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# BLOOD BASOPHIL ACTIVATION IS A RELIABLE BIOMARKER OF ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS IN CYSTIC FIBROSIS

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## SUPPLEMENTARY METHODS

### Patient characteristics

At the Stanford CF Center, the study enrolled 60 patients. Among the four CF categories, (1) there were 12 patients with CF (i.e., without *Af* colonization or ABPA); (2) 13 patients with CF-AC; (3) 20 patients with CF-ABPA; and (4) 3 patients with CF-ABPA-S. All patients were followed over 4 routine visits at six-month intervals as well as any sick (exacerbation) visits. A Stanford patient with asthma and APBA, and 11 healthy volunteer controls (HC), were also evaluated once each at single time points. At the Dublin Center, 25 patients with CF were evaluated in a cross-sectional analysis utilizing the same BAT protocol. According to the same *a priori* categorization, there were 11 patients with CF, 8 with CF-AC, and 6 with CF-ABPA. One Dublin asthma patient with APBA was also evaluated. Characteristics of the study subjects with cystic fibrosis are shown in Table 1 and Supplementary Table 1.

### Study design

Diagnosis of CF was by sweat chloride (>60 mmol/L by quantitative iontophoresis test) and/or documentation of two identifiable CFTR mutations and one or more clinical features consistent with CF. CFTR genotype was confirmed on all enrolled Stanford subjects using the Elucigene CF29 assay (Tepnel Diagnostics LTd., Manchester, UK). Lung function was tested by spirometry using American Thoracic Society criteria. Serum serologies (total IgE and *Af*-specific IgE antibody levels) were measured in all subjects by the Clinical Laboratories at Stanford Hospital and Clinics using standard ImmunoCAP assays according to the manufacturer's instructions (Phadia AB, Uppsala, Sweden). Serum IgG antibodies to *Af* were determined by the Platelia Aspergillus IgG enzyme-linked immunoassay *(*Bio-Rad*,* Marnes-la-Coquette, France). The Platelia assay uses a mix of recombinant *Af* antigens in an immunoenzymatic microplate assay.[Suppl ref 1] Samples were collected at each time point and stored at -80˚C until completion of

data collection. Samples were thawed and tested as one lot in the Platelia Aspergillus IgG ELISA kit according to manufacturer's instructions.[Suppl ref 2]

**Characteristics of the longitudinal analysis**

In the CF group, there were a total of 44 visits; for CF-AC, a total of 49 visits; and for CF-APBA, a total of 69 visits. We also followed 3 patients with CF-ABPA-S with a total of 11 visits. We also included 11 healthy adult volunteer controls and 1 asthma patient with ABPA; these non-CF individuals were evaluated at a single baseline visit. One CF patient was identified with IgE >1000 IU/mL at all visits (AB0039, Supplementary Table 1) but without evidence of ABPA/ABPA-S and was excluded from further analysis. The three CF patients with ABPA-S were also not included in most analyses, which were performed on the patients in the CF, CF-AC, and CF-ABPA categories.

**Sample collection and processing**

Within 30 minutes, blood was centrifuged at 400G for 10 minutes at 4ºC to separate the erythrocyte/leukocyte pellet (kept on ice) from platelet-rich plasma. Platelet-rich plasma was further spun at 3000G for 10 minutes at 4ºC. The erythrocyte/leukocyte pellet was reconstituted to its original volume with platelet-free plasma, yielding platelet-free, reconstituted blood. We used platelet-free reconstituted blood to assess blood basophil function in order to avoid platelet-triggered aggregation and clotting in the course of 10 and 30 minute 37°C incubation with saline or antigens (see below).

**Incubation with saline or antigens**

For each of the three incubations (described in the main manuscript), 100 µl of platelet-free, reconstituted blood (processed as above), was mixed with either 3 µl of PBS or relevant extracts for 10 and 30 minutes at 37ºC as previously described.[24] The incubation was stopped by adding

ice-cold PBS-2.5 mM EDTA as calcium chelator to block cell activation. The cells were pelleted by centrifugation (490G for 5 minutes at 4$^{o}$C) for flow cytometry. The supernatants were kept at -80°C. Cellular results presented in this paper are restricted to analyses of BAT responses although eosinophil and PMN activation markers have also been studied.[Suppl ref 3]

**Statistics**

For models that included both condition and visit, we first performed a preliminary test to test for an interaction between visit and category. No such interaction was found, and thus the interaction term was dropped from the model. Unadjusted longitudinal models were considered for the 3 CD203c outcomes (data not shown), as well as multivariate models including stimulation with heterologous antigen (peanut) and at 37°C for the same time points, treatment with oral steroids, exacerbations, and co-infections with *S. aureus*. Of these, only stimulation with heterologous antigen was statistically significant and included in final models. Other variables investigated in longitudinal analysis included total IgE; co-infections with *S. aureus*, mucoid and non-mucoid *P. aeruginosa*, and *S. maltophilia*; treatment with oral steroids, itraconazole, azithromycin, inhaled tobramycin, and inhaled corticosteroids; and spirometric lung function (FEV1 percent predicted) and age (evaluated together).

For comparisons with healthy controls, who were only evaluated at baseline, analysis of variance (ANOVA) and pairwise comparisons were used to compare the three CD203c outcomes. Genotype, number of pulmonary exacerbations, presence of insulin-dependent diabetes, and treatment at baseline were evaluated by a chi-square test for cross-classified frequencies. The IgG levels, which were obtained at visit 3, were also evaluated at a single time point using ANOVA and pairwise comparisons. *Af*-specific IgE levels were also evaluated at a single time point. Because many values were below the limits of detection, a non-parametric Kruskall-Wallis test based on ranks was used, supplemented with the Dwass, Steel, Critchlow-Fligner Method for pairwise comparisons.[Suppl ref 4]

Dublin BAT analyses were conducted using a log transform and one way ANOVA (Supplementary Table 2).

SUPPLEMENTARY RESULTS

Treatment with itraconazole and inhaled steroids were not significantly associated with category at baseline, nor were CFTR genotype (classified as F508del homozygote, F508del heterozygote, or two non-F508del mutations), FEV1, or FVC (Table 1). FEV and FVC were also not significantly associated with category in longitudinal analysis (Supplementary Figure 2). Treatment with azithromycin and inhaled tobramycin, number of pulmonary exacerbations, and presence of insulin-dependent diabetes were also not associated with category (data not shown).

**SUPPLEMENTARY REFERENCES**

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2. Guitard J, Sendid B, Thorez S, Gits M, Hennequin C. Evaluation of a recombinant antigen-based enzyme immunoassay for the diagnosis of noninvasive aspergillosis. J Clin Microbiol 2012;50:762-5.

3. Gernez Y, Everson C, Dunn CE, Fernandez-Becker N, Gudiputi L, Davies ZA, et al. Modulatory effects of *Aspergillus* colonization and ABPA on blood and sputum eosinophils and neutrophils in CF. J Cyst Fibros 2012;11:S20.

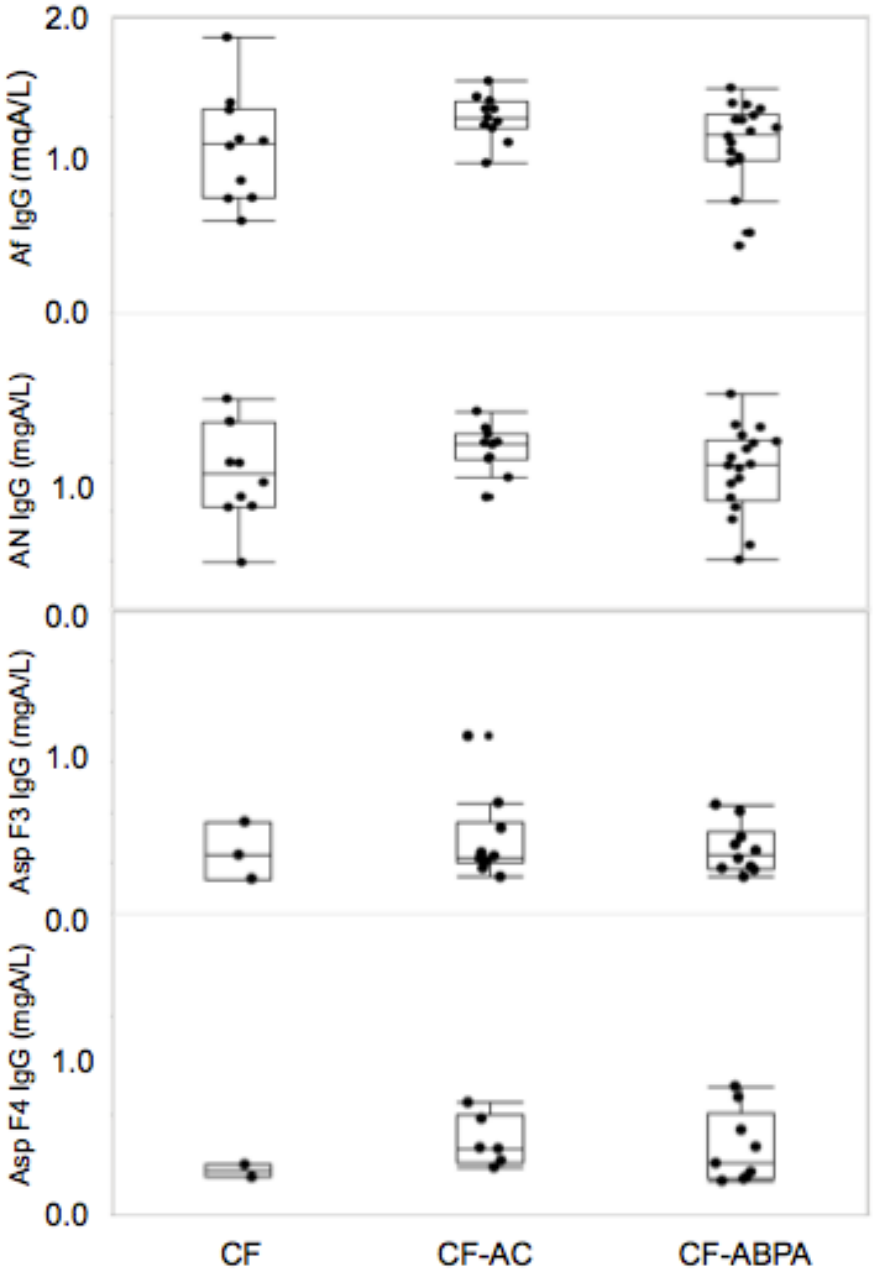
4. Hollander M, Wolfe D, Chicken E. Nonparametric Statistical Methods, Third Edition. John Wiley & Sons, Hoboken, NJ, 2014.

## LEGENDS TO SUPPLEMENTARY FIGURES

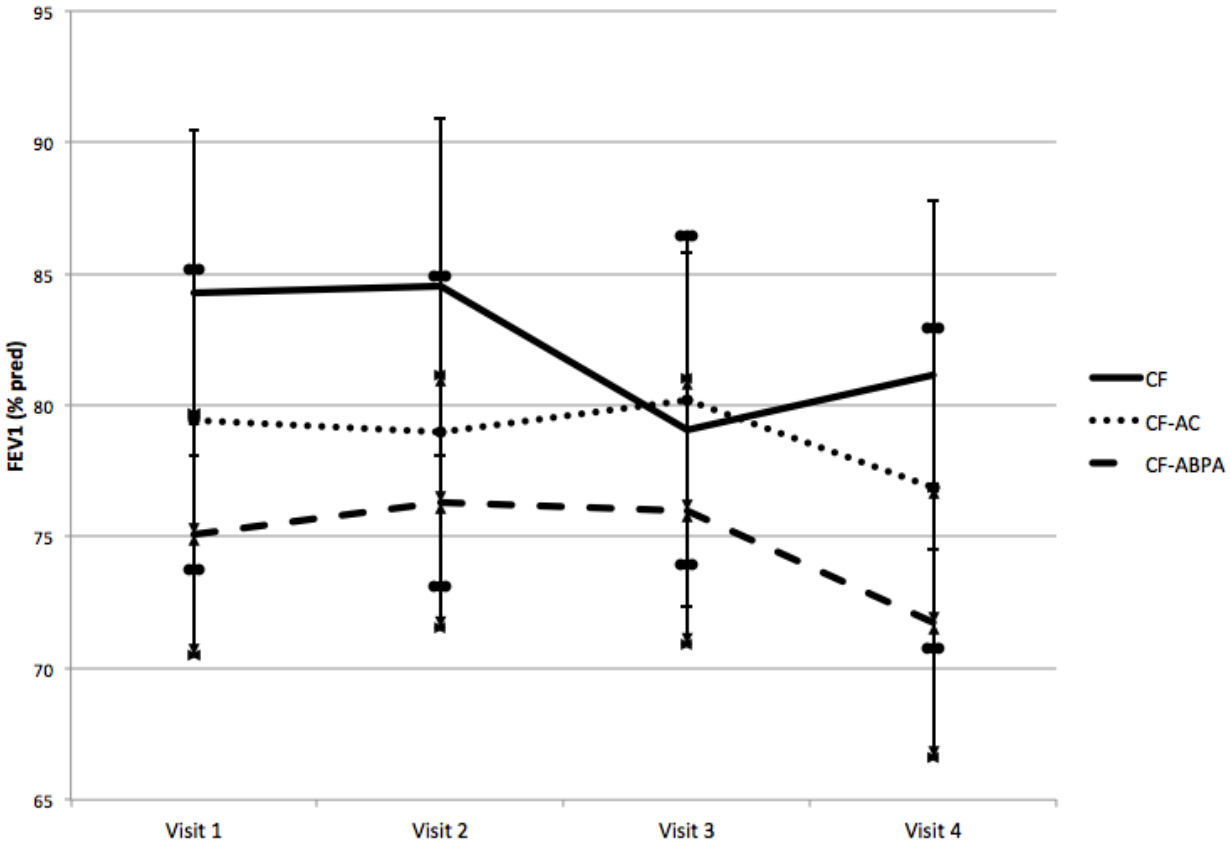
**Supplementary Figure 1**. Serum IgG antibody levels to *Aspergillus* antigens. *Af*, A.fumigatus extract; AN, *Aspergillus niger* extract; Asp F3, *Af* recombinant allergen f 3; Asp F4, Af. recombinant allergen f 4.

**Supplementary Figure 2.** FEV1 percent predicted over study course. There were no significant differences between CF-ABPA, CF-AC and CF categories.

**Supplementary Figure 1**




**Supplementary Figure 2**


95
90
85
80
75
70
65
FEV1 (% pred)
Visit 1
Visit 2
Visit 3
Visit 4
CF
CF-AC
CF-ABPA

**Supplementary Table 1. Baseline characteristics of individual cystic fibrosis subjects in the Stanford cohort..**

| Group | Sex | Age | Genotype | MPA | PA | SA | ITRA | OS | ZIT | INH | IgE (Ku/L) | FEV1 (% Pr.) | FVC % (Pr.) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CF | F | 17 | DF508/DF508 | N | N | Y | N | N | Y | Y | 33 | 90 | 100 |
| CF | M | 14 | DF508/DF508 | N | N | Y | N | N | Y | Y | 5 | 119 | 112 |
| CF | M | 10 | G178R/LA67P | N | N | Y | N | N | N | Y | 2 | 93 | 108 |
| CF | F | 14 | DF508/DF508 | N | N | Y | N | N | Y | N | 19 | 103 | 117 |
| CF | M | 7 | DF508/DF508 | N | N | Y | N | N | N | N | 51 | 78 | 80 |
| CF | M | 14 | DF508/1898+7G>A | Y | N | N | N | N | Y | Y | 34 | 76 | 79 |
| CF | F | 43 | DF508/S749N | N | N | Y | N | N | N | Y | 34 | 76 | 98 |
| CF | M | 15 | DF508/DF508 | Y | N | Y | N | N | Y | N | 8 | 82 | 105 |
| CF* | M | 18 | DF508/711 +1 G | N | Y | Y | N | N | N | N | 1008 | 87 | 100 |
| CF | F | 14 | DF508/E1371 | N | N | Y | N | N | N | Y | 2 | 88 | 97 |
| CF | M | 10 | N1303K/621+1G>T | N | N | Y | N | N | Y | Y | 105 | 59 | 68 |
| CF-AC | M | 12 | DF508/DF508 | Y | N | Y | N | N | Y | N | 35 | 95 | 99 |
| CF-AC | F | 18 | DF508/DF508 | N | Y | N | N | N | Y | Y | 123 | 36 | 58 |
| CF-AC | F | 14 | DF508/DF508 | N | N | N | N | N | N | Y | 18 | 97 | 107 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CF-AC | F | 19 | DF508/DF508 | N | N | Y | N | N | Y | N | 40 | 96 | 107 |
| CF-AC | M | 17 | DF508/DF508 | N | Y | N | Y | N | N | N | 204 | 85 | 92 |
| CF-AC | M | 20 | DF508/1717-1 | N | N | N | Y | N | Y | Y | 34 | 92 | 94 |
| CF-AC | F | 30 | DF508/DF508 | N | N | Y | N | N | Y | Y | 43 | 81 | 103 |
| CF-AC | F | 46 | W1282X/W1282X | N | N | N | Y | N | Y | Y | 2 | 93 | 99 |
| CF-AC | M | 51 | DF508/DF508 | Y | Y | N | N | N | Y | Y | 5 | | |
| CF-AC | M | 26 | DF508/DF508 | Y | Y | N | N | N | Y | Y | 35 | 81 | 103 |
| CF-AC | M | 35 | DF508/G551D | Y | N | N | N | N | Y | Y | 23 | 77 | 101 |
| CF-AC | F | 21 | DF508/G511D | N | Y | N | N | N | Y | Y | 3 | 65 | 71 |
| CF-AC | M | 14 | DF508/DF508 | N | N | N | N | Y | Y | Y | 301 | 67 | 79 |
| CF-ABPA | F | 44 | DF508/DF508 | N | Y | Y | N | N | N | Y | 171 | 90 | 91 |
| CF-ABPA | M | 27 | DF508/DF508 | Y | Y | N | Y | N | N | Y | 1126 | 55 | 76 |
| CF-ABPA | F | 11 | 3849+10kb C>T | N | N | Y | Y | Y | Y | Y | 571 | 97 | 112 |
| CF-ABPA | M | 19 | DF508/DF508 | N | Y | N | Y | N | Y | Y | 1204 | 56 | 92 |
| CF-ABPA | | | G542X/C1811+1643 | | | | | | | | | | |
| | F | 11 | G>T, 7T/9T | N | N | N | N | Y | N | N | 143 | | |
| CF-ABPA | F | 17 | DF508/DF508 | N | N | N | N | Y | Y | Y | 119 | 95 | 103 |
| CF-ABPA | F | 16 | DF508/R334W | Y | N | N | Y | N | Y | N | 198 | 50 | 73 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CF-ABPA | M | 69 | DF508/6194V, 7T/9T | Y | N | Y | N | N | Y | Y | 149 | 43 | 52 |
| CF-ABPA | F | 20 | DF508/G542X | N | N | Y | Y | Y | Y | N | 2105 | 63 | 84 |
| CF-ABPA | F | 51 | DF508/DF508 | Y | N | N | N | N | Y | Y | 875 | 71 | 90 |
| CF-ABPA | M | 25 | DF508/DF508 | N | N | N | N | N | Y | Y | 294 | 90 | 99 |
| CF-ABPA | M | 45 | DF508/DF508 | N | N | N | Y | Y | Y | Y | 1597 | 89 | 97 |
| CF-ABPA | F | 20 | DF508/G55ID | N | N | N | N | N | Y | Y | 291 | 94 | 96 |
| CF-ABPA | M | 35 | I507del/R117H | N | Y | Y | N | N | N | Y | 636 | 59 | 76 |
| CF-ABPA | F | 14 | DF508/R1162x | N | N | Y | N | N | Y | Y | 259 | 97 | 106 |
| CF-ABPA | M | 10 | G542X/unknown | N | N | Y | N | Y | Y | Y | 1202 | 94 | 103 |
| CF-ABPA | M | 16 | DF508/DF508 | N | N | Y | N | N | Y | Y | 859 | 68 | 75 |
| CF-ABPA | F | 9 | L206W/D1152H | N | N | N | Y | N | Y | Y | 1147 | 113 | 115 |
| CF-ABPA | M | 9 | DF508/DF508 | N | N | N | N | Y | Y | N | 1106 | 102 | 136 |
| CF-ABPA | M | 38 | F508/711+1G>T | N | N | Y | Y | N | Y | Y | 1129 | 40 | 53 |
| Asthma-ABPA | M | 8 | -- | N | N | N | N | Y | N | N | 2839 | 78 | 95 |
| CF-ABPA-S | F | 22 | DF508/DF508 | Y | N | Y | N | N | N | Y | 56 | 98 | 103 |
| CF-ABPA-S** | F | 18 | F508/W1282X | N | N | Y | N | N | N | Y | 256 | 93 | 95 |
| CF-ABPA-S | F | 14 | DF508/unknown | N | N | Y | N | N | Y | Y | 365 | 90 | 99 |

*Af*, *A. fumigatus*; CF-ABPA-S, CF serologic ABPA; F, female; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; IgE, immunoglobulin E; INH, inhaled corticosteroid; ITRA, itraconazole; M, male; med, medication; MPA, mucoid *P. aeruginosa*; OS, oral steroids; PA, non-mucoid *P. aeruginosa*; Pr., predicted; SA, *S. aureus*; ZIT, azithromycin.

*subject ID#AB0039 was excluded from analysis for persistent IgE >1000 IU/mL without other ABPA criteria.

**subject ID#AB00020, baseline CF-ABPA-S category, progressed to CF-ABPA.

**Supplementary Table 2. Statistical comparison of basophil CD203c levels upon 10-minute ex vivo stimulation with *Af* extract in the Stanford and Dublin cohorts.**

| | CENTER | CF-ABPA Vs HC | CF-ABPA Vs CF | CF-ABPA Vs CF-AC |
|---|---|---|---|---|
| **CD203c Levels** | **Stanford** | <0.001 | <0.001 | <0.001 |
| | **Dublin** | <0.001 | <0.001 | 0.042 |