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Proteomics as a Tool to Identify New Targets Against *Aspergillus* and *Scedosporium* in the Context of Cystic Fibrosis

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Abstract Cystic fibrosis (CF) is a genetic disorder that increases the risk of suffering microbial, including fungal, infections. In this paper, proteomics-based information was collated relating to secreted and cell wall proteins with potential medical applications from the most common filamentous fungi in CF, *i.e., Aspergillus* and *Scedosporium/Lomentospora* species. Among the *Aspergillus fumigatus* secreted allergens, β-1,3-endoglucanase, the alkaline protease 1 (Alp1/oryzin), Asp f 2, Asp f 13/15, chitinase, chitosanase, dipeptidyl-peptidase V (DppV), the metalloprotease Asp f 5, mitogillin/Asp f 1, and thioredoxin reductase receive a special mention. In addition, the antigens β-glucosidase 1, catalase, glucan endo-1,3-β-glucosidase EglC, β-1,3-glucanosyltransferases Gel1 and Gel2, and glutaminase A were also identified in secretomes of other *Aspergillus* species associated with CF: *Aspergillus flavus, Aspergillus niger*, *Aspergillus nidulans*, and *Aspergillus terreus*. Regarding cell wall proteins, cytochrome P450 and eEF-3 were proposed as diagnostic targets, and alkaline protease 2 (Alp2), Asp f 3 (putative peroxiredoxin pmp20), probable glycosidases Asp f 9/Crf1 and Crf2, GPI-anchored protein Ecm33, β-1,3-glucanosyltransferase Gel4, conidial hydrophobin Hyp1/RodA, and secreted aspartyl protease Pep2 as protective vaccines in *A. fumigatus*. On the other hand, for *Scedosporium/Lomentospora* species, the heat shock protein Hsp70 stands out as a relevant secreted and cell wall antigen. Additionally, the secreted aspartyl proteinase and an ortholog of Asp f 13, as well as the cell wall endo-1,3-β-D-glucosidase and 1,3-β-glucanosyl transferase, were also found to be significant proteins. In conclusion, proteins mentioned in this review may be promising candidates for developing innovative diagnostic and therapeutic tools for fungal infections in CF patients.

Keywords Fungus · Antigen · Allergen · Enzyme · Secreted · Protein · Cell wall

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Cystic Fibrosis, Fungi, and Proteomics

Cystic fibrosis (CF) is the most common lethal inherited disease among Caucasian people, affecting the production of several body secretions such as sweat, digestive fluids, and mucus. In consequence, these patients are susceptible to various diseases, mainly respiratory infections, since the accumulation of anomalous mucus in the respiratory tract promotes microbial growth. For many years, the association between numerous bacterial species and serious

outcomes in patients suffering from CF is clear, these include *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia,* and *Burkholderia cepacia*. On the contrary, although some studies correlate the presence of fungi with decreased lung function and increased frequency of disease exacerbations in CF patients [1–3], and there is no totally conclusive evidence that they are responsible for it [4, 5]. The use of prolonged antibacterial and corticosteroid therapy in these patients facilitates the presence and growth of fungi, and consequently their association may be secondary to clinical status and/or treatment.

However, the prevalence of fungi in CF patients, as in other diseases, was until recently underestimated because of the use of inadequate experimental approaches to detect and identify them [6]. In contrast, over the last few years, the use of selective media and molecular techniques proved that fungi are commonly present in more than 75% of the CF sputum samples [7–9], the most important being *Aspergillus* and *Scedosporium/Lomentospora* species among the filamentous fungi, and *Candida albicans* for yeasts. Therefore, it is important to detect fungal presence in CF patients, not only because it may cause respiratory decay, but also because it can lead to serious diseases such as bronchitis [10] and allergies [11]. To diagnose mycoses caused by these species, in addition to culture-based and molecular techniques, e.g., PCR, the detection of fungal antigens and antifungal host immunoglobulins (Ig) plays an important role [12, 13], and the latter also being useful to monitor the response to antifungal treatment. However, these methods still present some problems that must be solved.

Nowadays, one of the deficiencies in fungal diagnosis is the lack of assays to detect fungal pathogens with low prevalence, such as *Scedosporium* or *Exophiala*, because diagnosis kits are commercially available only for the most common species, mainly *Candida* and *Aspergillus*. Furthermore, there is still a worrying low sensitivity and specificity for the detection of both fungal antigens and host antibodies. Diagnostic assays offer a suboptimal sensitivity even for the detection of an invasive infection [13] and do not seem to be suitable for the detection of colonization or other less severe diseases such as bronchitis. Additionally, although commercially available assays offer standardized and reproducible tools, there is still a great problem with their specificity, since most of them are based on non-specific molecules, such as galactomannan and 1,3-β-D-glucan, or even on protein extracts or whole cells [12, 13]. Therefore, the cross-reactivity that may occur with other fungi makes these kits unsuitable for the discrimination between different fungal etiologic agents.

Moreover, antibody levels may differ between healthy individuals and patients with infection, colonization, or risk of infection. This is a problem, especially for CF patients who can present higher concentrations of antifungal antibodies than the commercially upper normal limit, as is the case with anti-*Aspergillus* IgG, for instance [14]. Therefore, to avoid false-positive or false-negative results in antibody detection, the use of disease-specific fungal antigens or an improved determination of the detection limits in each case are necessary.

Because of this, greater efforts must be made to identify new fungal molecules with medical applications which may result from proteomics studies. To do this, comparative studies between different microorganisms, conditions, or host samples are of special relevance. Comparison between different fungal species can be useful to identify not only species-specific molecules, but also putative panfungal targets depending on whether attention is focused on the differentially expressed proteins or on the ones that are shared, respectively. Moreover, infection-related proteins could be identified by looking for variations in their expression in pathogenic fungi in comparison with non-pathogenic isolates or by comparison of a strain after in vitro growth with the same strain during/after an infection. From all these studies, highly expressed molecules should stand out and can easily be targeted for therapy or diagnosis (Fig. 1a).

In addition, immunoproteomics-based studies using samples from healthy or infected individuals, such as serum, bronchoalveolar lavage or saliva, may also allow the identification of interesting molecules. The study of antigens differentially recognized by infected and non-infected patients has commonly been used with many pathogens to identify molecules that may be useful for serodiagnosis. However, few studies have focused on healthy populations that have been in contact with the fungus but have not suffered from any infection. These individuals may have developed a protective response and the antigens detected by their antibodies might be candidates for vaccine development, identification of therapeutic targets or even to

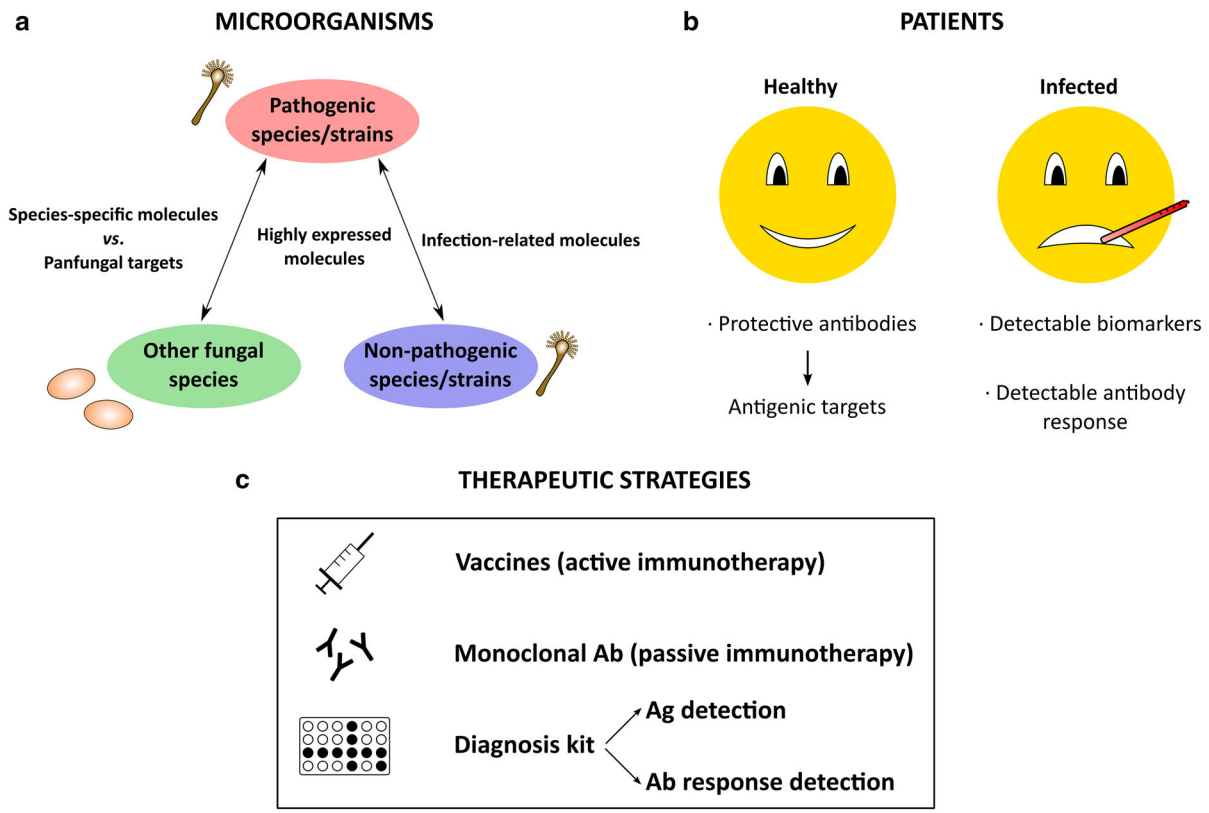

Fig. 1 Strategies to design proteomics studies for identification of potentially useful fungal molecules. **a** Comparative studies using different species of microorganisms or pathogenic versus non-pathogenic may identify species-specific or panfungal targets, highly expressed molecules and infection-related molecules. **b** Immunoproteomics studies using sera from healthy or infected individuals may also lead to the identification of interesting molecules with possible therapeutic interest. **c** Possibilities for medical use of the molecules identified in immunoproteomics-based studies

develop monoclonal antifungal antibodies to be used as passive immunotherapy (Fig. 1b).

All these possibilities, using both fungal and host samples, have been investigated by many researchers for the characterization and identification of new molecules of interest for medical approaches, such as active and passive immunotherapy and novel diagnosis methods (Fig. 1c). In fact, over the last decade proteomics-based studies have successfully identified a wide range of proteins involved in host-pathogen interactions [15, 16]. Among these, secretome-related proteins are of particular significance because many fungi secrete a vast number of proteins involved in nutrient acquisition, communication, colonization, detoxification, killing of competitors, and virulence [17]. Surprisingly, in many cases they are metabolic enzymes and have neither secretion signal peptide nor pathogenesis-related functions [18, 19]. However, many of them are well-known antigens and commonly modified during the post-translation step. Additionally, secreted proteins may be present in higher concentrations in body fluids than in the intracellular compartment and, more importantly, they shared very little to no homology with human proteins [20]. Hence, their identification is of interest for the selection of a range of specific and easy-detectable molecules as alternative candidates for the development of more sensitive diagnostic assays.

In addition to secreted proteins, cell wall-associated proteins are an important part of the dynamic response of pathogens to changes in the environmental conditions or interactions with the host [21]. Moreover, the fungal cell wall has a complex architecture and composition, which is completely different from any structure found in host cells. Therefore, the cell wall is an interesting target for the development of new drugs [22].

In summary, in spite of the fact that genomic studies nowadays are able to predict protein involvement and functionality, the actual presence of proteins must be analyzed to appreciate the complexity of pathogen interactions with the environment, above all in the host [23]. Furthermore, the most abundant or most specific proteins may act as potent immune stimulants, markers of a fungal infection or targets for inhibition [24, 25]. In consequence, their identification can contribute to the development of new treatment strategies, diagnosis or prevention of fungal infections. For all these reasons, in this paper we review the proteomics-based studies focused on the whole secretome and cell wall proteins of the most important CF-associated molds, specifically *Aspergillus* and *Scedosporium/Lomentospora* species, with a special focus on immunoreactive proteins.

Aspergillus Species

Aspergillus fumigatus

Aspergillus fumigatus is the most common filamentous fungus in patients suffering from CF. In fact, its prevalence in CF has been reported to be as high as 57% and to increase with patient age [26–32]. The most common clinical presentations are allergic diseases, the worst being allergic bronchopulmonary aspergillosis (ABPA), which occurs in approximately 7–10% of patients with CF and contributes to a worsening of their pulmonary disease [11, 33].

The secretome of *A. fumigatus* is made up of numerous molecules responsible for invading the host and causing pathogenesis [34–37]. Among these, it is worth highlighting the immunoreactive proteins recognized by IgG or IgE because of their importance in the diagnosis of different presentations of aspergillosis [12] and allergic diseases [38], respectively. Over the last 15 years, several proteomic researches focused on the whole secretome of *A. fumigatus* have been carried out, most of them on immunoreactive proteins. One of these studies, carried out in 2005, identified by N-terminal sequencing, aspergillopepsin I, chitinase, β-1,3-endoglucanase, mitogillin/Asp f 1, and chitosanase, the last two being postulated as promising candidates for the improvement of diagnostic tests because they were recognized by all assessed sera from eleven infected patients and not by control sera [39]. In fact, mitogillin/Asp f 1 was already tested for improving the serodiagnosis of *A. fumigatus*-associated diseases in a previous study, some years earlier [40].

Two years later the first study focused on the identification of the allergens secreted by *A. fumigatus* recognized by sera from patients with ABPA was carried out, using two-dimensional electrophoresis (2-DE) and western blotting (2-D WB), and by peptide mass fingerprint using MALDI–TOF–MS (matrix assisted LASER desorption ionization–Time of flight–mass spectrometry) and/or *de novo* sequencing [41]. The authors considered chitosanase, extracellular arabinase and one hypothetical protein as major allergens since they reacted with ≥75% of sera, and catalase and alkaline protease (Alp1/oryzin), among others, as minor allergens. Moreover, Asp f 2 and Asp f 13/15, previously described as allergens [42, 43], were also detected.

In 2010, in an intriguing study focusing on both IgG- and IgE-binding secreted antigens, thirty-five immunoreactive proteins were identified by 2-D WB followed by mass spectrometric (LC–MS/MS) analysis. The researchers achieved this result using, on the one hand, five individual sera from ABPA patients against one *A. fumigatus* isolate and on the other hand, a pool of different ABPA sera against two strains of the fungus [44]. Thus, seven important allergens were identified: mitogillin/Asp f 1, Asp f 2, Asp f 3 (putative peroxiredoxin pmp20), Asp f 4, Asp f 9/Crf1, Asp f 10, and Asp f 13/15. More important was the fact that seven proteins were recognized by 100% of the sera tested: β-glucosidase, FAD/FMN-containing isoamyl alcohol oxidase, mannosidase, chitosanase, dipeptidyl-peptidase V (DppV), Asp f 2, and one hypothetical protein. In addition, eight antigens were detected in the two isolates used, mitogillin/Asp f 1, Asp f 3, 1,3-β-glucanosyltransferase, catalase, lysophospholipase-3, Asp f 2, chitosanase, and mannosidase. The last three seemed to be significant as they were detected by all sera and in the two fungal isolates used.

Another interesting study, using 2-D WB coupled with MS/MS, identified twenty-four proteins from the whole secretome of *A. fumigatus*, fifteen of them being immunogenic in humans: β-glucosidase, catalase, DppV, α-1,3-glucanase/mutanase, chitinase, amidase, FG-GAP repeat protein, aspartyl aminopeptidase, Alp1/oryzin, extracellular serine rich protein, glutaminase, exo-β-1,3-glucanase, thioredoxin reductase,

FAD-dependant oxygenase, and GPI-anchored β-1,3-endoglucanase [45]. The last six were proposed for study as diagnostic markers because their homology with human proteins was less than 10%, especially the last three, which did not match any of them.

Apart from the proteomics studies carried out with medical purposes, several other studies have been performed regarding the secretome of *A. fumigatus* under different culture conditions, which may give information about the most abundant and constitutively secreted proteins. Wartenberg et al. [46] defined a representative secretome after growing the mold in *Aspergillus* minimal medium supplemented with either elastin, collagen, or keratin as carbon sources. Among the sixty-four secreted proteins identified by 2-DE followed by MS/MS, twenty-nine appeared in the three conditions mentioned above, including the proven allergens Asp f 9/Crf1, Asp f 18 (Alp2), Asp f 22 (enolase), Asp f 34 (cell wall protein phiA), and Mep/Asp f 5), but also catalases (Cat1 and Cat2), peptidases (Lap1 and DppV), superoxide dismutase, and chitinase. Moreover, the authors described Asp-hemolysin as a major secreted protein. One year later, another proteomic study of the secreted proteins in different culture conditions was performed by Farnell et al. [47] using Vogeĺs salt liquid medium supplemented with bovine milk casein, porcine lung, or porcine mucin. Twenty-one proteins were identified by LC–MS/MS from 1-D SDS-PAGE gels, and it was suggested that the substrate composition influences the secretion of proteases in *A. fumigatus,* for example, Lap1, Mep/Asp f 5 and Alp1/oryzin. In addition, other studies have identified a great number of proteins in different conditions and substrates, such as corn, wheat, or soybean, by isobaric tags for relative and absolute quantification (iTRAQ) or label-free quantitative proteomics [48–50]. Many of these proteins were related to degradation of vegetal tissues and might not be interesting for medical uses. However, although the immunoreactivity of the proteins identified in all these studies was not proven, some of them were coincident with the ones described in previous reports [44, 51].

Regarding cell wall proteins, their study seems to be crucial since they do not only act as a protective structural shield, but also contribute to interactive contacts with a wide range of environments inside the human host. Analysis of cell wall proteins is usually hampered by the presence of pigments and lipids that must be removed from the samples [52]. However, since most of the surface-exposed proteins are probably non-covalently bound to the cell wall or are just passing through the cell wall to be secreted, detailed studies can easily be performed using a specific extraction method, either chemically (e.g., SDS and β-mercaptoethanol) or enzymatically (e.g., trypsin). For example, one of the first analyses of the fungal cell surface proteins used endogenous GPI-phospholipase C after membrane solubilization with β-n-octylglucopyranoside [53]. This method allowed the identification, by 2-DE and peptide mass fingerprinting, of nine proteins mainly involved in cell wall building and remodeling, including proteins similar to *Candida* spp. or *Saccharomyces cerevisiae* glycosidases and 1,3-β-glucanosyltransferase as well as *Aspergillus* acid phosphatase PhiA and two β-1,3-glucanosyltransferases Gel1 and Gel4.

Five years later, Asif et al. [54] reported the first analysis of the conidial surface-associated subproteome. These proteins were extracted with 1,3-β-glucanase in a mild alkaline buffer, then separated by 2-DE in two ranges of pI (3–10 and 4–5) and analyzed by LC–MS/MS. By this way, twenty-six proteins were identified, all of them being considered by the authors as potential vaccine candidates or thought to act as allergens. From all identified proteins, twelve displayed a signal sequence for secretion. Among these putatively secreted proteins were hydrophobin Hyp1/Rod A, secreted aspartyl protease Pep2, one extracellular lipase, a putative disulfide isomerase and a protein from the family of phosphoglycerate mutases. Regarding the proteins identified without a signal for secretion, elongation factor 1, Asp f 3, CipC, tropomyosin, co-chaperon of heat shock protein (Hsp) 90 p21 protein, and one ribosomal protein were identified.

In an attempt to describe putative early diagnostic markers of invasive aspergillosis (IA), Virginio et al. [55] developed an immunoproteomic assay with 2-D WB and MS/MS to study the cell surface of *A. fumigatus* conidia in the early stages of growth. To do this, twenty-five serum samples from patients with proven IA, thirteen from patients with probable IA, six sera from patients with a fungal infection different to IA, and six sera from non-infected individuals were tested. Forty antigens were detected, but only 14 were recognized by IA proven serum samples: carbamoyl-phosphatase synthase, bifunctional purine biosynthetic protein Ade1, mitochondrial aconitate

hydratase, Hsp70 chaperones (HscA and Ssc70), mitochondrial processing peptidase β subunit, 60S ribosomal protein L3, cytochrome P450, G-protein complex β subunit CpcB, translation elongation factor eEF-3, succinate dehydrogenase subunit Sdh1, antigenic mitochondrial protein Hsp60, ATP synthase subunit β, and ATP-dependent RNA helicase eIF4A. However, after studying the homology of these antigens with human proteins and other pathogens capable of causing invasive fungal infections, they concluded that only cytochrome P450 and eEF-3 were potential diagnostic targets of IA.

Finally, with the aim of searching for drug targets and candidates for the development of an effective vaccine against *A. fumigatus* and other related fungi, Champer et al. [56] identified, via label-free quantitative MSE mass spectrometry, approximately ninety proteins in the cell wall fraction using two different culture media. Among the most abundant proteins identified in Czapek Dox media were, Asp f 2, Ecm33, Asp f 9/Crf1, catalase B, L-amino acid oxidase LaoA, alkaline protease 2 Alp2, and 1,3-β-glucanosyltransferases Gel1, Gel2 and Gel4. By contrast, in Potato Dextrose broth, the most abundant proteins included pheromone processing carboxypeptidase Sxa2, chitinases ChiA1 and ChiB1, BYS1 domain protein, putative glucan endo-1,3-β-glucosidase EglC, putative 1,4-β-D-glucan cellobiohydrolase A, putative α-N-arabinofuranosidase B, and also Asp f 9/Crf1, Ecm33, Gel1 and Gel4, the last four being identified in both culture media. More recently, the same research group identified in different fungal pathogens, several highly conserved proteins which showed low homology with human proteins [20]. Some of them were detected in all the CF-associated *Aspergillus* species, namely *A. flavus, A. fumigatus, A. nidulans, A. niger* and *A. terreus* (Fig. 2).

Non-*fumigatus* Species of *Aspergillus*

In addition to *A. fumigatus*, other species of the genus such as *A. terreus*, *A. flavus, A. niger* and *A. nidulans* have been reported, although many times transiently, in the context of CF [57, 58]. However, very few papers have studied their secretome or cell wall subproteome, the existing ones not focused on fungal pathobiology in general.

Information on the secretome of *A. flavus* is especially limited, even though it has been reported in some studies as the second most frequent filamentous fungus in CF [58]. To be more specific, the most remarkable study until now described the proteins secreted on three different culture media: flavonoid rutin, glucose medium, and potato dextrose broth [59]. Thus, fifty-one differentially secreted proteins were found by using 1- and 2-DE and MS/MS techniques,

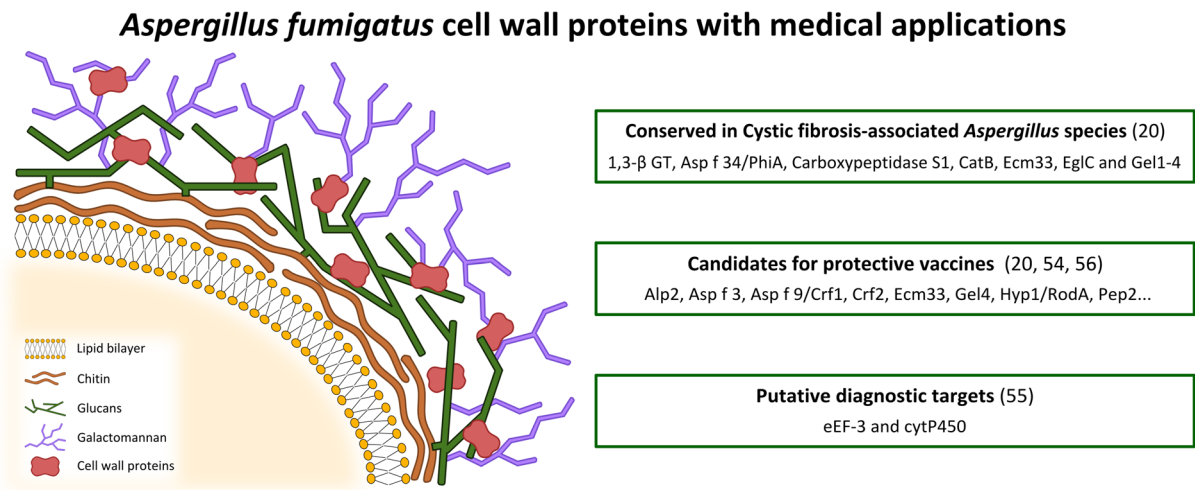

Fig. 2 Proteins from the cell wall of *Aspergillus fumigatus* described in proteomics-based studies and of interest for medical purposes. Alp2: alkaline protease 2; CatB: catalase B; ChiA1 and ChiB1: endochitinases; Crf1 and Crf2: probable glycosidases; CytP450: cytochrome P450; Ecm33: GPI-anchored protein; eEF-3: translation elongation factor 3; Gel1, Gel2 and Gel4: 1,3-beta-glucanosyltransferases; Hyp1/RodA: conidial hydrophobin

most of them being involved in carbohydrate metabolism and proteolysis.

Aspergillus terreus has been reported as a chronic colonizer of the airways and is the third most common filamentous fungus found in patients suffering from CF, with a prevalence ranging from 1.9 to 6.2% [60, 61]. In 2010, Han et al. [62] studied the secreted proteins utilizing sucrose, glucose, and starch as carbon sources. They identified eighty-two spots by 2-DE and nano-LC–MS/MS, which belonged to thirty-nine non-redundant proteins classified into three functional groups: hydrolases, proteins with miscellaneous functions and hypothetical/predicted proteins. Remarkably, some of these proteins are potential allergens, such as cerevisin, 24 kDa metalloproteinase, Alp1/oryzin, DppV, vacuolar protease A, catalase B, enolase, Asp f 13/15, Asp f 3, and Asp f 9/Crf1. However, only five proteins were detected in the three conditions: β-glucosidase 1, oryzin, putative endo-arabinase, putative aspartyl protease, and F0F1-type ATP synthase β subunit. One year later, Nayak et al. [63] suggested that the secreted hyphal exoantigens, leucine aminopeptidase and DppV, may be useful for serodiagnosis of *A. terreus* infections, the last protein having been reported previously as potential virulence factor in *A. fumigatus* [36] and as allergen in *Trichophyton* [64]. More recently, 63 secreted proteins were identified by LC–MS/MS method with the aim of comparing secreted proteins during different fermentation processes [65]. Due to the media used, most of these proteins were hydrolytic enzymes, and some of them were conserved in other *Aspergillus* species.

Regarding *A. niger*, it accounts for less than 5% of the filamentous fungi detected in CF patients [58], and it has been studied mostly because it is widely used in industry. Extensive studies of its secretome have been conducted during the degradation of several substrates using different proteomic approaches such as 2-DE/MALDI–TOF [66, 67], shotgun LC–MS/MS [68–71] and iTRAQ [72]. Some of the proteins identified, such as ABC multidrug transporter, Asp-hemolysin, hydrophobin Hyp1/RodA, and catalases have been reported as virulence factors of *A. fumigatus* [36]. However, despite the fact that many of these proteins are immunoreactive in *A. fumigatus*, their antigenicity or allergenicity has not been studied in *A. niger*.

The number of CF patients colonized by *A. nidulans,* similarly to *A. niger* and *A. flavus*, is low, most studies showing that it is found transiently [57]. It is unfortunate that, as reported for the other non-*fumigatus Aspergillus* species, no proteomics-based analyses have been carried out concerning secreted proteins from a medical point of view. In fact, three studies have identified a vast number of secreted proteins during the degradation of vegetable origin substrates such as starch, cork, and sorghum to find enzymes with potential interest for industry [73–75]. Among these proteins, mainly hydrolytic enzymes, there were also previously commented *A. fumigatus* immunoreactive proteins conserved in other species such as Gel1, Gel2, Asp f 13/15, Asp f 9/Crf1, and catalase (Fig. 3).

It is also worth mentioning that proteomics-based studies concerning secreted proteins by *A. oryzae* have focused mainly on the role of this species in food fermentation [76–78]. However, although *A. oryzae* has never been reported in CF, it is able to induce ABPA [79]. Hence, several proteins identified in this fungal species such as Asp f 13/15, catalase, Asp f 9/Crf1, and Alp1/oryzin have been reported as important allergens in other *Aspergillus* species as mentioned above, the latter being one of the most abundant extracellular enzymes of *A. oryzae* [77].

Regarding cell wall-associated proteins of non-*fumigatus* species, the only study focused on this subproteome so far was performed on *A. nidulans*. In this report, the authors used bioinformatic techniques to analyze in-depth the presence of genes related to the fungal cell wall in the genome of this species, as putative enzymes related to chitin or 1,3-β-glucan synthesis, GPI-anchored proteins for example [80]. More interestingly, they performed a free gel-based approach to identify proteins linked to the cell wall, which included eight transglycosylases belonging to four different protein families, Bgl2, Crh, Gas/Gel, and Ecm33, some of them previously mentioned as relevant secreted proteins with antigenic properties.

Finally, the recent study carried out by Champer et al. [20] is also remarkable. Proteins conserved not only in different *Aspergillus* species but also in other fungi were identified in both secretome and cell wall. By this way, the authors highlighted the most conserved proteins as cell wall proteins Gel 1–4, Bgt1, Ecm33, EglC, and Crf1.

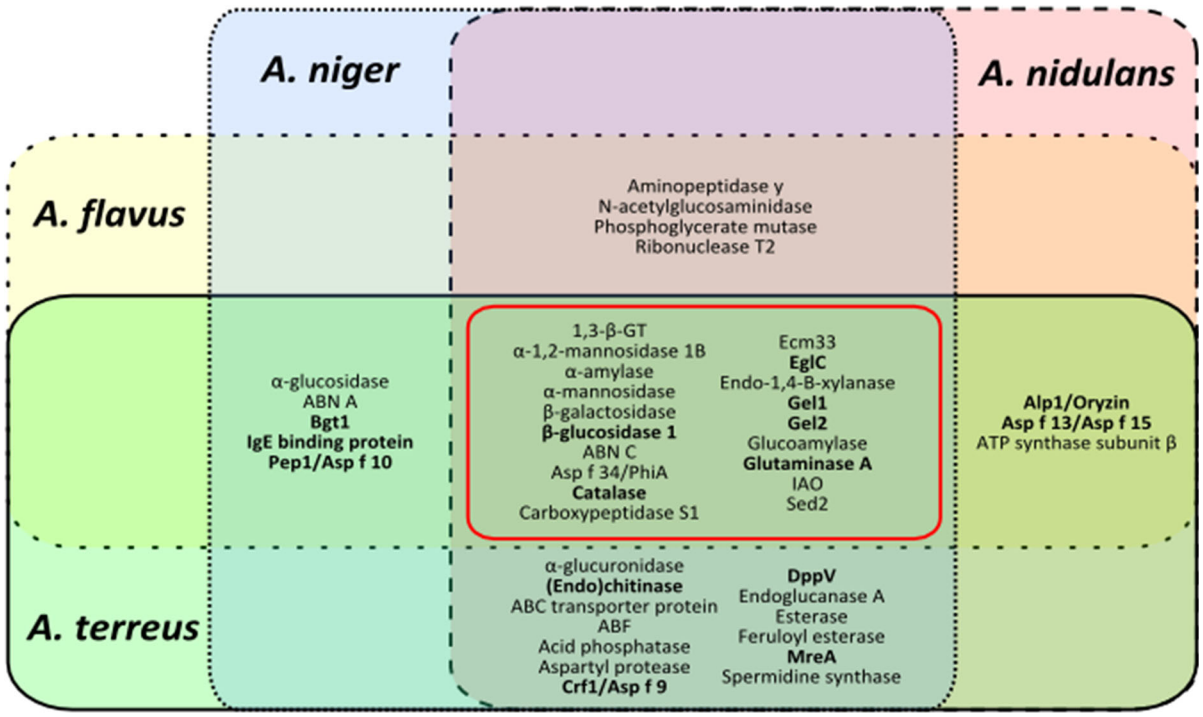

Fig. 3 Proteins identified in the secretome of at least three non-*fumigatus Aspergillus* species associated with cystic fibrosis. Proteins in *bold* type have also been identified in proteomics-based studies among *Aspergillus fumigatus* secreted immunoreactive proteins. 1,3-β-GT: 1,3-β-glucanosyltransferase; α-1,2-mannosidase 1B: Mannosyl oligosaccharide α-1,2-mannosidase 1B; ABF: α-L-arabinofuranosidase; ABN: Arabinan endo-1,5-α-arabinosidase; Asp f 34/PhiA: Cell wall protein phiA/Allergen Asp f 34; Bgt1: 1,3-β-glucanosyltransferase Bgt1; Crf1/Asp f 9: Extracellular cell wall glucanase Crf1/allergen Asp f 9; DppV: Dipeptidyl-peptidase V; Ecm33: GPI-anchored cell wall organization protein Ecm33; EglC: Glucan endo-1,3-β-glucosidase eglC; Endoglucanase A: Endo-β-1,4-glucanase A; Gel1: 1,3-β-glucanosyltransferase gel1; Gel2: 1,3-β-glucanosyltransferase gel2; IAO: Isoamyl alcohol oxidase; MreA: FAD/FMN-containing isoamyl alcohol oxidase MreA; Pep1/Asp f 10: Aspartic protease Pep1/Aspergillopepsin/Asp f 10; Sed2: Tripeptidyl peptidase Sed2; Xylanase: Endo-1,4-β-xylanase

Species of the *Scedosporium/Lomentospora* Complex

Patients suffering from CF may also carry a number of filamentous fungal species that are not *Aspergillus*. In fact, the prevalence of these non-*Aspergillus* species has been underestimated mainly due to the overgrowth of bacteria and other fungi in routine culture media [6]. Among others, the pathogens included in the genera *Scedosporium* and *Lomentospora,* which are considered as emerging pathogens [81], stand out as the second most prevalent filamentous fungi in CF patients after *Aspergillus*, being recovered from up to 17.4% of them, and causing allergic or invasive diseases after lung colonization [82–84]. Moreover, these species are among the most prevalent agents causing non-*Aspergillus* filamentous fungal infections in some countries, such as Spain or Australia [85, 86]. Interestingly, the prevalence of each species differs between countries, *S. apiospermum* and *S. boydii* being the most abundant species in European clinical settings [87–89], and *S. aurantiacum* and *L. prolificans* in Australia [7, 8]. In the last few years, interest in these emerging fungal species has increased due to their rising prevalence and high mortality rates (70–100%) [90, 91], which is related to the clinical situation of the patient, the difficulty of getting a rapid and specific diagnosis, and the resistance that many of these fungi present to antifungal drugs.

Analyses of the secretions during the growth of the *Scedosporium/Lomentospora* complex have shown that these fungi are able to produce a wide variety of compounds including antibiotics, such as tyroscherin and derivatives [92, 93], dithiodioxopiperazine derivatives [94, 95], fungistatics [96], and virulence-related polyketides [97]. Interestingly, some of these secreted

molecules have proved to be useful as biomarkers of mycoses caused by these species, as the cyclic non-ribosomal peptides, pseudacyclins [98], and the siderophore N^{α}-methyl coprogen B [99, 100].

Regarding the expression of secreted proteins by *Scedosporium*/*Lomentospora* species, the first experimental evidence related to their expression was focused on a 33 kDa exocellular serine peptidase produced by *S. apiospermum* which is able to degrade human fibrinogen, suggesting its involvement in virulence processes and its role as a mediator in severe chronic bronchopulmonary inflammation in CF patients [101]. Later, two distinct (metallo-type) peptidases released by *S. boydii* (previously *Pseudallescheria boydii* [102]) were also described. These may also be involved in the infection process, helping the fungus to cross human barriers and to evade the host defences because they have the ability to hydrolyze several human proteinaceous compounds, extracellular matrix components, and sialylated proteins [103].

However, up to now only two studies, not only focused on the purification and characterization of single secreted proteins, but on a global proteomics-based analysis of the extracellular subproteome of *Scedosporium*/*Lomentospora* species have been carried out. The first work showed that proteins secreted by *S. boydii* are able to induce irreversible damage and destruction in pulmonary epithelial cells in vitro. Interestingly, thirty proteins were identified by 2-DE and MS/MS, including the putative antigens Asp f 13, aspartyl proteinase, fructose-bisphosphate aldolase, glucanase, Hsp70, malate dehydrogenase, manganese superoxide dismutase, phosphoglycerate mutase, translationally controlled tumor protein, and triosephosphate isomerase [104]. The second study, which was focused on *S. aurantiacum*, identified several proteases present in the fungal secretome when the fungus is grown in a synthetic cystic fibrosis sputum medium containing mucin, showing differences between two strains, one clinical and one environmental [105]. Specifically, the authors identified peptidases S8 subtilisin family, putative leucine aminopeptidase M28 Zn-peptidase, and PA SaNapH-like protease in both strains; Peptidase C48, pepsin-like aspartate Asp, and trypsin-like serine protease secreted by the clinical strain; and pepsin-like aspartic protease and KLLA0E06711p protease only present in the secretome of the environmental strain.

By contrast, the cell wall of *Scedosporium*/*Lomentospora* species has been more extensively studied, in accordance with its importance in fungal biology and pathogenicity, as previously mentioned. Although the cell wall structure of these species is very similar to other pathogenic filamentous fungi, its composition is different. While galactomannan is very significant as a component of the carbohydrate matrix of *Aspergillus* spp. [106], rhamnomannans are the main components of the *Scedosporium*/*Lomentospora* cell wall [107, 108]. In fact, a peptidorhamnomannan extracted from *S. boydii* was proposed for use in diagnosis because of its high immunogenicity, but further research should be carried out in the future to determine its suitability [109]. More interestingly, the presence of melanin in the conidial wall of all species, and also in *L. prolificans* hyphae, impairs immune response since this compound acts as a potent antioxidant and may mask important molecules such as mannans, impeding the induction of immune responses [90, 110].

Additionally, only a few proteomics-based studies, mainly focused on *S. boydii* and *L. prolificans*, have been carried out so far to determine which proteins are present in the cell wall of these species. In the first one, the authors focused on GPI-anchored proteins on the cell wall of *S. boydii* conidia and germ tubes, among other aspects of cell wall changes during germination of the fungus [111]. Among the two-hundred and fifty-four proteins detected by nano-LC–MS/MS in the cell wall extracts of both morphotypes, only twenty carried a GPI-anchored domain, seven of them shared by conidia and germ tubes, while only one protein was solely present in conidia and twelve only found in hyphae. Many proteins identified in this study were related to pathways in cell wall biosynthesis, such as glucan endo-1,3-β-D-glucosidase, CRH1_transglycosylase, GH17 family protein, 1,3-β-glucanosyl transferase, and glucanosyl transferase Glyco-hydro 72, some of them found to be antigenic in other fungal species, as previously noted. Remarkably, several proteins related to fungus-host interactions were also identified, as several CFEM-like proteins, ceratoplatanin, and a Cu/Zn superoxide dismutase, the latter being the only one identified in conidia in this study.

In addition, it is worth mentioning that three studies have recently been published concerning the antigenicity of *L. prolificans* proteomes of conidia and hyphae [112–114], characterizing the immunomic

patterns via 2-D WB and mass spectrometry, using human salivary IgA (sIgA) and serum IgG. Regarding secreted proteins, the authors identified several highly prevalent antigens recognized by serum IgGs that were predicted to be secreted by using computational analysis of their sequences. Among these were malate dehydrogenase, cysteine transaminase, fumarate reductase, acetyl-CoA acetyltransferase, ATP synthase subunit α and β, glyceraldehyde-3-phosphate dehydrogenase, and 40S ribosomal S1-like protein.

Moreover, the antigenicity of proteins present in the cell surface of *L. prolificans* was analyzed. Relevant antigens present both in conidial and hyphal cell surfaces were identified including enolase, glyceraldehyde 3-phosphate dehydrogenase, putative ATP synthase subunit β, Hsp90, and Hsp70. Interestingly, Hsp70 was found to be recognized by both IgA and IgG isotypes and in the two morphotypes, proving the high immunogenic properties of this protein. Remarkably, Hsp70 has recently been shown to be essential in the tolerance of *A. fumigatus* and *A. terreus* to caspofungin and amphotericin B, respectively [115, 116], as it plays an important role in the Hsp90-calcineurin pathway related to stress responses. In addition, enolase and cyclophilins were also identified on the conidial surface of *S. apiospermum* and *S. aurantiacum*, showing high prevalence among sIgA-reactive antigens. More interestingly, it was shown that these proteins were cross-reactive with *A. fumigatus*. Therefore, more in-depth studies should be performed in order to determine the suitability of these proteins as therapeutic or diagnostic targets.

Discussion

Proteomics-based studies have identified a large set of proteins of interest not only for medical, but also for biological and industrial purposes. In this paper, we have reviewed studies focused on the secreted and cell wall subproteomes of the most common filamentous fungi in CF in order to find candidates that may improve diagnostic and treatment for CF patients suffering from fungal colonization or infection.

To organize and select the most interesting molecules, the difficulties arising from the limitations of databases and from the multiple synonyms used for some proteins must be taken into account; even today this continues to impede easy management of the relevant data. This is the case, for example, with the *A. fumigatus* alkaline serine protease or alkaline protease. Currently, the recommended name for this protein is Alp1, but it is also known as oryzin (Uniprot entry B0Y708), and it is sometimes considered as synonymous with Asp f 13 (Uniprot entry P28296). However, Asp f 13 sequence (Uniprot entry B0XSZ0) is totally different from that of Alp1/oryzin, and, surprisingly, is the same as Asp f 15 sequence (Uniprot entry O60022). Owing to this fact, protein identification is sometimes carried out without accuracy, and different names for the same protein may be obtained. This is of special relevance since the same protein can appear with different names not only in various studies but even within the same article. In our opinion, there is an urgent need for a definitive unification of the criteria for choosing and publishing names, and for a complete revision of the databases.

Even so, before highlighting some of the proteins from all the proteomics-based studies published, several of their characteristics should be considered. On the one hand, the expression level seems to be crucial since highly expressed proteins could be useful for sensitive antigen detection, particularly for species-specific proteins and the ones shared by various species of interest. Species-specific proteins can be of relevance for specific detection, and the proteins shared by pathogenic species for panfungal detection. On the other hand, it is significant when these molecules are absent, or present with low homology rates, in humans. Among all of them, immunoreactive proteins can also be used to diagnose fungal diseases by antibody detection, especially those which are differentially recognized by infected and non-infected individuals.

Taking into account the data reviewed in this work, it is difficult to describe specific proteins for each of the fungal species because up to now, many of them have not been studied in depth. However, there are some proteins, above all from the *Aspergillus* genus, which deserve a mention. For instance, concerning secreted proteins, twelve antigens of *A. fumigatus* have been identified in more than one of the proteomics-based investigations reviewed (Table 1).

Among these proteins, chitosanase and mitogillin have been considered promising candidates for the improvement of diagnostic tests [39–41, 44], and thioredoxin reductase, GPI-anchored β-1,3-endoglucanase, catalase, DppV and Asp f 13/15 have shown

Table 1 Bibliography related to non-*fumigatus Aspergillus* and *Scedosporium/Lomentospora* secreted antigens shared with *Aspergillus fumigatus*

Antigen/allergen*	*Aspergillus fumigatus*	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus terreus*	*Aspergillus nidulans*	*Aspergillus oryzae*	*Scedosporium/ Lomentospora*
β-glucosidase	[44, 45]	[66, 68, 70, 71]	[59]	[62, 65]	[73–75]	[76]	
Alp1/oryzin	[41, 45]		[59]	[62]	[73–75]	[76, 77]	
Asp f 2	[41, 44]						
Asp f 13/15	[41, 44]		[20]	[20, 62, 65]	[20, 73, 75]	[76]	[104]
Catalase	[41, 44, 45]	[20, 66–68, 71, 72]	[20, 59]	[20, 62, 65]	[73–75]	[76]	
Chitinase	[39, 44, 45]	[66, 70, 71]	[59]	[62]	[73, 74]	[76]	
Chitosanase	[39, 41, 44]					[77]	
Dipeptidyl-peptidase V	[44, 45]			[20, 62, 63]	[75]		
GPI-anchored β-1,3-endoglucanase	[44, 45]	[72]			[73]		
Mep/Asp f 5	[122, 139]					[77]	
Mitogillin/Asp f 1	[39, 44]						
Thioredoxin reductase	[44, 45]		[20]		[73, 75]		

Numbers in square brackets are the references where proteins have been identified for the corresponding species

* Antigens/allergens reported in two or more proteomics-based studies on the secretome of *Aspergillus fumigatus*

very low or no homology with human proteins [20, 45]. Of these, catalase, which is a main precipitin [34], has been reported as an allergen [41, 44], related to higher resistance in *A. terreus* to amphotericin B [117], and as a biomarker of *S. boydii* infections [118].

Furthermore, Asp f 13/15 and Alp1/oryzin have been reported as important antigens and allergens [41, 43, 44], produced in vitro by *A. fumigatus* in different experimental mediums [46, 47]. Of these two proteins, Asp f 13/15 has been identified as essential for airway inflammation and remodeling [119], and Alp1/oryzin as one of the most abundant *Aspergillus* secreted proteins, accounting for up to 50% of visible proteins in *A. oryzae* [46, 62, 77]. More interestingly, the presence of Alp1/oryzin in the lungs of infected mice has been detected by indirect immunofluorescence [120].

Other antigens that have been identified as secreted by *A. fumigatus* in different studies, and media are the elastinolytic metalloproteinase Mep/Asp f 5 and DppV. The first is secreted in lung infected tissue by *A. fumigatus* and by *A. flavus* [121, 122] and is recognized differently by sera from aspergilloma patients and control individuals [122]. As for DppV, it is a precipitin of *Aspergillus* [34] described as a virulence factor in other fungi [123, 124]. This protein has also been suggested for the serodiagnosis of ABPA [44], of invasive *A. terreus* infection [63] and, together with ribonuclease and catalase, of aspergilloma [125].

In addition, among other secreted proteins of interest, it is worth mentioning the allergens Asp f 1, Asp f 2, Asp f 3, Asp f 4, and Asp f 6 (Superoxide dismutase [Mn], mitochondrial), which have been tested to differentiate between ABPA and non-ABPA CF subjects, and to discriminate between stages of flares and remission [42, 44, 126–131]. Specifically, Asp f 1 has also been detected in the serum and/or urine of humans or animals infected with *A. fumigatus* [132, 133]. Another protein of interest is the cytotoxic Asp-hemolysin, which is a highly secreted protein [46] proposed as a target for detecting *A. fumigatus* by quantitative real-time PCR [134].

Apart from these *A. fumigatus* antigens, in this review, we have taken into account the results obtained regarding the secretome of *Aspergillus* species (Fig. 3). We have found nineteen proteins conserved in the four CF-associated non-*fumigatus* species, *i.e.*, *A. flavus, A. nidulans, A. niger,* and *A. terreus*, six of them being also detected for *A. fumigatus*: β-glucosidase, Gel1 and 2, catalase, EglC and glutaminase. Glutaminase, among others, has been suggested as a diagnostic marker [45].

Finally, it is important to note that some of the most abundant proteins in the secretome of these

fungi are of unknown function, hypothetical, or predicted proteins [46, 62], which highlights the importance of studying the function of these proteins in-depth, of having good identification rates and more importantly, a complete and detailed annotation of fungal genomes.

With regard to the cell wall proteins of *A. fumigatus*, it is important to mention the fact that they are antigens exposed to environmental conditions, drugs, and antibody-mediated recognition and therefore they appear to be good candidates as diagnostic and therapeutic targets (Fig. 2). Among the cell wall proteins identified in the different studies reviewed, some have been widely discussed in previous paragraphs since they have been detected also as commonly secreted proteins, namely chitinases, catalase, and Asp f 2. This fact is not surprising, since many proteins of the cell wall are also secreted proteins and, more interestingly, it points out these proteins as interesting diagnostic targets due to their immunoreactivity and wide localization.

In addition, other cell wall proteins deserve a mention. This is the case of the major conidial surface protein Hyp1/RodA, which is bound via a GPI anchor to cell wall polysaccharides and builds the conidial rodlet layer. Importantly, it was described as a virulence factor, inhibiting the Dectin-1- and Dectin-2-dependent immune system activation [135]. The acid protease Pep2 is also notable because it may be related to allergic diseases caused by *Aspergillus,* such as asthma, playing a pivotal role in the processing of allergens [54]. Moreover, proteolytic activity is a central biochemical property that endows molecules with intrinsic allergenicity [136].

Other cell wall proteins of interest are lipases, as they may be important for interaction with the host, and disulfide isomerase, which possibly plays an important role in the protein folding mechanisms of *A. fumigatus* antigens/allergens [137]. It is also worth-noting that Asp f 9/Crf1, Ecm33, Alp2, Gel4, and Crf2 were proposed as the best potential cross-protective vaccine candidates based on significant similarities between *A. fumigatus* and other fungal species [56]. In addition, Asp f 3, present in both secretome and cell wall, has been suggested as a candidate for vaccine development against IA [138]. Furthermore, two cell wall proteins from *A. fumigatus* have been reported as potential diagnostic targets of IA, because of their low homology with human proteins and other fungal pathogens, cytochrome P450 and translation elongation factor eEF-3 [55]. Also noteworthy is a group of ten cell wall proteins identified recently in all the CF-associated *Aspergillus* species by Champer et al. [20]. It might be interesting to study these very conserved proteins as potential diagnostic or therapeutic targets of aspergillosis in CF patients (Fig. 2).

Unfortunately, proteomics-based studies concerning *Scedosporium/Lomentospora* species are scarce, and mainly focused on three species of the complex, *S. boydii*, *S. aurantiacum*, and *L. prolificans*. However, very relevant antigens have been identified in the secretome of *S. boydii*, such as aspartyl proteinase or Asp f 13, both widely described in *Aspergillus* species. Likewise, a number of proteases have been identified in *S. aurantiacum*, with several differences between an environmental and a clinical strain [105].

Regarding cell wall-associated proteins, several carbohydrate biosynthesis- and host-pathogen interaction-related proteins were identified in *S. boydii*, including endo-1,3-β-D-glucosidase and 1,3-β-glucanosyl transferase, which have been also widely described as both antigens and as being present in the secretome of *Aspergillus* spp. Turning to *L. prolificans*, over the last two years, a large set of antigenic proteins has been identified, being reactive either with sIgA or serum IgG from immunocompetent individuals. Interestingly, Hsp70 was detected by both immunoglobulin isotypes and present in both conidia and hyphae, and was also identified in the secretome of *S. boydii* [104]. Therefore, this protein should be the target of future studies since it shows a great antigenic potential and a wide distribution in different fungal cell compartments.

In conclusion, this review provides all the relevant information concerning the secreted and cell wall proteins of the most common filamentous fungi in CF, *Aspergillus* and *Scedosporium/Lomentospora*. To be precise, we have highlighted some of the most important proteins, above all the immunoreactive ones, according to different criteria such as their abundance and presence in different studies. These proteins may be promising candidates to improve diagnostic systems and develop new therapeutic tools.

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Compliance with Ethical Standards

Conflict of interest The authors of this manuscript declare no conflicts of interest.

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