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CHAPTER 9

Cleaved secretory leukocyte protease inhibitor as a biomarker of chymase activity in allergic airway disease

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Abstract

Background: Secretory leukocyte protease inhibitor (SLPI), which is present in many physiological fluids including saliva, sputum and nasal discharge, is the most effective inhibitor of chymase. Previously, we demonstrated that chymase is able to cleave SLPI and that the cleaved portion, CSLPI, is a biomarker of chymase activity.

Objective: We investigated the potential of CSLPI as a biomarker of chymase activity in subjects with allergic rhinitis and asthmatic airway disease.

Methods: Baseline sputum samples were collected from atopic asthmatics and healthy controls. Nasal lavages (NAL) were performed in subjects with allergic rhinitis (AR) both at baseline and following a nasal challenge with allergen or placebo. Levels of CSLPI and chymase were determined by western analysis and tryptase and α 2-macroglobulin were measured by immunoassay.

Results: As compared with healthy controls, asthmatics showed a significant increase in baseline CSLPI/total SLPI ratios and an increase in chymase levels. There was a high correlation of CSLPI/SLPI ratios to chymase levels in normal individuals and untreated asthmatics. In the NAL of patients with AR, as compared with placebo, allergen challenge increased inflammatory biomarkers, including: CSLPI/SLPI ratios, chymase levels, tryptase levels, and α 2-macroglobulin levels. Correlations were observed between CSLPI/SLPI ratios and chymase levels and CSLPI/SLPI ratios and α 2-macroglobulin levels; no correlation was seen between CSLPI/SLPI ratios and tryptase levels.

Conclusion: Our data indicate that CSLPI reflects chymase activity in AR and asthma. Hence, CSLPI may serve as a biomarker for disease activity and for monitoring the efficacy of novel anti-inflammatory treatments in chymase-mediated diseases.

Introduction

The clinical hallmarks of asthma and allergic rhinitis (AR) are persistent airway inflammation and airway hyperresponsiveness (AHR) (1, 2). The observation that clinical symptoms and function do not fully reflect the severity of the underlying airway inflammation and AHR has initiated the search for more adequate biomarkers of asthma and AR (1, 3). Subsequently, the development and validation of non- and semi-invasive sampling techniques, including exhaled nitric oxide, induced sputum and nasal lavage, helped to identify several potential biomarkers of airway inflammation for both clinical practice and research purposes (4-6). However, there is still an unmet need to discover sensitive biomarkers from easily obtained samples that are closely linked to the inflammatory cascade that could serve to monitor the progress and treatment of airway diseases. The mast cell is an important effector-cell in the pathophysiology of allergic airway inflammation, AHR and airway remodeling (7-9). Degranulation of sensitized mast cells by relevant allergen causes the release of histamine and proteases (tryptase and chymase), and de novo synthesis of other inflammatory mediators (leukotrienes, prostaglandins, platelet activating factor and bradykinin) (10-12). On the basis of proteases content, mast cells can be subdivided into at least two phenotypically different subsets: connective tissue mast cells expressing chymase, tryptase and cathepsin G (MCTC) and mucosal mast cells producing tryptase, but no chymase (MCT) (13). Although not fully elucidated, chymase originating solely from MCTC cells has recently been implicated in the pathophysiology of airway inflammation, AHR and remodeling of allergic airway disease (9, 14). The proteinase inhibitors, α 1-antitrypsin, α 1-antichymotrypsin, α 2-macroglobulin and secretory leukocyte protease inhibitor (SLPI), released from neutrophils, macrophages and epithelial cells within the respiratory tract, have been shown to inactivate chymase, providing local protection against allergic stimuli (15-18). In a previous *in vitro* study we have shown that SLPI, is not only a chymase inhibitor, but also a substrate of chymase and that its cleaved product, CSLPI, is a biomarker of chymase activity *in vitro* (19). Based on these data, we hypothesized that CSLPI may be a biomarker of chymase activity in allergic airway disease *in vivo*. To this end, in two studies originally designed to assess allergic end-points in asthma and allergic rhinitis we retrospectively measured baseline levels of SLPI and CSLPI in relation to chymase levels. In the first study we examined SLPI, CSLPI and chymase levels in the sputum of subjects with allergic asthma in comparison with healthy subjects. In the second study, levels of CSLPI, SLPI, chymase, tryptase and α 2-macroglobulin were measured in nasal lavage (NAL) of individuals with AR, both under baseline conditions and following nasal allergen challenge.

Methods

SUBJECT GROUPS

Asthmatic subjects study

This part of the study was conducted at the University of Southampton, UK. On one study occasion, hypertonic saline-induced sputum was collected and analyzed from 12 participants (6M/6F, 18-70 years), subdivided into 4 study groups with the following characteristics:

1. Healthy non-atopic, non-smoking controls with no chronic respiratory symptoms and no evidence of upper or lower respiratory tract infection in the last 6 weeks ($FEV_1 > 80\%$ predicted, normal FEV_1/FVC ratio; group 1).
2. Non-smoking subjects with intermittent, atopic asthma ($FEV_1 > 80\%$ predicted, not on inhaled corticosteroids; group 2).
3. Non-smoking subjects with mild persistent, atopic asthma ($FEV_1 > 80\%$ predicted; regular use of inhaled corticosteroids (Beclomethasone (Qvar®) 100µg BD, Serevent 25µg BD; Fluticasone 100µg BD; group 3).
4. Non-smoking subjects with moderate-severe persistent atopic asthma (FEV_1 60-80% predicted; symptomatic despite regular treatment with corticosteroids: Becotide 200µg QDS; Seretide (1000µg fluticasone / 200µg salmeterol) BD; Seretide (125µg fluticasone / 25µg salmeterol) BD, Prednisolone 40mg OD; group 4).

All subjects gave written informed consent and ethical approval was obtained from the Joint Ethics Committee of Southampton Hospital and General Hospital.

Subjects with allergic rhinitis

Nasal lavage (NAL) specimens were obtained from 20 multi-sensitized, non-smoking patients (10M/10F; 19-51 years) with clinically stable AR who were not on maintenance therapy, participating in a clinical study at the Centre for Human Drug Research, Leiden, Netherlands (20). All subjects provided written informed consent and the study was approved by the Ethics Committee of Leiden University Medical Center, Leiden, Netherlands.

SPUTUM INDUCTION AND PROCESSING

In the asthma and healthy subject cohorts ($n = 3$ per subgroup) sputum was induced by hypertonic saline according to European Respiratory Society (ERS) recommendations (4, 21). Shortly, following salbutamol pretreatment, aerosolized hypertonic saline (4.5% NaCl) solution was inhaled at three 5-minute intervals by tidal breathing with the nose clipped. In more severe

asthmatics, a modified ERS protocol was used starting with normal saline and moving slowly to hypertonic saline only if there was no significant fall in FEV₁. At the end of each 5-minute interval, expectorated sputum was collected in a petri-dish and FEV₁ and PEF_R were recorded. If FEV₁ fell by $\geq 20\%$ from the post-salbutamol baseline, the procedure was discontinued. Sputum samples were kept on ice and processed within 1 hour of collection as previously described (4). In summary, sputum was teased from the sample using a forceps and collected into a fresh petri dish, avoiding contamination with saliva and squamous cells. One volume of selected sputum was diluted with four volumes of phosphate buffered saline (PBS). Four volumes of 6.3mM dithioerythritol (DTE) were added to one volume of selected sputum, to achieve a final concentration of 5mM DTE. Subsequently, sputum samples were incubated at room temperature for 30 minutes on a bench roller. Sputum samples were individually filtered through a 100 μ m nylon mesh cell strainer in order to remove any mucus and debris, and centrifuged at 4°C, 1500 rpm for 10 minutes. Subsequently, sputum supernatant was transferred to a new 15ml centrifuge tube and mixed well, samples were aliquotted and stored at -80°C pending analysis. For cytopins, an adequate sample of cell suspension was added into a cytospin tube to achieve a total number of 20-30,000 cells per slide, spun for five minutes, fixed and stained with Hemacolor staining to allow differentiation of cell types. Cytospin quality was regarded as adequate when squamous cell contamination was < 20%.

NASAL LAVAGE

The patients with AR randomly underwent a standardized nasal challenge with either a relevant allergen (house dust mite (HDM), cat or grass allergen; n=10) or its diluent (n=10) in a double-blind, parallel fashion (22). Selection of the intranasal allergen (HDM, grass pollen or cat dander) was based on the allergen causing the largest wheal in the skin prick test combined with the patient's history of clinical symptoms and the season. The nasal response to allergen was documented by symptom scores previously validated by Lebel (23). NAL collections were performed according to a modified protocol, sampling the same nostril at pre-challenge, and at 20 minutes post-challenge (22). NAL samples were collected, kept on ice and processed within 1 hour according to a validated protocol (22). Briefly, samples were centrifuged for 10 min at 400g; the supernatant was aliquotted and stored at -80°C pending analysis. Frozen samples were shipped to site of analysis.

REAGENTS

Recombinant SLPI (rSLPI) was obtained from R&D systems (Minneapolis, MN, USA). Rabbit anti-SLPI antibody was obtained from Alexis (San Diego,

CA, USA). Mouse anti-human mast-cell chymase was obtained from Serotec (Raleigh, NC, USA). The secondary antibodies goat anti-mouse and Goat anti-rabbit were obtained from Pierce Biotechnology (Rockford, IL, USA). Tryptase and α 2-macroglobulin were determined according to the manufacturers directions in the NAL samples with the following assay kits and detection limits: Tryptase by FEIA (Phadia, Uppsala, Sweden), 1 μ g/L; and α 2-macroglobulin by ELISA (R&D systems, Minneapolis MN, USA), 10 ng/mL.

SAMPLE PREPARATION FOR WESTERN ANALYSIS

During processing all samples were maintained at 4°C or less to insure stability of CSLPI and SLPI as determined previously (19). Ten μ l of human sputum supernatant or nasal lavage supernatant was diluted to a total volume of 20 μ l in loading buffer containing 50% Nupage LDS sample buffer from Invitrogen (Carlsbad, CA, USA), 30% water and 20% 1M DTT from Sigma-Aldrich (St. Louis, MO, USA) and was maintained on ice. The samples were boiled for 6 min at 95°C.

SLPI DETECTION BY WESTERN ANALYSIS

Denatured and reduced sputum supernatant and nasal lavage supernatant samples were applied to a 12% NuPage bis-tris gel from Invitrogen. The samples were electrophoresed and transferred to a PVDF membrane (Pierce Biotechnology). The blots were blocked in 5% milk /PBS-tween (PBS/0.4% tween-20) overnight at 4°C. Subsequently, the blots were washed for 10 minutes and incubated in the primary antibody (goat anti-SLPI at 1:1000) diluted in the blocking solution for 1 hour at room temperature. After three washes of PBS-tween the blot was incubated for 45 minutes at room temperature with the secondary antibody (goat anti-rabbit HRP) diluted 1:4000 in blocking solution. The blots were washed 6 times with PBS-tween and incubated for 1 minute at room temperature with Western Lightning chemiluminescence reagents (Perkin Elmer, Boston, MA, USA) and developed on Biomax Light Film (Eastman Kodak, Rochester, NY, USA) in the darkroom. Densitometry was performed on each film to determine the relative amount of intact SLPI (~15kD band) and CSLPI (~10kD). The data was expressed as a ratio of CSLPI/SLPI for normalization of each sample as done previously (19). The reliability of this assay was confirmed by using mass spectrometry analysis during its development (data not shown).

CHYMASE WESTERN ANALYSIS

Due to the lack of a commercially available chymase ELISA, chymase levels were determined by western analysis. In brief, denatured and reduced sputum supernatant and nasal lavage supernatant samples were applied to a 12%

NuPage bis-tris gel from Invitrogen. The samples were electrophoresed and transferred to a PVDF membrane (Pierce Biotechnology). The blots were blocked in 5% milk /PBS-tween (PBS/0.4% tween-20) overnight at 4°C. Subsequently, the blots were washed for 10 minutes and incubated in the primary antibody (mouse anti-human mast cell chymase at 1:1000) diluted in the blocking solution for 1 hour at room temperature. After three washes of PBS-tween the blot was incubated for 45 minutes at room temperature with the secondary antibody (goat anti-mouse HRP) diluted 1:4000 in blocking solution. The blots were washed 6 times with PBS-tween and incubated for 1 minute at room temperature with Western Lightning chemiluminescence reagents (Perkin Elmer) and developed on Biomax Light Film (Eastman Kodak) in the darkroom. Densitometry was performed on each film to determine the relative amount of chymase.

STATISTICAL ANALYSIS

Data was analyzed using a non-parametric Mann-Whitney and Student's t-test of endpoint data for the asthma trial. The chymase and α 2-macroglobulin data from the allergic rhinitis trial was log-transformed in order to stabilize variance. Welch two sample t-test was performed on the slopes of pre-post experimental values. Pearson correlation coefficient was determined to evaluate the relationship between CSLPI/SLPI ratios and other biomarkers.

Results

GENERAL

All sampling methods were well tolerated and no serious adverse events occurred. Analyzable sputum could be obtained from all but 1 asthmatic subject. Due to NAL-sample limitations, chymase levels could only be analyzed in 9 subjects challenged with placebo and 7 in the allergen arm.

ASTHMATIC SUBJECTS

In the sputum samples from asthmatic patients (A) CSLPI/SLPI ratios were determined. One subject from Group3 could not be analyzed due to low volume. As compared with healthy controls (HC) (group 1), CSLPI/SLPI ratios were increased in the sputum of asthmatic subjects (groups 2-4) (0.36 ± 0.02 (HC); 0.45 ± 0.02 (A); $p = 0.04$, student's t test; $p = 0.049$, non-parametric Mann Whitney test). On average sputum from untreated, atopic asthmatics (group 2) exhibited the highest CSLPI/SLPI ratio (0.50 ± 0.03), while treatment with corticosteroids (groups 3 and 4; $n = 5$) had a lower CSLPI/SLPI ratio

(0.41 ± 0.02 ; $p = 0.04$). (Figure 1) In addition, we measured chymase levels in the same sputum samples and found that the chymase levels followed a similar trend as the CSLPI/SLPI ratios (929.1 ± 301.0 relative units (HC); 1469.4 ± 141.5 relative units (A); $p < 0.17$) (Figure 2). Despite the low number of samples, a correlation between CSLPI/SLPI ratios and chymase levels in the normal and untreated atopic asthmatics was noted ($r=0.83$).

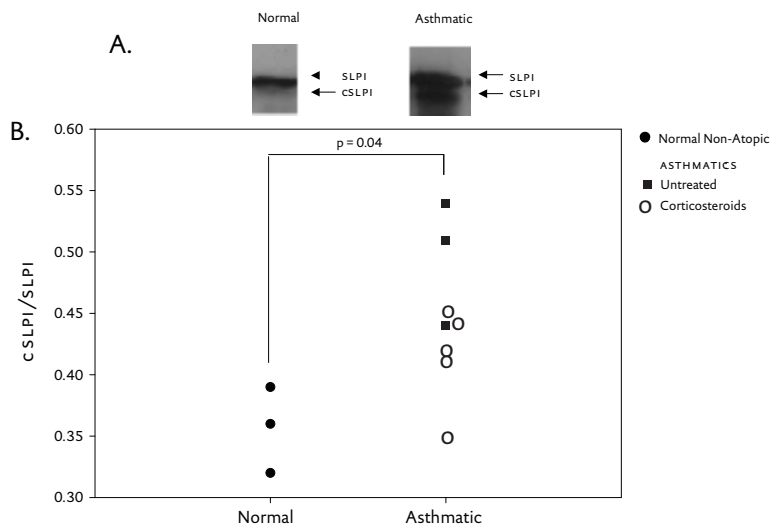


FIGURE 1 SPUTUM CSLPI/SLPI RATIOS. Example of western analysis of lowest and highest CSLPI expression (A.) as compared with healthy controls, sputum CSLPI/total SLP1 ratios were significantly increased in allergic asthmatics ($P=0.04$, student's t test; $p=0.049$, non-parametric mann whitney test). Corticosteroid use was associated with decreased CSLPI levels ($p=0.04$, student's t test) (B).

ALLERGIC RHINITIS

Nasal lavages were collected at baseline and 20 min post-challenge. As compared with sham challenge (P), intranasal allergen (A) produced an acute allergic response with a mean symptom score (mean $\pm$ SEM: 7.25 ± 0.56 (A); 0.70 ± 0.26 (P); $p = 0.001$) at 10 min post-challenge. In parallel, there was a significant increase in the CSLPI/SLPI ratio following allergen as compared with placebo challenge (Figure 3). Figure 3a shows the western analysis indicating

the CSLPI band in the nasal lavage from allergen challenged (highest level example shown) (left) and placebo-challenged (lowest level example shown) (right) individuals. Using densitometry, the CSLPI/total SLPI ratio was determined for each time point during both allergen (Figure 3b) and placebo (Figure 3c), respectively. As compared with placebo (0.22 ± 0.02), there was a significant increase in CSLPI/SLPI ratio in the nasal lavage of allergen challenged individuals (0.41 ± 0.05 ; $p=0.007$) at 20 min post-challenge. Similarly, there was a significant increase in the NAL-chymase levels in the allergen challenged individuals (1294.85 ± 688.50 relative units; Figure 4b) as compared with placebo-exposed individuals (706.01 ± 206.11 relative units; $p=0.038$; Figure 4c). Changes in CSLPI/SLPI levels and chymase levels from baseline to 20min post allergen challenge were highly correlated ($r = 0.91$) (Figure 5). If the highest point is removed the correlation is reduced to $r = 0.69$, which still shows a strong relationship between chymase levels and the CSLPI/SLPI ratio. Using regression analysis with all points the slope is significant ($p < 0.001$), with the highest point removed the trend is still significant ($p < 0.006$).

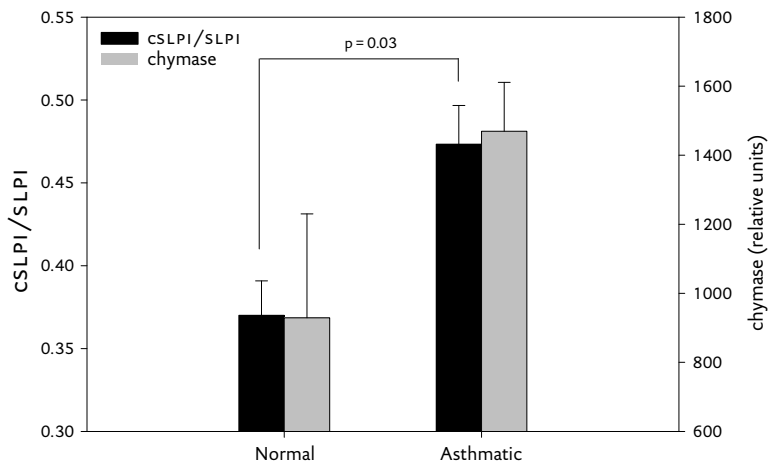


FIGURE 2 SPUTUM CHYMASE LEVELS AND CSLPI. Total slpi ratios, as compared with healthy controls, in patients with untreated allergic asthma sputum CSLPI/total SLPI ratios were significantly increased ($P=0.03$, student's t test). Chymase levels followed a similar trend with CSLPI/total SLPI ratios in both subject groups.

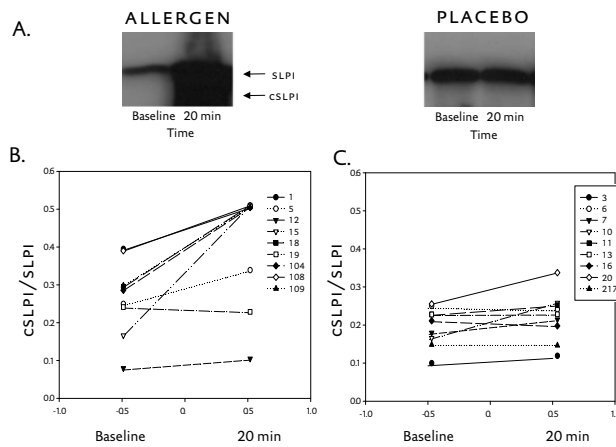


FIGURE 3 NAL CSLPI/SLPI RATIO FOLLOWING NASAL ALLERGEN COMPARED WITH PLACEBO. Example of western analysis of NAL from placebo (lowest level CSLPI) and allergen challenged AR-patients (highest level CSLPI) (A). densitometry analysis of CSLPI/total SLPI ratios in nasal lavage from allergen challenged (B) and placebo challenged human AR-patients (C). Welch T test, $P < 0.007$, allergen challenged as compared to placebo CSLPI/total SLPI ratio induction from baseline to 20 min post challenge. Legend refers to patient number.

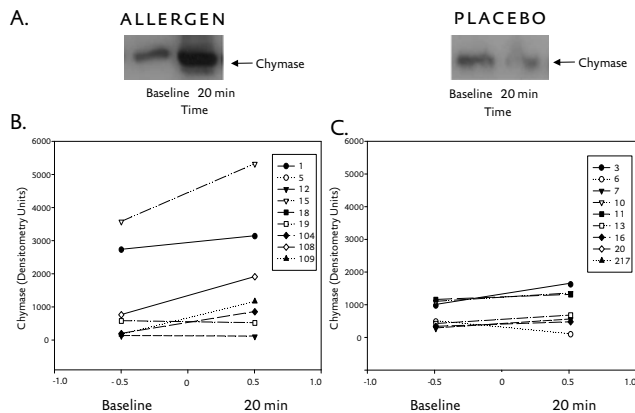


FIGURE 4 NAL CHYMASE LEVELS FOLLOWING NASAL ALLERGEN. Example of western analysis of nal from placebo (lowest level chymase) and allergen challenged AR-patients (highest level chymase) (A). Chymase levels (densitometry units) in nasal lavage from allergen challenged (B) and placebo challenged (C) human AR-patients. welch T test, $p < 0.038$, allergen challenged as compared to placebo from baseline to 20 min post challenge. Legend refers to patient number.



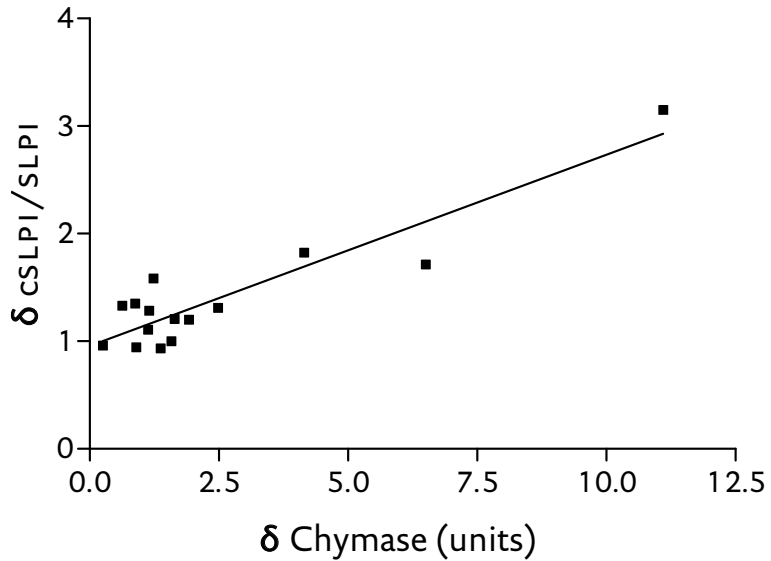


FIGURE 5 CORRELATION OF CSLPI/SLPI AND CHYMASE LEVELS IN ALLERGIC RHINITIS. Changes in NAL CSLPI/SLPI levels and chymase levels from baseline to 20 min post allergen challenge are highly correlated ($r = 0.91$).

In parallel to the increases in CSLPI and chymase, there was a significant raise in the NAL levels of $\alpha 2$ -macroglobulin (a protease inhibitor) (6630 ± 4240 ng/ml (A) vs. 50.75 ± 28.76 ng/ml (P); $p = 0.00035$) (figure 6a and b) at 20 min post-challenge. Similarly, tryptase (a mast cell protease) also increased in the allergen challenged group (4.3 ng/ml ± 2.01 ng/ml) as compared to placebo (undetectable levels; $p < 0.056$) (Figure 7a and b). A correlation analysis of CSLPI/SLPI ratios compared to each of the other biomarkers measured in this study shows a much stronger correlation between NAL CSLPI/SLPI ratios and chymase levels ($r=0.91$) and CSLPI/SLPI and $\alpha 2$ -macroglobulin levels ($r=0.41$) as compared to CSLPI/SLPI ratios and tryptase levels ($r=0.07$) (Figures 3-7).

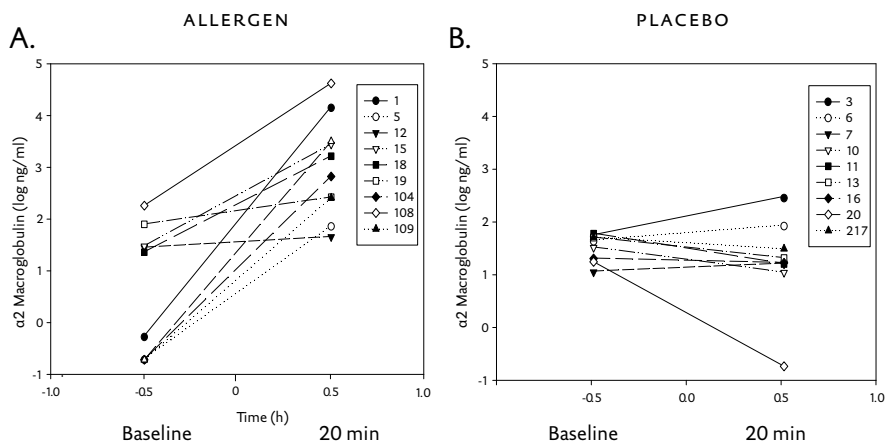


FIGURE 6 NAL $\alpha 2$ -MACROGLOBULIN LEVELS FOLLOWING NASAL ALLERGEN. $\alpha 2$ -macroglobulin levels are elevated in NAL following nasal allergen challenge AR-patients (A) versus placebo challenge (B). Welch T test, $P = 0.00035$, allergen challenged as compared to placebo from baseline to 20 min post challenge. Legend refers to patient number.

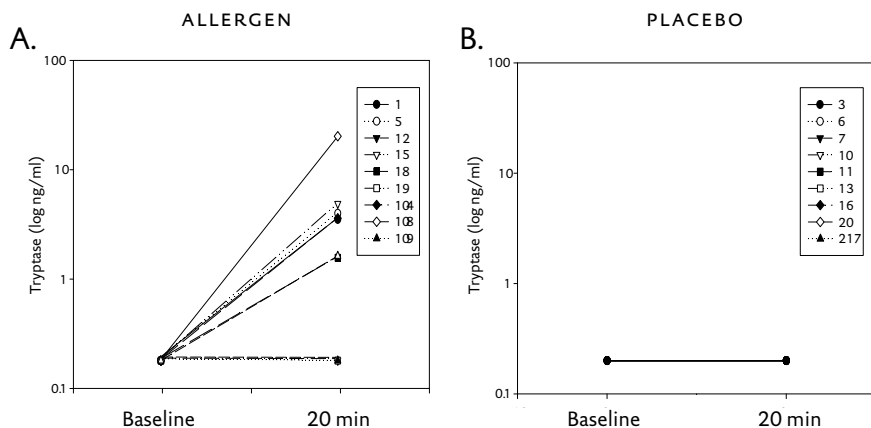


FIGURE 7 NAL TRYPTASE LEVELS FOLLOWING NASAL ALLERGEN. Tryptase levels are detectable only in NAL following allergen challenge (A) while none is detected following placebo treated AR-patients (B). students T test, $p = 0.056$, allergen challenged as compared to placebo from baseline to 20 min post challenge. Legend refers to patient number.

Discussion

The function of SLPI is to protect tissues against the harmful effects of environmental irritants through its anti-inflammatory, anti-protease and anti-microbial properties. Within the respiratory tract, SLPI is produced by a variety of cells including neutrophils, macrophages and epithelial cells (15). SLPI is cleaved by chymase at a specific site and the level of this cleavage product, CSLPI, is indicative of chymase activity (15). Here, we report increased baseline CSLPI levels in sputum and nasal lavage of subjects with untreated allergic asthma and allergic rhinitis (AR), respectively, with a further increase following nasal allergen challenge in the AR group. These findings are suggestive of an enhanced chymase activity in allergic airway disease *in vivo* and hence, extend our previous *in vitro* observations.

Based on *in vitro* data of our previous publication (19), CSLPI can be used as a measure of chymase activity in biological samples. We described, that out of a panel of mast cell proteases consisting of proteinase 3, elastase, tryptase, cathepsin G, chymotrypsin and chymase, only chymase is able to cleave SLPI (19). SLPI acts as a first line of inhibition but is also cleaved by the chymase. This allows us to see a record of chymase activity before α -2-macroglobulin and other inhibitors inhibit it irreversibly.

In the present study, we measured CSLPI levels in airway samples from patients with allergic airways disease. Increased CSLPI levels were demonstrated in sputum of patients with untreated allergic asthma, while use of corticosteroids was associated with reduced CSLPI levels. However, this finding is somewhat difficult to interpret since these individuals were also more severe asthmatics. Unfortunately, we are unable to obtain specimens from severe asthmatics who are not receiving either oral or inhaled corticosteroids. CSLPI was also increased in nasal lavage of subjects with allergic rhinitis at 20min following an intranasal allergen challenge, which elicited an early nasal response. In accordance with previous observations (19), CSLPI appeared closely correlated to chymase levels in both asthma ($r=0.83$) and AR ($r=0.91$). Taken together, these data suggest that CSLPI may act as a biomarker of chymase activity in allergic airways disease *in vivo*.

Apart from CSLPI and chymase, other markers of (mast-cell-related) airway inflammation were increased, including tryptase, a marker of mast cell activity, and α 2-macroglobulin, a key inhibitor of chymase in AR (24, 25). Following nasal allergen challenge, all these biomarkers were further increased. α 2-macroglobulin levels correlated well with the increased levels of CSLPI ($r=0.41$). This was expected, as α 2 macroglobulin is an inhibitor of chymase, and had a high correlation with chymase levels as well ($r=0.62$) (24). We observed an apparent 10-fold greater amount of tryptase as compared to

chymase and CSLPI in the nasal lavage following allergen challenge. We attribute this to the relatively greater amounts of tryptase stored in mast cells, up to 25% of cellular protein, as compared to chymase (26). Moreover, tryptase can originate from both MCTC and MCT cells, whereas chymase originates only from MCTC cells, and the relative degranulation of each of these mast cell types to the allergic response varies (27). In addition, some of the disparity in relative changes in chymase and tryptase can be related to the differences in methods of measurement – highly quantitative ELISA for tryptase and α -2 macroglobulin, while a qualitative Western was used for chymase and CSLPI. Consequently, it is not surprising to note that the rise in the tryptase levels although indicative of mast cell activity did not correlate with CSLPI/SLPI ratios (0.07) confirming that cleavage of SLPI is not based on presence of tryptase as observed in our previous study (19). Interestingly one third of the subjects in the present nasal challenge study did not experience an increase in NAL tryptase levels. It appears that tryptase is not necessarily elevated in all atopic individuals with airway disease. Bettiol *et al* showed that only 18% of asthmatics exhibited elevated tryptase levels (28).

Until now, no direct measure was available for chymase activity in biological fluids due to its short half-life, mainly caused by the high content of chymase inhibitors (25, 29). The advantages of the use of CSLPI as a biomarker of chymase activity are that SLPI cleavage is easily detectable in readily accessible human samples of saliva, sputum and nasal lavage. Additionally, known chymase inhibitors will inhibit the level of SLPI cleavage (19). Furthermore, disease states such as AR and asthma result in higher amounts of cleaved SLPI. Taken together, the data show that detection of these unique SLPI cleavage products can act as novel biomarkers of chymase activity and chymase-mediated disease.

However we do acknowledge several shortcomings of this particular study that begins with the sample size. Despite obvious relationships between the inflammatory markers, low patient numbers make it difficult to achieve adequate correlation values. Furthermore, in this population some asthma patients were using (inhaled) corticosteroids, whereas others were untreated. Even though significantly lower levels were detected in those on corticosteroids, the effect of these drugs on CSLPI levels needs to be prospectively evaluated in a well-powered, placebo-controlled studies.

In conclusion, our data show that CSLPI may be an *in vivo* biomarker of chymase activity in allergic airway disease. Our findings need confirmation from larger prospective trials in patients with well-defined disease characteristics and various treatments.

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