RESEARCH ARTICLE

Biomarker discovery in asthma-related inflammation and remodeling

Florence Quesada Calvo[1], Marianne Fillet[2]*, Dominique de Seny[3], Marie-Alice Meuwis[2], Raphael Maree[4], Céline Crahay[1], Geneviève Paulissen[1], Natacha Rocks[1], Maud Gueders[1], Louis Wehenkel[5], Marie-Paule Merville[2], Renaud Louis[6], Jean-Michel Foidart[1], Agnes Noël[1] and Didier Cataldo[1]*

[1] Laboratory of Biology of Tumours and Development, University of Liège and Centre Hospitalier Universitaire (CHU-Liège), Belgium
[2] Laboratory of Medical Chemistry, University of Liège and Centre Hospitalier Universitaire (CHU-Liège), Belgium
[3] Laboratory of Rheumatology, University of Liège and Centre Hospitalier Universitaire (CHU-Liège), Belgium
[4] GIGA-Management, Plateforme bioinformatique, University of Liège and Centre Hospitalier Universitaire (CHU-Liège), Belgium
[5] Department of Electrical Engineering & Computer Sciences, University of Liège and Centre Hospitalier Universitaire (CHU-Liège), Belgium
[6] Laboratory of Pneumology, GIGA-research (GIGA-I[3] and GIGA-cancer), University of Liège and Centre Hospitalier Universitaire (CHU-Liège), Belgium

Asthma is a complex inflammatory disease of airways. A network of reciprocal interactions between inflammatory cells, peptidic mediators, extracellular matrix components, and proteases is thought to be involved in the installation and maintenance of asthma-related airway inflammation and remodeling. To date, new proteic mediators displaying significant activity in the pathophysiology of asthma are still to be unveiled. The main objective of this study was to uncover potential target proteins by using surface-enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS) on lung samples from mouse models of allergen-induced airway inflammation and remodeling. In this model, we pointed out several protein or peptide peaks that were preferentially expressed in diseased mice as compared to controls. We report the identification of different five proteins: found inflammatory zone 1 or RELMα (FIZZ-1), calcyclin (S100A6), clara cell secretory protein 10 (CC10), Ubiquitin, and Histone H4.

Received: August 4, 2008
Revised: November 24, 2008
Accepted: November 26, 2008

Keywords:
Asthma / Lung tissue profiling / Mouse model / Proteomics / SELDI-TOF-MS

Correspondence: Dr. Marianne Fillet, University of Liège, GIGA (B34), 4000 Liège, Belgium
E-mail: marianne.fillet@ulg.ac.be
Fax: +3243664347

Abbreviations: CC10, clara cell secretory protein 10 or CCSP or clara cell secretory protein or uteroglobin; **CM10,** weak cation-exchanger; **FIZZ-1,** found inflammatory zone 1 or RELMα; **IM,** acute inflammation experiments; **RM,** chronic remodeling allergen exposure protocol; **S100A6**, Calcyclin

1 Introduction

Asthma is mainly characterized by reversible airway obstruction, airway hyperresponsiveness, and chronic airway inflammation [1]. As asthma becomes persistent, permanent changes of airway walls, referred to as "bronchial remodeling" caused by long-standing asthma-related-inflammation,

* These authors contributed equally to this study.

progressively develop [2–4]. These structural changes are thought to be responsible for asthma-related bronchial hyperresponsiveness [1] and to play key roles in the progressive decline of lung function reported in chronic asthma [5]. Albeit airway remodeling is a clinically relevant problem, mechanisms leading to this disease component through the activation of biological networks including airway epithelial cells, inflammatory cells, cytokines, other peptidic mediators, and proteases have not yet been elucidated. During the last decade, murine models of allergen-induced inflammation and hyperresponsiveness have been used to reliably explore disease mechanisms [2, 3]. Proteome studied under normal and diseased conditions provides a realistic view of different proteins taking part to biological processes and might link genome sequences with cellular behaviors.

In order to investigate which peptidic mediators are susceptible to take part to asthma progression, we used SELDI-TOF-MS technology. This ProteinChip platform was used to establish expression profiles for low molecular weight proteins and to highlight new potential biomarkers. Different ProteinChips have been designed to selectively retain a category of proteins from biological samples according to their biochemical properties and MS is applied on these samples leading to characteristic peak patterns [6–11].

The main objective of the current study was to define protein profiles in mouse models of allergen-induced airway inflammation and remodeling by using SELDI-TOF-MS in order to identify which peptides are the most relevant to discern inflamed or remodeled bronchi from normal lung tissue.

2 Material and methods

2.1 Mice

Specific pathogen-free Balb/c mice (6 wk old, male) were used. Two different protocols consisting in acute (short term) or chronic (long term) exposure to allergen were applied. Both protocols were approved by the Veterinary Ethical Committee of the University of Liège. In acute inflammation experiments (IM), mice were sensitized by intraperitoneal injection of 10 μg ovalbumin grade V (OVA) (Sigma, Germany) adsorbed on $Al(OH)_3$ (AlumInject©, Perbio, Belgium) on days 1 and 7. Animals were either challenged by 1% OVA – phosphate saline buffer (PBS) aerosols of suspended in or with PBS alone for 30 min on days 23 to 27 as previously described in Gueders *et al* [4]. Each experiment was performed with placebo mice ($n = 20$) and allergen-exposed mice ($n = 20$).

In the chronic remodeling allergen exposure protocol (RM), allergen sensitization, and exposure were followed by additional allergen or PBS exposures performed 5 days *per* even week, until day 90. Each experiment was performed with placebo mice ($n = 20$) and allergen-exposed mice ($n = 20$). For further details on the characterization of allergen-induced inflammation and airway responsiveness see Supporting Information.

2.2 SELDI-TOF-MS analysis

Total proteins were extracted from lungs by incubating crushed tissue with lysis buffer containing 7 M Urea, 2 M Thiourea, 2% CHAPS, and 50 mM Tris (pH 9). Each protein sample was diluted in acetate buffer pH 4 (100 mM) at a final protein concentration of 0.5 μg/μl. Weak cation-exchanger (CM10) ProteinChip® Arrays (BioRad, USA) were activated with 10 mM HCl for 5 min. CM10 arrays were then equilibrated with binding buffer (100 mM sodium acetate, 30 mM sodium chloride, pH 4) for 5 min. Five microliters of each sample were loaded *per* spot of CM10 arrays and incubated in a humidity chamber for 1 h. Spots were then washed twice with binding buffer for 5 min, in order to eliminate non-specifically retained proteins. Two additional washes with acetate buffer pH 4 for 5 min were followed by two quick rinses with deionized water. All surfaces were air-dried during 20 min. Then, 1 μL of satured solution of matrix (sinapinic acid (SPA) in 50% ACN, TFA 0.5% in water) was applied on each spot. During SELDI-TOF-MS experiments, proteins or peptides retained on the spot surfaces were ionized, desorbed, and detected by a Protein Biological System II ProteinChip reader (BioRad).

Protein chips were all analyzed with the same settings: laser intensity at 195, detection sensitivity at 8, and 180 shots *per* sample. Peaks were analyzed between m/z ranging from 2000 to 30 000 Da. Spectra were calibrated externally using All-in-one Peptide standard (BioRad) complemented by myoglobin (MW 16 951.5 Da) and cytochrome *c* (MW 12 360 Da) as described in ref. [6, 11]. Baseline substraction, normalization on TIC, and peak detection were performed as described previously [6, 11].

2.3 Protein purification for biomarkers identification

Spin columns CM ceramic HyperD F sorbent (BioRad) were centrifuged at 1000 rpm for 30 s. After washing steps in appropriate binding buffers, columns were loaded with 500 μg of total protein extract and were incubated for 40 min at room temperature. Bound proteins were eluted with a step gradient of sodium sulfate (100–500 mM) in binding buffer. Each eluate was split in two aliquots: one analyzed on CM10 array with SELDI-TOF-MS and the other one was used for protein identification (SDS-PAGE 1-D separation followed by identification by MS/MS). For further details on in-gel digestions and nano-LC-MS/MS see Supporting Information.

2.4 Immunoprecipitation

Proteins (300 μg) from lung were incubated with 20 μg of a found inflammatory zone 1 or RELMα (FIZZ-1) antibody (Chemicon, USA) for 1 h at 4°C. Then, proteins coupled to an antibody were added to 40 μL of Protein G PLUS-Agarose (Santa Cruz Biotechnology, USA) for 2 h at 4°C. After washes with PBS + 0.5% Triton, pellet and proteins were resus-

pended in acetic acid 100 mM containing 20% ACN. Proteins were completely air dried and resuspended in binding buffer (100 mM sodium acetate, 30 mM sodium chloride, pH 4).

2.5 Western blotting

Western blots were performed with lung tissue proteins that were also used for CM10 SELDI-MS profiling. Lung protein sample (20 μg) and molecular weight markers (Invitrogen, 'Multimark' 3–185 kDa, USA) were separated under reducing conditions using NuPage 12% Bis-Tris Gels (Invitrogen), transferred, and incubated with primary antibodies: rabbit anti-RELMα (FIZZ-1) (Chemicon) (1/1000), rabbit anti-clara cell secretory protein 10 (CC10) (Santa Cruz Biotechnology) (1/2000), rabbit anti-Calcyclin (S100A6) (Santa Cruz Biotechnology) (1/500), rabbit anti-Histone H4 (Upstate, Bioconnect, Netherlands), and rabbit anti-Ubiquitin (Santa Cruz Biotechnology) (1/200). Incubation with a secondary swine anti-rabbit (Dako, Denmark) antibody conjugated with HRP was followed by detection with the ECL kit (Perkin Elmer, USA) as recommended by the manufacturer.

The intensity of each band was measured with the Quantity One software (BioRad). Protein levels were normalized using β-actin signal used as an internal standard.

2.6 Immunohistochemistry

Endogenous peroxidase activity was blocked by incubation of tissue sections in 3% hydrogen peroxide (H_2O_2). For alpha smooth muscle actin (αSMA) staining, slides were permeabilized in 1% Triton X-100/PBS and nonspecific binding was blocked in normal goat serum (FIZZ-1, CC10 and S100A6) or in 10% BSA (αSMA and Ubiquitin). Tissue sections were then incubated with rabbit anti-RELMα (FIZZ-1) (1/150) (Chemicon), rabbit anti-CCSP (CC10) (1/2000) (Seven Hill bioreagent), rabbit anti-Calcyclin (S100A6) (1/100) (Santa Cruz Biotechnology), rabbit anti-Ubiquitin (Santa Cruz Biotechnology) (1/400), or mouse anti-α SMA (1/200) (Dako). Secondary antibodies were an anti-FITC-POD (Roche, USA) (αSMA) or biotin-coupled secondary antibody (Dako) (FIZZ-1, CC10, S100A6, and Ubiquitin). Peroxidase activity was revealed using 3-amino, 9-ethyl-carbazole (AEC) (Dako) staining (αSMA) or a 3-3'diaminobenzidine hydrochloride kit (DAB) (Dako) after incubation with a streptavidin/HRP complex (Dako).

2.7 Statistical analyses

2.7.1 SELDI-TOF-MS

Before statistical analysis, preprocessing of spectra involved calibration, baseline substraction, and normalization of all spectra [6]. Peak detection was performed using ProteinChip Biomarker Wizard software 3.0 (BioRad). For peak detection, S/N used was 5; for cluster completion, cluster mass selected was 0.3%, and the S/N for the second pass was set at 3.

2.7.2 Univariate analysis

This statistical approach determines if the intensity of a peak is significantly different in the experimental group spectrum as compared to controls. *p*-values associated with every peak were calculated using nonparametric Mann–Whitney *U*-test (labeled when significant: $p<0.05$).

2.7.3 Multivariate analysis

This approach was previously described by Geurts *et al* [12]. Machine learning method (Extra-Trees Decision Tree) was used to build classification models and rank biomarkers, to the information that they bring into the classification model [12]. Biomarkers were thus ranked according to their importance (%) provided by the machine learning method. Leave-one-out cross validation was used to provide the misclassification error rate, as well as, calculation of sensitivity and specificity. Sensitivity is defined as a percentage of samples from the target class that are correctly classified by the model (true positives), while specificity represents the percentage of samples from the other group that are well classified (true negatives). Error rate is the percentage of samples that are misclassified by the model.

2.7.4 Western blotting – immunohistochemistry – ELISA

Comparison between groups ($n = 20$/group) was performed by Mann–Whitney test. Results are expressed as mean ± SEM. *p*-values <0.05 were considered as statistically significant.

3 Results and discussion

Development of asthma implies complex networks involving different structural and inflammatory cell types and mediators of both peptidic (cytokines, growth factors. . .) and lipidic (eicosanoïds, leukotriens. . .) nature. In the present study, the use of an inbred mice disease model allowed substantial decrease of inter-individual variations and thorough disease characterization [13–15]; while studies using human samples are quite limited, being restricted to accessible fluids such as bronchoalveolar lavages or plasma [10, 16, 17]. Nevertheless, even in studies using inbred mice, an intrinsic variability is expected regarding the development of main asthma characteristics since genetic background is not the only factor that explains the development of this disease. Indeed, in human monozygotic twin pairs, the concordance rate for the development of asthma is only 19% [18]. For these reasons, we were prone to use groups of 20 mice in the present study in order to overcome the intrinsic variability of

this complex biological process. Proteins such as cytokines, chemokines, growth factors, and proteolytic fragments of larger proteins display low molecular weights. As these low molecular weight mediators are not easily accessible by SDS-PAGE studies, we chose to use the SELDI-TOF-MS technology. This technique is particularly suited to discern intensity changes in peaks corresponding to peptides and small proteins (<30 kDa). We therefore hypothesized that SELDI-TOF-MS could be a suitable tool for the identification of novel biomarkers in asthma. SELDI also offers the advantages of being compatible with high throughput screening, while requiring small sample volumes. We first characterized allergen-induced inflammation and airway responsiveness of both mice models (Supporting Information Fig. S1). Then, reproducibility of our proteomic studies was one of our main concerns. Multiple measurements (three replicates) were performed to assess the reproducibility of SELDI-TOF experiments. We used very standardized and controlled process for samples collection, storage, and preparation: storage at −80°C, without any defrost before proteomics analysis, standardization of procedures for sample, and quality control processing and ProteinChip assay preparation.

3.1 Lung tissue protein profiling

In order to determine which proteins (MW <30 kDa) were differentially expressed in both mice models, CM10 ProteinChip arrays were used as described in Materials and Methods section. After optimization of experimental conditions, a quality control lung sample was loaded on eight spots of this array and repeatability was assessed and found to be satisfactory: variation coefficients of quality control peak intensity were calculated on 10 peaks randomly taken within 2000–30 000 Da mass range (5.5–14.2%; $n = 8$). Throughout the study, variation coefficients of peak intensity were also measured to evaluate reproducibility of experiments (3.1–17.8%; 3 days). For this study, each sample was loaded in triplicate and 240 spectra were collected on CM10 arrays. One hundred and thirteen peaks ranging from *m/z* 2000 to 30 000 Da were detected per spectrum with the Biomarker Wizard software. *m/z* ranging from 0 to 2000 Da was excluded from analysis to eliminate saturating background noise from the matrix. Representative examples of spectra are shown in Fig. 1.

Spectra corresponding to allergen challenged mice were compared to those obtained from control mice. A multiple decision tree analysis (Extra-Trees) was used to assess the sensitivity and the specificity of classification model built with peaks profiles detected in protein extracts prepared with acute inflammation and chronic remodeling mice models. This statistical approach followed by a leave-one-out procedure, lead to very high sensitivity and specificity. We obtained a discriminatory model with a sensitivity of 95% and 80%, a specificity of 95% and 74% and an accuracy of 5% and 23% for acute model and remodeling model, respectively. Biomarkers for each model were ranked according to their discriminatory power or importance, expressed in percents (Table 1). For these potential biomarkers, *p*-values obtained by univariate analysis were also provided (Table 1).

In order to identify proteins corresponding to discriminatory peak obtained by SELDI-TOF-MS analysis, SDS-PAGE was used before tryptic digestion and fragmentation by LC-MS/MS of the peptide digests. A concentration step using CM10 columns was required to identify some of the proteins from chronic remodeling model. Indeed, CM ceramic HyperD F sorbent mimics binding characteristics of the ProteinChip CM10 array and thus isolates our potential biomarkers.

Searches on databases performed with MASCOT 56.0 allowed the identification of five different proteins. Peaks at *m/z* 9429, 6445, 4469, 4066, and 8134 were identified by MS/MS as FIZZ-1 (9429 Da), S100A6 (6445 Da), CC10 (4469 Da), Histone H4 (4066 Da), and Ubiquitin (8134 Da), respectively (Supporting Information Table S1). These peaks or biomarkers showed high discriminatory power in multi-

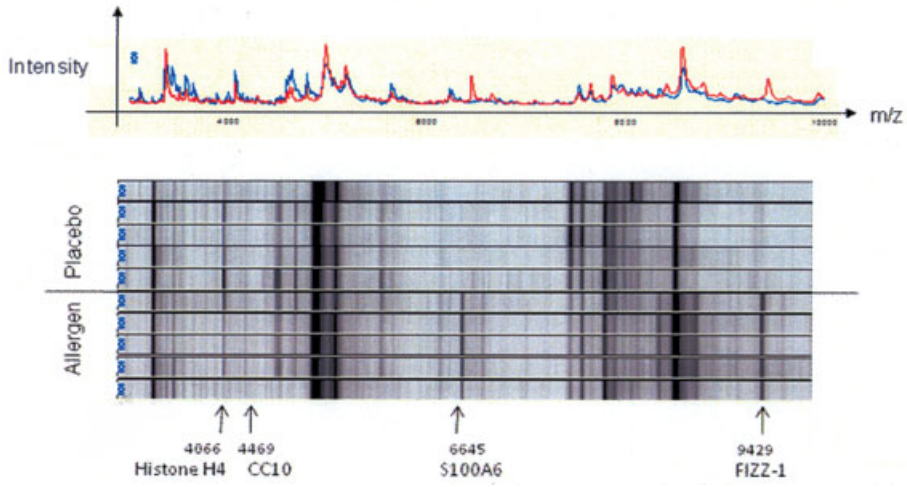


Figure 1. SELDI-TOF-MS profiles of mouse lung proteins. Lung protein extract of 2.5 μg from placebo and allergen-exposed mice (IM and RM) were profiled by SELDI-TOF-MS. Representative placebo and allergen-exposed mice from inflammation model in gel view and profiles ranging from *m/z* 2000 to 10 000 are shown.

Table 1. Potential biomarkers ranked according to their importance calculated for the IM and RMallergen-exposed mice *versus* placebo comparison

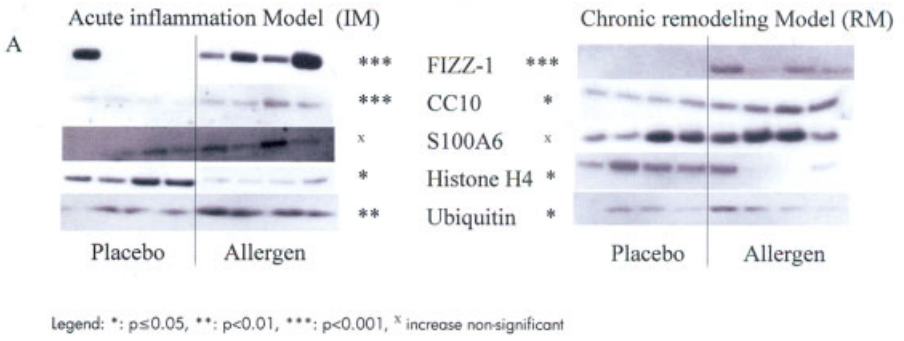
Inflammation model			Remodeling model		
m/z	% of importance	*p*-values	*m/z*	% of importance	*p*-values
9429	15.8	$<10^{-12}$	6490	5.7	2×10^{-9}
6445	11.5	$<10^{-12}$	6223	4.6	4×10^{-9}
6644	7.1	$<10^{-12}$	3749	3.9	9×10^{-7}
7081	6	$<10^{-12}$	**8134**	3.6	2×10^{-8}
6226	5.2	1.10^{-7}	3996	3	1×10^{-6}
8579	5.1	$<10^{-12}$	2947	3	1×10^{-8}
4469	3.8	$<10^{-12}$	3096	3	4.5×10^{-3}
4066	2.8	2.10^{-8}	4205	2.8	6×10^{-7}
2762	2.5	$<10^{-12}$	4450	2.8	2×10^{-6}
8407	2.3	$<10^{-12}$	3479	2.6	2×10^{-8}
4782	2.2	3.10^{-9}	8194	2.6	3×10^{-7}

Percentage of importance is the relative contribution of the *m/z* value or peaks obtained by Extra-Trees decision tree (multivariate analysis). *p*-values associated with every potential biomarker are determined by the Mann–Whitney *U*-test (univariate analysis). The *m/z* labeled in bold was identified in this work.

variate and univariate statistical analysis (Table 1). FIZZ-1, S100A6, CC10, and Ubiquitine displayed higher peak intensities on SELDI spectra in allergen-exposed *versus* control animals, while the inverse was observed for Histone H4. Ubiquitin showed a 1.1% of importance and a *p*-value of 3.6×10^{-4} in acute inflammation model. In chronic remodeling model, *p*-values for S100A6, CC10, Histone H4 were also significant (3.48×10^{-6}, 2.14×10^{-6}, 0.05, respectively; data not shown). As expressed in Table 1, we found a relatively low number of peaks undergoing an overlapping modulation in our two models (acute inflammatory model and chronic remodeling model). This is mainly due to the fact that only ten most significant peaks after multivariate analysis have been presented for each model in this table. However, when we consider greater numbers of potential biomarkers (with lower statistical scores in the multivariate analysis), many proteins appear to be detected in both models (data not shown). Another reason for this apparent discrepancy might be the intrinsic differences that exist between the two models displaying contrasted pictures regarding inflammation and airway remodeling rendering the finding of different mediators quite expected.

3.2 Validation of identified biomarkers by Western blot and immunohistochemistry

In order to confirm the relevance of proteins identified by SELDI-TOF-MS, Western blot analyses were performed on each protein sample used for SELDI studies. In our acute inflammation model, the peak displaying the highest importance in our discriminating multivariate model corresponds to FIZZ-1. Western blotting signal corresponding to FIZZ-1 protein was upregulated in allergen-exposed mice (Fig. 2A) and was found significantly higher in acute inflamed lung as compared to the model of chronic remodeling (Fig. 2A). Immunohistochemistry performed on lung tissue also showed increased binding of anti-FIZZ-1 antibody (Fig. 2B panels A–D). In the remodeling model (panels C–D), FIZZ-1 staining was restricted to the airway epithelium while mesenchymal and inflammatory cells were also positive in the acute model (panels A–B). In order to confirm that the peak at *m/z* 9429 actually corresponds to FIZZ-1 protein, we also performed a specific immunoprecipitation with an anti-FIZZ-1 antibody and further analyzed immunoprecipitates on CM10 ProteinChip array (Fig. 3). After immunoprecipitation, peak at *m/z* 9429 was observed in SELDI-TOF-MS profile while not in the negative control. PCR analyses were also carried out to assess FIZZ-1 mRNA expression and showed a significant increase ($p<0.01$) after acute allergen exposure as compared to controls (0.14 ± 0.04 and 0.5 ± 0.08, respectively, data not shown). It is the first time that FIZZ-1 is identified in tissue by SELDI-TOF-MS. FIZZ-1 is a secreted cytokine that was previously reported to be increased in bronchoalveolar lavage from infectious-exposed mice [19]. The production of FIZZ-1 was reported to be under the dependence of Th2 cytokines: IL-4 and IL-13, being themselves mainstay of asthma-related inflammatory cascade [20]. Moreover, the Th2 cytokine IL-21 stimulates FIZZ-1 production by alveolar macrophages under the dependence of IL-4 and IL-13 [21]. Therefore, our data indicate that FIZZ-1 could indeed be related to the Th2 cytokine network. More than being a biomarker, FIZZ-1 might also be an effector in asthma, since this protein has been reported to induce fibroblast differentiation into myofibroblasts, producing type I collagen and SMA and playing a role in airway remodeling in asthma [22]. Based on these data, we propose Fizz-1 as a biomarker of asthma.



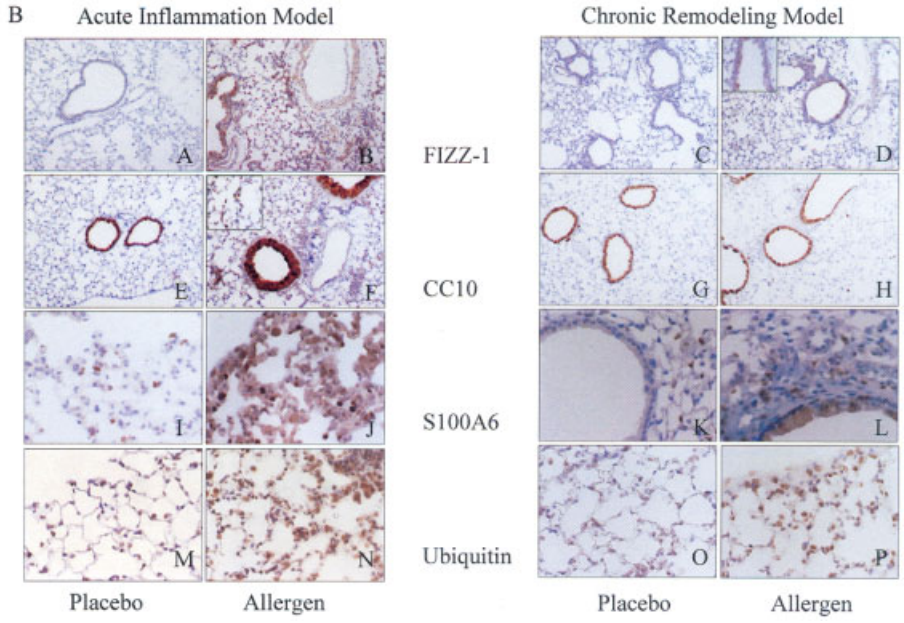


Figure 2. (A) Confirmation of biomarkers by Western blotting: Four representative samples in each group are shown. By contrast, statistical analyses were performed on all samples. Significant increase of FIZZ-1, CC10, and Ubiquitin protein signal in mice exposed to allergen confirmed SELDI-TOF-MS results. An enhanced S100A6 protein expression was observed after specific Western blotting but without reaching significance. Histone H4 protein expression is significantly decreased in allergen-exposed mice compare to the placebo-exposed mice, like obtained by SELDI-TOF-MS. All Western blotting signals were normalized against relevant β actin intensity (data not shown). (B) Histological analysis of lung: Panels A–D show Fizz-1 labeled lung sections (10 fields) from placebo-challenged mice (A and C) and from allergen-challenged mice (B and D). In bronchial epithelium, alveolar macrophages and type II pneumocytes are present in inflamed lung but not in the control lung, FIZZ-1 proteins were strongly positive. The protein expression was less pronounced in long-term allergen-exposed mice. An increased of CC10 signal can be found in lung tissue in short-term allergen-exposed mice (F). The allergen exposure leads to enhancement of S100A6 production (J and L). Quantification data of FIZZ-1, S100A6, and Ubiquitin showed significant differences ($p<0.001$) in both models, and CC10 signal was significantly different only in short term exposure. Ubiquitin staining was observed in IM and RM (N and P) in inflamed lung tissue, but to a lesser extent in placebo lung.

In this work, we also described that intensity of peak corresponding to CC10 is significantly increased in animals exposed to allergens. CC10 is produced mainly but not exclusively by Clara cells and can be detected in organs communicating with the external environment (*i.e.*, urogenital tract) [23]. The exact biological functions of CC10 are still unknown. By SELDI-TOF-MS, we detected CC10 as 5 kDa fragments in lung tissue. In line with our study, other authors have reported a 5 kDa isoform in nasal lavages from patients exposed to chemicals in professional environment [24]. Western blot analysis for CC10 revealed increased signal intensities in samples from allergen-exposed mice in both models ($p<0.001$ for IM and $p<0.05$ for RM, Fig. 2A), CC10 was detected by immunohistochemistry mostly in bronchial epithelium (Fig. 2B, panels E–H).

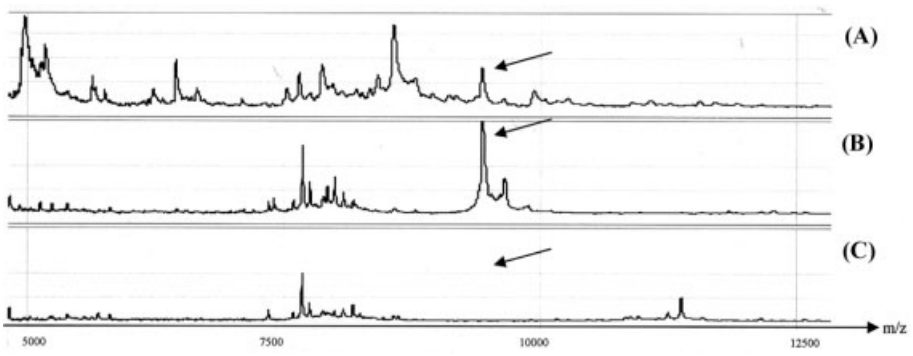


Figure 3. Confirmation of FIZZ-1 identification on CM10 array by specific immunodepletion. (A) Crude allergen exposed-mice lung extract; (B) FIZZ-1 immunoprecipited with a FIZZ-1 antibody and eluted, using the same allergen exposed-mice lung protein extract; (C) negative control immunoprecipited and eluted fraction with a non-relevant antibody.

Interestingly, the peak corresponding to *m/z* 6445 was identified as S100A6. This peak has been already detected but not identified in another SELDI-TOF-MS study on plasma samples from pregnant women with asthma [16]. The fact that such similarities can be found between a human study and our work on mouse model further supports the impact of our findings for potential human asthma applications. S100A6 is a member of the S100 family of Ca^{2+}-binding proteins and is associated with cell proliferation. The contribution of S100A6 in the control of cell proliferation is potentially important in asthma-related airway remodeling process. Indeed, Breen *et al* have demonstrated that S100A6 is a potent inducer of fibroblasts proliferation [25]. Regarding S100A6, Western blot did not reveal significant enhancement of protein levels in allergen-exposed mice (Fig. 2A). However, quantitative studies performed by immunohistochemistry confirmed that S100A6 protein was increased in allergen-exposed mice ($p<0.0001$) (Fig. 2B, panels I – L).

In our study, Histone H4 protein levels were also unexpectedly found to be decreased in samples from allergen-exposed animals. This difference was found by using CM10 protein chips but was also confirmed on IMAC chips study (data not shown). Further experiments should be conducted to assess potential Histone H4 metabolism during asthma. Indeed, Histone H4 hyperacetylation might lead to production of proinflammatory mediators [26, 27].

As found in our SELDI-TOF-MS profile studies, Histone H4 Western blot signal was also significantly lower in allergen-exposed mice than in placebo mice (Fig. 2A).

Finally, Ubiquitin was identified from the chronic remodeling mouse model by SELDI-TOF-MS as a relevant biomarker. Ubiquitin was also found by SELDI-TOF-MS as a discriminant peak in acute inflammation model (percentage of importance 1.1% and *p*-value 3.6×10^{-4}). Ubiquitin was detected as a monomer in our samples. This finding could suggest that the proteasome pathway is undergoing activation in the context of asthma-related inflammation participating in the inflammatory burden. This hypothesis is supported by the report of anti-inflammatory effects of the proteasome inhibitor P-51 [28]. Ubiquitin should thus be further explored to assess its exact role in asthma-related inflammation. Western blot analysis showed increased protein expression in allergen-exposed mice in both acute and chronic models but protein levels were from far higher after allergen exposure in the acute inflammation model. By immunohistochemistry, ubiquitin was detected in nuclei from alveolar epithelial and parenchymal inflammatory cells (Fig. 2B, panels M–P).

4 Concluding remarks

Taken together, our results show that new key biomarkers participating in the establishment of the asthmatic phenotype can be unveiled by proteomic studies aiming at comparing peptides issued from healthy and disease tissues. Our results suggest that SELDI-TOF-MS is a useful tool to identify new potential therapeutic targets in inflammatory diseases such as asthma.

This work was supported by grants of the Fonds National de la Recherche Scientifique (FNRS, Brussels, Belgium), the Walloon Region Government (DGTRE project #114/702), the Fondation Leon Fredericq (University of Liege), the CHU (Liege, Belgium), Action de Recherches Concertées, IAP6/35 network (funded by the Interuniversity Attraction Poles Programme, initiated by the Belgian State, Science Policy Office), the Centre Anticancéreux près de l'Université de Liège, the Interuniversity Attraction Poles Programme- Belgian Science Policy (Brussels, Belgium). M. F. and D. C. are research associates and M. P. M. Senior Research Associate of the FNRS (Belgium).

The authors have declared no conflict of interest.

5 References

[1] Louis, R., Lau, L. C., Bron, A. O., Roldaan, A. C. *et al.*, The relationship between airways inflammation and asthma severity. *Am. J. Respir. Crit. Care. Med.* 2000, *161*, 9–16.

[2] Bousquet, J., Chanez, P., Lacoste, J. Y., White, R. *et al.*, Asthma: a disease remodeling the airways. *Allergy* 1992, *47*, 3–11.

[3] Cataldo, D. D., Gueders, M. M., Rocks, N., Sounni, N. E. *et al.*, Pathogenic role of matrix metalloproteases and their inhibitors in asthma and chronic obstructive pulmonary disease and therapeutic relevance of matrix metalloproteases inhibitors. *Cell. Mol. Biol. (Noisy-le-grand)* 2003, *49*, 875–884.

[4] Gueders, M. M., Bertholet, P., Perin, F., Rocks, N. *et al.*, A novel formulation of inhaled doxycycline reduces allergen-induced inflammation, hyperresponsiveness and remodeling by matrix metalloproteinases and cytokines modulation in a mouse model of asthma. *Biochem. Pharmacol.* 2008, *75*, 514–526.

[5] Lange, P., Parner, J., Vestbo, J., Schnohr, P., Jensen, G., A 15-year follow-up study of ventilatory function in adults with asthma. *N. Engl. J. Med.* 1998, *339*, 1194–1200.

[6] de Seny, D., Fillet, M., Meuwis, M. A., Geurts, P. *et al.*, Discovery of new rheumatoid arthritis biomarkers using the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry ProteinChip approach. *Arthritis Rheum.* 2005, *52*, 3801–3812.

[7] de Seny, D., Fillet, M., Ribbens, C., Maree, R. *et al.*, Monomeric Calgranulins Measured by SELDI-TOF Mass Spectrometry and Calprotectin Measured by ELISA as Biomarkers in Arthritis. *Clin. Chem.* 2008, *54*, 1066–1075.

[8] Kriegova, E., Melle, C., Kolek, V., Hutyrova, B. *et al.*, Protein profiles of bronchoalveolar lavage fluid from patients with pulmonary sarcoidosis. *Am. J. Respir. Crit. Care. Med.* 2006, *173*, 1145–1154.

[9] Kwapiszewska, G., Meyer, M., Bogumil, R., Bohle, R. M. *et al.*, Identification of proteins in laser-microdissected small cell numbers by SELDI-TOF and Tandem MS. *BMC Biotechnol.* 2004, *4*, 30.

[10] Merkel, D., Rist, W., Seither, P., Weith, A., Lenter, M. C., Proteomic study of human bronchoalveolar lavage fluids from smokers with chronic obstructive pulmonary disease by combining surface-enhanced laser desorption/ionization-mass spectrometry profiling with mass spectrometric protein identification. *Proteomics* 2005, *5*, 2972–2980.

[11] Meuwis, M. A., Fillet, M., Geurts, P., de Seny, D. *et al.*, Biomarker discovery for inflammatory bowel disease, using proteomic serum profiling. *Biochem. Pharmacol.* 2007, *73*, 1422–1433.

[12] Geurts, P., Fillet, M., de Seny, D., Meuwis, M. A. *et al.*, Proteomic mass spectra classification using decision tree based ensemble methods. *Bioinformatics* 2005, *21*, 3138–3145.

[13] Kips, J. C., Tournoy, K. G., Pauwels, R. A., Gene knockout models of asthma. *Am. J. Respir. Crit. Care. Med.* 2000, *162*, S66–S70.

[14] Sakai, K., Yokoyama, A., Kohno, N., Hamada, H., Hiwada, K., Prolonged antigen exposure ameliorates airway inflammation but not remodeling in a mouse model of bronchial asthma. *Int. Arch. Allergy Immunol.* 2001, *126*, 126–134.

[15] Tournoy, K. G., Hove, C., Grooten, J., Moerloose, K. *et al.*, Animal models of allergen-induced tolerance in asthma: Are T-regulatory-1 cells (Tr-1) the solution for T-helper-2 cells (Th-2) in asthma? *Clin. Exp. Allergy* 2006, *36*, 8–20.

[16] Murphy, V. E., Johnson, R. F., Wang, Y. C., Akinsanya, K. *et al.*, Proteomic study of plasma proteins in pregnant women with asthma. *Respirology* 2006, *11*, 41–48.

[17] Wu, J., Kobayashi, M., Sousa, E. A., Liu, W. *et al.*, Differential proteomic analysis of bronchoalveolar lavage fluid in asthmatics following segmental antigen challenge. *Mol. Cell Proteomics* 2005, *4*, 1251–1264.

[18] Hall, I. P., Genetics and pulmonary medicine 8: Asthma. *Thorax* 1999, *54(1)*, 55–69.

[19] Nair, M. G., Gallagher, I. J., Taylor, M. D., Loke, P. *et al.*, Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infect. Immun.* 2005, *73*, 385–394.

[20] Liu, T., Jin, H., Ullenbruch, M., Hu, B. *et al.*, Regulation of found in inflammatory zone 1 expression in bleomycin-induced lung fibrosis: Role of IL-4/IL-13 and mediation via STAT-6. *J. Immunol.* 2004, *173*, 3425–3431.

[21] Pesce, J., Kaviratne, M., Ramalingam, T. R., Thompson, R. W. *et al.*, The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J. Clin. Invest.* 2006, *116*, 2044–2055.

[22] Davies, D. E., Wicks, J., Powell, R. M., Puddicombe, S. M., Holgate, S. T., Airway remodeling in asthma: New insights. *J. Allergy Clin. Immunol.* 2003, *111*, 215–225; quiz 226.

[23] Klug, J., Beier, H. M., Bernard, A., Chilton, B. S. *et al.*, Uteroglobin/Clara cell 10 kDa family of proteins: Nomenclature committee report. *Ann. N Y Acad. Sci.* 2000, *923*, 348–354.

[24] Lindahl, M., Svartz, J., Tagesson, C., Demonstration of different forms of the anti-inflammatory proteins lipocortin-1 and Clara cell protein-16 in human nasal and bronchoalveolar lavage fluids. *Electrophoresis* 1999, *20*, 881–890.

[25] Breen, E. C., Tang, K., Calcyclin (S100A6) regulates pulmonary fibroblast proliferation, morphology, and cytoskeletal organization *in vitro*. *J. Cell. Biochem.* 2003, *88*, 848–854.

[26] Barnes, P. J., Adcock, I. M., Ito, K., Histone acetylation and deacetylation: Importance in inflammatory lung diseases. *Eur. Respir. J.* 2005, *25*, 552–563.

[27] Tomita, K., Barnes, P. J., Adcock, I. M., The effect of oxidative stress on histone acetylation and IL-8 release. *Biochem. Biophys. Res. Commun.* 2003, *301*, 572–577.

[28] Elliott, P. J., Pien, C. S., McCormack, T. A., Chapman, I. D., Adams, J., Proteasome inhibition: A novel mechanism to combat asthma. *J. Allergy Clin. Immunol.* 1999, *104*, 294–300.

