ERJ Express. Published on September 26, 2007 as doi: 10.1183/09031936.00078107

Comparison of serum markers for allergic bronchopulmonary aspergillosis in cystic fibrosis

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Short title: TARC as serum marker for ABPA

Total word count: 2412 (3000)

Abstract word count: 196 (200)

Abstract

The diagnosis of allergic bronchopulmonary aspergillosis (ABPA) in cystic fibrosis (CF) is a challenge. Thymus- and activation-regulated chemokine (TARC) has recently been reported to play a role in ABPA. We aimed to compare the diagnostic value of TARC with known serological markers for diagnosis of ABPA in CF patients.

We followed longitudinally 48 CF patients, of whom 12 had a diagnosis of ABPA according to Nelson's criteria, for one to eight years with repeated measurements of serum total IgE, specific *Aspergillus fumigatus* IgE and IgG, recombinant *Aspergillus fumigatus* allergens f1, f3, f4 and f6 and TARC.

Median (IQR) TARC levels were 589 (465-673) pg/ml in ABPA patients and 232 (189-289) pg/ml in non-ABPA patients. Receiver operating characteristic (ROC) curves revealed that TARC was superior to the other markers for diagnosis of ABPA. The sensitivity, specificity and diagnostic accuracy were higher for TARC (92%, 95% and 93%) than for total IgE (65%, 81% and 74%), rAsp f4 (82%, 71% and 75%) or f6 (65%, 87% and 79%).

Our study indicates that TARC may be useful for the diagnosis of ABPA in CF patients. However, larger studies are needed before TARC can routinely be used in diagnostic algorithms.

Key words: Allergic bronchopulmonary aspergillosis, cystic fibrosis, diagnostic value, IgE, serum marker, TARC

Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a pulmonary hypersensitivity disease mediated by an allergic response to *Aspergillus fumigatus* (*A. fumigatus*) [1]. ABPA occurs in about 10% of cystic fibrosis (CF) patients and may lead to acute worsening of the respiratory status and ongoing decline of lung function [2], ultimately progressing to a chronic state and lung fibrosis without adequate treatment [3]. Despite the existence of the "gold standard" Nelson criteria [4], the diagnosis of ABPA in CF patients remains difficult.[5] A wide variation in diagnosis practices between clinics [6], different estimates of prevalence and a delay of recognition lead to undertreatment [7].

The main reason for the difficulties in diagnosing ABPA and ABPA exacerbations in CF patients is the overlap of diagnostic criteria for ABPA with common manifestations of CF. Pulmonary infiltrates, obstructive lung disease and bronchiectasis occur regularly in CF patients due to the underlying disease with bacterial colonization and thus are not specific for ABPA [8]. Further, lung colonization with *A. fumigatus* occurs in 20 to 25% of the CF patients [9-11]. Therefore, as stated in the most recent consensus document on diagnosis and therapy of ABPA in CF patients, serological findings should strongly contribute to confirming or excluding clinically suspected ABPA [5].

Animal studies suggest a pathophysiological role of the chemokine thymus- and activation-regulated chemokine (TARC) in ABPA by linking an anti-fungal immune response with the promotion of Th2-mediated hypersensitivity to *A. fumigatus* [12]. We have recently shown in a cross-sectional study that serum levels of TARC are elevated in CF patients with ABPA [13]. However, longitudinal clinical data on the usefulness of this marker are lacking and no

further study has been performed to validate TARC in comparison to other putative ABPA serum markers. The present study aimed at answering the following questions:

(i) Can we confirm the results of the previously published cross-sectional study [13] in another CF population?

(ii) What is the diagnostic value (sensitivity and specificity) of TARC compared to other serological markers for ABPA?

(iii) Is TARC useful for early detection of ABPA development?

Methods

Study design

From 1998 on, we started to systematically follow a group of 48 patients (23 female, 25 male, median (IQR) age 9 (7-14) years) with CF longitudinally [14]. All patients underwent careful clinical assessment, lung function testing and microbiological diagnostic at all visits during the study period. Skin testing against *A. fumigatus* was routinely performed using Bencard skin test antigens (SmithKline Beecham, Switzerland). Chest x-rays were performed at study entry and thereafter at least annually.

In addition we collected serum samples at the study visits resulting in an average of six serum samples per patient for analysis (see also table 1). The Ethics Committee of Berne, Switzerland approved the study and written consent was obtained at enrolment.

Clinical diagnosis of ABPA

According to Nelson's criteria, patients were diagnosed as having clinical ABPA when at least 6 out of the 7 following criteria were fulfilled: wheezing, positive *A. fumigatus* in sputum culture, presence of defined infiltrates in chest-x-ray, positive acute reaction to *A. fumigatus* in skin prick testing, elevated total IgE (cut-off 500 IU/ml), increased specific serum IgE (cut-off 17.5 IU/ml) and IgG (cut-off 20 kU/l) against *A. fumigatus* [4].

Serum markers

In the collected serum samples the following parameters were measured: Total IgE, specific IgE (RAST) to *A. fumigatus*, specific IgG against *A. fumigatus* extract (ELISA) and specific IgE against the recombinant *A. fumigatus* (rAsp) allergens f1, f3, f4 and f6 [14;15] and TARC.

TARC levels were analyzed in triplicates by sandwich ELISA according to the manufacturer's instructions (R&D Systems, MN, USA) and concentrations calculated from standard curves with detection limits of 7 pg/ml - 3000 pg/ml. Intra-assay variability was determined by evaluating 5 serum samples 10 times within the same assay run and showed a coefficient of variation (CV) between 6% and 9%. Inter-assay variability was determined by measuring 5 serum samples in 5 consecutive assay runs and showed a CV between 8% and 17%.

Statistical analysis

Data are given as medians (IQR) if not indicated otherwise. Diagnostic value of the serological markers and receiver operator characteristics (ROC) curves were calculated using STATA version 8.2 for Windows (STATA Corporation, College Station, TX, USA). Cut-off levels were set at the level that resulted in the optimal diagnostic accuracy, defined as correctly positive classified plus correctly negative classified as percentage of all.

Nested matched case-control analysis

In order to assess whether TARC elevation was specific for ABPA or was an epi-phenomenon of the hypersensitivity against *A. fumigatus* in ABPA patients, we performed a nested matched case-control analysis and compared TARC levels between cases (ABPA patients) and controls (non-ABPA patients), matched for total IgE levels and rAsp f6 levels, respectively.

Results

Twelve out of 48 CF patients were diagnosed with clinical ABPA based on Nelson criteria. Nine patients were diagnosed with ABPA before study entry, three patients developed their first episode of clinical ABPA during the time of the study; all were assigned to the ABPA group. The other 36 CF patients did not fulfill 6 out of 7 Nelson criteria for diagnosis at any time before or during the study period and were assigned to the non-ABPA group. Details on patients' characteristics are given in table 1.

TARC compared to other serological markers for the diagnosis of ABPA

Median (IQR) TARC levels were 589 (465-673) pg/ml in ABPA patients compared to 232 (189-289) pg/ml in non-ABPA patients (table 1). In the 16 non-ABPA patients with neither sensitisation to *A. fumigatus* nor elevation of total IgE, TARC levels were 207 (178-282) pg/ml.

When serological results of all available time points of the study period were included (n=265), the sensitivity, specificity and diagnostic accuracy for diagnosis of ABPA were 92%, 95% and 93% for TARC; 65%, 81% and 74% for total IgE; 68%, 83% and 78% for rAsp f1; 66%, 86% and 79% for rAsp f3; 82%, 71% and 75% for rAsp f4; 65%, 87% and 79% for rAsp f6 and 54%, 84% and 73% for IgG, respectively (table 2).

These results were confirmed using ROC curve analysis with TARC levels resulting in the largest area under the curve, as illustrated in figure 1 for TARC, total IgE, rAsp f4 and rAsp f6.

In a second approach we used only one serum sample of each patient for analysis, namely that from the time point with the highest total IgE level. Here again TARC levels resulted in the highest diagnostic accuracy, sensitivity and specificity as well as the largest area under the curve (table 3).

Nested matched case-control analysis

In the nested matched case-control design TARC levels discriminated well between 10 patients with clinical ABPA (cases) and 10 patients without clinical ABPA (controls) matched for total IgE levels (median total IgE levels 1375 IU/ml for the ABPA patients, 1152 IU/ml for the non-ABPA patients). The median TARC level was 673 pg/ml for the ABPA patients compared to 237 pg/ml for the patients without ABPA (figure 2). Two ABPA patients could not be included into the case-control analysis due to very high serum IgE levels (3710 IU/ml and 5263 IU/ml) without matching control. These patients had elevated TARC levels of 432 pg/ml and 721 pg/ml, respectively.

TARC levels also discriminated well between 11 ABPA patients (median TARC level 566 pg/ml) and 11 patients without ABPA (median TARC level 234 pg/ml) matched for rAsp f6 levels (median rAsp f6 13 IU/ml for the ABPA patients and 19 IU/ml for the non-ABPA patients; figure 3). One ABPA patient could not be included into the case-control analysis due to very high rAsp f6 levels (165 EU/ml) without matching control. This patient showed elevated TARC levels of 630 pg/ml.

Early elevation of TARC levels in the course of ABPA

In the subgroup of the three CF patients who developed their first episode of clinical ABPA during the study period, TARC levels were elevated prior to the full clinical picture of ABPA and before total IgE (figure 4).

Discussion

The present study confirms elevated TARC serum levels in ABPA patients in a CF population not previously investigated and shows that TARC is a highly sensitive and specific marker for discrimination of ABPA patients in comparison to other serum markers. Our results further suggest that TARC may be elevated early in the course of ABPA in CF patients.

In a pilot study investigating chemokines and cytokines in ABPA, TARC serum levels were elevated in CF and asthma patients with ABPA compared to several CF and non-CF control groups [13]. However, in this study, chemokines at different time points were measured in seven patients up to four months before and after an ABPA exacerbation only. We are now able to confirm elevated TARC levels in ABPA patients in a different CF population followed over a much longer period. It is well known that patients from different CF populations and centers show a great variability in their microbiological colonization [9], their atopic status [10] and their genetic background [16]. Studies examining the role of genetic modifiers have also found diverse results among different CF populations [16]. Thus, the confirmation of elevated TARC levels over a longer period of time in ABPA patients of a second CF cohort is very important regarding the possible diagnostic use of TARC in a clinical setting.

The development of recombinant antibodies against different allergens of *A. fumigatus* has facilitated ABPA diagnosis and commercially available kits are now used for the assessment of sensitization to *A. fumigatus* [17;18]. Several approaches have been undertaken to validate different serological markers in order to simplify the diagnosis of ABPA [14;15;17;19-23], mainly assessing the diagnostic value of recombinant *Aspergillus* antibodies, but also looking at other serum markers, such as Surfactant Protein-D [24]. Our group and others have previously shown that the combined use of increased total IgE, rAsp f4 and f6 allow a fair

discrimination between patients with ABPA and patients with *Aspergillus* sensitization without clinical ABPA [14;21]. Kurup et al. used both ELISA and CAP to determine rAsp f1, f2, f3, f4 and f6 levels and concluded that no single recombinant allergen is capable of differentiating between ABPA patients and non-ABPA patients [22]. In the present study, we compared TARC to other serological markers of ABPA using ROC curve analysis with two approaches. First, all serum samples of the total study period were used for analysis. This reflects real clinical practice with longitudinal follow-up of CF patients. In a second approach we evaluated the diagnostic value of the different serological markers using only the serum sample at the IgE peak of each patient. Our findings of TARC as a single marker being clearly superior to other serological markers with both approaches highlight the potential of this new marker for clinical practice in contrast to complicated combination analysis of several serological markers [14;22].

In our cohort, due to the study design the proportion of CF patients with ABPA was higher than in the typical CF population [11]. Therefore, we did not calculate positive or negative predictive values of the various serological markers, as these calculations take into account the prevalence of a disease in the study cohort. The high prevalence however does not weaken the strength of our findings; in contrary, despite this, TARC levels resulted in a very low negative likelihood ratio, indicating minimal false negative classification compared to the other serum markers.

In our study, the best cut-off value of TARC for discrimination of ABPA patients was around 400 pg/ml depending on whether all serum samples or only the serum samples at the IgE peak were taking into account. Further studies are needed to determine optimal cut-off values for TARC in the diagnosis of ABPA in CF, especially because such cut-off values may be also

influenced by methodological issues and differ between patient populations as it is known for IgE levels [5].

One patient without ABPA showed elevated TARC levels. Until now, this patient has not fulfilled the clinical criteria of Nelson for diagnosis of ABPA as listed in the methods section [4] but is being followed up carefully. So we can only speculate whether this patient will develop ABPA in the future or whether TARC is elevated for other unknown reasons, such as the recently described entity of *Aspergillus* bronchitis in CF patients [25].

Although numbers are small, the longitudinal study design allowed us to assess the value of TARC as an early marker of ABPA development in the subgroup of patients who experienced their first episode of clinical ABPA during the study period. TARC levels were elevated up to 30 months before the clinical picture of ABPA and much earlier than total IgE. These results need confirmation in a larger number of patients, but the early elevation of TARC levels make this new marker even more interesting for potential clinical use, as the chances of misdiagnosis and long-term consequences decrease with early awareness of possible ABPA [1].

As reviewed recently by Hartl et al. [26], *A. fumigatus* conidia are recognized via Toll like receptors on dendritic cells [27], which represent a major source of TARC [28]. TARC recruits Th2 cells via the chemokine receptor CCR4 to the pulmonary site of inflammation [29]. Th2 cells produce IL-4 that induces IgE production by B cells [30]. The secreted IgE, in turn, binds to mast cells, which release mediators ultimately leading to bronchoconstriction. Thus, both the presence of *A. fumigatus* and the development of a Th2 response seem to be the underlying reasons for the strong TARC response in ABPA patients. As it has been speculated before for acute eosinophilic pneumonia [31] and allergic asthma [32], a

pathophysiological role of TARC seems very likely also in the disease process of ABPA. However, we cannot exclude that TARC is only an epiphenomenon indicating individual patients prone to developing ABPA and should then be termed more correctly a risk factor. The IgE-independent mechanism of TARC activation might also explain the early increase of TARC compared to IgE levels and the elevation of TARC levels independently of IgE levels in the nested matched case-control analysis.

Taken together, we show that TARC levels are superior to other serum markers for discrimination of CF patients with and without ABPA followed longitudinally under clinical conditions. These findings might impact directly upon clinical practice, especially because TARC is a single marker and seems to be elevated early in the course of ABPA. Before TARC can be included in diagnostic algorithms for ABPA in CF patients, its contribution to clinical decision-making needs to be evaluated in further studies.

Acknowledgments

PL and DH contributed equally to the study.

Supported by Swiss national foundation grant 3200-B0-112099 to UF, a grant from the German Society for Pediatric Pneumology to DH and a grant of Münchener Medizinische Wochenschrift to PL.

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Tables

Table 1) Patient data and results of serum measurements.

Characteristics of Patients	**ABPA**	**Non-ABPA**
Number of patients	12	36
Age at study entry	10 (8-12)	9 (7-14)
Sex (M/F)	7/5	19/17
Study duration in months	40 (35-80)	40 (27-55)
Number of serum samples:		
total	87	178
median [range] of all patients	7 [4-12]	5 [1-8]
FEV1 (%-predicted) at study entry	73 (77-81)	89 (72-99)
A.fumigatus in sputum during study period*	12/12	20/36
P.aeruginosa in sputum during study period*	12/12	34/36
Serum measurements		
Total IgE (IU/ml)	965 (324-1961)	59 (21-433)
Specific IgG to *A.fumigatus* (kU/l)	145 (66-244)	51 (17-105)
Specific IgE to *A.fumigatus* [RAST class 0-6]	4 (3-5)	1 (0-3)
rAsp f1 (EU/ml)	128 (60-364)	21 (6-59)
rAsp f3 (EU/ml)	253 (99-739)	37 (14-94)
rAsp f4 (EU/ml)	23 (11-53)	4 (1-12)
rAsp f6 (EU/ml)	25 (8-67)	3 (2-8)
TARC (pg/ml)	589 (465-673)	232 (189-289)

Data are given in median (IQR) or in number of patients.

* number of patients with at least one positive sample during the study period.

Table 2) The value of TARC, total IgE, rAsp f1, f3, f4 and f6 and specific IgG for ABPA diagnosis using all serum samples.

	TARC	IgE	rAsp f1	rAsp f3	rAsp f4	rAsp f6	IgG
Sensitivity[1] [%]	91.8	64.7	68.3	65.9	81.7	64.6	53.7
Specificity[2] [%]	94.7	81.0	83.3	85.9	71.2	86.5	83.7
Diagnostic accuracy[3] [%]	93.4	74.3	78.1	79.0	74.8	79.0	73.2
Positive Likelihood ratio[4]	17.3	3.4	4.1	4.7	2.8	4.8	3.3
Negative Likelihood ratio[5]	0.09	0.44	0.38	0.40	0.26	0.41	0.55

Cut-off levels: 386 pg/ml for TARC, 514 IU/ml for IgE, 75 IU/ml for rAsp f1, 140 IU/ml for rAsp f3, 10 IU/ml for rAsp f4, 16 IU/ml for rAsp f6 and 140 kU/l for IgG.

[1] Sensitivity is defined as the probability that a patient with ABPA shows elevated serum levels of the respective marker at the cut-off level.

[2] Specificity is defined as the probability that a patient without ABPA shows serum levels of the respective marker below the cut-off level.

[3] Diagnostic accuracy is defined as the number of correctly positive categorized plus the number of correctly negative categorized as percentage of all.

[4] Positive Likelihood ratio is defined as the true positive rate divided by false positive rate. A higher ratio indicates a better test.

[5] Negative Likelihood ratio is defined as the false negative rate divided by true negative rate. A lower ratio indicates a better test.

Table 3) The value of TARC, total IgE, rAsp f1, f3, f4 and f6 and specific IgG for ABPA diagnosis using only serum samples at the total IgE peak level of each patient.

	TARC	IgE	rAsp f1	rAsp f3	rAsp f4	rAsp f6	IgG
Sensitivity [%]	100	83.3	63.6	54.6	54.5	72.7	90.9
Specificity [%]	97.2	94.4	100	100	90.3	100	86.2
Diagnostic accuracy [%]	97.9	91.7	90.5	88.1	80.9	92.9	87.5
Positive Likelihood ratio	36.0	15.0	19.7	16.9	5.6	22.5	6.6
Negative Likelihood ratio	0.00	0.18	0.36	0.45	0.50	0.27	0.11
Area under the ROC	0.99	0.94	0.92	0.89	0.85	0.83	0.87

Cut-off levels: 487 pg/ml for TARC, 1624 IU/ml for IgE, 274 IU/ml for rAsp f1, 739 IU/ml for rAsp f3, 51 IU/ml for rAsp f4 and 65 IU/ml, for rAsp f6 and 87 kU/l for IgG.

See table 2 for explanation of sensitivity, specificity, diagnostic accuracy and likelihood ratio.

Figure legends

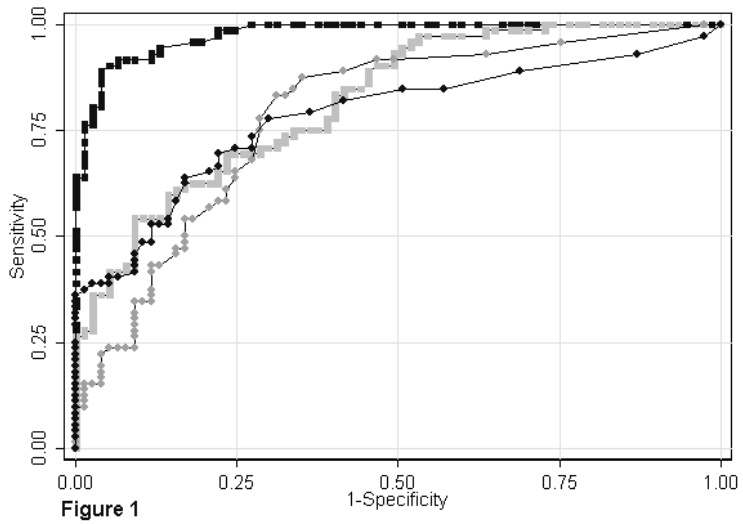

Figure 1. Receiver-operator characteristic (ROC) curves for TARC, total IgE, rAsp f4 and rAsp f6 for diagnosis of ABPA.

ROC curves are shown for TARC (black squares), total IgE (gray squares), rAsp f4 (gray circles) and rAsp f6 (black circles) using all available serum samples of the cohort (n=265). The resulting area under the curve is 0.98 for TARC, 0.84 for total IgE, 0.79 for rAsp f4 and 0.80 for rAsp f6.

Figure 2

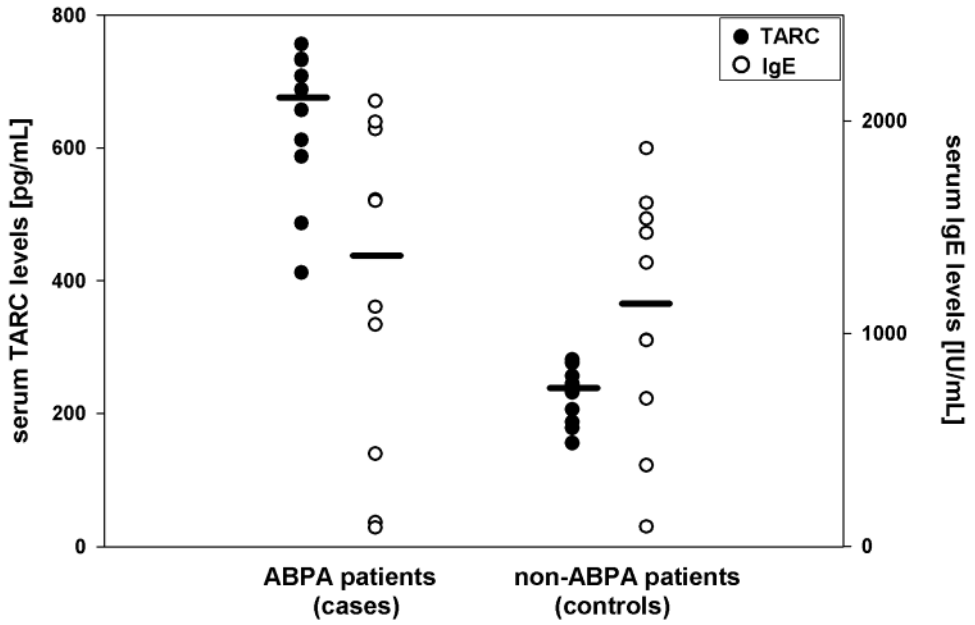

Figure 2. Nested case-control analysis: Comparison of serum TARC levels in ABPA and non-ABPA patients matched for total IgE serum levels.

Serum samples of 10 CF patients with clinical ABPA (cases) and 10 CF patients without clinical ABPA (controls), matched for IgE levels. 2 ABPA patients were excluded due to very high serum IgE levels (3710 and 5263 IU/ml) without matching control. Both showed elevated TARC serum levels (721 and 432 pg/ml). The bars indicate median TARC and IgE levels.

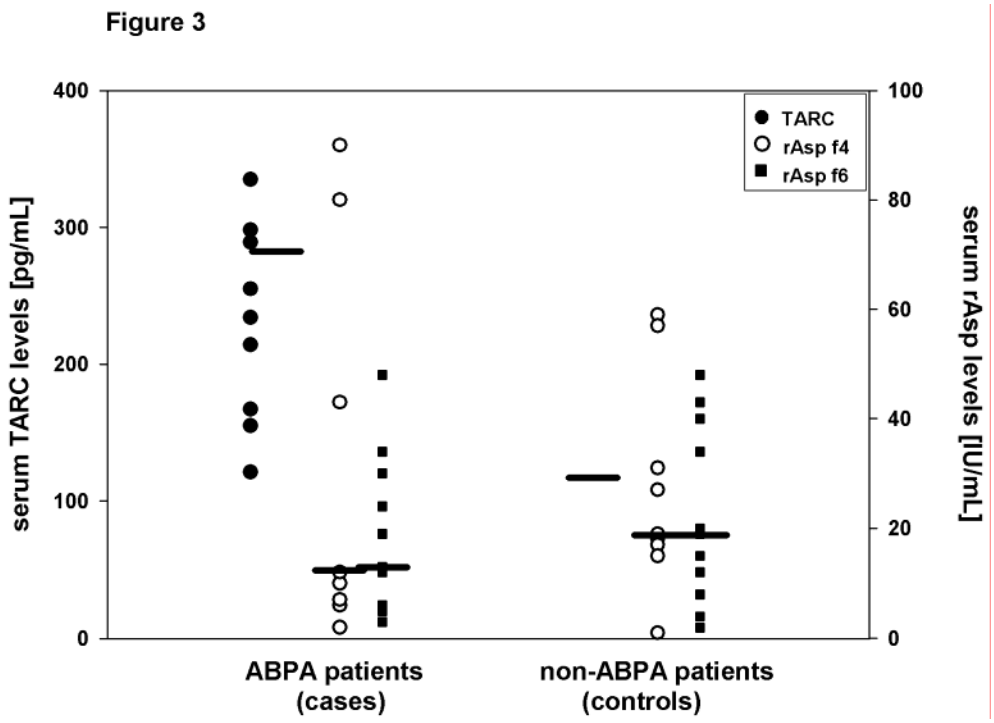

Figure 3. Nested case-control analysis: Comparison of serum TARC levels in ABPA and non-ABPA patients matched for rAsp f6.

Serum samples of 11 CF patients with clinical ABPA (cases) and 11 CF patients without clinical ABPA (controls), matched for rAsp f6 levels. One ABPA patient was excluded due to very high rAsp f6 levels (165 EU/ml) without matching control. He showed elevated TARC serum levels (630 pg/ml). The bars indicate median TARC and IgE levels.

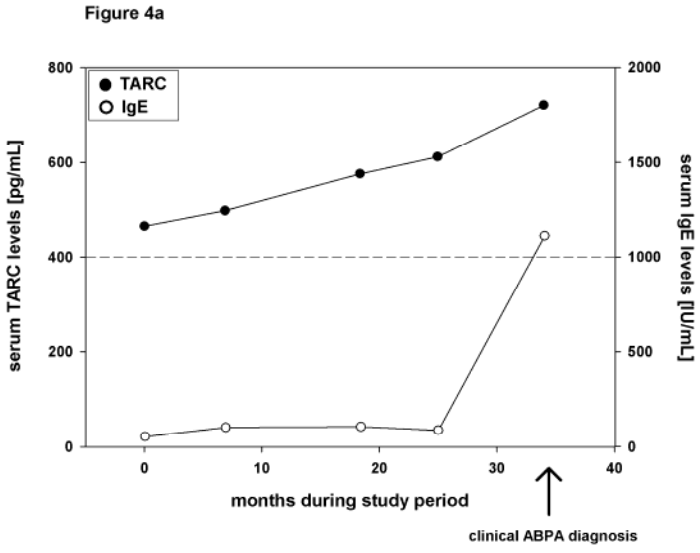

Figure 4b

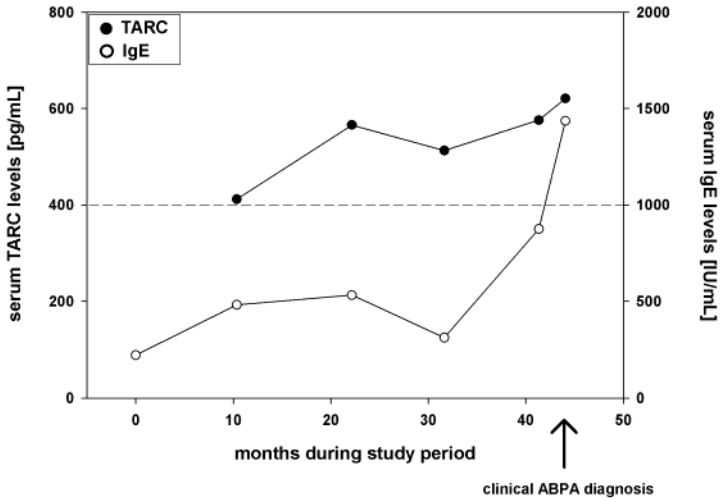

Figure 4c

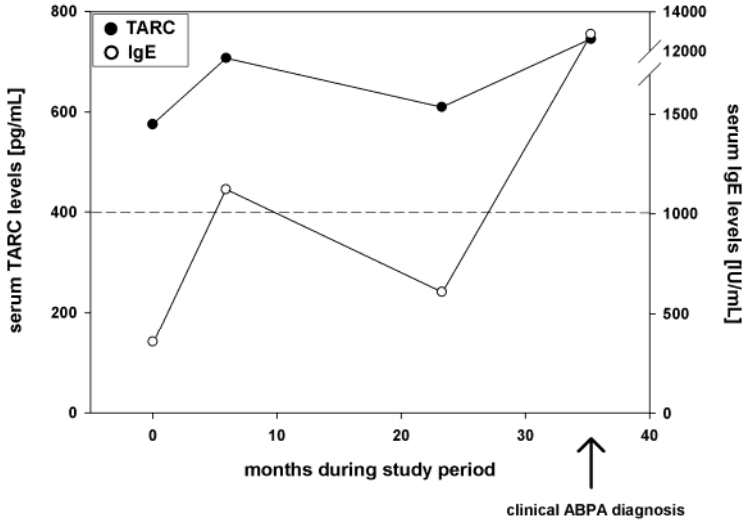

Figure 4. Longitudinal course of TARC levels compared to total IgE levels before the development of ABPA during the time of the study.

TARC levels are given as black circles; total IgE levels are given as white circles. The dashed line indicates a TARC cut-off level of 400 pg/ml.