or probable diagnosis. A comparable combination of criteria is used in the guidelines from the European Society for Clinical Microbiology and Infectious Diseases and European Respiratory Society for diagnosing chronic pulmonary aspergillosis (CPA).[2] A microbiological test result can be meaningless if it does not fit the right clinical picture. Autopsy studies show that diagnosis of IPA is still often missed, demonstrating the persisting limitations of our diagnostic tests and approaches.[3–6] Even more challenging is the diagnosis of fungal coinfections. Coinfections with *Aspergillus* and Mucorales are more common than previously thought. The presence of a positive *Aspergillus* antigen test may prevent the clinician to search further for other mold pathogens being involved.[7–9] As fungal biomarkers play a crucial role in diagnosing invasive aspergillosis (IA), a good knowledge of the diagnostic possibilities and especially their limitations is necessary for every clinician working with patients at-risk (►**Tables 1** and **2**). Therefore, in this review, we aim to critically reflect on microbiological diagnostic possibilities for pulmonary aspergillosis.

The Traditional Methods: Histopathology, Microscopy, and Culture

Histological detection of fungal hyphae within inflamed or necrotic tissue or a culture from a normally sterile site remains the gold standard for making a diagnosis of IPA. For a better yield, lung tissue sampling should be guided by CT findings. But as the patients at-risk are in general very vulnerable and may have a high risk of bleeding or respiratory deterioration, clinicians are often reluctant to pursue invasive tissue sampling. Diagnosis, thus relies often on other sample types such as bronchial aspirates and bronchoalveolar lavage fluid (BALF). As *Aspergillus* is ubiquitous and often causes contamination and colonization, culture results of these samples might be misleading.

With standard hematoxylin and eosin (H&E) staining all fungi show pink cytoplasm, blue nuclei, and no wall discoloration. Moreover, H&E may give an idea of the host response. The more specific Grocott–Gomori's (or Gömöri) methenamine silver stain causes reduction of silver ions which renders the *Aspergillus* cell wall black or dark brown. With periodic acid–Schiff staining, the cell wall will be pink to purple. Thin (2.5–12 µm), septate hyphae with dichotomous acute angle (<45 degrees) branching is typical for aspergillosis on microscopic examination, but also for other fungal infections, although not for mucormycosis. On microscopy, necrotizing pneumonia with areas of hemorrhage and granulomatous inflammation in non-neutropenic patients or fungal hyphae occluding the lumen of pulmonary arteries with associated infarcted area can be seen.[10,11] In the microbiology laboratory, Gram staining is generally used to stain samples for the microscopic detection of bacteria. This staining is, however, not reliable for the detection of fungal elements. Instead, staining with optical brighteners such as Calcofluor white or Blankophor is preferred. Culture has the additional advantage of allowing fungal species identification and determining antifungal susceptibility. But, BALF cultures have only moderate sensitivity and blood cultures are nearly always negative in IPA even in disseminated IA.[12]

Table 1 An overview of the sensitivity of the most used diagnostic tests for invasive pulmonary aspergillosis

Test	Sensitivity for IPA
In BALF	
Culture	+
Microscopy	+
Aspergillus antigen	+++
Beta-D-glucan	Not to be performed
PCR	+++
In blood	
Culture	1/2−
Aspergillus antigen	++ (highly dependent on patient population)
Beta-D-glucan	++
PCR	++

Abbreviations: BALF, bronchoalveolar lavage fluid; IPA, invasive pulmonary aspergillosis; PCR, polymerase chain reaction.
−, +, ++, and +++ represent a general grading of sensitivity.

Expanding Antigen Detection Testing

Often, proof of IPA is lacking, and therefore algorithms for presumed diagnosis have been developed taking into account the presence of different microbiological markers.

Beta-D-glucan (BDG) is a glucose polymer and a component of the cell wall of many pathogenic fungi including *Aspergillus* spp., *Candida* spp., *Fusarium* spp., and *Pneumocystis.* Classically, the Fungitell assay (Associates of Cape God, East Falmouth, MA) is used to detect BDG, but more recently the Fungitell STAT and the Wako (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) were introduced, providing the possibility for single-sample testing, enhancing speed and cost-efficiency. Other assays for the detection of BDG are the Fungitec G assay (Seikagaku Corporation), the Dynamiker Fungus (1-3)-beta-D-glucan assay (Dynamiker Biotechnology), and the GKT-25M assay (Tianjin Era Biology Technology Co.). Different assays have similar accuracy for the diagnosis of invasive fungal infections (IFI).[13] The Fungitell assay uses a colorimetric reaction with limulus amebocyte lysate (LAL), resulting in a change in color to yellow in the presence of BDG. The Wako assay also uses LAL but in a turbidimetric reaction, with a change in turbidity in the presence of BDG. The turbidimetric reaction showed less interference than the colorimetric reaction and also exhibited less inter- and intrarun variability.[14] BDG is tested in blood, as it has very poor specificity in BALF. Sensitivity and specificity of BDG in blood to diagnose proven and probable IA in a large meta-analysis with adult hemato–oncological patients were 57% (33–83%) and 97% (96–98%), respectively, with a single value above the cutoff, and 46% (21–73%) and 97% (95–98%),

Table 2 Advantages and disadvantages of microbiological diagnostic tests for *Aspergillus* infections

		Pros	Cons
Histopathology, microscopy		- Actual golden standard when performed on normally sterile samples - Well-established and widely used technique - Low cost - PCR for species determination possible on formalin-fixed paraffin-embedded tissue specimens	- Material difficult to obtain (invasive sampling in mostly vulnerable, often cytopenic patients necessary) - Good specimen quality necessary (fungi are not homogenously distributed in tissue—sampling error may occur) - Expertise required for microscopic interpretation of stained samples
Culture		- Possibility to identify on a species level - Possibility to perform antifungal susceptibility testing - Well-established and widely used technique - Low cost	- Invasive sampling - Time-consuming and labor-intensive - Risk of contamination - Low diagnostic yield
Aspergillus antigen		- Noninvasive testing possible (blood testing) - Early detection (in some patient populations possibility of screening) - Evaluation of treatment response - Risk stratification in certain patient groups - Widely available	- No species identification - Sometimes unclear infection site - Performance dependent on host factors, sample type, timing, and therapy
	EIA	- More recently also single-sample testing possible	- Most assays only available in batch testing format
	LFT	- Short turnaround time - Single-sample testing - Possibly feasible in low-income countries	- Limited information on clinical performance - Limited information on interpretation of quantitative results generated by different assays - Still a high cost for low-income countries
	Urinary Ag test	- Noninvasive - New antibody test under development	- Very limited clinical evidence - Influence of renal function, underlying diseases, and other urinary proteins to be investigated
Beta-D-glucan		- Noninvasive - Broad coverage of fungal species (role in screening) - More recently also rapid and single-sample testing possible - Still useful in patients under antifungal therapy	- No genus/species identification - Many factors related with false-positive results
Aspergillus PCR		- Ability to detect some resistance markers but sensitivity for resistance detection is limited - Early diagnosis - Still useful in patients under antifungal therapy - Standardization strongly improved	- Best performance in invasive samples - Lack of standardization (in-house vs. commercialized) - Susceptibility to antifungal agents cannot be proven - Difficult discrimination between contamination and colonization versus infection - More expensive than conventional methods - Not widely available - Extraction method often labor-intensive
NGS cfDNA		- Noninvasive - Pan-infectious test - Possibility to differentiate on a species level - Detection of antifungal resistance markers is possible - Unexpected pathogens can be detected	- Very expensive - Lack of standardization - Limited clinical evidence - Also detects pathogens with low fungal burden, difficult interpretation of the results: colonization versus infection versus commensal - Implementation limited by accessibility (specialized equipment and staff) - Complex bioinformatics - Dependent on quality of the reference database

(*Continued*)

Table 2 (*Continued*)

	Pros	Cons
Omics	- Focus also on the host and host response (providing holistic understanding of the disease) - May allow personalized medicine	- Lack of standardization - Very expensive - Limited clinical evidence (to be further explored in the field of aspergillosis) - Difficult interpretation of the results - Specialized equipment - Complex bioinformatics
Aspergillus IgG and precipitins	- Noninvasive - Rapid tests such as LFAs exist (feasible in low-income countries) - Low cost	- Only useful for the diagnosis of CPA

Information on diagnostic performance is not included as this is already discussed in the text (CPA, chronic pulmonary aspergillosis; EIA, enzyme immunoassay; ICU, intensive care unit; IgG, immunoglobulin G; LFT, lateral flow test including lateral flow assays (LFAs) and lateral flow devices (LFDs); NGS cfDNA, next-generation sequencing of cell-free DNA; PCR, polymerase chain reaction; VOC, volatile organic compound).
General remark: Clinical correlation and the combination of different diagnostics are always indicated.

respectively, with two consecutive values above the cutoff.[13] Specificities are lower in other analyses.[15–18] A negative BDG is not reliable to rule out IPA.

Galactomannans (GMs) are polysaccharides that consist of a mannose backbone and galactofuran side chains of different lengths. GM forms part of the fungal cell wall and is released only by certain molds such as *Aspergillus* during growth, in contrast to BDG. GM is an umbrella term for all molecules containing cross-reactive galactofuranose polymers.[19] After first being detected by the Pastorex *Aspergillus* antigen latex agglutination test historically, it is now most commonly detected by the commercial Platelia *Aspergillus* enzyme immunoassay (EIA; Bio-Rad, Marnes-la-Coquette, France). The Platelia is based on the EB-A2 rat monoclonal antibody (mAb) which recognizes the 1→5-beta-galactofuranose side chains of the GM molecule. A survey in Europe showed that 88% of participating centers had a GM EIA available, either on-site or outsourced.[20] However, more recently, tests based on other *Aspergillus* antigens are trending and diagnostic possibilities are expanding.

Pooled sensitivity of GM in serum is moderate to good (48–92%) and specificity is good (85–95%).[21–23] Pooled sensitivity of GM in BALF is better (61–92%) and specificity is also good (89–98%).[24–26] A positive GM antigen test can precede clinical or radiologic features by several days but performance is related to the tested matrix and to patient characteristics. Sensitivity of GM detection in serum but not of BALF is the highest in neutropenic patients and is lower in non-neutropenic patients such as solid organ transplant recipients and critically ill patients.[22] Cutoffs used in diagnostic algorithms are different from the ones recommended by the manufacturer (optical density index (ODI) = 0.5) to improve the specificity.[19] Although GM is marked for testing in serum and BALF only, studies show that performance in plasma is as good or even better than in serum.[27]

Cross-reactivity with non-*Aspergillus* molds such as *Fusarium* spp., *Penicillium* spp., *Acremonium* spp., *Alternaria* spp., and *Histoplasma capsulatum*, may occur but rarely interferes in daily clinical practice. Semisynthetic beta-lactam antibiotics, multiple myeloma, blood products collected using Fresenius Kabi bags, gluconate-containing plasma expanders, flavored ice pops, and frozen dessert containing sodium gluconate, *Bifidobacterium* spp. as part of the gut microbiome or present in probiotics, severe gastrointestinal mucositis, and enteral nutritional supplements were all reported to be the cause of false-positive GM results.[28] Nowadays, the amount of GMs in infusion fluids is carefully checked by the manufacturers and reduced in a way that they can be eliminated as a cause of false-positive GM results.[29,30] Concomitant use of antifungal prophylaxis or empiric mold-active antifungal agents or mucolytic agents can cause false-negative GM results.[21,31]

The EB-A2 antibody was the only available antibody to detect *Aspergillus* antigens for a long time, but since 2008 the mouse IgG3 mAb JF5 was introduced. The JF5 mAb detects an epitope present on an extracellular glycoprotein of *Aspergillus* secreted during active growth. It was developed in the form of a lateral flow device (LFD; OLM Diagnostics, Newcastle Upon Tyne, UK) providing the possibility for more rapid antigen testing. In 2018, the Soña *Aspergillus* GM lateral flow assay (LFA; IMMY, Norman, OK) came into the market. IMMY also commercialized an EIA to detect GM (Clarus *Aspergillus* galactomannan EIA). More recently, other lateral flow tests (LFTs) received Conformite Européenne (CE)- and *in vitro* diagnostics (IVD)-marking (QuicGM *Aspergillus* galactomannan Ag LFA [Dynamiker, Tianjin, China]), the FungiXpert *Aspergillus* Galactomannan Detection K-set LFA (Genobio [Era Biology Technology], Tianjin, China), and the TECO®Fast *Aspergillus* galactomannan Ag LFA (TECOmedical Group, Sissach, Switzerland; Dynamiker, Tianjin, China), although clinical validation trials for these tests are still lacking.

LFTs are often presented as point-of-care tests and are currently validated for serum and BALF samples. Although they provide rapid results, they often need pretreatment and experienced lab personnel, restricting them to the laboratory instead of the bedside. A European survey showed that the LFA is available in 33% of institutions and the LFD in 24%,[20] but this can vary between different countries.[32] However, with multiple LFTs being available it is not desirable to speak

of the LFA and the LFD test but rather to see LFTs as a group of different tests. Expert groups have decided to not yet incorporate these new tools as a microbiologic criterion in the consensus definitions awaiting more assessment of their performance and the correct cutoffs to use,[19] however, at the 9th European Conference on Infections in Leukaemia, *Aspergillus*-specific LFTs (IMMY and OLM) were suggested to be clinically useful as an alternative to the GM EIA to diagnose IA on serum or BALF with a grade B strength of recommendation, level II quality of evidence (BII) recommendation.[33] In addition to the longer turnaround time, another disadvantage of the Platelia GM is that batching is necessary. This limits its use in smaller hospital laboratories. The newer VirClia *Aspergillus* galactomannan Ag Monotest (Vircell SL, Granada, Spain) is an automated chemiluminescence immunoassay that can test individual serum and BALF samples and can provide a quantitative result within 1.5 hours. In a large multicenter study including 141 patients of which 66 cases are probable IPA, the VirClia correlated well with the Platelia for detecting GM in BALF.[34] This was also found in two smaller cohorts in BALF and serum[35,36] but more extensive validation of the test and its cutoffs in serum are needed.

Urine as a Sample for the Diagnosis of Aspergillosis

After the JF5 mAb, until 2012, no "new" antibodies or targets were studied for a long time to implement into a new diagnostic test. Then a new IgM mAb (mAb476) was described. The antibody detects small molecular weight galactofuranose-containing glycans secreted in urine of animals and humans infected with *Aspergillus*.[37] After further optimization, the MycoMEIA® test (Pearl Diagnostics, Baltimore) was developed using mAbs detecting free glycoproteins and extracellular vesicles in urine.[38] In the first studies including 310 people with suspected invasive fungal disease (IFD), per subject sensitivity for IA was 91.2% (95% confidence interval [CI] 76–98%) and specificity was 89.2% (95% CI 82–94%).[39,40] The same test is currently being developed in LFT format, called the MycoFLOW.

Molecular Testing

Detection of *Aspergillus* DNA is a complementary test to antigen detection. *Aspergillus* PCR testing has the potential additional advantage of detection of resistance mutations in the *A. fumigatus Cyp51A* gene. Initially, because of the lack of commercial options, many hospitals used in-house PCR tests and standardization over different centers and studies was lacking. But now, many expert initiatives, such as the Fungal PCR Initiative (FPCRI, formerly the European *Aspergillus* PCR Initiative), have worked on the standardization of *Aspergillus* PCR testing for the diagnosis of IA, of which fungal DNA extraction is the most important step.[41–43] When methods compliant with the FPCRI recommendations were used, a trend towards improved sensitivity but a significant improvement in specificity is seen.[42,44]

Aspergillus DNA has been found in 37% of lung biopsy specimens of healthy adults and the microbiome and mycobiome of the respiratory tract is dynamic and influenced by external factors.[45,46] False-positive results in the setting of colonization, therefore, complicate the interpretation of a single positive PCR result in bronchoscopy. Meta-analyses of *Aspergillus* PCR on BALF demonstrate sensitivity and specificity values of 76.8 to 79.65% and 93.7 to 94.5%, respectively.[47–49] Sensitivity of *Aspergillus* PCR in blood is generally lower than PCR tests for the detection of Mucorales DNA.[8] Meta-analyses of *Aspergillus* PCR on blood demonstrate sensitivity and specificity values of 84 to 88% and 75 to 76%, respectively.[50–52] The presence of two consecutive positive *Aspergillus* PCRs increases specificity to 95%.[52,53] A subgroup analysis of a meta-analysis showed, in contrast to its influence on the GM, that antimould prophylaxis significantly decreases *Aspergillus* PCR specificity, without affecting sensitivity.[54] This could be since mold-active treatment limits the invasiveness of IA, but its influence on GM could also lead to a reduction in classification of cases of probable IA.

A recent prospective study in Belgium and The Netherlands including hematology patients with a suspect chest CT in whom a bronchoscopy was performed, showed that in patients with an isolated positive PCR, the median cycle threshold (Ct) value was higher (36.4, interquartile range [IQR] 35.1–37.5) compared with patients with a positive GM or culture (33.8, IQR 31.8–36.1 and 33.4, IQR 32.6–36.4 247, respectively).[55] Probably, a second sample and/or a cutoff to define PCR positivity is necessary to improve the role of *Aspergillus* PCR in the diagnostic criteria, but initiatives to do so are currently ongoing. Combining the use of *Aspergillus* PCR with GM in BALF or on blood improves performance for both confirming and excluding IA.

Most *Aspergillus* PCR assays are designed to detect *A. fumigatus*. The capability to detect other *Aspergillus* species should be verified.[51]

Search for New Biomarkers

Sequencing of cell-free DNA (cfDNA) in the bloodstream—sometimes called "liquid biopsy"—has already proven its utility in the oncology field, but now metagenomic next-generation sequencing (NGS) of microbial cfDNA also seems to be a promising noninvasive method for diagnosis of infectious diseases. With metagenomic NGS all nucleic acids in a sample, which may contain mixed populations of microorganisms, are run and then assigned to their reference genomes to understand which microbes are present and in what proportions.[56] There are some commercialized tests using this method of which most studies are published with the Karius test (Karius Inc., Redwood City, CA).[57] After cfDNA is extracted from plasma, it is sequenced, and human reads are removed. The remaining sequences are then aligned to a large pathogen database including more than 1,000 different pathogens of which more than 400 are fungi. Reporting is based on certain predefined thresholds.

Studies have been performed using the Karius test in plasma for diagnosing IFD in high-risk patients. In a study including hemato–oncology patients and hematopoietic cell transplant (HCT) recipients including six patients with proven IFD and one with probable IFD, cfDNA NGS detected six fungal pathogens.[58] In a similar study including 35 HCT recipients with 39 mold infections (16 *Aspergillus* and 23 non-*Aspergillus* infections), the test could detect mold in 38, 26, 11, and 0% of samples collected during the first, second, third, and fourth week before clinical diagnosis, respectively.[59] Likewise, in a cohort of 114 coronavirus disease 2019 (COVID-19) intensive care unit patients of which 6 patients had probable COVID-19-associated pulmonary aspergillosis (CAPA), the Karius test had a sensitivity of 83% and a specificity of 97%.[60] One of the benefits of microbial cfDNA NGS is its possibility to detect rare fungal pathogens that may be missed or misclassified by conventional diagnostics, which can be important given the higher incidence of *Aspergillus* and Mucorales coinfections than previously thought.[8,9,60] However, the clinical diagnostic and therapeutic impact of the test seems to be rather low.[61,62]

Another research group developed a PCR test based on plasma cell-free microbial DNA for the detection of mold infections. Comparing the performance of *Aspergillus* plasma cfDNA PCR to serum GM for the diagnosis of IA in 238 patients showed a better sensitivity and specificity for the *Aspergillus* plasma cfDNA PCR in comparison to the serum GM in these patients (86.0 and 93.1% vs. 67.9 and 89.8%, respectively).[63] To perform the test, the investigators started with a high volume of 4 mL of plasma for the DNA extraction.

Another relatively new diagnostic methodology based on analysis of the profile of volatile organic compounds (VOCs) in exhaled breath—sometimes called "breath biopsy"—has gathered interest in the diagnosis of several diseases in recent years, both respiratory (e.g., asthma, COPD, respiratory infections) and nonrespiratory (e.g., different types of cancer, diabetes).[64,65] VOCs can be detected by the electronic nose, selective ion flow mass spectrometry, ion mobility spectrometry, and gas chromatography with mass spectrometry. A small study in prolonged neutropenic patients showed a sensitivity of 100% and a specificity of 83.3%, but different profiles are being identified, dependent on the pathogen and the host, and not all of them can be replicated in vivo.[66,67] There is currently no standardized methodology for VOC analysis in this context. Although being an interesting technique already proven very helpful in other diseases, more information about influencing factors such as food or smoking, coinfections, medication, and environmental factors, and more information about what may arise from the pathogen and what from the inflammatory host response is necessary.

A Shift Towards the Host and its Immunology with More Omics

The central paradigm in current laboratory testing for infectious diseases is to look for the pathogen as sign or proof of infection. Indeed, while clinicians use biomarkers of inflammation such as C-reactive protein daily, immunological tests detecting signatures associated with specific infectious diseases are currently not validated or not available in clinical practice. This paradigm might shift, as specific immunological assays are being developed to distinguish infectious inflammation from sterile inflammation,[68,69] to discriminate viral from bacterial infections,[70] or to even identify infection by specific pathogens such as severe acute respiratory distress syndrome coronavirus type 2.[71] With the growing insight into the pathophysiology of IPA and the advent of many "omics" strategies that start to find their way to the clinic, host-based immunological strategies to diagnose IPA might become available in the future. Thus far, only a few studies have addressed this topic. Although most studies only show associations and not one unique signature has been identified, results are promising.

Targeted studies showed that measuring specific molecules such as pentraxin 3, or measuring expression of genes such as *S100B*, in BALF or serum might have diagnostic value.[72–75] Yet, more unbiased approaches are becoming increasingly popular. For instance, studies of multiple proteins in BALF and serum led to the discovery of the diagnostic potential of increased proinflammatory cytokines and decreased alpha diversity in lung microbiota in IPA.[76–79]

Importantly, the patient's background must be considered when using the host response to identify fungal infections.[80] This drawback of different behavior of host–response-based assays in different patient populations may be overcome by including more fungal infection-specific proteins, genes, or other response parameters in the assay signatures, but this will require large patient populations. Moreover, the validation of identified host–response markers and translation into easy-to-use tests are other major hurdles to overcome.

Biomarkers as Risk Stratification and Treatment Follow-up

Although the incidence of IA is on the rise and is non-negligible, the incidence in general is low which leads to a low pretest probability. A combination of negative biomarkers in the setting of screening (in selected patients), therefore, excludes the presence of IA and can limit the use of antimould treatment. As mentioned above, novel biomarkers and "multiomics" evaluating host immune response can help in evaluating the risk profile of certain patients.

For example in a multinational case–control study including CAPA patients, positive serum GM and BDG were associated with increased mortality compared to serum biomarker-negative CAPA patients (87.5 vs. 41.7%, $p = 0.046$; 90.0 vs. 42.1%, $p = 0.029$, respectively).[81]

In addition, biomarkers can also be used to follow-up on treatment efficacy or patient status.

In a study evaluating 206 hematological patients with proven and probable IA, a model for survival at week 6 was created based on the serum GM taken at baseline and on early serum GM kinetics. In patients with a baseline serum galactomannan (sGM) index > 1.4, who failed to lower that index to <0.5 after 1 week, 6-week mortality was significantly higher (48.1%) than in patients with a baseline serum

GM index $\leq$ 1.4 that obtained a negative serum GM (<0.5) after 1 week (10.1%).[82] In another study including hematological patients, a positive GM also implied a higher mortality ($p = 0.004$). However, a positive *Aspergillus* PCR was not related to a higher mortality (24 vs. 19% at week 6, $p = 0.324$; 31 vs. 27% at week 12, $p = 0.457$).[55] This limitation could be overcome by working with a stricter quantitative polymerase chain reaction (qPCR) threshold.[83]

In high-risk hematology patients, biomarkers can have a role in preemptive screening prior to the development of symptoms. In a large randomized controlled trial, patients with acute myeloid leukemia or myelodysplastic syndrome and HCT recipients were randomly assigned to receive caspofungin empirically or preemptively. Overall survival at day 42 was not inferior in the preemptive treatment arm (96.7 vs. 93.1%), and the rate of IFD at day 84 was not significantly different. However, the rate of patients who received antifungal treatment was halved.[84] In addition, a meta-analysis showed that by screening high-risk patients for IA with GM and PCR tests, the absence of any positive test can obviate the need for antifungal agents.[23] Biomarkers could, thus, indeed play an important role in antifungal stewardship.

Different Patient Populations with Different Needs and Possibilities

Most studies regarding diagnostic performances of biomarkers for IA are performed on adult patients with hematological malignancies and those undergoing hematopoietic stem cell transplantation. Data in other patient groups at-risk such as patients with other malignancies, undergoing solid organ transplantation, or requiring intensive care treatment, or such as pediatric populations are much more limited, although the test performance can be different in these populations.

Critically ill patients developing IPA in the intensive care unit form a specific patient cohort regarding *Aspergillus* diagnostics, as they may lack the host factors defined by the EORTC/MSGERC. Specific definitions for *Aspergillus* superinfections in viral pneumonia (influenza-associated pulmonary aspergillosis [IAPA] and CAPA, respectively, together with virus-associated pulmonary aspergillosis [VAPA]) have been constructed.[85,86] Work-up for VAPA relies heavily on bronchoscopy-based diagnostics, including visualization of the bronchial tree to detect invasive *Aspergillus* tracheobronchitis (IATB) and BAL sampling for culture, PCR, and GM testing.

Histopathology of lung tissue is virtually impossible in alive VAPA patients given the inherent respiratory failure that accompanies this disease. In patients without increased risk for bleeding complications, biopsies may be obtained from tracheobronchial plaques, ulcers, or pseudomembranes if IATB is suspected. The role of tracheal aspirates (TAs) in the diagnosis of VAPA is currently unclear due to lack of high-quality data. Given that a positive TA culture or GM may reflect colonization rather than invasive disease, a higher GM cutoff (e.g., ODI $\geq$ 2.0) has been proposed to increase specificity for VAPA.[87] Verweij and colleagues considered *Aspergillus* detection in TA as insufficient evidence to support CAPA diagnosis in their task force report.[88] Likewise, tests on sputum and nonbronchoscopic lavage, which were implemented in the "possible" CAPA European Confederation of Medical Mycology (ECMM)/International Society for Human & Animal Mycology (ISHAM) criteria due to reluctance to perform bronchoscopy early in the COVID-19 pandemic out of fear for contamination of health care workers, are not recommended anymore given concerns on their diagnostic performance and lack of validation.[86,88] While BAL GM and PCR perform well regarding sensitivity and specificity for VAPA, serum GM has a poor sensitivity of approximately 60% in IAPA and 20% in CAPA, reflecting less angioinvasion in the latter.[89–91] Several studies have investigated the use of LFTs in CAPA patients on serum and BALF (and TA), which showed good accordance with GM on the same samples.[92–95] In summary, bronchoscopy-based diagnostics remain the mainstay for diagnosis of VAPA.

Chronic Pulmonary Aspergillosis

Most studies on diagnostic biomarkers focus on IPA. Methods for diagnosing CPA are mostly extrapolated from IPA, but their performance can differ as it generally affects more immunocompetent patients but with a preexisting pulmonary condition. Moreover, as CPA is a spectrum of disease on itself this will also lead to a different interpretation.[96] Like IPA, the diagnosis of CPA is also based on a combination of suggestive symptoms, typical radiological findings and direct evidence of *Aspergillus* infection or an immunological response to *Aspergillus*, with the exclusion of some alternative diagnoses and the disease being present for at least 3 months.[2] Tissue allows a distinction between subacute invasive aspergillosis (SAIA), chronic cavitating pulmonary aspergillosis, and chronic fibrosing pulmonary aspergillosis. SAIA is classified within the spectrum of CPA but as it is the only category which presents with tissue invasion, patients mostly have some level of immunosuppression and its diagnosis similar to IPA.

To make a diagnosis in case of a fungus ball or cavities, *Aspergillus* IgG or precipitins, *Aspergillus* antigen or DNA detection in respiratory samples, or a percutaneous or excision biopsy are useful tests.

The presence of *Aspergillus* antibodies differentiates between infected and colonized patients.[97] Anti-*Aspergillus* IgG antibodies (by EIA) in combination or not with precipitins (by immunodiffusion or counterimmunoelectrophoresis) should be tested in any patient suspected of having CPA, testing *Aspergillus* IgA and IgM is not recommended.[2] Sensitivity of *Aspergillus* IgG is much better than that of precipitins, ranging from 85 to 98.4%.[2,98] Many different commercial and in-house serological assays are available. Sensitivity and specificity of *Aspergillus* IgG EIA is in general 83.8 to 98.2% and 92.9 to 99.3%, respectively.[99,100] Cross-reactivity with *Histoplasma* and *Coccidioides* spp. can occur. A sudden rise in quantitative result can imply therapy failure. Also LFTs for *Aspergillus* IgG have been introduced in

diagnosis of CPA, mainly intended for low-resource settings. The LDBIO *Aspergillus* ICT LFA (LDBIO Diagnostics, Lyon, France) is a commercially available LFA for detecting *Aspergillus*-specific antibodies and has similar sensitivity and specificity to the EIA.[101–103] Also, IMMY (Norman, OK) has an LFD for *Aspergillus* immunoglobulins.

Sputum culture is often positive in CPA but it is not diagnostic as colonization in this patient population is frequent.[45,104] However, a positive culture in a bronchoscopic specimen is suggestive of infection, but sensitivity is low.[98] The value of serum and BALF GM in diagnosing CPA remains unclear.

Sensitivity of GM in serum of CPA patients is low due to the less invasive nature of the infection.[105] BALF GM is more sensitive and may be helpful for diagnosing CPA when culture and *Aspergillus* IgG and precipitins are negative, but its role in CPA is still under consideration.[2,98,106] A meta-analysis to evaluate the diagnostic performance of serum and BALF GM showed an optimal cutoff for serum GM of 0.96 with a sensitivity of 29% and specificity of 88% and for BALF GM of 0.67 with a sensitivity of 68% and specificity of 84%.[107] Only very few studies have been performed evaluating the GM LFTs in CPA, with one study showing a sensitivity for the OLM LFD of 7% in BALF.[108] Another study, Japanese, showed a surprisingly high sensitivity (62%) for the OLM LFD in serum but *Aspergillus* IgG EIA was not used for comparison raising concern for false-positive test results.[109] Sensitivity in BALF was 66.7%.

Molecular methods (PCR) are more sensitive than culture. Sensitivity and specificity of *Aspergillus* PCR in CPA is around 80%.[99] A lower Ct value suggests higher fungal load and is more suggestive of possible invasion.

Conclusion

As for every diagnostic test, but especially for the diagnosis of IA, it is of utmost importance to be aware of the performance and limitations of the test being used and the influence of patient population, clinical context, matrix, and test strategy on performance. The diagnosis of IA cannot be made on a positive biomarker test alone. Results should always be interpreted in the clinical context taking into account the risk factors of the patient as well as signs of infection and imaging results. The main limitation of available biomarkers in general is their sensitivity. A sound diagnosis leading to the correct approach in vulnerable patients at-risk for aspergillosis can only be reached by combining information as different pieces of a puzzle.

Conflict of Interest
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