Author's Final Submitted Version: The original article was published in "Current Protein & Peptide Science" and is available at the Bentham Science website "<http://www.eurekaselect.com/122087/article>"

Allergic aspergillosis and the antigens of *Aspergillus fumigatus*

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Abstract

Incidence of fungal infections has increased alarmingly in past few decades. Of the fungal pathogens, the *Aspergillus fumigatus* has been a major cause of allergic bronchopulmonary aspergillosis (ABPA) which has five main stages - the acute, remission, exacerbation, glucocorticoid dependent and fibrotic stage. The diagnosis of ABPA remains difficult due to its overlapping clinical and radiological features with tuberculosis and cystic fibrosis. From past few decades, the crude fractions of *A. fumigatus* have been used for immunodiagnosis of ABPA. Most of the detection kits based on crude fractions of *A. fumigatus* are quite sensitive but have low specificity. Till date 21 known and 25 predicted allergens of *A. fumigatus* have been identified. Of these allergens, only five recombinants (rAsp f1-f4 and f6) are commercially used for diagnosis of allergic aspergillosis. Remaining allergens of *A. fumigatus* have been restricted for use in specific diagnosis of ABPA, due to sharing of common antigenic epitopes with other allergens. Complete sequencing of *A. fumigatus* genome identified 9926 genes and the reports on the proteome of *A. fumigatus* have shown the presence of large number of their corresponding proteins in the pathogen. The analysis of immunoproteomes developed from crude fractions of *A. fumigatus* by IgG/IgE reactivity with ABPA patients and animal sera have identified the panel of new antigens. A brief description on the current status of *A. fumigatus* antigens is provided in this review. The implementation of advance recombinant expression and peptidomic approaches on the *A. fumigatus* antigens may help in the selection of appropriate molecules for the development of tools for more specific early diagnosis of ABPA, and desensitization therapies for patients of allergic disorders.

Keywords: Allergic aspergillosis, allergens, recombinants, diagnosis, desensitization

List of abbreviations :

Allergic bronchopulmonary aspergillosis (ABPA)

Aspergillus hypersensitivity (AH)

Computed tomography (CT)

Enzyme linked immunosorbent assay (ELISA)

Non invasive aspergillosis (NIA)

Radio allergosorbent test (RAST)

Skin prick test (SPT)

Thioredoxin (Trx)

Tuberculosis (TB)

Two dimensional gel electrophoresis (2DE)

1. Introduction

Among the pathogenic species of *Aspergillus*, *A. fumigatus* is the most common cause of aspergillosis which includes a spectrum of diseases. The primary route of infection with *Aspergillus* has been via inhalation of airborne conidia. The average size of *A. fumigatus* conidia has been found to be 2.0 to 3.0 μm . Because of such a small size, the conidia can easily get airborne. Also the conidial production of *A. fumigatus* has been highly prolific and, therefore, the human respiratory tract always remains at risk of acquiring *Aspergillus* infection [1]. The germination of conidia in the respiratory tract and the extent of mycelial colonization in lungs has been shown to be influenced by the competence of the host immune system [2]. It was reported recently that change in temperature during the growth of *A. fumigatus* significantly influenced the allergenicity of *A. fumigatus* through differential production of allergenic proteins [3]. In upper airways, the conidia may cause respiratory discomfort due to contact irritation and allergic responses. Aimagani et al (2009) demonstrated that the hydrophobic nature of rodlet layer prevents immune response against dormant conidia of *A. fumigatus* [4]. Thus it has been suggested that the immunogenic responses may get induced after the release of cellular proteins including virulence factors and allergens from metabolically active germinating conidia. Such initial allergic reactions have been accountable for development of different forms of allergic aspergillosis. Repeated exposure of lungs to conidia, their germination, expansion of hyphal mass and focal growth of *A. fumigatus* in pre-existing lung cavities such as the lesions in patients with pulmonary tuberculosis may lead to the development of noninvasive aspergillomas. The *A. fumigatus* may invade the lung tissues and disseminate to the deeper body parts to cause systemic infection. The increased severity and incidence of allergic and nonallergic aspergillosis need a better understanding of the interplay between host and fungus that contributes to *A. fumigatus* pathogenesis [6]. Also, detailed knowledge of allergenic and antigenic molecules of *A. fumigatus* is required for the selection of molecules for application in specific diagnosis and immunotherapy of aspergillosis.

2. Forms of aspergillosis

2.1. Allergic

The *A. fumigatus* may cause various kinds of allergic conditions in the body of the host. These allergic reactions due to *A. fumigatus* may lead to the development of four distinct clinically recognizable forms of hypersensitivity respiratory disorders i.e., allergic bronchopulmonary aspergillosis (ABPA), allergic *Aspergillus* sinusitis, IgE-mediated asthma, and hypersensitivity pneumonitis [5].

In the atopic individuals with diseases of altered lung function such as asthma and cystic fibrosis, *A. fumigatus* can cause ABPA, a hypersensitive response to fungal products. The ABPA is most common among the other forms of allergic aspergillosis and considered to be an immunologically mediated lung disease, which is very frequently associated with asthma. ABPA has been an idiopathic inflammatory lung disease characterized by hypersensitive allergic response to the colonization of *Aspergillus* in the airways. It has been the most severe allergic pulmonary complication caused by *A. fumigatus*. The first case of ABPA was reported from England [7]. The disease existed in USA also and was reported first by Patterson and Golbert in the year of 1968 [8]. Shah (1971) was the first to report three cases of ABPA from India [9]. But it was soon realized that allergic aspergillosis was world wide in distribution. The clinical course of ABPA is known to vary from mild asthma to fatal destruction of lungs with bronchiectasis and fibrosis [10]. Several attempts have been made to determine the prevalence of ABPA but due to lack of uniform diagnostic criteria and standardized tests, it has remained a tough task [11]. The allergic response against *Aspergillus* was characterized by the presence of an immediate type hypersensitivity reaction to *A. fumigatus* antigens and has been the first step in the development of ABPA [8]. Only a small number of patients with *Aspergillus* hypersensitivity (AH) have been found to develop the complete clinical picture of ABPA [12]. Chest X-ray and computed tomography (CT) have been found to be useful for establishing diagnosis of ABPA by visualizing the infiltration and bronchiectasis in the lungs of patients. Although, various studies have reported the prevalence of ABPA to be 1.0-2.0% in asthmatic individuals [5, 9, 11-13], the prevalence of ABPA in severe asthma has been considered to be much higher. In a study of 357 patients with acute severe asthma admitted in the respiratory intensive care unit, the prevalence of ABPA was reported to be 39.0% [14]. The prevalence of ABPA in patients with cystic fibrosis

was found to be 2.0-15.0% [5, 11-13]. In Indian population, the *Aspergillus* cutaneous type I sensitization was shown to be as high as 28.5-50.0% [15-17] in asthmatics. In a study by Kumar and Gaur (2000), the prevalence of ABPA was shown to be 16% in patients of asthma [18]. Maurya et al (2005) carried out a study on 105 patients with bronchial asthma and found 7.5% prevalence of ABPA [16]. Agarwal et al (2010) reported ABPA prevalence of 27.2% in a study of 564 asthmatics from North India [14]. High prevalence of ABPA has been reported from various parts of the world and has remained a disease of health importance for clinicians globally especially in Asia.

The overlapping clinical features of ABPA with other respiratory disorders, mainly with tuberculosis (TB) and the difficulties in establishing differential diagnosis, and thereby misdiagnosis quite often, have been the main reasons for wrongly considering ABPA less important [19-21]. It was found that TB has been the most common type of chronic pulmonary infections in developing countries [22]. According to a recent World Health Organization report (2011), the incidence of TB was reported to be 0.185%. In India the incidence of TB has been very high and that contributed over one fifth of the global incidence of TB cases in 2009. Studies from India showed that ABPA was misdiagnosed as TB in 17.0% to 50.0% of subjects examined at various hospitals and clinics [14, 21, 23-25]. Free availability of immunosuppressants and their gross misuse in asthmatics increases the risk of *Aspergillus* infection. Under these conditions the ABPA gets masked and would be diagnosed only at the late stages of disease. There is lack of a universal diagnostic test with high sensitivity and specificity to distinguish between ABPA and TB. The lack of awareness about ABPA among general practitioners, high cost and lack of widespread availability of myco-serological tests, and scarcity of advanced facilities at all clinical centers, have made it difficult to diagnose ABPA in patients of respiratory distress including TB. The diagnosis of ABPA depends on a set of clinical, serological and radiological criteria.

Although typical presentation of ABPA is observed in patients with a history where one finds it difficult to control asthma, the spectrum of presentation has been highly variable and needs to be considered in any patient with moderate to severe asthma and hypersensitivity to *A. fumigatus*. The complaints of ABPA patients have been nonspecific and include dyspnea, wheezing, poor asthma control, cough (commonly productive of thick, brown mucus plugs),

malaise, low-grade fever and occasionally, hemoptysis. There may be an antecedent history of recurrent asthma exacerbations in conjunction with pneumonia without a culture identified bacterial source. In addition, a history of atopy with rhinitis, drug allergy, and/or allergic conjunctivitis has been common. Very often, the patient remains ill over weeks to months and shows unresponsiveness to standard treatments prescribed on the basis of suggestive diagnosis.

2.2. Non allergic

The aspergilloma generally occur in preexisting pulmonary cavities that develop during the course of TB, sarcoidosis, or other bullous lung disorders [26, 27, 28]. About 10.0 to 15.0% of patients with cavitating lung diseases may develop this syndrome [29]. The aspergilloma consists of an accumulated mass of hyphae embedded in a proteinaceous matrix with sporulating outgrowths on the margin. Hemoptysis was found to be quite frequent in the case of aspergilloma [30, 31]. The chest radiographs show spheroid mass of hypha generally bordered by a radiolucent crescent [32]. Lesions of aspergillomas in the lung of patients have been superlative demonstrated by CT scans of the chest [33, 34]. The chronic pulmonary aspergillosis is described as a slightly different condition than aspergilloma due to slow progression of infection by damaging the lung tissues which may result in fibrosis and inflammation in the lungs of host [35]. The occurrence of invasive infection has been usually seen in the setting of an immunocompromised state [36]. The invasive aspergillosis has been recognized as the main fungal infection in cancer patients, however, its true incidence was probably underestimated because of the low sensitivity of diagnostic tests [37]. It also occurs in patients with nonhematogenous underlying conditions such as acquired immunodeficiency syndrome [38] and chronic granulomatous disease [39].

3. Therapy of aspergillosis

3.1. Chemotherapy

The drugs are available for treatment of *Aspergillus* induced infections, however, it has been considered that they can only reduce the fungal burden in the host and the cure of infection can rarely be achieved [40]. The drugs currently available for treatment of *Aspergillus* induced diseases, belong to three major groups (a) Polyenes: Amphotericin, Nystatin, (b) Azoles: Voriconazole, Itraconazole, Posaconazole and (c) Echinocandins: Caspofungin, Micafungin. In

addition to these molecules, high doses of corticosteroids are nonspecifically prescribed for treatment of ABPA. Although the long-term benefits of corticosteroids are not clear, their many side effects have been the matter of concern. Nebulized corticosteroids with amphotericin B was suggested to improve the outcome of treatment in ABPA [41]. The azoles also have been proposed as an alternative treatment for ABPA. Of this group, the itraconazole was found to be the most active [42]. Both voriconazole and posaconazole are potentially effective alternative treatment options for severe asthma with fungal sensitization and ABPA. Although these drugs may improve asthma control and reduce severity of symptoms, larger prospective studies are required to support the retrospective findings [43]. The glucocorticoids and itraconazole nanosuspension represented an interesting formulation for inhaled administration in CF patients suffering from ABPA [44].

Recently a new approach was explored, where recombinant DNA-derived humanized IgG (omalizumab) which is an anti-IgE monoclonal antibody, was used to treat 2 patients of cystic fibrosis, in whom frequency of ABPA exacerbations was markedly reduced with treatment, and free titre of IgE was also decreased significantly [46]. Thus it may be considered that omalizumab is useful in treating ABPA in patients with CF and measurement of free IgE may help in monitoring the response to therapy. It was further demonstrated that omalizumab was also useful in patients of severe asthma with fungal sensitization and ABPA [47].

3.2. Limitations of chemotherapy

The molecules available for treatment of aspergillosis are not only fewer in number, they have limited efficacy. The amphotericin B is an effective antifungal agent but its therapeutic utility has been hindered due to high toxicity for host cells by channel formation across cell membrane and disruption of postendocytic trafficking [48]. Other anti-*Aspergillus* drugs also have been found to cause toxic effects as well [49]. Most drugs used for treating aspergillosis have been shown to be immunosuppressive [50]. The development of drug resistance in *A. fumigatus* has become an increasingly serious problem [42, 45]. The drug resistance can be primary (fungal pathogen inherently resistant to antifungal drug) or secondary (development of resistance during treatment). The resistance in *Aspergillus* to polyenes has been shown to be primary rather than secondary resistance. The primary resistance to polyenes in *A. fumigatus* has

been less frequent [51] than in *A. terreus*. Most *A. terreus* isolates were found to be resistant to amphotericin B *in vitro* but were susceptible to itraconazole or voriconazole [52]. *In vitro* susceptibility of 156 clinical isolates of *A. fumigatus* to amphotericin B and itraconazole was investigated by microbroth dilution assay. It was observed that 2.0 µg/ml concentration of amphotericin B was lethal to all isolates whereas 4 isolates of *A. fumigatus* were found to be resistant against a high dose (16.0 µg/ml) of itraconazole [53]. There are reports which have demonstrated resistance in *Aspergillus* species against voriconazole among stem cell transplant recipients [54-56]. The development of resistance in *A. fumigatus* against azoles and other antifungal agents [45], even if increasing noticeably but still remains a rare event. However, the limited efficacy of current drugs, their toxicity and the issue of development of resistance in pathogen have emphasized the work on identification of novel antifungal molecules, or alternatively the development of immunotherapy.

3.3. Immunotherapy

The efforts have been concentrated at global level for identification of antigenic preparations and/or molecules of *A. fumigatus* which could serve as potential vaccine candidates. In this context, immunization of experimental animals by antigens of *Aspergillus* has been shown to be effective in desensitization and treating *Aspergillus* related allergies [57]. It was demonstrated by Fukahori et al (2010) that *A. fumigatus* conidia could regulate the functions of airway dendritic cells in the development of mite allergen-induced allergic airway inflammation [58]. Cenci et al (2000) showed the capability of *A. fumigatus* antigens to elicit Th1 and Th2 type of responses, and it was suggested that crude culture filtrate of *A. fumigatus* might fulfill the requirement of a candidate vaccine for aspergillosis [59]. A comparative study on culture filtrate and sonicated conidia of *A. fumigatus* as potential vaccine for invasive pulmonary aspergillosis demonstrated that sonicated conidial vaccine was more effective when administered through subcutaneous route [60]. Few individual proteins of *A. fumigatus* have also been tested for their potential as vaccine candidates. These proteins included rAsp f3, rAsp f9/Asp f16, 1,3-glucanotransferase and aspartic endoprotease which elicited protective immunity against experimental aspergillosis [61-63]. The rAsp f3 provided immunity in cortisone acetate-immunosuppressed mice against experimentally induced pulmonary aspergillosis [61]. A recent study from the same group demonstrated the role of antibodies and T cells in providing

immunoprotection. They, however, observed that Asp f3 located in the peroxisomes of *A. fumigatus* hyphae remained inaccessible to antibodies, unless both cell wall and cell membrane have been permeabilized. The Asp f3 primed CD4⁺ T cells were not expectedly involved in clearance of fungal pathogen directly, instead they might locally activate immunosuppressed phagocytes to elicit antifungal effect [64]. It was reported very recently that *A. fumigatus* vaccine based on rAsp f3 has the potential to prevent aspergillosis in humans [65]. Most studies carried out so far on vaccination, have been limited to the experimental animals only and not even a single protein of those examined for immunoprotection, has reached the level of clinical trial. Therefore, identification of new immuno-therapeutic molecules may serve as potential vaccine candidates.

4. Diagnosis

This issue has been haunting clinicians since the recognition of the disease as a distinct entity and even today there has been no single test which can establish the diagnosis of aspergillosis. The diagnosis of ABPA usually relies on a set of criteria and depends on a combination of clinical observations and laboratory investigations.

4.1. Clinical diagnosis

A number of major and minor clinical criteria have been proposed to diagnose ABPA [10, 66]. The major criteria include asthma, presence of transient pulmonary infiltrates (fleeting shadows) in chest X-ray, immediate cutaneous reactivity to *A. fumigatus*, elevated total serum IgE, precipitating antibodies against *A. fumigatus*, peripheral blood eosinophilia, elevated serum IgE and IgG specific to *A. fumigatus* and central/proximal bronchiectasis with tapering of distal bronchi. The minor criteria include expectoration of golden brownish sputum plugs, positive sputum culture for *Aspergillus* species and late (Arthus-type) skin reactivity to *A. fumigatus*.

Even with these criteria, there is no consensus regarding the number of conditions to be satisfied to diagnose ABPA. Also patients may not satisfy all these criteria at one point of time and positivity may vary according to the stage of the disease. The basic criteria of Rosenberg et al (1977) subsequently revised by Vlahakis et al (2001) and Greenberger (2002) have been followed widely for diagnosis of ABPA [10, 67, 68]. Accordingly the patients of bronchial

asthma with positive skin prick test against *A. fumigatus* may be diagnosed as ABPA if they meet any four of the following criteria: elevated total IgE, presence of specific IgE and IgG against *A. fumigatus*, serum precipitating antibodies against *A. fumigatus*, peripheral blood eosinophilia, transient pulmonary infiltrates on chest X-ray and proximal or central bronchiectasis seen on CT scan.

4.2. Immunodiagnosis

The immunodiagnosis of *A. fumigatus* induced diseases has been based on the *in vivo* (skin prick and intradermal tests) and *in vitro* tests (radioallergosorbent test, enzyme linked immunosorbent assay and Western blotting). The sensitization of host with allergens of *A. fumigatus* may induce elevated levels of total serum IgE and *Aspergillus* specific IgE and/or IgG antibodies. The detection of total IgE and *Aspergillus* specific IgE and IgG antibodies in sera has been considered to be important immunodiagnostic criteria for ABPA [10, 19, 66-69]. The determination of anti-*Aspergillus* antibodies in sera of suspected subjects was dependent on sensitivity of the test. The skin prick and intradermal tests performed with antigens of *Aspergillus* have been found to be nonspecific in significant number of individuals [70]. For the skin prick test (SPT) positive allergic patients, the radio allergosorbent test (RAST) and enzyme linked immunosorbent assay (ELISA) were used regularly in laboratories for detection of *Aspergillus* specific IgE antibodies which show high specificity and sensitivity to detect ABPA. There are few robotic platforms (i.e. ImmunoCap, Phadia, Sweden and Immulite, Siemens, USA) that exist at multispecialty testing centers to perform immunoassays based on crude and specific recombinant antigens of *A. fumigatus* for detection of allergic aspergillosis. The determination of IgG in the sera of patients against antigens of *A. fumigatus* by manual ELISA was also used for detection of ABPA at the commercial level (De-Meditec, Germany, and DRG International, Inc, USA). However, there has been a lack of antigenic standardization among different laboratories because of the use of local antigen preparations [71]. With no choice of specific antigens for detection of antibodies, only crude antigenic fractions of *A. fumigatus* were commonly used for diagnosis of ABPA. The crude extracts used at different centers for immunodiagnosis of ABPA have not been comparable in their protein composition due to use of different strains [72] and batch-to-batch variations even with same strains, has been quite common [73]. The fungal extracts mainly produced from mycelial cells and/or spores may differ in their protein

composition [74]. Further, the culture conditions, protein extraction methods and storage procedures have been critical with respect to the quality and quantity of individual allergen in the crude extracts of *A. fumigatus* [75]. These factors may also affect the degradation rate of the extracted proteins in antigenic preparations [76]. There have been instances of frequent variability in antigenic preparations and that may lead to variable sensitivity and specificity of the test [77]. Additionally, the ELISA kits for detection of *Aspergillus* antigens like, mannan and galactomannan in IA have been considered to be good in specificity but sensitivity was found to be quite variable [78]. Hence in the absence of optimally characterized allergens, the use of locally prepared crude antigenic fractions of *A. fumigatus* for diagnosis of ABPA is continued at different diagnostic centers.

5. Allergens of *A. fumigatus*

5.1. Known allergens

The *A. fumigatus* produces a large number of allergenic molecules which show reactivity with IgE antibodies derived from patients of ABPA [73, 79, 80]. According to an allergen database, allergome (www.allergome.org), only 23 molecules of *A. fumigatus* (Asp f1-f13, Asp f15-f18, Asp f22, Asp f23, Asp f27-f29, and Asp f34) have been recorded as allergens. One of these allergens, the Asp f15, has been proposed to be removed from the list due to its sequence similarity with Asp f13. Also the allergen Asp f16 has been shown to have a high degree of homology with Asp f9, thus, making the effective list of 21 allergens of *A. fumigatus*. Some of these allergens are known for structural, toxic and enzymatic functions and their relationship with virulence also has been reported. However, other allergens do not have virulence properties, except being as immunologically active molecules [81]. These antigenic components increase the risk of allergic aspergillosis because they may redirect the immune response to Th2 type, a response that does not seem to be efficient in eliminating the fungus from the body of host [82].

The description of known allergens of *A. fumigatus* demonstrated that the Asp f1 has been a major 18 kDa allergen belonging to member of the mitogillin family of cytotoxins [83] and recombinant Asp f1 was used to detect *Aspergillus* specific antibodies in the sera of patients [84]. The 37 kDa recombinant protein (Asp f2) has been an allergen of *A. fumigatus* which was evaluated for its immunological properties [85]. The Asp f2 showed IgE binding with ABPA and

cystic fibrosis patients having ABPA [86]. Hemmann et al (1997) cloned Asp f3 from a cDNA library displayed on phage surface. In an ELISA, serum IgE antibody reactivity to rAsp f3 was detected in 72.0% of 89 individuals sensitized to *A. fumigatus* [87]. There are studies which showed that the rAsp f3 provided protective immunity in a mice model against experimentally induced aspergillosis [61] which later on was proposed to be a potential vaccine molecules against aspergillosis [64, 65]. The deletion mutation analysis of Asp f4 performed by Ramachandran et al (2004) showed differential reactivity pattern with IgE antibodies derived from sera of ABPA patients [88]. The Asp f5 has been a 43 kDa serine protease of *A. fumigatus*. Experiments were conducted to study, if Asp f5 contributes to the virulence. Results showed that the gene-disrupted mutants for Asp f5 of *A. fumigatus* retained virulence and it has been considered to be involved in degradation of lung structural architecture by enzymatic activity [89]. Cramer et al (1996) used serum IgE antibodies from *A. fumigatus*-sensitized individuals to enrich phage-expressing allergenic proteins [90]. They identified one cDNA encoding for 26.7 kDa manganese superoxide dismutase called Asp f6 sharing 67.2% homology with human manganese superoxide dismutase which also showed autoreactivity in allergic persons sensitized to Asp f6 of environmental *A. fumigatus*. The allergen Asp f8 was identified as acidic ribosomal phosphoprotein type 2 (P2 protein) which also shared 84.0% sequence homology with human P2 protein. Both recombinant P2 proteins showed strong IgE reactivity and Th1 type skin reactions in *A. fumigatus* sensitized individuals. These observations suggested that the patients suffering from chronic *A. fumigatus* allergy be evaluated carefully for *in vitro* and *in vivo* humoral and cell-mediated responses to human P2 protein [91]. Another allergen (Asp f9/Crf) of *A. fumigatus* had significant sequence homology with Asp f16 which was identified as cell wall glucanase involved in cell wall assembly. The Asp f9 also showed positive IgE reactivity in allergic patients as demonstrated by ELISA and SPT [79, 92]. The gene encoding for Asp f10 (aspergillopepsin F) was cloned and sequenced by Lee and Kolattukudy [93]. It was reported that Asp f10 can catalyze the hydrolysis of major structural proteins of basement membrane such as elastin, collagen, and laminin in *A. fumigatus* infected organs of humans. The Asp f11 (cyclophilin) was identified as peptidyl-prolyl cis-trans isomerase and reported as cross-reactive protein across species including humans [94]. Kumar et al (1993) identified a heat shock protein (Hsp 90, Asp f12) of 65 kDa in *A. fumigatus* [95]. The amino acid sequence of Asp f12 was homologous with Hsp 90 family of heat shock proteins in humans as well as other organisms. The rAsp f12 reacted with IgE and IgG antibodies in the sera of patients with ABPA. The Asp

f13, which was renamed as Asp f15 (alkaline protease) of *A. fumigatus* was found to share IgE and IgG epitopes present in allergens of other *Aspergillus* species [96]. The serine protease of *A. flavus* was found to be cross-reactive with the antibodies prepared against Asp f13/Asp f15 of *A. fumigatus* [97]. Recent studies implied that the complement-degrading activity of Asp f15 was similar with Asp f13 [98]. The gene *AFMP1* of *A. fumigatus* encodes the antigenic cell wall galactomannoprotein (Asp f17) having molecular weight of 31.2 kDa. It was also reported that patients with aspergilloma and IA due to *A. fumigatus* develop a specific antibody response against Asp f17 [99]. Shen et al (2001) cloned and expressed a 34 kDa major allergen (Asp f18) of *A. fumigatus* which was identified as a vacuolar serine proteinase. The results of immunoblot inhibition assay showed IgE cross-reactivity of Asp f18 among major allergens of *A. fumigatus*, *Penicillium notatum* and *P. oxalicum* [100]. Using 2DE immunoblotting, Lai et al (2002) identified a 47 kDa IgE reactive component (Asp f22) in the extracts of *A. fumigatus* and *P. citrinum*. The IgE cross-reactivity of rAsp f22 was also detected by immunoblot inhibition experiments with enolases of *Alternaria alternata* [101]. The Asp f23 of *A. fumigatus* showed specific binding with IgG and IgE antibodies present in sera of ABPA patients and was further characterized as an allergen (60S ribosomal protein L3) of *A. fumigatus*, by cDNA library screening approach using pooled sera of ABPA patients [102]. The 60S ribosomal protein L3 is found in humans also with 67% sequence homology to Asp f23. It was, therefore, suggested that there exists a possibility of involvement of *A. fumigatus* 60S ribosomal protein L3 in autoimmune reactions. A cyclophilin (Asp f27) from *A. fumigatus* has been cloned, expressed and shown to exhibit cross-reactivity *in vitro* and *in vivo*. Glaser et al (2006) identified conserved solvent-exposed residues in the fungal and human cyclophilin that were potentially involved in the IgE-mediated cross-reactivity [103]. The thioredoxins (Trx) of *Malassezia sympodialis* and *A. fumigatus* were identified as novel IgE-binding proteins. The Trx, including the human enzyme, represent cross-reactive domains recognized by binding with serum IgE from individuals sensitized to Trx of *M. sympodialis*. The Mala s13 (a Trx of *M. sympodialis*) shares 45.0% sequence identity with human Trx [104]. Glaser et al (2008) cloned human Trx, Mala s13 and 2 thioredoxins (Asp f28 and Asp f29) of *A. fumigatus* to produce them as recombinant proteins [105]. All recombinant thioredoxins, including that of human, did bind to IgE from patients with ABPA. Inhibition experiments also showed that the thioredoxins were cross-reactive. The same group of investigators described another allergen (Asp f34) from cDNA library screened for cell wall components. The recombinant Asp f34 showed binding in western blots with IgE antibodies

derived from *A. fumigatus* sensitized individuals. It was also found to be involved in the induction of allergen-specific proliferation of PBMCs of patients sensitized to the allergen [106].

5.2. Predicted allergens

In addition to the 21 known allergens of *A. fumigatus*, 25 allergens were predicted on the basis of their cross-reactivity and sequence similarities with other potential allergens from several fungal and bacterial sources [107]. These predicted allergens have been identified as alcohol dehydrogenase, α -amylase, β -xylosidase XylA, glutathione S-transferase GliG, molecular chaperone Hsp70, thioredoxin, 60S acidic ribosomal protein P1, aldehyde dehydrogenase AldA, alkaline serine protease Alp1, aryl-alcohol dehydrogenase, β -N-acetylhexosaminidase NagA, conidial hydrophobin Hyp1/RodA, extracellular lipase, glucan 1,4- α -glucosidase, histidine acid phosphatase, HLH transcription factor GlcD- γ , Hsp70 chaperone Hsp88, malate dehydrogenase NAD-dependent, NADH-quinone oxidoreductase Pst2, nuclear transport factor NTF-2, protein disulfide isomerase Pdi1, RNA binding protein MSSP-2, secreted dipeptidyl peptidase DppV, thioredoxin and translation elongation factor 1 subunit Eef1- β . These predicted allergens are involved in diverse cellular processes such as cellular maintenance and metabolism, and few of them have been characterized as pathogenic determinants. Of the predicted allergens the secreted dipeptidyl peptidase DppV, nuclear transport factor NTF-2, thoredoxins, protein disulfide isomerase Pdi1 and alkaline serine protease Alp1 of *A. fumigatus* have been investigated for their antigenic potential but found to be nonspecifically reactive. Many of the predicted allergens of *A. fumigatus* were found to have significant amino acid sequence homology with potential allergens that were identified from other organisms (http://fermi.utmb.edu/SDAP/sdap_ppm.html). Thus it may be suggested from the available literature on predicted allergens of *A. fumigatus* that they do not have enough potential to solve the problem of cross reactivity.

5.3. Use of allergens of *A. fumigatus* in diagnosis

Of the known and predicted allergens of *A. fumigatus* 5 namely rAsp f1, rAsp f2, rAsp f3, rAsp f4 and rAsp f6, were shown to have high IgE reactivity [69, 108] and have been used commercially for diagnosis of allergic aspergillosis (Immunocap, Phadia, Uppsala, Sweden). An enzyme immunoassay (the Platelia) developed by Bio-Rad for detection of anti-*A. fumigatus* IgG

antibodies, was based on a recombinant *A. fumigatus* antigen. It was evaluated by Guitard et al, (2012) using sera of 551 patients of non invasive aspergillosis (NIA) in 5 different groups that included subjects having definite diagnosis of NIA (group 1; n = 64), bronchial *Aspergillus* colonization (group 2; n = 26), probable aerial *Aspergillus* contamination (group 3; n = 44), patients suspected of NIA with negative serological and mycological findings (group 4; n = 49) and patients not suspected for NIA (group 5; n = 222). It was found that the methodology was a highly useful screening tool for the diagnosis of NIA [109]. Comparison of conventional diagnostic criteria (detection of anti-*A. fumigatus* IgE, IgG, and precipitins) with recently described biomarkers, such as IgE towards recombinant allergens of *A. fumigatus*, revealed that later was an useful tool for diagnosing ABPA in patients of cystic fibrosis [19]. But the application of recombinant allergens has been limited mostly to the IgE detection only. The cross-reactivity of other recombinant allergens of *A. fumigatus* due to sharing of common antigenic epitopes with antigens of other organisms has made it difficult to use them for diagnostic purpose universally [110]. Therefore, to overcome the limitations associated with *A. fumigatus* recombinant molecules or crude antigens based assays, the identification of new and specific allergens has become essential for the development of more specific diagnostic assays to detect allergic aspergillosis in the suspected patients at an early stage of infection.

6. Complex genome and proteome of *A. fumigatus*

The genome of *A. fumigatus* has been quite complex and so is the proteome. The release of complete genome sequence of *A. fumigatus* (29.4 megabases), has identified the presence of 9,926 genes in this pathogenic mold [111]. Till date the products of many of these genes have not been identified and are just only predicted. Recent update on genomic data of various *Aspergillus* species provided resource material for *in silico* predictions and among them a web based tool, “The Central *Aspergillus* Resource” enabled to analyze the molecular content of *A. fumigatus* [112]. Also “The *Aspergillus* genome database” has included complete comprehensive review of the entire published literature on *A. fumigatus* as well as information on other pathogenic species of *Aspergillus*. The *Aspergillus* genome database facilitates comparative genomics by providing a full-featured genomic viewer, as well as matched and standardized sets of genomic information for the sequenced species of *Aspergillus* [113]. This information could be considered as a background for identification of molecules which are

unknown yet, but their sequences have been identified in *A. fumigatus* genome and which might play crucial role in the development of *Aspergillus* induced allergic and invasive diseases.

The analysis of known and predicted allergens of *A. fumigatus*, suggested that a single molecule may not be sufficient for universal use in diagnosis of ABPA at initial stages of infection [70]. Glaser et al (2008, 2009) used cDNA library screening approach in order to improve available diagnostic tools, but selecting the right protein target using cDNA screening proved to be difficult [105, 106]. Thus the multiple antigens from less explored cytosolic antigenic fraction in combination with secreted molecules may form a panel of potential key determinants for the selection of universally reactive molecules for wider application in early and more specific diagnosis of *A. fumigatus* induced disorders. Recent advances in technologies introduced microarray based allergen chip diagnosis that made it possible to detect IgE against multiple allergens in small amounts of serum [114]. Though the recombinant allergen based chip diagnosis approach appeared to be very promising but still needs evaluation and optimization with regard to selection of allergens for use in the chip assay [115]. In fact cloning and expression of all antigens/allergens of *A. fumigatus* to elucidate their biological functions has not been feasible and the dependence on available less useful few recombinants continues. It is, therefore, important to identify new functional molecules by advanced techniques like proteomic tools, clone them and produce recombinantly for various applications.

It was evident from literature that proteomic characterization of micro organisms became possible after introduction of two dimensional gel electrophoresis (2DE) by O'Farrell (1975) who resolved the complex proteome of *E. coli* [116]. Since then 2DE has been considered as a powerful tool for separation of proteins from any biological samples. Coupling 2DE with the advanced proteomic analysis techniques like multidisciplinary chromatography and mass spectrometry, served as key to unveil complex pathogenic contents of several microbes. However, the first report on the proteomic based identification of *A. fumigatus* molecules appeared only a decade back [117]. The possible reasons for such delay in availability of proteomic based information on *A. fumigatus*, were (i) the difficulties in sample preparation from various fractions i.e. cell wall, secreted, cytosolic and mycelia [118], and (ii) inherent properties responsible for poor separation of fungal proteins by 2DE [119]. Vodisch et al (2009) generated the proteome map of *A. fumigatus* from cytosolic and mitochondrial fractions [120]. Teutschbein

et al (2010) identified proteins from dormant conidia to provide complete profiling of *A. fumigatus* conidial fraction [121]. Proteomes of *A. fumigatus* from different early developmental stages [122] and conidial fraction [123] to identify stage specific abundant proteins have been published recently. The above mentioned proteomic studies so far carried out on *A. fumigatus* indicated that various fractions of this pathogenic mold could be explored for identification of new antigenic proteins. For the purpose of improved and early diagnosis of aspergillosis, a thorough description of immunoproteome of *A. fumigatus* is required because antigens of *A. fumigatus* so far reported are of limited value in detecting the allergic aspergillosis at early stages of infection.

7. Immunoproteomics of *A. fumigatus*

In the context of identification of allergens/antigens of *A. fumigatus* few attempts have been made in recent past by using immunoproteomic approach. Gautam et al identified panels of secreted allergens of *A. fumigatus* by IgE reactivity with sera of ABPA patients [124, 125]. Kumar et al (2011) also identified secreted antigens of *A. fumigatus* by immunoreactivity with sera from ABPA patients and those obtained from immunized animals [126]. An account of potential vaccine candidates from cell wall associated [127] and cytosolic fraction [128] of *A. fumigatus* has been reported.

The studies on immunoproteomes of various fractions i.e. cytosolic and secreted of two geographically diverse strains (Indian and German) of *A. fumigatus* for identification and characterization of novel immunodominant antigenic molecules that specifically bind to IgE and IgG antibodies of ABPA patients, were undertaken. The late stage secreted [129], and early stage specific conidial fractions containing cytosolic and cell wall associated proteins [130], were used to identify a panel of new antigenic molecules. A list of 98 antigenic proteins of *A. fumigatus* identified by Singh et al [129, 130] is provided in the Table. These proteins included 9 known (Asp f1-f4, f9, f10, f12, f13/15 and f22) and 6 predicted (dipeptidyl peptidase-V precursor, nuclear transport factor-2, malate dehydrogenase, Hsp70, Hsp88 and alcohol dehydrogenase) allergens of *A. fumigatus*. Importantly, 19 proteins from the panel identified by Singh et al [129, 130], which are marked with an asterisk [*] in the Table, were used as new molecules by an

allergen database called allergome (http://www.allergome.org/script/search_step2.php) to update the list of potential allergens of *A. fumigatus*.

There have been few studies which investigated the effect of antifungal molecules on the proteome of *A. fumigatus* [131, 132]. Quite interestingly, 34 immunogenic proteins marked with psi [ψ], in the Table were found to show altered expression in the proteome of *A. fumigatus* due to treatment with an antifungal synthetic coumarin SCD-1 [132], indicating that some of the antigens/allergens of *A. fumigatus* may be important targets for new antifungal therapy. The type of immuoreactivity (IgE, IgG and IgG/IgE both) and the immunodominance are important criteria to be considered for determination of the usability of antigenic molecules of *A. fumigatus* in diagnosis and therapy of the allergic aspergillosis. Also the production of useful antigens/allergens of *A. fumigatus* recombinantly and their immunological characterization would be essential for possible commercial application.

8. Strategies for the recombinant expression

To prepare various antigenic proteins recombinantly, the choice of suitable expression system was always a matter of concern. However, a variety of prokaryotic and eukaryotic expression systems have been evolved to solve expression associated issues. In this section we briefly discuss the expression related problems and their alternate approaches. The prokaryotic systems have remained as first choice for expressing recombinant proteins. Among the prokaryotes, the *E. coli* has been considered as the most efficient and widely used host for manipulation of DNA and proteins which can express recombinants up to 30% of its cell mass [133]. The well-known genetics, high transformation efficiency, cultivation simplicity, rapid cell cycle and inexpensiveness, are the main factors that make *E. coli* to be the preferred host for the recombinant expression of proteins. It was reported that expression of many recombinant allergens of *A. fumigatus* such as Asp f3 [87], Asp f6 [91], Asp f9/Asp f16 [92], Asp f10 [93], Asp f17 [99] and Asp f23 [102] has been carried out successfully in the *E. coli*. We also produced 1 protein as full length recombinant, selected from a panel of potential allergens of *A. fumigatus*. This allergen has an mRNA (*AFUA_5G03520*) of 972 bp which coded for a 37 kDa hypothetical protein (Uniprot ID: Q4WEM3). The recombinant 37 kDa protein was found to be reactive with IgE antibodies of ABPA patients (Unpublished data).

Although the expression of 37 kDa full length hypothetical protein was achieved, we faced several difficulties while attempting the expression of other selected immunodominant allergens as full length proteins. Therefore, instead of expressing complete gene, reduction of the length of recombinant construct by removing the incompatible regions of DNA and selecting the functional region of target gene has been followed to express partial protein. It was speculated that the expression of partial protein may enhance the solubility of recombinants. It is evident from the literature that many of the genes severely interfere with the survival of *E. coli* cells during the process of their expression in bacteria and lead to death of host cell or cause significant defects in bacterial growth that dramatically decrease expression capabilities [134]. On the other hand less solubility of the recombinant proteins due to their interaction with inclusion bodies and associated toxicity also remained as major problem which is usually encountered many a times [135]. The occurrence of hydrophobic regions in the antigens is a major feature that caused toxicity to host cells and hindered their expression in *E. coli*. However, it was also reported that the presence of hydrophobic regions are crucial of the generation of efficient vaccine moieties [136]. The presence of hydrophobic domains in *A. fumigatus* allergens might have been responsible for inhibiting the full length expression in *E. coli*. Therefore, the removal of hydrophobic regions from proteins (by gene cloning methods) and retaining a minimal functional domain may allow high level expression of functional proteins in *E. coli*. To resolve this issue the hydrophobicity index of the allergens of *A. fumigatus* was predicted using a web based tool TopPred v 0.01 (<http://mobyli.pasteur.fr/cgi-bin/portal.py?#forms::toppred>) and accordingly selected the less hydrophobic polypeptide of the proteins for recombinant expression. Interestingly, employing this approach, we had been achieved the expression of fructose biphosphate aldolase class-II (Uniprot ID: Q4WY39) and alcohol dehydrogenase (Uniprot ID: Q4WTV5) in *E. coli* [130]. It was reported in other pathogens also that the plasmid constructs encoding for truncated parasite antigens (TSOL18 of *Taenia solium* and EG95 of *Echinococcus granulosus*) with deleted N- and C-terminal hydrophobic domains significantly improved heterologous expression from insoluble to soluble form of proteins in *E. coli* [137].

It was suggested that the fusion partner neutralizes the toxic effect of the target protein to some extent and increases the stability of recombinant protein in the expression cells. Some of these fusion partners include glutathione-S-transferase, maltose binding protein, thioredoxin, small ubiquitin modifier, and N-utilization substrate. Of them glutathione-S-transferase and

maltose-binding protein have an advantage to remain as affinity purification tag also. It was noticed that a single fusion tag will not work for all recombinant proteins, and multiple fusion partners may be needed to be used for successful expression of the soluble recombinants. However, it is important to note that the neutralizing partner of fusion protein may not be enough to eliminate the toxicity of target protein completely. But a strong interaction of fusion partner with target protein, may lead to a suppression of the toxic effect [134]. Recombinant proteins that are toxic to the expression host can be challenging to produce, but this difficulty can usually be overcome by using expression of the recombinant gene in the regulation of an inducible promoter, transcription terminators, control of the plasmid copy number, and/or modification of the coding sequence of the recombinant gene [138]. If the recombinant protein is toxic, it is often useful to determine whether the problem is host cell specific. If so, then the protein can be expressed in a more compatible expression host. Another option could be to use a tightly regulated prokaryotic inducible expression system. The expression of the mammalian apoptosis modulator protein Bax in *E. coli* is an important example illustrating the rationale design of a neutralizing partner. The Bax protein leads to the lysis of *E. coli* cells at concentrations as low as 0.01% of total proteins [139]. Attempts to express Bax protein as full length into the periplasm of *E. coli* failed to diminish its toxic effects, therefore, several strategies have been employed to express it as a fusion protein [140]. We also attempted to express one antigenic protein (sorbitol/xylulose reductase Sou1 like) in its full length or partial form but recombinant expression was not achieved. Thereafter, employing the strategy to express sorbitol/xylulose reductase Sou1 like, with a fusion partner resulted in recombinant production of protein of 67 kDa which included a part of 38 kDa of fusion partner which was maintained on its C-terminus (Unpublished data).

Although *E. coli* has been the first choice over all other expression systems, it has certain limitations. It was reported that the *E. coli* usually has been incapable of providing the environment suitable for the correct formation of disulfide bonds at the time of expression of the recombinant proteins in the cytoplasm [141]. Formation of disulfide bond in *E. coli* takes place only in the periplasm in presence of Dsb system [142] and specific periplasmic expression in *E. coli* reduces yield of protein significantly. It is also seen that *E. coli* based expression systems were less efficient for post-translational modifications as compared to eukaryotic host. Moreover, the secondary post-translation modifications such as enzyme-mediated N-linked glycosylation,

O-linked glycosylation, amidation, hydroxylation, myristoylation, palmitation, or sulfation were not supported by *E. coli*.

To overcome limitations associated with *E. coli* based systems, the eukaryotic expression systems are the alternate approach. *Yeast* is the most powerful organism for the manipulation of DNA and expression of recombinant proteins after *E. coli*. Among yeasts, the *Saccharomyces cerevisiae* [143] and *Pichia pastoris* [144] are the routinely used systems because they typically permits higher levels of recombinant protein expression with secondary post translation modifications. The *P. pastoris* has been used to obtain both intracellular and secreted recombinant proteins. Apart from *Yeast* based expression system, *Baculovirus* (a lytic, 130 kb long double-stranded DNA, virus) mediated expression in insect cells (fall armyworm *Spodoptera rugiperda*, *Sf 9*, *Sf 21*) offers another useful tool for generating recombinant proteins with secondary modifications [145]. As the last choice, mammalian expression methods have conventionally been taken as the least efficient machinery for expressing recombinant proteins. But due to the excellent property of expressing recombinants with most superior folding and disulfide bond formation in comparison to other expression hosts, the mammalian cells have been used oftenly. Recent advances have significantly improved the expression efficiency of mammalian cell lines. It was reported that the stably transected Chinese hamster ovary cells expressed recombinant antibodies up to a level of a few grams per liter [149, 147].

Employing these strategies on the expression of new antigens of *A. fumigatus* and developing recombinant based microarray will be helpful for providing new molecules and/or methods with superior applications in diagnostics and desensitization therapies. It was shown that the microarrayed recombinant allergens can be used to determine and monitor the profile of disease-eliciting allergens using single tests that require minute amount of serum from allergic patients [148]. In a cross sectional survey of IgE reactivity in 23077 patients against a panel of 75 potential allergens was carried out by using microarray based detection system [149]. A chip microarray based on 1205 proteins of *Burkholderia pseudomallei*, probed with 88 melioidosis patient sera, identified 170 antigens which included 49 specifically reactive antigens and 59 cross-reactive antigens. On the basis of these results, authors devised a test that could classify melioidosis positive and negative individuals with sensitivity and specificity of 95% and 83%, respectively [150].

9. Antigenic epitopes and peptide library based approaches

The prediction of antigenic epitopes and exploration of peptide library screening approaches have several advantages. It was found that full length sequence of a protein in terms of immune-recognition may contain several non antigenic and hypo-antigenic regions. For the characterization of total antigenic structure of a protein, identification of IgE and/or IgG binding epitopes is a determinant factor. The dissimilarity in the linear and conformational epitopes in the same protein, is a confounding factor for type of reactivity. A link between paratope, epitope and mimotope influences the definition of a specific interaction between antigen and antibody [151]. The mimotope can bind antibody without any relation in the sequence of protein antigen used to induce antibody [152]. It was suggested that the IgE binding epitopes are more cross reactive than the IgG binding epitopes. This might be due to the presence of structural mimics (mimotopes) of the hyper-allergenic epitopes of the antigenic proteins. The mimotopes have the capabilities to mimic not only peptides, even they can recognize carbohydrate, lipid or haptens including biotin and they can not be easily mapped on the 3 dimensional structures of antigens [151]. The phenomenon of cross reactivity which occurs mostly with the allergens of *A. fumigatus* may be due to presence of mimotopes of other proteins which hamper the specific reactivity of the target allergens. Thus, avoiding the regions of antigens which contain mimotopes has to be an important criterion while designing the peptide libraries from the newly identified allergens. The peptide microarrays have become increasingly usable in recent years for diagnostic applications [153].

In a recent report, the use of a bioinformatic approach for epitope prediction, their synthesis and evaluation on peptide microarray was described. It was shown that such array was a powerful method for the selection of *Toxoplasma gondii* epitopes for serological diagnosis of toxoplasmosis with diagnostic specificity of 84% [154]. The replacement of lengthy polypeptides of the antigenic proteins with short, synthetic peptides is virtually a straightforward approach to capture antigen-specific antibodies from serum samples, however, both the immuno-biological display of peptides on support and their performance in microarray based immunoassay are often unsuccessful on application [155]. Antibody epitope mapping using synthetic peptide array has been shown to be useful for mapping of full sequence of antigens for the identification of hyper-antigenic epitopes [156]. Several strategies were suggested to generate hypoallergic variants of

allergens to use them as vaccine candidates [157]. The peptide microarray data was also used for the analysis of target specificity of protein [158]. Recently Chen et al, (2012) identified immunodominant epitopes of Pen c13, a major allergen of *P. citricum* by generation of peptides by enzymatic cleavage of full length protein [159]. Further the mutant (G270A) generation of Pen c13, containing IgE binding epitopes revealed their application in diagnosis/prognosis of mold allergy, whereas another hypoallergenic mutant (K274A) of the Pen c13, may provide a framework for the design and development of a safe and efficient therapeutic strategy for treating human allergic diseases.

10. Conclusion

It could be inferred from the above description that the diagnosis of allergic aspergillosis at the early stages of infection, and its effective treatment is extremely difficult. Therefore, ABPA remains an encumbrance on human health globally. The limitations in terms of cross-reactivity of known and predicted allergens of *A. fumigatus* diminished their diagnostic relevance. Also the molecules characterized as pathogenic/virulence factors of the *A. fumigatus* are not very much promising in development of strategies for detection of ABPA. Furthermore, the difficulties have been encountered while expressing several biologically functional and immunodominant antigenic/allergenic molecules of *A. fumigatus* recombinantly. The immunoproteomic studies on crude secreted and cytosolic fractions of *A. fumigatus* identified a panel of new immunogenic molecules. *In silico* assessment of these molecules provided predictive information on the topology, antigenicity, linear and/or conformational architecture of the epitopes in their amino acid sequences. Characterization as full length recombinant and generation of peptide libraries on the basis of newly identified antigens of *A. fumigatus* may have potential applications in the diagnostic and therapeutics. However, the selection of the specific allergen and thereafter, identification of peptides from single or multiple proteins is the utmost difficult task. Hence a thorough screening of the peptides derived from novel antigens, and preparation of their different compositions are essentially required to minimize the occurrence of mimotopes. The effective methods for immobilization of the selected IgE and IgG reactive peptides on the microchips to construct the tools for the development of peptide based chip array of customized molecules will be useful in specific and early diagnosis of allergic forms of aspergillosis. Also the thoroughly

characterized antigenic/allergenic molecules of *A. fumigatus* and the suitable peptides from them may have immunotherapeutic potential against *A. fumigatus* induced disorders.

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Table. List of newly identified potential immunoreactive molecules of *A. fumigatus*

S. No.	Uniprot ID	Identification in <i>A. fumigatus</i>	Biological function	Immuno-reactivity	Antigenicity			Characterized as	Reference	Potential use
					Antigenic Index	B-Cell Epitope	T-cell Epitope			
1	P67875	Asp f1-ribonuclease	Purine-specific ribonuclease	IgG/IgE	High	Multiple	Several	Recombinant, Allergen	[83, 84, 129]	Diagnosis
2 ψ	P79017	Asp f2-hypothetical protein	Not known	IgG/IgE	High	Multiple	Several	Recombinant, Allergen, Drug target	[85, 86, 125, 129, 132]	Diagnosis, Therapy, Drug development
3	O60024	Asp f4- hypothetical protein	Not known	IgG/IgE	High	Multiple	Several	Recombinant, Allergen	[88, 129]	Diagnosis, Therapy
4	Q8J0P4	Asp f 9-probable glycosidase, crf1	Glycosidase, Hydrolase	IgE	High	Multiple	Several	Recombinant, Allergen	[79, 92, 129]	Diagnosis
5	O60022	Asp f 13/15-hypothetical protein	Not known	IgG/IgE	High	Multiple	Several	Recombinant, Allergen	[129, 160, 161]	Diagnosis, Therapy
6*	Q4WEM3	Hypothetical protein	Not known	IgG/IgE	High	Multiple	Several	Native, MS based, Antigen	[129]	Diagnosis, Therapy
7*	Q4WTF6	Aminotransferase-class V, putative	Amino transferase	IgG/IgE	High	Multiple	Several	Native, MS based, Antigen	[129]	Diagnosis, Therapy
8*	P0C959	Dipeptidyl-peptidase-V precursor, DppV	Hydrolase, Protease	IgG/IgE	High	Multiple	Several	Recombinant, Predicted allergen	[126, 129, 162]	Diagnosis, Therapy
9*	Q9Y760	Chitosanase	Chitinolysis	IgG/IgE	High	Multiple	Several	Recombinant, Enzyme, Antigen.	[126, 129, 163]	Diagnosis, Therapy
10	Q4WSV9	1,3- β -glucanoyltransferase, Bgt1	Glycosidase, Hydrolase Transferase	IgG/IgE	High	Multiple	Several	Native, Cell wall molecule, Antigen	[129, 164]	Diagnosis, Therapy
11	Q4WXR8	Nuclear transport factor 2, NTF-2	Transcription factor	IgG	High	Multiple	Several	Recombinant, Cross reactive, predicted allergen	[129, 165]	Immuno-therapy
12	Q4WW81	Fucose specific lectin, FleA	Sugar binding	IgG/IgE	High	Multiple	Several	Recombinant, Cell wall molecule, Antigen	[129, 166]	Diagnosis, Therapy
13	Q4WK08	FG-GAP repeat protein	Ligand binding	IgG/IgE	High	Multiple	Several	Native, MS based, Cell wall molecule, Antigen	[126, 129]	Diagnosis, Therapy
14	P41748	Aspf10 aspergillopepsin-F	Aspartyl protease	IgE	High	Multiple	Several	Recombinant, Allergen	[93, 129, 167]	Diagnosis
15	B0XT72	1,3- β -glucanoyltransferase, gel1	Transferase activity	IgG/IgE	High	Multiple	Several	Native, Enzyme, Cell wall molecule, Antigen	[129, 168, 169, 170]	Diagnosis, Therapy
16*	B0Y8H9	1,3- β -glucanoyltransferase, gel2	Transferase activity	IgG/IgE	High	Multiple	Several	Native, Cell wall molecule, Antigen	[129, 169, 170]	Diagnosis, Therapy
17	A4D9F7	Class V chitinase	Glycosidase, Hydrolase	IgG/IgE	High	Multiple	Several	Recombinant, Cell wall degrading enzyme, Antigen	[126, 129, 171, 172]	Diagnosis, Therapy
18*	Q92405	Catalase-B, Cat-B	Heme binding	IgG/IgE	High	Multiple	Several	Recombinant, Antigen	[125, 126, 129, 173]	Diagnosis, Therapy

19 ψ	E9RAH5	Thioredoxin reductase, GliT	Reductase	IgE	High	Multiple	Several	Recombinant, Toxin, Antigen, Drug target	[126, 129, 132, 174, 175]	Diagnosis, Drug development
20*	B0XZV8	Lysophospholipase-3, Plb3	Lipolysis	IgG/IgE	High	Multiple	Several	Recombinant, Enzyme, Antigen	[129, 170, 176]	Diagnosis, Therapy
21*	B0Y665	Lysophospholipase-1, Plb1	Lipolysis	IgG/IgE	High	Multiple	Several	Recombinant, Enzyme, Antigen	[117, 129, 170, 176]	Diagnosis, Therapy
22*	Q4WRZ5	Mannosidase, MsdS	Glycosidase, Hydrolase	IgG/IgE	High	Multiple	Several	Recombinant, Morphogenetic factor, Antigen	[126, 129, 177, 178]	Diagnosis, Therapy
23	Q870C0	Chitinase, Chi-B	Glycosidase	IgG/IgE	High	Multiple	Several	Recombinant, Enzyme, Cell wall molecule, Antigen	[126, 129, 171, 172]	Diagnosis, Therapy
24 ψ	O43099	IgE binding protein/Asp f3/pmp20	Cell-redox, homeostasis	IgG/IgE	High	Multiple	Several	Recombinant, Allergen, Vaccine candidate, Drug target	[61, 64, 65, 87, 127, 128, 129, 132]	Diagnosis, Therapy, Drug development
25	Q00050	Asp-hemolysin, Asp-HS	Cytolysis, Hemolysis	IgG	High	Multiple	Several	Recombinant, Invasion enzyme, Antigen	[129, 179, 180]	Therapy
26	Q4WG16	GPI-anchored cell wall beta-1,3-endoglucanase EglC	Glycosidase, Hydrolase	IgG/IgE	High	Multiple	Several	Native, MS based, Cell wall degrading enzyme, Antigen	[126, 129]	Diagnosis, Therapy
27	Q4WFY5	Hypothetical protein AFUA_3G00600	Not known	IgG/IgE	High	Multiple	Several	Native, MS based, Antigen	[129]	Diagnosis, Therapy
28	B0Y2B3	Conserved hypothetical protein	Not known	IgG	High	Multiple	Several	Native, MS based, Antigen	[129]	Immuno-therapy
29*	Q4WIT0	Pectate lyase A	Lyase	IgG/IgE	High	Multiple	Several	Native, <i>In silico</i> , MS based, Antigen	[129, 181]	Diagnosis, Therapy
30* ψ	Q4WDJ0	NAD-dependent formate dehydrogenase, AciA/Fdh	Oxido-reductase activity	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[128, 129, 132]	Diagnosis, Drug development
31*	Q4WGP3	Malate dehydrogenase, NAD-dependent	Oxido-reductase	IgE	High	Multiple	Several	Native, MS based, Predicted allergen	[128, 129, 130]	Diagnosis
32*	Q7Z7W6	Bifunctional catalase- peroxidase, Cat2	Catalase Peroxidase	IgE	High	Multiple	Several	Native, MS Based, Antioxidant, Antigen	[129, 182, 183]	Diagnosis
33*	Q4WJJ3	Beta-glucosidase	Glycosidase Hydrolase	IgG/IgE	High	Multiple	Several	Native MS based, Cell wall molecule, Antigen	[129]	Diagnosis, Therapy
34*	Q4WD61	FAD/FMN-containing isoamyl alcohol oxidase, MreA	FAD binding Oxido-reductase	IgG/IgE	High	Multiple	Several	Native MS based, Cell wall molecule, Antigen	[129]	Diagnosis, Therapy
35*	B0XS20	Glucose-6-phosphate isomerase	Isomerase	IgE	High	Multiple	Several	Native MS based, Enzyme, Antigen	[129, 130]	Diagnosis
36* ψ	Q4WZX5	Sorbitol/xylulose reductase Sou1-like	Oxidoreductase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130]	Diagnosis, Drug development
37	Q4WY02	Proteasome component Prs2	Hydrolase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis

38	Q4WMR7	Proteasome component Pre9	Hydrolase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
39ψ	A4D9S0	6-phosphogluconolactonase	Hydrolase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
40ψ	Q4WUD7	Pyridoxine biosynthesis protein	Catalytic activity	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
41ψ	E9R3W5	Spermidine synthase	Transferase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
42	Q4WWK6	Phosphoribosyl-aminoimidazole-succinocarboxamide synthase	Ligase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
43	Q4WX65	Inorganic diphosphatase	Hydrolase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
44	Q4WJR3	60S ribosomal protein P0	Ribosomal ribonucleoprotein,	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
45ψ	Q4WTX0	Adenosine kinase	Kinase, transferase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
46ψ	E9R9J7	Thiamine biosynthesis protein (Nmt1)	Function not known	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[128, 130, 132]	Diagnosis, Drug development
47ψ	Q4WY39	Fructose-bisphosphate aldolase, class II	Lyase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[128, 130, 132]	Diagnosis, Drug development
48ψ	Q4WUP8	Transaldolase	Transferase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[128, 130, 132]	Diagnosis, Drug development
49	Q4WT41	S-adenosylmethionine synthetase	Transferase,	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
50	Q4WQK3	Glutamine synthetase	Ligase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
51ψ	Q96X30	Asp f22/ Enolase	Lyase	IgE	High	Multiple	Several	Recombinant, Allergen, Drug target	[101, 128, 130, 132]	Diagnosis, Drug development
52	Q6A3P8	Putative flavohaemoglobin	Electron carrier, Oxidoreductase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
53ψ	Q4WT69	Phosphoglycerate kinase PgkA	Transferase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[128, 130, 132]	Diagnosis, Drug development
54ψ	Q4WT91	Adenosylhomocysteinase	Hydrolase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
55	Q4WQ64	Phosphatidyl synthase	Hydrolase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
56ψ	Q4WM98	ATP citrate lyase subunit (Acl), putative	Lyase, Ligase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
57	Q4WFT3	GMP synthase [glutamine-hydrolyzing]	Ligase, ATP binding	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
58ψ	Q4WN06	6-phosphogluconate dehydrogenase Gnd1	Oxidoreductase, NADP binding	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[128, 130, 132]	Diagnosis, Drug development
59	Q4X282	Gamma-glutamyl phosphate reductase	Oxidoreductase, NADP binding	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis

60ψ	Q4WWD5	Glutamatecarboxy peptidase	Carboxypeptidase, metallo-peptidase protein dimerizaion	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
61	Q4X155	Secretory pathway gdp dissociation inhibitor	Rab-GDP dissociation	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
62	Q4WCS2	Glucokinase GlkA	Kinase, ATP binding	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
63	Q4X0J6	Bifunctional tryptophan synthase TRPB	Pyridoxal phosphate binding,	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
64	Q4X144	3-isopropylmalate dehydratase	Iron sulfur cluster binding	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
65ψ	Q4WRZ4	Phosphoribosyl-AMP cyclohydrolase	Dehydrogenase, Hydrolase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
66ψ	Q4WUD3	Aminopeptidase P	Metallopeptidase, Hydrolase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
67	Q4X1H5	Mitochondrial Hsp70 chaperone (Ssc70)	ATP/unfolded protein binding	IgE	High	Multiple	Several	Native MS based, Antigen	[128, 130]	Diagnosis
68	Q4WAM8	Heat shock protein (Sti1)	Stress response	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
69	Q4WCM2	Hsp70 chaperone (HscA)	ATP binding	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
70ψ	E9RAB9	Hsp70 chaperone Hsp88	ATP binding	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
71ψ	P40292	Asp f12/ Heat shock protein P90/ /Hsp90/Hsp1	ATP/unfolded protein binding	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
72	Q4WJ30	Molecular chaperone Hsp70	ATP binding	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
73ψ	Q4WV25	ATP synthase F1, β subunit	Hydrolase, ATP/metal ion binding	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
74*ψ	Q4WJK8	40S ribosomal protein S3	Ribonucleoprotein	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
75ψ	Q4WLN1	Mitochondrial aconitate hydratase	Tricarboxylic acid cycle mediator	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[128, 130, 132]	Diagnosis, Drug development
76ψ	O42630	Aspartic endopeptidase PEP2	Protease	IgE	High	Multiple	Several	Recombinant, Enzyme, Vaccine candidate, Antigen, Drug target	[128, 130, 132, 184]	Diagnosis, Immuno-therapy, Drug development
77ψ	Q4WH99	Protein disulfide isomerase Pdi1	Ca ion binding, Isomerase	IgG	High	Multiple	Several	Native MS based, Antigen, Drug target	[127, 130, 132]	Immuno-therapy, Drug development
78ψ	Q4X0G7	Translation elongation factor EF-2	GTPase,	IgG	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Immuno-therapy, Drug development
79ψ	Q4WNY2	Cobalamin-independent methionine synthase Meth/D	Methyltransferase Zn ion binding	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development

80ψ	E9QYY1	Isocitrate dehydrogenase, NAD-dependent	Oxidoreductase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
81ψ	Q4WP16	Phosphoribosylaminoimidazole carboxamide formyltransferase/IMP cyclohydrolase	Hydrolase, Transferase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
82	Q4WTN7	Nucleosome assembly protein Nap1	Nucleosome assembly	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
83	Q4WWZ8	Ran GTPase activating protein 1 (RNA1 protein)	Protein binding	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
84	Q4WZI4	Curved DNA-binding protein	DNA binding, Hydrolase, Aminopeptidase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
85	Q4WXF1	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	Isomerase, Mn ion binding	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Diagnosis, Drug development
86	Q4WGP1	Pyruvate dehydrogenase complex, dihydrolipoamide acetyltransferase component	Acetyl transferase, Protein binding	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
87*	P20359	ACT_BOTCI Actin	ATP/protein binding	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
88	Q4WTK5	UPF0160 domain protein MYG1	Function not known	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
89	Q4WPU2	Homocitrate synthase	Acyltransferase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
90	Q4X099	Dihydroxy acid dehydratase Ilv3	Hydratase	IgE	High	Multiple	Several	Native MS based, Antigen	[130]	Diagnosis
91	Q4WIQ6	Myo-inositol-phosphate synthase	Isomerase	IgE	High	Multiple	Several	Native MS based, Antigen	[128, 130]	Diagnosis
92ψ	Q4WQN8	Glutamate/Leucine/Phenylalanine/Valine dehydrogenase	Oxidoreductase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[128, 130, 132]	Diagnosis, Drug development
93ψ	Q4WMS1	Assimilatory sulfite reductase	Oxidoreductase	IgG	High	Multiple	Several	Native MS based, Antigen, Drug target	[130, 132]	Immuno-therapy, Drug development
94	Q4WXF4	Serine hydroxymethyltransferase	Transferase,	IgE	High	Multiple	Several	Native MS based, Antigen	[128, 130]	Diagnosis
95	Q4WTV5	Alcohol dehydrogenase	Oxidoreductase, Zn ion binding	IgE	High	Multiple	Several	Native MS based, Antigen	[128, 130]	Diagnosis
96	Q4WE70	Glyceraldehyde 3-phosphate dehydrogenase GpdA	Oxidoreductase, NAD binding	IgE	High	Multiple	Several	Native MS based, Antigen	[128, 130]	Diagnosis
97ψ	Q4WEU5	Citrate synthase (Cit1)	Acyltransferase	IgE	High	Multiple	Several	Native MS based, Antigen, Drug target	[128, 130]	Diagnosis, Drug development
98	B0Y617	Zinc-containing alcohol dehydrogenase, putative	Oxidoreductase, Zn ion binding	IgE	High	Multiple	Several	Native MS based, Antigen	[128, 130]	Diagnosis

Proteins marked with an asterisk (*) have been updated in allergen database allergome.org as potential allergens of *A.fumigatus*.

Proteins marked with psi (ψ) apart of their immuno-reactivity they were targeted by antifungal drugs in the proteome of *A. fumigatus*.