Progress in diagnosing ABPA in patients with CF by using biomarkers and recombinant allergens.

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Abstract

Aspergillus *fumigatus* is an opportunistic fungus responsible for Allergic Bronchopulmonary Aspergillosis (ABPA), often also seen in patients with cystic fibrosis (CF) (Nikolaizik et al., 1991). The disease is progressive, and there is often difficulty with diagnosis, ultimately leading to respiratory failure. Diagnosis is difficult due to many reasons, including overlapping symptoms and no official agreement for cut-offs with criteria, leading to differences between clinics (Zirbes & Milla, 2008). This thesis describes the need for progress in diagnostic criteria as well as two relatively new methods that seem to be promising. Fricker-Hidalgo et al., sheds light on using recombinant antigen rAspf4 detection against IgE, which gave significant results with high sensitivity. IgE levels are one of the most important criteria in diagnosing ABPA in CF patients, thus new types of criteria are also sought after. Keown et al., describes the usage of measuring exhaled nitric oxide levels in children with CF to diagnose ABPA, leading to significant results and promising new information for more accurate diagnostic criteria in the future.

Key words: Aspergillus *fumigatus*, Allergic Bronchopulmonary Aspergillosis, ABPA, Cystic Fibrosis, CF, Diagnosis, rAspf4, rAspf6, recombinant allergens, Nitric oxide, IgE, eNose.

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1. Introduction

In recent years, many have come to realize the extent of the problem that Aspergillus *fumigatus* brings. This opportunistic fungus establishes in immunodeficient individuals, and is thus dangerous in a hospital environment. Guidelines for infection prevention have since become more strict, nonetheless nosocomial outbreaks still happen (Doll et al., 2017; Furtwängler et al., 2017). A. *fumigatus* is found outdoors and indoors, on a variety of surfaces including soil and building materials and also indoor walls (Weber, 2010). Although its natural niche is soil, this fungus is one of the most ubiquitous species with airborne conidia. Their size (2-3 µm) is small enough to keep the conidia buoyant both in- and outdoors, leading to easy distribution into the lungs. Surveys show that humans inhale at least several hundred conidia per day, which is usually not a problem in the healthy individual, but can lead to detrimental effects in immunodeficient patients (Goodley et al., 1994).

1.1 An introduction to allergic bronchopulmonary aspergillosis and cystic fibrosis

One of the disorders caused by A. *fumigatus* is allergic bronchopulmonary aspergillosis (ABPA), and is currently known as the most severe allergic pulmonary complication caused by this species. Because of its small spore size and the ability to grow in temperatures ranging from 15℃ till 53℃, A. *fumigatus* can easily germinate in the bronchi once inhaled (Slavin, 1996). ABPA is characterized by pulmonary infiltrates, recurring wheezing episodes, bronchial hyperreactivity, and bronchiectasis that has a chance of progressing to fibrosis. Lastly, the growth of hyphae may be seen in sputum as mucus plugs (Moss, 2005).

A commonly found phenomenon is that patients with ABPA already have been diagnosed with a different disease, as A. *fumigatus* is opportunistic. It was shown that ABPA occurs in 10%, till up to 30% of patients with cystic fibrosis (Laufer et al., 1984; Nikolaizik et al., 1991). CF itself is also a common disease, with numbers ranging to 1 in 3000 births in caucasian communities from Northern Europe (O'Sullivan & Freedmandue, 2009). This disease causes severe damage to the lungs, but also to the digestive system and other organs in the body. CF is progressive, and gives patients a significantly lower quality of life. However, these individuals are usually able to attend school and work and may live to their forties or even their fifties (O'Sullivan & Freedman, 2009).

In contrast to diagnosis of ABPA, diagnosis of CF is not difficult. CF can already be diagnosed during the first month after birth, before symptoms begin. Some patients do not experience symptoms until they are in their teenage years, but these individuals often have a milder disease form (Bergeron & Cantin, 2019). Pulmonary symptoms include wheezing, cough with sputum, recurrent sinusitis and stuffy nose, and repeated lung infections. The thick mucus can block enzymes that travel from the pancreas to the small intestine. This leads to symptoms like stool with a foul smell, poor weight gain or growth, intestinal blocking and severe constipation.

In 1988, Lap Chee Tsui and his team identified a gene that seemed to be involved in disease origin. Located on chromosome 7, the CF locus helps generate a gene product that is mutated in the same way in over two-thirds of patients with CF. The product, named CFTR, lacks a phenylalanine residue because of a deletion mutation (Tsui et al., 1988). CFTR is involved in fluid homeostasis, and its primary function is regulation of transport of chloride ions. Abnormal CFTR generates a relative increase in chloride ion concentration, leading to an elevation in levels of sodium chloride and water absorption by osmosis (Bergeron & Cantin, 2019).
As can be seen in figure 1, the CFTR protein is found in the cell membrane and consists of 12 transmembrane segments. The deletion of the phenylalanine group at position 508 decreases the channel opening rate. Consequently, this leads to thick, adherent and viscous mucus that reduces microbial clearance of the lungs and thus increases risk of infection (Chen et al., 2012).

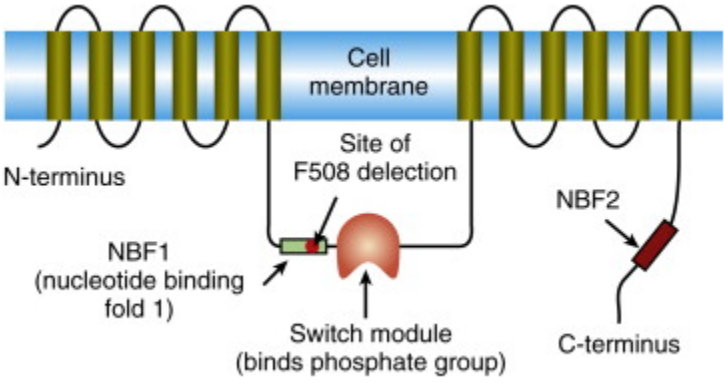


Figure 1: CFTR protein showing the F508 site that is often mutated in CF patients (Clark & Pazdernik, 2016).

1.2 Morphology and Immunology:

Once inhaled, A. *fumigatus* conidia remain in an environment which is perfect for growth, as 37°C is their optimum temperature. This species is a saprotrophic fungi, which means that A. *fumigatus* obtains nutrients from decomposing organic matter. A. *fumigatus* is morphologically quite variable, which makes it difficult to identify on morphology alone. However, when identifying using morphology, conidia and conidiophores are taken into account. The species is characterised by green conidia that can grow as long as 8 µm. Some isolates also produce white or pigmentless conidia (Nayak et al., 2018). At a disadvantage to us, A. *fumigatus* grows fast. The colony size can reach 5 cm within a week grown on agar at 25°C.

ABPA is the allergic form of an A. *fumigatus* infection. An infection by this species is also known as aspergillosis, and besides the allergic form there are also the invasive and the chronic type. (Latgé & Chamilos, 2019). In all three types, the course of infection with aspergillosis starts with the breaching of the epithelial layer. A. *fumigatus* is known to be able to cross the epithelial barrier through actin tunnels without harming the epithelial layer extensively. Besides that, there is more overlap in how the disease progresses, which makes it a lot harder to diagnose, however there are some differences (Fernandes et al., 2018).

The first type of aspergillosis, chronic pulmonary aspergillosis (CPA), is recognised by weight loss, chest pain, night sweats and fungal balls on chest imaging. Whereas in ABPA levels of IgE increase vastly, CPA is known to mostly have an increase in levels of IgG (Denning et al., 2016). Invasive pulmonary aspergillosis (IPA) is the second and the most extreme of the three types. The most serious cases end up in intravascular thrombosis and pulmonary infarctions. Symptoms are acute, and include shortness of breath and chest pain. Especially a fever that persists despite treatment with antibiotics raises suspicion for IPA (Zmeili & Soubani, 2007). Often it is seen that T cell dysfunction predisposes IPA, which includes impaired T cell responsiveness and depletion of naïve T cells (Cramer et al., 2011).
The third and last type of aspergillosis infection is ABPA. The immune reaction in Allergic bronchopulmonary aspergillosis is, as the name suggests, mostly allergic. Currently, ABPA is known as the most severe allergic pulmonary disorder caused by any Aspergillus species. The presence of the fungus in the lungs and its continuous release of allergens and proteases leads to the activation of an immune response mainly driven by Th2-type cells, as well as high production of IgE antibodies (fig. 2). Whenever A. *fumigatus* breaches the epithelial cell layer, Antigen presenting cells come into contact with Th2 cells that will produce IL-13 and IL-4 (Kauffman, 2003).

These interleukins lead to local and systemic eosinophilia. Eosinophils are the cause of damage to the epithelial layer since they produce toxic granular proteins. In addition, the previously mentioned interleukins induce the release of TGFb2, which helps transform fibroblasts into myofibroblasts that end up producing endothelin-1 (ET-1) and vascular endothelial growth factor (VEGF). Consequently, these secretions lead to irreversible remodeling of the lungs, or even bronchiectasis/fibrosis. Patients can also be observed with a Th1 response and the formation of IgG and IgA to A. *fumigatus* antigens (Kauffman, 2003). IgE is of special interest as it is important in the process of diagnosis, which will be discussed in the next part. This is because the adaptive immune response has elevated levels of A. *fumigatus*-specific IgE antibodies (Moss, 2005).

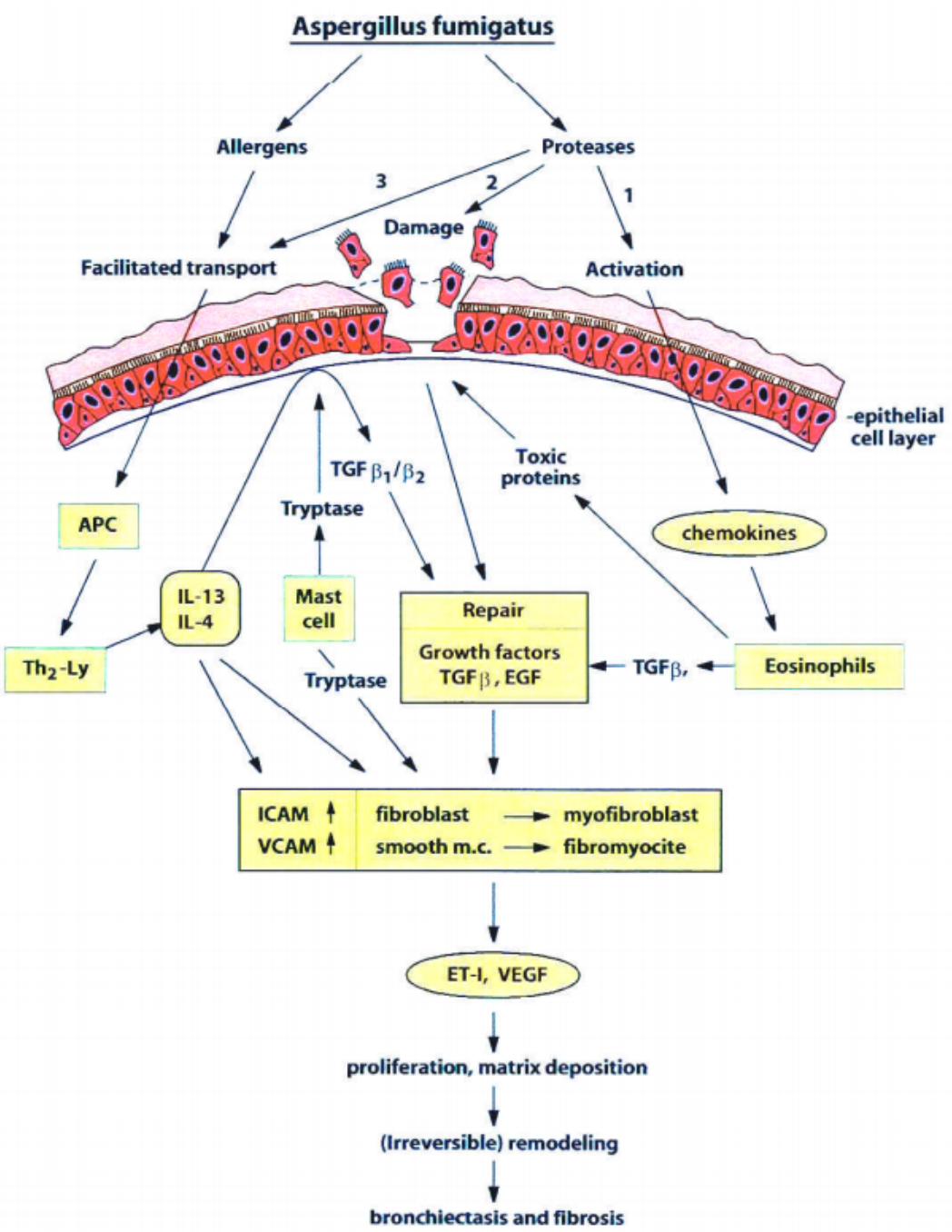
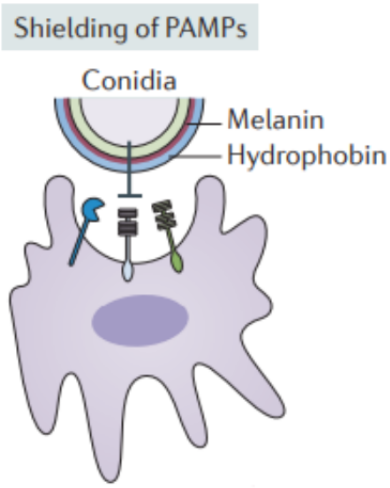


Figure 2: Schematic overview of the immune response to an Aspergillus *fumigatus* infection (Kauffman, 2003).



The human body uses pattern-recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) on invading species, and start an immune response to induce phagocytosis of infected cells, as well as regulating cytokine signalling to activate the immune system. Whenever a microorganism is able to evade recognition, it makes it easier for the species to colonize and spread disease. A. *fumigatus* is able to evade this by masking PAMPs on the cell wall with conidial hydrophobin. A second evading mechanism is the production of melanin, this blocks the acidifying of phagosomes and inhibits the NADPH oxidase complex, which is responsible for the production of antifungal ROS (Wassano et al., 2020).

Figure 3: Two ways A. Fumigatus evades the immune system by shielding of PAMPs (van de Veerdonk et al., 2017)

1.3 The big problem with ABPA: diagnosis

Diagnosis of ABPA in patients is difficult, as all criteria are rarely fulfilled, and features of disease are not stagnant overtime. Take for example bronchiectasis, which is only detected in later stages of the disease (De Soyza & Aliberti, 2017). Limitations in diagnosis have led to patients developing damage to the respiratory mucosa because of A. *fumigatus*, but not being diagnosed. This has also been referred to as 'silent' ABPA. In these patients, symptoms progress into respiratory failure, with few patients actually having remissions (Schønheyder et al., 1988).

Traditionally, there are 5 essential criteria to clinically diagnose ABPA: (1) airflow obstruction/asthma, (2) skin reactivity to A. *fumigatus*, (3) elevated serum of IgE and IgG specific or A. *fumigatus*, (4) total IgE serum levels above 1000 ng/mL, and lastly, (5) central bronchiectasis (Greenberger & Patterson, 1986). The problem is that patients with CF already have pulmonary function abnormalities to begin with. The minimal diagnostic criteria might not be enough to confidently diagnose patients with ABPA that already have CF, and the need for distinct criteria has long been recognized. The criteria to diagnose have been through changes over time, and were ultimately formulated by the cystic fibrosis Foundation (CFF) in 2003, as seen in Table 1 (Janahi et al., 2017). The criteria from table 1 are more specific, which is necessary for successful diagnosis, but as mentioned before, many characteristics are shared with other lung infections. This thesis underlines the need for progress to acquire more specific criteria to diagnose ABPA in CF patients, and describes new methods to do so by looking into research performed by Fricker-Hidalgo et al. and Keown et al.

Criteria classically listed for diagnosing ABPA

Classic case	Minimal diagnostic criteria
Acute or subacute clinical deterioration that is not attributable to another etiology	Acute or subacute clinical deterioration that is not attributable to another etiology
A serum total IgE level of >2400 ng/mL unless patient is receiving systemic steroids*	A serum total IgE level of >1200 ng/mL*,†
Presence of IgE antibodies to *A. fumigatus in vitro* or immediate cutaneous hypersensitivity to *Aspergillus*‡	Immediate cutaneous hypersensitivity to *Aspergillus*‡ or presence of IgE antibodies to *A. fumigatus*
Precipitating antibodies to *A. fumigatus* or serum IgG antibody to *A. fumigatus* by an *in vitro* test	One of the following
	Precipitins to *A. fumigatus* or IgG antibody to *A. fumigatus in vitro*
New or recent infiltrates (or mucus plugging) on chest radiography or computed tomography that do not respond to antibiotics and standard physiotherapy	New or recent abnormalities on chest radiography or computed tomography that do not respond to antibiotics and standard physiotherapy

Adapted from: Stevens *et al.*[10] *If a patient is receiving steroids, check serum total IgE levels when the patient is off steroid treatment, †If allergic bronchopulmonary aspergillosis is suspected and serum total IgE level is between 480 ng/mL and 1200 ng/mL, repeat testing in 1-3 months, ‡Cutaneous reactivity to *Aspergillus* is indicated by a wheal of 3 mm (or more) in diameter with surrounding erythema following a skin prick test in a patient who is currently off systemic antihistamines. *A. fumigatus* = *Aspergillus fumigatus*

Table 1: Criteria for diagnosing allergic bronchopulmonary aspergillosis in patients with cystic fibrosis, formulated by the Cystic Fibrosis Foundation in 2003 (Janahi et al., 2017).

2. Progress in diagnosis of ABPA.

Early diagnosis of ABPA in cystic fibrosis patients is important to prevent as much serious damage to the lungs as possible. However, diagnosis remains difficult as criteria are not always met and different diagnostic practices are used among clinics (Zirbes & Milla, 2008). Research has been performed to find new ways of diagnosis, including the usage of recombinant allergens and biomarkers, which will be discussed below.

2.1: Diagnosis with the use of recombinant antigens.

Fricker-Hidalgo et al. researched the usage of recombinant antigens to get a more precise diagnostic tool. Recombinant antigens are manufactured artificially, in contrast to native antigens. After evaluation of several recombinant allergens, rAsp f6 and f4 were chosen for research, as IgE levels against these proteins increased almost exclusively in ABPA patients (Crameri et al., 2006). 5 samples of each of 20 patients were taken and analysed using several biological tests. Total IgE and IgE specific against the two recombinants were measured. The latter with a cut-off corresponding to the previously mentioned criteria (2.4 ng/L). Furthermore, IgG specific against the recombinants using ELISA, and aspergillus colonies in the sputum were measured. These biological tests were performed in 13 cystic fibrosis patients with proven ABPA, before analysing and comparing the results to the results of patients with bronchitis/sinusitis and patients without ABPA (fig. 1).

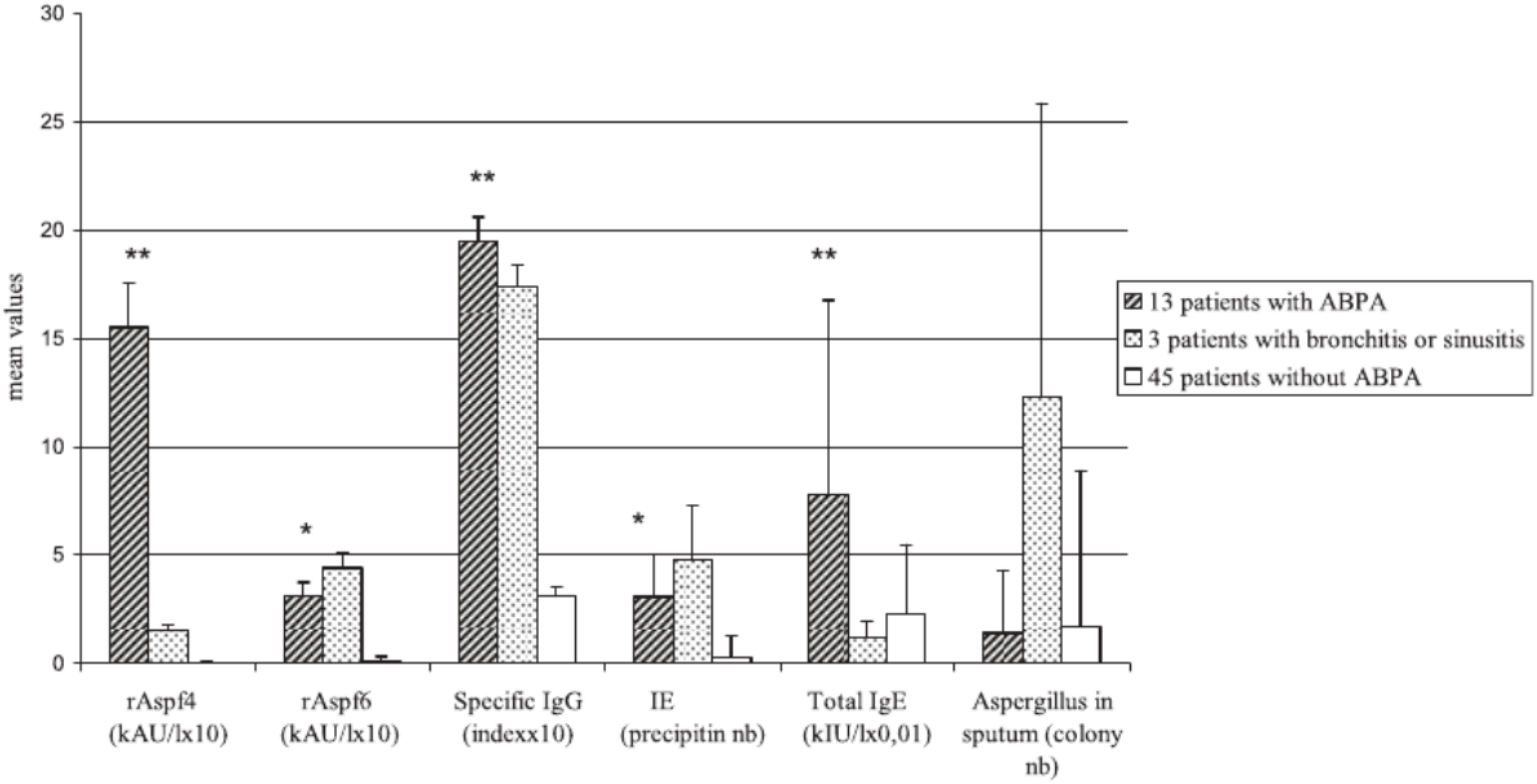


Figure 3**: Results of recombinant antigen analysis obtained of the three groups of cystic fibrosis patients**. Values of IgE detection with rAsp f4 and f6 and specific IgG detection were multiplied by 10, and total IgE was multiplied by 10^{-2}. Significant results can be seen in the ABPA patient and patients without ABPA groups ($P < 0.001$ for rAsp4, specific IgG and total IgE, and $P < 0.05$ for rAspf6) (Fricker-Hidalgo et al., 2010).

As can be seen in figure 1, the sensitivity of rAspf4 is significantly higher than that of rAspf6. Among the 13 patients with proven ABPA, 12 had at least 1 out of 5 samples positive for rAspf4, which is the case for 7 individuals in the context of rAspf6. There were no samples that were positive for rAspf6, that were not positive for rAspf4. Total IgE values fluctuated over time, but the mean values proved to be significantly higher in the individuals with ABPA. 4 patients had a lower IgE value than the cut-off. These patients were on corticosteroid treatment, and would probably have higher values before their treatment. The specific IgG levels were shown to be very high in patients with ABPA compared to patients without ABPA. However, the levels of IgG were also very high in the group of individuals with other forms of Aspergillus disorders. In short, the most specific and sensitive marker in this study is rAspf4 against IgE. The detection was 92.3% compared to rAspf6, which was 53.8%.

2.2: The need for diagnostic criteria besides using IgE levels.

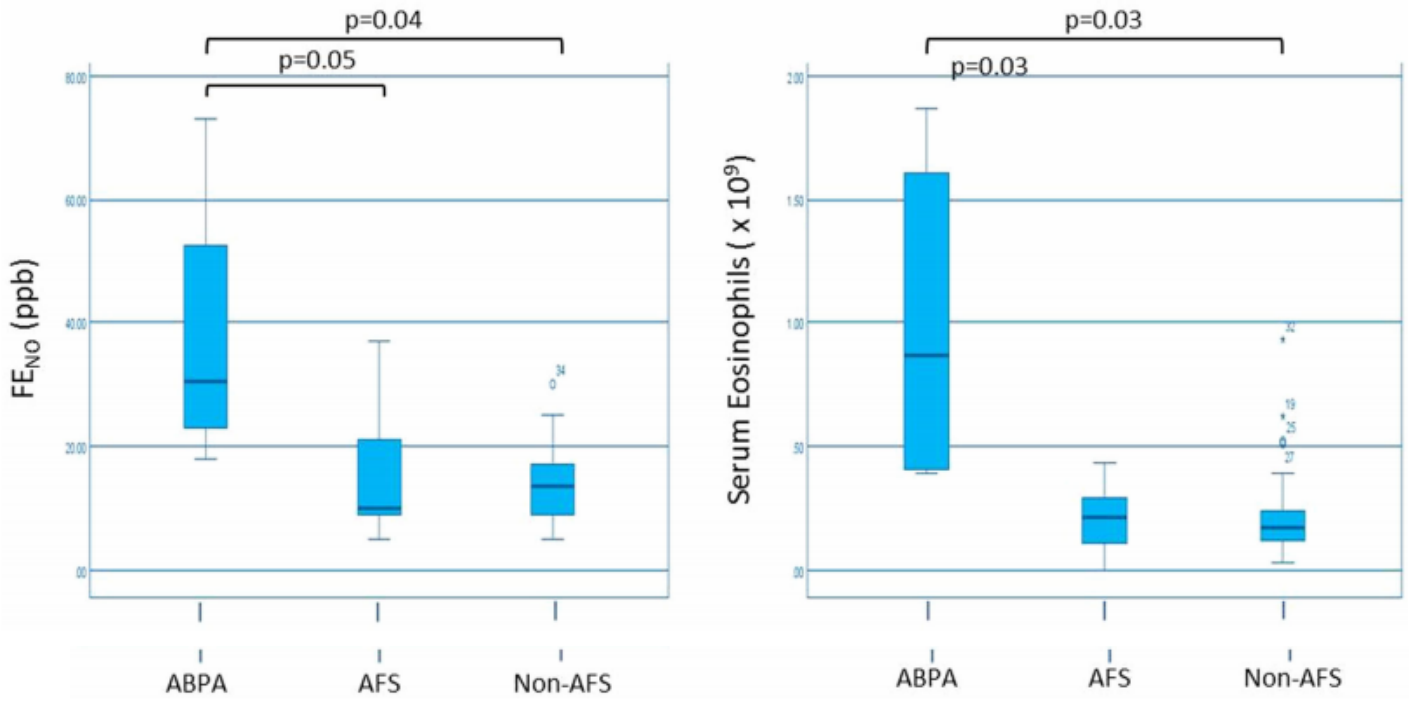
In contrast, there are several articles that have steady arguments for being cautious with the use of IgE levels in ABPA diagnosis in CF patients. Various levels of diagnostic cut-offs have been suggested (>500 IU/mL, >1000 IU/mL), and a staging system of different levels during different stages of progress of disease has also been suggested. The latter is used in patients with asthma and ABPA, but until now it is not often applied in combination with CF (Chotirmall et al., 2008). Instead, IgE should be more useful to exclude ABPA when normal IgE levels are observed.

The problem with focusing on IgE for diagnosing does exist the other way around, where some CF patients have been diagnosed with especially low levels of IgE (<500 IU/mL). In one of these cases the patient was further diagnosed using bronchoalveolar lavage as well as looking at genetic risk factors and interleukin 4 sensitivity (Knutsen et al., 2005). Even in the previously discussed study, 5 patients had high IgE levels over the cutoff used, which was 500 IU/mL. These patients were either atopic with allergies, and two of them had intestinal helminthiasis. Intense and ongoing stimulation of the immune system in patients with CF may also lead to high levels of IgE in the absence of ABPA. However, the situations where ABPA exists in patients with low levels (<500 IU/mL) of IgE are known and have been documented, which still makes IgE levels more useful for excluding ABPA than for diagnosing ABPA (Knutsen et al., 2005)

2.3: Diagnosis with the use of biomarkers including exhaled nitric oxide and eosinophil counts.

In a new study performed by Keown et al., progress has been made using eosinophil levels, eosinophilic cationic protein (ECP) and exhaled nitric oxide (FE_{NO}). The finding of diagnosing using eosinophil levels is promising, as eosinophil counts are not part of the consensus criteria, and the study showed significant results. The study focused on children, with 62 patients of 6 years and older with CF, of which 13% had ABPA. The other two groups were controls, with 19% of patients being sensitized to A. fumigatus, and 68% not sensitized to the species. Exhaled nitric oxide was measured performing a test called NIOX MINO, where individuals have to exhale at 50mL/s for 10 seconds with visual feedback (Dweik et al., 2011). Eosinophil counts were measured during the patients' annual blood work tests, and the ECP concentrations were measured using an enzyme-linked immunosorbent assay.

Previous research proved that CF patients with no signs of ABPA showed relative low eosinophil counts, with most of them under 1000 cells/μL and 30% under 400 cells/μL (Laufer et al., 1984). This was shown to be opposite in the study of Keown et al., where 75% of peripheral eosinophil counts were higher than 400 cells/μL and 40% being over 1000 cells/μL in CF patients with ABPA. ECP was of special interest as it has been proven to be a sensitive marker of eosinophil activity (Koller et al., 1994). Serum ECP is released from activated eosinophils. This granular protein was measured with a mean of 61.8 mg/L in patients with ABPA, and lower in sensitized (46.8 mg/L) and non sensitized patients (43.1 mg/L) (Fig. 4).



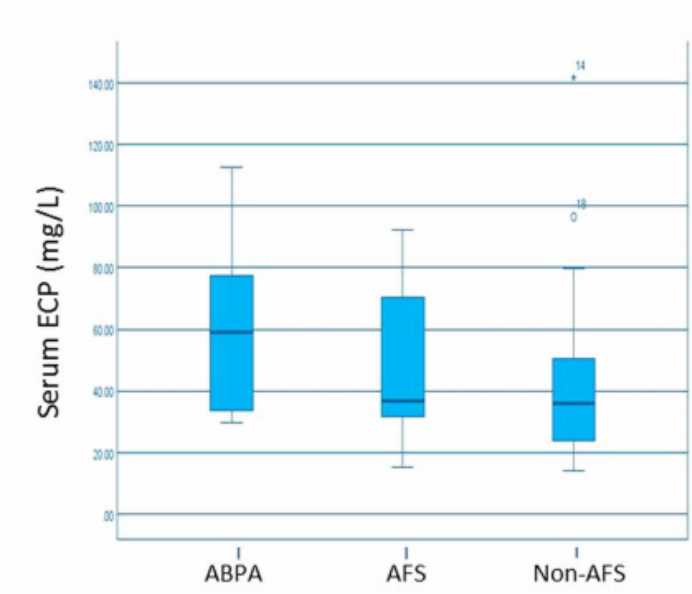


Figure 4: **Results of experiments performed on FE_{NO} , serum eosinophil and serum ECP levels.** Boxplot distributions (25%ile, median and 75%ile) of FE_{NO} (ppb), serum eosinophils (x 10^9) and serum ECP (mg/L) in ABPA patients, and individuals sensitized and non sensitized to A. *fumigatus*. Results are considered significant when P<0.05 or less.

The last analysis performed was that of nitric oxide levels. This gas is produced by several cells in the lower respiratory tract including inflammatory cells. Consequently, when inflammation is induced, more inflammatory cells are present here, and thus more exhaled NO gas can be observed. This type of measuring has already been used in patients with asthma, and could be promising for CF patients with ABPA symptoms too (Kharitonov et al., 1994). FE_{NO} levels were proven to be higher in CF patients with ABPA too, compared to control groups. The mean of FE_{NO} in the ABPA group was 37.8 ppb, significantly higher than the sensitized (15.1 ppb) and the non sensitized (13.7 ppb) group (fig. 4).

As can be seen in figure 4, measuring FE_{NO} levels and serum eosinophil counts showed statistical significance. Levels of serum ECP were lower in individuals without ABPA, but the results were not statistically significant. All patients were treated with corticosteroids, which might have affected the latter to be not significant. Corticosteroid treatment is known to attenuate eosinophilic and Th2 inflammation as well, thus the results found in this study for FE_{NO} and eosinophilic levels might have been underestimated too.

3. Discussion

Aspergillus *fumigatus* is a fungus known for its ability to cause several disorders in immunocompromised individuals. One of the diseases caused by this species is allergic bronchopulmonary aspergillosis (ABPA). This is a hypersensitivity lung disease characterized by wheezing and excessive viscous mucus due to inhalation and germination of conidia. Patients with ABPA often have underlying problems as this fungus is opportunistic. Cystic fibrosis (CF) is one of these problems, since up to 30% of CF patients get ABPA (Nikolaizik et al., 1991). Considering the fact that a lot of diagnostic criteria overlap between CF and ABPA, disease symptoms are not stagnant over time and cut-offs of criteria are not completely agreed upon, has led to the diagnosis of ABPA in CF patients being very difficult. Over the years, criteria for diagnosis have changed, however diagnosis remains difficult and progress is necessary as chance of survival lowers when ABPA is only diagnosed in later stages (Schønheyder et al., 1988).

One of the most important points during diagnosis is serum IgE levels, with the official criteria of the Cystic Fibrosis Foundation having a cut-off of >1200 ng/mL. Looking at IgE levels is not very specific, and thus Fricker-Hidalgo et al. tried to build on this criterion by looking at reactivity of recombinant antigens rAspf4 and rAsp6 to IgE. Results showed that especially rAspf4 was highly sensitive (92.3%). This corresponds with previously conducted studies which hypothesized that diagnosis using rAspf4 might be a very specific solution to provide a tool for accurate diagnosis (Kraemer et al., 2006). The study mentions that previously, other researchers have concluded that diagnosis using serum IgE against recombinant allergens was not helpful (Oliveira et al., 2007). In the future, this type of tool should be investigated more to find a way to make diagnosis using IgE more specific and reduce differences in criteria among clinics.

A different approach was taken by Keown et al., where instead of focussing on IgE levels, the main findings were on eosinophil counts, ECP levels and nitric oxide levels. Significant results were found in peripheral eosinophil counts, however the researchers were unsure about the specificity, as peripheral eosinophil counts are not part of the CF criteria, and because they are not in the compartment of interests for ABPA diagnosis, which would be the airways (Stevens et al., 2003). Given the significant results for eosinophil counts, it was surprising that this was not repeated in the results for serum ECP. However, sampling processing problems led to a reduced sample cohort and thus this experiment should be repeated in order to completely exclude ECP levels as a good tool for diagnosis.
The findings using FE_{NO} levels for diagnosis seem to be the most promising. This is the first study to systematically assess nitric oxide levels in this context, and only one study before this had looked into NO levels in A. *fumigatus* sensitized adult patients (Lim et al., 2003). In that study, overall FE_{NO} levels were lower (1.2-18 ppb in adults) compared to the study of Keown et al. (5- 73 ppb), with a cohort of children. This might mean that diagnosis in adults using exhaled nitric oxide can be less specific, as overall levels were a lot lower. More research is necessary to find out whether this tool for diagnosis is as helpful in adults as would be in children.

Additionally, these findings could possibly contribute to the progression of diagnosis using an analytic device called eNose. eNose uses an array of sensors that are able to detect a variety of chemical compounds. The algorithm it uses can pick up on difficult patterns and clusters of metabolic signals, which is what ABPA and many other respiratory infections consist of (Vries & Sterk, 2020). The device can detect many volatile organic compounds (VOCs) including but not limited to alkanes, alcoholes, ketones and aromatic compounds. This method is already in use for detecting lung cancer for example, with relatively high sensitivity, but not yet completely perfect (Farraia et al., 2019). There is already proof that A. *fumigatus* can be detected this way. A study found that the eNose can distinguish the species from bacteria and yeasts (Heer et al., 2016). The detection of exhaled nitric oxide is possible with eNose, as found by Montuschi et al. To make diagnosis even more accurate, perhaps the FE_{NO} levels can be used as one of the VOCs detected for, to diagnose ABPA.

In short, diagnosis of ABPA in CF patients remains difficult and more research is needed to progress. The main criterion of IgE levels could be optimized by making it more specific by looking at reactivity with recombinant antigen rAspf4. More criteria could be added, for example looking at FE_{NO} levels in children seemed to be promising. In the future the experiments using nitric oxide levels should be repeated to find out whether the positive results remain the same, and the possible combination of eNose and FE_{NO} levels should be looked into.

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