CONTROL OF HUMAN AND ANIMAL ASPERGILLOSIS – A HEALTH APPROACH

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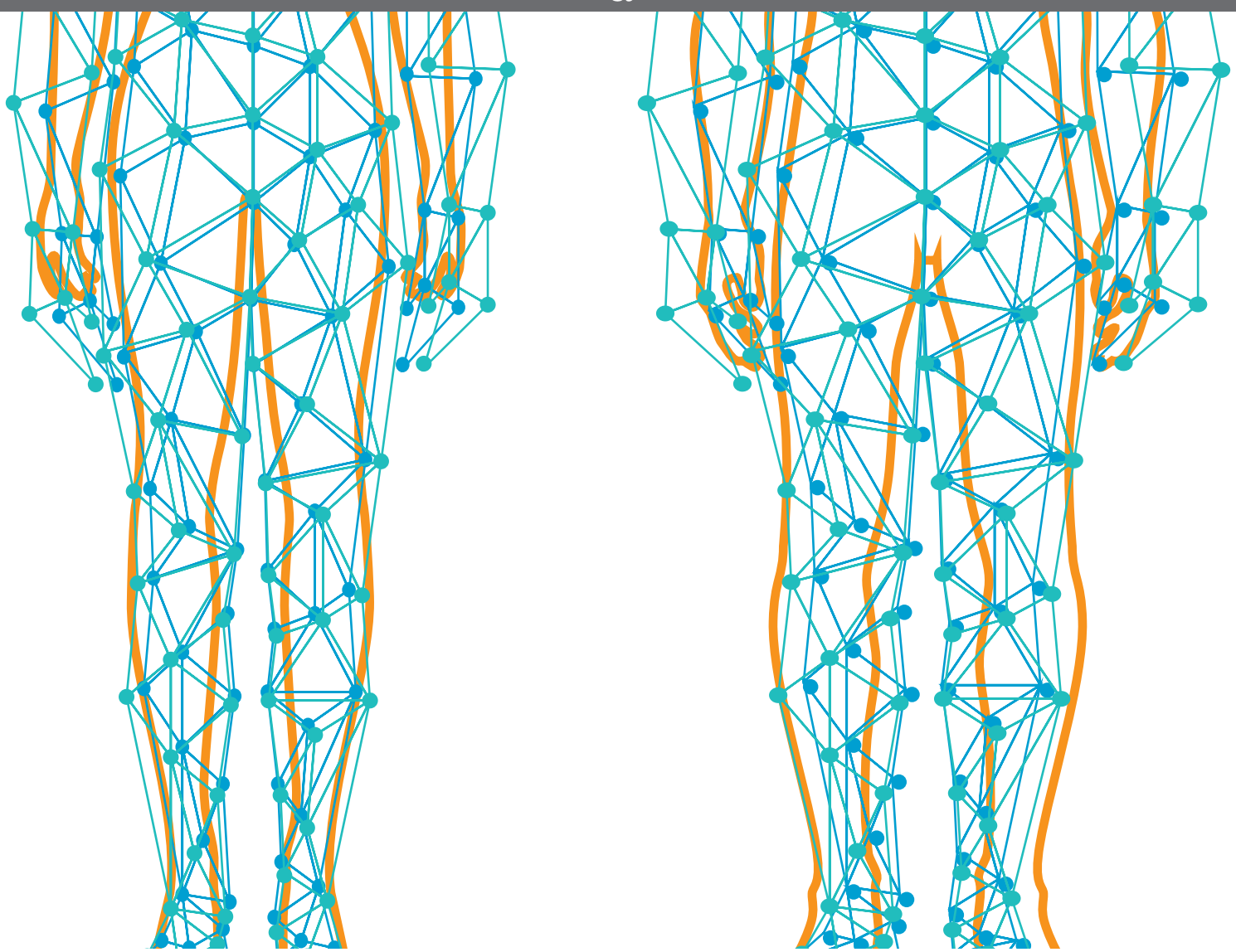

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CONTROL OF HUMAN AND ANIMAL ASPERGILLOSIS – A HEALTH APPROACH

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The Clinical Characteristics of Patients With Nonneutropenic Invasive Pulmonary Aspergillosis

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Objective: The goal of this study was to reveal the clinical manifestations of nonneutropenic invasive pulmonary aspergillosis (IPA), which are different from those of neutropenic patients.

Methods: The clinical data of patients with nonneutropenic IPA were collected at the Department of Respiratory and Critical Care Medicine, Jinling Hospital, from February 2009 to November 2019. We analyzed the general conditions, clinical manifestations, imaging findings, and laboratory tests of these IPA patients.

Results: A total of 116 patients with nonneutropenic IPA (31 proven and 85 probable) were included. They had an average age of 59.8 years. The most common underlying disease was chronic obstructive pulmonary disease (COPD, $n = 33$). The common clinical symptoms included cough (93.1%, $n = 108$), expectoration (59.5%, $n = 69$), fever (57.8%, $n = 67$), hemoptysis (30.2%, $n = 35$), and dyspnea (40.5%, $n = 47$). The common CT imaging manifestations included consolidation (47.4%, $n = 55$), cavities (47.4%, $n = 55$), air crescent sign (14.7%, $n = 17$), and nodules (8.6%, $n = 10$). Multiple lesions (74.1%, $n = 86$) were more common than single lesions (17.2%, $n = 20$) and diffuse lesions (8.6%, $n = 10$). The positive rate of laboratory tests was 88.2% (30/34) for BALF galactomannan (GM), 55.4% (56/101) for serum GM, 45.3% (48/106) for 1,3-β-D-glucan (BDG), 43.3% (46/106) for sputum culture, and 36.4% (20/55) for BALF culture. Patients who had high serum GM level [GM optical density index (ODI) >1] were more likely to have severe respiratory symptoms and higher serum ferritin. Further investigation showed that there was a positive correlation between serum GM level and serum ferritin level.

Conclusion: The clinical symptoms and radiological manifestations of nonneutropenic IPA are diverse and often lead to delayed diagnosis. It is important to become more vigilant of aspergillosis in nonneutropenic patients in order to achieve early diagnosis and treatment and to reduce mortality.

Keywords: invasive pulmonary aspergillosis, clinical manifestation, serum ferritin, serum GM, nonneutropenic

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INTRODUCTION

Aspergillus is a ubiquitous fungus that is abundant in nature air and is tiny enough (2–3 μm) to be inhaled into the small airway. *Aspergillus fumigatus* is the most common species of this genus, and it may cause invasive infection. The patients most at risk for invasive pulmonary aspergillosis (IPA) are neutropenic patients (1, 2). Due to the increasing prevalence of aging-related diseases and the widespread use of glucocorticoids and broad-spectrum antibiotics, nonneutropenic patients have experienced an increasing incidence of IPA. According to statistics from the American Healthcare Cost and Utilization Project—Nationwide Inpatient Sample database in 2004, the top three groups of patients most prone to invasive fungal infection (IFI) are those with chronic obstructive pulmonary disease (COPD), diabetes, and hematological malignancies (3). In contrast to patients with neutropenia, nonneutropenic patients have diverse clinical manifestations and often are ignored by clinicians for the consideration of IPA. So, they are easily misdiagnosed with other infectious diseases. Despite advances in both diagnosis and therapy, their mortality rate remains high. This article aims to explore the clinical features of IPA in nonneutropenic patients and provide valuable information for clinical diagnosis and treatment.

MATERIALS AND METHODS

Data from a total of 116 nonneutropenic IPA patients were collected from February 2009 to November 2019 at the Department of Respiratory and Critical Care Medicine, Nanjing Jinling Hospital. IPA was divided into proven and probable according to the EORTC/MSG criteria (4). Nonneutropenic: The absolute counts of peripheral blood neutrophils was $>1.8*10^9$/L, which was consistent with nonneutropenia. Proven: Histopathology for *Aspergillus* was positive. Probable: There were dependable evidence of host factors, clinical manifestations, imaging findings on chest CT scan, and microbiological evidence [serum galactomannan (GM) or BALF GM, sputum culture]. We performed analysis of the general conditions, clinical manifestations, laboratory tests, and imaging features of 116 nonneutropenic IPA cases. Elevated serum ferritin was defined as values ≥1,000 μg/L.

Data are shown as median with interquartile range (IQR) for quantitative variables and as numbers (percentages) for qualitative variables. The Chi-square and Fisher's exact tests were used for categorical variables. An effect was considered to be statistically significant when the *P*-value was <0.05, and all significance tests were two tailed. The data were statistically analyzed using SPSS 22.0, and graphs were generated using GraphPad Prism.

RESULTS

Patient Characteristics and Clinical Presentations

A total of 116 patients with nonneutropenic IPA were included in this study. Thirty-one cases were diagnosed as proven IPA

TABLE 1 | Characteristics of all 116 nonneutropenic invasive pulmonary aspergillosis (IPA) patients.

	All IPA (*n* = 116)
BASELINE FACTORS	
Women	32
Age, y	59.8 (19–84)
UNDERLYING PULMONARY DISEASE, *N* (%)	
Lung cancer	7 (6)
Bronchiectasis	9 (7.8)
Pulmonary tuberculosis	21 (18.1)
Chronic obstructive pulmonary disease (COPD)	33 (28.4)
EXTRAPULMONARY DISEASE, *N* (%)	
Diabetes	22 (19)
Autoimmune disease	6 (5.2)
Cardiovascular disease	27 (23.3)
RESPIRATORY SYMPTOMS, *N* (%)	
Cough	108 (93.1)
Expectoration	69 (59.5)
Fever	67 (57.8)
Hemoptysis	35 (30.2)
Dyspnea	47 (40.5)
IMMUNOSUPPRESSIVE MEDICATION, *N* (%)	
Long-term (>2 weeks) glucocorticoid	13 (11.2)

TABLE 2 | The microbiological, laboratory, and CT thorax findings of 116 nonneutropenic IPA patients.

	All IPA (*n* = 116)
LABORATORY FINDINGS, *N* (%)	
IL-6, ng/L (45)	52.1 (10.08–266.5)
D-Dimer, mg/L (70)	1.24 (0.06–8.42)
Ferritin, μg/L (34)	544.80 (92.1–1,015.7)
CRP, mg/L (98)	71.31 (0.5–398.9)
ESR, mm/h (71)	54.49 (6–140)
Neutrophil, 10^9/L (116)	9.63 (2.4–42.3)
MYCOLOGICAL FINDINGS, *N* (%)	
BALF galactomannan (GM) > 0.7 optical density index (ODI) (34)	30 (88.2)
Serum GM ≥ 0.5 ODI (101)	56 (55.4)
1,3-β-D-glucan (BDG) (106)	48 (45.3)
Sputum culture (106)	46 (43.3)
BALF culture (55)	20 (36.4)
Biopsy (45)	31 (68.9)
CT THORAX FINDINGS, *N* (%)	
Consolidation	55 (47.4)
Cavity	55 (47.4)
Ground-glass opacity	29 (25)
Nodule	10 (8.6)
Air crescent sign	17 (14.7)
Halo signs	4 (3.4)
Single lesion	20 (17.2)
Multiple lesions	86 (74.1)
Diffuse lesions	10 (8.6)

TABLE 3 | Clinical factors associated with serum GM levels in nonneutropenic IPA patients.

	Serum GM		*P*-value	Serum GM		*P*-value
	<0.5 ODI (*n* = 45)	≥0.5 ODI (*n* = 56)		≤1 ODI (*n* = 79)	>1 ODI (*n* = 22)	
BASELINE FACTORS						
Women	13	15	0.814	22	6	0.957
Age, y	59.0 (19–84)	61.3 (21–84)	0.208	60 (19–84)	61.2 (32–84)	0.37
UNDERLYING PULMONARY DISEASE						
Lung cancer	5	2	0.138	6	1	0.618
Bronchiectasis	5	5	0.715	9	1	0.342
Pulmonary tuberculosis	11	6	0.067	14	3	0.651
COPD	13	19	0.588	28	4	0.05
EXTRAPULMONARY DISEASE						
Diabetes	9	12	0.86	15	6	0.397
Autoimmune disease	2	3	0.834	4	1	0.921
Cardiovascular disease	10	14	0.744	19	5	0.897
RESPIRATORY SYMPTOMS						
Cough	41	53	0.487	74	20	0.652
Expectoration	26	31	0.807	46	11	0.491
Fever	25	34	0.601	45	14	0.422
Hemoptysis	14	18	0.912	21	11	0.037
Dyspnea	25	42	0.04	57	10	0.019
LABORATORY FINDINGS						
IL-6, ng/L	31.0 (25)	78.6 (20)	0.005	48.8 (38)	70.2 (7)	0.371
D-Dimer, mg/L	1.4 (30)	1.1 (40)	0.288	1.3 (56)	1.0 (14)	0.395
Ferritin, μg/L	447.8 (14)	612.7 (20)	0.298	410.3 (27)	1,063.7 (7)	<0.0001
CRP, mg/L	60.4 (44)	80.2 (54)	0.136	68.6 (77)	81.3 (21)	0.432
ESR, mm/h	53.1 (29)	57.7 (34)	0.588	55.2 (50)	57.2 (13)	0.85
Neutrophil, 10^9/L	8.2 (45)	9.6 (56)	0.26	9.1 (79)	8.5 (22)	0.71
CT THORAX FINDINGS						
Consolidation	18	30	0.175	35	13	0.219
Cavity	14	30	0.024	34	10	0.84
Nodule	5	5	0.715	8	2	0.886
Air crescent sign	3	9	0.147	11	1	0.229
Halo signs	2	2	0.823	3	1	0.847
Single lesion	11	5	0.034	12	4	0.734
Multiple lesions	32	44	0.388	60	16	0.757
Diffuse lesions	2	7	0.158	7	2	0.973

by histopathologic evidence, while 85 cases were diagnosed as probable IPA. The demographic and clinical characteristics of nonneutropenic IPA are summarized in **Table 1**. The study population was 84 males and 32 females with an average age of 59.8 (19–84) years. COPD was the most common underlying disease among the patients (33 of 116 patients, 28.4%). A high rate of diabetes was also found (22 of 116 patients, 19%). The most common respiratory symptom was cough (108 cases, 93.1%) in these nonneutropenic IPA patients. Thirteen patients had received long-term (>2 weeks) glucocorticoid therapy.

Microbiological and Laboratory Findings

Detailed microbiological, laboratory, and CT thorax findings and are shown in **Table 2**. A serum GM optical density index (ODI) ≥ 0.5 was observed in 56 patients (55.4%), and a BALF GM ODI >0.7 (5) was observed in 30 patients (88.2%). The 1,3-β-D-glucan (BGD) test in serum samples had a lower positive rate (48 of 106 patients, 45.3%). Sputum *Aspergillus* cultures were performed in 106 patients, and 46 out of the 106 BALF cultures (43.3%) were positive for *Aspergillus*. The positive rate of BALF *Aspergillus* culture was 36.4% (20 of 55 patients). Histopathology was done in 45 patients, and the positive rate was 68.6%.

Clinical inflammatory-related indicators in IPA patients were above the normal values. In all patients, the median IL-6 concentration was 52.1 (10.08–266.5) ng/L, D-dimer was 1.24 (0.06–8.42) mg/L, CRP was 71.31 (0.5–398.9) mg/L, and ESR was 54.49 (6–140) mm/h. In addition to the inflammatory indicators, we also observed serum ferritin (median: 544.8 μg/L) above normal values in nonneutropenic IPA patients.

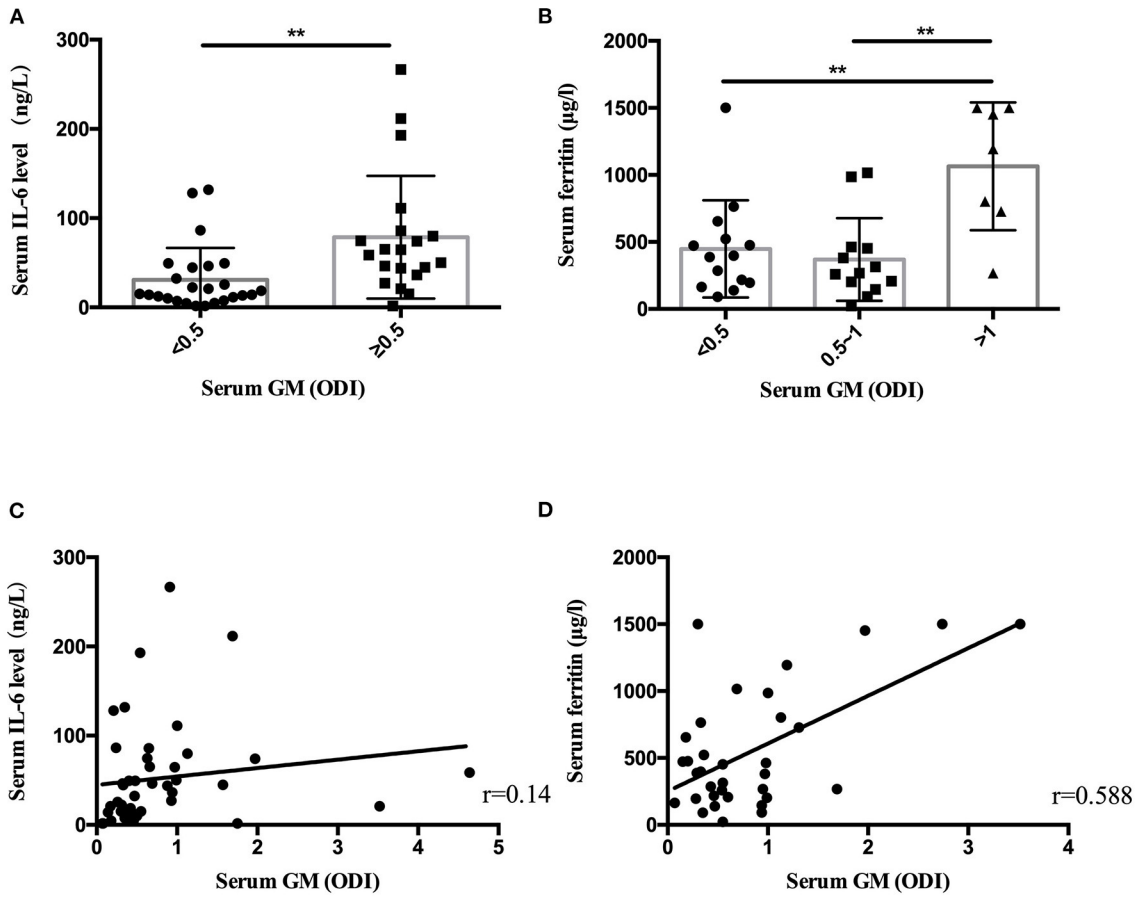

FIGURE 1 | The serum IL-6 level and ferritin level in nonneutropenic invasive pulmonary aspergillosis (IPA). **(A)** The patients with serum galactomannan (GM) ≥ 0.5 optical density index (ODI) had higher serum IL-6 levels. **(B)** The patients with serum GM > 1 ODI had higher ferritin levels. **(C)** Scatter diagrams of serum GM vs. serum IL-6 level. **(D)** Scatter diagrams of serum GM vs. serum ferritin level. The value of *r* represents the correlation coefficients between the GM values and the IL-6 level and ferritin level. $^{**}P < 0.01$.

The Relationship Between Serum GM Level and Clinical Factors

At present, the main clinical laboratory method used to diagnose pulmonary aspergillosis is serum GM testing. Therefore, we grouped the patients according to the serum GM level (<0.5 ODI vs. ≥0.5 ODI; ≤1 ODI vs. >1 ODI). **Table 3** shows that patients with higher serum GM levels (GM >1 ODI) were more likely to have COPD and had more serious respiratory symptoms, hemoptysis, and dyspnea. In terms of imaging findings, patients with low serum GM (GM <0.5 ODI) had more single lesions, but multiple lesions were not necessarily more likely to appear in patients with high serum GM levels.

Association of Elevated Ferritin With Serum GM

Statistical analysis found that the patients with serum GM ≥0.5 ODI had higher serum IL-6, and the patients with serum GM >1 ODI had higher serum ferritin (**Table 3**). Further analysis revealed (**Figure 1**) that there was a positive correlation between serum GM level and serum ferritin level ($r = 0.588$, $P = 0.0003$), but serum GM had no significant correlation with IL-6 ($r = 0.14$, $P = 0.358$). Therefore, we compared the difference between the low-ferritin (<1,000 μg/L) and high-ferritin groups (≥1,000 μg/L) (6). Forty-two patients were tested for serum ferritin. Diabetes and expectoration were significantly more common in patients with high ferritin (**Table 4**).

DISCUSSION

IPA usually occurs in patients with hematological malignancies or patients who are severely immunocompromised (7). However, recent studies have found that the incidence of IPA is increasing year by year in nonneutropenic patients (8). The sample sizes of most studies of nonneutropenic IPA (9, 10) are small. This article included more nonneutropenic IPA patients and increased the reliability of our conclusion. A retrospective survey of pulmonary fungal epidemiology covering 16 studies in China (1997–2008) showed that, instead of hematological malignancies, the most common underlying diseases were solid tumors (14%), COPD (11%), tuberculosis (10%), and diabetes (10%) (11). Due to

TABLE 4 | Differences in clinical characteristics according to serum ferritin.

Characteristics	Ferritin<1,000 μg/L (*n* = 34)	Ferritin≥1,000 μg/L (*n* = 8)	*P*-value
BASELINE FACTORS			
Women	14	0	0.037
Age, y	60.9 (28–84)	66.4 (51–81)	0.151
UNDERLYING DISEASE			
Lung cancer	4	0	0.572
Autoimmune disease	3	0	1.0
Diabetes	4	4	0.03
Pulmonary tuberculosis	7	1	1.0
COPD	7	4	0.174
Bronchiectasis	5	2	0.601
Cardiovascular disease	7	4	0.174
RESPIRATORY SYMPTOMS			
Cough	32	8	1.0
Expectoration	20	8	0.037
Fever	16	5	0.697
Gasp	16	3	0.709
Hemoptysis	12	0	0.08
Dyspnea	9	1	0.655

the large population and increasing morbidity, COPD is an increasingly common underlying disease in IPA patients, and the mortality rate of COPD with IPA is high (12, 13). We also found that COPD was the highest-incidence (33 of 116 patients, 28.4%) underlying disease in our nonneutropenic IPA patients.

Nonspecific clinical symptoms are one of the main causes of a delayed diagnosis of IPA. Fever is the most common clinical symptom in patients with neutropenic IPA (14). However, we found that the incidence of fever in nonneutropenic patients ranked third (57.8%), and the most common clinical symptom was cough (93.1%). In previous studies, it was found that the positive rate of halo signs was 95% in IPA patients with neutrophil deficiency, and the positive rate of air crescents was 33% (15). In this study, we found that consolidation (47.4%) and cavities (47.4%) were the most common imaging findings in nonneutropenic IPA patients, while typical halo signs and air crescent signs accounted for only 3.4 and 14.6%, respectively. Atypical clinical symptoms and chest CT imaging often lead to misdiagnosis of nonneutropenic IPA.

Biopsy is necessary to prove a diagnosis of pulmonary aspergillosis, but it is often difficult and risky to accomplish, especially in critically ill patients. Out of our 116 patients, only 45 patients underwent lung biopsy through percutaneous or transbronchoscopic lung puncture, and the positive rate was 68.9%. At present, *Aspergillus* antigen tests, such as the GM test, are common and reliable laboratory tests for the diagnosis of IPA. The positive rate of serum GM in our study was 55.4%; BALF GM had a higher positive rate (88.2%). Previous studies have also shown that the value of the BALF GM test was higher than that of the serum GM test in high-risk patients with IPA (16, 17). Although the positive rate of BAFL GM was higher, only 34 patients had the chance to be tested, because the bronchoscopy is not suitable for many critically ill patients. Respiratory cultures of *Aspergillus* from sputum (43.3%) and BALF (36.4%) had lower positive rates. Therefore, new diagnostic methods for detecting pulmonary *Aspergillus*, such as PCR and *Asp*LFD, are worth being carried out in clinical practice to help diagnose IPA (18–20).

We conducted a subgroup analysis to explore the relationship between GM level and different clinical manifestations. The results showed that patients with high serum GM level (>1 ODI) had more serious respiratory symptoms (hemoptysis and dyspnea), and patients with low GM level (<0.5 ODI) mainly showed a single lesion on thoracic CT imaging, suggesting that serum GM level might be related to the severity of the disease. Woods et al. (21) found a strong correlation between serum GM and aspergillosis outcome in both neutropenic and nonneutropenic patients. The survival of the patients whose serum GM normalized was significantly better than that of persistently positive patients. Other authors have confirmed that an increase in GM level at the time of diagnosis increases the risk of all-cause mortality at 6 weeks (22, 23).

In laboratory findings, we found that only the level of serum ferritin increased significantly in the high-GM group. Some small-sample-size studies showed increased ferritin levels or iron stores in patients who were diagnosed with invasive mold infections after allogeneic hematopoietic stem cell transplantation (24, 25). The decrease in GM values can be used to monitor the efficacy of a treatment (26). A recent study showed that elevated serum ferritin (>1,000 ng/ml) conferred an increased risk of fungal pulmonary infections (27). Our results suggest that serum ferritin was increased in nonneutropenic IPA patients (544.80 μg/L). In patients with serum GM >1 ODI, the ferritin level was significantly higher than in those patients with serum GM ≤1 ODI. We found that serum ferritin had a positive linear relationship with serum GM ($r = 0.588$, $P = 0.000$) in nonneutropenic IPA patients. In patients with elevated serum ferritin (≥1,000 μg/L), diabetes was more common ($P = 0.03$). Ford et al. (28) found that elevated serum ferritin levels were associated with insulin resistance and an increased risk of diabetes. The number of patients who received a ferritin test in our study was still small. Further research is necessary.

Taking our results together, we believe that the clinical symptoms and imaging manifestations of patients with nonneutropenic IPA are atypical. For patients with relevant underlying diseases and who failed to respond to antibiotic treatment, *Aspergillus* infection must be taken into consideration. Appropriate laboratory tests and prompt antifungal treatment are important to decrease the mortality.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for

participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

XS and LL designed the study and drafted the manuscript. LL collected patients' data and analyzed the data. YG analyzed the data. YW and KS were critically involved in the data collection and the revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Early-Warning Immune Predictors for Invasive Pulmonary Aspergillosis in Severe Patients With Severe Fever With Thrombocytopenia Syndrome

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Aspergillus-related disease was confirmed to be associated with immune disorders in patients, severe patients with severe fever with thrombocytopenia syndrome (SFTS) infected by novel phlebovirus were confirmed to have severe immune damage including cellular immunosuppression and cytokine storms. Secondary invasive pulmonary aspergillosis (IPA) in severe SFTS patients can increase fatality rate. This study investigated early-warning predictive factors of secondary IPA in severe SFTS patients. Receiver operating characteristic analysis was used to assess the value of immune parameters to predict IPA in SFTS patients. The cut-off values of $CD4^+$ and $CD8^+$ T-cell counts to predict IPA were 68 and 111 cells/mm^3, with sensitivities of 82.6% and 72%, and specificities of 56.7% and 83.3%, respectively. Cut-off values of IL-6, TNF-α, IL-8, and IL-10 to predict IPA incidence in critically ill SFTS patients were 99 pg/mL, 63 pg/mL, 120 pg/mL, and 111 pg/mL, with sensitivities of 90.0%, 86.7%, 83.3% and 90.0% and specificities of 80.4%, 71.7%, 82.6% and 65.2%, respectively. Lower $CD4^+$ and $CD8^+$ T-cells counts, higher levels of IL-6, TNF-α, IL-8 and IL-10, higher incidence of pancreatic and renal damage, early antibacterial therapy of carbapenems, and intensive care unit admission were risk factors of IPA in SFTS patients. Multivariate logistic regression analysis indicated counts of $CD4^+$ T-cells <68 cells/mm^3 combined with $CD8^+$ T-cells <111 cells/mm^3 (odds ratio [OR] 0.218, 95% confidence interval [CI] 0.059–0.803, p=0.022), IL-6 >99 pg/ml combined with IL-10 >111 pg/ml (OR 17.614, 95% CI 2.319–133.769, p=0.006), and brain natriuretic peptide level >500 pg/ml (OR 13.681, 95% CI 1.994–93.871, p=0.008) were independent risk factors for IPA in SFTS patients. The mortality in the IPA group was significantly higher than in the non-IPA group (p=0.001). Early antifungal treatment of IPA patients was significantly associated with improved survival (log-rank, p=0.022). Early diagnosis of IPA and antifungal treatment can

improve the prognosis of SFTS patients. Besides, we speculate SFTS may be as a host factor for IPA.

Keywords: novel phlebovirus, severe fever with thrombocytopenia syndrome, invasive pulmonary aspergillosis, immunity, risk factors

INTRODUCTION

Severe fever with thrombocytopenia syndrome (SFTS) is caused by a novel phlebovirus in the Bunyaviridae family named SFTS virus (SFTSV) (1). Contact with ticks appears to be a major risk factor for acquiring SFTSV, SFTSV could also be transmitted from person to person *via* infected blood (2). SFTS has been becoming an increasing public health threat in East Asia due to high morbidity and mortality (3–9). Secondary infection has contribution to the fatality of virus infection. Invasive pulmonary aspergillosis (IPA) have been reported to improve mortality in SFTS patients (10, 11). However, why severe SFTS patients are at risk for invasive pulmonary aspergillosis is not yet clear. Secondary infection as IPA should be concerned in severe SFTS patients.

Aspergillus-related disease associated with immune disorders in critically ill patients has been reported (12). Severe SFTS patients were confirmed to have immune cell dysfunction, and humoral immune system dysregulation which may predispose for IPA (13–16), however, measurable real-time indicators of predictor factors for IPA in severe SFTS patients are lack. Diagnosis and appropriate antifungal therapy of IPA are often delayed due to lack of factors to evaluate IPA in severe SFTS patients. This study was to investigated the early-warning predictors for IPA occurrence in severe SFTS patients to reduce mortality.

METHODS

Study Population

SFTS patients with abnormal findings by CT scan of the lungs were included in the study at the First Affiliated Hospital of Anhui Medical University from March 2015 to December 2019. Patients with chronic lung disease, chronic kidney disease, and chronic liver disease were excluded. All SFTS patients were diagnosed with SFTSV RNA-positive *via* blood samples. SFTSV was amplified from serum samples using specific primers and probes by real-time reverse-transcription polymerase chain reaction (RT-PCR) under the conditions previously described (17). The demographic data, clinical manifestations, antibacterial therapy, antifungal therapy, and laboratory test results of patients were analyzed. Severely ill patients were diagnosed based on the guidelines for the prevention and treatment of fever with thrombocytopenia syndrome (2010 Edition) (18). Written informed consent was provided by all patients following the Declaration of Helsinki. The study and relevant experiments were approved by the local Ethics Committee of Anhui Medical University.

Laboratory Tests

The functions of the liver, kidney, and pancreas, and myocardial enzymes were detected by routine biochemistry tests in a hospital laboratory after patient admission. Laboratory tests including routine blood test, B-type natriuretic peptide (BNP), C-reactive protein (CRP), procalcitonin (PCT), galactomannan (GM), and (1, 3)-β-D-glucan (G) were detected by routine tests after patient admission.

As described in our previous study (13), inflammatory mediators including interleukin (IL)-6, IL-10, IL-8, and tumor necrosis factor (TNF)-α were measured in plasma samples from patients using MILLIPLEX MAP human cytokine/chemokine magnetic bead panel kits (Merck Millipore, Germany) according to the manufacturer's instructions (Luminex 200 System, Life Technologies, Grand Island, NY, USA).

Peripheral EDTA blood was stained with the following mouse anti-human monoclonal antibodies (Beckman Coulter Immunotech SAS, Marseille, France) according to the manufacturer's recommendations: CD45-Krome orange (KO), CD3-fluorescein isothiocyanate (FITC), CD4-phycoerythrin (PE), CD8-allophycocyanin (APC), and fluorospheres. White blood cells were washed and resuspended in phosphate-buffered saline (PBS), then analyzed for $CD4^+$ and $CD8^+$ T lymphocytes on a Beckman Coulter Navios flow cytometer (Beckman Coulter, Miami, FL, USA).

Definition of Invasive Aspergillosis Among SFTS Patients

Based on the 2019 EORTC-MSG consensus definitions (19), IPA was diagnosed based on clinical, radiological, and mycological criteria as the presence of the following criteria: 1) signs and symptoms of IPA such as cough, expectoration, and abnormal findings by CT scan of the lungs; 2) mycological criteria: histopathologic evidence of hyphae with recovery of Aspergillus from tissue, *Aspergillus fumigatus* recovered by culture from sputum, broncho alveolar lavage (BAL), or bronchial brush, positive GM as any 1 of the following: single serum or plasma: ≥1.0, BAL fluid: ≥1.0, single serum or plasma: ≥0.7 and BAL fluid ≥0.8. Severe neutropenia is defined as an absolute neutrophil count less than 500×10^6/L. All the SFTS patients included in this study were sorted into IPA and non-IPA groups. According to the EORTC/MSG diagnostic criteria, patients with proven and probable IPA were classified as the IPA group.

Statistical Analysis

Data were analyzed using SPSS, version 20.0 (SPSS Inc., USA). Quantitative variables were expressed as means ± standard deviation (SD) or as medians (range). Univariate analysis was performed by utilizing the independent Student's *t*-test or the Mann-Whitney *U*-test for between-group comparisons of

continuous variables and chi-squared tests for between-group comparisons of qualitative data. Multiple logistic regression analysis was used for between-group comparisons of differences in clinical and biochemical variables. Survival analysis comparisons between groups were analyzed by the log-rank (Mantel-Cox) test using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA). All significance tests were two-tailed, and differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Characterization of SFTS Patients

Among 169 SFTS patients from March 2015 to December 2019, 76 severe SFTS patients who had abnormal findings by CT scan of the lungs with a new or progressive radiographic infiltrate were included to be studied. Among 76 SFTS patients, the mean age was 66.75 ± 9.55 years (47–87 years), 44 patients were male, 6 patients had diabetes mellitus, and all the patients were from rural areas. According to the 2019 EORTC-MSG diagnostic criteria, 30 (39.5%) of the 76 severe SFTS patients were diagnosed as IPA. As shown in **Figure 1**, twenty patients were diagnosed as probable IPA with positive culture of *Aspergillus fumigatus* in sputum or BAL, the GM levels were 0.95 ± 0.49 μg/L in serum among the 20 patients and 1.05 ± 0.25μg/L in BAL among 7 patients. Ten patients were diagnosed as probable IPA with positive GM (1.27 ± 0.23μg/L) in the serum.

All the 76 severe SFTS patients had fever with a mean duration of 10 ± 5 days, showed in **Table 1**, the severe patients had clinical symptoms and signs as cough, hemoptysis, dyspnea, gastrointestinal symptoms, and central nervous system symptoms. Thirty-five (46.1%) patients had severe neutropenia, 71 (93.4%) had multiple organ damage (liver damage 96.1%, pancreatitis 86.8%, kidney damage 50%, and myocardial damage 90.7%). Damage of the anticoagulant system was demonstrated by a prolonged anginal partial thromboplastin time (APTT) of 71 seconds (interquartile range 55-94 seconds).

All severe SFTS patients had immune damage in this study. the $CD4^+$ and $CD8^+$ T lymphocytes were reduced obviously. Among the 76 severe patients, the median levels of $CD4^+$ and $CD8^+$ T lymphocytes were 96(interquartile range, 56-139) and 111(interquartile range, 61-168) cells/mm^3, respectively. The proinflammatory cytokines IL-6 and TNF-α, and anti-inflammatory mediator IL-10 and IL-8 were upregulated. The median levels of IL-6, TNF-α, IL-10 and IL-8 were 95 (interquartile range, 35-197), 65(interquartile range, 35-205), 136(interquartile range, 44-413.5) and 89(interquartile range, 35-256.8) pg/mL, respectively.

Risk factors for IPA in SFTS patients

Among the 76 severe SFTS patients, 24(31.5%) patients including 16 in the IPA group and 8 in the non-IPA group died. The mortality rate in the IPA group was significantly higher than that in the non-IPA group (53.3% *vs* 17.4%, p=0.001).

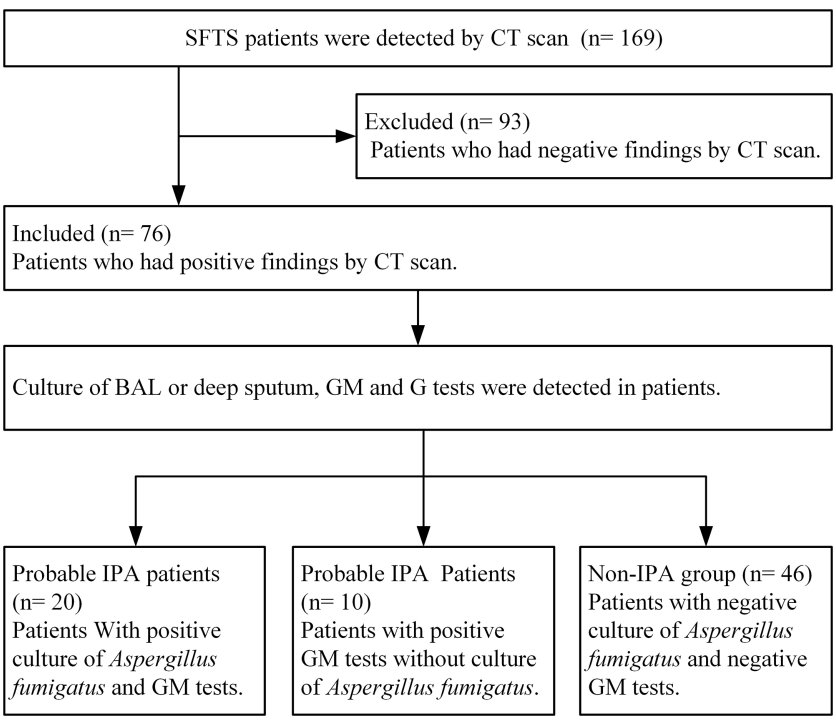

FIGURE 1 | Diagram flow of severe patients with severe fever with thrombocytopenia syndrome included in this research. IPA, invasive pulmonary aspergillosis; SFTS, severe fever with thrombocytopenia syndrome; GM, galactomannan; G, (1,3)-β-D-glucan; BAL, broncho alveolar lavage.

TABLE 1 | Comparisons of clinical and laboratory characteristics between IPA and non-IPA patients with severe fever with thrombocytopenia syndrome.

Index	All patients (N = 76)	IPA patients (N = 30)	Non-IPA patients (N = 46)	*P* value
Clinical characteristics, symptoms or signs				
Age, years	66.75 ± 9.55	66.53 ± 9.05	66.89 ± 9.87	0.874
Male, N (%)	46(60.5)	20(66.6)	26(56.5)	0.376
Duration of fever, (days)	10.55 ± 5.07	11.63 ± 5.76	9.85 ± 4.5	0.135
Cough, N (%)	48(63.1)	24(80)	24(52.2)	0.014
Hemoptysis, N (%)	14(18.4)	10(33.3)	4(8.7)	0.007
Dyspnea, N (%)	19(25)	12(40)	7(15.2)	0.015
CNS symptoms, N (%)	47(61.8)	26(86.6)	21(45.6)	0.000
Alimentary symptoms, N (%)	33(43.3)	13(43.3)	20(43.4)	0.924
Laboratory parameters				
Neutropenia, N (%)	35(46.1)	20(66.6)	15(32.6)	0.004
Platelet count ($x10^9$/L)	33(20-48)	21(16-33)	38(28-53)	0.000
$CD4^+$ T-cells, (cells/mm^3, normal range 410–1590)	96(56-139)	58(44-97)	122(75-200)	0.000
$CD8^+$ T-cells, (cells/mm^3, normal range 190–1140)	111(61-168)	67(41-104)	142(99-218)	0.000
IL-6,(pg/mL, normal range 0–5.9)	95(35-197)	172(132-297)	44(25-94)	0.000
TNF-α, (pg/mL, normal range 0–8.1)	65(35-205)	137(73-232)	44(27-80)	0.000
IL-8, (pg/mL, normal range 0–62)	89(35-256.8)	223(126-365.5)	40(29-98)	0.000
IL-10, (pg/mL, normal range 0–9.1)	136(44-413.5)	260(135.7-509.2)	86(29-221)	0.000
BNP, (pg/mL, normal range 0–100)	174(75-512)	567(254-1016)	94(55-189)	0.000
G test, (pg/mL)	9.7(5-28.8)	19(9.8-56)	5(5-10)	0.000
GM test, (μg/L)	0.65 ± 0.45	1.06 ± 0.44	0.38 ± 0.17	0.000
PCT, (mg/L)	0.7(0.27-1.4)	0.49(0.18-1.52)	0.86(0.3-1.3)	0.127
CRP, (ng/mL)	15.1(6.5-43.1)	23.4(9-50.7)	10.8(3.3-35.3)	0.031
APTT, (seconds, normal range 28-42)	71(55-94)	89(58.9-104.4)	64.2(50-84)	0.005
MODS, N (%)	71(93.4)	30(100)	41(89.1)	0.062
Pancreatic damage, N (%)	66(86.8)	29(96.6)	37(80.4)	0.041
Renal damage, N (%)	38(50)	25(83.3)	13(28.3)	0.000
Myocardial damage, N (%)	69(90.8)	29(96.6)	40(86.9)	0.152
ICU admission, N (%)	7(9.2)	7(23.3)	0	0.001
DM, N (%)	6(7.9)	3(10)	3(6.5)	0.583
Carbapenems application, N (%)	28(36.8)	21(70)	7(15.2)	0.000
Death, N (%)	24(31.6)	16(53.3)	8(17.4)	0.001

Data are presented as the mean ± standard deviation, median (interquartile range), and proportion.

IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-8, interleukin-8; IL-10, interleukin-10; MODS, multiple organ dysfunction syndromes; ICU, intensive care unit; DM, diabetes mellitus. BNP, brain natriuretic peptide; G, (1,3)-β-D-glucan; CRP, C-reactive protein; PCT, procalcitonin; GM, galactomannan.

A comparison of patients (**Table 1**) indicated the incidence of cough (80.0% *vs* 52.2%, p=0.014), hemoptysis (33.3% *vs* 8.7%, p=0.007), dyspnea (40.0% *vs* 15.2%, p=0.015), CNS symptoms (86.6% *vs* 45.6%, p=0.000), and neutropenia (66.6% *vs* 32.6%, p=0.004) were significantly higher in patients with IPA than in patients without IPA. Thirty patients with IPA had lower levels of $CD4^+$ and $CD8^+$ lymphocytes and platelets (all p=0.000), higher levels of IL-6, TNF-α, IL-8 and IL-10 (all p=0.000), higher levels of G, GM, CRP, and APTT (all p<0.05), and a higher incidence of pancreatic and renal damage (p<0.05) compared with non-IPA patients. No significant differences in underlying disease such as DM were found between IPA and non-IPA patients.

As showed in **Table 2** and **Figures 2**, **3**, receiver operating characteristic (ROC) analysis was used to assess the value of immune parameters to predict IPA in SFTS patients. The cut-off values of $CD4^+$ and $CD8^+$ T-cell counts to predict IPA were 68 and 111 cells/mm^3, with sensitivities of 82.6% and 72%, and specificities of 56.7% and 83.3%, respectively. Cut-off values of IL-6, TNF-α, IL-8, and IL-10 to predict IPA incidence in severe SFTS patients were 99 pg/mL, 63 pg/mL, 120 pg/mL, and 111 pg/mL (**Table 2** and **Figures 2**, **3**), with sensitivities of 90.0%, 86.7%, 83.3% and 90.0%, and specificities of 80.4%, 71.7%, 82.6% and 65.2%, respectively.

Because all SFTS patients were diagnosed with pulmonary infection, antibiotic treatment as carbapenems were used in the early stage of disease in 28 patients. Among the 28 patients, 21 developed IPA. Early antibacterial therapy of carbapenems was associated with higher incidence of IPA in SFTS patients (70% *vs* 15.2%, p=0.000). Seven patients were admitted to the intensive care unit, and they all developed IPA during hospitalization, indicating intensive care unit admission was a risk factor for IPA. In this study, no patients used corticosteroids.

As shown in **Table 3**, multivariate logistic regression analysis indicated that $CD4^+$ T-cells <68 cells/mm^3 combined with $CD8^+$ T-cells <111 cells/mm^3 (OR 0.218, 95% CI 0.059–0.803, p=0.022), IL-6 >99 pg/mL combined with IL-10 >111 pg/mL (OR 17.614, 95% CI 2.319–133.769, p=0.006), and BNP level >500 pg/mL (OR 13.681, 95% CI 1.994–93.871, p=0.008) were independent risk factors for IPA in SFTS patients.

Prognostic Factors for Patients With IPA

In our study, the median time from disease onset to IPA diagnosis was 10 days (interquartile range, 8–14). The median time from admission time to IPA diagnosis was 5 days (interquartile range, 3–9). As shown in **Figure 4**, the case fatality rate was significantly reduced in patients with IPA

TABLE 2 | Receiver operating characteristic curve analysis of immune parameters predicting invasive pulmonary aspergillosis insevere patients with severe fever with thrombocytopenia syndrome.

Index	Cut off value	AUC (95%CI)	*P* value	sensitivity	specificity
$CD4^+$T-cells, cells/mm^3	68	0.777(0.673-0.881)	0.000	82.6	56.7
$CD8^+$T-cells, cells/mm^3	111	0.772(0.662-0.881)	0.000	72.0	83.3
IL-6, pg/ml	99	0.843(0.750-0.937)	0.000	90.0	80.4
TNF-α, pg/ml	63	0.755(0.641-0.869)	0.000	86.7	71.7
IL-8, pg/ml	120	0.797(0.690-0.905)	0.000	83.3	82.6
IL-10, pg/ml	111	0.752(0.644-0.860)	0.000	90.0	65.2

AUC, area under the curve; CI, confidence interval; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-8, interleukin-8; IL-10, interleukin-10.

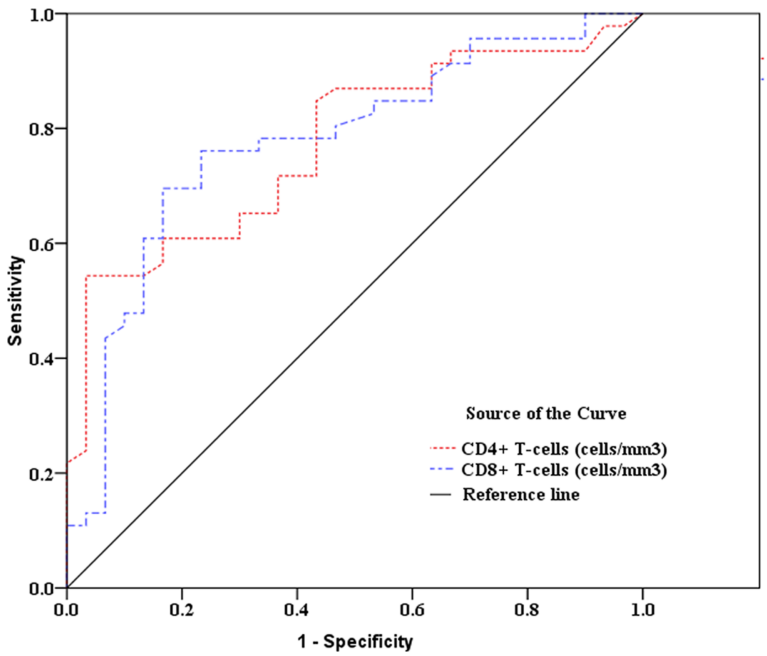

FIGURE 2 | Receiver operating characteristic curve analysis of $CD4^+$ and $CD8^+$ T-cell counts to predict invasive pulmonary aspergillosis in severe patients with severe fever with thrombocytopenia syndrome.

occurrence 14 days after onset compared with IPA occurrence 6–13 days after onset (log-rank, p=0.0001). The earlier occurrence of IPA was associated with a higher fatality rate. Early antifungal treatment against IPA was significantly associated with improved survival (log-rank, p=0.022) in severe SFTS patients (**Figure 5**).

DISCUSSION

Non-neutropenic critically ill patients with COPD, cirrhosis, severe influenza and neutropenic critically ill patients have been described in association with invasive aspergillosis (20–22). SFTSV has the ability to attenuate cellular and humoral immune responses of host, the dysregulated immune system as cellular immunosuppression and immune paralysis may contribute to the development of IPA in severe SFTS patients. In this study, severe SFTS patients with secondary IPA were studied.

IPA contributed to increase mortality of SFTS patients. Clinically, when critically ill patients suffered from secondary infection, bacterial infection was considered firstly, followed by fungal infection, and therefore antibacterial treatment was emphasized. However, we found critically ill SFTS patients can be infected with aspergillus in the early-stage of disease in this study. Besides, the occurrence of IPA at the early-stage of disease course was identified to be associated with a higher mortality rate. What should be noted is that, all our patients were from rural areas where the tick-borne was epidemic, the living characteristics of SFTS patients indicated they were easily to be exposed to aspergillus in the environment in daily life. Under severely ill condition caused by SFTSV, they would be more prone to aspergillus infection. Besides, the pathogenic characteristics of cellular immunosuppression, humoral immune regulation disorders, severe neutropenia and MODS in SFTS patients may increase susceptibility to IPA. Early

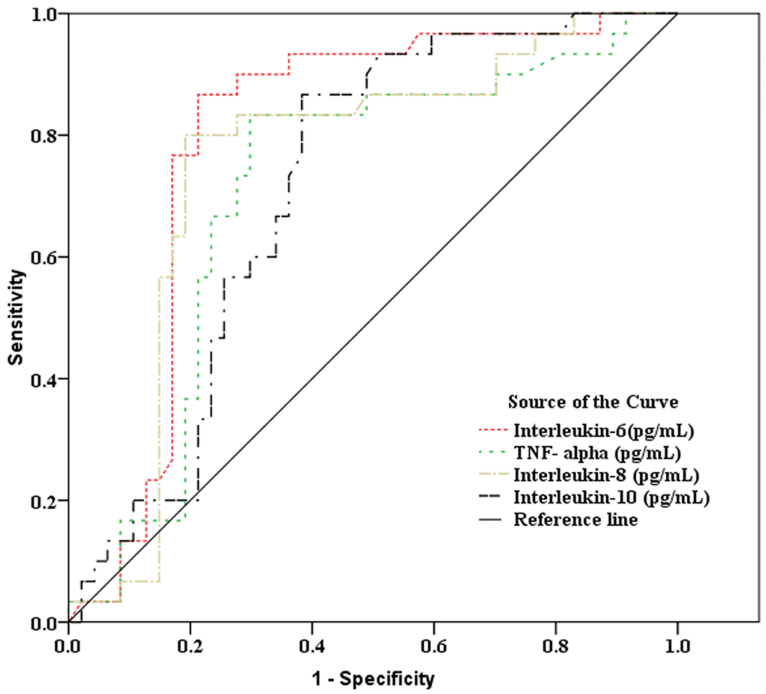

FIGURE 3 | Receiver operating characteristic curve analysis of inflammatory mediators to predict invasive pulmonary aspergillosis in severe patients with severe fever with thrombocytopenia syndrome.

TABLE 3 | Multivariate logistic regression analysis of factors predicting invasive pulmonary aspergillosis in severe patients with severe fever with thrombocytopenia syndrome.

Index	β	OR	95%CI	*P*-value
$CD4^+$T-cells <68 cells/mm^3 and $CD8^+$T-cells <111 cells/mm^3	-1.523	0.218	0.059-0.803	0.022
IL-6>99 pg/ml and IL-10>111 pg/ml	2.869	17.614	2.319-133.769	0.006
BNP>500 pg/ml	2.616	13.681	1.994-93.871	0.008

CI, confidence interval; OR, odds ratio; IL-6, interleukin-6; IL-8, interleukin-8; BNP, brain natriuretic peptide.

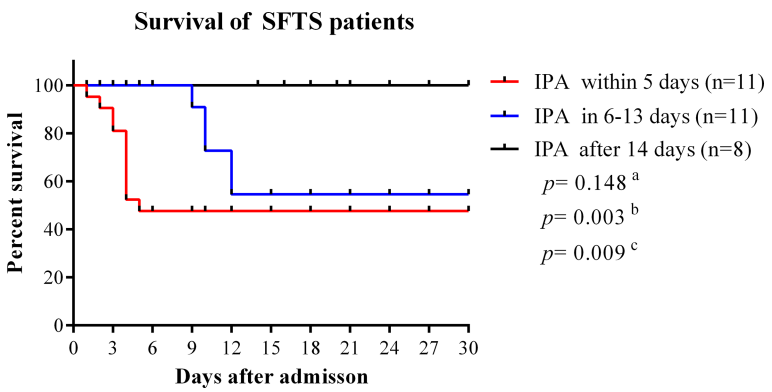

FIGURE 4 | Survival curves were compared among SFTS patients with different time points of IPA occurrence. IPA, invasive pulmonary aspergillosis; SFTS, severe fever with thrombocytopenia syndrome. [a]Comparison of survival curves between patients with IPA occurrence within 5 days and 6–13 days from disease onset. [b]Comparison of survival curves between patients with IPA incidence within 5 days and after 14 days from disease onset. [c]Comparison of survival curves between patients with IPA incidence 6–13 days after onset and 14 days after disease onset.

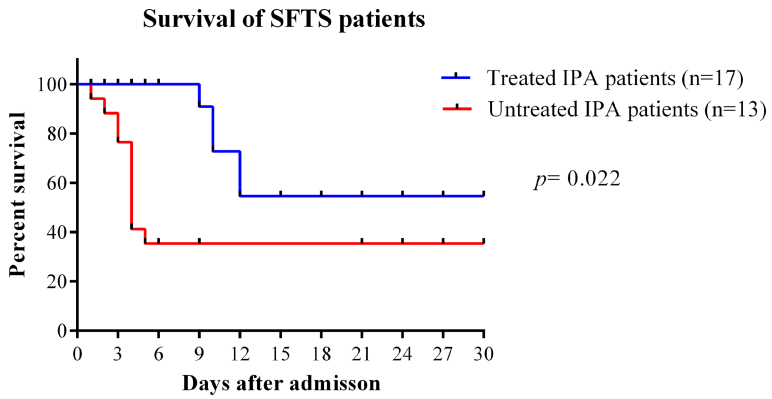

FIGURE 5 | Survival curves were compared between SFTS patients with IPA treated with antifungal treatment or untreated. IPA, invasive pulmonary aspergillosis; SFTS, severe fever with thrombocytopenia syndrome.

diagnosis and effective antifungal treatment against IPA have important value to improve the prognosis of SFTS patients. Therefore, identifying predisposing factors of IPA in severe SFTS patients as an early warning of IPA is very important. According to our results, agranulocytosis, attenuation of $CD4^+$ and $CD8^+$ T-cell, humoral immune imbalance, pancreatic and renal damage can predict the occurrence of IPA.

In this research, $CD4^+$ and $CD8^+$ T-cell counts were confirmed to be useful parameters to early prediction of IPA in SFTS patients with high sensitivities and specificities of cut-off values. T lymphocyte cells in SFTS patients decreased seriously by SFTSV invasion in severe SFTS patients. We found lower counts of $CD4^+$ and $CD8^+$ T-cells were independent predictors for a high risk of IPA in severe SFTS patients. Previous study has showed that fungal infections were associated with impaired cell-mediated immunity and $CD4^+$ T-cell responses were critical for protection against invasive fungal infections (IFIs) (23). $CD8^+$ T-cells were also reported to be independent predictors for a high risk of IPA and early mortality in critically ill immunocompromised patients with IPA (12). Our research showed that $CD4^+$ T-cell counts <68 cells/mm^3 combined with $CD8^+$ T-cell counts <111 cells/mm^3 were independent risk factors for IPA in critically ill SFTS patients. To the best of our knowledge, this is the first report to evaluate the important early warning role of $CD4^+$ and $CD8^+$ T-cell counts for IPA in critically ill SFTS patients.

Our study confirmed the early-warning value of inflammatory mediators to predict IPA in severe SFTS patients with high sensitivities and specificities of cut-off values. The inflammatory response in SFTS patients was confirmed to play an important role in the pathogenesis of SFTSV (13, 14). In the acute-stage in severe SFTS patients, many pro- and anti-inflammatory mediators are produced, resulting in systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARS). SIRS lead to cell death and organ dysfunction, CARS lead to immunosuppression which increases susceptibility to secondary infection (24, 25). When SIRS and CARS coexist, a more serious inflammatory disorder will occur, termed mixed antagonistic response syndrome, which increases the occurrence of secondary infection. Our previous research has revealed cytokine storm with high levels of pro- and anti-inflammatory cytokines in severe SFTS patients (13). Cytokine storms have been reported as a major pathophysiologic mechanism that aggravates leukopenia and thrombocytopenia (26). Leukopenia is a well-known risk factor for IPA, platelets have the ability to block *Aspergillus fumigatus* germination and hyphal elongation (27). All these characteristics in severe SFTS can make patients more vulnerable to IPA. Pro-inflammatory cytokine such as IL-6, and anti-inflammatory cytokine such as IL-10, have key roles in maintaining the immune balance. Overexpression of IL-10 has been reported to be a risk factor for invasive aspergillosis in patients after stem-cell transplantation (28). Measurement of immunocompetence would be possible to detect SFTS patients who are at high risk of developing IPA at an earlier time. In this study, we've got quantitative indicators of inflammatory factors to predict IPA, IL-6 >99 pg/mL combined with IL-10 >111 pg/mL in SFTS patients was confirmed to be an independent predictor for the high risk of IPA.

BNP are known to reflect cardiac damage and circulation overload, it was also reported to be independently associated with risk of infection including pneumonia, urinary tract infections, bloodstream infections, and cellulitis (29). In addition, the results in this research concluded that BNP was an important independent predictor for IPA occurrence in severe SFTS patients. Clinically, monitoring BNP in critical SFTS patients is of great significance for disease condition evaluation and the early diagnosis of IPA.

The course of SFTS patients can be divided into the initial stage of fever, acute stage, and recovery period or death. According to our results, patients with IPA occurrence within 2 weeks from disease onset had a significantly higher mortality rate than those with IPA occurrence after 2 weeks from onset, suggesting the risk of death from IPA is higher in the acute phase. Within the first 2 weeks of the disease, severe SFTS patients have impaired immune system and MODS. Occurrence of IPA within 2 weeks would be fatal for severe SFTS patients. Our results

revealed that early antifungal therapy significantly reduced mortality in SFTS patients with IPA, similar to a previous report (11). However, in clinical practice, early diagnosis and antifungal therapy of IPA represents a challenge due to lack of diagnostic algorithm. SFTS patients who are considered at risk for IPA, should raise clinician awareness.

In conclusion, severe SFTS patients have more risk factors to develop IPA in the early stage of disease, which can increase mortality. $CD4^+$ and $CD8^+$ T-cell counts, IL-6 and IL-10 levels, and BNP level have important predictive values for the early diagnosis of IPA, which in combination with appropriate antifungal treatment can contribute to a better prognosis for severe SFTS patients. Moreover, we speculate SFTS may be as a host factor for IPA. Our results will be of great value to the field of fungal infections and optimal practices for diagnosis and treatment of SFTS disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by the local Ethics Committee of the First Affiliated Hospital of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LH and JL conceived and designed the project, and composed the manuscript. QK and CY collected clinical data and analyzed the data. YYL, HZ, and TB performed the laboratory experiments. QK, CY, and XM performed the flow cytometry data analyses. LH, XX, LX, YY, HY, QS, YG, and JL gave diagnosis and treatment of the patients. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Efficacy of Ebselen Against Invasive Aspergillosis in a Murine Model

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Invasive aspergillosis is one of the major causes of morbidity and mortality among invasive fungal infections. The search for new antifungal drugs becomes imperative when existing drugs are not able to efficiently treat these infections. Ebselen, is an organoselenium compound, already successfully approved in clinical trials as a repositioned drug for the treatment of bipolar disorder and prevention of noise-induced hearing loss. In this study, we aimed to reposition ebselen for the treatment of invasive aspergillosis by showing ebselen effectiveness in a murine model. For this, BALB/c mice were immunosuppressed and infected systemically with *Aspergillus fumigatus*. Animals were divided and treated with ebselen, voriconazole, or drug-free control, for four days. The kidneys were used for CFU count and, histopathological and cytokine analysis. Ebselen was able to significantly reduce the fungal burden in the kidneys of infected mice with efficacy comparable with voriconazole treatment as both had reductions to the same extent. The absence of hyphae and intact kidney tissue structure observed in the histopathological sections analyzed from treated groups corroborate with the downregulation of IL-6 and TNF. In summary, this study brings for the first time *in vivo* evidence of ebselen efficacy against invasive aspergillosis. Despite these promising results, more animal studies are warranted to evaluate the potential role of ebselen as an alternative option for the management of invasive aspergillosis in humans.

Keywords: ebselen, *Aspergillus*, murine model, antifungal, systemic infection

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INTRODUCTION

Invasive aspergillosis (IA) remains one of the major causes of morbidity and mortality among invasive fungal infections, especially in intensive care unit patients (Ostrosky-Zeichner and Al-Obaidi, 2017). Voriconazole is the gold standard for treatment of IA (Patterson et al., 2016). Despite this, the mortality in patients who received appropriate initial voriconazole therapy is up to 24% (Lestrade et al., 2019). Patients with invasive aspergillosis caused by azole-resistant *A. fumigatus* showed 100% all-cause mortality at 100 days (Cho et al., 2019).

Facing this critical scenario, in an attempt to optimize the process of searching for new drug options, repositioning drugs has become an interesting approach to speed up the discovery of new antifungal drugs. This approach decreases the conventional time of drug discovery from 10–17 to 3–12 years for repurposed compounds, as it bypasses much of the discovery and preclinical stages and phase I studies of safety (Farha and Brown, 2019).

Ebselen [2-phenyl-1,2-benzoselenazol-3(2H)-one; EbSe] is an organoselenium compound already successfully tested in human clinical trials for neuroprotective effect (Saito et al., 1998), treatment for bipolar disorder (Masaki et al., 2016), and prevention of noise-induced hearing loss (Kil et al., 2017) with no reported side effects or toxicity. Recently it also has been approved for clinical trials on moderate COVID-19 patients (NCT04484025) (ClinicalTrials.gov, 2020). In addition, the antifungal *in vitro* activity of EbSe against *Candida* spp., *Trichosporon asahii*, and *Cryptococcus* spp. has been demonstrated (Thangamani et al., 2017; Kubiça et al., 2019). Recently, Marshall and colleagues (2019) proved the ability of EbSe to block *A. fumigatus* thioredoxin reductase (TrxR) activity (Marshall et al., 2019). This flavoenzyme has been largely studied by our group as a promising target for antifungal drugs (Capoci et al., 2019; Rodrigues-Vendramini et al., 2019; Faria et al., 2020). In fact, differences between TrxR from humans and fungi lead EbSe to exert different effects in fungi TrxR by accumulation of reactive oxygen species (ROS) and cell death (Ren et al., 2018). Recently, Binder et al. (2020) showed that the *trxR* gene is essential for *A. fumigatus* survival and has only 28% of homology to its human ortholog.

To the best of our knowledge, this is the first study demonstrating the efficacy of ebselen antifungal treatment *in vivo*. Thus, the aim of this study was to bring evidence of EbSe effectiveness in invasive aspergillosis using a murine model.

MATERIALS AND METHODS

Antifungal Agents

The following compounds were used for susceptibility tests: ebselen (EbSe; $C_{13}H_9NOSe$; TargetMol), voriconazole (VOR; Pfizer Incorporated, New York, NY, USA), and amphotericin B (AMB; Sigma-Aldrich, St. Louis, MO, USA). Stock solution of voriconazole was prepared in Dimethyl Sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). DMSO and Pluronic® F-127 (Sigma-Aldrich, St. Louis, MO, USA) were used for the solubilization of ebselen. For *in vivo* treatment, we used voriconazole injectable solution (VOR; Cristalia Prod. Quim. Farm. Ltda., Itapira, SP, Brazil) diluted in phosphate saline buffer (PBS) and the ebselen stock solution (50 $mg.ml^{-1}$ in DMSO) prepared in PBS with Pluronic® F-127 (1.25%). The control group was treated with vehicle (PBS, DMSO, and 1.25% Pluronic® F-127).

Organisms and Inoculum Preparation

Aspergillus fumigatus reference strain (ATCC 64026) and two clinical isolates of *A. fumigatus* isolated from sputum and bronchoalveolar lavage (Af1 and Af2) were used. The collection of isolates was carried out in accordance with the regulations of the *Comitê de Ética em Pesquisa Envolvendo Seres Humanos* of the *Universidade Estadual de Maringá*, Brazil (Approval n° 2.748.843). The sample collection was performed by healthcare professionals at the *Hospital Universitário de Maringá* (HU) and at the *Laboratório de Ensino e Pesquisa em Análises Clínicas* (LEPAC). For inoculum preparation, the strains were grown on potato dextrose agar (PDA) at 35°C for 7 days. Conidia were harvested with 0.1% Tween 80 in saline (0.85%). Homogenous conidial suspensions were collected following filtration through a sterile syringe with cotton and then adjusted to the desired concentration.

Minimum Inhibitory Concentration Determination

The procedures were performed according to the broth microdilution protocol from the clinical & laboratory standards institute (CLSI) M38-A2. For the interpretation of results, 0.02% of resazurin sodium salt ($C_{12}H_6NNaO_4$; R7017, Sigma, St. Louis, MO) was added after 24 h and incubated for an additional 24 h at 35°C. A blue color was interpreted as the absence of metabolic activity (no spore germination). A fluorescent pink color was interpreted as the presence of metabolic activity (spore germination), and a purple color was interpreted as a trailing result, which means that some metabolic activity was present and a longer incubation time would allow the purple color to change to pink.

Experimental Model of Invasive Aspergillosis *In Vivo*

The procedures were carried out in accordance with the regulations of the Institutional Ethics Committee for animal experimentation of the State University of Maringá, Brazil (Approval n° CEUA 9067030518). A total of 21 female BALB/c mice, weighing 22–25 g were used. Animals were housed in filter top cages and allowed access to food and water *ad libitum*. To induce an immunosuppressed state, intraperitoneal injections of cyclophosphamide (200 $mg.kg^{-1}$ on day −3, on day 0 (day of infection), and every 3 days until the end of the experiment) were applied. Animals were infected with $1–2 \times 10^4$ conidia of *A. fumigatus* (strain ATCC 64026) suspended in 100 µl of saline (0.85%) by lateral tail vein injection and were left for 24 h before starting the treatment.

The infected mice (n = 21) were randomly divided into three experimental groups: Ebselen (seven mice treated with 10 $mg.kg^{-1}$/765.8 µmoles per mouse of ebselen), voriconazole (seven mice treated with 10 $mg.kg^{-1}$/572.5 µmoles per mouse of voriconazole), and control (seven mice treated with solubilization buffer used as a placebo). All treatments were intraperitoneally administered, twice daily for four days. On day 5 post-infection, animals were anesthetized with isoflurane (Isoforine®, Cristália, SP, BR), and blood samples were collected in microtubes and centrifuged (5,000 rpm for 5 min). The serum was then stored at −80°C for cytokine measurement. After that, the animals were euthanized, and the right kidneys

were aseptically removed, weighed, and mechanically homogenized in sterile saline (0.85%). Serial 10-fold dilutions of the homogenates in saline were placed on PDA and incubated for 48 h at 35°C to quantify the fungal burden in the kidneys measured as $\log_{10}$ CFU per gram of tissue. The kidney homogenates were centrifuged (11,000 rpm for 13 min), and tissue supernatants were collected and stored at −80°C for cytokine measurement.

Histopathological Analysis

For histopathological evaluations, the left kidneys of all animals were collected, immediately fixed in 4% paraformaldehyde, paraffin-embedded, and cut into thin sections (5 μm). The sections were stained by Grocott–Gomori's methenamine silver (GMS) to visualize fungi and counterstained with hematoxylin and eosin (H&E) for characterization of host cells. Slides were observed and photographed using a binocular light microscope (Motic BA310) with a camera (Moticam 5) coupled to a computer using Motic Images Plus 2.0 software.

Cytokines

Cytokines in serum samples and kidney homogenate supernatants of five animals per group were measured with a BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Bioscience, San Jose, CA, USA). The kit was used for the simultaneous detection of mouse interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin-12p70 (IL-12p70) in a single sample following the manufacturer's protocol. Samples were measured on the BD FACSCalibur Flow Cytometer and analyzed by FCAP Array™ Software Version 3.0 (BD Bioscience).

Statistical Analysis

The statistical significance of the differences observed between mice treated with placebo and EbSe or voriconazole was analyzed by applying an unpaired t-test using the GraphPad Prism 5 software package (GraphPad Software Inc., San Diego, CA, USA). P <0.05 was considered significant in these analyses.

RESULTS

Antifungal Susceptibility Testing

In general, all strains tested showed the same susceptibility profile standard *in vitro* (**Table 1**). *A. fumigatus* reference strain and Af1 and Af2 clinical isolates showed the same MIC values for EbSe and voriconazole: 4.0 μg.ml^{-1} (14.6 μM) and 0.25 μg.ml^{-1} (0.27 μM), respectively.

Ebselen Was Able to Significantly Reduce the Fungal Burden in a Model of Invasive Aspergillosis *In Vivo*

The immunosuppressed condition of each mouse was monitored by counting the polymorphonuclear cells from the blood on days −3, 0, and +4 post-infection (d.p.i.). All animals were immunosuppressed on day 0 and continued in this condition until day +4 (data not shown).

TABLE 1 | Susceptibility profile of *A. fumigatus* reference and clinical strains against ebselen and standard antifungals.

A. fumigatus Strains	MIC (μg.ml^{-1}/μM)		
	Ebselen (0.5–256/1.82–933.6)	Amphotericin B (0.03–16/0.03–17.3)	Voriconazole (0.03–16/0.08–45.8)
ATCC 64026	4.0/14.6	0.25/0.27	0.25/0.71
Af1	4.0/14.6	0.25/0.27	0.25/0.71
Af2	4.0/14.6	0.12/0.13	0.25/0.71

Minimum inhibitory concentration; Af1, A. fumigatus clinical isolate 1; Af2, A. fumigatus clinical isolate 2.

The amount of CFUs recovered after 4 days post-infection from the kidneys of mice treated with ebselen was similar to that recovered from the kidneys of mice treated with voriconazole ($p > 0.05$) and significantly lower than that recovered from the kidneys of mice treated with placebo ($p < 0.05$) (**Figure 1**).

Histopathological analyses showed a massive dissemination of hyphae in the kidneys from the control group (**Figure 2A**). Fungal hyphae were numerous and centered on the pelvis and secondarily extended to renal tubes of the medulla and cortex with presence of hyphae across the Bowman's capsule and intact glomerulus. Additionally, the control group showed severe lesions with an extended area of coagulative necrosis and bleeding. Only in this group was there a diffuse inflammatory infiltrate with a predominance of mononuclear cells.

Concerning the groups treated either with voriconazole or ebselen (**Figures 2B, C**, respectively), in both, no fungal elements were detected on the entire kidney section observed. Kidney tissue was intact, and no evidence of inflammation was noted.

Systemic cytokines (retrieved from the serum) (**Figure 3A**) and local cytokines (retrieved from kidney homogenates) (**Figure 3B**) showed similar expression patterns. The ebselen treatment was able to downregulate the expression of proinflammatory cytokine

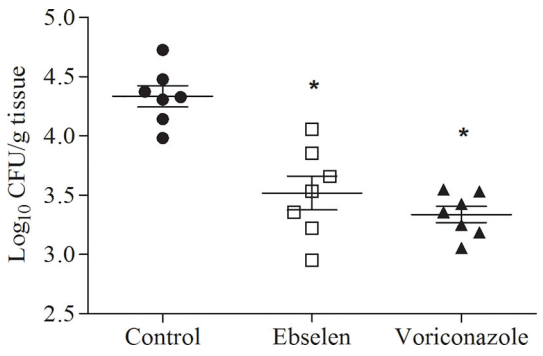

FIGURE 1 | Fungal burden in the kidney after systemic infection by *A. fumigatus* (ATCC 64026). Control: mice treated with placebo; ebselen: mice treated with 10 mg.kg^{-1} (765.8 μmoles per mouse) of ebselen; voriconazole: mice treated with 10 mg.kg^{-1} (572.5 μmoles per mouse) of voriconazole. All groups were treated intraperitoneally twice daily for 4 days starting 1 day after infection. *$p < 0.05$. Error bars correspond to the standard deviation.

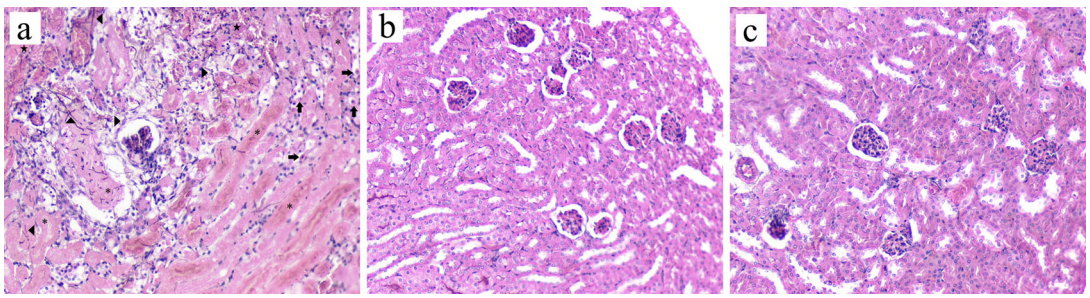

FIGURE 2 | Histological findings in the kidney of immunocompromised BALB/c mice inoculated with *Aspergillus fumigatus* after five days of systemic infection. **(A)** Control: mice treated with placebo; **(B)** voriconazole: mice treated with 10 mg.kg^{-1} (572.5 µmoles per mouse); **(C)** ebselen: mice treated with 10 mg.kg^{-1} (765.8 µmoles per mouse) of ebselen. The treatments were performed intraperitoneally, twice a day, for four days. Tissues were stained with Grocott–Gomori's methenamine silver (GMS) and hematoxylin and eosin (H&E); magnification, ×400. Asterisk: coagulative necrosis; arrow's head: hyphae; arrow: mononuclear cell; star: hemorrhage.

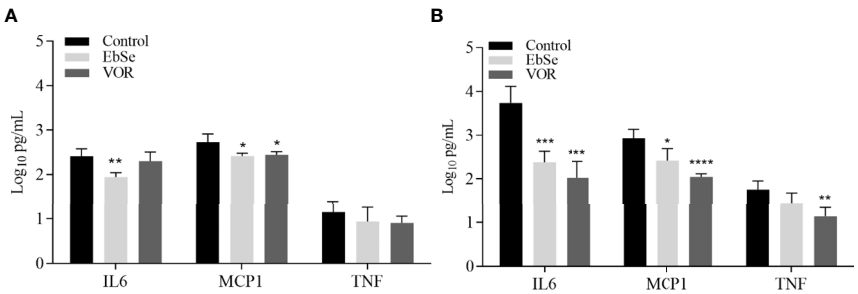

FIGURE 3 | Systemic and local inflammatory cytokine evaluation in mice after treatment with EbSe or voriconazole. **(A)** Cytokines recovered from serum and **(B)** kidney homogenates from mice treated with placebo, EbSe, or voriconazole (VOR). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$, significant difference compared with control from each cytokine. TNF, tumor necrosis factor; MCP-1, macrophage/monocyte chemoattractant protein-1; IL-6, interleukin-6. Error bars correspond to the standard deviation.

IL-6 and MCP-1 in both systemic and local responses ($p < 0.05$) (**Figure 3**). In the systemic response, only EbSe showed a significantly reduced expression of IL-6 ($p = 0.0073$) and MCP-1 ($p = 0.0377$). In this situation, voriconazole modulated only MCP-1 ($p = 0.0132$). In contrast, EbSe exhibited its greater effect on lowering the production of IL-6 ($p = 0.0004$) and MCP-1 ($p = 0.0249$) in kidney homogenates, although at a lower extent than in mice treated with voriconazole (IL-6, $p = 0.0003$; MCP-1, $p < 0.0001$; TNF, $p = 0.0057$) (**Figure 3B**).

DISCUSSION

Aspergillosis remains one of the main causes of death by invasive fungal infections (Lestrade et al., 2019). The incidence of azole-resistant strains has increased, mainly associated with the acquisition of resistant environmental strains which challenges the limited antifungal arsenal available (Cho et al., 2019; Lestrade et al., 2019). Therefore, the search for new treatment against aspergillosis is essential, and the drug repositioning tools have accelerated this process. Recently, Binder et al. (2020) showed that the TrxR protein is encoded by an essential gene for *A. fumigatus*, the *trxR* gene. Suppression of the *trxR* gene causes growth deficiency that is not supplied by supplementation of glutathione or other organic sources of sulfur, as occurs in yeasts. In addition, Marshall et al. (2019) elucidated the crystal structure of *A. fumigatus* thioredoxin reductase (AfTrxR) and described that the main mechanism of action of EbSe over *A. fumigatus* is the inhibition of AfTrxR. However, only *in vitro* studies were performed.

Our research group has been exploring the thioredoxin system as a promising drug target, with the selection of promising molecules for other pathogenic fungi (Capoci et al., 2019; Rodrigues-Vendramini et al., 2019; Faria et al., 2020). In this search for new mechanisms of action, different from those that are currently available, ebselen fitted our proposal well. Marshall and colleagues described that EbSe binds to Cys148 in the active site of thioredoxin reductase from *A. fumigatus*, locking AfTrxR in a catalytically nonproductive conformation (Marshall et al., 2019). This target of inhibition is totally different from those addressed in the commercial antifungal treatment, highlighting the possibility of EbSe in the treatment of refractory strains alongside the commonly used antifungals with usual targets (e.g., ergosterol). In addition, the selective manner in

which EbSe links to human and fungi/prokaryotes TrxR confers TrxR as an excellent drug target (Ren et al., 2018).

EbSe has already been approved in a phase I clinical trial, in which safety, pharmacokinetic profile, and oral bioavailability in healthy humans were tested (Kil et al., 2017). In addition, this promising drug overcomes the hematoencephalic barrier acting in the central nervous system (Singh et al., 2016), an interesting feature for antimicrobial agents. Another clinical trial for the prevention of noise-induced hearing loss and treatment of mania or hypomania showed that doses of up to 600 mg twice daily did not change the hematological, serum chemistry, or radiological assessments between EbSe treatment and placebo groups also showing EbSe to be effective in the proposed treatment (Singh et al., 2013; Sharpley et al., 2020). Previous study of this group used 10 $mg.kg^{-1}$ i.p. of EbSe to show its efficacy in the treatment against bipolar disorder (Singh et al., 2013). In an attempt to reproduce good results with safety and well tolerability in future human use, we treated mice infected with *A. fumigatus* by using 10 $mg.kg^{-1}$ i.p. twice daily which allowed for a significant reduction of fungal burden. Just one *in vivo* study demonstrating the antimicrobial activity of ebselen using a model of *Caenorhabditis elegans* infection is described in the literature. The results showed that EbSe was more effective in reducing the fungal load of *Candida* and *Cryptococcus* over conventional antifungals such as amphotericin, fluconazole, and flucytosine (Thangamani et al., 2017). So far, there is no murine model showing antimicrobial EbSe efficacy.

In this study, a murine model allowed us to verify certain important points related to the host's response to infection and treatment with EbSe, especially with histopathological and cytokine analyses. The treatments were shown to be efficient in reducing the fungal burden without exacerbating immune response which could be explained by the fast killing kinetics of EbSe as it was previously shown *in vitro* for *Candida* and *Cryptococcus* (Thangamani et al., 2017), which could also prevent the emergence of kidney lesions in the treated groups.

The decrease of proinflammatory cytokines could be associated with a reduction of infection and absence of hyphae, once the marked release of IL-6 occurs due to the exposition of hyphal fragments of *A. fumigatus* (Øya et al., 2019) TNF plays an important role in host immune defense against invasive fungal infections (Filler et al., 2005). In mice, the amount of TNF increases after 24 h, the acute phase response, and is associated with accumulation of large numbers of leukocytes at the foci of infection (Herbst et al., 2013). In this sense, the decrease of TNF levels could be correlated with kidney clearance and corroborated with histopathological analysis results.

Although the IV route does not mimic the natural route of infection in humans and involves organs that are not usually affected, such as the kidneys, this methodology provides greater accuracy and reproducibility of results, especially with assertive fungal inoculum for a reduced group of animals (Desoubeaux and Cray, 2017; Desoubeaux and Cray, 2018). In addition, this systemic proposed treatment can be extrapolated to a situation of invasive and systemic aspergillosis in antifungal EbSe activity evaluation. Thus, this study brings for the first time *in vivo* evidence of EbSe efficacy for invasive aspergillosis treatment, especially with a reduction of fungal burden. As a repurposing drug candidate, EbSe showed similar antifungal efficacy to conventional drugs, with a good safety profile and effectiveness. However, more animal studies are warranted in order to evaluate the potential role of EbSe as an alternative option for management of disseminated aspergillosis in humans.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Ethics Committee for animal experimentation of the State University of Maringá, Brazil (Approval n° CEUA 9067030518). Collection and storage of *Aspergillus* clinical isolates were authorized by the Ethics Committee on Human Research of the State University of Maringá (Approval n° 2.748.843).

AUTHOR CONTRIBUTIONS

KS, IC, PC, FR-V, DF, and GA contributed to conception and design of the study. KS, PB-M and EK organized the database. PB-M and EK performed the statistical analysis. KS wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Evaluation of a Prototype of a Novel Galactomannan Sandwich Assay Using the VIDAS® Technology for the Diagnosis of Invasive Aspergillosis

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Objectives: To evaluate the analytical and clinical performance of a prototype of a VIDAS® Galactomannan (GM) unitary test (bioMérieux, Marcy l'Etoile, France) and compare to that of the Platelia™ *Aspergillus* Ag assay (Bio-Rad, CA, USA).

Methods: Repeatability, reproducibility, and freeze-thaw stability of VIDAS®GM were evaluated. Sera from patients at risk of IA were concurrently tested with both the VIDAS®GM and Platelia™ *Aspergillus* Ag assays. Correlations between the two assays were assessed by Passing Bablok (PB) regression and performance by ROC analysis.

Results: The correlations between the VIDAS®GM indexes after one and two cycles of freezing/thawing were r=1.00 and r=0.989, respectively. The coefficients of variation for negative, low-positive, and positive sera were 13%, 6%, and 5% for repeatability and 14.4%, 7.2%, and 5.5% for reproducibility. Overall, 126 sera were tested with both assays (44 fresh and 82 frozen). The correlation between VIDAS®GM and Platelia™ *Aspergillus* Ag was r=0.798. The areas under the curve of the ROC analyses were 0.892 and 0.894, for VIDAS®GM and Platelia™ *Aspergillus* Ag, respectively.

Conclusions: This new VIDAS®GM prototype assay showed adequate analytical and clinical performance and a good correlation with that of Platelia™ *Aspergillus* Ag with 126 sera, although these results need to be confirmed in a larger prospective and multicentric study. As for the other VIDAS® assays, VIDAS®GM is a single-sample automated test using a solid reagent strip and receptacle. It is easy to use and suitable for rapid on-demand test results.

Keywords: diagnosis, invasive aspergillosis, galactomannan, single-sample test, VIDAS®

INTRODUCTION

Invasive aspergillosis (IA) is an opportunistic infection that occurs mainly among immunocompromised patients. Its incidence has increased with the increasing use of immunosuppressive therapies (Vallabhaneni et al., 2017). IA is associated with high morbidity and mortality, especially if diagnosis and treatment are delayed (Cornely et al., 2017). Since 1990, biological

markers, mainly galactomannan (GM), have considerably improved IA diagnosis and its precocity (Guo et al., 2010; Jenks et al., 2019). Until very recently, Platelia™ *Aspergillus* Ag (BioRad), based on a sandwich enzyme immuno-assay (EIA) technique, was the most widely used for GM detection. A meta-analysis showed a pooled sensitivity and specificity for proven cases of 0.71 and 0.89, respectively (Pfeiffer et al., 2006). However, well-known limitations include poor reproducibility and repeatability and the need to batch the samples in series, resulting in a loss of speed (Oren et al., 2012). Several lateral-flow devices assays for detecting either GM or another antigen have been recently commercialized to respond to the need of a rapid and easy-to-use single-sample test (Thornton, 2008; Jenks and Hoenigl, 2020; Mercier et al., 2020). These tests performed better on broncho-alveolar lavages than sera, in which the sensitivity was lower than that of Platelia™ *Aspergillus* Ag (Donnelly et al., 2020). Here, we evaluated the analytical and clinical performance of a prototype of a VIDAS®GM (BioMérieux) unitary test. As for the other VIDAS tests, it is a fluorescent EIA packaged in ready-to-use disposable strips.

METHODS AND RESULTS

This monocentric retrospective and prospective study included 126 sera from 30 patients at risk of IA at the University Hospital of Grenoble (France). The patients had mainly hematological malignancies (n=27) including allogeneic bone marrow (n=21) and solid organ transplantation. Eighteen probable and 6 possible IA were diagnosed by a local multidisciplinary Aspergillosis committee. The EORTC/MSG criteria were used to classify the patients (Donnelly et al., 2020). Probable cases were mostly classified as such on the basis of the GM (Platelia™ *Aspergillus* Ag) results and/or *Aspergillus* PCR. The possible cases (only radiological/clinical criteria) were not considered as IA cases. Patients were screened for GM detection with Platelia™ *Aspergillus* Ag and the samples collected as part of routine clinical care and registered in the certified biological collection DC-2008-582. Both frozen and fresh samples were analyzed. Frozen and fresh samples were tested the same day with the two assays to assess the effect of storage at -80°C on GM detection. Platelia™ *Aspergillus* Ag was performed according to the manufacturer's instructions using an automated EVOLIS Premium® system (BioRad) and a GM index cut-off value of 1 was considered for positive samples as recommended in the recent revision of the EORTC/MSG criteria (Donnelly et al., 2020). VIDAS® GM is an automated qualitative sandwich assay with a coated solid-phase receptacle that also serves as a pipetting device. Samples are heat pre-treated with EDTA, as for the Platelia™ *Aspergillus* procedure. In the instrument, after a dilution step, GM is captured between the coated mouse monoclonal antibody (mAb) and the detection rat mAb conjugated to biotin. Alkaline phosphatase linked to an anti-biotin antibody hydrolyzes the substrate into a fluorescent product at 450 nm. The assay prototype uses a standard (S1) and a positive control. A relative fluorescence value (RFV) is generated and automatically calculated by the instrument, according to S1, and an index value (I) is calculated as I=RFVsample/RFVS1. IA cases were classified as proven or probable according to the 2020 EORTC/MSG criteria (Donnelly et al., 2020). Appropriate permissions have been obtained from BioMérieux for the copyright of the VIDAS® trademark.

Overall, 126 sera were tested with both assays (44 fresh and 82 archived at -80°C). We evaluated the stability of VIDAS®GM after one and two cycles of freezing after seven days (at -80°C) and thawing (at room temperature) using 9 and 11 samples, respectively. We used the Passing Bablok (PB) test and analyse-it 5.0 software. The PB showed excellent correlations between the VIDAS®GM indices after one (r=1.00) and two cycles (r=0.989) of freezing/thawing. The repeatability (precision within run) and reproducibility (total precision) of VIDAS®GM were evaluated from four sera (one negative, one low-positive, and two positive) measured in triplicate twice a day for 3 days, totalizing 18 measurements per sera. The coefficients of variation for negative, low-positive, and positive sera were 13%, 6%, and 5% and 14.4%, 7.2%, and 5.5%, respectively (SAS Add-in 9.2 software). The PB correlation between VIDAS®GM and Platelia™ *Aspergillus* Ag levels was r=0.798 (**Figure 1**). There was a good agreement, with a Cohen kappa index of 0.82 (95% CI=0.71-0.93) between the two assays for the positive and negative results based on a VIDAS®GM cut-off of 1, calculated from the PB equation (**Figure 1**; Y=0.1166+0.8811X), and a 1 Platelia™ *Aspergillus* Ag cut-off. The performance of the assays assessed by ROC curves is shown in **Figure 2**. Considering all 126 sera, the areas under the curve (AUC) were 0.808 and 0.827 for the VIDAS®GM and Platelia assays, respectively. For the sera collected 15 days before or after the date of the IA diagnosis AUC of the two assays were better and similar (0.892 and 0.894, respectively). The cut-offs and results that correspond to the higher Youden index (the best balance between

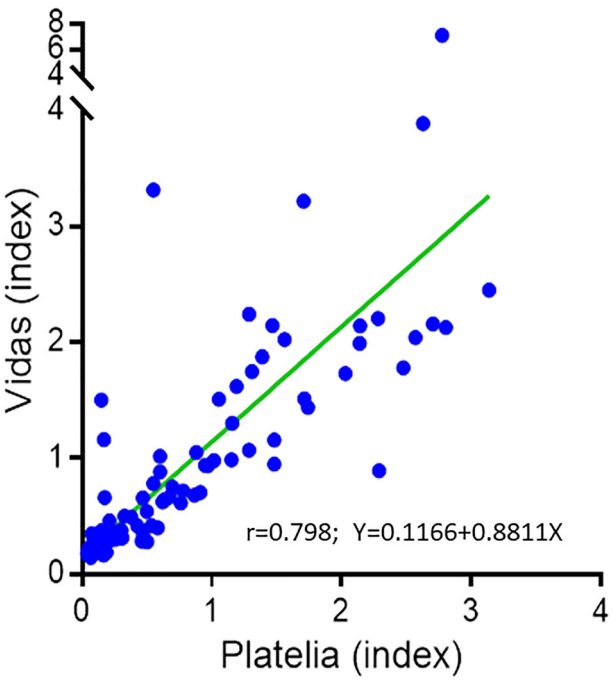

FIGURE 1 | Correlation between VIDAS®GM and Platelia™ *Aspergillus* Ag using serum samples. N = 118 (42 fresh and 76 frozen, samples with Platelia results ≥ 3.5 were excluded).

sensitivity and specificity) for both tests are shown in **Table 1**. A cut-off of 0.36 for the VIDAS®GM assay can thus be proposed based on these ROC analyses and Youden index.

The median VIDAS®GM and Platelia™ *Aspergillus* Ag index levels of the IA cases were 0.98 and 0.88 for IA cases and 0.26 and 0.125 for non-IA patients, respectively.

DISCUSSION

GM detection has been used for IA diagnosis since the early 90's. The EIA Platelia™ *Aspergillus* Ag kit rapidly supplanted the latex agglutination Pastorex *Aspergillus*® kit (BioRad), which was commercialized first. Newly developed single-sample tests have addressed the need to reduce the time to results for early-targeted therapy and an improved outcome of IA patients (Thornton, 2008; Jenks and Hoenigl, 2020; Mercier et al., 2020). Here, we evaluated a prototype of a novel single-sample GM assay, VIDAS®GM and compared it to Platelia™ *Aspergillus* Ag. VIDAS®GM showed excellent stability, repeatability, and reproducibility. The correlation between the two assays was also high (r=0.798) and their diagnostic performance comparable, with the AUC under ROC curves of 0.892 and 0.894 for the VIDAS®GM and Platelia assays, respectively (**Figure 2**). In **Figure 1** showing the correlation the three points with low indices of Platelia™ *Aspergillus* Ag and high indices of the VIDAS GM correspond to false negative of the Platelia in probable IA patients (these 3 patients presented other sera positive with the Platelia™ *Aspergillus* Ag). Importantly, the IA diagnosis was established according to the revised EORTC/MSG criteria (Donnelly et al., 2020), which include GM itself. Thus, the results of the diagnostic performance of the two assays should be interpreted with caution, as the sensitivity may have been overestimated. Nevertheless, the IA diagnosis remains possible when excluding GM from the diagnostic criteria, as our patients fulfilled the risk factors, as well as the clinical and radiological EORTC/MSG features.

The ROC curves and best Youden index revealed a VIDAS®GM cut-off of 0.36, corresponding to a sensitivity of 0.957, a specificity of 0.857, and an AUC of 0.892 when selecting the sera surrounding the IA diagnosis. This cut-off needs to be confirmed in further larger studies.

The new VIDAS®GM single-sample assay provides a semi-quantitative measurement of GM, a widely used biomarker for which biologists and physicians have developed substantial

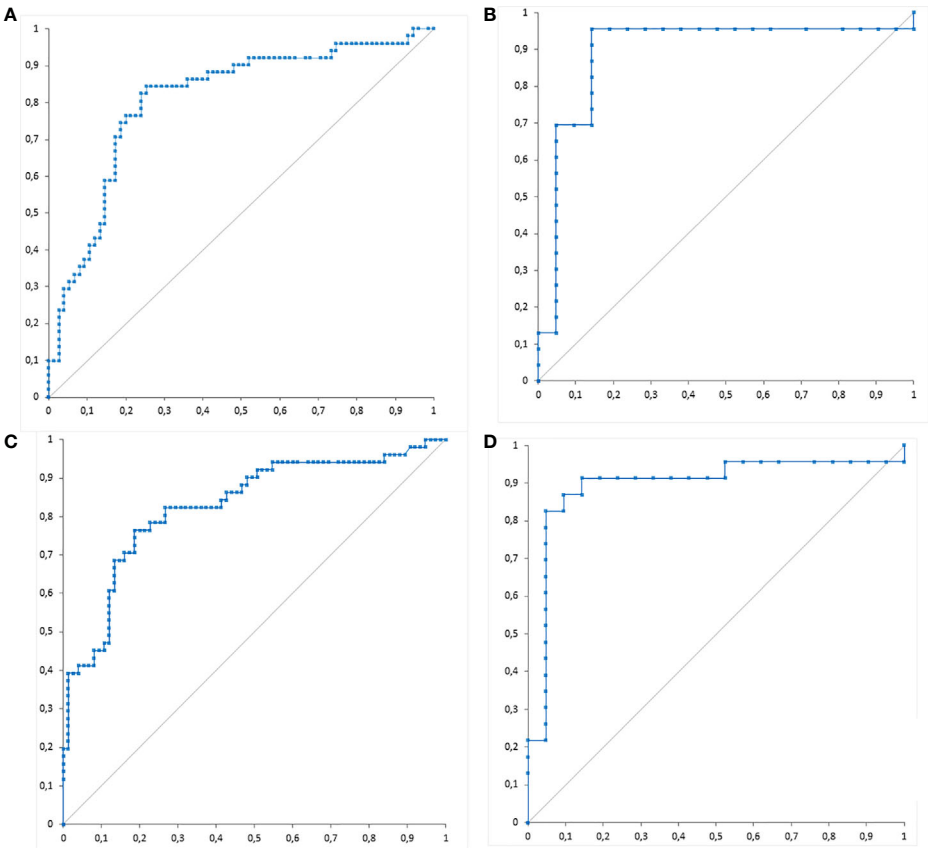

FIGURE 2 | Receiver operating characteristic (ROC) curves of VIDAS®GM for **(A)** total serum samples and **(B)** serum samples collected 15 days before or after the date of invasive aspergillosis diagnosis. ROC curves of Platelia™ *Aspergillus* Ag for **(C)** total serum samples and **(D)** serum samples collected 15 days before or after the date of invasive aspergillosis diagnosis.

TABLE 1 | Clinical performance of the VIDAS®GM and the Platelia™ *Aspergillus* Ag assays using ROC analyses and best Youden index and serum samples of patients at risk for invasive aspergillosis.

	Sensitivity	Specificity	PPV*	NPV*	AUC*	Youden	Cut-off
All sera (n = 126)							
VIDAS®GM	0.843	0.747	0.694	0.875	0.808	0.590	0.36
Platelia™ Aspergillus Ag							
cut-off close to 0.5	0.765	0.813	0.736	0.836	0.827	0.578	0.47
cut-off close to 1	0.471	0.880	0.730	0.710		0.350	1.01
Sera collected 15 days before or after the IA diagnosis (n = 44)							
VIDAS®GM	0.957	0.857	0.880	0.947	0.892	0.814	0.36
Platelia™ Aspergillus Ag							
cut-off close to 0.5	0.870	0.905	0.909	0.864	0.894	0.774	0.55
cut-off close to 1	0.609	0.952	0.930	0.690		0.561	0.97

**PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve.*

expertise in interpreting. During the course of infection and treatment, fluctuations of the level of the VIDAS®GM indexes were consistent with those of Platelia™ *Aspergillus* Ag and the patient's outcome (data not shown). The main benefit of VIDAS®GM is that it is a simple ready-to use system adapted for VIDAS instruments, thus providing rapid results (70 min).

This novel GM single-sample assay showed suitable analytical (stability, repeatability, reproducibility) and clinical performance and good correlation with that of the Platelia™ *Aspergillus* Ag assay. These results need to be confirmed in a larger prospective, multicentric study in which the diagnosis may be defined by composite criteria without GM. Such future studies will allow refinement of the cut-off for sera and the analysis of respiratory samples.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The samples collected in this study are part of routine clinical care and registered in the certified biological collection DC-2008-582. This collection is approved by the ethical committee of the Centre Hospitalier Universitaire of Grenoble. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Risk Factors for Invasive Aspergillosis in Patients Admitted to the Intensive Care Unit With Coronavirus Disease 2019: A Multicenter Retrospective Study

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Background: Invasive pulmonary aspergillosis (IPA) is a life-threatening complication in coronavirus disease 2019 (COVID-19) patients admitted to intensive care units (ICUs), but risk factors for COVID-19-associated IPA (CAPA) have not been fully characterized. The aim of the current study was to identify factors associated with CAPA, and assess long-term mortality.

Methods: A retrospective cohort study of adult COVID-19 patients admitted to ICUs from six hospitals was conducted in Hubei, China. CAPA was diagnosed via composite clinical criteria. Demographic information, clinical variables, and 180-day outcomes after the diagnosis of CAPA were analyzed.

Results: Of 335 critically ill patients with COVID-19, 78 (23.3%) developed CAPA within a median of 20.5 days (range 13.0–42.0 days) after symptom onset. Compared to those without CAPA, CAPA patients were more likely to have thrombocytopenia (50 vs. 19.5%, $p < 0.001$) and secondary bacterial infection prior to being diagnosed with CAPA (15.4 vs. 6.2%, $p = 0.013$), and to receive vasopressors (37.2 vs. 8.6%, $p < 0.001$), higher steroid dosages (53.9 vs. 34.2%, $p = 0.002$), renal replacement therapy (37.2 vs. 13.6%, $p < 0.001$), and invasive mechanical ventilation (57.7 vs. 35.8%, $p < 0.001$). In multivariate analysis incorporating hazard ratios (HRs) and confidence intervals (CIs), thrombocytopenia (HR 1.98, 95% CI 1.16–3.37, $p = 0.012$), vasopressor use (HR 3.57, 95% CI 1.80–7.06, $p < 0.001$), and methylprednisolone use at a daily dose $\geq$ 40 mg (HR 1.69, 95% CI 1.02–2.79, $p =$ 1.02–2.79) before CAPA diagnosis were independently associated with CAPA. Patients with CAPA had longer median ICU stays (17 days vs. 12 days, $p = 0.007$), and higher 180-day mortality (65.4 vs. 33.5%, $p < 0.001$) than those without CAPA.

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Conclusions: Thrombocytopenia, vasopressor use, and corticosteroid treatment were significantly associated with increased risk of incident IPA in COVID-19 patients admitted to ICUs. The occurrence of CAPA may increase the likelihood of long-term COVID-19 mortality.

Keywords: COVID-19, CAPA, methylprednisolone, mortality, thrombocytopenia

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, and has now been responsible for millions of hospitalizations (1, 2). Most patients recover from COVID-19 but some—mainly critically ill patients—are vulnerable to dangerous secondary infection by opportunistic pathogens (3–5). Among the relevant microbes, *Aspergillus* spp. is considered very serious and is potentially deadly, particularly if it induces invasive pulmonary aspergillosis (IPA) (3, 6, 7).

COVID-19-associated IPA (CAPA) is a life-threatening complication in intensive care unit (ICU) patients, and the reported incidence of CAPA varies from 4 to 35% (4, 5, 8–13). The variation in estimates may be partly due to different diagnostic approaches and definitions (3, 4, 6). Regardless of the definition used, IPA is reportedly associated with both viral and host factors (3–6). For example, SARS-CoV-2 can induce severe lung or immune damage that impairs the host's ability to clear the *Aspergillus* spp. Some critical patients have been immunocompromised due to diabetes and chronic kidney disease (14), which are associated with an increased risk of developing IPA (4, 14). Some drugs used to treat COVID-19 including steroids may also render patients more vulnerable to *Aspergillus* spp. infection (15). Notably however, few studies have focused on these host and infection variables in an effort to facilitate early stratification of COVID-19 patients at high risk of developing CAPA.

The current study was conducted to determine the incidence of CAPA in COVID-19 patients admitted to ICUs, and investigate risk factors associated with CAPA occurrence and long-term outcomes.

METHODS

Study Design and Participants

This retrospective study was conducted at six ICUs of Union Hospital, Union Hospital West Campus, Jinyintan Hospital, Wuhan Pulmonary Hospital, Xiangyang Central Hospital, and Taihe Hospital in China. Patients aged $\geq$ 18 years were screened if they had a confirmed diagnosis of SARS-CoV-2 infection between 29 December 2019 and 01 April 2020. The ICU admission criteria and treatment decisions for all patients were as previously described (16, 17). Patients were tested for invasive fungal infections if they were suspected of having IPA or there was evidence of clinical disease progression while they were in the ICU (12). All patients with one or more mycological tests of serum or bronchoalveolar lavage (BAL), bronchial aspiration (BA), galactomannan culture, or *Aspergillus* spp. PCR were analyzed as potential cases.

CAPA was diagnosed if *Aspergillus* spp. was cultured from the patient's BAL, or if their serum galactomannan index was $\geq$0.5, or if two of the following three conditions were met in accordance with recent CAPA studies (6, 11, 12): Positive *Aspergillus* spp. culture of BA, BAL galactomannan index $\geq$ 1.0, and positive BAL *Aspergillus fumigatus* qPCR. In patients without CAPA during hospitalization, the day of IPA diagnosis was identified as the day on which the first diagnostic microbiological test after ICU admission was performed. In all six hospitals galactomannan index testing was performed using the Bio-Rad enzyme linked immunosorbent assay (ELISA) test. Cases in which the only positive mycological evidence for IPA was a culture from sputum or tracheal aspirate from the lower respiratory tract were deemed to represent colonization (18).

Patients were excluded if they had a history of IPA and exhibited pulmonary aspergillosis. Research approval was granted by the institutional review board of Wuhan Union Hospital, as the central coordinating center (approval number 2020-0041-1). The requirement for written for informed consent was waived.

Data Collection

Patient identification in the ICUs was achieved by reviewing admission logs from available medical records as previously described (2, 7, 19). Data were extracted from local servers by experienced research physicians at each center. Patients' demographic data, preexisting comorbidities, vital signs at ICU admission, laboratory values at the time of CAPA diagnosis, galactomannan antigenemia data, microbiology findings, and data pertaining to secondary bacterial infection, complications, and known IPA risk factors including immunosuppressive drugs (steroids and others) were analyzed. With respect to discharged patients, phone calls were made by 15 January 2021 to determine their living status. Mortality at 180 days after the diagnosis of CAPA was analyzed.

Definitions

Secondary pulmonary bacterial infection was diagnosed if a patient returned a positive culture of a new pathogen from a lower respiratory tract pathology specimen (bacterial pneumonia) acquired $\geq$48 h after ICU admission (2). Neutropenia was defined as an absolute neutrophil count of $<0.5 \times 10^9$/L within the 3 days before the diagnosis of IPA. European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses

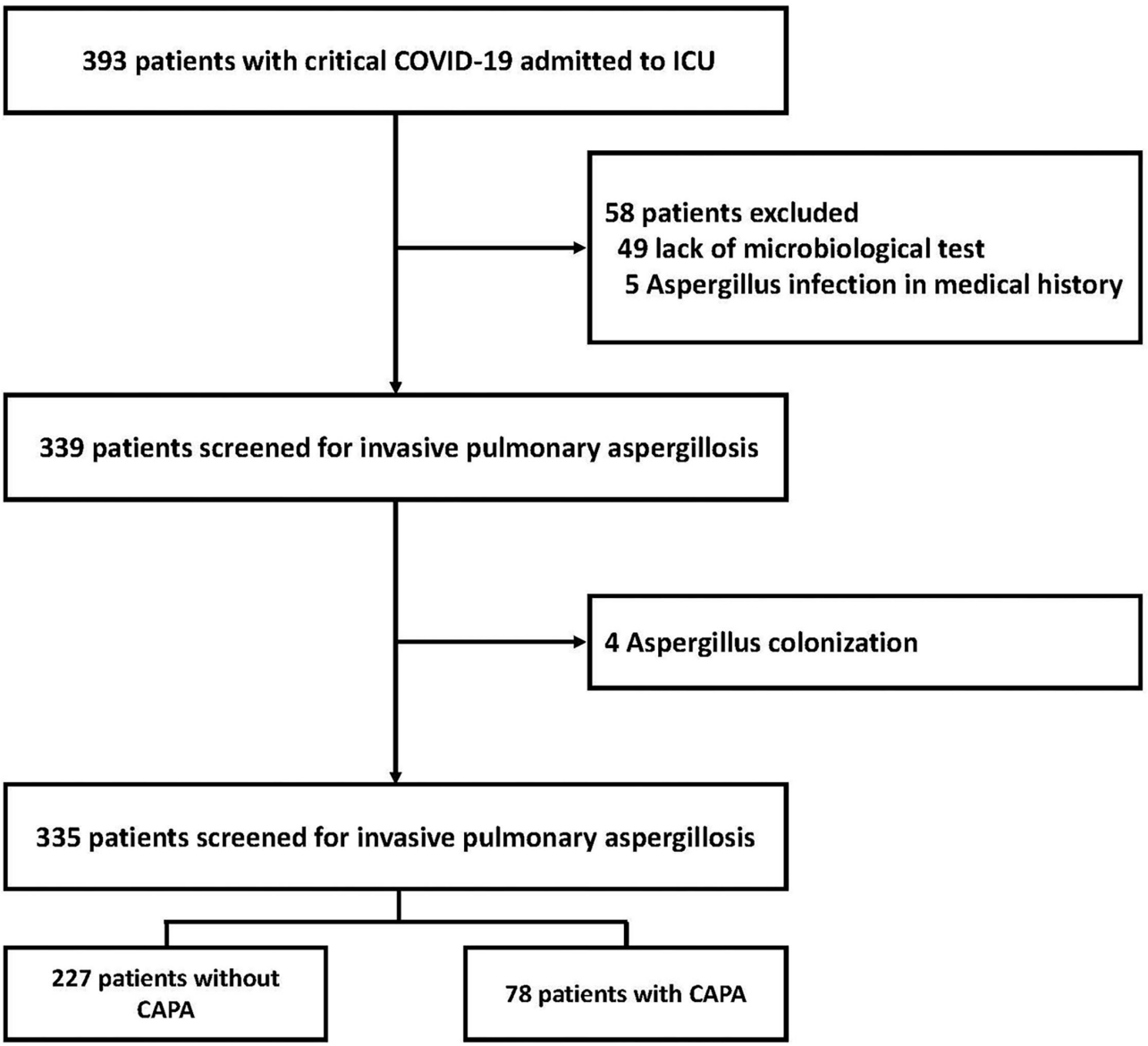

FIGURE 1 | Flowchart of the study including patients with CAPA. CAPA, coronavirus disease 2019-associated invasive pulmonary aspergillosis; COVID-19, coronavirus disease 2019.

Study Group (EORTC/MSG)-host factors were identified as previously described (18). Thrombocytopenia is defined as platelets less than 125×10^9/L (2, 19).

Statistical Analysis

Due to the exploratory nature of the study as many eligible cases as possible were included. Values were expressed as means ± the standard deviation, medians and interquartile ranges (IQRs), or medians and ranges for continuous variables, and as numbers and percentages for categorical variables. Differences between patients with and without CAPA were investigated using the two-sample *t*-test for parametric variables, the Wilcoxon rank-sum test for non-parametric variables, and Fisher's exact test for categorical variables. To explore independent risk factors for the development of CAPA, age, lymphocyte and platelet counts, treatments, comorbidities, and dichotomous complications associated with a *p*-value of <0.1 in univariate analyses were included in Cox proportional hazards regression analysis. Age was dichotomized at 60 years, lymphocyte counts at the median value, and platelet counts at 125×10^9/L. Survival probabilities 180 days after the diagnosis of CAPA were analyzed *via* Kaplan-Meier curves, and compared using log-rank tests. Tests were two-sided, and $p < 0.05$ was deemed to indicate statistical significance. Stata/IC 15.1 software (StataCorp, College Station, TX, USA) was used for all analyses.

TABLE 1 | Invasive pulmonary aspergillosis characteristics of COVID-19 patients admitted to ICU.

	CAPA *n* = 78(%)
ICU admission to first cultures or GM tests, days[¶]	0 [0-1]
Mycological tests	
Day of first indication after ICU admission	1 [0-3]
Day of first determination after ICU admission	1 [0-9]
Cultures	62 (79.5)
BAL culture positive	36 (46.2)
Aspergillus fumigatus	21 (28.8)
Aspergillus flavus	15 (20.6)
Aspergillus niger	1 (1.4)
Serum GM tests	66 (84.6)
Positive serum GM (index > 0.5)	49 (62.8)
Serum GM value, index, median (IQR)[#]	0.41 [0.12-1.01]
BAL GM	12 (15.4)
Positive BAL GM (index > 1.0)	9 (75.0)
BAL GM value, index, median (IQR)[*]	2.30 [1.25-5.32]
Aspergillus PCR test	15 (19.2)
Positive Aspergillus PCR	3 (20)
Time from illness onset to CAPA diagnosis	20.5 [13.0-42.0]

BAL, bronchoalveolar lavage; COVID-19, coronavirus disease 2019; CAPA, coronavirus-associated invasive pulmonary aspergillosis; GM, galactomannan; ICU, intensive care unit; PCR, polymerase chain reaction; IQR, interquartile range.

[¶]Days of first cultures or GM tests after ICU admission, the tests were available in 78 patients.

[#]Serum GM tests were conducted in 66 patients.

[*]BAL GM value was from the 9 patients with GM index > 1.0.

Data were expressed as count (%) unless otherwise.

RESULTS

Clinical Characteristics and Test Results

A total of 393 COVID-19 patients admitted to ICUs at the six participating centers and screened between 31 December 2019 and 01 April 2020 were initially considered for inclusion in the final analysis. Fifty-eight patients were subsequently excluded; 49 due to unavailability of sufficient information pertaining to microbiological testing while in the ICU, 5 with a medical history of IPA, and 4 with *Aspergillus* spp. colonization. The final analysis set thus included 335 patients with COVID-19 (**Figure 1**). Their mean age was 60.1 ± 14.1 years, 191 (57%) were male, and 25 (7.5%) were EORTC/MSG host-factor positive. The mean acute physiology and chronic evaluation II score was 15 (IQR 12–19). Based on the CAPA definition applied, 78 (23.3%) cases were diagnosed a median 20.5 days after COVID-19 symptom onset (IQR 13.0–42.0 days). BAL culture tests were performed in 62/78 (79.5%) patients with CAPA, 35 (44.9%) of which were positive for *Aspergillus spp*. Serum galactomannan tests were performed in 66/78 (84.6%) CAPA patients, and were positive as determined via an optical density ≥ 0.5 in 49 patients (62.8%) (**Table 1**).

Differences in characteristics, risk factors, and outcome parameters between patients with and without CAPA are shown in **Table 2**. A greater proportion of CAPA patients were aged > 60 years (71.8 vs. 52.5%, $p = 0.002$). There were no significant differences in underlying diseases between patients with and without CAPA, with the exception of hypertension ($p = 0.046$). Compared with patients without CAPA, more patients with CAPA had lymphocyte counts $< 0.8 \times 10^9$/L (76.9 vs. 56.8%, $p = 0.047$) and platelet counts $< 125 \times 10^9$/L (50 vs. 19.5%, $p < 0.001$). Patients with CAPA were more likely to have secondary bacterial infections (15.4 vs. 6.2%, $p = 0.013$).

Compared to those without CAPA, patients with CAPA were more likely to require vasopressors (37.2 vs. 8.6%, $p < 0.001$), mechanical ventilation support (57.7 vs. 35.8%, $p < 0.001$), and renal replacement therapy (37.2 vs. 13.6%, $p < 0.001$). There were no significant differences in mean daily methylprednisolone equivalent dose (61.8 ± 24.6 mg vs. 60.7 ± 22.8 mg, $p > 0.6$) or the duration of methylprednisolone (8 days vs. 7 days, $p > 0.2$) between the two groups. Patients with CAPA were significantly more likely to use a higher daily dose (≥40 mg) of methylprednisolone than those without CAPA (53.9 vs. 34.2%, $p = 0.002$) (**Table 2**).

Risk Factors for CAPA Development

Data pertaining to risk factors for the occurrence of CAPA derived from pooled data from all patients are shown in **Table 3**. Thrombocytopenia (HR 1.98, 95% CI 1.16–3.37, $p = 0.012$), vasopressor use (HR 3.57, 95% CI 1.80–7.06, $p < 0.001$), and the use of methylprednisolone at a daily dose of ≥40 mg (HR 1.69, 95% CI 1.02–2.79, $p = 1.02–2.79$) before CAPA diagnosis were independently associated with an increased risk of developing CAPA. Conversely, EORTC/MSG host factor, a lymphocyte count lower than the median of all patients included in the analysis, renal replacement therapy, and secondary pulmonary bacterial infection were not.

Clinical Outcomes

All patients returned negative CAPA test results before they were discharged. Median ICU stays were significantly longer in CAPA patients than in those without CAPA (17 days vs. 12 days, $p = 0.007$), as were median hospital stays (21 days vs. 13 days, $p < 0.001$). ICU mortality was higher in CAPA patients than in those without CAPA (52.6 vs. 28.4%, $p < 0.001$). At 180 days after the initial diagnosis of CAPA no patients tested positive for CAPA and were still being hospitalized, and 51 (65.4%) patients with CAPA had died. Kaplan-Meier curves depicted significantly higher 180-day mortality after aspergillosis diagnosis in CAPA patients compared with patients without CAPA ($p < 0.001$) (**Figure 2**).

DISCUSSION

Early identification of CAPA in critically ill COVID-19 patients admitted to ICUs will facilitate the optimal use of medical resources. The current multicenter retrospective study investigated risk factors associated with CAPA, and long-term outcomes in COVID-19 patients admitted to ICUs. Patients with thrombocytopenia, vasopressor use before CAPA diagnosis, and the use of methylprednisolone at a daily dose of ≥40 mg were much more likely to develop CAPA.

TABLE 2 | Demographic characteristics, underlying diseases, and clinical characteristics of COVID-19 patients with or without CAPA.

	Total *N* = 335 (%)	With CAPA *N* = 78 (%)	Without CAPA *N* = 257 (%)	*P*-value
Demographics				
Age, years, mean (±SD)	60.1 ± 14.1	64.3 ± 13.6	58.8 ± 14.0	0.0024
Age ≥ 60 years	191 (57.0)	56 (71.8)	135 (52.5)	0.002
Male	198 (59.1)	49 (62.8)	149 (57.9)	0.265
APACHE II admission*	15 [12-19]	16 [15-21.5]	15 [12-19]	0.0076
SOFA admission*	5 [4-7]	5 [4-8]	5 [4-6]	0.0325
Underlying diseases				
Any	197 (58.1)	56 (71.8)	141 (54.9)	0.005
Hypertension	129 (38.5)	38(48.7)	91 (35.4)	0.046
Diabetes mellitus	63 (18.1)	16(20.5)	47 (18.3)	0.385
Coronary disease	28 (8.4)	8 (10.3)	20 (7.8)	0.314
Chronic cardiac disease	37 (11.0)	13 (16.7)	24 (9.3)	0.058
Autoimmune disease	3 (0.9)	2 (3.3)	1 (0.4)	0.137
Cerebrovascular disease	37 (11.0)	10 (12.8)	27 (10.5)	0.542
Chronic liver disease	22 (6.6)	5 (6.4)	17 (6.6)	0.593
Liver cirrhosis	3 (0.9)	1 (1.3)	2 (0.8)	0.550
Chronic kidney disease	18 (5.4)	3 (3.9)	15 (5.8)	0.362
Chronic pulmonary disease	14 (4.2)	5 (6.4)	9 (3.5)	0.206
Symptoms at hospital admission				
Fever	276 (82.3)	65 (83.3)	211 (82.1)	0.476
>38°C	21 (6.3)	5 (6.4)	16 (6.2)	0.566
Cough	237 (70.8)	60 (76.9)	177 (68.9)	0.109
Dyspnoea	122 (36.4)	35 (44.9)	87 (33.9)	0.052
Known risk factors				
EORTC/MSG host factor	25 (7.5)	9 (11.5)	16 (6.2)	0.097
Malignancy	16 (4.8)	3 (3.9)	13 (5.1)	0.466
Human immunodeficiency virus	4 (0.9)	2 (2.6)	4 (0.8)	0.137
Immunodeficiency	42 (12.5)	9 (11.5)	33 (12.8)	0.467
Neutropenia ($<0.5 \times 10^9$/L)	1 (0.3)	1 (1.3)	0 (0)	0.233
COPD	14 (4.2)	4 (5.1)	10 (3.9)	0.418
Chronic intermittent hemodialysis	9 (2.7)	2 (2.6)	7 (2.7)	0.649
Studied risk factors				
Methylprednisolone 7 days before ICU	46 (13.7)	11 (14.1)	35 (13.6)	0.522
CS 7 days before fungal	63 (18.8)	19 (24.4)	44 (17.1)	0.104
Corticosteroid duration, d	7 [4-13]	7 [4-14]	7 [4-12]	0.4397
>5	74 (66.7)	27 (69.2)	47 (65.2)	0.419
Laboratory tests at admission				
Leukocyte count, cells/μl	7.6 [5.1-11.9]	8.2 [5.2-12.7]	7.2 [5.1-11.4]	0.2013
Leukocyte count $< 4.0 \times 10^9$/L	33 (9.9)	6 (7.7)	27 (10.5)	0.313
Neutrophils, 10^9/L	6.3 [3.7-10.6]	7.1 [4.2-11.2]	5.9 [3.5-9.9]	0.1150
Neutrophils $< 1.5 \times 10^9$/L	18 (5.4)	5 (6.4)	13 (5.1)	0.412
Lymphocytes, 10^9/L†	0.8 [0.5-1.1]	0.7 [0.5-1.1]	0.8 [0.5-1.1]	0.2279
Lymphocytes $< 0.8 \times 10^9$/L	206 (61.5)	60 (76.9)	146 (56.8)	0.001
Platelets, 10^9/L‡	186 [144-254]	165 [116-219]	193 [150-261]	0.0013
Platelets $< 150 \times 10^9$/L	89 (26.6)	39 (50.0)	50 (19.5)	<0.001
Creatinine, mg/dl	71.2 [57.5-87.3]	71.5 [54.4-103.0]	71.2 [58.4-85.7]	0.5072
C-reactive protein, mg/dl	42 [13.8-116.0]	79.3 [19.7-152.8]	34.0 [9.3-97.9]	0.0185
C-reactive protein> 250 μ/L	170 (50.8)	50 (64.1)	120 (46.7)	0.005
LDH, IU/L	354 [263-487]	398 [294-574.5]	325 [251-464]	0.0063
LDH > 250 μ/L	257 (76.7)	64 (82.1)	193 (75.1)	0.130

(Continued)

TABLE 2 | Continued

	Total *N* = 335 (%)	With CAPA *N* = 78 (%)	Without CAPA *N* = 257 (%)	*P*-value
IL-6, pg/ml	8.9 [6.7-13.4]	10.2 [6.8-16.1]	8.7 [6.6-12.5]	0.0787
Elevated IL-6 level[¶]	251 (74.9)	60 (76.9)	191 (74.3)	0.381
Secondary bacterial infection at time of diagnosis				
Sputum or BAL fluid	28 (8.4)	12 (15.4)	16 (6.2)	0.013
Gram-negative bacteria, *n* (%) Respiratory nonfermenting bacteria[c]	28 (8.4)	12 (15.4)	16 (6.2)	0.013
Pseudomonas spp.	2 (7.4)	1 (1.3)	1 (0.4)	0.412
Gram-positive bacteria, *n* (%)	0 (0)	0 (0)	0 (0)	
Blood	17 (5.1)	6 (7.7)	11 (4.3)	0.179
Hydrothoraxa	4 (1.2)	2 (2.6)	2 (0.8)	0.232
Fungus culture results[⁋]				
Candida sp., *n* (%)	75 (22.4)	15 (19.2)	60 (23.4)	0.275
Mucormucedo sp., *n* (%)	1 (0.3)	1 (1.3)	0 (0.0)	0.233
Treatment before CAPA diagnosis				
Commutative dosages, median [IQR], mg	440 [230–680]	480 [240–740]	430 [180–660]	0.4550
Dose, methylprednisolon equivalent/days, mg	61.1 ± 23.3	61.8 ± 24.6	60.7 ± 22.8	0.6595
≥20	130 (38.8)	42 (53.9)	88 (34.2)	0.002
≥40	27 (8.1)	8 (10.3)	19 (7.4)	0.04
Duration of methylprednisolon, days	7 [4-12]	8 [4-14]	7 [4-11]	0.2094
Tocilizumab	6 (1.8)	3 (3.9)	3 (1.2)	0.141
Thymosin α1	159 (47.5)	37 (47.4)	122 (47.5)	0.550
Organ support at time of diagnosis				
Vasopressors use	51 (15.2)	29 (37.2)	22 (8.6)	<0.001
Mechanical ventilation	142 (42.4)	46 (59.0)	96 (37.4)	0.001
Non-invasive	82 (24.5)	21(26.9)	61 (23.7)	0.332
Non-invasive mechanical ventilation, days	5 [2-8]	8 [2-15]	5 [2-7]	0.2089
Invasive	137 (40.9)	45 (57.7)	92 (35.8)	<0.001
Invasive mechanical ventilation, days	10 [4-20]	15 [8-26]	7 [3-15]	0.002
Renal replacement therapy, *n* (%)	64 (19.1)	29 (37.2)	35 (13.6)	<0.001
Renal replacement therapy days	5 [2-9.5]	6 [2-9]	3 [1-10]	0.3097
Outcome				
Alive at ICU discharge, *n* (%)	221 (66.0)	37 (47.4)	184 (71.6)	<0.001
Length of ICU stay, days	13 [8-20]	17 [10-29]	12 [8-17]	0.007
Length of hospital stay, days	15 [9-24]	21 [15-33]	13 [9-21]	<0.001
ICU mortality	114 (34.0)	41 (52.6)	73 (28.4)	<0.001
180-day mortality	137 (40.9)	51 (65.4)	86 (33.5)	<0.001

APACHE II, Acute Physiology and Chronic Health Evaluation II; CAPA, coronavirus-associated invasive pulmonary aspergillosis; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; HFNC, High-flow nasal cannula; ICU, intensive care unit; IQR, interquartile range; IL 6, interleukin 6; LDH, lactate dehydrogenase; pO_2, partial pressure of oxygen; FiO_2, fraction of inspired oxygen; pCO_2, partial pressure of carbon dioxide; SOFA, sequential organ failure assessment.

Data were expressed median [interquartile range] or as mean ± standard deviation.

**Scores at ICU admission were available in 155 patients, because arterial blood gas analysis was conducted in 103 non-CAPA and 52 CAPA.*

[†] The lower limit of normal range of lymphocyte count was 1.1 × 10^9/L.

[‡] The lower limit of normal range of platelet count was 125 × 10^9/L.

[¶] The upper limit of normal range was 7 pg/ml.

[⁋] The fungus was from the culture of tracheal aspirate or sputum.

[c] Pseudomonas spp., Acinetobacter spp., Stenotrophomonas spp., Burkholderia spp., Escherichia coli.

The reported incidences of CAPA in patients admitted to ICUs vary widely from 2.5 to 39.1%, and in the current cohort it was 23.3% (8, 9, 20, 21). Differences may be associated with different diagnostic approaches or interpretations of IPA markers (3). BAL culture and galactomannan testing of BAL are a gold standard for IPA diagnosis, but BAL and autopsy are usually lacking in patients with COVID-19 due to fear of viral particles spreading. In the present cohort BAL culture was only performed in 62 (79.5%) CAPA patients, and in 48.7% of patients the BAL cultures were *Aspergillus* spp.-positive. Given the limited sensitivity of BAL *Aspergillus* spp. culture (13, 22–24), a serum galactomannan test—which was performed in 316 (94.3%) of

TABLE 3 | Risk factors for the development of CAPA in critically ill patients with COVID-19.

Variables	HR (95% CI)	*P*-value
Age ≥ 60	1.16 (0.67-2.01)	0.596
Underlying disease	1.33 (0.78-2.27)	0.302
EORTC/MSG host factor	1.03 (0.49-2.18)	0.486
Lymphocyte count $< 0.8 \times 10^9$/L†	1.34 (0.74-2.45)	0.337
Thrombocytopenia‡	1.98 (1.16-3.37)	0.012
C-reactive protein	1.40 (0.87-2.27)	0.166
Vasopressors use	3.57 (1.80-7.06)	<0.001
Corticosteroid dosage		
≥20 mg/d	0.34 (0.04-2.56)	0.292
≥40 mg/d	1.69 (1.02-2.79)	0.043
Renal replacement therapy	0.79 (0.37-1.71)	0.552
IMV	0.65 (0.34-1.24)	0.189
Secondary bacterial infection	1.35 (0.68-2.69)	0.392

CAPA, coronavirus-associated invasive pulmonary aspergillosis; COVID-19, coronavirus disease 2019; CI, confidence interval; HR, hazard ratio; IMV, invasive mechanical ventilation.

† The low limit of normal range of lymphocyte count was 1.1×10^9/L.

‡ The low limit of normal range of platelet count was 125×10^9/L.

335 patients including 66 (84.6%) of 78 patients with CAPA—was used to classify patients in the present study. Of the patients with CAPA in the current cohort 61.5% yielded positive serum galactomannan tests.

In a study of 630 patients with influenza in the ICU, invasive aspergillosis was detected in 17%. The slightly lower incidence in an influenza cohort could be explained by the different pathogenesis of SARS-CoV-2 infection (18). In most influenza patients respiratory epithelium damage and mucociliary clearance dysfunction may facilitate invasion by *Aspergillus* spp. Unlike influenza however, in addition to respiratory system damage SARS-CoV-2 sends parts of the immune system into overdrive or depletes certain immune cells, impairing the lung's capacity to clear *Aspergillus* spp. or leaving the patient less able to fight off other infections (3, 6).

Steroids such as dexamethasone (6 mg daily dose) have been shown to curb overactive immune responses and improve survival rates in severely ill COVID-19 patients (25), but steroids are a double-edged sword because they can render the patient susceptible to other infections, particularly *Aspergillus* spp. In the current cohort the use of ≥40 mg of methylprednisolone (equivalent to 7.5 mg of dexamethasone) before CAPA diagnosis was independently associated with the development of invasive pulmonary aspergillosis, consistent with corticosteroid treatment increasing the risks of hyperglycemia and infection in severe COVID-19 (15). In a recent observational study COVID-19 patients received higher than recommended steroid doses and were likely have *Aspergillus* spp. infections (26), therefore IPA infections in critically ill COVID-19 patients should be inversely related to the dosage of immunosuppressive therapy.

Platelets have versatile antifungal immune functions, and thrombocytopenic hosts are highly susceptible to IPA and can rapidly succumb to infection (27–29). To the best of our knowledge no study has investigated the correlation between thrombocytopenia in COVID-19 patients and IPA susceptibility. In the present study thrombocytopenia was an independent risk factor for CAPA, but the underlying mechanisms are not clear. Mechanisms of IPA-related thrombocytopenia have been suggested (27–29). One is that when infected with *Aspergillus* spp. the galactosaminogalactan secreted by the pathogen is deposited on the surfaces of platelets, inducing their activation and resulting in fungal damage (27, 29, 30). Another is that the altered platelet surface triggers a complement cascade reaction that attracts and activates phagocytes to eliminate the invading *Aspergillus* spp. (27, 28). In the current cohort platelets were extremely low in critically ill patients with COVID-19, thus their susceptibility to IPA was increased.

In the present study mortality in patients with CAPA was 65.4% at 180 days after CAPA diagnosis, and the study is the first report on the long-term outcomes of CAPA. The rate is higher than that previously reported in patients admitted to the ICU with influenza-associated IPA at 90 days (51%) (18), and in other cohorts such as aspergillosis patients with phlebovirus (∼40%) (31). The different incidence may be because the pathophysiology of COVID-19 differs substantially from that of influenza and phlebovirus infections. On 04 April 2021 the European Centre for Disease Prevention and Control (CDC) stated that in 186 patients with CAPA the mortality rate was 52.2%, which was similar to the ICU mortality in the current study (52.6%). The study was somewhat cross-sectional in that it included data at 6 and 12 weeks after CAPA diagnosis. The number of patients with CAPA had decreased by the 90-day timepoint because a lot of them had died by that time. In the present cohort, a number of patients with CAPA decreased after 90 days. Mortality was also considerably high in case series from France, Germany, Belgium, and the Netherlands, ranging from 44.5–66.7% (10, 11, 13, 32). Of particular importance was the 100% fatality rate of patients with underlying diseases reported in the Netherlands (10).

The current study had several limitations. Due to its retrospective design confounding cannot be ruled out and a standardized diagnostic approach toward IPA was not used. Considerations of mycological criteria and antigen testing were included in case classifications however, and the galactomannan index was beneficial for the early identification of CAPA patients at risk of a poor outcome. Thus, we believe that the diagnosis of CAPA in the study was more accurate and clinically pertinent. Another potential limitation was that 13 patients with only positive serum galactomannan data were categorized as CAPA patients, and this was likely to have included some false-positive cases. A higher serum galactomannan index represents an increased *Aspergillus* spp. burden however, because severe SARS-CoV-2 infection is likely to contribute to *Aspergillus* spp. micro-angioinvasion at the interface of the alveoli and blood vessels. Indeed, there was no difference in mortality between the galactomannan-positive and the galactomannan-negative groups among the CAPA patients in the study.

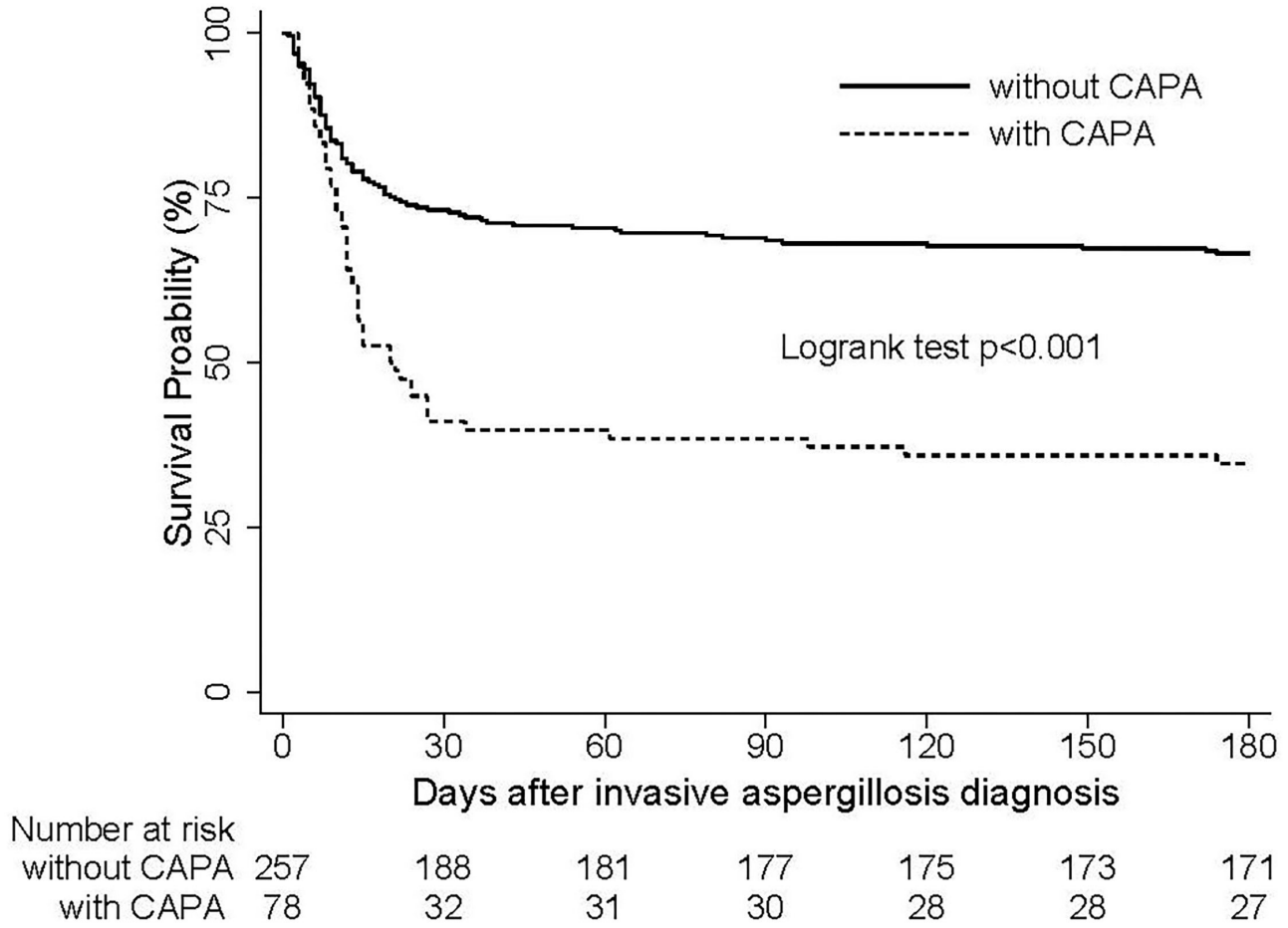

FIGURE 2 | Survival curves of 335 critically ill patients with or without CAPA. The number of patients with CAPA was 78, and the number without CAPA was 257. CAPA, coronavirus disease 2019-associated invasive pulmonary aspergillosis; COVID-19, coronavirus disease 2019.

Whether serum galactomannan should be considered as a standardized diagnostic approach should be a focus of future prospective studies.

CONCLUSION

Thrombocytopenia, vasopressor use before CAPA diagnosis, and the use of methylprednisolone at a daily dose $\geq$ 40 mg are independent predictors of the development of CAPA. The occurrence of CAPA increases the long-term mortality of critically ill COVID-19 patients. The results of the current study may help physicians to optimize the management of critically ill COVID-19 patients, and improve antifungal management.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**. Deidentified participant data will be provided after approval from the corresponding author and study center.

ETHICS STATEMENT

Research approval (2020-0041-1) was granted by the institutional review board of Wuhan Union Hospital as the central coordinating center. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JX, ZL, XZ, LZ, WC, and BL collected the epidemiological and clinical data. JX, XY, and TZ summarized all data. JX, XY, HL, YY, and YS contributed to literature search and writing of the manuscript. YS, CH, SY, and MH designed the study, had full access to all data in the study, and

take full responsibility for accuracy of the analyses and their interpretation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.753659/full#supplementary-material

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Clinical Characteristics and Prognostic Risk Factors of Patients With Proven Invasive Pulmonary Aspergillosis: A Single-Institution Retrospective Study

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Background: The mortality and burden of medical costs associated with invasive pulmonary aspergillosis (IPA) is very high. Currently, the clinical features and prognostic factors of patients with proven IPA are not very clear, especially in the Chinese population. In this retrospective analysis, we aimed to identify the clinical features and prognostic factors of patients with proven IPA.

Methods: The diagnostic criteria for proven IPA were based on the international consensus of the EORTC/MSG. Data of patients with proven IPA at the West China Hospital of Sichuan University between January 2012 and December 2018 were collected. The optimal cut-off value of continuous variables was determined by Receiver Operating Characteristic curve and maximum Youden's index. Finally, using the Cox regression analysis to identify correlations between the clinical parameters associated with morbidity.

Results: A total of 117 patients with proven IPA were included in the study, and 32 (27.4%) patients died during the follow-up period. Compared with the survivor group, elderly, patients with comorbidities, and patients undergoing chemotherapy and the level of inflammatory biomarkers [erythrocyte sedimentation rate, platelet count, interleukin-6, C-reactive protein (CRP)] in the non-survivor group were higher, while the albumin level was lower ($P = 0.018$). The imaging features were consolidation, nodules, cavities, pleural effusion, ground-glass shadows, and halo signs in order. Overall, 41.0% patients had mixed imaging features. The results suggested the most appropriate cut-off value of age and CRP were 60 years and 14.1 mg/L, respectively. The multivariate Cox regression analysis suggested that advanced age (>60 years) [hazard ratio (HR): 10.7, confidence interval (CI): 2.5–44.9, $P < 0.001$), undergoing chemotherapy (HR: 9.5, CI: 2.7–32.9, $P < 0.001$), presence of pleural effusion (HR: 5.74, CI: 1.6–20.8, $P = 0.008$), and increased CRP levels (>14.1 mg/L) (HR: 6.3, CI: 1.2–34.3, $P = 0.033$) were risk factors for all-cause mortality in patients with proven aspergillosis.

Conclusions: This study showed that the prognosis of proven IPA is poor, and the age >60 years, undergoing chemotherapy, pleural effusion on CT image, and CRP levels >14.1 mg/L may be as risk factors for mortality in patients with proven IPA. large samples and real-world studies are needed to confirm these results in the future.

Keywords: clinical characteristics, imaging features, prognosis, risk factor, IPA

INTRODUCTION

Aspergillus spp. is ubiquitous in the environment. The global burden of pulmonary aspergillosis cannot be underestimated. Invasive aspergillosis is the least common, with 0.2 million cases each year. However, it likely represents only 50–60% of actual cases (1). Aspergillus mainly affects immunocompromised individuals, has an extremely high mortality rate between 40 and 90% (2). In addition, Zilberberg et al. showed that the hospital cost of patients with aspergillosis as the principal diagnosis increased from $440 million in 2004 to $590 million in 2013 after adjusting for inflation (3). Despite the increased awareness, increasing number of susceptible individuals and subspecies, and improvement in antemortem diagnosis, China has reported a steady increase in the number of aspergillosis cases for decades (4). In addition, the unaffordability of antifungal treatment, ineffectiveness of antifungal treatment, or failure of patients to adhere to antifungal treatment has resulted in severe outcomes, which have had a huge impact on public health. Nonetheless, epidemiological data on aspergillosis remain scarce in China.

Early diagnosis of pulmonary aspergillosis remains a challenge and should be based on the integration of clinical, microbiological, and radiological data (5). In fact, there is a large proportion of patients with "probable diagnoses" because there are no pathological results. Few studies have focused on the clinical features and prognostic factors of patients with proven aspergillosis. Therefore, in this study, we retrospectively investigated the case data of patients with invasive pulmonary aspergillosis (IPA) confirmed by pathology (proven IPA) at the West China Hospital of Sichuan University to gain a better understanding of its clinical features and risk factors. In addition, this study also provides a reference basis for establishing a prospective multicenter cohort of pulmonary aspergillosis in Western China.

MATERIALS AND METHODS

Patients, Setting and Definitions

This study retrospectively collected the available medical records and data of patients with proven IPA at the West China Hospital, Sichuan University, from January 2012 to December 2018. The diagnostic criteria for IPA were mainly based on the international consensus of the European Organization of Treatment and Research of Cancer and the Mycosis Study Group (EORTC/MSG) (6). Proven IPA identification requires histopathological or cytopathological examination of a specimen obtained by needle aspiration or biopsy in which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage, or positive culture for *Aspergillus* from a sample obtained by sterile procedure from the lung (6).

In this study, all patients underwent lung or bronchoscopy biopsy (including needle aspiration), and the pathological findings (*Aspergillus* infection) were confirmed by two experienced pathologists. All included patients were inpatients at the West China Hospital of Sichuan University, while outpatients and patients who underwent pathological consultations from other hospitals were excluded. Follow-up and prognostic data were obtained *via* telephone interviews or hospital electronic information systems. If a patient's follow-up data were not available or a patient could not be contacted by telephone after more than three attempts, the patient was excluded. All-cause death was defined as the endpoint of this study. The final follow-up of this study was on January 1, 2020.

This retrospective study was approved by the Biomedical Ethics Committee of the West China Hospital of Sichuan University (No. 2021-305). We confirm that all the experiment protocol for involving human data was in accordance to guidelines of national/international/institutional and Declaration of Helsinki in the manuscript. The Biomedical Ethics Committee waived the requirement for informed consent (No. 2021-305), and all original data used in the analyses were anonymized and de-identified.

Data Collection

The history information system (HIS) was queried to identify all patients with proven IPA. Demographic information, clinical parameters, underlying diseases, and medications for aspergillosis were included in the data extracted from the HIS. Laboratory examinations were obtained from the initial results of patients within 3 days after admission, including white blood cell count (WBC), albumin (ALB) count, erythrocyte sedimentation rate (ESR), platelet count, C-reactive protein (CRP) level, glycated hemoglobin, interleukin (IL)-6, galactomannan (GM) test, and pathogen culture. Radiological features were identified in chest computed tomography (CT) images by two experienced radiologists. All included patients were followed-up to obtain the follow-up data.

Abbreviations: IPA, invasive pulmonary aspergillosis; CRP, C-reactive protein; CPA, chronic pulmonary aspergillosis; HIS, history information system; ALB, albumin; ESR, erythrocyte sedimentation rate; IL-6, interleukin-6; PLT, platelet; GM, galactomannan; CT, computed tomography; ROC, receiver operator characteristic curve; COPD, chronic obstructive pulmonary disease; BALF, bronchoalveolar lavage fluid; HR, hazard ratio; CI, confidence interval.

Statistical Analysis

Continuous variables with normal distribution are expressed as mean ± standard deviation, and the *t*-test was used for comparisons between two groups. Continuous variables with non-normal distribution are described as median with interquartile (25th percentile, 75th percentile), and the Mann-Whitney U-test was used for comparisons among groups. Frequency and percentage were used to describe categorical variables, which were analyzed using the chi-square test or Fisher's exact test. The optimal cut-off value of continuous variables and their sensitivity and specificity were determined by Receiver Operating Characteristic (ROC) curve and maximum Youden's index. Statistical significance was set at $p < 0.05$, and all statistical analyses were two-sided. Kaplan–Meier survival curves and Cox regression analysis were used to identify the risk factors associated with all-cause death in patients with aspergillosis, and significance was determined using the log-rank test. Statistical analysis was performed using the SPSS software (version 21.0; IBM Corp., Armonk, NY, USA).

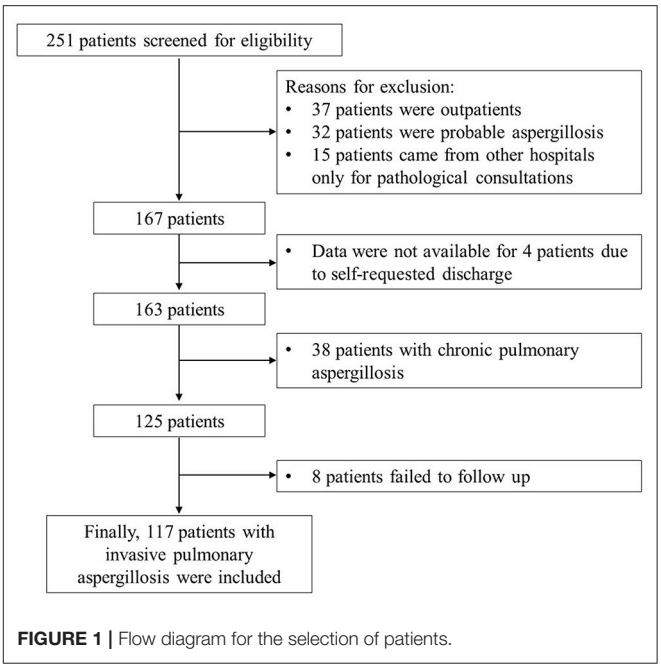

FIGURE 1 | Flow diagram for the selection of patients.

RESULTS

Study Population

All 125 patients with proven IPA who met the inclusion criteria (6) were enrolled, and eight patients were lost to follow-up. Finally, 117 patients were included in this retrospective analysis (**Figure 1**). Among them, 74 (63.2%) patients were males, the average age was 52.6 ± 12.9 years. There were 85 (72.6%) survivors and 32 (27.4%) non-survivors. The most common underlying comorbidities were diabetes mellitus (25.6%), malignancy (20.5%), bronchiectasis (17.1%), and chronic obstructive pulmonary disease (COPD) (12.0%). It is worth noting that one patient was HIV positive, one patient underwent lung organ transplantation, and 39 (27.4%) patients had two or more comorbidities. Additionally, 16 patients (13.7%) had a history of tuberculosis, and 15 patients (12.8%) underwent chemotherapy. In terms of etiology, a total of 31 patients (26.5%) were positive for *Aspergillosis spp.* of sputum or bronchoalveolar lavage fluid (BALF) culture, of which two patients were combined with mucor.

Difference in the Clinical Characteristics Between the Survival and Non-survival Groups

The clinical characteristics of the survival and non-survival (overall) groups are summarized in **Table 1**. Patients with proven aspergillosis who died were more likely to be older ($P < 0.001$). Symptoms such as dyspnea ($P = 0.007$), comorbidities such as COPD ($P = 0.002$), and malignancy ($P < 0.001$) were more common in the non-survival group. There were more all-cause deaths were associated with two or more comorbidities ($P = 0.020$). Moreover, patients who did not undergo chemotherapy had a higher chance of survival ($P < 0.001$). In terms of laboratory test results, patients with lower WBC ($P = 0.004$), higher ALB ($P = 0.018$), lower ESR ($P = 0.007$), lower platelet (PLT) ($P = 0.037$), lower IL-6 ($P = 0.018$), and lower CRP ($P = 0.003$) were likely to survive.

Difference in the Radiologic Characteristics Between the Survival and Non-survival Groups

The most common abnormalities were consolidation ($n = 71$, 60.7%), nodules ($n = 59$, 50.4%), cavity ($n = 46$, 39.3%), pleural effusion ($n = 31$, 26.5%), ground-glass opacity ($n = 20$, 17.1%), and the halo sign ($n = 14$, 12.0%). Overall, 48 (41.0%) patients had mixed imaging features. For all-cause death analysis, on comparison of the different imaging features, survivors were less likely to present with consolidation ($P = 0.006$) and pleural effusion ($P = 0.004$). The differences in other imaging features between the groups are shown in **Table 1**.

Difference in the Treatment Drugs Between the Survival and Non-survival Groups

The treatment strategy is mainly treatment with voriconazole alone ($n = 94$, 80.3%), followed by amphotericin B ($n = 11$, 9.4%), Echinocandins ($n = 6$, 5.1%), Posaconazole ($n = 4$, 3.4%), and Itraconazole ($n = 2$, 1.7%). The number of patients treated with two drugs was small ($n = 2$, 1.7%). As shown in **Table 2**, for overall cause death analysis, there was no significant difference between the survival and non-survival groups, regardless of whether they were treated with a single-agent or combination therapy.

Determination of Optimal Cut-Off Value of Continuous Variables

In this study, the ROC analyses and maximum Youden's index were used to determine the most appropriate cut-off value

TABLE 1 | Baseline and clinical characteristics of all patients with proven IPA.

Parameters	Survivors ($n = 85$)	Non-survivors ($n = 32$)	*P*-value
Male/Female, *n*	49/36	25/7	0.053
Age, years (mean ± SD)	49.1 ± 12.2	61.8 ± 10.1	<0.001
Chemotherapy, *n* (%)	2 (2.4)	13 (40.6)	<0.001
History of tuberculosis, *n* (%)	13 (15.3)	3 (9.4)	0.552
Symptoms, *n* (%)			
Cough	64 (75.3)	30 (93.8)	0.035
Fever	30 (35.3)	9 (28.1)	0.516
Hemoptysis	46 (54.1)	16 (50.0)	0.681
Dyspnea	10 (11.8)	11 (34.4)	0.007
Chest congestion	15 (17.6)	8 (25.0)	0.438
Others	15 (17.6)	2 (6.3)	0.225
Comorbidity, *n* (%)			
COPD	4 (4.7)	10 (31.3)	<0.001
Diabetes mellitus	21 (24.7)	9 (28.1)	0.813
Bronchiectasis	18 (21.2)	2 (6.3)	0.060
Malignancy	7 (8.2)	17 (53.1)	<0.001
Two or more diseases	18 (21.2)	14 (43.8)	0.020
WBC (median (interquartile), $\times 10^9$ cells/L)	6.4 (4.6–9.6)	8.2 (6.8–11.8)	0.004
ALB (mean ± SD, g/L)	36.8 ± 7.7	33.1 ± 6.4	0.018
ESR (mean ± SD, mm/h)	50.9 ± 32.1	73.9 ± 37.1	0.007
PLT (mean ± SD, $\times 10^9$ cells/L)	208.6 ± 102.8	254.8 ± 106.4	0.037
CRP (median (interquartile), mg/L)	14.1 (4.0–78.7)	86.3 (18.6–157.5)	0.003
IL-6 (median (interquartile), pg/ml)	17.5 (6.8–43.0)	113.4 (10.4–196.2)	0.018
Glycated hemoglobin (mean ± SD)	8.3 ± 2.5	8.0 ± 2.2	0.785
GM test positive, *n* (%)	13 (15.3)	4 (12.5)	1.0
Imaging site, *n* (%)			
Left lobe	62 (72.9)	17 (53.1)	0.049
Right lobe	22 (25.9)	15 (46.9)	0.044
More than three lesions	34 (40.0)	15 (46.9)	0.534
Imaging features, *n* (%)			
Consolidation	45 (52.9)	26 (81.3)	0.006
Cavity	36 (42.4)	10 (31.3)	0.297
Halo sign	9 (10.6)	5 (15.6)	0.525
Nodule	42 (49.4)	17 (53.1)	0.837
Ground-glass opacity	15 (17.6)	5 (15.6)	1.0
Pleural effusion	16 (18.8)	15 (46.9)	0.004
Mixed	33 (38.8)	15 (46.9)	0.528
Antibiotic use, *n* (%)	29 (34.1)	16 (50.0)	0.138
Etiology positive, *n* (%)	22 (25.9)	9 (28.1)	0.817

IPA, invasive pulmonary aspergillosis; SD, standard deviation; COPD, chronic obstructive pulmonary disease; WBC, white blood cell; ALB, albumin; ESR, erythrocyte sedimentation rate; PLT, platelet; CRP, C-reactive protein; IL-6, interleukin-6; GM, galactomannan.

of continuous variables for overall survival in patients with proven IPA. The results suggested the most appropriate cut-off value of age, WBC, ALB, ESR, PLT, IL-6, and CRP were 60 years, 6.6 $\times$ 10^9 cells/L, 38.8 g/L, 86.0 mm/h, 190.0 $\times$ 10^9 cells/L, 44.6 pg/ml, 14.1 mg/L, respectively. According to the cut-off value, we converted these continuous variables into dichotomous variables, and used them in the subsequent Cox regression analysis.

Correlations Between All-Cause Death and Parameters of Patients With Proven IPA

The median follow-up time was 1,559 days, and the all-cause mortality over the follow-up period was 27.4% in patients with proven IPA. For all-cause death analysis, in the multivariate Cox regression analysis (**Table 3**), when the cut-off value of age was set at 60 years and the CRP count cut-off was 14.1 mg/L, age [hazard ratio (HR): 10.7, confidence interval (CI): 2.5–44.9, $P < 0.001$),

TABLE 2 | Difference in the treatment drugs between the survival and non-survival groups.

Drugs (*n*, %)	Survivors (*n* = 85)	Non-survivors (*n* = 32)	*P*-value
Voriconazole	70 (82.4)	24 (75.0)	0.278
Amphotericin B	8 (9.4)	3 (9.4)	1.0
Echinocandins	3 (3.5)	3 (9.4)	0.343
Posaconazole	3 (3.5)	1 (3.1)	1.0
Itraconazole	1 (1.1)	1 (3.1)	0.481
Combination therapy	2 (2.4)	0 (0)	0.560

TABLE 3 | Multivariate cox regression analysis of all-cause death over the entire follow-up period in patients with proven IPA.

Parameters	HR (95% CI)	*P*-value
Age (>60 years)	10.67 (2.54–44.90)	<0.001
Chemotherapy	9.46 (2.72–32.94)	<0.001
Consolidation	1.23 (0.15–9.81)	0.846
Pleural effusion	5.74 (1.59–20.78)	0.008
Albumin (<38.75)	1.03 (0.18–5.93)	0.973
CRP (>14.05)	6.31 (1.16–34.33)	0.033
ESR (>86.0)	1.19 (0.34–4.16)	0.785
Two or more comorbidity	0.91(0.22–3.77)	0.897

IPA, invasive pulmonary aspergillosis; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HR, hazard ratio; CI, confidence interval.

undergoing chemotherapy (HR: 9.5, CI: 2.7–32.9, $P < 0.001$), pleural effusion on CT image (HR: 5.74, CI: 1.6–20.8, $P = 0.008$), and CRP count (HR: 6.3, CI: 1.2–34.3, $P = 0.033$) were found to be independent risk factors for mortality. Additionally, it cannot be proven that consolidation on the CT image, albumin level, ESR level, or having two or more comorbidities is correlated with survival. Kaplan–Meier curves of overall patient survival according to age category, chemotherapy status, presence of pleural effusion, and CRP levels are shown in **Figure 2**.

DISCUSSION

In this single-center study, 117 patients with proven IPA were included. The main research results are as follows: first, 27.4% patients died during the follow-up period, and comorbidities were more common among the patients who died. The all-cause mortality of patients with proven IPA in this study is similar to that reported in previous studies involving Chinese patients (7, 8); second, chest CT showed that pulmonary consolidation and pleural effusion were more common in the non-survival group; third, advanced age (>60 years), increased CRP levels (>14.1 mg/L), undergoing chemotherapy, and presence of pleural effusion were risk factors for all-cause mortality in patients with proven IPA.

In this study, the most common underlying comorbidities were diabetes mellitus, malignancy, bronchiectasis, and COPD. Uncontrolled diabetes patients are usually considered to be immunocompromised because hyperglycemia may lead to impaired neutrophil function, antioxidant system, and humoral immune function (9). Several previous studies have shown that the incidence and mortality of pulmonary aspergillosis are significantly increased in immunocompromised patients (10, 11). Aspergillus can colonize and grow in the injured area of the lung in patients with bronchiectasis, and fungal infection can also lead to bronchiectasis. Aspergillosis is common not only in severely immunosuppressed hosts, but also in patients with structural lung diseases, including bronchiectasis and COPD (12, 13). In addition, studies have shown that diabetes and bronchiectasis are risk factors for the prognosis of aspergillosis (14, 15).

We found that the levels of inflammatory biomarkers (WBC, CRP, IL-6, and ESR) were significantly increased in the non-survivor group, and the multivariate Cox regression analysis found that the elevated CRP level (>14.1 mg/L) was closely correlated with poor prognosis of patients with proven aspergillosis (HR: 6.3, CI: 1.2–34.3, $P = 0.033$), which was similar to the results of a previous study (16). Chai et al. found that the continuously elevated levels of IL-6, IL-8, and CRP may be an early predictor of the adverse outcome of invasive aspergillosis, which can be used for the early identification of patients with poor response to antifungal therapy, and may help in the optimization of antimicrobial therapy (16). Pang et al. found that the CRP level was closely correlated with the prognosis of aspergillosis after the secondary analysis of two multicenter randomized cohorts. However, the study found that an increase in PLT may be positively correlated with the survival of patients with aspergillosis (17). However, our study found that although the number of PLT in the non-survival group was higher than that in the survival group at admission, they were all within the normal reference range.

Advanced age and chemotherapy are correlated with the impairment of immune function. Therefore, they are recognized risk factors for infectious diseases, including aspergillosis (7, 11, 18–20). In this study, we also found that advanced age and chemotherapy increased the overall mortality of patients with proven IPA (HR: 10.7, CI: 2.5–44.9, $P < 0.001$; HR: 9.5, CI: 2.7–32.9, $P < 0.001$; respectively). It is worth noting that 24 patients with malignant tumors were included in this study; however, only two patients with hematological malignancies were included. It is well known that hematologic malignancies and stem cell transplantation are important risk factors for IPA (21). The main reason for the inclusion of a small number of patients with hematological malignancies in our study may be that many of these patients cannot undergo puncture or biopsy, and usually rely on clinical data and etiological examination results; therefore, these severe patients were likely diagnosed with hematological malignancies and excluded from this study. In addition, in this study, it was found that the positive rate of pathogenic culture in patients with proven IPA was relatively low (26.5%). In combination with all the above findings, we recommend that when patients have a high mortality risk and can tolerate biopsy, pathological diagnosis should be made as early as possible to identify the disease, so as to avoid underestimating the disease and leading to increased mortality.

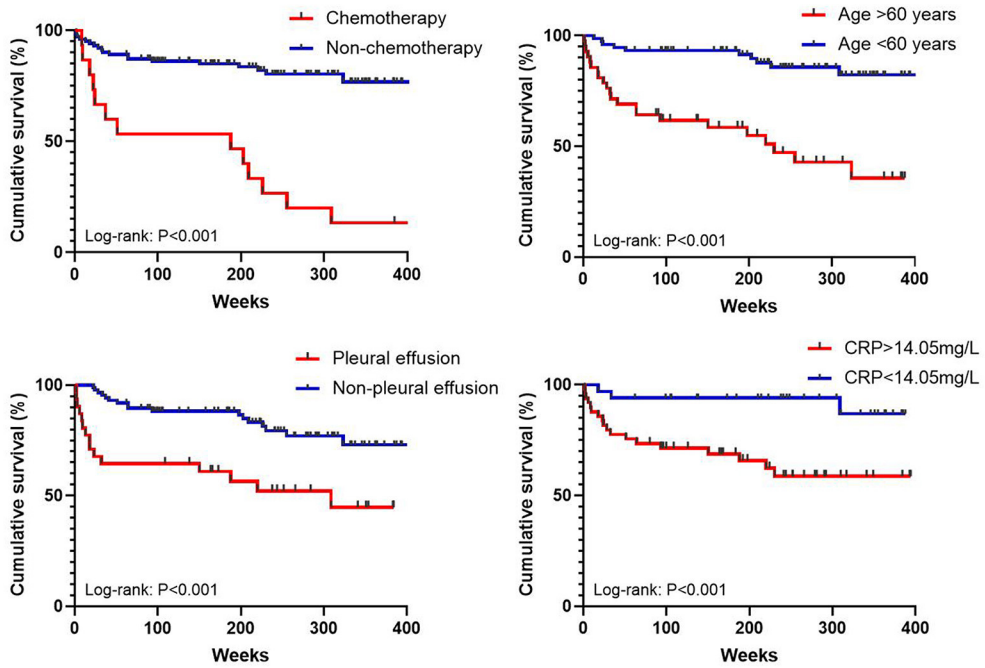

FIGURE 2 | Kaplan–Meier curves of overall patient survival according to age category, chemotherapy status, presence of pleural effusion, and CRP levels.

In this study, we found that there was no difference in the distribution of lung lesions between the two groups; however, consolidation and pleural effusion were more common in the non-survival group. Interestingly, our results suggest that pleural effusion could increase the overall mortality of patients with proven IPA, which is consistent with previous reports (22, 23). Nivoix et al. suggested that the proportion of invasive aspergillosis patients with pleural effusion was as high as 47.8%, while the proportion of aspergillosis patients with pleural effusion in our study was 26.5%. However, another study reported that the proportion of patients with invasive aspergillosis and pleural effusion was only 2.2% (24). Although we were unable to confirm whether Aspergillus was present in the pleural effusion, our results and those of previous studies suggest that pleural effusion increased the overall mortality of patients with IPA, which may be due to the more severe inflammatory reaction and disease condition in IPA patients with pleural effusion.

This study has several limitations. First, the GM assay is broadly accepted as a valuable tool for the diagnosis of patients with IPA (25, 26), including patients in the ICU (27). Additionally, the GM samples from the BALF were more than GM samples from the serum (28). However, the BALF-GM test was officially launched in our hospital in 2016; therefore, some patients did not have the BALF-GM test results. There may be bias in the results value of the serum and BALF-GM tests in patients with proven IPA. Second, as the nature limited value of retrospective studies on long-term prognosis, it is difficult to make further hierarchical analysis on some possible influencing factors, which may have a certain impact on the real results. Third, it is difficult to further analyze the cause of aspergillosis-specific death in detail; therefore, all-cause mortality was used in this study.

CONCLUSIONS

In summary, our study suggests that advanced age (>60 years) (HR: 10.7, $P < 0.001$), undergoing chemotherapy (HR: 9.5, $P < 0.001$), presence of pleural effusion (HR: 5.74, $P = 0.008$), and elevated CRP levels (>14.1 mg/L) (HR: 6.3, $P = 0.033$) may be risk factors for all-cause mortality in patients with proven IPA. We strongly suggested that pathological diagnosis should be made as early as possible for patients with a high mortality risk and who can tolerate to biopsy. In the future, large samples and real-world studies are needed to verify these results.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Biomedical Ethics Committee of the West China Hospital of Sichuan University. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HF, XT, and YW designed the study, coordinated the study, and directed its implementation. XT, TL, KJ, DW, and SL collected data and conducted the follow-up work. XT, TL, and KJ wrote the manuscript. All the authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Metagenomic Next-Generation Sequencing of Cerebrospinal Fluid for the Diagnosis of Cerebral Aspergillosis

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Purpose: Cerebral aspergillosis (CA) is a rare but often fatal, difficult-to-diagnose, opportunistic infection. The utility of metagenomic next-generation sequencing (mNGS) for diagnosis of CA is unclear. We evaluated the usefulness of mNGS of the cerebrospinal fluid (CSF) for the diagnosis of CA.

Methods: This prospective study involved seven consecutive patients with confirmed CA in whom CSF mNGS was performed. Serum (1→3)-β-D-glucan and galactomannan levels were determined, and histopathological examination and mNGS of the CSF were conducted. CSF specimens from three non-infected patients were used as positive controls.

Results: mNGS of the CSF was positive in six of the seven confirmed CA cases (85.71% sensitivity). In the cryptococcal meningitis group (control), mNGS of the CSF was positive for *Aspergillus* in two patients (84.62% specificity). The positive likelihood ratio, negative likelihood ratio, and Youden's index of mNGS for CA in the CSF were 5.565, 0.169, and 0.7, respectively. Among the six mNGS-positive cases, more than two *Aspergillus* species were found in four (4/6, 66.67%). In the positive controls, the addition of one *A. fumigatus* spore yielded a standardised species-specific read number (SDSSRN) of 25.45 by mNGS; the detection rate would be 0.98 if SDSSRN was 2.

Conclusion: mNGS facilitates the diagnosis of CA and may reduce the need for cerebral biopsy in patients with suspected CA.

Trial Registration Number: Chinese Clinical Trial Registry, ChiCTR1800020442.

Keywords: cerebral aspergillosis, metagenomic next-generation sequencing, pathogens, cerebrospinal fluid, diagnosis

INTRODUCTION

Cerebral aspergillosis (CA) is a rare and life-threatening opportunistic infection caused by *Aspergillus* species. This notorious complication of invasive aspergillosis, which accounts for 5–10% of all intracranial fungal pathologies (Ellenbogen et al., 2016), is associated with a >90% mortality rate (Walsh et al., 2008). The major risk factors for invasive aspergillosis include bone marrow transplant (32%), haematological malignancy (29%), solid organ transplant (9%), pulmonary diseases (9%), and acquired immunodeficiency syndrome (8%) (Patterson et al., 2000). CA typically involves haematogenous dissemination from pulmonary lesions, iatrogenic inoculation during surgery or spinal anaesthesia, or direct extension from infections of the ear, orbital, or paranasal sinuses (McCarthy and Walsh, 2017; Winterholler et al., 2017). The gold standard for diagnosing CA is histopathological evidence or a positive culture result for a biopsy or cerebrospinal fluid (CSF) specimen (Walsh et al., 2008). However, these methods are time-consuming, laborious, and have variable sensitivity and specificity (McCarthy and Walsh, 2017). Therefore, confirmation of CA is problematic, and the misdiagnosis rate is high (Wang et al., 2017).

Metagenomic next-generation sequencing (mNGS) enables diagnosis of infectious diseases of the central nervous system (CNS) (Ramachandran and Wilson, 2020) and is increasingly being used in the clinic (Wilson et al., 2014, 2019; Guan et al., 2016; Fan et al., 2018; Wang et al., 2019). mNGS of the CSF can identify pathogens of infectious diseases of the CNS (Xing et al., 2020). However, whether mNGS can detect *Aspergillus*, which is widespread in the environment (Cadena et al., 2016), and assist the diagnosis of CA is unclear. Here, we present seven cases of biopsy-confirmed CA to evaluate the performance of mNGS of CSF for detecting CA.

MATERIALS AND METHODS

Participant Recruitment

We prospectively identified seven consecutive patients with confirmed CA admitted to two teaching hospitals in Beijing, China between November 2016 and September 2019. We recorded the patients' clinical data, including relevant medical history, physical examination findings, routine blood examinations, CSF parameters, and neuroimaging findings. Diagnosis of CA was confirmed by CSF culture or histopathological evidence. Because of the similar risk factors for CA and cryptococcal meningitis (CM), 13 patients with CM confirmed by CSF India ink smear and/or culture were used as controls.

Sterile CSF was collected from three non-infected patients who required lumbar puncture and divided into three 0.6-mL tubes. Next, different concentrations of *Aspergillus* spores (*A. fumigatus* B5233 wild type) were added to sterile CSF for sensitivity testing of mNGS in spiked specimens and as positive controls. The number of *A. fumigatus* spores in the three 0.6-mL amounts of CSF was 50, 250, and 500.

Metagenomic Next-Generation Sequencing of Cerebrospinal Fluid

Cerebrospinal fluid specimens were collected from the CA and CM patients in accordance with standard aseptic procedure and subjected to mNGS within 24 h. Next, CSF samples were subjected to bead beating, DNA extraction, DNA library construction, and sequencing (BGISEQ 50 platform; BGI-Tianjin, Tianjin, China). Nucleic acids extracted from blood of healthy volunteers were mixed with sterile water as negative controls. CSF specimens from three non-infected patients were sequenced as positive controls using the MGI DNBSEQ platform (BGI-Tianjin). The procedure has been described in detail elsewhere (Xing et al., 2018, 2019, 2020).

Interpretation of Metagenomic Next-Generation Sequencing Data

The sequencing data were analysed in terms of species-specific read number (SSRN), genome coverage (%), and depth. *Aspergillus* species with an SSRN ≥ 2 were considered positive. The data interpretation method has been described in detail elsewhere (Xing et al., 2020).

Statistical Analysis

Continuous data were subjected to non-parametric tests. Quantitative variables are expressed as medians (ranges) and qualitative variables as percentages. Data processing was performed using SPSS software (version 23.0; IBM Corp., Armonk, NY, United States). We performed a linear regression of SSRN and the number of spores that transected the origin. Because the total number of reads obtained from mNGS varied among samples, we first standardised the SSRN to obtain the standardised species-specific read number (SDSSRN) for comparison purposes. The standardised ratio (SR) was calculated as the number of total reads × (1-adaptor ratio)/20,000,000 (Huang et al., 2020). Twenty million was the expected number of total reads after the removal of all adaptors. The SDSSRN was calculated as SSRN/SR, and the spores (n = 50, 250, and 500) were mapped to SDSSRNs.

We assessed the probability of *A. fumigatus* detection given an SDSSRN of 2, the former standard for *A. fumigatus* detection. We hypothesised that the probability of mapping a single PMseq read to the *A. fumigatus* genome is a Bernoulli process, as suggested by Ebinger et al., meaning that the read either mapped correctly or incorrectly (Van Borm et al., 2021). Therefore, we expected the detection probability of *A. fumigatus* to follow a binomial mass function with increasing SDSSRN. A binomial distribution function takes into account two parameters: the probability of an event (p; a single read successfully mapped to the genome) and the number of trials (n; the number of SDSSRNs obtained). For a PMseq read, a DNA sequence of 150 bp on average is produced, and 50 bp at one end is used for genome mapping. Hrant et al. suggested that a 50 bp read obtained by shotgun-sequencing gave a 6% probability of mapping to multiple genomes and 0.52% probability of erroneous mapping (Hovhannisyan et al., 2020), while Hajibabeai et al. suggested that a 109 bp mini-barcode DNA sequence could be used to identify a species with

92% accuracy. We estimated the probability, *p*, that a single read mapped correctly to the *A. fumigatus* genome (indicating *A. fumigatus* positivity) was 0.85. Therefore, we defined the detection probability as $[1 - (0.15)^{SDSSRN}]$, where 0.15 is the likelihood that one SDSSRN read was mapped incorrectly.

RESULTS

Patients' Characteristics

Of the seven non-HIV-infected patients identified, three (42.86%) were males. The median age at presentation was 46 (27–80) years. The patients' characteristics are listed in **Table 1**. The major symptoms and neurological signs of the seven cases included paralysis of the cranial nerve (6/7, 85.71%), facial pain or headache (4/7, 57.14%), and weakness of the limbs (3/7, 42.86%). Of the seven CA cases, five (5/7, 71.43%) had a history of underlying conditions, including nasal surgery, mastoiditis, diabetes mellitus, excessive alcohol consumption, and septic shock. Of the 13 cases of confirmed CM, 7 (53.85%) had a history of underlying conditions (**Table 2**).

All of the patients with CA (7/7, 100%) underwent cranial magnetic resonance imaging (MRI) and exhibited space-occupying lesions, including in the paranasal sinuses, cavernous sinus, or skull base in five cases (5/7, 71.43%) and frontal lobe and insular lobe in two cases (2/7, 28.57%) (**Figure 1**). Leptomeningeal enhancement was noted in seven patients (7/7, 100%). Diffusion-weighted imaging (DWI) hyperintensity was found in two patients (2/7, 28.57%), which was considered as CA-related acute cerebral infarction.

Laboratory Results

The serum tumour markers of the seven patients with CA were normal. Six patients underwent serum (1→3)-β-D-glucan (BDG) testing, and all were positive (6/6, 100%). Five patients underwent serum galactomannan (GM) testing, and three (3/5, 60%) were positive (**Table 1**). The CSF laboratory results and histopathological findings of the seven patients are summarised in **Table 3**. Elevated intracranial pressure (≥200 mmH_20) was found in six patients (6/7, 85.71%). The CSF white blood cell count ranged from 0×10^6 to $530 \times 10^{6/}$L (median = $18 \times 10^{6/}$L), the CSF glucose level from 2.4 to 5 mmol/L (median = 3.2 mmol/L), and the CSF protein level from 0.2 to 1.238 g/L (median = 0.878 g/L). All fungal cultures of the CSF were negative (7/7, 100%), and fungal culture of the brain tissue of case 7 was positive for *A. fumigatus*. Twelve of the 13 cases of CM are described in detail elsewhere (Xing et al., 2019). The seven CA patients underwent craniocerebral biopsy, and the histopathological findings showed granulomatous inflammation or inflammatory cell infiltration and *Aspergillus* hyphae (**Figure 1**). Periodic acid–Schiff (PAS) staining of specimens was positive in six patients (6/7, 85.71%).

Of the seven patients with confirmed CA, six exhibited positive mNGS results, for a sensitivity of 85.71%. Species-specific reads mapped onto *A. flavus* (SSRN 2–8) in three cases. Species-specific reads mapped onto *A. sydowii* (SSRN 11–92) and

TABLE 1 | Clinical features of seven patients with cerebral aspergillosis.

Case no./age (years) /gender	Symptoms and neurological signs	Underlying conditions	MRI findings	Serum BDG (<10 pg/mL)	Serum GM (<0.65 μg/L)
1/40/F	Facial pain, headache, paralysis of cranial nerves (II–VI)	Nasal surgery	Space-occupying lesion of right paranasal sinuses, cavernous sinus, foramina lacerum anterius and temporal lobe, leptomeningeal enhancement	982	ND
2/54/F	Fever (39.5°c) paralysis of cranial nerves (IX, X), limb weakness	DM, mastoiditis	Space-occupying lesion of left tentorium cerebelli, acute cerebral infarction (pons), leptomeningeal enhancement	95.0	0.67
3/46/M	Paralysis of cranial nerves (VI, VII, IX, X), instability of gait, numbness of limbs	Excessive alcohol consumption	Space-occupying lesion of right cerebellum, leptomeningeal enhancement, acute cerebral infarction (right cerebral hemisphere)	108.4	0.937
4/59/F	Headache, ophthalmodynia, proptosis, paralysis of cranial nerves (II–V1)	DM	Space-occupying lesion of left sphenoid sinus and nasopharynx, leptomeningeal and left optic nerve sheath enhancement	27.3	Neg
5/80/F	Headache, paralysis of cranial nerves (II–VI), behavioural change, neck stiffness	Infection of biliary tract and septic shock	Space-occupying lesion of left posterior orbital, cavernous sinus, temporal lobe and anterior skull base, leptomeningeal and optic nerve sheath enhancement	176.3	0.884
6/38/M	Headache, memory impairment, weakness of limbs	Neg	Space-occupying lesion of left frontal and insular lobe, leptomeningeal enhancement	ND	ND
7/27/M	Memory impairment, aphasia, paralysis of cranial nerve (VII), decreased consciousness, weakness of limbs, epilepsy, neck stiffness	Neg	Space-occupying lesion of left frontal lobe, insular lobe and basal ganglia, leptomeningeal enhancement	60.2	<0.25

BDG, (1→3)-β-D-glucan; DM, diabetes mellitus; F, female; GM, galactomannan; M, male; MRI, magnetic resonance imaging; ND, no data; neg, negative.

TABLE 2 | Characteristics of 13 patients with cryptococcal meningitis.

Case no./age (years)/gender	Underlying diseases	India ink staining/CSF culture	mNGS of CSF for *Aspergillus*			
			***Aspergillus* identified**	**SSRN**	**Coverage, %**	**Depth**
1/55/M	DM	+/+	–	ND	ND	ND
2/68/F	Polymyalgia rheumatica, IST	+/+	–	ND	ND	ND
3/60/F	–	+/+	–	ND	ND	ND
4/41/M	–	+/+	–	ND	ND	ND
5/66/F	Membranous nephropathy, IST	+/−	–	ND	ND	ND
6/62/F	SLE, IST	+/+	–	ND	ND	ND
7/56/M	DM, CHB	−/+	–	ND	ND	ND
8/15/M	Years of chronic diarrhoea (aetiology unknown)	+/−	–	ND	ND	ND
9/27/M	–	+/+	–	ND	ND	ND
10/54/F	IgA nephropathy, IST	−/+	–	ND	ND	ND
11/30/M	–	+/+	*A. sydowii*	2	0.0003	1
12/41/M	Renal transplantation, IST	+/+	*A. flavus*	5	0.044%	2.8
13/60/M	–	+/+	–	ND	ND	ND

CHB, chronic hepatitis B; DM, diabetes mellitus; IST, immunosuppressive therapy; SLE, systemic lupus erythematosus; MRI, magnetic resonance imaging; ND, no data.

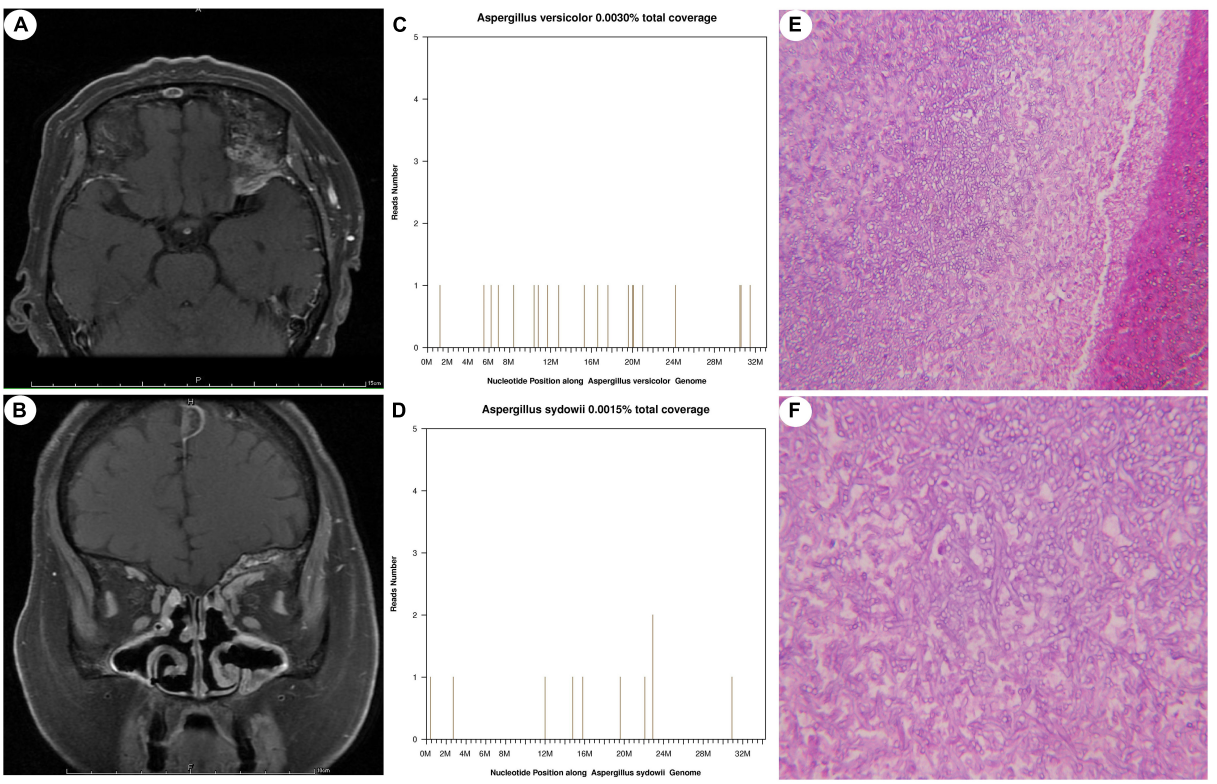

FIGURE 1 | Neuroimaging, mNGS results, and histopathological findings of case 4. T1-weighted MRI with contrast depicting a space-occupying lesion in the left sphenoid sinus and nasopharynx **(A,B)**. The species-specific read numbers of *Aspergillus versicolor* and *Aspergillus sydowii* genomes were 20 and 11, with coverages of 0.0030 and 0.0015%, respectively **(C,D)**. PAS stain demonstrating *Aspergillus* hyphae branching at 45°. Magnification, ×200 **(E)**, ×400 **(F)**.

A. oryzae (SSRN 21–23) in two cases. Among the six mNGS-positive cases, four (4/6, 66.67%) had more than two *Aspergillus* species. In the six cases with CA, the percentage of SSRNs of *Aspergillus* species (i.e., relative species abundance) was 0.90% (7/774), 31.82% (7/22), 90.91% (30/33), 19.35% (18/93), 73.64% (95/129), and 43.75% (28/64), respectively. Of the 13 patients with CM, *Aspergillus* was found in the CSF of two (**Table 2**). The specificity, positive likelihood ratio, negative likelihood ratio, and Youden's index of mNGS for CA in CSF were 84.62%, 5.565, 0.169, and 0.7, respectively.

TABLE 3 | Results of CSF analysis and histopathological findings in seven patients with cerebral aspergillosis.

Case no./age (years)/ gender	Routine laboratory CSF evaluations				mNGS of CSF					Histopathological finding/fungal culture
	Pressure (mmH_2O)	WBC ($\times 10^6$/L)	Glucose (mmol/L)	Protein (g/L)	Time from onset to CSF collection day	Pathogen identified	SSRN	Coverage, %	Depth	
1/40/F	200	0	3.46	0.2	351	*A. fumigatus*	3	0.0043	1	Granulomatous inflammation; *Aspergillus* hyphae, PAS (+)
						A. flavus	2	0.0064	2.4	
						A. nidulans	2	0.0038	1	
2/54/F	330	530	5.0	1.114	243	*A. niger*	7	0.0028	1	Granulomatous inflammation; *Aspergillus* hyphae, PAS (+)
3/46/M	280	110	3.2	1.016	228	*A. oryzae*	21	0.0368	1	Granulomatous inflammation; *Aspergillus* hyphae; PAS (+)
						A. flavus	8	0.0289	1	
4/59/F	242	5	2.4	0.274	183	*A. versicolor*	20	0.0030	1	Granulomatous inflammation; *Aspergillus* hyphae; PAS (+)
						A. sydowii	11	0.0015	1	
5/80/F	75	20	2.6	1.238	272	*A. sydowii*	92	0.0158	1	Inflammatory cell infiltration; *Aspergillus* hyphae; PAS (−)
6/38/M	230	0	3.1	0.572	104	Neg	ND	ND	ND	Inflammatory cell infiltration; *Aspergillus* hyphae; PAS (+)
7/27/M	330	18	3.4	0.878	29	*A. oryzae*	23	0.0279	1	Granulomatous inflammation; *Aspergillus* hyphae; PAS (+); fungal culture of brain tissue (*A. fumigatus*)
						A. flavus	4	0.0262	1.01	

A, Aspergillus; CSF, cerebrospinal fluid; mNGS, metagenomic next-generation sequencing; ND, no data; PAS, periodic acid-Schiff; SSRN, species-specific read number; WBC, white blood cell.

After dividing SSRN by SR, case 1-P (case1-positive control) had SDSSRNs of 7,754, 11,157, and 27,253 for 50, 250, and 500 added spores, respectively. For case 2-P, the corresponding SDSSRNs were 847, 2,794, and 8,403; and for case 3-P, they were 200, 1,421, and 3,874 (**Figure 2**). As no SDSSRN should be detected if no spore is added, a linear regression that minimises the total distance of points from the line was drawn through the origin. The linear model had the function SDSSRN = 25.45 × (number of spores), suggesting that the addition of one spore gave an SDSSRN of 25.45 by mNGS. We investigated the *A. fumigatus* detection probability according to SDSSRN. At a *p*-value of 0.85, the detection rate would be 0.98 for an SDSSRN of 2 (**Figure 3**). Therefore, an SDSSRN of 2 is suitable for *A. fumigatus* detection by mNGS.

DISCUSSION

Most studies of CA are case reports or case series (Ruhnke et al., 2007; Ellenbogen et al., 2016). In this prospective study, we enrolled seven consecutive CA patients confirmed by biopsy, and 13 confirmed CM, to evaluate the utility of mNGS for the diagnosis of CA. mNGS of CSF contributed to the diagnosis of CA. CA may be caused by several *Aspergillus* species. Moreover, although *Aspergillus* may be present at low abundance, it should not be regarded as background contamination because of the high mortality rate.

Under the updated classification, the genus *Aspergillus* contains 446 species (Houbraken et al., 2020), which are distributed worldwide in various habitats (Samson et al., 2014). The most common pathogenic species are *A. fumigatus, A. flavus, A. niger, A. terreus, A. nidulans*, and *A. sydowii* (Lockhart et al., 2011; Chen et al., 2018). The overall incidence of infections caused by *Aspergillus* is increasing (Chen et al., 2018). Aspergillosis is a life-threatening infection uncommon among the immunocompetent (Bao et al., 2014) but common in the immunocompromised (Patterson et al., 2016).

The clinical presentation of patients with CA is variable and non-specific (Kourkoumpetis et al., 2012), and MRI findings can be helpful for the clinical diagnosis but are nonspecific (Ruhnke et al., 2007). (1→3)-β-D-glucan (BDG), a polysaccharide fungal cell wall component and not specific for *aspergillosis*, can be regarded as a panfungal marker (Lass-Flörl, 2019); the positivity rate was 100% in this study. GM, a carbohydrate component of the cell wall of *Aspergillus* and other fungal species (Ray et al., 2019), is a diagnostic marker for invasive aspergillosis (Patterson et al., 2016); the positivity rate was 60% in this study. Furthermore, GM is not specific to *Aspergillus* and can be positive in infections by other fungi, including *Penicillium marneffei*, *Fusarium*, *Alternaria*, *Histoplasma*, and *Blastomyces* (Barton, 2013).

In view of the non-specificity and low sensitivity of fungal antigen tests, accurate aetiological diagnosis is crucial for the management of CA. *Aspergillus* is rarely detected in cultures of CSF from suspected fungal intracranial infection (Hummel et al., 2006; Ray et al., 2019). The sensitivity and specificity of CSF *Aspergillus* PCR were reported as 75 and 98.3%, respectively, in a case series including five confirmed and seven

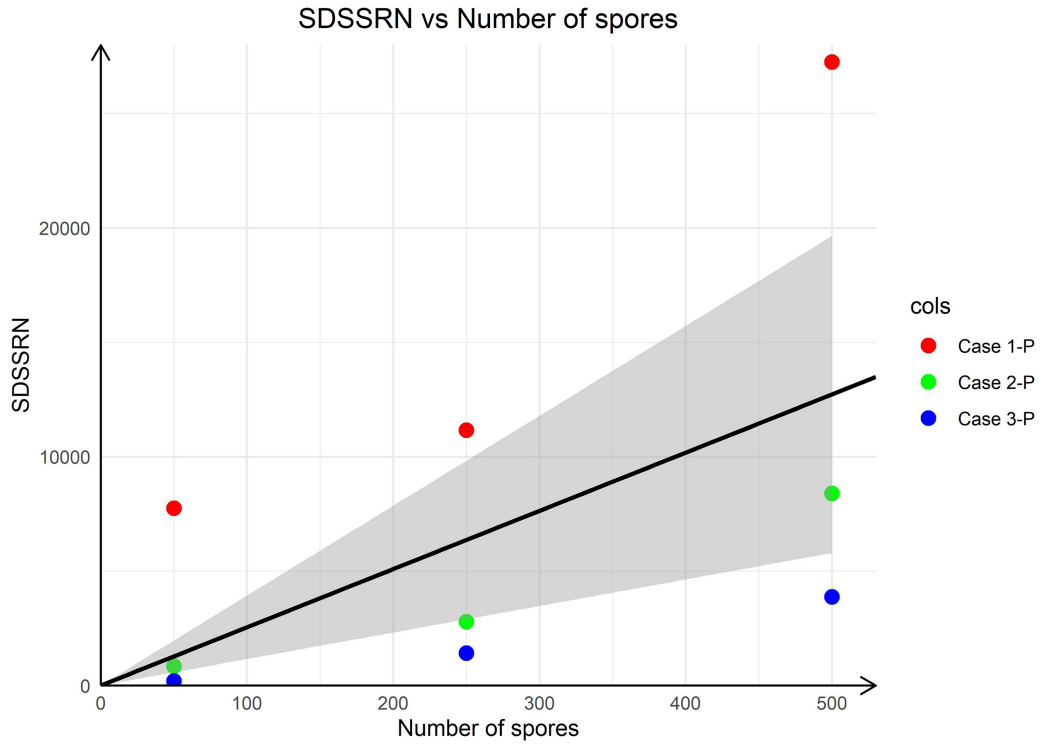

FIGURE 2 | Linear regression of SDSSRN *versus* the number of spores added. The linear regression has the function SDSSRN = 25.45 × (number of spores); the grey area corresponds to the 95% confidence level.

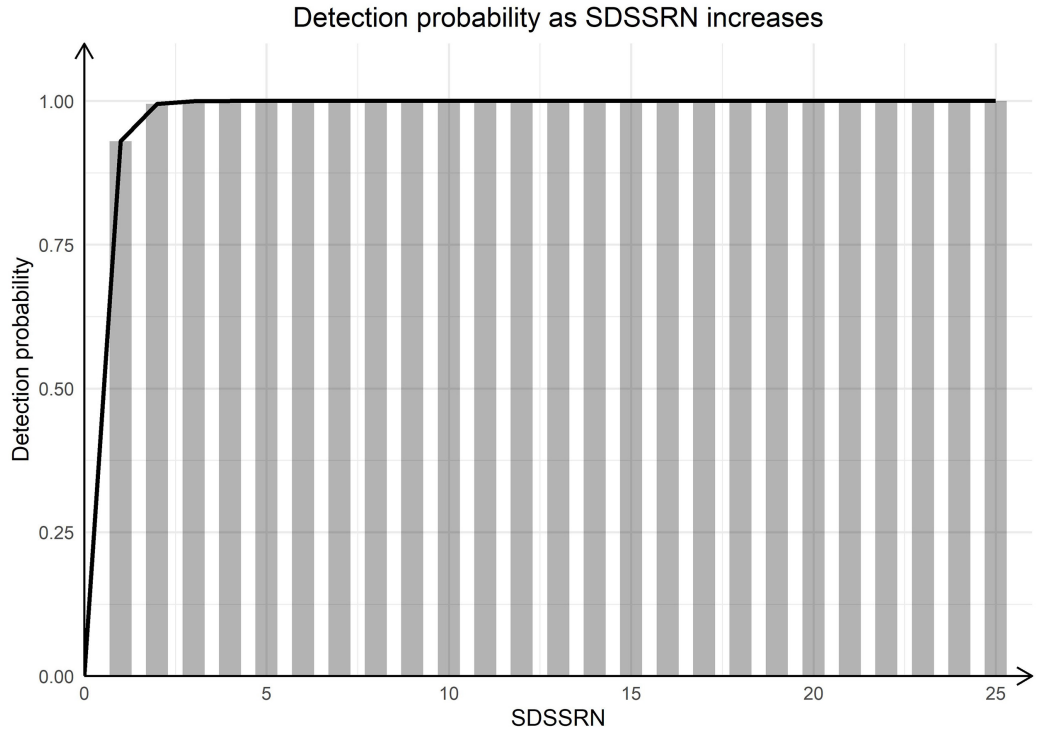

FIGURE 3 | Detection probability with increasing SDSSRN. Bars were calculated as $(1–0.15^{SDSSRN})$.

probable CA cases (Imbert et al., 2017). Nevertheless, *Aspergillus* infection is usually considered only after failure of initial antibiotic treatment for common CNS pathogens (Winterholler et al., 2017). mNGS overcomes these limitations and allows simultaneous and unbiased identification of all microorganisms in human samples (Goldberg et al., 2015).

Our findings show that mNGS enables accurate diagnosis of CA. However, because *Aspergillus* is widely distributed (Samson et al., 2014) and difficult to distinguish from invasive disease, the question arises as to how can we determine that *Aspergillus* detected by mNGS is not a background microorganism. Although CSF is considered aseptic, there may be contaminants, e.g., from skin or laboratory reagents (Ramachandran and Wilson, 2020). Therefore, a strict aseptic and nucleic acid-free standard operating procedure and use of appropriate controls are required for CSF collection and laboratory processing. Also, the clinical significance of detection of *Aspergillus* at low abundance is unclear. Although *Aspergillus* is an opportunistic pathogen, it should not be regarded as background contamination because of the poor outcome of CA. Also, the clinical context is an important matter.

This study involved a relatively large consecutive series of confirmed CA cases. No probable or possible cases were enrolled, enhancing the robustness of the evidence. However, this study had several limitations. First, relatively few patients were enrolled. Second, all *Aspergillus* detected were considered positive mNGS results in the present study. However, whether these opportunistic fungal pathogens are intracranial pathogens is debatable. Third, the utility of an SSRN cut-off value of 2 is unclear. The limited sample size and concentrations of *Aspergillus* spores in the positive controls mean that further study is necessary.

In conclusion, our findings highlight the utility of mNGS of the CSF for non-invasive identification of CA. However, strict aseptic and nucleic acid-free processing and elimination of background contamination are necessary. Pathogen identification should be considered together with the clinical context, such as underlying conditions, symptoms and signs, radiographic evidence, and results of smear, culture, BDG, GM, and other relevant tests.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Chinese PLA General Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

J-TZ designed the study. S-YY organised the experts' meeting. R-ST, Y-BM, R-FW, G-EY, and FC contributed to the acquisition of clinical data. XT and Q-PG conducted the pathological analysis. S-FY performed the mNGS analysis of positive controls. X-WX conducted analyses and wrote the manuscript. All authors read and approved the final manuscript.

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CASE REPORT
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Case Report: Chronic Pulmonary Aspergillosis—An Unusual Long-Term Complication of Lung Cancer Treatment

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Background: Chronic pulmonary aspergillosis (CPA) is a rare complication of radiochemotherapy for lung cancer. It may develop months or years after radical treatment. The diagnosis of CPA is challenging and complex. Not only fungal infection but also cancer relapse always have to be taken under consideration. Antifungal therapy is the base treatment, especially in the case when a surgical procedure is not possible. Standard treatment for at least 6 months is recommended but the optimal duration of the antifungal therapy is unknown. We present the clinical case of CPA, in which we had to perform multidirectional diagnostic tests to confirm the diagnosis and modified treatment due to the recurrence of the disease.

Case Presentation: We report a patient who developed CPA three and a half years after concurrent radiochemotherapy for locally advanced non-small-cell lung cancer. Non-specific symptoms were the cause of delayed diagnosis of fungal infection. Samples collected during bronchoscopy allowed to exclude the recurrence of lung cancer and establish the diagnosis of CPA. The patient was treated with itraconazole for 6 months. A few months later, controlled chest CT scans revealed the progression of CPA. Initially, retreatment with itraconazole was implemented. Due to the progression of fungal infection, voriconazole was used in the second line of treatment. Unfortunately, this therapy was complicated by the side effects and deterioration of the patient's condition. The reintroduction of itraconazole resulted in clinical and radiological improvement. Treatment is scheduled for at least 12 months.

Conclusion: Chronic pulmonary aspergillosis (CPA) was the cause of clinical deterioration and radiological progression in a patient after the radical treatment of lung cancer. In the described case, the diagnosis of CPA was delayed because of the suspicion of the recurrence of lung cancer. As the surgery was not possible, antifungal therapy with itraconazole was implemented and the proper dosage and duration led to significant clinical improvement.

Keywords: lung, cancer, aspergillosis, itraconazole, personalized treatment

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INTRODUCTION

Chronic pulmonary aspergillosis (CPA) is a rare disease (1). It is usually diagnosed in immunocompromised patients with other chronic respiratory disorders (2, 3). CPA risk factors include a history of pulmonary tuberculosis or non-tuberculous infections, lung cancer treated radically with surgery or radiochemotherapy, chronic lung disease, and emphysema (2–4). The diagnosis of CPA is challenging due to non-specific symptoms and it is based on clinical, radiological, and microbiological criteria, and the exclusion of other, more frequent causes of the reported symptoms (5, 6). Accurate diagnosis is crucial for initiating appropriate treatment. Antifungal treatment with itraconazole, voriconazole, and amphotericin B is recommended as a single or combination therapy in selected cases. The optimal duration of therapy is unknown (5). The duration of the treatment should be based on clinical and radiological improvement. In selected cases, surgical treatment is possible (5, 7, 8).

We present a history of a 54-year-old female patient, with adenocarcinoma of the right lung (cT3N2Mo CS IIIA), treated radically with chemoradiotherapy, who developed CPA as a long-term complication after oncological treatment. After the first six-month course of antifungal therapy, the disease recurred. The next course of treatment was complicated by side effects of the drug and affected the patient's quality of life.

CASE DESCRIPTION

A 54-year-old female patient, active smoker (35 pack-years), with a history of pulmonary tuberculosis in adolescence, was admitted to the Department of Lung Diseases and Tuberculosis due to productive cough, weakness, low exercise tolerance, weight loss (about 7 kg). Symptoms exacerbated during last 6 months. She was treated empirically with oral antibiotics in standard doses (amoxicillin, amoxicillin-clavulanate, levofloxacin, and cefuroxime) without clinical improvement. Three and a half years earlier, the patient underwent radiochemotherapy for locally advanced non-small-cell lung cancer of the right upper lobe (adenocarcinoma, the clinical stage of the tumor was T3N2Mo CS IIIA /T—tumor, N—lymph node, M—metastasis/, **Figure 1a**). Platinum-based chemotherapy with etoposide (three cycles) was combined with radical radiotherapy of the right lung tumor area, hilum, and mediastinal lymph nodes (66 Gy in 33 fractions). This treatment was well-tolerated and no early complications were observed. Since then, the patient has been under strict oncological control. Follow-up CT images of the chest and abdomen were taken regularly every 6 months. A cavitary lesion developed at the site of the tumor of the right lung 2 years after the end of lung cancer treatment (**Figure 1b**).

Chest CT scans showed an irregular cavity in the upper part of the right lung with thick walls and accompanying mass of the posterior wall, surrounded by solid consolidation, dilated surrounding bronchus, and emphysema (**Figure 1c**). Imaging diagnostics was extended to PET-CT, which revealed the unclear nature of the hypermetabolic masses in the right lung, more likely inflammation (**Figure 1d**).

Physical examination revealed numerous wheezing and rales during lung auscultation. During bronchoscopy, purulent bronchitis was confirmed (**Figures 2a,b**). Laboratory tests showed increased concentration of C-reactive protein (CRP) (90 mg/dl, normal range <5 mg/dl). Lung function tests revealed a moderate impairment on pulmonary transfer coefficient for carbon monoxide (TLCO; 41% predicted value). No obstruction or restriction was observed.

Microbiological tests (smears, aerobic and anaerobic cultures) of the sputum and bronchial wash were negative for bacteria and fungi. Ziehl-Neelsen staining, cartridge-based nucleic acid amplification test (CBNAAT), and culture of sputum and bronchial wash did not show evidence of Mycobacterial infection. *Aspergillus fumigatus* precipitins were positive in serum. The test was performed using the qualitative Ouchterlony double immunodiffusion method in an agarose gel. The test was developed with its own research methodology and performed in the Department of Biological Health Hazards and Parasitology, Institute of Rural Health, Lublin, Poland. The result was assessed on the basis of the formed precipitation lines, validated with a negative control serum test. Ceftriaxone (2.0 g per day) and levofloxacin (500 mg twice a day) were administered empirically intravenously with a moderate clinical effect. Control bronchoscopy revealed a partial reduction of purulent mucus.

Due to the suspicion of lung cancer recurrence, a transthoracic, CT-guided fine-needle aspiration of the peripheral margin of the right lung cavity was performed. Only macrophages and inflammatory cells such as lymphocytes and neutrophils were observed in aspirated cytological samples. No microbiological tests were performed because of the small amount of the collected sample. Also, forceps biopsy of bronchial tissue collected from the second right bronchus during the bronchoscopy was negative for neoplastic cells. Moreover, no other clinical or radiographic signs of malignancy were observed.

The low value of transfer factor of the lung for carbon monoxide (TLCO) disqualified the patient from surgical resection of the right lung (pneumonectomy).

The patient met diagnostic criteria for CPA and antifungal treatment with itraconazole was initiated (200 mg orally twice a day). The therapy was well-tolerated. Two months later, sputum culture was positive for *A. fumigatus*. A sabouraud dextrose agar (SDA) medium with chloramphenicol and gentamicin was used as a selective medium for the isolation of fungi. The species was determined on the basis of both macro- (colony morphology in SDA) and microscopic morphology (conidiophores, vesicles, metules, phialides, and conidia). Drug susceptibility was determined by the microdilution method (Thermo Scientific). The minimum inhibitory concentration (MIC) for itraconazole was 0.12 μg/ml. Every 8 weeks, the patient was re-evaluated clinically by physical examination and radiologically on the basis of chest CT (**Figures 3a–d**) and x-rays

Abbreviations: CBNAAT, cartridge-based nucleic acid amplification test; CPA, chronic pulmonary aspergillosis; CRP, C-reactive protein; CT, computed tomography; MIC, minimum inhibitory concentration; PCR, polymerase chain reaction; PET-CT, positron emission tomography combined with computed tomography; SDA, Sabouraud dextrose agar; TLCO, transfer coefficient for carbon monoxide.

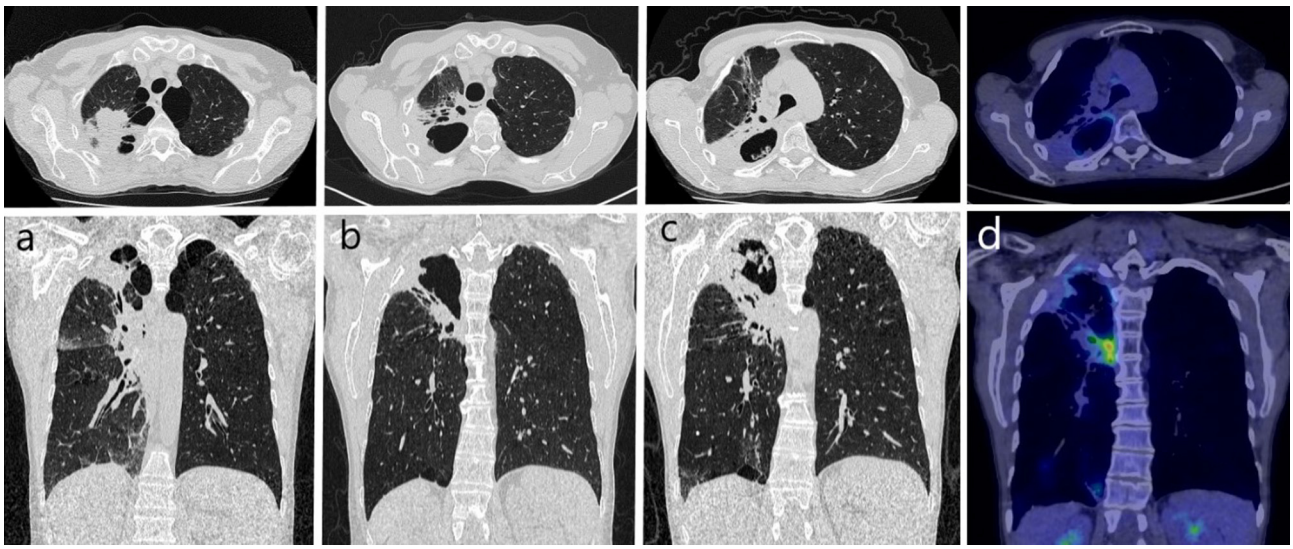

FIGURE 1 | Chest CT in years 2016–2020: **(a)** 2016 - 3 months after chemoradiotherapy: solid tumor in the upper lobe of the right lung, emphysema in the apical parts of both lungs; **(b)** 2018 - cavity with the thick wall has formed at the site of the tumor in the right lung; **(c)** 2020 - progression of an irregular cavity in the upper part of the right lung with thick walls (concomitant mass on the posterior wall), surrounded by solid consolidation, dilated bronchus, and emphysema; **(d)** 2020 - positron emission tomography combined with computed tomography: unclear character of hypermetabolic masses in the right lung, more likely inflammatory, SUVmax = 2.5.

(**Figures 4a–d**). Follow-up bronchoscopy and microbiological tests of bronchial wash were also performed. Four months after initiation of itraconazole treatment, sputum culture was positive for itraconazole sensitive *A. flavus* (MIC = 0.25 μg/ml). Taking into account the well-graded clinical response, radiological improvement, and good tolerability, therapy was continued for up to 6 months.

Six months later, the patient was re-admitted to the Department of Lung Diseases and Tuberculosis with symptoms of increasing weakness, productive cough, night sweats, chest pain on the right side, and weight loss (3 kg). Chest imaging confirmed CPA progression (**Figures 5a**, **6a**). Microbiological tests for fungi and bacteria were negative. A bronchial biopsy ruled out cancer recurrence. Once again antifungal treatment with itraconazole (200 mg orally twice a day) was initiated. After 3 months, antifungal medication was switched to orally administered voriconazole (200 mg twice daily) due to radiographic progression (**Figures 5b**, **6b**). The tolerance of the therapy was poor. Four weeks after starting the voriconazole treatment, the patient reported pain in the chest and right upper abdominal quadrant, further weight loss (a total of 7 kg), and temporary visual disturbances. On bronchoscopy, massive purulent bronchitis and concentric stenosis of the right upper lobe were detected. In the cytological examination of the bronchial wash, fungal hyphae were detected for the first time during the entire diagnostic process (**Figure 2c**). Chest CT images showed further progression. The cavity in the upper right lobe was larger, with a level of thickened, heterogeneous fluid and air in the upper part (**Figures 5c**, **6c**). Physical examination and CT images of the abdominal cavity confirmed the hepatomegaly. The laboratory test revealed elevated transaminases (>5x upper limit of normal), high CRP (90 mg/dl), and anemia (red blood cells−3.4 x 10^6/μl, hemoglobin−10.2g/dl).

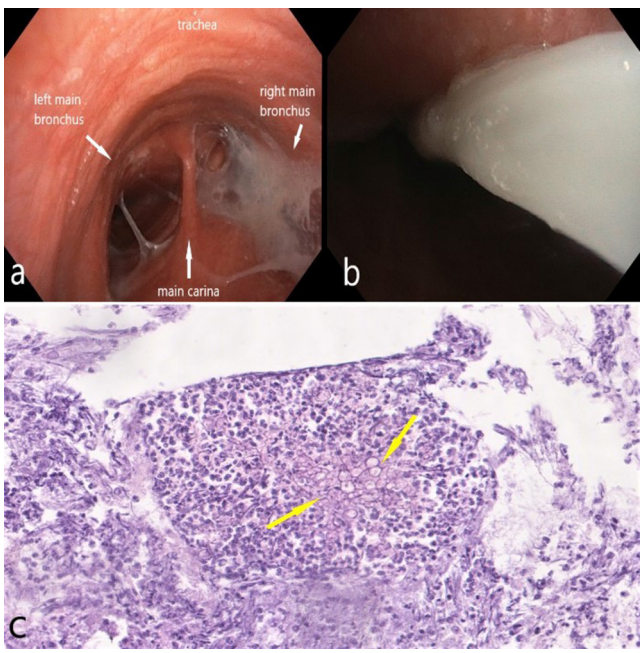

FIGURE 2 | Bronchoscopy: **(a)** main trachea - purulent bronchitis, more severe in the right main bronchus; the bronchial mucosa is dull, red, with dilated vessels; **(b)** thick, purulent mucus in the right main bronchus; **(c)** fungal hyphae (yellow arrows) in the bronchial wash (original magnification × 100).

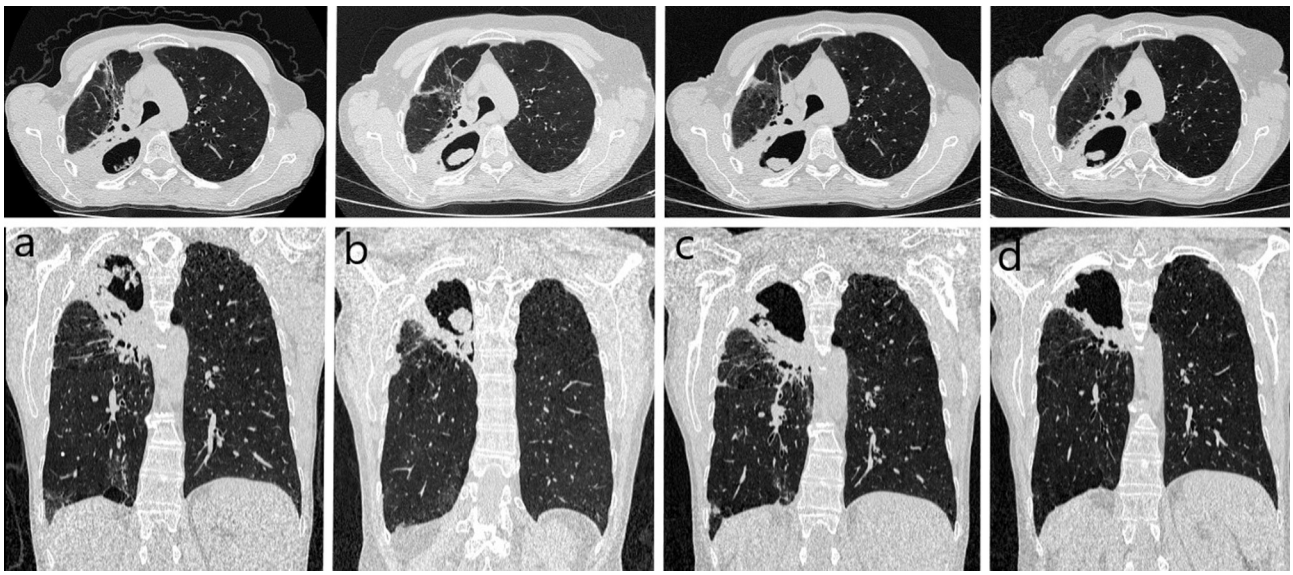

FIGURE 3 | Controlled chest computed tomography during first-line treatment in 2020: **(a)** at the beginning of the therapy – irregular cavity size 102 x 59 mm in apical parts of the right lung, surrounded by solid consolidations widely adhere to the parietal pleura, connecting to the right hilum; **(b)** after 2 months – cavity with thick walls size 71 × 49 mm, concomitant mass on the posterior wall-size 29 x 10 mm; **(c)** after 4 months – cavity with thick walls size 69 × 47 mm, concomitant mass on the posterior wall-size 14 x 5 mm; **(d)** after 6 months – image stabilization.

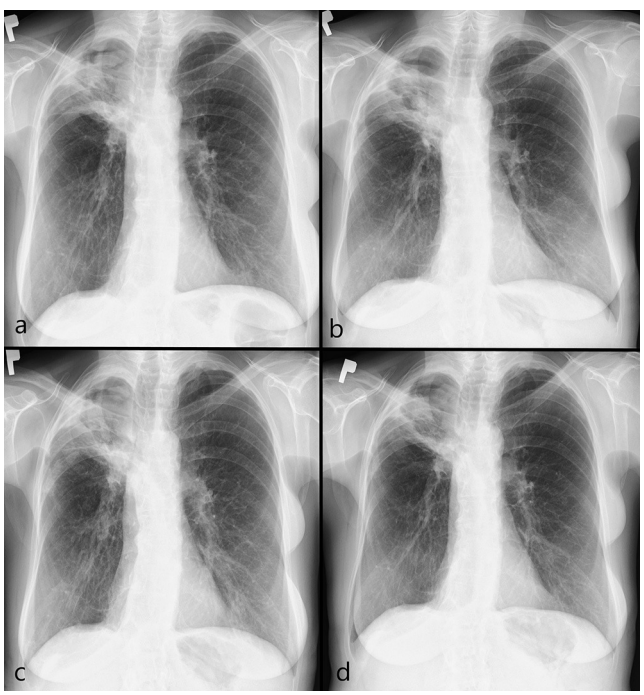

FIGURE 4 | Controlled chest x-ray during first-line treatment in 2020: **(a)** at the beginning of the therapy – thick-walled cavity with internal consolidations in apical parts of the right lung; **(b)** after 2 months – cavity consolidation; **(c)** after 4 months – further cavity consolidation; **(d)** after 6 months – image stabilization.

The antifungal treatment was modified again. Itraconazole (200 mg orally twice a day) bridging therapy was used in anticipation of the unavailability of liposomal amphotericin B as part of emergency access to therapy.

After 1 month of itraconazole treatment, clinical and radiological improvement was achieved. The patient confirmed her well-being, an increase in appetite and body weight (2 kg), and good tolerance of the antifungal therapy. Control imaging showed partial regression (**Figures 5d**, **6d**). It was decided to continue treatment with itraconazole for at least 12 months under close clinical and radiological monitoring. The antifungal treatment is still ongoing. Follow-up visits are scheduled. A timeline with all relevant data from this clinical case is available in **Figure 7**.

DISCUSSION

Lung cancer is the most common cause of death in both men and women (9). The five-year survival rate for all stages of non-small lung cancer together is only around 20% which is rather disappointing (9). Follow-up after radical treatment should be based not only on monitoring the possibility of tumor recurrence but also on identifying possible late infectious complications in potentially immunosuppressed survivors.

CPA is a rare complication of lung cancer treatment. It is observed in patients who have undergone surgical resection or are treated radically with radiochemotherapy (4, 5, 10). The clinical course of CPA is chronic and progressive (5). Our patient developed CPA three and a half years after the

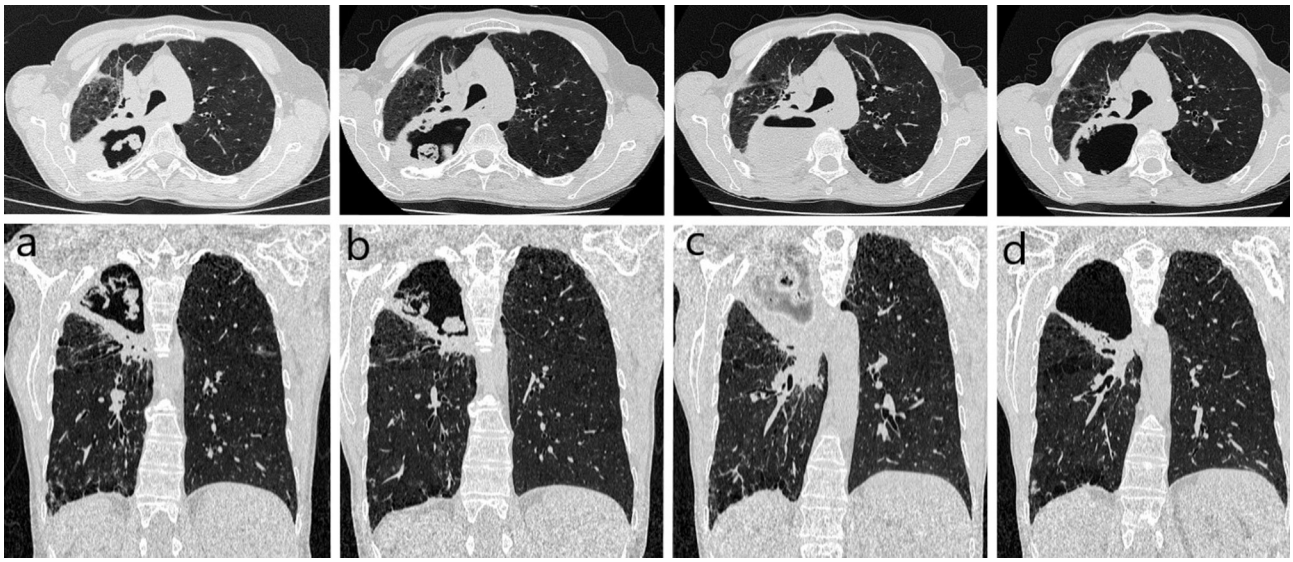

FIGURE 5 | Controlled chest CT during second-line treatment in 2021: **(a)** at the beginning of the therapy – cavity with thick walls size 69 × 47 mm, concomitant mass on the posterior wall-size 22 × 9 mm, quantitative progression of the surrounding consolidations compared to the previous image; **(b)** after 3 months – cavity size 70 × 66 mm, the mass inside larger and more irregular (progression); **(c)** after 4 months – circumscribed opacity with an air-fluid level size 95 x 75 mm in the place of the previously described cavity (further progression, abscess); **(d)** after 5 months – cavity with thick wall much smaller, the mass inside size 28 × 20 mm, no fluid was visualized (regression).

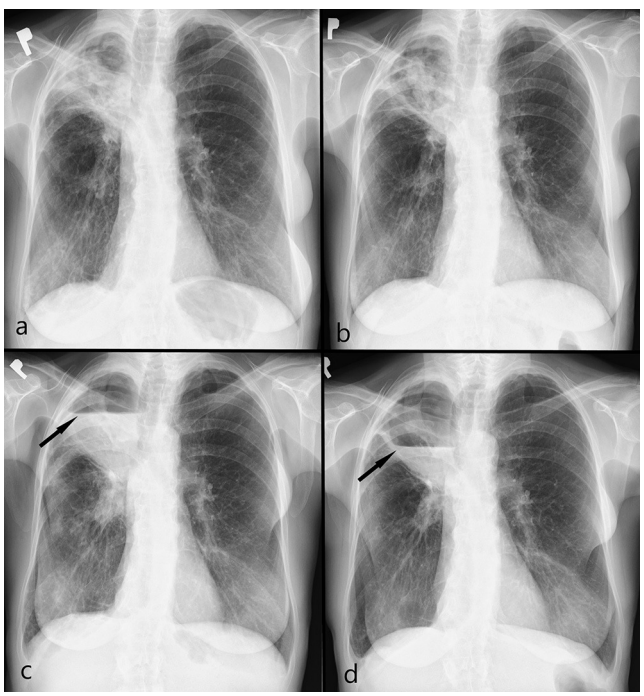

FIGURE 6 | Controlled chest x-ray during second-line treatment in 2021: **(a)** at the beginning of the therapy - a thick-walled cavity in apical parts of the right lung, progression internal consolidations and surrounding opacities; **(b)** after 3 months – cavity consolidation, regression of surrounding opacities; **(c)** after 4 months – cystic lesion with an air-fluid level (arrow) (progression, abscess); **(d)** after 5 months – regression of an air-fluid level (arrow).

completion of oncological treatment. CPA symptoms, such as coughing, weakness, night sweats, and weight loss are non-specific (5, 6). Therefore, they require multidirectional diagnostic methods in the field of imaging, endoscopy, microbiology, immunology, molecular biology, and pathology (5–7). Bronchoscopy, as a basic procedure in pulmonary patients, plays an important role in collecting samples for microbiological and histopathological examination. Cultures of bronchoalveolar lavage and bronchial washing fluid are more sensitive than spontaneously expectorated sputum cultures (5). For the diagnosis of CPA, culture and microscopy are essential. In patients with a high suspicion of CPA but with negative cultures samples, a supporting role is played by the detection of *Aspergillus* serum precipitin, IgG antibodies, and molecular tests such as PCR (5, 6, 11). In our patient, we had no direct evidence of *Aspergillus* infection but we confirm immunological response to *Aspergillus* species (7). The results of these tests brought us closer to the diagnosis of CPA and allowed to start the antifungal therapy.

The radiological features of CPA are the combination of the findings resulting from the underlying lung disease and changes secondary to the ongoing infection (12). CPA most often occurs as one or more pulmonary cavities possibly containing one or more aspergillomas, as well as irregular intraluminal material. Accompanying pleural thickening, surrounding consolidations, and adjacent bronchiectasis have also been reported (5, 7). To confirm the diagnosis, radiological progression confirmed after at least 3 months of follow-up observation is required (5). Our patient's CT and PET-CT images were ambiguous and progressive. They suggested a recurrence of lung cancer or CPA.

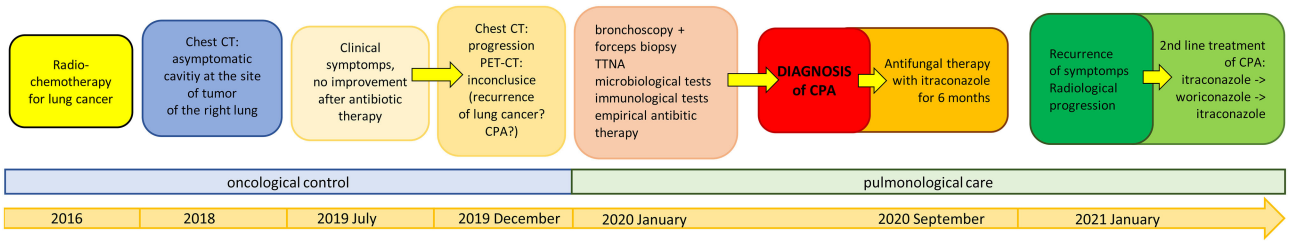

FIGURE 7 | A timeline with all relevant data from this clinical case.

Azoles are the first-line drug for CPA. In the second line of treatment, liposomal amphotericin B or echinocandins are used (5, 7). The optimal duration of the antifungal therapy is unknown. Standard treatment for at least 6 months is recommended (7, 8). The implementation of the therapy results in a slow but gradual clinical recovery and radiological improvement (5). Bongomin et al. suggest that extended therapy may be required to achieve clinical improvement (8). They demonstrated among a cohort of 206 patients with CPA that azoles are modestly effective, especially if given for 12 months. After 6 months of treatment with itraconazole, the described patient had a recurrence of the infection. Therefore, the duration of the second-line treatment is planned for at least 12 months. The use of alternative drugs such as liposomal amphotericin B was limited due to a lack of availability at the time.

Antifungal treatment is often associated with side effects. Common side effects during antifungal treatment are hepatotoxicity, cytopenia, photosensitivity, gastrointestinal symptoms, and allergic reactions (1). Therefore, careful monitoring is crucial for them. The frequency of monitoring should be based on the patient's age, concomitant medications, comorbidities, drug toxicities, and resources. Immediate identification and management of side effects reduce the risk of treatment and possibly improve changes at the end of treatment (11, 13). Despite strict monitoring in the described case, treatment with voriconazole had to be discontinued due to hepatotoxicity.

For symptomatic patients unresponsive to medical therapy, surgical resection of simple aspergilloma is strongly recommended (6). The type of surgery should be based on clinical indications, the extent of the disease, and the results of pulmonary function tests (5). If the estimated postoperative TLCO is <40% of predicted, the risk of morbidity and mortality is quite significant (14). In our case, surgical treatment was not possible due to the high risk of postoperative respiratory failure.

CONCLUSION

Chronic pulmonary aspergillosis (CPA) was the cause of clinical deterioration and radiological progression in a patient after radical treatment of lung cancer. In the described case, the diagnosis of CPA was delayed because of the suspicion of recurrence of lung cancer. As the surgery was not possible, antifungal therapy with itraconazole was implemented and the proper dosage and duration led to significant clinical improvement.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Written informed consent for the publication of identifying images or other personal or clinical details of participant that compromise anonymity was obtained.

AUTHOR CONTRIBUTIONS

KG and RM: analyzed and prepared the data and wrote the manuscript. UB: performed the pathomorphological study. KK: was the leading doctor. All authors read and approved the final version of the manuscript.

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Prospective Evaluation of Positivity Rates of *Aspergillus*-Specific IgG and Quality of Life in HIV-Negative Tuberculosis Patients in Lagos, Nigeria

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Background: Pulmonary tuberculosis (PTB) often results in residual anatomical and functional changes despite microbiological cure and may be complicated by chronic pulmonary aspergillosis (CPA). In this study, we determined the perceived health-related quality of life (HRQoL) of patients during and after PTB therapy and compared it with their quantitative *Aspergillus*-specific IgG positivity rates.

Methodology: We conducted a longitudinal study among TB patients attending two directly observed therapy short-course (DOTS) clinics in Lagos, Nigeria. Two hundred and four confirmed TB patients were recruited over 9 months, with five visits at baseline and 3, 6, 9, and 12 months. They were all acid-fast bacilli smear, GeneXpert, or culture positive for *Mycobacterium tuberculosis*. Two HRQoL questionnaires translated into Yoruba were self-administered. Chest X-ray and *Aspergillus* IgG were collected at each visit.

Results: A total of 204 participants were recruited into this study. Most (70.6%) were age 18–39 years, and only 3.9% were above 60 years; 66.7% of all participants were males. A total of 189 (92.6%) participated in the 3-month assessment, 174 (85.3%) at 6 months, 139 (68.1%) at 9 months, and 99 (48.5%) at 12 months. At baseline, only 60.9% scored "good" or "very good" QoL and health on the WHOQOL-Bref, which improved to 77% at 6 months. At baseline, 10.4% had positive *Aspergillus* IgG levels, 15.1% at 3 months, 11.5% at 6 months, 16.7% at 9 months, and 19.3% at 12 months. Those with a positive *Aspergillus* IgG at 6 months had worse physical health ($p = 0.001$), psychological state ($p = 0.002$), social relationships ($p = 0.006$), and environmental QoL ($p = 0.001$) domains of the WHOQOL-Bref. Probable CPA was 10.4% at baseline and 19.3% at 6 months post-PTB therapy. Thirty-eight (18.6%) relocated after 6 months of treatment, 16 (7.8%) were lost to follow-up, and 11 (5.4%) died.

Conclusion: Our findings reveal a significant relationship between the QoL and *Aspergillus* IgG levels of TB patients. Further follow-up studies and additional imaging are required to determine when patients develop CPA and its clinical impact.

Keywords: *Aspergillus* IgG, tuberculosis, quality of life, LMICs, Nigeria, chronic pulmonary aspergillosis

INTRODUCTION

Globally, tuberculosis (TB) is a major public health problem and remains one of the world's deadliest communicable diseases. The World Health Organization (WHO) launched the "End TB Strategy" aimed at reducing TB incidence by 90% and mortality by 95% by 2030 (Uplekar et al., 2015). Each year, nearly 6 million people are diagnosed with pulmonary tuberculosis (PTB), of whom about 55% have their diagnosis confirmed with laboratory testing (World Health Organization, 2020). The recent WHO global TB report 2020 ranked Nigeria as the first in Africa and sixth globally among the 30 high TB burden countries, with a TB incidence of 219/100,000 population and 120,266 cases notified. Nigeria is on the list of 14 countries with the triple burden of TB, HIV-associated TB, and multidrug-resistant (MDR) TB with a population incidence of MDR-TB of 11/100,000 (World Health Organization, 2020).

Tuberculosis patients face various challenges such as physical restrictions, psychological and emotional issues, and economic and social problems. These known challenges impact on the quality of life of the patients (Hansel et al., 2004; Guo et al., 2009). Clinically, the therapeutic modalities, the side effects of the drugs, and the sequelae of the disease also impact on the quality of life (QoL) of the patients (Hansel et al., 2004; Muniyandi et al., 2007). PTB also results in residual anatomical and functional changes, despite microbiological cure (Pasipanodya et al., 2007). These changes are associated with post-TB lung disease (Pasipanodya et al., 2007). PTB patients often have pulmonary cavities, which can become colonized and infected by inhaled *Aspergillus* spores resulting in chronic pulmonary aspergillosis (CPA) (Denning et al., 2018). Other structural sequelae include fibrosis, chronic obstructive pulmonary disease (COPD) (Denning et al., 2018), and bronchiectasis, as well as sensitization to *Aspergillus* (IgE response), itself linked to worse lung function, independently of CPA (Dhooria et al., 2014). Worsening clinical status can be assumed to be MDR-TB.

TB services and clinical research in Nigeria have focused on the outcomes of mortality and microbiologic cure and have neglected the preferences of the patient, such as the quality of life of the patient, a crucial component of outcome. Health-related quality of life (HRQoL) involves assessing the perceptions of a person of his or her physical and mental health. Both physical and mental distress are common in TB patients leading to decreased compliance with medical care (Liefooghe et al., 1995; Rajeswari et al., 1999). The impact of PTB on HRQoL has been reported in a systematic literature review (Kastien-Hilka et al., 2016). This systematic review found that PTB had a negative impact on the HRQoL and overall wellbeing of the patients, even after PTB cure.

Quality of life assessment tools can be used in both high resource settings and in LMICs. Despite the availability of these standard instruments for assessing health-related quality of life, the feasibility, reliability, and validity of such instruments among TB patients in different populations of sub-Saharan Africa, where the burden of TB is of concern, are still limited.

This longitudinal study aimed to evaluate patient-reported HRQoL in pulmonary TB in Lagos, Nigeria, using two QoL assessment tools, namely, the generic WHOQOL-Bref (The WHOQOL Group, 1998; Skevington et al., 2004) and the specific St. George's Respiratory Questionnaire (SGRQ) (American Thoracic Society (ATS); Welling et al., 2015). The study sought to identify persons with post-TB lung disease following "microbiological cure" 6 months post-therapy and understand the overall impact of TB on the QoL and important domains of qualities of life identified by the WHO.

MATERIALS AND METHODS

Study Design

We conducted a longitudinal study among TB patients attending two directly observed treatment short-course (DOTS) clinics (National Institute of Medical Research and Lagos University Teaching Hospital) in Lagos, Nigeria, from March 2016 to February 2018. Ethical approval for this research was obtained from the HREC of both institutions. Confirmed (smear and/or Xpert (PCR) and/or culture positive for *Mycobacterium tuberculosis*) TB patients were recruited. Patients were excluded if they were diagnosed with multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) and/or had HIV co-infection. Informed written consent was obtained from each study participant. Eligible participants received a 6-month standard TB treatment with rifampicin, isoniazid, ethambutol, and pyrazinamide. Excluded from the study were patients with extrapulmonary TB and patients with any significant associated pulmonary diseases such as lung cancer, chronic obstructive pulmonary disease, and asthma and other chronic diseases likely to affect quality of life including diabetes mellitus and cardiovascular diseases.

Two QoL assessment tools were collected: the WHOQOL-Bref and SGRQ questionnaires. Sociodemographic factors including age, gender, educational status, and work status were captured. Mean transformed QoL scores were calculated from the 26 items of the WHOQOL-Bref, for its four QoL domains (physical, psychological, social, and environment) and for general overall QoL and health (Skevington and Epton, 2018). High scores mean very good QoL. Domain scores are sensitive to change during interventions (Skevington and Epton, 2018).

SGRQ is a disease-specific instrument designed to assess patients with respiratory tract and immune system diseases, especially asthma, pulmonary diseases, and COPD (American Thoracic Society (ATS); Welling et al., 2015). SGRQ comprises 50 items in three domains (symptoms, activity, and impacts on daily life). Both scores are scaled from 0 to 100, with higher scores indicating worse QoL. A minimally important difference (MID) for SGRQ was defined as an improvement of 4 points in the domain scores and the total score (American Thoracic Society (ATS); Welling et al., 2015).

Questionnaires were administered five times altogether, at the start of TB treatment and then at 3-month intervals until 6 months post-therapy (**Supplementary Table S1**). The questionnaires were incorporated into clinical care visits for the convenience of the participants and were self-administered although supervised. Patients completed the questionnaire themselves in a quiet area, free from distraction, but a research nurse was available to give explanations, if required. The questionnaire was designed to elicit the perceptions of the patient of his/her health, not others views, so family, friends, or healthcare staff did not influence the responses of the patient. An accompanying spouse or partner was asked to wait in a separate area. Similarly, patients were not allowed to take the questionnaires home. It was emphasized that they should complete the questionnaire as honestly as possible and that there are no right or wrong answers: simply the answer that best applies to them. They were asked to answer all questions. Since literacy rates in Nigeria are relatively low, translation of the questionnaires into the local dialect Yoruba was available and a nurse was available to read it aloud for those who could not read Yoruba either.

Five milliliters of blood was collected at each visit and serum was separated for *Aspergillus* IgG levels testing using ELISA (Bordier affinity products SA, Switzerland, Bordier® Kit), with a cutoff of 1.0. Sputum was not collected for culture. Chest X-ray (CXR) was done at recruitment, then again at 3, 6, 9, and 12 months. Two independent radiologists, who had no immediate access to the *Aspergillus* IgG results, interpreted the CXR. CT scans were not performed and so CPA was not definitively diagnosed or confirmed. As it was highly likely in many patients, we refer to it as "probable CPA".

Data Analysis

The first analysis assessed measurement (psychometric properties) in the sample to find out whether the WHOQOL-Bref is a valid, reliable measure to use in this population. The second step compared the QoL of people at different intervals after starting treatment for TB. The third step compared those with and without positive *Aspergillus* antibodies, especially those with documented chronic pulmonary aspergillosis. Descriptive statistics were applied to sociodemographic data and furthermore to all HRQoL data at baseline and in all follow-up visits, to understand the HRQoL impairment of TB patients. Descriptive statistics tested the following: frequencies (*N*, *N* missing, %), central tendency (mean, median), and confidence interval (set at 95%). The distribution of data was examined by standard deviation (SD), minimum and maximum values, and frequency plots.

Significance testing (paired-samples *t*-test), multivariable analysis, and repeated measures analysis of variance (ANOVA) were based on observations that contained all data points required for a specific analysis. Overall changes in HRQoL between baseline and 6-month treatment (visit 4) were calculated as frequencies (%). Longitudinal changes were determined by the change in mean scores between all follow-up visits and baseline. The change in mean scores was examined by paired-samples *t*-test, with a statistical significance (two-tailed) set *a priori* at $p < 0.05$. Changes in mean scores in the intensive treatment phase (baseline to visit 2) were compared with changes in the continuous treatment phase (visit 2 to visit 5) based on paired-samples *t*-test with a statistical significance (two-tailed) set *a priori* at $p < 0.05$. The change in mean scores at each time point from baseline was also compared with the reported MID for each measure, to understand if longitudinal changes in HRQoL were clinically meaningful. A paired-samples *t*-test was applied to examine the difference between changes in mean scores and MID at a significance level $p < 0.05$.

Differences in QoL mean scores among all these measures over time (baseline and follow-up visits 1–4) were examined by repeated measures ANOVA with a Bonferroni correction applied to adjust for multiple tests. Responsiveness over time for each HRQoL dimension was measured as an effect size partial eta-squared, providing information of the effect of time on changes in QoL. The resulting candidate factors were further assessed in multivariable models to understand the impact of sociodemographic factors over time [change from baseline to 6 months post-treatment (visit 5)]. The univariate and multivariate analyses included a general linear model and ANOVA.

RESULTS

Sociodemographic Data

A total of 204 participants with confirmed PTB were recruited into this study, of which 144 (70.6%) were aged 18–39 years (**Table 1**). One hundred and thirty-six (66.7%) were men giving a male:female ratio of 2:1. Educational levels varied substantially (from illiteracy to tertiary education levels), with 3.9% having no formal education. About half (49.8%) of the participants were single, and 44.8% were married (**Table 1**).

Overall Quality of Life and General Health (WHOQOL-Bref)

Of the 204 participants, 189 (92.6%) participated in the baseline assessment, 174 (85.3%) at 3 months, 139 (68.1%) at 6 months, 99 (48.5%) at 9 months, and 88 (43.1%) at 12 months. Of the participants, 60.9% had a good or very good perception of their QoL at baseline assessment, 78.7% at 3 months, 77% at 6 months, 79.8% at 9 months, and 87.5% at the 12-month assessment (**Table 2**). In contrast, at baseline, 15.3% described their QoL as very poor or poor, 4.0% at 3 months, 4.4% at 6 months, 1.0 at 9 months, and 1.1%

TABLE 1 | Sociodemographic data of the study participants.

Variable	Frequency	Percentage (%)
Age	*N* = 204	
18–39	144	70.6
40–49	36	17.6
50–59	16	7.8
>60	8	3.9
Gender	*N* = 204	
Male	136	66.7
Female	68	33.3
Educational level	*N* = 203	
None	8	3.9
Primary	33	16.3
Secondary	90	44.3
Tertiary	72	35.5
Marital status	*N* = 203	
Single	101	49.8
Married	91	44.8
Divorced	1	0.5
Widowed	2	1.0
Separated	4	2.0
Living as married	4	2.0

at 12 months (**Table 2**). These response trends were mirrored by general health and overall QoL questions (**Table 2**).

Changes in the Dimensions of HRQOL Measures

The mean scores increased with the length of TB therapy in the domains of the WHOQOL-Bref, with the highest at the 12-month assessment and the lowest during the baseline assessment (**Table 3**). Over the course of 12 months, there was significant improvement in quality of life as measured by both the WHOQOL-Bref and SGRQ. Scores decreased by 9–25 points in the SGRQ, while scores increased by 12–20 points depending on the domain of the WHOQOL-Bref.

Comparison of HRQoL Scores in Those With and Without a Positive *Aspergillus* IgG

Among the participants, 10.4% had a positive *Aspergillus* IgG at baseline assessment, 15.1% at 3 months, 11.5% at 6 months, 16.7% at 9 months, and 19.3% at 12 months (**Figure 1**). There was a statistically significant difference in the mean WHOQOL-Bref domain scores between participants with positive and negative *Aspergillus* IgG at the 6-month assessment, in physical health ($p = 0.001$), psychological state ($p = 0.002$), social relationships ($p = 0.006$), and environmental ($p = 0.001$) quality of life domains (**Table 4**). At 6 months, WHOQOL-Bref scores were significantly higher in patients with positive *Aspergillus* IgG levels compared with those with negative *Aspergillus* IgG levels, in all four domains ($p < 0.05$). At 12 months, QoL scores were higher in those with elevated *Aspergillus* IgG levels, compared with low *Aspergillus* IgG levels in physical, psychological, and environmental QoL domains. Although social QoL scores were lower in probable CPA patients compared with those in non-CPA patients, the difference was not significant ($p > 0.05$). To remove the confounding effect, linear regression was done using Asp IgG levels and total QoL scores, and it was statistically significant (*p*-value 0.029) at 9 months.

Symptoms Measured by the SGRQ

Statistical difference was observed between mean symptoms scores from successive assessments over 12 months, using one-way ANOVA ($F = 15.058$, p-value < 0.001) (**Table 4**). A Tukey *post-hoc* test revealed that the mean baseline score was significantly higher than all the other months of assessment and significantly higher at 3 months than at 12 months ($p < 0.05$) (**Table 5**). A significant difference was also found between mean symptoms, impacts, activity, and overall score and between months of assessment (one-way ANOVA, $p < 0.001$) (**Table 5**).

Comparison of SGRQ in *Aspergillus* IgG-Positive Versus *Aspergillus* IgG-Negative Participants

At 6 months, participants with positive *Aspergillus*-specific IgG levels had lower SGRQ scores indicative of worse health compared with those that had negative *Aspergillus*-specific IgG levels, but this difference was not statistically significant ($p > 0.05$) (**Table 6**). Scores were slightly higher in those with positive *Aspergillus* IgG at 12 months, but this was also not significant ($p > 0.05$).

TABLE 2 | Overall quality of life and general health.

	Very poor (%)	Poor (%)	Neither poor nor good (%)	Good (%)	Very good (%)
How would you rate your quality of life?					
Baseline	6.3	9.0	23.8	42.9	18.0
3 months	0.0	4.0	17.2	60.3	18.4
6 months	2.2	2.2	18.7	48.2	28.8
9 months	0.0	1.0	19.2	50.5	29.3
12 months	1.1	0.0	11.4	55.7	31.8
How satisfied are you with your general health?					
Baseline	12.2	25.4	27.5	26.5	8.5
3 months	1.7	13.3	19.1	50.9	15.0
6 months	2.2	9.4	19.4	51.1	18.0
9 months	3.0	3.0	22.2	46.5	25.3
12 months	4.5	3.4	11.4	50.0	30.7

TABLE 3 | Mean scores for different domains of the HRQoL.

	Responders (*N*)	WHOQOL-Bref (mean ± SD)	SGRQ (mean ± SD)
Physical health			
Baseline	189	51.43 ± 18.90	37.89 ± 26.05
3 months	174	67.06 ± 16.40	25.86 ± 23.49
6 months	139	69.95 ± 18.81	18.33 ± 20.57
9 months	99	73.84 ± 18.18	16.93 ± 20.00
12 months	88	73.63 ± 18.23	16.83 ± 23.12
Psychological health			
Baseline	189	59.79 ± 18.29	37.89 ± 26.05
3 months	173	68.00 ± 14.58	26.03 ± 23.47
6 months	139	71.85 ± 15.00	18.33 ± 20.57
9 months	99	75.04 ± 14.11	16.93 ± 20.00
12 months	88	72.65 ± 15.76	16.83 ± 23.12
Social relationships			
Baseline	189	58.32 ± 22.24	37.89 ± 26.05
3 months	173	68.76 ± 19.98	25.98 ± 23.53
6 months	139	71.35 ± 20.91	18.33 ± 20.57
9 months	98	73.60 ± 19.89	16.93 ± 20.00
12 months	87	73.77 ± 20.74	17.18 ± 23.23
Environment			
Baseline	189	57.57 ± 16.84	37.89 ± 26.05
3 months	174	64.90 ± 14.66	25.86 ± 23.49
6 months	139	68.14 ± 15.35	18.33 ± 20.57
9 months	99	70.98 ± 14.03	16.93 ± 20.00
12 months	87	71.75 ± 14.73	15.72 ± 22.05

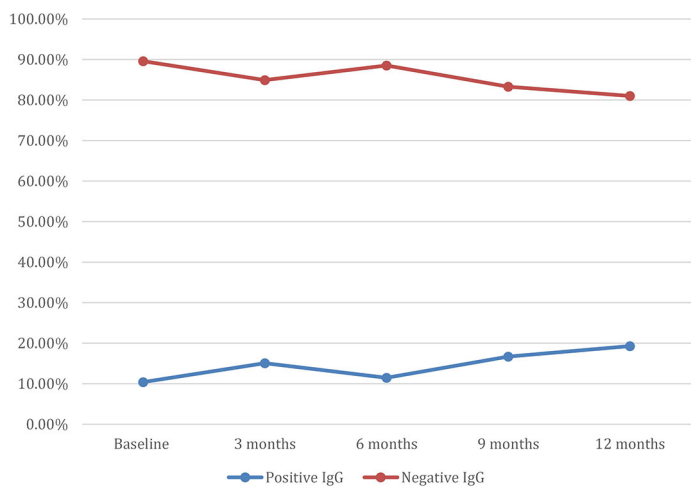

FIGURE 1 | Trends in Proportion of Aspergillus IgG being positive at different time points.

Radiological Findings

At baseline, 69.5% of the participants had CXR features reported as PTB, while 14.7% had features suggestive of CPA or PTB/CPA co-infection, and 12.4% had normal CXR (**Figure 2A**). There was improvement at 3 months on anti-TB therapy, with 41.9% showing improvement in CXR findings with only 23.4% with classical features of PTB. At 6 months when anti-TB therapy was completed, both normal CXR features and features of improvement were found in 41.2% of the participants (**Figure 2B**). Further improvement was documented at 3 months after anti-TB therapy, with normal CXR features documented in 50% (**Figure 2C**). However, at 6 months after anti-TB therapy, normal CXR features were still found in only 50% of participants, but 20% had features suggestive of either PTB or CPA or both (**Figure 2D**).

Probable CPA

Integrating the combination of persistent symptoms (>3 months), CXR features of CPA and raised *Aspergillus* IgG enabled a diagnosis of probable CPA at different time points

TABLE 4 | Mean HRQoL scores in Asp IgG +ve and Asp IgG +ve participants.

Timing	Asp IgG positive	Asp IgG negative	*t*-test	*p*-value
6 months				
Physical health	81.36 ± 13.21	69.46 ± 17.86	2.754	0.016*
Psychological health	86.00 ± 11.88	70.81 ± 14.12	3.974	0.002*
Social relationships	86.36 ± 14.83	70.62 ± 20.78	3.227	0.006*
Environment	82.00 ± 10.31	67.04 ± 14.72	4.399	0.001*
12 months				
Physical health	74.09 ± 22.45	72.83 ± 18.11	0.175	0.864
Psychological health	73.27 ± 24.79	72.25 ± 14.96	0.133	0.897
Social relationships	70.00 ± 27.22	75.79 ± 17.61	–0.648	0.531
Environment	77.00 ± 15.45	72.13 ± 14.48	0.923	0.374

**Statistically significant, $p < 0.05$.*

TABLE 5 | Mean scores of participants answering the SGRQ.

	No. of patients	Mean ± SD	*F*	*p*-value
Symptoms scores				
Baseline	196	41.55 ± 27.59	15.058	<0.001*
3 months	155	30.50 ± 25.29		
6 months	102	25.47 ± 23.31		
9 months	72	22.75 ± 26.89		
12 months	50	16.72 ± 22.98		
Impact scores				
Baseline	196	32.28 ± 27.51	15.121	<0.001*
3 months	155	21.11 ± 23.51		
6 months	102	13.72 ± 19.46		
9 months	72	13.82 ± 20.58		
12 months	50	15.49 ± 23.15		
Activity scores				
Baseline	196	39.00 ± 31.76	11.080	<0.001*
3 months	155	30.58 ± 29.51		
6 months	102	20.86 ± 25.88		
9 months	72	21.10 ± 25.32		
12 months	50	17.83 ± 27.41		
Overall scores				
Baseline	196	36.04 ± 26.29	16.496	<0.001*
3 months	155	25.53 ± 23.53		
6 months	102	17.76 ± 20.15		
9 months	72	17.48 ± 20.24		
12 months	50	16.43 ± 22.75		

****means the p-values are statistically significant.*

(**Figure 2**). Overall probable CPA was found in 20/193 (10.4%) at baseline, 17/148 (11.5%) at 6 months, and 17/88 (19.3%) at 6 months post-TB therapy. There was no statistically significant relationship between any of the domains of HRQoL and probable CPA.

DISCUSSION

The WHO ascertains that patient involvement in their healthcare is a social, economic, and technical necessity (Guo et al., 2009; Kastien-Hilka et al., 2016). Disease-specific health status questionnaires have proven their ability to discriminate between different levels of disease severity (Hays et al., 1995; Kastien-Hilka et al., 2016). However, generic measures like the WHOQOL-Bref (Skevington et al., 2004) are designed to be completed by patients with almost any disease or condition and may be designed to be completed by healthy people, too. Such generic instruments provide a systematic way to usefully compare patient and proxy assessments of QoL and health status across different organ systems and geographies. They also provide information beyond those obtained from clinical and microbiological parameters. Patient experience is assessed using a patient-reported outcome measure (PRO). PROs for CPA have included SGRQ, but this can only be compared among other lung diseases, not other conditions, and is poorly validated in countries with English as a second or third language.

In this study, we observed that most participants were ill at enrollment, with the lowest WHOQO-Bref and the highest SGRQ scores. Scores improved in relation to the length of TB therapy in the different QoL domains, with the highest at 12 months. Moreover, most respondents at 3 months had good

TABLE 6 | SGRQ in those with *Aspergillus* IgG-positive versus *Aspergillus* IgG-negative participants.

	IgG positive	IgG negative	*t*-test	*p*-value
6 months				
Symptoms score	23.92 ± 26.78	26.39 ± 23.93	−0.252	0.808
Impacts score	6.75 ± 10.78	15.47 ± 20.59	−1.975	0.071
Activity score	10.95 ± 21.65	23.25 ± 26.70	−1.502	0.167
Total score	10.71 ± 14.97	19.59 ± 21.08	−1.541	0.155
12 months				
Symptoms score	20.38 ± 21.30	13.36 ± 19.89	0.935	0.366
Impacts score	19.55 ± 24.16	11.03 ± 18.64	1.032	0.322
Activity score	24.14 ± 30.83	14.47 ± 24.03	0.918	0.377
Total score	21.10 ± 22.75	12.48 ± 19.05	1.097	0.293

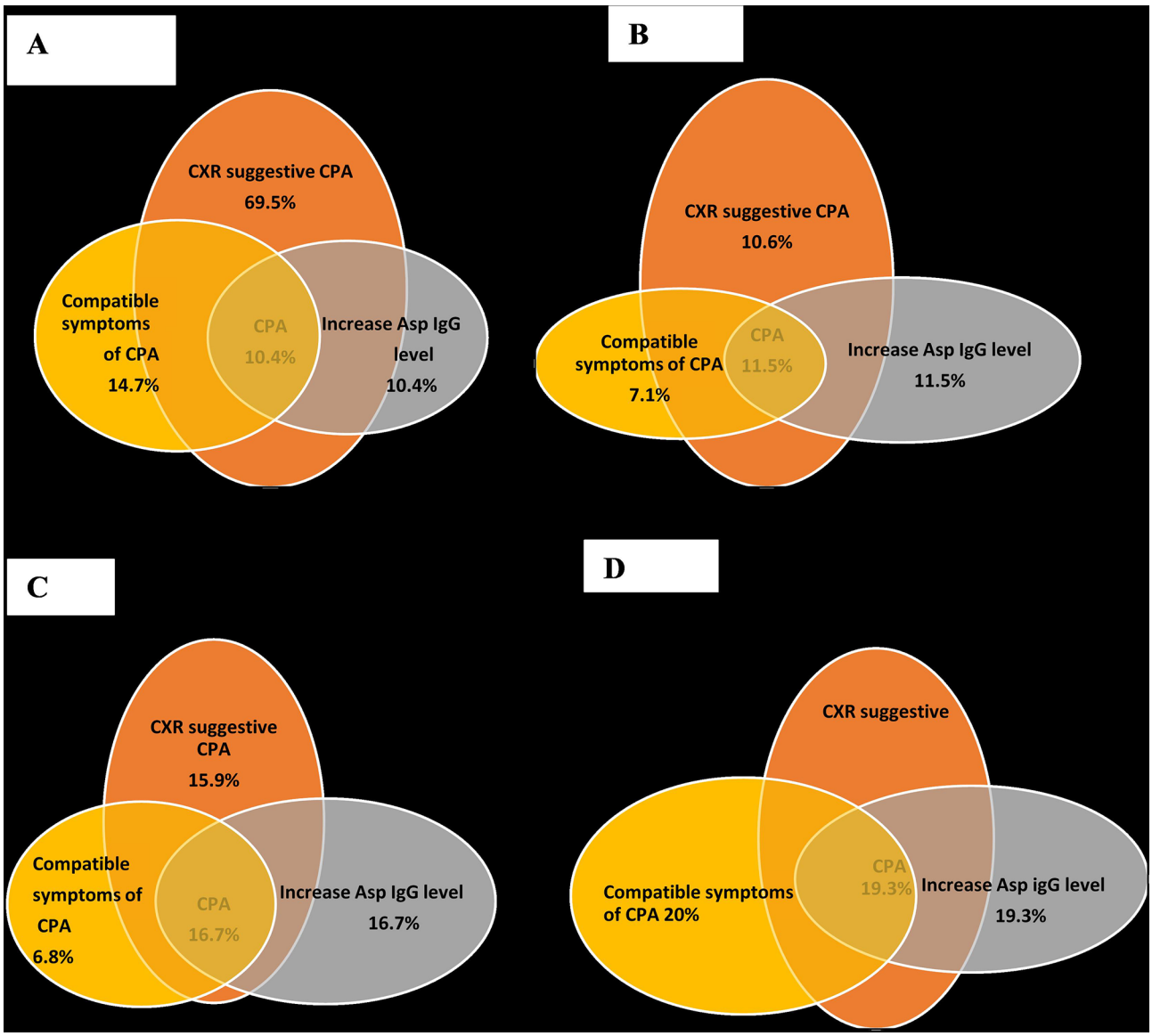

FIGURE 2 | Assessment of CXR findings in studied population. **(A)** Baseline, **(B)** 6 months, **(C)** 9 months, **(D)** 12 months.

perceptions of their overall QoL and general health. However, during this same period, a significant number of respondents were found to have positive *Aspergillus* IgG (**Figure 1**). Overall QoL and general health demonstrated the highest elevation of scores from baseline to 6 months. This most likely reflects the known efficacy of anti-TB therapy and the compliance of the patients.

Research studies using the HRQoL among patients treated for TB in Africa are few (Adeyeye et al., 2014), and to our knowledge, none has been longitudinal. Furthermore, this study is the first to investigate both TB and *Aspergillus* IgG levels and CPA and its effect on HRQoL. Studies like these are urgently needed (Brown et al., 2015) to better manage these large populations.

The finding of progressive improvement in QoL due to anti-TB therapy accords with the findings from similar studies that used the WHOQOL-Bref, for <12 months (Dhuria et al., 2009; Balgude and Sontakke, 2012; Aggarwal et al., 2013; Aggarwal, 2019). This was also the trend in other studies that did not use the WHOQOL-Bref (Babikako et al., 2010; Kruijshaar et al., 2010; Atif et al., 2014). All these studies revealed a gradual increase in scores and improved QoL, as treatment progressed.

Some societal factors that could influence QoL were assessed in our study. Domain scores were generally better among men, urban residents, younger patients, and those with higher socioeconomic status, less severe disease, higher education, better social security, or close family support (Duyan et al., 2005; Babikako et al., 2010; Adeyeye et al., 2014). Independent predictors of low quality of life scores included depression, illiteracy, self-stigma, low monthly income, duration of illness, concomitant illnesses, unemployment, advancing age, no family support, and sociodemographic and other economic factors (Duyan et al., 2005; Adeyeye et al., 2014). Although some of our findings are comparable to other studies, the impact of gender is not clear. We recruited more men, and being male has been associated with good domain scores (Adeyeye et al., 2014; Brown et al., 2015), although not universally (Hays et al., 1995). As expected, treatment with antituberculosis drugs improves the HRQoL of patients (Deribew et al., 2009). From a programmatic perspective, it is important to ensure adherence to medications and avoid treatment default.

In our study, the 12-month means of physical and psychological QoL in this study were lower than the means at the 9-month visit. This finding was previously reported in a study by Mamani et al. in 2014 (Mamani et al., 2014) and may be attributed to a recurrence of symptoms. If so, these symptoms could be due to the re-emergence of TB or the presence of aspergillosis; this is further supported by CXR reports (**Figures 2B, C**). Alternatively, it could represent co-infection of TB and aspergillosis, bronchiectasis, or *Aspergillus* bronchitis complicating bronchiectasis. Further studies involving fungal culture and chest imaging would be helpful to measure the prevalence of pulmonary aspergillosis complicating tuberculosis among this cohort.

There was, however, a significant difference ($p < 0.05$) between respondents with positive and negative *Aspergillus* IgG at 6 months of assessment, in all the domains of the WHOQOL-Bref. This could result from the continuous presence of symptoms, as reported by a previous longitudinal study of CPA (Bongomin et al., 2018). A Ugandan study has reported that *Aspergillus*-specific IgG antibodies were elevated in 4% of HIV-infected adults at the start of TB treatment and in 9% at the end (Kwizera et al., 2017). This is likely due to colonization and either the development of CPA, a reflection of *Aspergillus* co-infection that needs to be resolved, or *Aspergillus* bronchitis complicating bronchiectasis among TB patients. Given the slow genesis of CPA, longitudinal follow-up and CT scanning are required, but our radiological data appear to supports this hypothesis (**Figure 2**). Reduced pulmonary function persists in patients cured of TB (Pasipanodya et al., 2007).

A drop in the number of respondents with each visit was observed in this study. Reasons include transfers of patients to sites closer to home, treatment default, death, and hospitalization. Other studies have reported that the dropout at the second and third follow-up visits was mostly among younger patients with no physical impairment as well as among male patients and due to poverty, severe psychological distress, or alcohol misuse (Louw et al., 2012; Atif et al., 2014; Olufemi et al., 2018). It is important to note that the treatment of TB was completed for these patients at 6 months. Thus, the need to return to the clinic may have not been vital to them visiting the facility again, as they may have achieved a complete cure to the disease. Also with improved QoL, there is a higher likelihood that treatment default may be high due to reduced motivation to improve it.

Some limitations of the study were the continuous drop in respondent numbers at 9 and 12 months, which may have had some impact on the results. A defined cutoff for *Aspergillus* IgG levels has not been established for Africans, although the European-derived cutoff (Wilopo et al., 2020) performs well in Vietnam (Nguyen et al., 2021). CT scan, which is a better representative imaging tool, was not used due to cost, and its inclusion would have allowed confirmation of the diagnosis of CPA. Also, patients underwent HIV testing when diagnosed with TB before starting treatment. HIV testing was not repeated during the treatment and any change in HIV status was therefore unknown. The severity of PTB was not accessed since it was not the focus of the study.

In conclusion, different domains of HRQoL can be a helpful tool for the assessment of the patient and outcome prediction. HRQoL is hindered in patients with PTB and improves significantly with program-based treatment. HRQoL-based disease appraisals in resource-limited countries have become an important instrument to grasp health outcomes and provide focused and empirically informed ways to manage care and treatment better. *Aspergillus* IgG levels were significantly raised in patients being managed for PTB, probably reflecting colonization by *Aspergillus* or early CPA, which alters treatment outcomes among TB patients. Routine screening of *Aspergillus* IgG at the DOTS facility would assist with improving the QoL of patients who may have been misdiagnosed as having

PTB. Further follow-up studies and additional imaging, preferably CT scan, are required to determine when these patients develop CPA and its clinical impact.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Research Ethics Committee (HREC), Lagos University Teaching Hospital, Lagos, Nigeria; HREC, National Institute of Medical Research, Lagos, Nigeria; and HREC, The University of Manchester. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RO, SS, and DD conceptualized and designed the study. RO, TG, and NI collated the data. RO, SS, and DD analyzed and interpreted the data. All authors contributed to the writing and review of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.790134/full#supplementary-material

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Clinical Features, Diagnostic Test Performance, and Prognosis in Different Subtypes of Chronic Pulmonary Aspergillosis

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Objective: The aim of this study was to describe clinical features in different subtypes of chronic pulmonary aspergillosis (CPA)-simple aspergilloma (SA), chronic cavitary pulmonary aspergillosis (CCPA), chronic fibrosing pulmonary aspergillosis (CFPA), aspergillus nodule (AN), and subacute invasive aspergillosis (SAIA), respectively, and identify long-term prognosis of CPA.

Methods: We reviewed patients diagnosed with different subtypes of CPA from 2002 to 2020 at Nanjing Jinling Hospital, China. We analyzed the clinical and survival information of five different subgroups. A Cox regression model was used to explore proper antifungal duration and long-term survival factors of CCPA and SAIA.

Results: A total of 147 patients with CPA were included, consisting of 11 SA, 48 CCPA, 5 CFPA, 12 AN, and 71 SAIA. The most common underlying pulmonary disease was pulmonary tuberculosis ($n = 49$, 33%), followed by bronchiectasis ($n = 46$, 31.3%) and chronic obstructive pulmonary disease (COPD) or emphysema ($n = 45$, 30.6%), while in SAIA and CFPA groups, the most common was COPD or emphysema (45.1 and 100%). Cough (85%), expectoration (70.7%), hemoptysis (54.4%), and fever (29.9%) were common symptoms, especially in CCPA, CFPA, and SAIA groups. The common imaging manifestations included cavitation ($n = 94$, 63.9%), fungal ball ($n = 54$, 36.7%), pleural thickening ($n = 47$, 32.0%), and bronchiectasis ($n = 46$, 31.3%). SAIA and CFPA groups had a lower value of hemoglobin (HB) and serum albumin (ALB) with higher C-reactive protein and erythrocyte sedimentation rate. The positive rate of sputum culture, serum galactomannan (GM), and bronchoalveolar lavage fluid GM was 32.7% (36/110), 18.4% (18/98), and 48.7% (19/39), respectively. There were 64.6% (31/48) patients with CCPA and 25.4% (18/71) patients with SAIA who received surgery and the 5-year cumulative survival rate was 92.1 and 66.6%, respectively. SAIA, old age, male, low body mass index (BMI), COPD or emphysema, multiple distribution, low serum ALB, and positive sputum culture were adverse prognosis factors for SAIA and CCPA group, and BMI $\leq$ 20.0 kg/m^2 was independently associated with increased mortality (hazard ratio (*HR*) 5.311, 95% *CI* 1.405–20.068, $p = 0.014$). Multivariable Cox regression indicated

that surgery (*HR* 0.093, 95% *CI* 0.011–0.814, $p = 0.032$) and antifungal duration >6 months (*HR* 0.204, 95% *CI* 0.060–0.696 $p = 0.011$) were related to improved survival.

Conclusion: The clinical features and laboratory test performance are different among SA, CCPA, CFPA, AN, and SAIA. Low BMI was an independent risk factor for survival. Selective surgery and antifungal duration over 6 months were associated with improved survival.

Keywords: chronic pulmonary aspergillosis, clinical features, prognosis factors, surgery, antifungal duration

INTRODUCTION

Chronic pulmonary aspergillosis (CPA) was thought to affect over 3 million people worldwide (1). It is caused by the Aspergillus species, usually, *Aspergillus fumigatus*, whose conidia are present in indoor and outdoor environments and tiny enough (2–3 μm) to be inhaled into the small airway (2). CPA always complicates many other respiratory disorders such as pulmonary tuberculosis, non-tuberculous mycobacterial infection, allergic bronchopulmonary aspergillosis (ABPA), and chronic obstructive pulmonary disease (COPD) or emphysema (3, 4). If not been treated properly, it may progressively exacerbate.

According to the European guidelines by Denning et al. in 2016 (4), CPA is divided into 5 different subtypes. Chronic cavitary pulmonary aspergillosis (CCPA) is one of the most common, if untreated, which may progress to chronic fibrosing pulmonary aspergillosis (CFPA). Simple aspergilloma (SA) and Aspergillus nodule (AN) are less common and have few symptoms. The above four categories are often found in non-immunocompromised patients, while subacute invasive aspergillosis (SAIA), a more rapidly progressive infection, is usually found in moderately immunocompromised individuals. Since Denning et al. proposed the above guideline, only few small simple studies described all ungrouped CPA cases in China (5). However, the definition of CPA, especially with regard to the classification of subtypes, still remains uncertain, and the therapy and outcome of these subtypes are different. Therefore, it is necessary for us to further identify the characteristics and prognosis of different subtypes to guide proper clinical treatment, especially between CCPA and SAIA which are similar in symptom and imaging. Recently, there are quite a few studies trying to clarify the risk factors affecting the prognosis of patients with CPA, and a 5-year survival rate range from 45 to 62% (6–8), but no exact consensus is come up with. Furthermore, the optimal antifungal duration is still unclear so far. The aim of this study was to describe clinical features and diagnostic performance in different subtypes of CPA, identify potential prognostic factors for 10-year survival and explore proper antifungal duration especially in CCPA and SAIA groups.

MATERIALS AND METHODS

Study Design

This retrospective study reviewed hospitalized patients with CPA referred to the Nanjing JinLing Hospital from January 2002 to December 2020. In addition, patients were reassessed by at least two professors of respiratory medicine. The survival data were finally collected in June 2021 or follow-up time reached 10 years, resulting in a minimum survival data of 6 months for surviving patients. The diagnosis and classification of CPA were based on the European guidelines published in 2016 by Denning et al. All patients had the following characteristics (4): (1) one or more cavities with or without a fungal ball present or nodules on thoracic imaging; (2) microbiological evidence of Aspergillus infection (microscopy or culture from biopsy, sputum, or bronchoalveolar lavage fluid (BALF) culture, positive serum or BALF galactomannan (GM) test or serum anti-Aspergillus immunoglobulin G (IgG) antibody and exclusion of alternative diagnoses; and (3) all present for at least 3 months (SAIA may present for 1–3 months). Patients who did not fulfill these diagnostic criteria were excluded.

According to clinical and radiological findings, patients were divided into 5 different subtypes including SA, CCPA, CFPA, AN, and SAIA. The specific classification methods were as follows: (1) SA occurred in a non-immunocompromised group and presented as a single pulmonary cavity or bronchiectasis containing a fungal ball on imaging. They had mild symptoms such as cough, sputum, and hemoptysis or no symptom and had no radiological progression over at least 3 months of observation. (2) Patients with CCPA presented as one or more pulmonary cavities containing one or more aspergillomas or irregular intraluminal material and had radiological progression such as new cavities, increasing pericavitary infiltrates, or increasing fibrosis. They often had significant pulmonary and/or systemic symptoms such as cough, sputum, hemoptysis, or fever and lasted for at least 3 months, but the disease progression was slower than SAIA. (3) CFPA suffered severe fibrotic destruction of at least two lobes of the lung which manifested as consolidation, reduced volume, or large cavities with surrounding fibrosis on imaging. They all had very poor lung function and significant pulmonary and systemic symptoms. Those who involved only one lobe were classified as CCPA. (4) AN presented as one or more nodules on imaging. They often had minor or no symptoms and could only be definitively diagnosed by histology in which aspergillus complicating necrosis could be seen but there was no tissue invasion. (5) SAIA had a similar clinical manifestation as invasive pulmonary aspergillosis (IPA) but the disease progression was slower than that and the natural course lasted for 1–3 months. They usually occurred in mildly immunocompromised patients, such as diabetes or use of glucocorticoids or immunosuppressants, and had variable

TABLE 1 | Baseline characteristics of patients with chronic pulmonary aspergillosis (CPA) in different subtypes.

Characteristic	ALL ($n = 147$)	SA ($n = 11$)	CCPA ($n = 48$)	CFPA ($n = 5$)	AN ($n = 12$)	SAIA ($n = 71$)
Sex, male	85 (57.8)	4 (36.4)	27 (56.3)	4 (80.0)	4 (33.3)	46 (64.8)
Age, years	55.5 ± 13.8	46.5 ± 13.7	51.8 ± 14.0	60.6 ± 6.8	51.3 ± 11.5	59.7 ± 13.0
BMI, kg/m^2	21.9 ± 3.0	22.4 ± 1.6	22.0 ± 2.9	20.9 ± 4.9	23.2 ± 3.1	21.6 ± 3.2
Underlying pulmonary disease, *n* (%)	104 (70.7)	4 (36.4)	34 (36.4)	5 (100.0)	5 (41.7)	56 (78.9)
Tuberculosis	49 (33.3)	2 (18.2)	20 (41.7)	3 (60.0)	3 (25.0)	21 (29.6)
Bronchiectasis	46 (31.3)	2 (18.2)	9 (18.8)	4 (80)	2 (16.7)	29 (40.8)
COPD or/and emphysema	45 (30.6)	1 (9.1)	6 (12.5)	5 (100)	1 (8.3)	32 (45.1)
ABPA	3 (2.0)	0	1 (2.1)	0	0	2 (2.8)
Asthma	1 (0.7)	0	0	0	0	1 (1.4)
Lung cancer	6 (4.1)	0	0	0	1 (8.3)	5 (7.0)
History of pulmonary surgery	3 (2.0)	0	2 (4.2)	0	0	1 (1.4)
Others	5 (3.4)	0	2 (4.2)	0	0	3 (4.2)
Underlying systemic disease, *n* (%)						
Diabetes	17 (11.6)	0	1 (2.1)	1 (20.0)	0	15 (21.1)
Circulation system disease	24 (16.3)	1 (9.1)	3 (6.3)	1 (20.0)	3 (25.0)	16 (22.5)
Chronic hepatitis	7 (4.8)	0	2 (4.2)	1 (20.0)	0	4 (5.6)
Extra-pulmonary malignancy	10 (6.8)	1 (9.1)	2 (4.2)	0	0	7 (9.9)
Autoimmune disease	14 (9.5)	0	5 (10.4)	1 (20.0)	1 (8.3)	7 (9.9)
Ankylosing spondylitis	5 (3.4)	0	3 (6.3)	0	0	2 (2.8)
Long term use of glucocorticoid or immunosuppressants (over 3weeks)	13 (8.8)	0	3 (6.3)	0	1 (8.3)	9 (12.7)

BMI, body mass index; COPD, chronic obstructive pulmonary disease; ABPA, allergic bronchopulmonary aspergillosis.

radiological features, including cavitation, nodules, infiltrates, or consolidation. The biopsy of SAIA shows hyphae in invading lung tissue.

We performed an analysis of the baseline characteristic, clinical, and radiological manifestation, laboratory and microbiology data, treatment and survival, especially surgery and anti-fungal duration information within 10 years of patients with CPA in different subtypes. The baseline collected was before or at admission. Positive serum or BALF GM test was defined as ≥1.0 pg/ml (ELISA, Bio-Rad Laboratories, CA, USA), and positive aspergillus-specific IgG antibody was >80 AU/ml (ELISA, Dynamiker, China). Due to CCPA and SAIA groups being the most common form of CPA, and deaths of CCPA and SAIA account for almost 90% of all, it is significant to conduct the survival analysis to explore risk factors for prognosis among the above two groups. The study protocol was approved by the Institute Ethics Committee of Nanjing Jinling Hospital. Informed consent was waived because of the retrospective nature of the study.

Statistical Analysis

Data are shown as mean ± *SD* in normal distribution or median with interquartile range (*IQR*) in non-normal distribution for quantitative variables and as numbers (percentages) for qualitative variables. The chi-square and Fisher's exact tests were used for categorical variables. Quantitative variables with normal distributions were compared with Student's *t*-test, while non-normally distributed variables were compared with the Mann–Whitney *U*-test. Survival curves were used to analyze the prognosis of patients from diagnosis to year 10. Survival analysis was performed with the Kaplan–Meier method with the log-rank test. A multivariable Cox analysis was performed to explore independent risk factors for 10-year mortality in CCPA and SAIA groups. An effect was considered to be statistically significant when the *p*-value was <0.05, and all significance tests were two-tailed. The data were statistically analyzed using SPSS 25.0.

RESULTS

Clinical Characteristics

A total of 147 patients proven with CPA were included in this study. The most common subtypes of CPA were SAIA ($n = 71$) and CCPA ($n = 48$) in this study, followed by AN ($n = 12$), SA ($n = 11$) and CFPA ($n = 5$). The baseline characteristics of CPA in different subtypes are summarized in **Table 1**. There were 85 men and 62 women with an average age of 55.5 (18–89) years. In CCPA, CFPA, and SAIA groups, men were in the majority, and patients with CFPA were older than other subtypes. The mean body mass index (BMI) was 21.9 ± 3.0 kg/m^2, and the CFPA and SAIA groups had lower BMI.

Approximately, 82.0% (121) of patients had either underlying pulmonary disease or systematic disease, the most common was pulmonary tuberculosis ($n = 49$, 33.0%) including 5 patients with existing tuberculosis and 45 cases with previous tuberculosis, followed by bronchiectasis ($n = 46$, 31.3%) and COPD or emphysema ($n = 45$, 30.6%). Tuberculosis was the most common pulmonary disease in CCPA group ($n = 20$, 41.7%), so it was in SA ($n = 2$, 18.2%) and AN ($n = 3$, 25.0%), while in SAIA group was COPD or emphysema ($n = 32$, 45.1%) which all 5 patients with CFPA also suffered. Six patients with lung cancer were found

TABLE 2 | The clinical and radiological manifestation of patients with CPA in different subtypes.

Clinical feature	ALL $n = 147$	SA $n = 11$	CCPA $n = 48$	CFPA $n = 5$	AN $n = 12$	SAIA $n = 71$	*P*-value
Clinical manifestations, *n* (%)							
Cough	125 (85.0)	4 (36.4)	44 (91.7)	5 (100.0)	5 (41.7)	67 (94.4)	
Sputum	104 (70.7)	2 (18.2)	35 (72.9)	5 (100)	3 (25.0)	59 (83.1)	
Hemoptysis	80 (54.4)	1 (9.1)	34 (70.8)	3 (60.0)	5 (41.7)	37 (52.1)	0.041[#]
Fever	44 (29.9)	0	13 (27.1)	0	0	31 (43.7)	0.066[#]
Chest distress/asthma/dyspnea	46 (31.3)	0	7 (14.6)	4 (80.0)	2 (16.7)	33 (46.5)	
Chest pain	22 (15.0)	1 (9.1)	7 (14.6)	1 (20.0)	1 (8.3)	12 (16.9)	
Weak or night sweat	14 (9.5)	0	6 (12.5)	1 (20)	2 (16.7)	5 (7.0)	
Imaging manifestations, *n* (%)							
Cavitation	94 (63.9)	7 (63.6)	48 (100.0)	4 (80.0)	1 (8.3)	34 (47.9)	
Fungal ball	54 (36.7)	11 (100.0)	23 (47.9)	4 (80.0)	3 (25.0)	13 (18.3)	
Nodule	31 (21.1)	0	9 (18.8)	1 (20.0)	12 (100.0)	9 (12.7)	
Consolidation	16 (10.9)	0	4 (8.3)	1 (20.0)	0	11 (15.5)	
Pleural thickening	47 (32.0)	1 (9.1)	20 (41.7)	3 (60.0)	2 (16.7)	21 (29.6)	
Bronchiectasis	46 (31.3)	2 (18.2)	9 (18.8)	4 (80)	2 (16.7)	29 (40.8)	
Single lesion	65 (44.2)	11 (100.0)	22 (45.8)	0	9 (75.0)	23 (32.4)	
Multiple lesion	82 (55.8)	0	26 (54.2)	5 (100.0)	3 (25.0)	48 (67.6)	

[#]*Means comparing between CCPA and SAIA.*

in CPA, one of them had coexistent AN, and five were diagnosed with SAIA after chemotherapy or radiotherapy. A high rate of diabetes was also found ($n = 17$, 11.6%) mainly in SAIA group ($n = 15$, 21.1%). Fourteen patients suffered autoimmune disease, such as ankylosing spondylitis, and 13 patients had received long-term (>3 weeks) glucocorticoid or immunosuppressants therapy.

The Clinical and Radiological manifestation data were shown in **Table 2**. The most common symptoms were cough ($n = 125$, 85%) and expectoration ($n = 104$, 70.7%), followed by hemoptysis ($n = 80$, 54.4%) and fever ($n = 44$, 29.9%), most of these symptoms focused on CCPA, CFPA, and SAIA groups, while occasionally occurring in the SA and AN groups. Hemoptysis was more common in the CCPA group than that in SAIA (70.4 vs. 52.1%, $p < 0.05$), while fever was mainly seen in the SAIA group compared with CCPA (43.7 vs. 27.1%, $p = 0.066$). Cavitation ($n = 94$, 63.9%) was the most common imaging finding in CPA, followed by fungal ball ($n = 54$, 36.7%), pleural thickening ($n = 47$, 32.0%), and bronchiectasis ($n = 46$, 31.3%). Sixty-five patients (44.2%) presented with a single lesion on imaging, while eighty-two (55.8%) with multiple lesions. Except in SA and AN groups, multiple distribution is more common in other groups.

Laboratory and microbiology data were summarized in **Table 3**. Inflammation indicators including C-reactive protein (CRP, $n = 115$) and erythrocyte sedimentation rate (ESR, $n = 65$) significantly increased in the SAIA group compared with CCPA, SA, and AN groups (CRP $p < 0.001$, ESR $p = 0.017$), and the CFPA group also had a high level above normal. The patients with CFPA (105 g/L, *IQR* 99–138) and SAIA (117 g/L, *IQR* 105–126) both had lower hemoglobin (HB), while the other three groups had significantly higher HB. The median albumin (ALB) was 33.5 (*IQR* 32.4–41.8) g/L and 36.5 (*IQR* 28.0–47.1) in CFPA and SAIA groups, respectively, which was lower than that in SA, CCPA, and AN groups (SAIA $p < 0.001$, CFPA $p = 0.036$).

Pathology evidence was found among 86 patients by lung biopsy ($n = 27$) and/or surgical excision ($n = 66$). Sputum culture was performed in 110 patients and was found positive in 36 (32.7%) cases, including 22 (35.5%) patients with SAIA and 11 (32.4%) patients with CCPA. Approximately, 98 patients performed serum GM test and had 18 (18.4%) positive results. BALF GM test was performed in 39 patients mainly with CFPA and SAIA, the positive rate was 48.7% which was higher than serum GM ($p < 0.001$) and sputum culture ($p = 0.075$), and the CCPA group had a similar positive rate of BALF GM with SAIA (64.3 vs. 47.4%, $p = 0.335$). Unfortunately, there were only 24 patients who conducted Aspergillus-specific IgG antibody test and the median was 125.33 (*IQR* 47.97–293.96) AU/ml, and unexpectedly, the level in patients with SAIA (312.75 AU/ml, *IQR* 270.03–757.57) was significantly higher than patients with CCPA (70.57 AU/ml, *IQR* 33.88–163.35) ($p = 0.010$).

Therapy and Prognostic Factors

As was shown in **Table 4**, antifungal treatment was administrated in 83% (122 out of 147 patients) patients with CPA, including 24 cases before surgery, 20 after surgery, and 78 cases using the drug alone. The most two common first-line anti-fungal drugs were voriconazole ($n = 70$, 57.4%) and itraconazole ($n = 35$, 28.7%), 7 patients changed triazoles from itraconazole to voriconazole during treatment. The duration of 32 cases (30.5%) lasted over 6 months, while 73 cases (69.5%) were <6 months. Surgery was performed in 66 patients (44.9%). The proportion of surgery is high especially in the patients with SA (81.8%), CCPA (64.6%), and AN (66.7%).

TABLE 3 | Laboratory and microbiology data of patients with CPA in different subtypes.

Variable	ALL ($n = 147$)	SA ($n = 11$)	CCPA ($n = 48$)	CFPA ($n = 5$)	AN ($n = 12$)	SAIA ($n = 71$)	*P*-value
Laboratory data, median (IQR)							
WBC count, 10^9/L (146)	6.2 (4.9–8.0)	5.5 (4.4–6.2)	5.8 (4.5–7.2)	7.8 (6.7–9.8)	5.3 (4.6–6.0)	6.9 (5.2–8.8)	
Neutrophil count	3.6 (2.8–5.8)	3.3 (2.0–4.0)	3.1 (2.6–5.2)	5.5 (4.4–6.9)	3.1 (2.2–3.6)	4.1 (3.0–6.9)	
HB, g/L (146)	123 (111–136)	131 (115–139)	131 (119–139)	105 (99–138)	133 (119–144)	117 (105–126)	
ALB, g/L (147)	40.5 (33.5–44.3)	42.7 (40.7–44.0)	42.2 (37.9–45.2)	33.5 (32.4–41.8)	42.5 (40.0–46.9)	36.5 (28.0–47.1)	<0.001
CRP, mg/L (115)	4.1 (1.0–28.2)	1.0 (0.5–2.5)	2.6 (0.6–17.0)	19.1 (8.7–57.6)	0.9 (0.6–2.7)	13.8 (2.5–80.6)	<0.001
ESR, mm (65)	34 (16.5–70.5)	4 (3–4)	22 (12.5–57.0)	47 (/)	/	39 (27–80)	0.017
Microbiology data, *n* (%)							
Sputum culture (110)	36/110 (32.7)	0/3	11/34 (32.4)	1/4 (25.0)	2/7 (28.6)	22/62 (35.5)	
Serum GM (102)	18/98 (18.4)	1/5 (20.0)	7/33 (21.2)	1/4 (25.0)	0/6	9/50 (18)	
BALF GM (39)	19/39 (48.7)	0/2	9/14 (64.3)	0/1	1/3 (33.3)	9/19 (47.4)	
1,3-β-D-glucan (BDG) (91)	30/91 (33.0)	0/4	8/22 (36.4)	2/5 (40.0)	0/5	20/55 (36.4)	
NGS or PCR (7)	7	1	1	0	0	5	
Pathology (86)	86	10	36	0	12	28	
Aspiration biopsy	27	1	8	0	5	13	
Surgical excision	66	9	31	0	8	18	
Aspergillus specific IgG antibody, AU/ml (24)	125.33 (47.97–293.96)	/	70.57 (33.88–163.35)	/	/	312.76 (270.03–757.57)	0.010[#]
Aspergillus specific IgG antibody, *n*	13/24	0/1	4/11	1/1	1/3	7/8	

IQR, interquartile range; WBC, white blood cell; HB, hemoglobin; ALB, albumin; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; GM, galactomannan; G BALF, bronchoalveolar lavage fluid; NGS, next generation sequencing; PCR, polymerase chain reaction.

[#]*Means comparing between CCPA and SAIA.*

Among all 147 patients, we successfully followed up 140 patients from diagnosis to 10-year or June 2021. While 7 patients lost follow-up completely. During the follow-up, 34 patients died, including 26 cases of SAIA, 3 of CCPA, 3 of CFPA, one of SA, and one of AN, respectively. In the AN group, only one patient died because of unilateral pneumonectomy which was excessive treatment for this case. For patients with SA followed up, there was only one case complicating tuberculosis dead because of massive hemoptysis. However, 60% of patients with CFPA died by the end of follow-up time.

We analyzed 10-year survival-related factors in the CCPA and SAIA groups because they were the most common subtypes of CPA in this study. The Kaplan–Meier cumulative survival curve of different factors in CCPA and SAIA groups was presented in **Figure 1**. As we can see, the cumulative survival rate was significantly lower in the SAIA group than in the CCPA group (log-rank test, $p = 0.001$, **Figure 1A**), and the 1-, 5-, and 10-year cumulative survival rates in the CCPA group were 95.6, 92.1, and 92.1%, respectively, and 82.3, 66.6, and 51.8%, respectively, in SAIA group. According to the Univariate Cox regression shown in **Figure 2**, age > 57 years, male, BMI ≤ 22.0 kg/m^2, SAIA, COPD or emphysema, multiple distributions on imaging, ALB ≤ 40.3 g/L, and positive sputum culture were the risk factors for 10-year mortality.

In CCPA and SAIA group, 45 patients accepted surgical treatment, 60 cases were treated by antifungal drugs for <6 months and 28 cases beyond 6 months and Cox regression indicated that surgery and antifungal duration > 6 months were protective factors. As was presented in **Figure 1**, the cumulative survival rates of patients treated with surgery and/or antifungal duration > 6 months were significantly higher than those without surgery or antifungal duration ≤ 6 months groups (log-rank test, $p < 0.0001$ and $p = 0.023$, **Figures 1B,C**). Although antifungal duration > 12 months had lower mortality than duration ≤ 12 months (**Figure 1D**), we found no statistical difference finally ($p = 0.11$). There was no significant difference in overall mortality between voriconazole and itraconazole antifungal therapy. Further adjusting confounding factors with multivariable Cox regression, we found that BMI ≤ 20.0 kg/m^2 (*HR* 5.311, 95% *CI* 1.405–20.068, $p = 0.014$) was still independently associated with higher 10-year mortality, meanwhile, surgery (*HR* 0.093, 95% *CI* 0.011–0.814, $p = 0.032$) and antifungal duration >6 months (*HR* 0.204, 95% *CI* 0.060–0.696 $p = 0.011$) were related to improved survival.

DISCUSSION

Chronic pulmonary aspergillosis is a potential chronic pulmonary infection disease, which includes SA, CCPA, CFPA, AN, and SAIA, and the overlap of them is often seen (4). In this retrospective study involving 147 patients, we illustrated the characteristics of five subtypes and explored the risk factors for the long-term prognosis of patients with CCPA and SAIA.

The distribution of the CPA subgroup varied in different areas. Many research studies indicated that CCPA was the most common type of CPA (4, 9, 10), but in this study, the most common was SAIA (48.3%), and CCPA accounted for 32.7%, followed by SN (8.2%), SA (7.5%), and CFPA (3.4%). Previous

TABLE 4 | Treatment and outcomes of patients with CPA in different subtypes.

Variable	ALL ($n = 147$)	SA ($n = 11$)	CCPA ($n = 48$)	CFPA ($n = 5$)	AN ($n = 12$)	SAIA ($n = 71$)
Treatment method						
Drug alone	78	2	17	4	3	52
Surgery alone	16	5	8	0	2	1
Drug + surgery	44	4	21	0	5	14
Before surgery	24	1	13	0	3	7
After surgery	20	3	8	0	2	7
Surgery therapy	66	9	31	0	8	18
Lobectomy	50	6	26	0	5	13
Wedge resection	9	3	3	0	2	1
Multiple lobectomy	2	1	0	0	0	1
Unilateral pneumonectomy	1	0	0	0	1	0
Unknown	4	0	1	0	0	3
Antifungal drug						
VCZ	70	4	23	4	5	33
ITZ	35	0	11	0	3	21
Changing triazoles	7	0	1	0	0	6
Others or unknow	10	2	2	0	0	7
Never antifungal treatment	18	5	8	1	3	1
Antifungal time, months						
≤6	73	5	23	2	6	37
>6	32	0	8	2	2	20
Deaths	33	1	3	3	1	25

VCZ, voriconazole; ITZ, itraconazole.

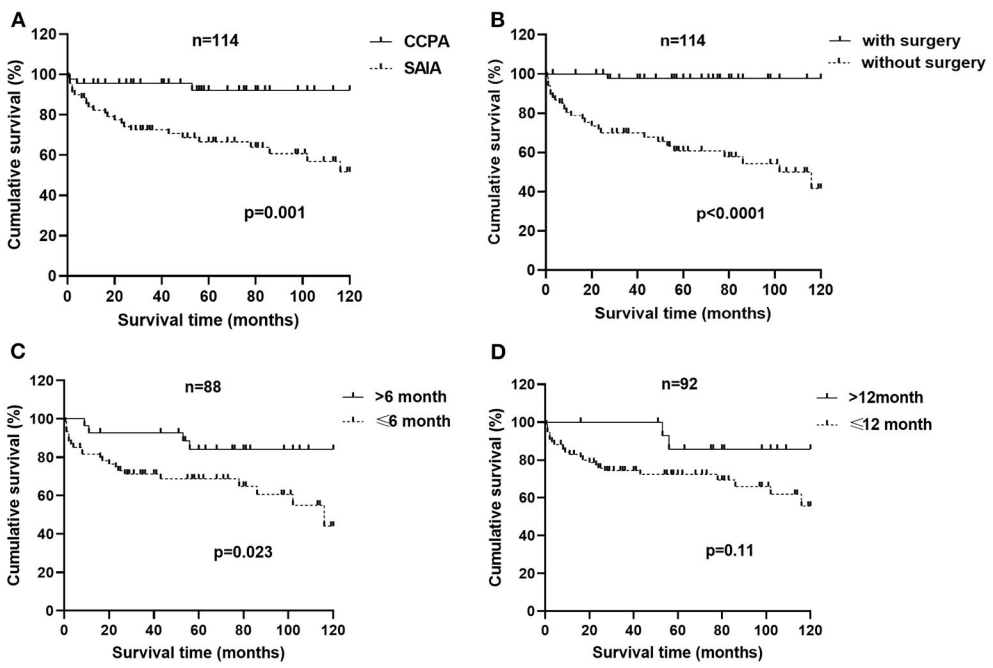

FIGURE 1 | Factors associated with a 10-year survival of patients with chronic cavitary pulmonary aspergillosis (CCPA) and subacute invasive aspergillosis (SAIA). **(A)** Survival from diagnosis to month 120 in patients of CCPA and SAIA groups according to Kaplan–Meier (K–M) analysis with log-rank test ($p = 0.001$). **(B)** K–M cumulative survival curve of patients with chronic pulmonary aspergillosis (CPA) with and without surgical therapy with the log-rank test ($p < 0.0001$). **(C)** K–M cumulative survival curve of patients with CPA who accept anti-fungal therapy for ≤6 months and >6months with log-rank test ($p = 0.023$). **(D)** K–M cumulative survival curve of patients with CPA who accept anti-fungal therapy for ≤12 months and >12months with log-rank test ($p = 0.11$).

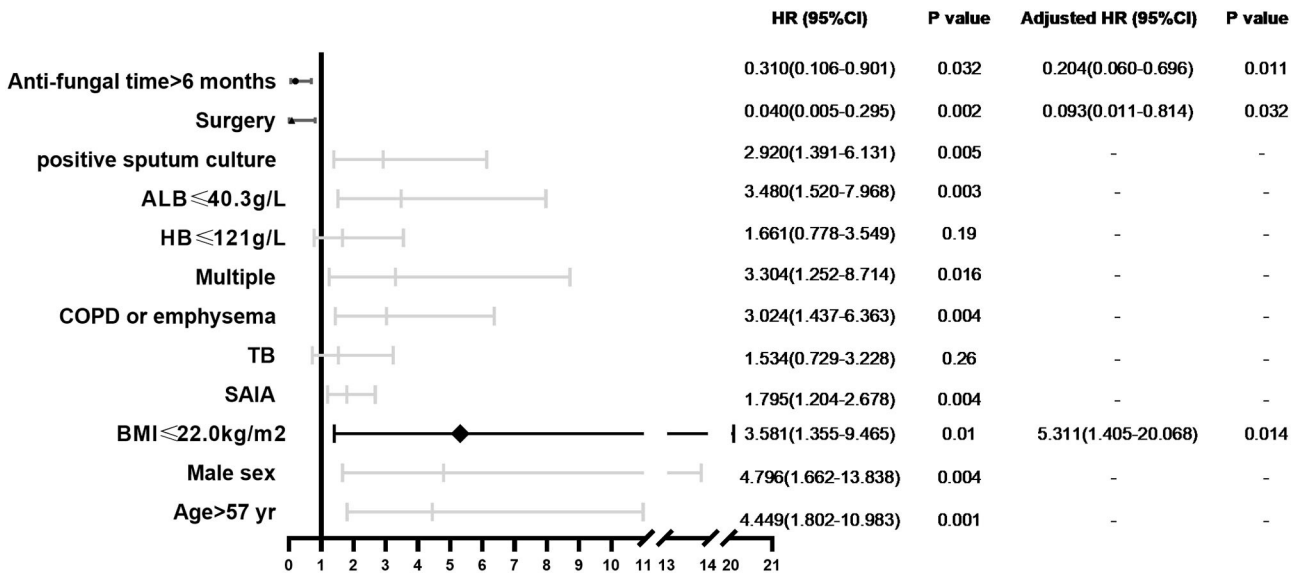

FIGURE 2 | Forest plot of prognostic factors independently associated with 10-year mortality in patients with CCPA and SAIA.

tuberculosis (33.3%) was the most common underlying disease in CPA, especially in the CCPA group, which was similar to some previous studies (3, 4, 6, 9, 11). In recent years, COPD and emphysema became increasingly common in patients with CPA (7, 8, 12), we also found that COPD or emphysema was the highest-incidence disease in SAIA and CFPA groups (45.1 and 100%, respectively). As we know, SAIA often occurred in the mild-immunosuppression population (4), a moderate rate of diabetes (21.1%), and autoimmune disease (9.9%) were also found in the SAIA group. It is noticeable that subtle immunodeficiency seems to be present in some patients with CCPA too. Colombo et al. found that patients with CPA show impaired IFN-γ production in peripheral blood in response to stimuli, and immunotherapy with IFN-γ could be beneficial for those patients (13), which was consistent with what Kelleher et al. shared before (14). We should pay attention to this in the future. Non-tuberculous mycobacteria (NTM) pulmonary disease was common in CPA (15), but our study was lack of information about it, which may result in ignoring patients combined with NTM infection indeed.

Clinical presentation of CPA is non-specific, and hemoptysis is the most common and life-threatening symptom (4, 16, 17). However, we found that the incidence of hemoptysis ranked third in our study (54.4%), and the most common symptom was cough (85.0%). Among all five groups, hemoptysis was more often seen in the CCPA group (70.8%) while fever was more frequently seen in SAIA (43.7%). In SA and SN groups, cough and hemoptysis were also common, but 11 out of 23 patients (47.8%) had no clinical symptom, whose lesions were found by healthy examination occasionally and were diagnosed by surgical resection. Radiological presentation of CPA based on cavitation with or without aspergilloma (4). In our study, common imaging findings of CPA were cavitation, fungal ball, pleural thickening, and bronchiectasis. SA usually had only one cavity with aspergilloma on imaging which could be distinguished from other groups easily. CFPA involved multiple lung lobes and manifested as fibrosis or consolidation, which was consistent with our study. SN presented as a single (9 out of 12 patients, 75%) or multiple nodules (3 out of 12 patients, 25%), but it is difficult to distinguish them from other lung pathology on CT findings alone and we can only rely on pathology finally (4, 18). Sometimes, SAIA and CCPA are difficult to distinguish from each other based on a single radiology result. CCPA may present as pre-existing cavities showing peri-cavitary infiltrates and SAIA commonly had progressive lesions within weeks (19).

Inflammation factors such as WBC count, CRP, and ESR are not always high in CPA. Usually, they increased in CCPA, CFPA, and SAIA groups along with systemic symptoms, while that in patients with SA and AN had no increase (4). We found that SAIA and CFPA groups had more pronounced abnormalities in blood test results such as higher CRP and ESR, lower ALB and HB; meanwhile, they were also accompanied by old age and low BMI. These findings may help us distinguish CCPA and SAIA to some degree. It is worth mentioning that Asians with CPA especially Chinese and Korean showed lower age and BMI than Europeans (5, 7, 8, 10, 12, 20–22), and this was in accordance with our study. In 2014, the American Thoracic Society highlighted the increased susceptibility to lung diseases in the older population (23). We also found CPA frequently occurred in elderly individuals and survival analysis indicated that old age (>57 years) had lower 10-year mortality in CCPA and SAIA groups. Low BMI and serum ALB usually reflect poor nutritional status. The previous studies indicated that lower BMI was a useful indicator of poor long-term survival in chronic progressive respiratory illnesses such as COPD and tuberculosis (24–26) and there were similar results for prognosis

in patients with CPA (6, 7, 11, 21). Our study found that low BMI ($\leq$20.0 kg/m^2) is independently associated with higher 10-year mortality. Lower serum ALB was also related to a poorer outcome in patients with CCPA and SAIA in our study, which was in agreement with the previous studies (6, 7). Vanstraelen et al. illuminated that hypoalbuminemia increased unbound voriconazole plasma concentrations and possibly caused adverse events (27). Therefore, it is necessary for patients with CPA to improve nutritional status to acquire better survival, especially for older and with lower BMI CCPA patients, we should treat them more aggressively, or they may turn into CFPA eventually.

Microbiological evidence of Aspergillus infection is essential for the diagnosis of CPA. A biopsy is reliable to prove a diagnosis of pulmonary aspergillosis and was taken in 86 cases in our study. About 91% (10 out of 11) of patients with SA and all AN had biopsy evidence of microbiology. Serum GM and BALF GM tests have been demonstrated to be valuable for the diagnosis of IPA (28). For patients with CPA, the diagnosis value of the BALF GM test was better than the serum GM test (29–31), and this was also verified in our study, the positive rate of the BALF GM test (48.7%) was higher than that of serum GM test (18.4%). Sputum culture, a frequently used and traditional measure, was performed in 110 patients and the positive rate was 32.7%. All three methods mentioned above had no statistical difference between CCPA and SAIA groups. Aspergillus-specific IgG antibody test was crucial for the diagnosis of CPA and could be followed up to monitor the effectiveness of therapy (4). In our study, patients with SAIA (312.75 AU/ml) had higher antibody levels than CCPA (70.57 AU/ml), which is different from the previous studies (4, 32) probably because the sample size was too small and some antibody tests were conducted after antifungal treatment. In addition, the sensitivity of the Dynamiker Aspergillus antibody test ranged from 77 to 84.1% according to the previous studies (32–35). In recent years, new diagnostic methods such as next generation sequencing and PCR are playing an increasingly important role, but they were not routinely carried out in this study.

Antifungal drugs and surgery are two main therapies for CPA. In SA and AN groups, what we care about is whether patients need surgery or antifungal therapy. Partial previous studies recommend early surgical resection to prevent progression especially massive hemoptysis (4, 36, 37), but there was little evidence that whether patients need antifungal therapy before or after surgery, so we need more prospective study in the future. The patients with CFPA had a poor outcome, and what we need to do is control risk factors and actively treat CCPA before they deteriorate into CFPA. Voriconazole and itraconazole are the most common drugs for CPA in our study, and it is necessary to monitor therapeutic drug levels and adverse effects (4, 38).

To explore risk factors for the long-term prognosis of CCPA and SAIA and optimal antifungal treatment time, we conducted a 10-year survival analysis. The 1-, 5-, and 10-year cumulative survival rates in the CCPA group were 95.6, 92.1, and 92.1%, respectively, and 82.3, 66.6, and 51.8%, respectively, in the SAIA group, which were much higher than previous studies (6–8). The high survival rate of the CCPA group may be related to lower average age, appropriate surgical treatment, and long normative antifungal treatment in these patients. According to univariate analysis, old age, man, low BMI, COPD or emphysema, multiple distributions on imaging, low serum ALB, and positive sputum culture were all adverse prognosis factors for CPA, and patients with SAIA had a better outcome than CCPA. We found that surgery was an independent protective factor for CPA, patients with surgery had a significantly better prognosis than patients without surgery, which was consistent with the previous studies (36, 37, 39, 40). A retrospective study involving 61 patients with CPA indicated that surgical treatment accompanied with antifungal therapy before surgery could minimize the recurrence rate of CPA (41), but we did not find a significant survival difference between preoperative and postoperative antifungal therapy. In addition, some patients could not tolerate surgery due to poor cardiopulmonary function or comorbidities. Although surgery might bring a better long-term prognosis in some patients with CPA, it is necessary to carefully assess the condition of patients with CPA before making the decision. So far, there were no acknowledged criteria for treatment discontinuation in CPA, the efficacy and the adverse effects of drugs are difficult to balance and the duration of antifungal therapy remains unclear. The European CPA rationale in 2016 recommended a minimum of 4–6 months of oral triazole therapy (4). A recent retrospective study involving 196 patients from South Korea indicated that prolonging antifungal therapy beyond 12 months could reduce the recurrence rate in CPA patients (42). Another retrospective cohort from the UK of 206 patients with CPA showed that patients with extended antifungal treatment for 12 months experienced a greater improvement in the quality of life (12). In our study, we found that patients with CCPA and SAIA whose antifungal duration was beyond 6 months had a higher long-term survival rate than patients who received antifungal drugs no more than 6 months. However, we found no statistical survival difference between antifungal duration over 12 months and no more than 12 months. In general, we consider that antifungal therapy beyond 6 months was favorable for patients with CCPA and SAIA.

Our research described the clinical characteristics of CPA in different subgroups, suggested appropriate antifungal duration, and presented key prognostic factors that would assist in identifying patients at risk of poor prognosis, which could improve clinical diagnosis and treatment to some degree. But there were indeed some limitations in our study. First, this was a retrospective study conducted in a single referral hospital, and some patients lost to follow-up. Second, the diagnosis, classification, and treatment of cases many years ago are immature, which may influent the assessment of prognosis, but we remedy for it by reassessing all patients with CPA included by at least one professor of medicine. In addition, our study did not present a specific cause of death and overlooked the adverse effect of antifungal drugs. Finally, we still did not provide optimal antifungal treatment time and we need further study.

In conclusion, the clinical characteristics differ among SA, CCPA, CFPA, AN, and SAIA and clinicians can distinguish them by combining symptom, radiology, laboratory, and microbiology. SAIA, old age, male, low BMI, COPD or emphysema, multiple distributions on imaging, low serum ALB, and positive sputum

culture were adverse prognosis factors for CCPA and SAIA, and low BMI was an independent risk factor after adjusting for confounding factors. Selective surgery and antifungal duration over 6 months could improve long-term survival.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institute Ethics Committee of Nanjing Jinling Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XS, HZ, and YaW designed the study and drafted the manuscript. HZ, YaW, and YG collected the data of patients and analyzed the data. YN, YuW, and KS were critically involved in the data collection and the revision of the manuscript. YS revised the manuscript. All authors contributed to the article and approved the submitted version.

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Synergy Between *Pseudomonas aeruginosa* Filtrates And Voriconazole Against *Aspergillus fumigatus* Biofilm Is Less for Mucoid Isolates From Persons With Cystic Fibrosis

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Persons with cystic fibrosis (CF) frequently suffer from *Pseudomonas aeruginosa* and *Aspergillus fumigatus* co-infections. There is evidence that co-infections with these interacting pathogens cause airway inflammation and aggravate deterioration of lung function. We recently showed that *P. aeruginosa* laboratory isolates synergistically interact with the anti-fungal azole voriconazole (VCZ), inhibiting biofilm metabolism of several *A. fumigatus* laboratory strains. Interaction was usually mediated *via* pyoverdine, but also *via* pyocyanin or pyochelin. Here we used planktonic filtrates of 7 mucoid and 9 non-mucoid *P. aeruginosa* isolates from CF patients, as well as 8 isolates without CF origin, and found that all of these isolates interacted with VCZ synergistically at their IC50 as well as higher dilutions. CF mucoid isolates showed the weakest interactive effects. Four non-mucoid *P. aeruginosa* CF isolates produced no or very low levels of pyoverdine and did not reach an IC50 against forming *A. fumigatus* biofilm; interaction with VCZ still was synergistic. A VCZ-resistant *A. fumigatus* strain showed the same level of susceptibility for *P. aeruginosa* anti-fungal activity as a VCZ-susceptible reference strain. Filtrates of most *Pseudomonas* isolates were able to increase anti-fungal activity of VCZ on a susceptible *A. fumigatus* strain. This was also possible for the VCZ-resistant strain. In summary these data show that clinical *P. aeruginosa* isolates, at varying degrees, synergistically interact with VCZ, and that pyoverdine is not the only molecule responsible. These data also strengthen the idea that during co-infections of *A. fumigatus* and *P. aeruginosa* lower concentrations of VCZ might be sufficient to control fungal growth.

Keywords: *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, voriconazole, microbial interaction, cystic fibrosis, drug interaction, therapy

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INTRODUCTION

Co-infections with bacteria and fungi can worsen the course of infection, e.g., in persons suffering from cystic fibrosis (CF). Among such co-infections, combinations of *Aspergillus fumigatus* with *Pseudomonas aeruginosa*, members of the *Burkholderia cepacia* complex, respiratory syncytial virus, and influenza virus are prominent and have severe impact on lung function (King et al., 2016; O'Brien and Fothergill, 2017). *A. fumigatus* and *P. aeruginosa* co-infections trigger more severe outcomes than each mono-infection (Amin et al., 2010; Reece et al., 2017). To control such infections multiple drugs are used, e.g., targeted therapy to correct CFTR (cystic fibrosis transmembrane conductance regulator) deficiency in persons with CF and eliminate mucus accumulation as the basis for severe infections, or drugs targeting individual pathogens. To treat *A. fumigatus* infections, different azoles, e.g., voriconazole (VCZ), itraconazole, or posaconazole are used (Chishimba et al., 2012; Hogan and Denning, 2011). Occasionally, *Aspergillus* develops resistance against azoles, mainly based on mutations in *cyp51A* gene (Arastehfar et al., 2021).

Pseudomonas competes with *Aspergillus* for crucial resources, e.g., iron, *via* a variety of their products, such as phenazines, its major siderophore pyoverdine (Sass et al., 2017), pyocyanin (5-N-methyl-1-hydroxyphenazine) (Kerr et al., 1999; Briard et al., 2015; Sass et al., 2020), 1-hydroxyphenazine (Kerr et al., 1999; Briard et al., 2015), phenazine-1-carboxamide (Briard et al., 2015), phenazine-1-carboxylic acid (Briard et al., 2015), and rhamnolipids (Briard et al., 2017; Sass et al., 2021). Using laboratory reference isolates (PA14, PAO1) we recently showed that the *Pseudomonas* products pyoverdine, pyocyanin, and pyochelin interacted synergistically with the anti-fungal drug VCZ against *A. fumigatus* forming biofilm *in vitro* (Sass et al., 2021). As correctly emphasized in a recent excellent review, "Pathogens are known to adapt to the host environment during chronic infections; therefore, testing reference strains alongside clinical isolates is extremely important in polymicrobial communication studies" (Reece et al., 2021). Here we studied the relevance of microbial interaction on drug effects using clinical isolates. Given the heterogenicity of clinical *Pseudomonas* isolates, particularly of those from persons with CF (Ferreira et al., 2015), we compared CF and non-CF isolates.

MATERIALS AND METHODS

Materials

2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), menadione, rhamnolipids, and RPMI 1640 medium were purchased from Sigma-Aldrich (St. Louis, MO). Iron content in RPMI 1640 medium was below the detection limit (<1 μM, measured by inductively coupled plasma optical emission spectroscopy by Paolo Visca, Rome, Italy, personal communication). Voriconazole (VCZ) was obtained from Pfizer, New York City. Stock was prepared in DMSO and was further diluted to test conditions in RPMI. DMSO concentration in our combination experiments was 0.01%. DMSO concentrations below 1% do not affect *A. fumigatus* biofilm metabolism, thus did not require separate DMSO controls. Large batches of the reagents were prepared in aliquots and frozen, and a fresh aliquot was used in each experiment.

Strains and Isolates

The use of all microbes in our laboratory is approved by the CIMR Biological Use Committee (approval no. 001-03Yr.16). Assays were performed using the A. *fumigatus* virulent patient isolate 10AF [ATCC 90240 (Denning et al., 1990; Denning and Stevens, 1991)], or a VCZ-resistant isolate (AF21-23) with the most common mutation, TR34/L98H, in the promotor and within the *cyp51A* gene, which encodes the 14-demethylase enzyme critical to ergosterol synthesis (Sabino et al., 2021). Sixteen *P. aeruginosa* isolates from CF patients (7 with mucoid phenotype and 9 with non-mucoid phenotype) and eight isolates of *P. aeruginosa* recovered from non-CF patients were randomly chosen from a library of patient cultures, and obtained after written informed consent, for biobanking of the specimens and subsequent use of the patients, approved by the Stanford University (SU) Institutional Review Board. Another isolate was obtained following clinically indicated cultures from the Santa Clara Valley Medical Hospital (VMC). All isolates are shown in **Table 1**. In CF airways *P. aeruginosa* evolves into

TABLE 1 | Clinical *P. aeruginosa* isolates used in this study.

CIMR#	Clinical Laboratory ID # Santa Clara Valley Medical Center (VMC) or Stanford University (SU)	Specimen	Phenotype
14-79	SU20060455	Respiratory	CF mucoid
14-92	SU09710807	Respiratory	CF mucoid
14-97	SU16242976	Respiratory	CF mucoid
14-112	SU40721045	Respiratory	CF mucoid
14-115	SU40943938	Respiratory	CF mucoid
14-118	SU08215535	Respiratory	CF mucoid
14-122	SU60141132	Respiratory	CF mucoid
14-81	SU09710807	Respiratory	CF non-mucoid
14-82	SU16242976	Respiratory	CF non-mucoid
14-84	SU7841943	Respiratory	CF non-mucoid
14-89	SU41053570	Respiratory	CF non-mucoid
14-113	SU23373137	Respiratory	CF non-mucoid
14-114	SU23373137	Respiratory	CF non-mucoid
14-116	SU40943938	Respiratory	CF non-mucoid
14-117	SU60908696	Respiratory	CF non-mucoid
14-119	SU06034359	Respiratory	CF non-mucoid
14-75	VMC	Respiratory	Non-CF
14-78	SU60370871	Respiratory	Non-CF
14-86	SU42266353	Respiratory	Non-CF
14-90	SU27917939	Respiratory	Non-CF
14-91	SU27917939	Respiratory	Non-CF
14-93	SU21548292	Respiratory	Non-CF
14-98	SU41082579	Non-Respiratory	Non-CF
14-101	SU28674323	Non-Respiratory	Non-CF

Abbreviations: CF, Cystic Fibrosis; Pa, *Pseudomonas aeruginosa*; VCZ, Voriconazole; Af, *Aspergillus fumigatus*; S, Synergy; I, Independence; A, Antagonism; Y_a, inhibition of fungal metabolism by drug A; Y_b, inhibition of fungal metabolism by drug B; $Y°_{ab}$, observed combined antifungal effect; Y^p_{ab}, predicted combined antifungal effect; inhibitory concentration of 50% (IC50).

variants, such as mucoid colony types, which are adapted to chronic residence there (Davis et al., 1967; Davies and Bilton, 2009; Folkesson et al., 2012; Ferreira et al., 2015). For comparison, the *P. aeruginosa* laboratory reference isolates PA14 (O'Toole and Kolter, 1998; Lee et al., 2006; Fischer et al., 2016) and PAO1 (ATCC 15692) (Stover et al., 2000) were used.

P. aeruginosa Planktonic Filtrate Production and Pyoverdine Measurement

P. aeruginosa filtrates were prepared as detailed previously (Ferreira et al., 2015). Briefly, *P. aeruginosa* bacteria (5×10^7 cells/ml) were incubated in RPMI 1640 medium (Sigma-Aldrich) at 37°C and 100 rpm for 24 h. Bacterial growth was measured at 600 nm using a spectrophotometer (Genesys 20, Thermo Fisher Scientific Inc., Waltham, MA). Bacterial cultures were centrifuged at 200×*g* for 30 min at room temperature, and filtered for sterility (0.22 μm). Pyoverdine production was measured at 405 nm. Measurements were normalized to bacterial growth using the formula: Relative pyoverdine production = OD 405/OD600, forming a pyoverdine quotient (Sass et al., 2017).

Assay for Measurement of *Aspergillus* Forming Biofilm Metabolism

A. fumigatus conidia (10^5/ml final concentration) were distributed into the wells of sterile flat-bottom 96-well culture plates at 50 μl/well. Bacterial supernatants or test substances and VCZ were combined in equal parts by volume (25 μl each) to the final concentrations indicated. Final volumes in wells during assays were 100 μl. RPMI 1640 medium served as the negative control. The assay plates were incubated at 37°C overnight and hyphae growth was verified by optical microscopy before performing XTT assays.

All experiments were evaluated by XTT metabolic assay as detailed previously (Scudiero et al., 1988; Ferreira et al., 2015). Briefly, 150 μl of an XTT/menadione mixture (150 μg/ml XTT, 30 μM menadione) were added to each test well and incubated at 37°C for 1 h. Supernatants from each well were transfered to a fresh 96-well plate (100 μl), and assayed using a plate reader (Vmax, Molecular Devices, San Jose, CA) at 490 nm.

Determination of the Isolate Dilution With 50% Anti-Fungal Activity (IC50)

Filtrates were diluted in RPMI in 1:2 steps with final concentrations ranging from 1:2 to 1:1,024. The concentration closest to inhibiting 50% of fungal metabolism here is referred to as the IC50 of an isolate.

BLISS Independence Model for Analysis of Drug Combination Effects

Combined drug effects were calculated using the BLISS Independence Model as described previously (Zhao et al., 2014) when combining drugs at their IC50, the optimal concentration for reagent interactions. Briefly, if drugs A (VCZ) and B (*P. aeruginosa* supernatant) inhibit Y_a and Y_b % of growth, respectively, their predicted combined effect (considering they work independently) is given by the formula: $Y^p_{ab} = \mathbf{Y_a + Y_b - Y_a Y_b}$. The predicted combined effect was compared to the observed combined effect ($Y°_{ab}$, anti-fungal activity by the drug combination in XTT assays). Results were interpreted as:

Observed > Predicted: Synergy (S)

Observed = Predicted: Independent (5% range of Y^p_{ab}) (I)

Observed < Predicted: Antagonism (A)

(Abbreviations: Y_a = inhibition of fungal metabolism by VCZ, Y_b = inhibition of fungal metabolism by Pa sup, $Y°_{ab}$ = observed combined antifungal effect, Y^p_{ab} = predicted combined antifungal effect).

Statistical Analysis

Results were analyzed using Student's *t* test, if two groups were compared, and 1-way ANOVA combined with a Tukey's post-test for multiple comparisons. All data in this study are expressed as a mean ± SD. Data are reported as the percent of control. Each assay was performed with three to four biological and technical replicates. Representative experiments are shown.

RESULTS

Determination of IC50s for Filtrates of Clinical *P. aeruginosa* Isolates Against *A. fumigatus* 10AF Biofilm Formation

Planktonic filtrates of seven randomly chosen mucoid or nine non-mucoid clinical *P. aeruginosa* isolates, derived from persons with CF (CF mucoid, CF non-mucoid), or from eight non-CF patients (described in **Table 1**) were produced in RPMI medium under iron-limiting conditions (Sass et al., 2017). Isolate filtrates were tested for anti-fungal activity against 10AF biofilm metabolism in 1:2 dilution steps from 1:2 to 1:1,024. Dose–response curves allowed the determination of dilutions that were within a 2-fold step of the individual IC50 (concentration of agent inhibiting 50%, compared to control). **Table 2** shows that for most isolates an IC50 could be determined, with the dilution closest to the IC50 for individual isolates being between 1:16 and 1:256. Four isolates did not reach an IC50 at any dilution (**Table 2**: isolates 14-82, 14-89, 14-116, and 14-119). The anti-fungal strength of isolates, as assessed by the dilution of filtrate approximating the IC50, closely correlated with their pyoverdine content (**Table 2** and **Figure 1**). These results are in agreement with previous findings (Sass et al., 2017). **Table 2** also shows that mean pyoverdine production and IC50 in the CF mucoid group were lower than observed for the CF non-mucoid or non-CF groups, or for laboratory isolate PA14.

Synergy Between VCZ and Clinical *P. aeruginosa* Isolates Against *A. fumigatus* 10AF Biofilm Formation

In a previous study we determined the IC50 for VCZ against 10AF forming biofilm metabolism within a 2-fold dilution step of 125 nM (Sass et al., 2021). We now combined filtrates of each clinical isolate at the dilution closest to its individual IC50 (**Table 2**) with VCZ at its IC50. Combination experiments were performed for all isolates with the exception of the four isolates that did not reach an IC50 (**Table 2**: isolates 14-82, 14-89, 14-116, and 14-119). **Figure 2** gives

TABLE 2 | IC50, BLISS score and pyoverdine content determination for isolates used in this study.

CIMR#	Filtrate dilution closest to IC50	Interaction filtrate IC50/ VCZ IC50 BLISS score	Pyoverdine quotient
CF mucoid			
14-79	1:64	S	0.90
14-92	1:16	S	0.57
14-97	1:256	S	1.73
14-112	1:256	S	3.78
14-115	1:64	S	0.72
14-118	1:64	S	1.29
14-122	1:256	S	1.72
CF mucoid group mean	**1:128**	**S**	**1.53**
CF non-mucoid			
14-81	1:256	S	2.83
14-82	No IC50	–	0.06
14-84	1: 128	S	2.01
14-89	No IC50	–	0.04
14-113	1:64	S	0.18
14-114	1:512	S	3.41
14-116	No IC50	–	0.06
14-117	1:256	S	3.97
14-119	No IC50	–	0.17
CF non-mucoid group means	**1:256**	**S**	**2.48**
	no IC50	**-**	**0.08**
Non-CF			
14-75	1:256	S	1.99
14-78	1:256	S	2.80
14-86	1:128	S	2.24
14-90	1:256	S	1.82
14-91	1:256	S	2.48
14-93	1:256	S	2.78
14-98	1:16	S	0.13
14-101	1:256	S	1.63
Non-CF group mean	**1:256**	**S**	**1.98**
PA14	**1:256**	**S**	**2.23**
PAO1	**1:512**	**S**	**3.23**

BLISS score: S: synergy.
As indicated in the Table the bold values are 'group means'.

an example of an isolate (14–92), tested for interaction with VCZ at concentrations surrounding its own IC50 (1:8 to 1:32), in combination with VCZ (**Figure 2A**: 0.25 μM, **Figure 2B**: 0.125 μM, **Figure 2C**: 0.063 μM). Our results show that synergistic effects were achieved, unless anti-fungal activity of one of the components alone was very strong (**Figure 2**).

We show each clinical isolate filtrate as well as PA14 filtrate at concentrations closest to their individual IC50s (**Table 2**), combined with VCZ at its IC50 (**Figure 3**). This shows that all individual filtrates had significantly stronger anti-fungal activity when combined with VCZ, independently of the phenotype or origin of the isolates; mucoid (**Figure 3A**), non-mucoid CF (**Figure 3B**), or non-CF (**Figure 3C**). Using the BLISS Independence Model we calculated the type of interaction (synergistic (S), independent (I) or antagonistic (A)) for all filtrate/VCZ combinations. **Table 2** summarizes that all interactions were synergistic.

When all isolates per group were combined, combinations with VCZ were synergistic as well (**Figure 4A**: mucoid CF, **Figure 4B**: non-mucoid CF, **Figure 4C**: non-CF; **Table 2**).

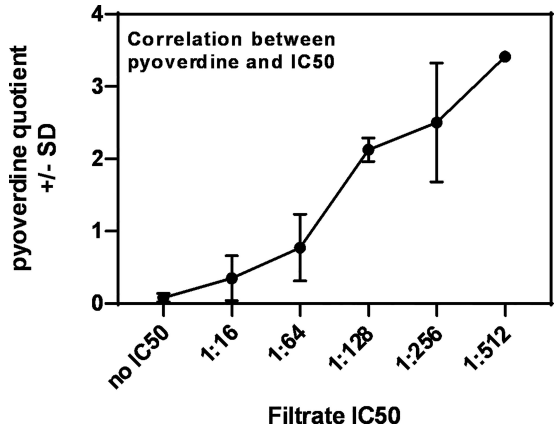

FIGURE 1 | Correlation between pyoverdine content in *Pseudomonas* filtrates, and their IC50s. Pyoverdine quotients shown in **Table 2** were plotted against the individual filtrate's IC50, revealing a correlation between high pyoverdine content and strong anti-fungal activity.

When comparing mucoid CF, non-mucoid CF and non-CF isolates, synergy of mucoid CF isolates with VCZ was found weaker than synergy between VCZ and non-mucoid CF or non-CF isolates (**Figure 4D**). Synergy between isolate filtrates and VCZ was strongest in the non-CF group (**Figure 4D**).

High Dilutions of Clinical Isolate Filtrates Still Support Anti-Fungal Activity of VCZ

To determine how potent interactions of isolates of each group (CF mucoid, CF non-mucoid, non-CF) were with VCZ, we further combined each isolate at the same high filtrate dilution (1:256), with VCZ at its IC50. This also enabled comparisons of the data from studying clinical isolates to those previously published with reference laboratory isolates (Sass et al., 2021), as the latter study investigated filtrates at a 1:256 dilution. **Figure 5** shows that in each group (**Figure 5A**: mucoid CF, **Figure 5B**: non-mucoid CF, **Figure 5C**: non-CF) all isolates acted synergistically with VCZ when used at a 1:256 dilution. The same result was obtained when all isolates per group were combined (**Figure 6A**: mucoid CF, **Figure 6B**: non-mucoid CF, **Figure 6C**: non-CF). Again, combinations of CF mucoid filtrates showed significantly less synergy with VCZ than non-mucoid filtrates or non-CF filtrates, and synergy of non-CF isolates with VCZ was strongest (**Figure 6D**).

Isolates Not Showing an IC50 Still Support VCZ Anti-Fungal Activity

In the non-mucoid CF group we found four isolates that did not produce pyoverdine, a major *Pseudomonas* anti-fungal factor (Sass et al., 2017), and did not reach an IC50 (14-82, 14-89, 14-116, 14-119 in **Table 2**). This is a phenomenon not previously encountered, in studying wildtype laboratory isolates. We combined 1:2 (**Figure 7A**) or 1:256 dilutions of these filtrates (**Figure 7B**) with VCZ close to its IC50, and found that all 1:2 diluted filtrates interacted synergistically with VCZ (**Figure 7A**). When filtrates were diluted to 1:256 final

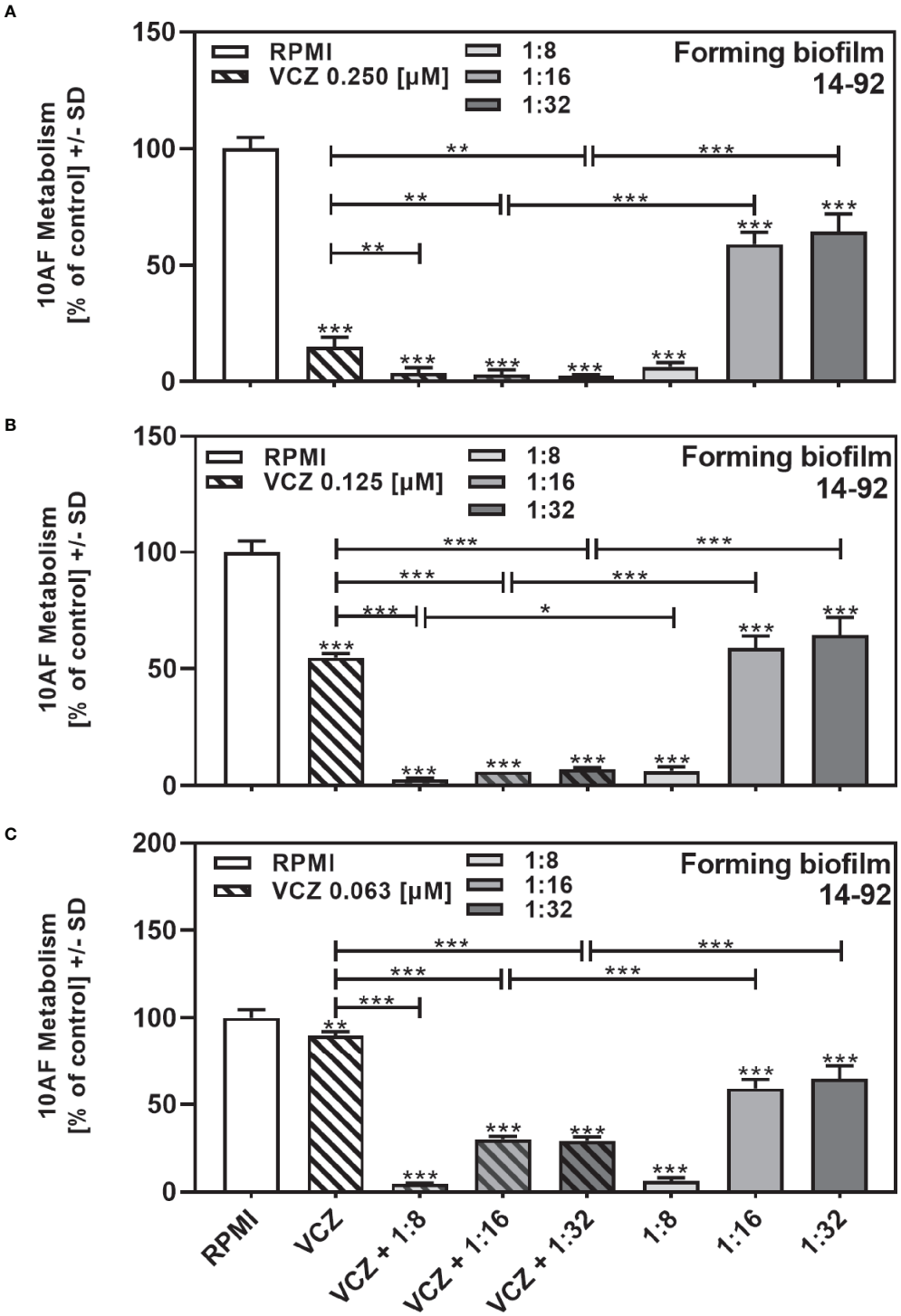

FIGURE 2 | Examples for synergistic anti-fungal effects of the combination of *P. aeruginosa* clinical isolate filtrate and VCZ. Isolate 14-92 filtrate was diluted to final concentrations of 1:8 to 1:32, encompassing its inhibitory concentration of 50% (IC50), and combined with VCZ at 0.25 µM **(A)**, 0.125 µM **(B)**, or 0.063 µM **(C)** to test their combined antifungal activities against 10AF forming biofilm (10^5 conidia/ml in RPMI 1640 medium). Assay plates were incubated at 37°C overnight. 10AF fungal metabolism was measured by XTT assay. Metabolism in the presence of RPMI alone (white bar) was regarded as 100%. Statistical analysis: Unpaired t-test for VCZ (white striped bar), or each filtrate dilution (solid gray bars), vs. combinations of VCZ and filtrate (striped gray bars): one, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

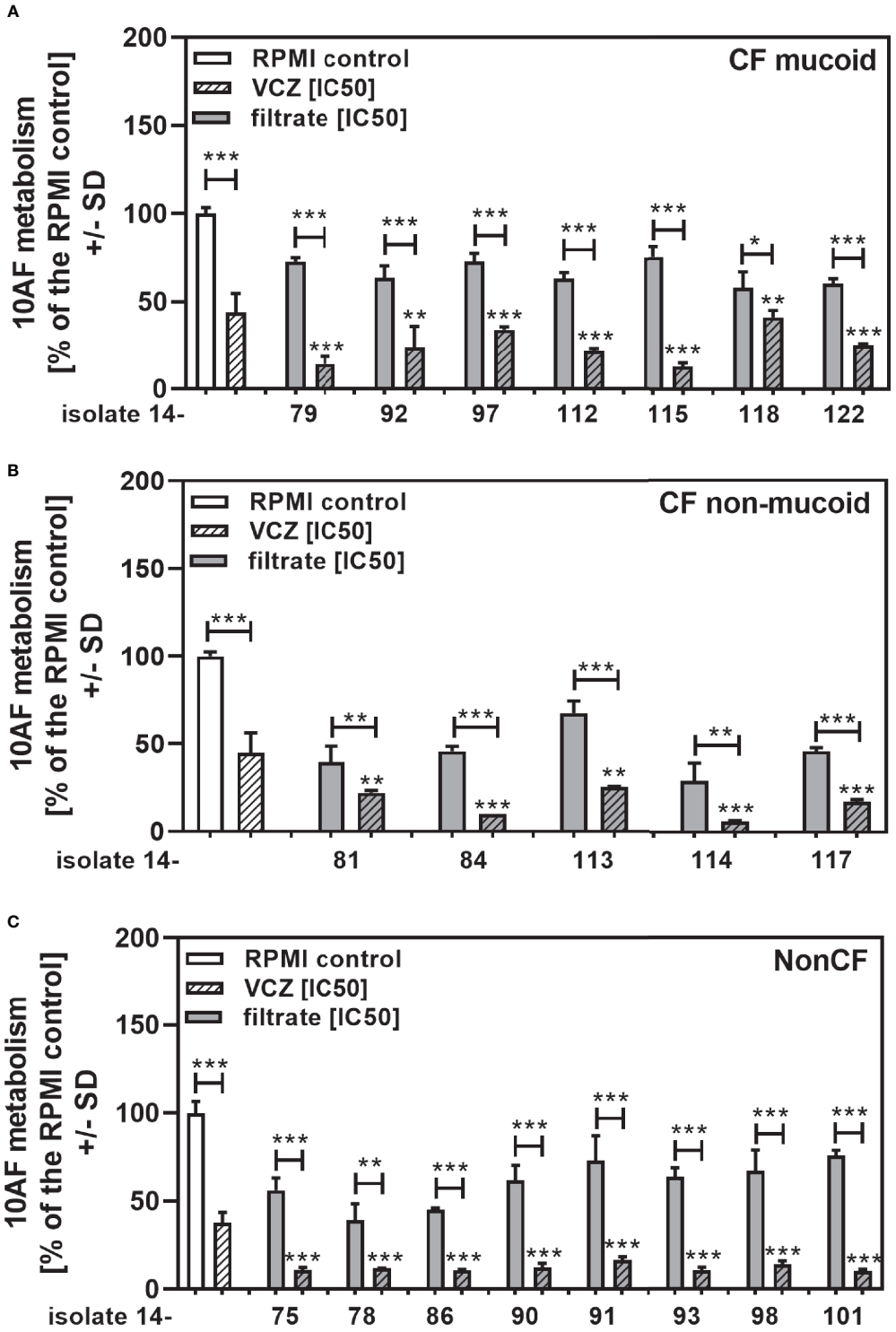

FIGURE 3 | Anti-fungal effects of the combination of CF mucoid, CF non-mucoid, or non-CF *P. aeruginosa* clinical isolate filtrates and VCZ at their IC50s. CF mucoid **(A)**, CF non-mucoid **(B)**, or non-CF isolate filtrates **(C)** were diluted to be used at final concentrations closest to their IC50, and combined with VCZ close to its IC50, to test their combined antifungal activities against 10AF forming biofilm (10^5 conidia/ml in RPMI 1640 medium). Assay plates were incubated at 37°C overnight. 10AF fungal metabolism was measured by XTT assay. Metabolism in the presence of RPMI alone (white bar) was regarded as 100%. X-axis shows isolate numbers. Comparisons without brackets: VCZ alone (striped white bar) vs. the individual isolate filtrate combination with VCZ (gray striped bar). Other comparisons as indicated by the ends of the brackets. Statistical analysis: Unpaired t-test: one, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

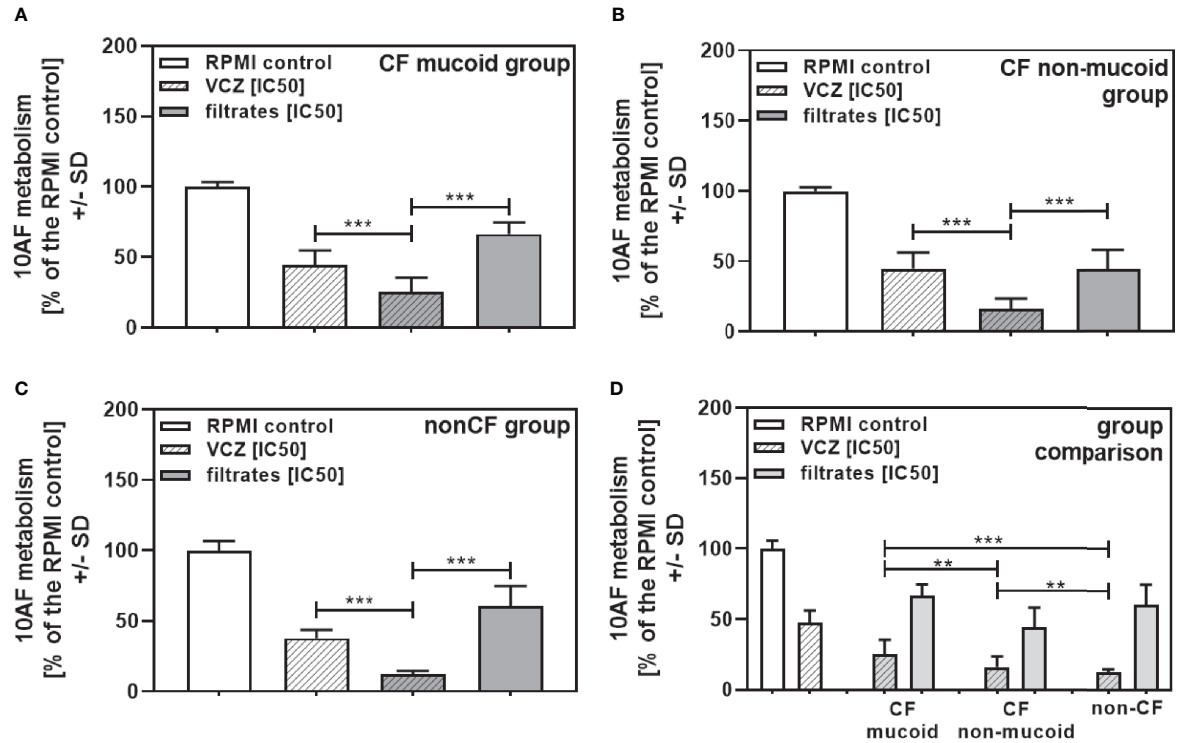

FIGURE 4 | Group comparisons for anti-fungal effects of the combination of CF mucoid, CF non-mucoid, or non-CF *P. aeruginosa* clinical isolate filtrates and VCZ at their IC50s. Individual isolate interactions with VCZ shown in **Figure 3** were combined per group [CF mucoid **(A)**, CF non-mucoid **(B)**, or non-CF isolate filtrates **(C)**]. **(D)** Comparison among effects of VCZ/filtrate combinations, shown in **(A–C)**, on 10AF biofilm formation. Metabolism in the presence of RPMI alone (white bar) was regarded as 100%. Comparisons as indicated by the ends of the brackets. Statistical analysis: Unpaired t-test: two or three asterisks = $p \leq 0.01$ or $p \leq 0.001$, respectively.

concentration, two of the isolates still interacted with VCZ synergistically (**Figure 7B**: 14-82 and 14-119), whereas two isolates no longer did (**Figure 7B**: 14-89 and 14-116). These data suggest that pyoverdine is not solely responsible for combined anti-fungal activity of filtrates and VCZ, and that different amounts of one or more other factors are present in filtrates that interact with VCZ. We studied rhamnolipid production (Sass et al., 2021), and found that isolates 14-82 and 14-119 were capable of producing rhamnolipids, whereas isolates 14-89 and 14-116 were not. This ability to produce rhamnolipids correlated with their abilities to interact synergistically with VCZ at high dilutions. We therefore tested effects of rhamnolipids on 10AF forming biofilm metabolism, and also synergy with VCZ near its IC50, and found the IC50 for anti-fungal activity of rhamnolipids was about 160 μM, and synergy with VCZ at rhamnolipid concentrations below 39 μM, the lowest concentration tested (**Figure 7C**).

Clinical *Pseudomonas* Isolate Filtrates Boost Anti-Fungal Activity of VCZ at Sub-Optimal Concentrations of VCZ, or in VCZ-Resistant *Aspergillus*

When clinical isolate filtrates close to their individual IC50s were combined with VCZ at VCZ concentrations that on their own allowed about 25% of anti-fungal activity, we still found synergistic interaction for most of the tested isolates (**Figure 8A**: mucoid CF, **Figure 8B**: non-mucoid CF, **Figure 8C**: non-CF). When all isolates in each group were combined, interactions were synergistic, compared to the single agents (**Figure 9A**: mucoid CF, **Figure 9B**: non-mucoid CF, **Figure 9C**: non-CF). CF mucoid filtrates showed significantly less synergy with VCZ than non-mucoid filtrates, but not less than non-CF isolates, whereas interactions with non-mucoid CF isolates were strongest (**Figure 9D**).

As an extreme example of poor VCZ anti-fungal activity we used a clinical *Aspergillus* isolate resistant to VCZ concentrations. We studied concentrations that produced an IC50 in 10AF (**Figure 10A**), and in another wildtype laboratory reference strain, AF13073 (Sass et al., 2021). The VCZ-resistant strain AF21-23 showed an IC50 of 4–8 μM, which is about 50× higher than the IC50 of a susceptible strain (**Figure 10A**). Susceptibility towards *Pseudomonas* anti-fungal activity was similar for the VCZ-susceptible and the VCZ-resistant *Aspergillus* strain (**Figure 10B**). Combination with *Pseudomonas* filtrate synergistically increased VCZ anti-fungal activity even for the resistant fungus (**Figure 10C**).

DISCUSSION

Co-infection with *A. fumigatus* and *P. aeruginosa* has been described to result in more severe outcome than mono-

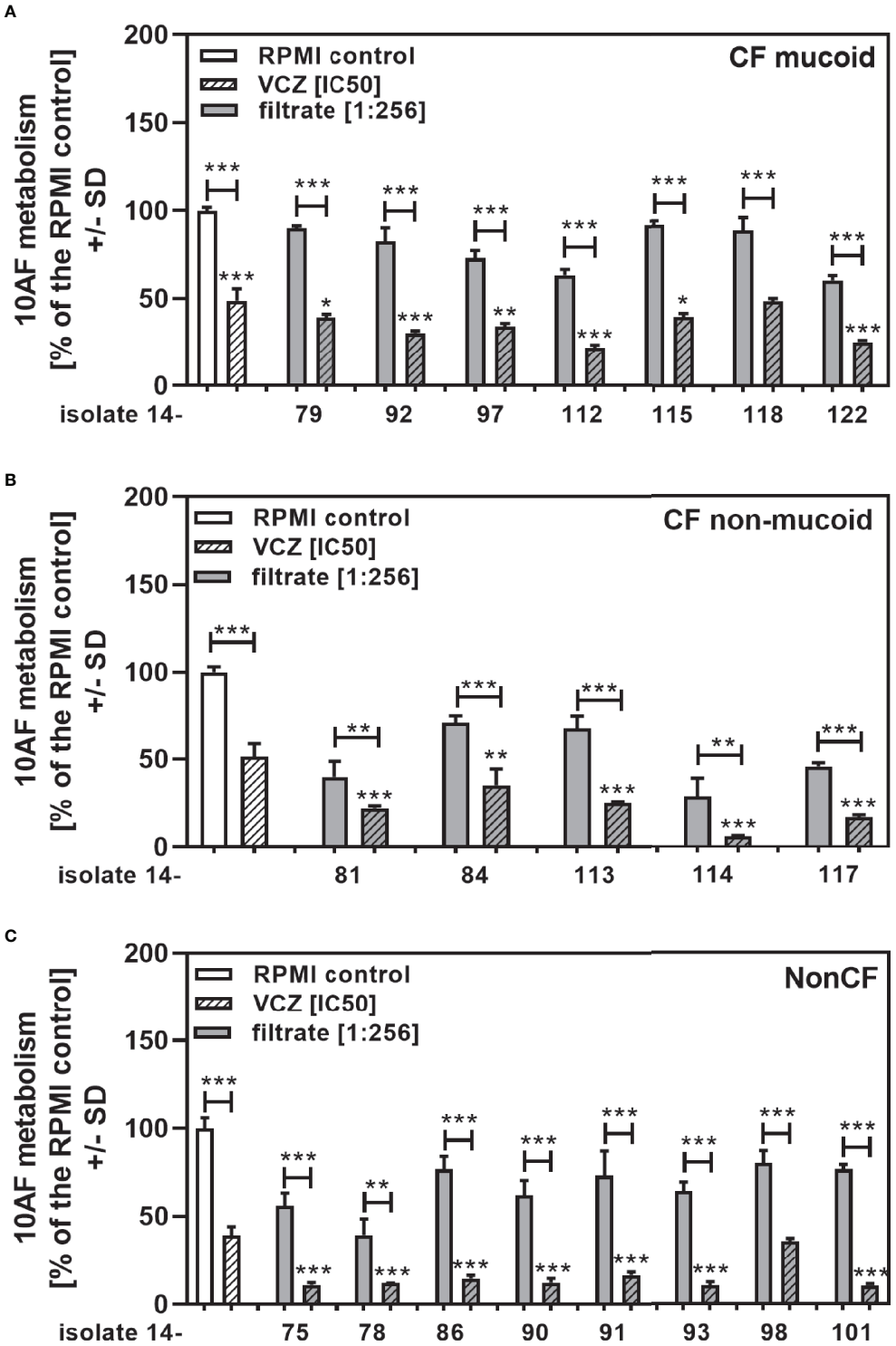

FIGURE 5 | Anti-fungal effects of the combination of CF mucoid, CF non-mucoid, or non-CF *P. aeruginosa* clinical isolate filtrates at 1:256 dilutions with VCZ at its IC50. CF mucoid **(A)**, CF non-mucoid **(B)**, or non-CF isolate filtrates **(C)** were diluted to be studied at final concentrations of 1:256, and combined with VCZ close to its IC50, to test their combined antifungal activities against 10AF forming biofilm (10^5 conidia/ml in RPMI 1640 medium). Assay plates were incubated at 37°C overnight. 10AF fungal metabolism was measured by XTT assay. Metabolism in the presence of RPMI alone (white bar) was regarded as 100%. X-axis shows isolate numbers. Comparisons without brackets: VCZ alone (striped white bar) vs the individual isolate filtrate combination with VCZ (gray striped bar). Other comparisons as indicated by the ends of the brackets. Statistical analysis: Unpaired t-test: one, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

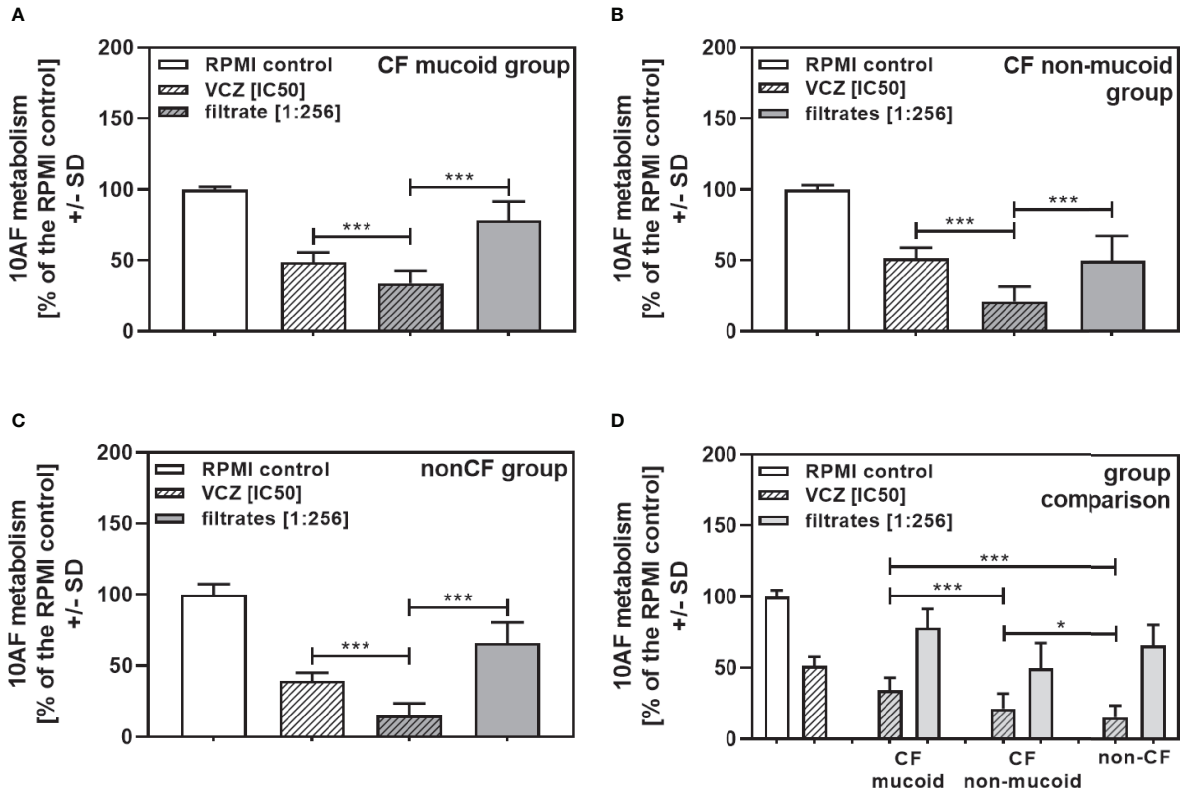

FIGURE 6 | Group comparisons for anti-fungal effects of the combination of CF mucoid, CF non-mucoid, or non-CF *P. aeruginosa* clinical isolate filtrates at 1:256 dilutions with VCZ at its IC50. Individual isolate interactions with VCZ shown in **Figure 4** were combined per group [CF mucoid **(A)**, CF non-mucoid **(B)**, or non-CF isolate filtrates **(C)**]. **(D)** Comparison among effects of VCZ/filtrate combinations, shown in **(A-C)**, on 10AF biofilm formation. Metabolism in the presence of RPMI alone (white bar) was regarded as 100%. Comparisons as indicated by the ends of the brackets. Statistical analysis: Unpaired t-test: one or three asterisks = $p \leq 0.05$ or $p \leq 0.001$, respectively.

infection (Amin et al., 2010; Reece et al., 2017), possibly as a result of inflammatory signals caused by intermicrobial competition. On the other hand, numerous *P. aeruginosa* molecules, such as pyoverdine (Sass et al., 2017), phenazines, e.g., pyocyanin (Kerr et al., 1999; Sass et al., 2020), or di-rhamnolipids (Briard et al., 2017) have been shown to interfere with fungal metabolism or growth. The major siderophore pyoverdine was found to be the primary anti-fungal molecule under iron-limited conditions (Sass et al., 2017), whereas phenazine anti-fungal activity was triggered under non-limiting iron conditions (Chatterjee et al., 2020; Sass et al., 2020).

In a recent study, using *P. aeruginosa* laboratory strains PA14 and PAO1, we found synergistic anti-fungal activity of bacterial filtrates and VCZ, independently of the *A. fumigatus* strain used (Sass et al., 2021). The study showed that mediators of synergy encompass pyoverdine and pyocyanin, but also pyochelin, and suggested that the support of VCZ anti-fungal activity by soluble bacterial factors should be taken into consideration when treating *Aspergillus-Pseudomonas* co-infections. Such co-infections are not uncommon in persons with CF and other immunocompromised patients (King et al., 2016; O'Brien and Fothergill, 2017). It has been shown that in persons with CF two major *P. aeruginosa* phenotypes exist, mucoid, and non-mucoid CF isolates, of which the mucoid phenotype is associated with lower anti-fungal activity, compared to the non-mucoid phenotype (Ferreira et al., 2015; Nazik et al., 2020). The present study supports the finding of mucoid *P. aeruginosa* isolates from CF patients having less anti-fungal activity than non-mucoid CF isolates by showing that also their interaction with VCZ is weaker. Nevertheless, it is important to stress, all clinical isolates in the present study showed synergistic anti-fungal activity with VCZ, whether used at their individual IC50s, or at a dilution of 1:256, as previously used for testing interaction of VCZ with laboratory isolates (Sass et al., 2021). Synergy was observed over a wide range of filtrate, as well as VCZ, dilutions, increasing the likelihood of such interactions also taking place in the body. Many experiments (not shown) studying VCZ concentrations flanking the VCZ IC50 on both sides (example in **Figure 2**) also affirm the synergy illustrated in this paper. The exceptions were when VCZ or *Pseudomonas* filtrate concentrations, higher than the individual IC50 on their own, already had very strong effects on fungal metabolism. In such cases, synergy could not be calculated using the BLISS Independence Model. Animal studies now have to confirm if our *in vitro* observations of synergy reflect bacterial factor–drug interactions in the body. If stronger VCZ effects are observed

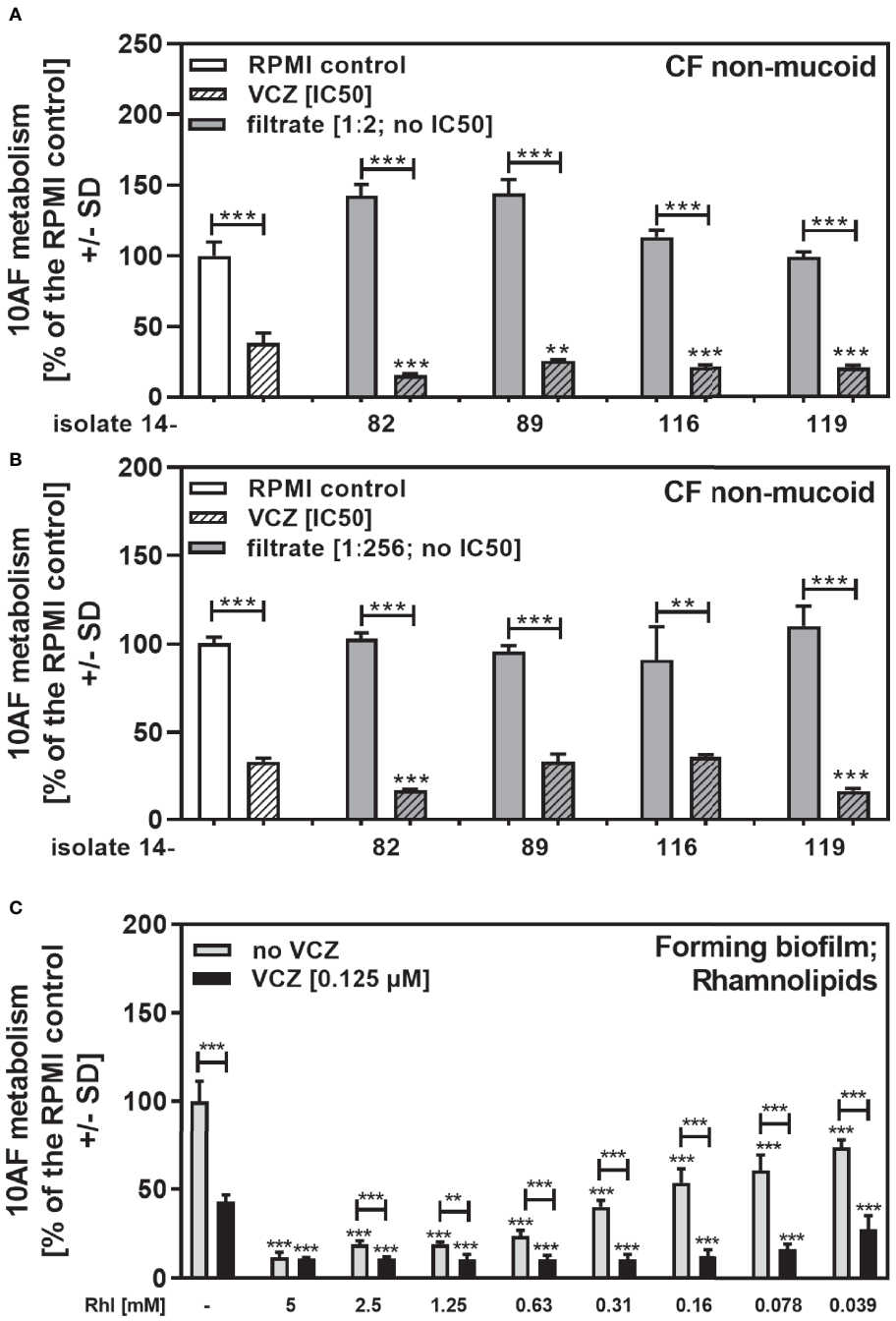

FIGURE 7 | Anti-fungal effects of the combination of pyoverdine-negative *P. aeruginosa* clinical isolate filtrates with VCZ. Four pyoverdine-negative CF non-mucoid isolate filtrates (no IC50) were diluted 1:2 **(A)** or 1:256 **(B)**, and combined with VCZ close to its IC50, to test their combined antifungal activities against 10AF forming biofilm (10^5 conidia/ml in RPMI 1640 medium). Assay plates were incubated at 37°C overnight. 10AF fungal metabolism was measured by XTT assay. Metabolism in the presence of RPMI alone (white bar) was regarded as 100%. X-axis shows isolate numbers. Comparisons without brackets: VCZ alone (striped white bar) vs. the individual isolate filtrate combination with VCZ (gray striped bar). Other comparisons as indicated by the ends of the brackets. Statistical analysis: Unpaired t-test: two or three asterisks = $p \leq 0.01$ or $p \leq 0.001$, respectively. **(C)** Rhamnolipids were diluted to final concentrations of 5 to 0.039 mM, and tested against 10AF forming biofilm metabolism (10^5 conidia/ml in RPMI 1640 medium) either alone (gray bars) or in combination with VCZ close to its IC50. Assay plates were incubated at 37°C overnight. 10AF fungal metabolism was measured by XTT assay. Metabolism in the presence of RPMI alone (leftmost bar) was regarded as 100%. Comparisons: RPMI vs all rhamnolipid concentrations, or VCZ (leftmost black bar) vs. all rhamnolipid/VCZ combinations. Other comparisons as indicated by the ends of the brackets. Statistical analysis: 1-way ANOVA for dose-response-curves, unpaired t-test for comparisons indicated by brackets: two or three asterisks = $p \leq 0.01$ or $p \leq 0.001$, respectively.

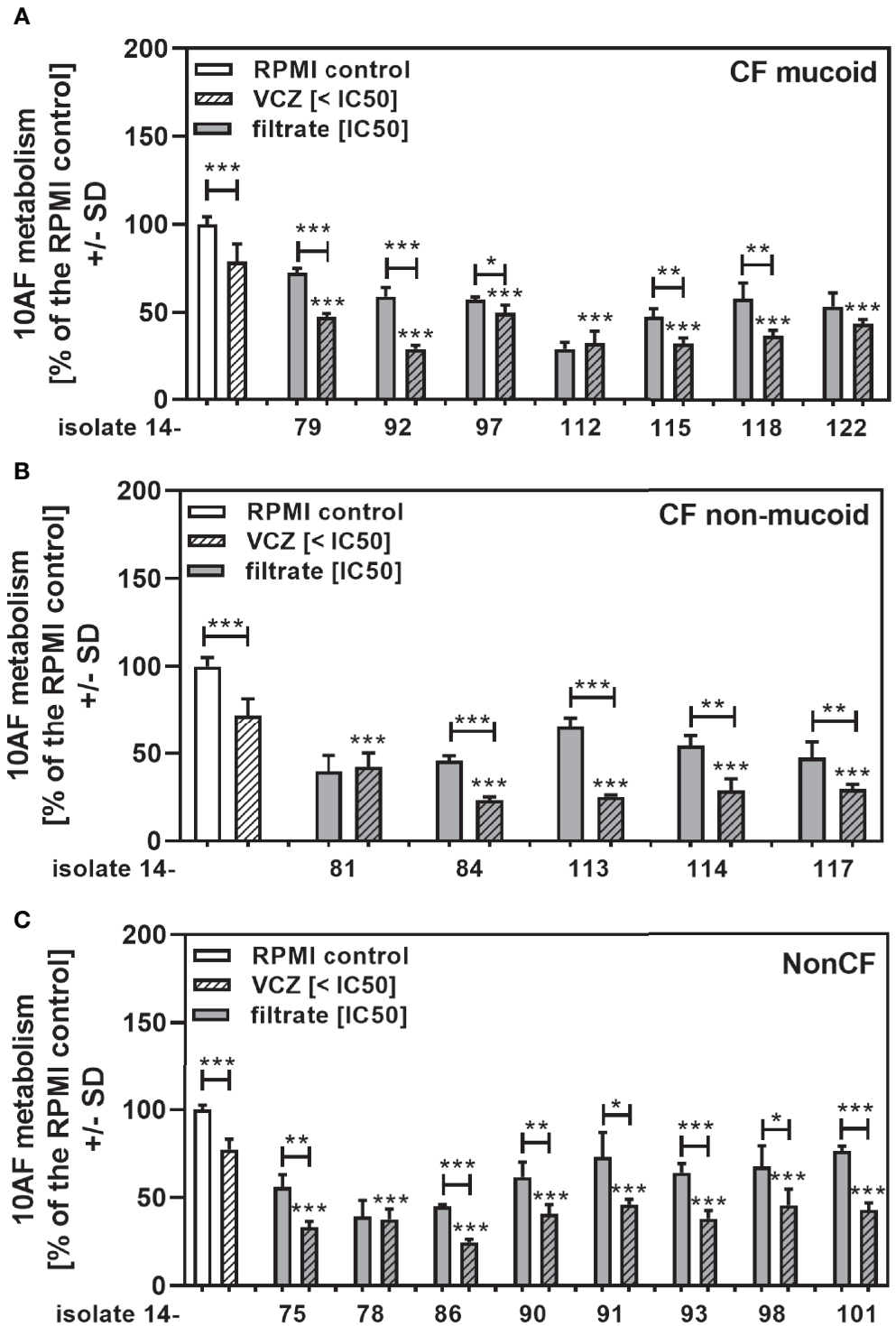

FIGURE 8 | Anti-fungal effects of the combination of CF mucoid, CF non-mucoid, or non-CF *P. aeruginosa* clinical isolate filtrates at their IC50s with VCZ below its IC50. CF mucoid **(A)**, CF non-mucoid **(B)**, or non-CF isolate filtrates **(C)** were diluted to be used at final concentrations closest to their IC50, and combined with VCZ at concentrations below its IC50, to test their combined antifungal activities against 10AF forming biofilm (10^5 conidia/ml in RPMI 1640 medium). Assay plates were incubated at 37°C overnight. 10AF fungal metabolism was measured by XTT assay. Metabolism in the presence of RPMI alone (white bar) was regarded as 100%. X-axis shows isolate numbers. Comparisons without brackets: VCZ alone (striped white bar) vs. the individual isolate filtrate combination with VCZ (gray striped bar). Other comparisons as indicated by the ends of the brackets. Statistical analysis: Unpaired t-test: one, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

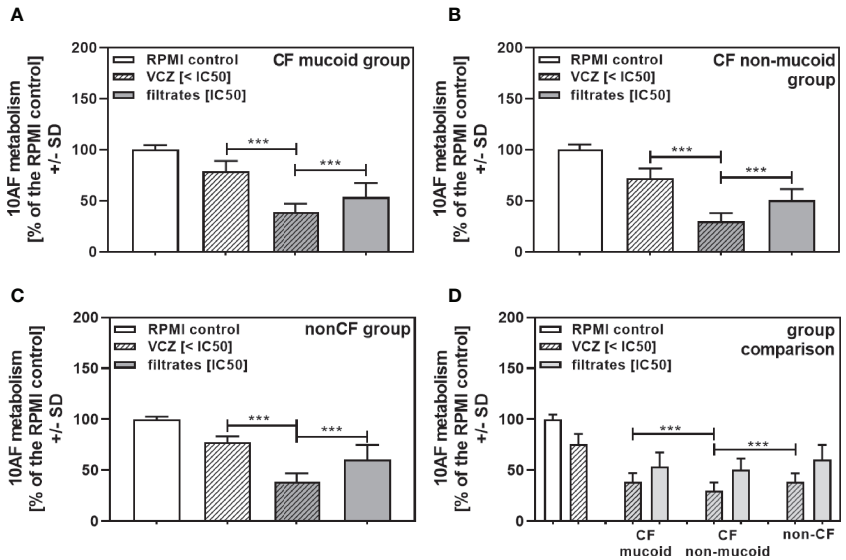

FIGURE 9 | Group comparisons for anti-fungal effects of the combination of CF mucoid, CF non-mucoid, or non-CF *P. aeruginosa* clinical isolate filtrates at their IC50s with VCZ below its IC50. Individual isolate interactions with VCZ shown in **Figure 7** were combined per group [CF mucoid **(A)**, CF non-mucoid **(B)**, or non-CF isolate filtrates **(C)**]. **(D)** Comparison among effects of VCZ/filtrate combinations, shown in **(A–C)**, on 10AF biofilm formation. Metabolism in the presence of RPMI alone (white bar) was regarded as 100%. Comparisons as indicated by the ends of the brackets. Statistical analysis: Unpaired t-test: three asterisks = $p \leq 0.001$.

during *Aspergillus–Pseudomonas* co-infections *in vivo* it might imply the possibility of reducing VCZ therapeutic doses, reducing side effects. On the other hand, treating *Pseudomonas* infection during co-infection with *Aspergillus* might result in the necessity for augmenting VCZ dosing. It is becoming more common to isolate VCZ-resistant *A. fumigatus* strains from CF patients (Hamprecht et al., 2018). Therefore, the observed synergistic effect with the mutant TR34/L98H pan-azole resistant isolate highlights the potential importance of these findings in future therapeutic options. Future studies of interest would include exploring the interaction of VCZ with *A. fumigatus* strains of increased drug vulnerability, such as variants that lack wall galactosaminogalactan.

In general, anti-fungal and synergistic activity of clinical isolates is weaker than that of laboratory isolates, although pyoverdine production is comparable (compare **Table 2** to **Figure 6D** and **Supplemental Figure 1**). In our previous study using laboratory isolates of *Pseudomonas* we identified three bacterial factors that interacted synergistically with VCZ, i.e., pyoverdine, pyocyanin, and pyochelin (Sass et al., 2021). In the present study we encountered four isolates with markedly deficient pyoverdine production (isolates 14-82, 14-89, 14-116, and 14-119) for which we observed similar growth to pyoverdine-positive isolates, measured by OD610. With the loss of pyoverdine production these isolates lost most of their anti-fungal activity, which could be expected, as pyoverdine is a major anti-fungal factor under iron-limiting conditions (Sass et al., 2017). Although pyoverdine seemed to be a major supporter of VCZ anti-fungal activity (Sass et al., 2021), all 4 isolates showed strong synergy with VCZ when used at high concentrations. When used at low concentrations two of the 4 isolates still acted synergistically with VCZ, which suggests the presence of high amounts of anti-fungal factors other than pyoverdine in bacterial filtrates that support VCZ anti-fungal activity. Under the experimental conditions used here (iron-limited medium) it is unlikely that these molecules are phenazines, such as pyocyanin, as pyocyanin induction requires elevated amounts of iron in growth medium (Sass et al., 2020). We also observed that under non-limiting iron conditions very few of our clinical isolates produced pyoyanin, and of the four pyoverdine-negative isolates only 14-119 was able. Synergy of filtrates produced in the presence of iron with VCZ was reduced, compared to filtrates produced under iron-limiting conditions. The third previously identified molecule that supports VCZ anti-fungal activity is pyochelin (Sass et al., 2021). We here could show that rhamnolipids as well are VCZ-synergistic *Pseudomonas* molecules. There are other anti-fungal *Pseudomonas* molecules known that are present in bacterial filtrates, such as 3,4-dihydroxy-2-heptylquinoline (PQS, Nazik et al., 2020), or 4-hydroxy-2-heptylquinoline (HHQ, Nazik et al., 2021), which might add to synergy with VCZ.

Further preliminary data show that harsh heating of pyoverdine negative isolates (95°C for 30 min) significantly reduced their synergistic anti-fungal activity with VCZ ($p \leq 0.01$ for one isolate, $p \leq 0.001$ for 3 isolates). These data indicate that besides the predominant synergistic factor pyoverdine there are other heat-sensitive and heat-stable *Pseudomonas* molecules present that synergistically interact with VCZ against *Aspergillus* forming biofilm metabolism. The repertoire of an individual *Pseudomonas*

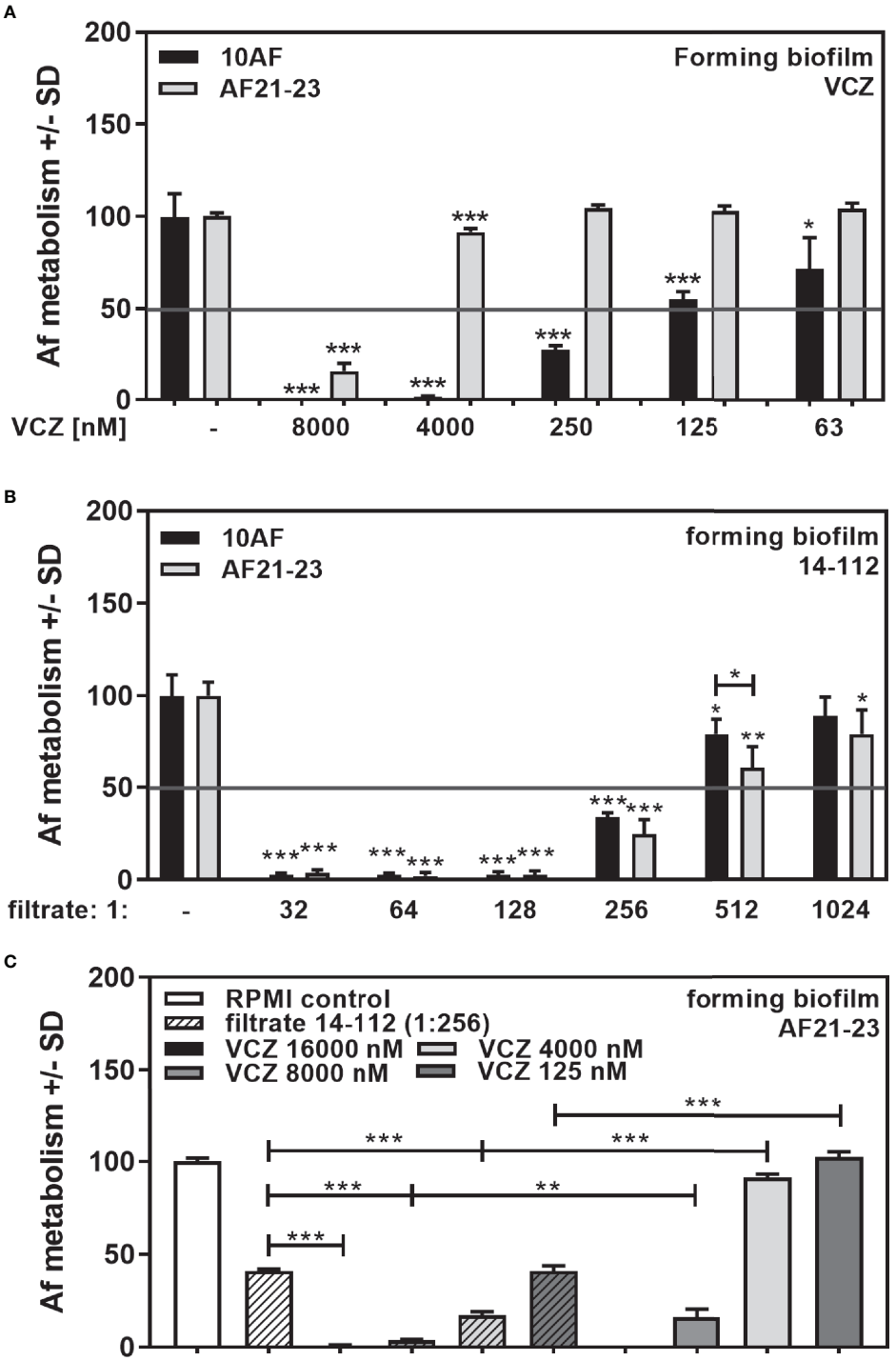

FIGURE 10 | Anti-fungal effects of the combination of *P. aeruginosa* clinical isolate filtrate at 1:256 dilution with VCZ on a VCZ-resistant fungus. VCZ-susceptible (10AF) or VCZ-resistant (AF21-23) *A. fumigatus* were challenged with VCZ [**(A)** 63, 125, 250, 4,000 or 8,000 nM], *Pseudomonas* filtrate [**(B)** isolate 14-112 at 1:32 to 1:1,024 dilution], or **(C)** a combination of VCZ (125. 4,000, 8,000, or 16,000 nM) with 14-112 filtrate (1:256). Comparisons without brackets: untreated fungus vs all treated samples of the same groups. Other comparisons as indicated by the ends of the brackets. Statistical analysis: Unpaired t-test: one, two or three asterisks = $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

isolate of produced molecules varies, and it has to be seen if there are common variations within clinical isolate phenotype groups, e.g., mucoid or non-mucoid.

In summary, these data show that clinical *P. aeruginosa* isolates, at varying degrees, synergistically interact with VCZ, and that pyoverdine is not the only molecule responsible.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization, DAS. Data curation, DAS, GS, PS, and JJM. Formal analysis, DAS, GS, PS, and JJM. Funding acquisition, DAS. Investigation, DAS, GS, PS, and JJM. Methodology, DAS, GS, PS, and JJM. Project administration, DAS and GS. Resources, DAS and RS. Software, GS. Supervision, DAS and GS. Validation, DAS, GS, PS, and JJM. Visualization, DAS, GS, PS, and JJM. Writing—original draft, GS. Writing—review & editing, DAS, GS, PS, JJM and RS. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.817315/full#supplementary-material

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Challenges to establish the diagnosis of aspergillosis in non-laboratory animals: looking for alternatives in veterinary medicine and demonstration of feasibility through two concrete examples in penguins and dolphins

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Aspergillosis remains difficult to diagnose in animals. Laboratory-based assays are far less developed than those for human medicine, and only few studies have been completed to validate their utility in routine veterinary diagnostics. To overcome the current limitations, veterinarians and researchers have to propose alternative methods including extrapolating from human diagnostic tools and using innovative technology. In the present overview, two specific examples were complementarily addressed in penguins and dolphins to illustrate how is challenging the diagnosis of aspergillosis in animals. Specific focus will be made on the novel application of simple testing in blood based on serological assays or protein electrophoresis and on the new information garnered from metabolomics/proteomics to discover potential new biomarkers. In conclusion, while the diagnostic approach of aspergillosis in veterinary medicine cannot be directly taken from options developed for human medicine, it can certainly serve as inspiration.

KEYWORDS

Sphenisciformes, *Spheniscus, Tursiops*, western blot, cetaceans, mass spectrometry, iTRAQ (Isobaric tagged for relative and absolute quantitation), protein electrophoresis

Introduction

Aspergillosis is a fungal airborne disease caused by ubiquitous molds belonging to the *Aspergillus* genus, and primarily by *Aspergillus fumigatus* species (Desoubeaux et al., 2014). In animals, aspergillosis can infect a wide range of species from invertebrates, such as corals, to higher vertebrates (Seyedmousavi et al., 2015). In the latter, the course of the disease and the clinical signs can vary greatly. For instance in penguins, aspergillosis is mostly observed in individuals managed under human care and is represented by the subacute development of granuloma and plaques at the surface of lungs and air sacs (Desoubeaux et al., 2018; Cateau et al., 2022). In contrast in cetaceans, aspergillosis course is based on a chronic invasive process of lungs, and then other organs like brain, which is generally indicative of another disease and/or (sub-)acute physiologic stress (Bunskoek et al., 2017; Desoubeaux et al., 2018), but it is rarely associated with severe immunosuppression and profound neutropenia (Seyedmousavi et al., 2015)..

For veterinarians and all staff that takes care of animals, the diagnosis of aspergillosis is often quite challenging because laboratories tools are neither numerous nor accurate enough (Cray et al., 2009; Desoubeaux et al., 2018; Elad and Segal, 2018; Tell et al., 2019), and medical imaging is not readily available in every facility (Jones and Orosz, 2000). Furthermore, there is no approved classification for helping to rank the cases according to the level of evidence, as is found in human medicine (Donnelly et al., 2020).

In order to more accurately address the diagnosis of aspergillosis in animals, a novel approach can consist in trying to extrapolate new tools initially-intended for human medicine to veterinary one. Another possibility is identifying high-put screening potential biomarkers by the means of innovative technology such as metabolomics or proteomics. Thus, in the present article primarily intended to veterinarians and staff of animal diagnostic laboratory, we will specifically discuss two complementary examples in penguins and dolphins to illustrate studies of aspergillosis in animals and diagnostic options which have been defined in this research.

What are the options to achieve a more accurate diagnosis in penguins?

A large number of avian species can be infected with *Aspergillus* (Seyedmousavi et al., 2015). Penguins are especially at risk (Desoubeaux et al., 2018)3 Several reasons have been raised for explaining this finding. First, most species belonging to Spheniscidae family that have burrowing behavior, *e.g.* Magellanic (*Spheniscus magellanicus*), Humboldt (*Spheniscus humboldti*) or African penguins (*Spheniscus demersus*), so that the birds are potentially exposed to fungal spore clouds when disturbing soil. Secondly, captive conditions in zoological parks or aquaria can enhance the presence of stress related factors which may predispose penguins to aspergillosis: for example, massive afflux of visitors, long transfers between two facilities, dirty habitats with dampness, and bad ventilation or excess of ammonium derivatives (Miller and Fowler, 2014; Terio et al., 2018). Moreover, aspergillosis seems more commonly diagnosed in juveniles – potentially more fragile birds – than adults (Xavier et al., 2007; Terio et al., 2018; Cateau et al., 2022). Altogether, its relative prevalence can reach 20-27% in some penguin colonies under managed care (Filho RP da et al., 2015; Krol et al., 2020), and its incidence was recently measured at ~3.4% case-years in a French zoological park (Cateau et al., 2022). However, one should be aware that great discrepancies can be observed between centers depending on the occurrence variations of co-morbidities and the availability of diagnostic means of diagnosis (e.g. access to medical imaging).

The clinical course of aspergillosis is mostly subacute or chronic in birds (Seyedmousavi et al., 2015). It can lead to weight loss and lethargy, open mouth-breathing, wheezing, coughing, altered vocalization and dyspnea (Miller and Fowler, 2014), and eventually death in up to 50% infected penguins (Filho RP da et al., 2015). *Antemortem* diagnostics can involve a host of ancillary testing including routine hematology, biochemistry, and radiography among others many of which can only provide supportive or suggestive results to help form the diagnosis (Jones and Orosz, 2000). The curative treatment is based on azole drugs, like voriconazole tablets hidden in the food or itraconazole suspension administrated by pipette directly into the esophagus. Mass antifungal prophylaxis is not practiced in penguins and usually restricted only to individuals that are fragile or at risk (Xavier et al., 2007; Miller and Fowler, 2014).

Necropsy can offer the possibility to observe nodules in the lung parenchyma. In addition, whitish plaques are sometimes visible in the air sacs and at the inner surface of the trachea providing conclusive confirmation of the fungal etiology (Desoubeaux et al., 2014). The direct examination through histopathology or cytology can reach up 90% sensitivity (personal data; under submission). In contrast, *ante mortem* diagnostics usually have poor performance. First, biopsy sampling can be difficult to perform in ill or debilitated subjects. Colony-forming unit (CFU) counting based on *in vitro* mycological culture is not reliable enough to estimate the actual *Aspergillus* burden, because it does not reflect the total amount of viable, dormant and dead fungus within the tissue or the fluid that is investigated (Desoubeaux et al., 2014; Melloul et al., 2014). Since the penguin respiratory system may be colonized by *Aspergillus* spp., it may be difficult to distinguish between true infection and simple colonization on the basis of the culture alone (Desoubeaux et al., 2014). Detection of galactomannan antigen in plasma is not reliable in some birds

and is frequently found falsely negative or only slightly positive in infected penguins (Cray et al., 2009). For example, Desoubeaux et al. reported no significant difference in mean galactomannan index of 0.6, 0.5 and 0.2 in 47 *Aspergillus*-diseased African penguins, 29 control penguins with miscellaneous inflammatory conditions, and 96 clinically-normal penguins, respectively (P=0.14) (Desoubeaux et al., 2018). Some lateral flow devices with murine JF5 and MAb476 antibodies were developed to detect *Aspergillus* antigens in human blood, relying on the same technical principle as for a pregnancy test in urine (Savelieff et al., 2018). These devices were tested in birds, mostly penguins, but with poor results to date (personal data unpublished).

The (Desoubeaux et al., 2014; Seyedmousavi et al., 2015; Cateau et al., 2022)-β-D-glucan is a cell wall pan-fungal component with a high negative predictive value (Donnelly et al., 2020), but with no specificity to *Aspergillus* genus. Highly variable levels of this marker were reported in experimentally and naturally infected birds, as well as control birds, negating then its potential ready application to avian medicine (Burco et al., 2012). Its sensitivity and specificity were assessed at 60.0 and 92.7% with an elevated cutoff established at 461 pg/dL (*vs*. 80 in human medicine). Measurement of anti-*Aspergillus* antibody by the means of ELISA kits based on a crude antigen preparation has been reported to be consistently positive in penguin blood regardless of clinical status (Cray et al., 2009). In the aforementioned study, the mean indices of antibody were 1.8, 1.7 and 1.7 for the infected population, the inflammatory controls, and the healthy subjects (according to a positive cutoff established at 1.4 index), respectively (Desoubeaux et al., 2018). Recently listed among the diagnostic options acknowledged for the use in humans (Donnelly et al., 2020), *Aspergillus* qPCR has been rarely reported in birds (Melloul et al., 2014). Usually, it targets the ribosomal RNA subunits, like the 28S subunit (Chauvin et al., 2019). In a recent study performed in a large French zoological park, its sensitivity and specificity performance were evaluated at ≈84% and 100% in lung biopsies obtained from deceased individuals belonging to a colony of ≈130 Humboldt penguins (Cateau et al., 2022).

As simple and low-cost alternative, some authors suggested to focus on plasma protein electrophoresis (EPH) to address the diagnosis of aspergillosis (Cray et al., 2009). Valid quantitation of certain protein fractions through EPH was demonstrated to provide a reflection of acute phase responses (Cray et al., 2011): a decreased albumin was often observed in tandem with the increase of four globulin fractions, including α1-, α2-, β- and γ-globulins (Kaneko, 2008) (Figure 1, 2). In another study, a moderate decrease in percent albumin was supportive of its designation as a negative acute phase protein and showed a strong negative predictive value for assessing survival in gentoo penguins (*Pygoscelis papua*) (Naylor et al., 2017). The clinical significance of prealbumin changes in birds has not been clearly defined, but a decrease was observed in experimentally-infected falcons (Kummrow et al., 2012; Fischer et al., 2014), and in naturally-infected African penguins (0.32 *vs*. 0.39g/dL in healthy controls; P=0.006) (Desoubeaux et al., 2018). Elevation of haptoglobin is proposed to be related to the approximate +0.33 g/dL change of α2-globulin fraction that was observed in some recent studies (Desoubeaux et al., 2018). Greater levels of γ-globulins are consistent with the stimulation of humoral immunity, and two fold increased *vs*. control animals were observed (Desoubeaux et al., 2018). Overall, the presence of these EPH based abnormalities is sufficient to initiate preemptive antifungal treatment. Semi-automated methods of protein fractionation have been commercialized for veterinary purposes over the last 20 years (Tatum et al., 2000; Kaneko, 2008; Cray et al., 2009; Cray et al., 2011).

Based on laboratory animal models, several studies suggest that the detection of gliotoxin could have a strong potential as diagnostic signature of aspergillosis (Lewis et al., 2005; Cerqueira et al., 2014; Sugui et al., 2017). Gliotoxin is produced by *Aspergillus* during its hyphal growth and is the most abundant mycotoxin playing the role of key-virulence factor that results in far-reaching immune suppression of the host (Kwon-Chung and Sugui, 2009; Arias et al., 2018). Through measurement with high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) methodology, we recently tested the relevancy of gliotoxin detection for the diagnosis of aspergillosis in blood samples drawn from African, Humboldt, gentoo and Magellanic penguins among other avian species (Reidy et al., 2022). In all, gliotoxin was detected in almost 74% of the clinical samples obtained from birds with proven or probable aspergillosis, but was not detected in samples from clinically-normal penguins. A poor prognostic was associated to repeated measures from birds undergoing unsuccessful treatment. Gliotoxin positive rates were higher in confirmed rather than probable cases.

Recently, proteomic and metabolomic studies provided new opportunities of exploration for discovering potential biomarkers of infection including aspergillosis (Desoubeaux et al., 2014). For example, a significant increase of some ketone bodies, lipoprotein, and fatty acids, including 3-hydroxybutyrate, was firstly described by metabolomics in the blood of infected falcons (Pappalardo et al., 2014). Elevation of 3-hydroxybutyrate in plasma was subsequently confirmed in a cohort of 47 infected African penguins *vs*. 115 controls (Desoubeaux et al., 2018). Notably, it was associated with the increase of the absolute concentrations of β-globulins and α2-globulins. Using these measures in tandem resulted in high specificity (>90%) and high negative predictive value (≥80%), thus suggesting that basic EPH testing in combination with 3-hydroxybutyrate can provide reliable evidence for the absence of aspergillosis diagnosis, when all are negative. In the same study, when 3-hydroxybutyrate concentration was elevated > 1.9 mmol/L, the clinical prognosis was quite poor. In contrast, the

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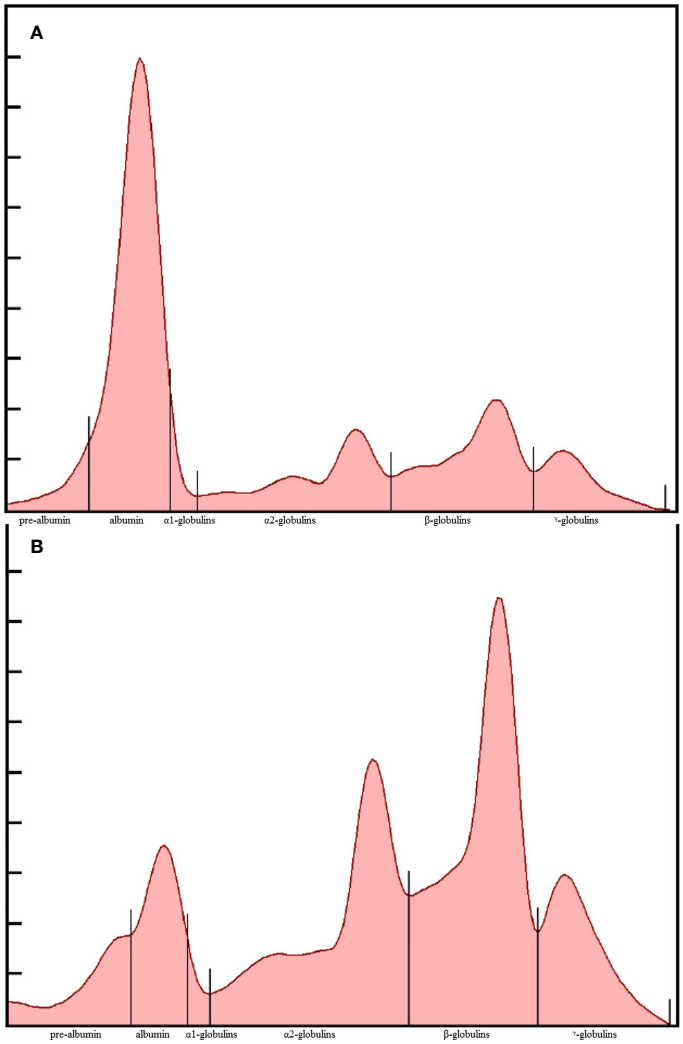

FIGURE 1
Representative plasma protein electrophoretograms of a clinically-normal **(A)** and *Aspergillus*-diseased African penguin (*Spheniscus demersus*) **(B)**. The fractions, from left to right, are prealbumin, albumin, α1, α2, β, and γ - globulins.

levels returned to normal when penguins recovered. Through its great sensitivity, proteomics may contribute to the discovery of new markers of aspergillosis (Desoubeaux et al., 2014). Interestingly, a second example of exploratory study based on proteomics evidenced several significant changes in protein representation in infected penguins. For instance, F-box/LRR-repeat protein 4, THAP domain-containing protein 1, histidine-tRNA cytoplasmic ligase and AIM1 (absent in melanoma-1) protein were found 4.4-, 2.5-, 2.5-, 2.2-fold overexpressed in the blood of diseased subjects *vs.* non-*Aspergillus* inflammatory controls (Desoubeaux et al., 2018). Globally, it is noteworthy to report that three protein pathways were significantly enriched during aspergillosis processes: cadherin, Wnt and FGF signaling pathways. Cadherin pathway is involved in cell adhesion by forming *adherens* junctions to bind cells within tissues together (Brüser and Bogdan, 2017). Wnt signal stimulates several intracellular signal transduction cascades, including the canonical or Wnt/β-catenin dependent pathway and the non-canonical or β-catenin-independent pathway (Habas and Dawid, 2005). FGF pathway plays a role during metabolic disorders or in injured tissues, where it mediates metabolic functions, tissue repair, and regeneration, often by reactivating developmental signaling pathways. In another study using high resolution capillary electrophoresis and mass spectrometry methods, several changes in acute phase proteins including fibrinogen and haptoglobin, as well as lipoproteins, were identified in the plasma of an African penguin with confirmed aspergillosis (Valdivia et al., 2020).

In managed care scenarios, as are common to penguins, ideal testing should provide diagnostic impact as well as an option to

use as a health surveillance tool to monitor the population for potential outbreaks so that early action may be taken. In addition, given the cost and labor involved in treatment, such tools should also provide prognostic value. As discussed here, it is doubtful that there will be a single tool that can provide such a broad implementation. Galactomannan and gliotoxin can aid in diagnosis but electrophoresis, hydroxybutyrate and antibody titers may best reflect prognosis. Overall, while the diagnostic role of new proteins and tools in penguins is unknown, their description broadens the perspectives of investigations and warrants additional studies to confirm their potential interest. In addition, various proteomic tools and methods may provide differing results; their use should be equally considered to aid in the potential identification of novel biomarkers.

What are the diagnostic means in dolphins?

In marine mammals, aspergillosis is considered rare, but it has been recently reported with increasing frequency with more than two thirds of cases have been published after the year 2000 (Barley et al., 2007; Dagleish et al., 2008; Abdo et al., 2012). Cetaceans like dolphins live in aquatic media and *Aspergillus* spores are known to be also present in water. It is plausible that aspergillosis can occur in dolphins *via* water contamination although those in managed care may be further exposed due to environmental/air contamination in enclosed facilities. In dolphins, the risk factors are not clearly elucidated. A case report indicated a likely immune suppression related to a morbillivirus infection may have resulted in fatal aspergillosis in a free-ranging dolphin (Cassle et al., 2016). Overall, however, reports are not associated with severe neutropenia as seen in humans (Seyedmousavi et al., 2015), and instead, the development of aspergillosis is thought to be based on a chronic invasive process which is usually secondary to another condition like stress or other co-infection (Bunskoek et al., 2017), as it was seen in non-neutropenic humans with severe COVID-19 and/or influenza diseases. Underlying pulmonary disease may also affect host defense mechanisms in dolphins, leading to colonization and potential invasion of bronchial tissue by *Aspergillus* spp., generating one or several nodule(s) (Reidarson et al., 1998). In such a context, coughing, abnormal vocalizations, hard chuffing, radiological changes, and all signs related to tracheitis, bronchitis, pneumonia, pleurisy, can be reported. Other organs, like the brain, may also be infected following bloodstream dissemination (Dagleish et al., 2006; Dagleish et al., 2008; Abdo et al., 2012). *Aspergillus fumigatus* species is mostly involved in cetacean aspergillosis (Lamoth, 2016), followed by species belonging to the *Nigri* section and *Terrei* section, respectively (Balajee, 2009). For the curative treatment, several options exist, like nebulizing amphotericin B or some azole drugs directly into the blow hole (Bunskoek et al., 2017) or oral administration of voriconazole.

For the diagnosis of aspergillosis in dolphins, *antemortem* laboratory tools are largely less developed than for humans and not validated (Dagleish et al., 2008; Delaney et al., 2013; Cassle et al., 2016). Some diagnostic procedures are difficult to perform (Desoubeaux et al., 2014), especially because medical imaging (*e.g.* computed tomography (CT), magnetic resonance imaging (MRI), or even endoscopy) is not readily available. Positive culture from respiratory specimens may reflect a simple colonization of the upper airways or represent an environmental contaminant (Desoubeaux et al., 2014). Also, as reported in humans (Desoubeaux et al., 2014), blood cultures are usually also non diagnostic for aspergillosis in dolphins even in disseminated cases. Detection of galactomannan antigen is not sensitive: in a cohort of 87 common bottlenose dolphins (*Tursiops truncatus*), there were no differences between the *Aspergillus*-diseased cases *vs*. the controls (mean indices of 0.2 *vs*. 0.3) (Desoubeaux et al., 2018). Rare studies regarding the use of *Aspergillus* qPCR (usually targeting repeated regions of the ribosomal RNA subunits) in dolphins are available (Groch et al., 2018), so that it is difficult to definitively conclude on the diagnostic potential.

In contrast, as dolphins are not specifically immunocompromised during aspergillosis, serological testing to detect anti-*Aspergillus* antibodies is postulated as a reasonable option as reported in chronic infection in humans (Kurup, 2005; Persat, 2012). With a lab developed ELISA assay, antibody levels were higher in 32 diseased dolphins *vs.* 55 controls, at 1: 1024 *vs.* 1: 256 median titer dilution. The same study also reported the accuracy of a commercial western blot (WB) assay developed for use in humans that was adapted to dolphin serum *via* the use of a species specific conjugate antibody. Focusing on reactivity to four distinct WB bands that are also present in human samples at 30, 22, 18-20, and 16 kDa (Oliva et al., 2015), a minimum score of 5 (of a maximum of 16) was proposed to distinguish between the infected and non-infected dolphins (**Figure 2**). Seroconversion was consistently observed before positive fungal culture, and the specificity was 93%, regardless of the *Aspergillus* species involved. The cross-reactivity with other fungal genera was not observed and the WB testing also exhibited a valuable prognostic value.

To understand more about the pathophysiology of infection and possibly identify new tools, some studies have used mass spectrometry to study aspergillosis in dolphins (Desoubeaux et al., 2019). Several over-represented proteins which play a role in the adaptive immune response were identified, including MHC (major histocompatibility complex) proteins and others involved in catalytic activity like the NADPH-ubiquinone oxido-reductases or cytochrome b. The former are believed to be required for catalysis which functions in the transfer of electrons to the respiratory chain required for enzymatic activity to fight against aspergillosis. Noteworthy in the same study, no markers were shared with blood samples from infected humans, except one, so called Testis expressed 11 (Fragment)

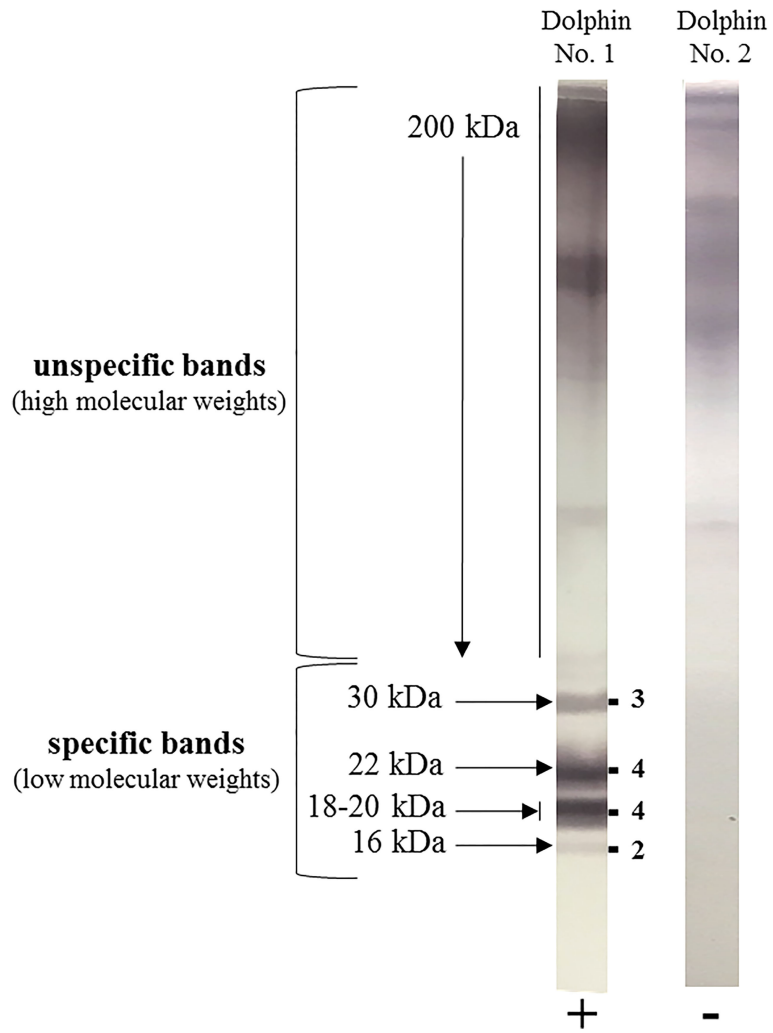

FIGURE 2
Example of two blood samples from common bottlenose dolphins (*Tursiops truncatus*) tested for anti-*Aspergillus* antibody by the *Aspergillus* Western blot IgG® kit (LDBio Diagnostics, Lyon, France). Dolphin N°1 was found positive (+) with a global Western blot score of 13/16, as indicated on the right side of the immunoblot strip by the sum of the respective band intensities observed at 30, 22, 18-20 and 16 kDa. Dolphin N°2 was found negative (-).

protein, >3-fold increased in both species and involved in the cell organization and biogenesis; metabolic process; regulation of biological process (Desoubeaux et al., 2018; Desoubeaux et al., 2019). Further studies are warranted to validate the relevancy of these proteins in the diagnostic process of aspergillosis.

Conclusion

This brief overview highlights the difficulty in the diagnosis of aspergillosis in animals (Elad and Segal, 2018; Tell et al., 2019). *Antemortem* lab-based methods derived from human medicine need to be implemented and validated with the goal to increase the accuracy of the diagnosis in animals. Proteomic studies may reveal new biomarkers which may be unique to infection in some animal species or possibly aid in enhanced diagnosis in mammals including humans. When one can do without invasive specimens and advanced imaging, using blood for such investigations represents great opportunity to commence a pathway to diagnosis. However, results from these studies need to be carefully interpreted (Desoubeaux et al., 2018; Desoubeaux et al., 2019). Animals may more likely to be colonized and/or chronically infected than humans. Moreover, the clinical signs depend on the host species and the underlying conditions or comorbidities may greatly vary. In addition, the lack of consensus regarding the disease classification in animals can have great impact on the reliability of all the studies published thus far (Donnelly et al.,

2020), and that protein libraries available are lacking data related to non-traditional species *vs.* humans and laboratory animals (Mi et al., 2017). Lastly, in the absence of neutropenia and severe immune compromise as seen in transplant recipients, the pathology and immune response of aspergillosis in birds and dolphins should be rather compared to those of subacute/chronic human aspergillosis and any diagnostic tools which are developed and/or adapted address these opportunities for sensitive and specific options for antemortem diagnosis.

Data availability statement

The original contributions presented in the study are included in the article/**supplementary material**. Further inquiries can be directed to the corresponding author.

Author contributions

GD led and supervised the writing, CC edited the text (English native speaker) and brought specific comments, AC corrected the manuscript and proposed some improvements. All authors contributed to the article and approved the submitted version.

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Conflict of interest

GD was specifically invited by the editorial board to write this manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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