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## What happens to basophils and tryptase, LXA<sub>4</sub>, and CysLTs during aspirin desensitization?

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### ABSTRACT

**Introduction:** Aspirin desensitization (AD) is an effective treatment in patients with non-steroidal anti-inflammatory drugs (NSAID)-exacerbated respiratory disease (NERD) by providing inhibitory effect on symptoms and polyp recurrence. However, limited data is available on how AD works. We aimed to study comprehensively the mechanisms underlying AD by examining basophil activation (CD203c upregulation), mediator-releases of tryptase, CysLT, and LXA<sub>4</sub>, and LTB<sub>4</sub> receptor expression for the first 3 months of AD.

**Methods:** The study was conducted in patients with NERD who underwent AD (group 1:  $n=23$ ), patients with NERD who received no desensitization (group 2:  $n=22$ ), and healthy volunteers (group 3,  $n=13$ ). All participants provided blood samples for flow cytometry studies (CD203c and LTB<sub>4</sub> receptor), and mediator releases (CysLT, LXA<sub>4</sub>, and tryptase) for the relevant time points determined.

**Results:** All baseline parameters of CD203c and LTB<sub>4</sub> receptor expressions, tryptase, CysLT, and LXA<sub>4</sub> releases were similar in each group ( $p>0.05$ ). In group 1, CD203c started to be upregulated at the time of reactions during AD, and continued to be high for 3 months when compared to controls. All other study parameters were comparable with baseline and at the other time points in each group ( $p>0.05$ ).

**Conclusion:** Although basophils are active during the first 3 months of AD, no releases of CysLT, tryptase or LXA<sub>4</sub> exist. Therefore, our results suggest that despite active basophils, inhibition of mediators can at least partly explain underlying the mechanism in the first three months of AD.

**Abbreviations:** AD: aspirin desensitization; BTRL: LTB<sub>4</sub> receptor; CysLT: cysteinyl leukotrienes; FEV1: forced expiratory flow in one second; NERD: nonsteroidal antiinflammatory drugs exacerbated respiratory diseases

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## Introduction

Nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (N-ERD) is a clinical phenotype of asthma in which aspirin (ASA) and other NSAIDs cause asthma attacks and rhinoconjunctivitis (1–3). Intractable nasal polyps and/or chronic sinusitis requiring recurrent multiple nasal surgeries exist in almost all patients (1–3).

The pathophysiology of N-ERD is attributed to the dysregulation of arachidonic acid metabolism and eosinophilic inflammation. The dysregulation of this pathway results in overproduction of Cysteinyl

leukotrienes (Cys-LTs) and prostaglandin (PG)D<sub>2</sub> and decreased levels of PG E<sub>2</sub> (3–5). Although *in vitro* studies revealed conflicting results, *in vivo* studies performed on human samples such as bronchoalveolar lavage (BAL) and urine provided good evidence for Cys-LTs enhancement and possible involvement in this disease. Cys-LTs, the most potent bronchoconstrictors described thus far, induce mucus secretion, airway edema, and eosinophil influx (6–8). Another putative mechanism is a deficiency in the generation of lipoxygenase interaction products, otherwise known as lipoxins (LX), which are regarded as important

antiinflammatory mediators involved in N-ERD (9,10). Considering the nature of the hypersensitivity reactions, as well as the mediators involved during these reactions, mast cells and basophils emerged as the potential cellular sources of these reactions.

Aspirin Desensitization (AD) is a procedure when tolerance of aspirin is induced in a hypersensitive patient by increasing doses of aspirin given orally or intranasally in short time intervals (11). Aspirin treatment after desensitization (ATAD) was first described in 1982 in a patient with NERD who had good clinical outcomes at Scripps Clinic (12). Subsequent reports on ATAD confirmed the clinical efficacy of ATAD in this patient group (13–15). These studies showed an improvement in both sinonasal outcomes (nasal symptoms, sense of smell, sinusitis, and delayed occurrence of nasal polyps) and asthma outcomes (decline in asthma attacks, medication use due to asthma) (16–18). Our recent study on clinical effectiveness of ATAD in patients with N-ERD in a controlled manner also provided similar clinical outcomes (19). On the other hand, despite the increased number of studies that showed the clinical benefits of this treatment, limited data are available on how ATAD works. Owing to the paucity of literature on the underlying mechanisms of aspirin desensitization (AD), as a part of our evaluation, we performed a study on several aspects of the mechanism of ATAD in patients with NERD. With this, basophil activation (CD203c expression), mediator releases (tryptase, Cys-LTs, and LXA<sub>4</sub>), and LTB<sub>4</sub> receptor expression were studied. The study was performed in a controlled manner in which two main groups served as controls (NERD without ATAD and healthy volunteers).

## Material and methods

### Study subjects

The study involved consecutive subjects with N-ERD who were admitted to our tertiary care clinic. Diagnosis of aspirin hypersensitivity based mainly on history with/without aspirin provocation tests. If the patients had at least two convincing episodes with aspirin and/or other NSAIDS, they were not provoked by aspirin (11,20,21). However, if the patients have unconvincing history, aspirin provocation was performed in the same doses given in aspirin desensitization. Asthma diagnosis was based on a history of recurrent symptoms of wheezing, shortness of breath, cough, and demonstration of objective signs of reversible airway obstruction by means of at least  $a > 12\%$  increase in forced expiratory volume in one second (FEV<sub>1</sub>) after 15 min with inhalation of 400 µg

salbutamol (22). Nasal polyps were diagnosed through endoscopic examinations with or without computed tomography of paranasal sinuses. None of the patients with asthma were on specific immunotherapy or anti-IgE treatments. Patients aged under 18 years were not included.

### Study design

Subjects with N-ERD who were admitted to our clinic underwent an initial clinical evaluation to determine whether they were eligible for ATAD. The severity of asthma, treatment level, nasal polyp status, and the presence of any contraindications for ATAD was assessed. Indications for ATAD were a history of severe asthma despite optimum treatment and/or recurrent nasal polyps in patients with N-ERD (19–21). Contraindications were previous history of bleeding, peptic ulcer, and FEV<sub>1</sub> values less than 1.5 L. Candidate patients who were not willing to undergo ATAD were also not desensitized. After this evaluation, if there was any indication and no contraindications, the patients were invited to be desensitized with aspirin. The remaining subjects who had an indication but were not willing to undergo ATAD or had a contraindication for ATAD were followed up as control patients. Therefore, the patients with N-ERD who were indicated for ATAD were assigned into two groups: group 1, ATAD, and group 2, no ATAD. For *in vitro* studies, a second control group of healthy volunteers without asthma nasal polyps and NSAII hypersensitivity served as healthy controls (group 3).

The study was approved by the ethics committee (No: 136–3997) and each subject provided written informed consent before the study began.

ATAD consisted of 3 parts (11,22). In this sense acute procedure consist of Aspirin provocation and aspirin desensitization as recommended (21). In this sense, the procedure started as AP and continued as desensitization once the patient developed reaction during AP. AP/AD was performed under strict surveillance where emergency equipment and trained personnel were available. In general, recommendations of the American AD Joint Task Force (21) were followed during AP/AD with a slight dose modification because of the available aspirin doses in our country. The starting dose and dose intervals varied depending on the history of the patient. The starting dose was either 20 or 40 mg of aspirin. After a light breakfast, increasing doses of aspirin were given every 1.5 to 3 h unless a reaction occurred. Sequential doses for aspirin were 20, 40, 80, 100, 150, 300, and 600 mg. If no reaction occurred, the next dose was given.

Positive reaction was considered as development of nasal symptoms and/or conjunctivitis and/or worsening of asthma ( $a \geq 20\%$  decrease in FEV1) (11,21). Provoking dose of aspirin was recorded. Once a reaction occurred, the patients were treated adequately and the procedure continued as AD in group 1.

if the reaction was mild and the patient was fully recovered after at least 3 h, the same dose of aspirin was given. If the reaction was moderate, the patient was administered the previous dose of aspirin. Therefore, AD continued with the same principles throughout the procedure. After safely completing 600 mg aspirin, patients were instructed to start 600 mg aspirin twice daily for the first month in terms of ATAD.

The patients were reviewed at 1 and 3 months of ATAD (Figure 1). At the visit in the first month, the aspirin dose was dropped to 600 mg daily. These patients continued their regular treatment for asthma and nasal polyps. Patients with NERD who did not undergo ATAD (group 2) were also followed up with their regular treatment and scheduled for visits at 1 and 3 months (Figure 1).

### *In vitro* studies

All participants provided blood samples for *in vitro* studies. Blood was collected for flow cytometry studies

(CD203c, and LTB<sub>4</sub> receptor-BTRL), and mediator releases (Cys-LTs, LXA<sub>4</sub>, and tryptase). Group 1 provided blood for *in vitro* studies at baseline (before desensitization, at the time a reaction was seen during the AP procedure, at the end of dose escalation of AD, and the 1st and 3rd months while the patients were under regular aspirin treatment daily, whereas group 2 was assessed for the same parameters at baseline, and the 1st and 3rd months (Figure 1). Healthy volunteers provided baseline blood samples and 1 h after 600 mg aspirin ingestion for *in vitro* studies.

### *Flow cytometry studies*

CD203c expression in basophils and BTRL expression in lymphocytes and other cells were studied using flow cytometry. Ten-milliliter peripheral venous blood samples were drawn into EDTA-containing tubes.

**Cd203c.** Three-color direct immunofluorescence was used for staining. CD203c-induced expression was evaluated using an Allergenicity Kit (Cellular Analysis of Allergy, Beckman Coulter) in accordance with the manufacturer's instructions. To evaluate the background basal values without stimulation (negative control), 20  $\mu$ L of phosphate-buffered saline (PBS) (Sigma Diagnostics, St. Louis, Mo., USA) was added to whole blood in another tube. As a positive control, 20  $\mu$ L of a monoclonal anti-IgE receptor

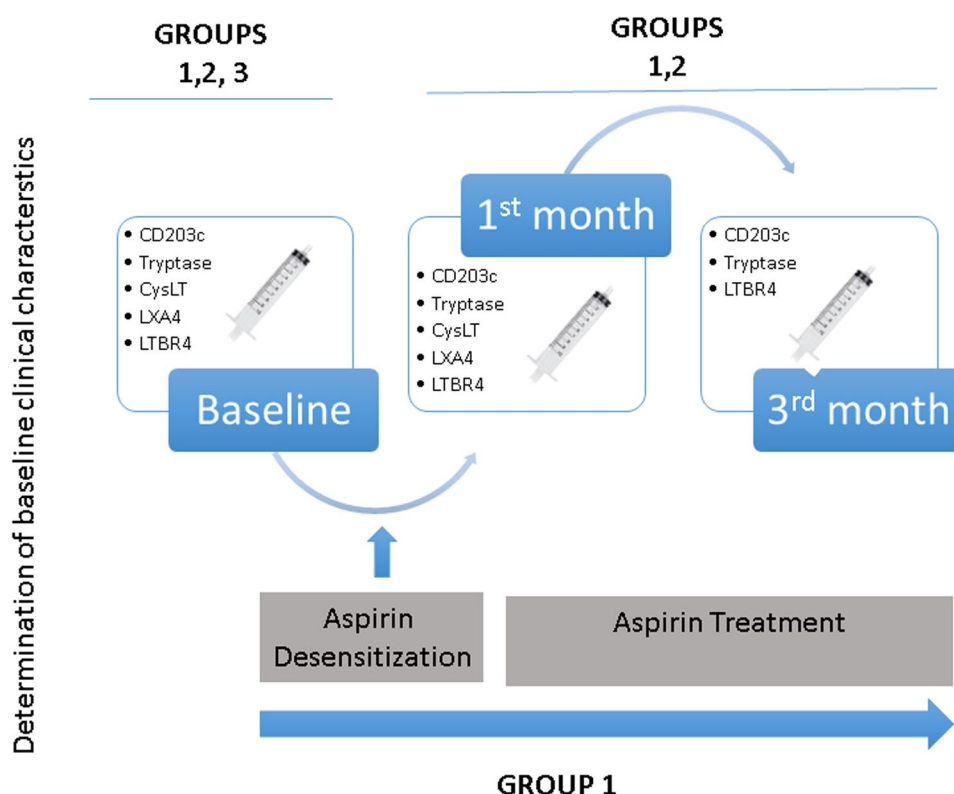


**Figure 1.** Schematic view of the study design.

(FcεRI) antibody, and 20 µL of CRTH-2 (clone: BM16)-FITC/CD203c (clone: 97A6)-PE/CD3 (clone: UCHT-1)-PC7 monoclonal antibodies were used (23,24). One hundred microliters of activation solution containing calcium were added and incubated at 37°C in a water bath for 15 min. The reaction was halted by adding 100 µL of stop solution. Tubes were then incubated for another 10 min for lysis at room temperature. Three-color staining using CRTH-2-FITC/CD203c-PE/CD3-PC7 (Beckman Coulter) of surface markers was performed at 488 nm on a NAVIOS Flow Cytometer (Beckman Coulter) equipped with an argon laser and analyzed using Kaluza software. On the histogram defined by forward scatter and side scatter, the initial gate was mapped around all peripheral blood cells. The second gate was defined as cells that did not express CD3. Cells expressing both CRTH-2-FITC and CD203c-PE were identified as basophils. At least 500 basophils were assessed in each assay. The percentage of cells expressing CD203c was given. The results are expressed as the percentage of CD203c and as the stimulation index (SI), calculated as the ratio between the percentage of degranulated basophils (CD63<sup>fl</sup> cells) with different allergens and the negative control. The BAT result was considered to be positive if the SI was over or equal to 2.

**Leukotriene B<sub>4</sub> receptor (LTBR).** Leukotriene B<sub>4</sub> receptor (HBLTR-1) expression on total lymphocytes, CD3<sup>+</sup>45<sup>+</sup>, and CD15<sup>+</sup>45<sup>+</sup> gates was analyzed using flow cytometry with whole-blood lysis (25,26). One hundred microliters of peripheral blood were put into 12 × 75-mm polystyrene tubes (Falcon Labware, Meylan Cedex, France) and incubated in the dark at room temperature for 15 min with appropriate monoclonal antibodies. Then, red blood cell lysis, leukocyte stabilization, and fixation were performed using Immunoprep A, B, and C solutions with a Coulter-Q-Prep Epics Immunology Workstation (Coulter Corp., 4235825-B, Hialeah, FL, USA) and analyzed using five-color staining flow cytometry (Cytomics FC500 Flow Cytometer, Beckman Coulter Inc. Miami, FL, USA). The monoclonal antibodies used in this study were CD45 PC5 (Beckman Coulter, Marseille, France), CD3 FITC (Beckman Coulter, Marseille, France), and CD15 FITC (Beckman Coulter, Marseille, France). Leukotriene B<sub>4</sub> receptor (HBLTR-1) and PE (BD Pharmingen) were used as negative controls.

### Mediator releases

#### Cys-LTs and LXA<sub>4</sub>

Cys-LTs and LXA<sub>4</sub> were studied from the peripheral blood samples.

**Cell preparation.** A 3-ml peripheral venous blood sample was drawn into venipuncture tubes containing EDTA and then transferred into polypropylene tubes (27,28). Blood was centrifuged at 280 g at +4°C for 15 min. After discarding the supernatant, physiologic saline at the same volume was added to the tubes. We added 0.55 ml of 3.5% dextran solution per mL and the sample was vortexed and incubated at 37°C for 20 min. The supernatant, which contained blood leukocytes, was then transferred into another tube and centrifuged at 1500 g at +4°C for 15 min. The supernatant was discarded and the cells lysis solutions were added (2.5 ml lysis A [0.2% NaCl], followed by 2.5 ml lysis B [3.98 g NaCl, 500 mg saccharose, 250 ml distilled water]). Finally, the samples were centrifuged at 280 g at +4°C for 15 min. The supernatant was discarded and the number of cells was counted. A buffer solution was added to all samples to provide 6,250,000 cells/mL.

**Cell priming.** A buffer solution (3 ml) (20 mM HEPES, 0.025% HSA, 125 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.5 mM glucose) containing 1.5 pg/mL IL-3 (human recombinant IL-3, Biosource International, Camarillo, CA, USA) was immediately added to the cell-containing tube at room temperature and vortexed. Before cell stimulation, 250 µL of this suspension was pipetted into the first tube, 200 µL into a second Eppendorf tube, and immediately stimulated.

**Cell stimulation.** No stimulant was added to the first tube and this was considered the baseline unstimulated level. A 50 µL of 5 × 10<sup>-5</sup> M A23187 (Sigma, Istanbul, Turkey) used as nonspecific leukocyte stimulants were added to tube 2. The samples were incubated at 37°C for 40 min and centrifuged at 1000 g at 4°C for 5 min. The supernatant was stored at -80°C until required for analysis.

### Enzyme immune assay studies

#### Cys-LT

Cys-LT release was evaluated using a competitive enzyme immunoassay (EIA; SPI-BIO, Massy Cedex, France). In the measurement, Cys-LT antiserum, Cys-LT acetylcholine esterase tracer, and standard for Cys-LT were used (27,28). The minimal detectable level of Cys-LT was 30 pg/mL. Cross-reactivity for several LO and COX products was 0.01%. A 50-ml volume of each sample, tracer, and antiserum was pipetted into microplates and incubated at 48°C for 20 h. After removal of unbound materials by washing with EIA washing solution, 200 µl of Ellmann's reagent

was added to the microplates. Absorbance was measured at 414 nm after  $60 \pm 90$  min. A standard curve was achieved using seven concentrations between 15.6 pg/mL and 1000 pg/mL for each microplate. Duplicate EIA assays were performed. Cys-LT concentration was calculated using a four-parameter logistic equation.

#### LXA<sub>4</sub>

LXA<sub>4</sub> was measured using an enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's instructions (Neogen Corp., Lexington, KY, USA). The detection limit was 20 pg/mL for LXA<sub>4</sub> (29).

#### Statistics

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 16.0; Chicago, Illinois). Numeric values are expressed as mean  $\pm$  SEM and nominal values are given as number and percentage (n, %). For the comparison of numeric values among the three groups, one-way analysis of variance (ANOVA) was used. The comparison of two groups was made using the independent samples t-test if the distribution of data was homogeneous. Non-parametric tests were used when the distribution of the groups was not homogenous. Numeric values within groups were compared using Wilcoxon's paired-sample t-test, and the groups were compared using the Mann-Whitney *U* test. Nominal values were compared using the Chi-square test. *P* values less than 0.05 were considered statistically significant.

## Results

A total of 23 subjects with NERD were in group 1 and 22 were in group 2. There were 13 healthy volunteers in group 3. All groups had comparable age and sex distribution. Groups 1 and 2 also had similar demographics and clinical features (Table 1). The provoking doses of aspirin were between 40–100 mg and all were mild-to-moderate reactions.

#### In vitro studies

##### Flow cytometry studies

**Cd203c.** Baseline CD203c expressions were similar in each group ( $p > 0.05$ ) (Figure 2). In group 1, CD203c expression increased at the time of reactions during AD ( $p = 0.044$ ) and was still higher compared with baseline at the 1st-month follow-up ( $p = 0.033$ ). In the 3rd month, although the higher expressions of CD203c continued, this was not statistically significant (Figure 2). CD203c expression at 1 month was higher in group 1 than in group 2 ( $p = 0.033$ ).

In group 2, there were no differences in CD203c expression at any time point ( $p > 0.05$ ) (Figure 2). In group 3, 600 mg of aspirin did not cause a significant change in CD203c expression from basophils.

#### Mediator releases

##### Tryptase

Baseline tryptase levels were comparable in each group ( $p > 0.05$ ) (Figure 3). Tryptase levels did not change in groups 1 and 2 at any time points of the study.

**Table 1.** Demographics and disease characteristics of the patients in Group 1 and Group 2.

| Variable                                                              | Group 1        | Group 2         | p   |
|-----------------------------------------------------------------------|----------------|-----------------|-----|
| n                                                                     | 23             | 21              | NS* |
| Sex (F/M) n, %                                                        | 14/9           | 14/7            | NS  |
| Age (mean $\pm$ SEM) (years)                                          | 44.8 $\pm$ 2.3 | 44.6 $\pm$ 3.3  | NS  |
| Blood eosinophil count                                                | 252 $\pm$ 50   | 400 $\pm$ 17    | NS  |
| Total IgE level (IU/ml)                                               | 89.7 $\pm$ 4.6 | 110.6 $\pm$ 3.2 | NS  |
| Presence of asthma n (%)                                              | 21 (91)        | 21 (100)        | NS  |
| Asthma duration (mean $\pm$ SEM) (year)                               | 12.4 $\pm$ 1.8 | 13.1 $\pm$ 1.7  | NS  |
| FEV <sub>1</sub> (%), (mean $\pm$ SEM)                                | 92.3 $\pm$ 3.6 | 87.6 $\pm$ 5    | NS  |
| Severe asthma attacks in the last year (mean $\pm$ SEM)               | 0.5 $\pm$ 0.2  | 0.4 $\pm$ 0.1   | NS  |
| Number of patients with severe asthma attack in the last year, n(%)   | 18 (78.2%)     | 20 (95.2%)      | NS  |
| Presence of nasal polyps n (%)                                        | 22/23          | 21/21           | NS  |
| Duration of nasal polyps (mean $\pm$ SEM) (year)                      | 10.4 $\pm$ 1.6 | 10.04 $\pm$ 1.5 | NS  |
| Nasal surgery (mean $\pm$ SEM/year)                                   | 4.95 $\pm$ 0.9 | 4.33 $\pm$ 1.08 | NS  |
| Duration of hypersensitivity to Aspirin/NSAID (mean $\pm$ SEM) (year) | 8.8 $\pm$ 1.4  | 8.4 $\pm$ 1.8   | NS  |
| Number of sinusitis episodes in the last 1 year (mean $\pm$ SEM)      | 6.1 $\pm$ 1.2  | 5.8 $\pm$ 2.2   | NS  |
| <b>Treatment</b>                                                      |                |                 |     |
| GINA treatment level                                                  |                |                 |     |
| Step 3                                                                | 13 (56%)       | 12 (57.1%)      | NS  |
| Step 4                                                                | 10 (44%)       | 9 (42.9%)       | NS  |
| ICS/LABA                                                              | 20 (86.9%)     | 19 (90.4%)      | NS  |
| Anti-leukotrien                                                       | 19 (82.6%)     | 12 (57.1%)      | NS  |
| Nasal corticosteroids                                                 | 17 (73.9%)     | 17 (80.9%)      | NS  |
| Antihistamines                                                        | 11 (47.8%)     | 6 (28.5%)       | NS  |

\*NS: Not significant.

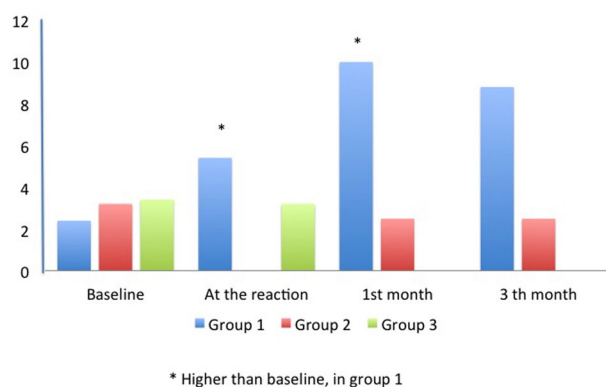


**Figure 2.** CD203c expressions of the study groups at baseline (all groups), at the reaction (2 groups), and 1 and 3 months.

Aspirin intake did not change tryptase levels in group 3 (Figure 3).

### Cys-LT

Cys-LT levels were assessed at baseline and the 1st month of treatment. Baseline Cys-LT levels were similar in each group (Figure 3). No significant change was detected after the 1st month of treatment in group 1. Aspirin intake did not change Cys-LT levels in group 3 (Figure 3).

### Lipoxin A<sub>4</sub>

Ca-ionophore-stimulated baseline LXA<sub>4</sub> generation was comparable in each group ( $p > 0.05$ ) (Figure 3). No changes occurred after the 1st month of AD in group 1 ( $p > 0.05$ ).

### Btrl

Baseline BTRL expression was similar in each group ( $p > 0.05$ ) (Table 2). In group 1, no changes occurred at any observation time during AD ( $p > 0.05$ ) (Table 2). Aspirin caused no changes in BTRL expression in group 3 ( $p > 0.05$ ) (Table 2).

### Results in responders and nonresponders

In our series; 2 patients had nasal polyps recurrence at 1 and 3rd months of ATAD, respectively. This data is a part of a project in which both immunological and clinical effectiveness of ATAD was investigated. Therefore, we had clinical data of these cases for 12 months (19). In this sense; 4 patients stopped ATAD due to GIS side effects and two patients because of eosinophil increase within one year. Therefore, remaining 15 patients continued ATAD for 12 months and were considered clinically responsive. Therefore, we can consider only 2 patients as nonresponder to

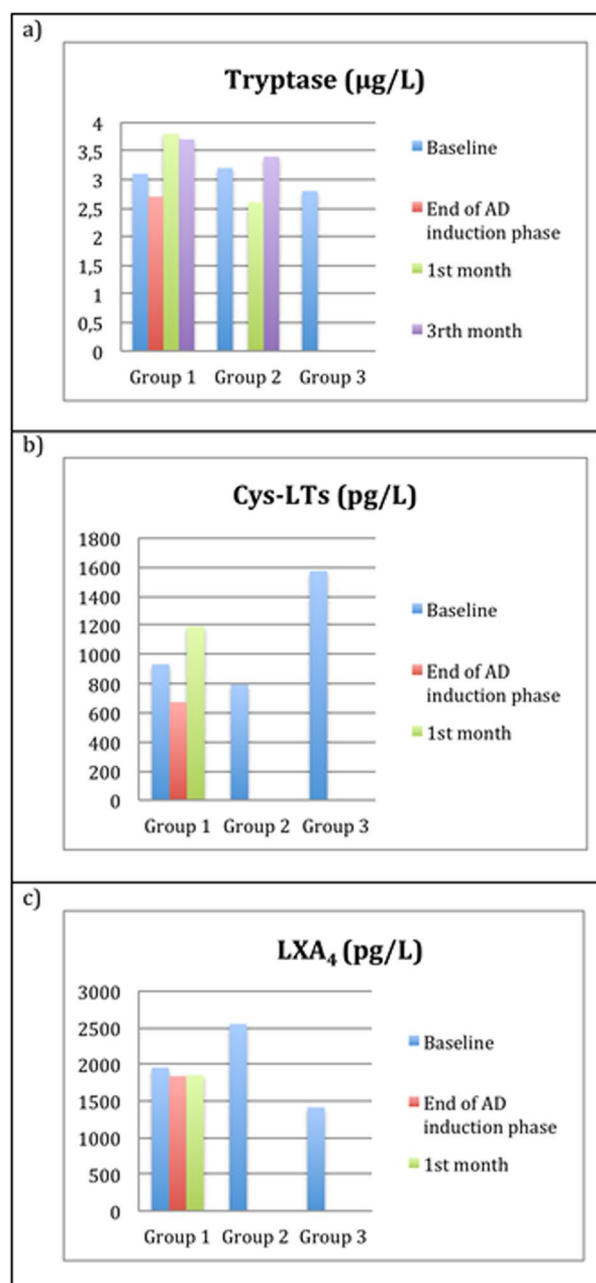


**Figure 3.** Comparison of mediator releases in 3 study groups.

ATAD and remaining 15 cases as responder (excluding the ones having side effects). As we had only 2 non-responder cases, no statistical analysis in terms of comparison of responders versus nonresponders were not performed. Our 12 month analysis in that study (19) showed that peripheral blood eosinophil count in responder cases showed an tendency to increase at 12 months .

### Discussion

In this study, we comprehensively investigated the underlying mechanisms of ATAD during the first

**Table 2.** BTRL expression by the groups. Values are given as mean  $\pm$  SEM.

|                               | Group 1  | Group 2   | Group 3  | P  |
|-------------------------------|----------|-----------|----------|----|
| <b>CD3-negative cells</b>     |          |           |          |    |
| Baseline                      | 40.6±4.4 | 41.6±2    | 40.5±2.3 | NS |
| 1 <sup>st</sup> month         | 44.9±5.9 | 38.8±5    |          |    |
| 3rd month                     | 36.9±6.8 | 46.3±15.5 |          |    |
| <b>CD3-positive cells</b>     |          |           |          |    |
| Baseline                      | 3.1±1.2  | 8.0±2.1   | 0.9±0.13 | NS |
| 1 <sup>st</sup> month         | 2.5±1.4  | 1.2±0.2   |          |    |
| 3rd month                     | 1.3±0.2  | 2.1±1.2   |          |    |
| <b>CD15-45-positive cells</b> |          |           |          |    |
| Baseline                      | 94.4±1.7 | 92.4±4.1  | 93.1±2.3 | NS |
| 1 <sup>st</sup> month         | 92.4±1.7 | 92.9±3.1  |          |    |
| 3rd month                     | 92.0±3.2 | 94.7±1.5  |          |    |

3 months, in a controlled manner. *In vitro* studies in this experiment aimed to document the natural effect of ongoing *ex vivo* aspirin exposure in patients with N-ERD. According to our results, basophils, in terms of upregulation of CD203c expression, seemed to be active in the first 3 months of ATAD. However, no significant mediator releases of tryptase, Cys-LT, or LXA<sub>4</sub> existed in measurements at any established time points. No other study parameters of LTB<sub>4</sub> receptors changed in the first 3 months of ATAD either.

Beyond the studies showing tryptase and Cys-LTs releases, recent studies focused on flow cytometric evaluation of basophil activation in N-ERD (23,24,30–32). Although conflicting results exist, there are some suggestive data on basophil involvement in the pathogenesis of N-ERD (30–34). Altered basophil identification markers have been discovered to associate with allergic asthma in recent years. In this sense, in a recent study finding of upregulated expressions of CD123, CD203c, and CD63 in granulocytes of patients with Allergic Asthma and Allergic rhinitis suggested that CD123<sup>+</sup>HLA-DR<sup>+</sup> cells may contribute to the development of AA and ARA (35). In terms of basophil identification markers; so far, only a single study performed by Silberman et al. examined basophil activation during ATAD for 6–8 weeks (36). This study showed persistent basophil activation at 6–8 weeks after ATAD in patients with NERD who had coronary artery disease. They also documented that urinary excretion of thromboxane-B2 diminished substantially in desensitized patients but remained higher than in controls (36). Similar to this data, our findings also suggested activation of basophils during AD, starting from the first reaction during AD and lasting till 3 months of treatment. However, there were no mediator releases of either tryptase or cys-LT, indicating nonfunctional activation of basophils in our trial. Taken together, our findings suggested the data on involvement of basophils without releasing inflammatory mediators in the pathogenesis of N-ERD.

Our results related to CD203c upregulation provided some important findings. First, baseline values were similar in all groups indicating no basophil activity in resting status in N-ERD. Secondly, neither 600 mg aspirin intake in healthy subjects nor measurements at established time points of 1 and 3 months in subjects with N-ERD who were not desensitized revealed any changes in CD203c expression showing upregulation of CD203c expression was only seen in AD group. In this sense; CD203c expression started to be upregulated at the first reaction seen during the dose-escalation phase of ATAD and continued to be high until the 3rd month, although the latter did not reach a statistically significant level when compared with baseline.

We also studied mediators of tryptase and Cys-LTs, both of which are assumed to be released from basophils to show the functioning of basophils during ATAD. Interestingly, we found no releases of either tryptase or Cys-LTs in any groups at any measurement time points. Finding no releases of mediators especially Cys-LTs is particularly important to show the role of ATAD on this release as many *in vivo* and *in vitro* studies showed an increase in Cys-LTs release after aspirin exposure/provocation in patients with N-ERD (5–8,27,28). Based on these data, we may assume that although basophils are active, there is a suppression of relevant mediator releases during ATAD. Considering that Cys-LTs are very potent bronchoconstrictors that lead to mucus secretion, airway edema, and eosinophil influx, we may assume that clinical improvement seen during ATAD can, at least in part, be explained by this suppression. However, a conflict exists about cys-LT release under ATAD. Studies are showing increased, not changed, or diminished levels of Cys-LTs during ATAD (25,26,37–40). However, supporting our findings, a recent study by Makowska et al. also demonstrated that there were no changes in urinary LTE<sub>4</sub> during the first 3 months of ATAD (38). A longer period of ATAD (52 weeks) was also shown to not affect eicosanoid concentrations (38). Considering our results on Cys-LT levels, it is interesting why these mediators are not increased despite active basophils and continuous exposure to aspirin. Both Cys-LTs and LTB<sub>4</sub> act through GPCR coupled to G proteins, thereby increasing intracellular calcium. Inhibition of signal transmission such as STAT 6 could be responsible for this inhibition in mediator releases (40–42).

In this study, we also evaluated the level of LXA<sub>4</sub> in the study subjects. As a member of the arachidonic acid pathway, LXs, as a class of eicosanoids, exert endogenous antiinflammatory actions, are produced

by a variety of cells (43). Diminished LXA<sub>4</sub> generation has been shown in N-ERD when compared with aspirin-tolerant asthma (9,28). Our results showed a similar baseline value of LXA<sub>4</sub>. Neither the level at the 1st-month follow-up between group 1 and group 2 nor the progress of the level at different time points in desensitized patients showed any differences. Similar results were provided by another recent study (44). Therefore, we may suggest that no decrease in LXA<sub>4</sub> generation occurs during the early phases of ATAD. On the other hand, more attention can be given to an imbalance between proinflammatory cysteinyl-leukotrienes and anti-inflammatory 15-epi-LXA<sub>4</sub> in the pathogenesis of NERD, which was elegantly shown by another recent study (45).

ATAD was shown to inhibit LTB<sub>4</sub> from monocytes in patients with NERD (25). However, another study performed on nasal biopsy specimens in a limited number of patients who underwent ATAD failed to show a decrease in the percentage of the leukocytes expressing LTB<sub>4</sub> receptors (26). Our results also showed no changes in LTB<sub>4</sub> receptor expressions at each time point of the groups.

In this study, owing to short term duration of analysis, we weren't able to provide any clinical data in terms of effectiveness of ATAD. However, as a part of this project, these cases continued to use ATAD for 12 months. In this sense, we had only 2 nonresponder cases with nasal polyp recurrancy at 1 and 3 months, and 15 cases with clinical response to ATAD at the end of 12 months (excluding cases who drop out because of side effects of aspirin). Therefore, based on our clinical data for 12 months (19) we may consider that immunological data in this study consist of the results of the responder subjects and basophil activation in terms of increased CD203c expression seem to immunologically determine clinical response in our series.

Recently, high-dose aspirin therapy was reported to increase global markers of T2 inflammation, such as peripheral blood eosinophilia, mast cell activation assessed by plasma tryptase levels, and urinary leukotriene E4 production in N-ERD (46). In their study a significant decrease was shown in sputum eosinophil percentage in responders, while no changes were observed in non-responders. Our 12 month analysis didn't include sputum eosinophil count but showed that peripheric blood eosinophil count in responder cases showed an tendency to increase at 12 months (19). But we may say that diminished CysLT release could partly explain the clinical benefit of ATAD at 12 months in our series. It is also of interest whether clinical response varies depending on the underlying phenotypes of N-ERD (47–49).

We believe, studying several aspects of the underlying mechanisms of ATAD is the strength of our study. However, we studied only CD203c for basophil activation. Addition of CD63, another basophil marker could show more data about basophil involvement in these patients. Moreover, peripheral basophils count and percentage were not included in the statistical calculations. These can be considered as limitations of our study.

In conclusion, in this study, we comprehensively investigated the mechanisms that underlie ATAD and showed that basophils were active in terms of CD203c upregulation in the first 3 months of ATAD; with no releases of mediators of tryptase and Cys-LTs. Our results also indicated no increment of either LXA<sub>4</sub> levels or LTB<sub>4</sub> receptor expressions in this process. We believe our data complete a part of the puzzle of ATAD by showing important data on basophils and mediators, all of which are considered to have high importance in the pathogenesis of NERD. Documentation that the increment of major mediators such as Cys-LTs is suppressed during ATAD can partly explain the good clinical outcomes because Cys-LTs are involved in the mechanisms of NERD. However, it is of interest how this response is influenced by long-term ATAD. Much is certainly needed to clarify the other complex mechanisms that participate in this unique disease.

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## Clinical trial registration

This study is registered to ISRCTN Registry (BMC, Springer Nature) with registry number ISRCTN27096541.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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