

# Expression of S100A12 (EN-RAGE) in cystic fibrosis

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**Background:** Chronic airway inflammation and recurrent infections are a core phenomenon in cystic fibrosis (CF). Diagnosing acute infectious exacerbations is difficult in the presence of chronic inflammatory processes. S100A12 exhibits proinflammatory functions via interaction with the multiligand receptor for advanced glycation end products. Blocking this interaction inhibits inflammatory processes in mice.

**Methods:** The expression of S100A12 in lung specimens of patients with end stage lung disease of CF was investigated, and S100A12 levels in the serum of patients with acute infectious exacerbations of CF were measured.

**Results:** Immunohistochemical studies of CF lung biopsy specimens revealed a significant expression of S100A12 by infiltrating neutrophils. High S100A12 levels were found in the sputum of patients with CF, and serum levels of S100A12 during acute infectious exacerbations were significantly increased compared with healthy controls (median 225 ng/ml v 46 ng/ml). After treatment with intravenous antibiotics the mean S100A12 level decreased significantly. There was also a significant difference between S100A12 levels in patients with acute infectious exacerbations and 18 outpatients without exacerbations (median 225 ng/ml v 105 ng/ml).

**Conclusions:** S100A12 is extensively expressed at local sites of inflammation in CF. It is a serum marker for acute infectious exacerbations. High local expression of S100A12 suggests that this protein has a proinflammatory role during airway inflammation and may serve as a novel target for anti-inflammatory treatments.

Lung injury due to chronic airway inflammation is the primary cause of morbidity and mortality in cystic fibrosis (CF). Pulmonary infections with a variety of bacteria including *Staphylococcus aureus* and *Pseudomonas aeruginosa* account for a large number of complications. Neutrophilic inflammation occurs early in life and contributes to progressive tissue damage.<sup>1,2</sup> Acute infectious exacerbations are a common reason for admission to hospital and treatment with antibiotics.

There is a lack of agreement about the criteria for defining acute episodes. Conventional parameters normally used to identify acute infections—for example, fever, leucocytosis, C reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR), deterioration of lung function, and sputum cultures—are frequently unhelpful.<sup>3,4</sup> It would be useful to have more reliable markers indicating inflammatory exacerbations to monitor disease and guide treatment. Ideal sensitive markers should indicate local bronchial processes before systemic responses occur.

S100A12 (p6, calgranulin C) belongs to the S100 family of calcium binding proteins.<sup>5</sup> These proteins share EF-hand domains (helix-loop-helix motifs) and are involved in binding of calcium and zinc ions. Intracellular functions include alteration of the cytoskeleton, cell polarisation, signal transduction, and regulation of phagocytosis. S100A12 is expressed by granulocytes, whereas its expression by monocytes remains a subject of debate.<sup>6-9</sup> It exhibits several proinflammatory properties, and its chemotactic activity is comparable to that of other strongly chemotactic agents.<sup>8</sup> Bovine S100A12 is a ligand for the receptor for advanced glycation end products (RAGE) expressed on macrophages, endothelium, and lymphocytes.<sup>7</sup> Intracellular signalling via protein kinases induces nuclear factor (NF)- $\kappa$ B dependent secretion of different cytokines.<sup>10,11</sup> It is hypothesised that binding of S100A12 to RAGE mediates the proinflammatory properties of this protein. The name EN-RAGE (for extracellular newly identified RAGE binding protein) has been proposed to emphasise

its central role in a receptor mediated signalling pathway which might offer attractive targets for intervention with blocking agents.<sup>7</sup>

The aim of this study was to investigate the role of S100A12 in CF. S100A12 levels in patients admitted to hospital for antibiotic treatment were analysed and compared with serum levels in outpatients without exacerbations.

## METHODS

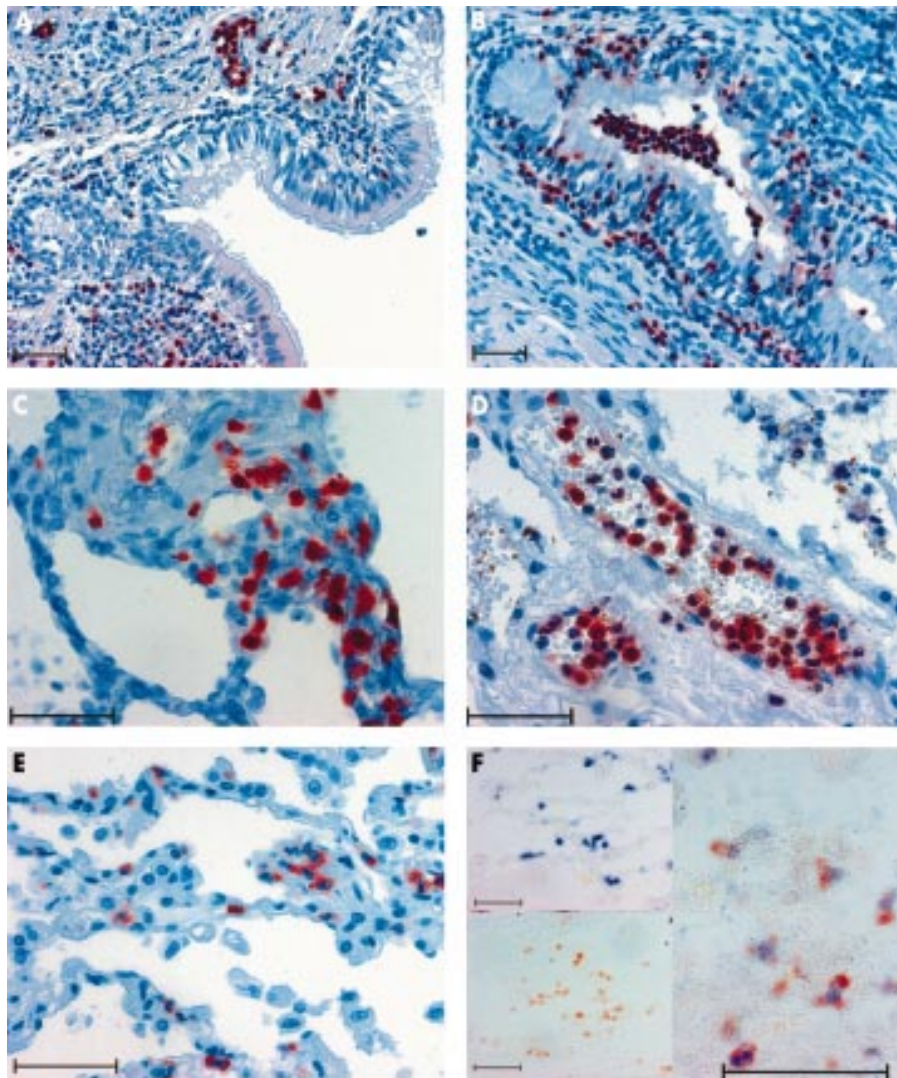
### Immunohistochemical study

Paraffin embedded sections of explanted lungs of patients with CF undergoing lung transplantation were used to detect S100A12 expression by anti-human S100A12 antibody. Monoclonal mouse anti-human granulocyte associated antigen CD15 antibody (Dako, Hamburg, Germany) was used to determine the subset of cells in the infiltrates, and double labelling procedures were performed to detect co-expression of S100A12 and CD15. Secondary antibodies and substrate peroxidase and phosphatase were used for colour reaction as described previously.<sup>12</sup>

### Patients and controls

Serum S100A12 levels were determined in 17 CF inpatients with 21 acute infectious exacerbations at the beginning and end of intravenous antibiotic treatment and in 18 outpatients with CF without acute infectious exacerbations. Sputum samples from eight inpatients with acute infectious exacerbations were also analysed, and CRP levels and ESR were documented. The upper limit of normal range was 0.49 mg/dl for CRP and 20 mm/h for ESR.

Serum levels of S100A12 were measured in 18 healthy adults of median age 31.5 years (range 19–43) and 16 children of median age 11.5 years (range 3–17) without signs of inflammation. A total of 34 normal controls of median age 22 years (range 3–43) were therefore investigated. Healthy adults were volunteers from our laboratory or from the University Children's Hospital of Muenster. Healthy children were those



**Figure 1** Expression of S100A12 by granulocytes in CF lung tissue. (A) Bronchial respiratory epithelium and interstitial tissue with infiltrating granulocytes ( $\times 200$ ). (B) Bronchiolus with subepithelial granulocytes; viscous mucus containing S100A12 positive cells ( $\times 200$ ). (C) Alveoli with interstitial S100A12 positive cells ( $\times 400$ ). (D) Granulocytes adherent to endothelium of small pulmonary vessels ( $\times 400$ ). (E) Distribution of neutrophil marker CD15 resembling S100A12 expression ( $\times 400$ ). (F) Double labelling technique showing simultaneous expression of S100A12 and CD15 ( $\times 630$ ). Inserted images show single labelling of S100A12 (blue) and CD15 (red), respectively ( $\times 400$ ). Scale bars indicate 50  $\mu\text{m}$ .

who presented for routine blood testing, predominantly for evaluation of suspected deficiency of growth hormones or before minor surgery. All controls were without any signs or symptoms of acute or chronic disorders with normal ESR values. There were no significant differences in age or sex distribution between patients and healthy controls.

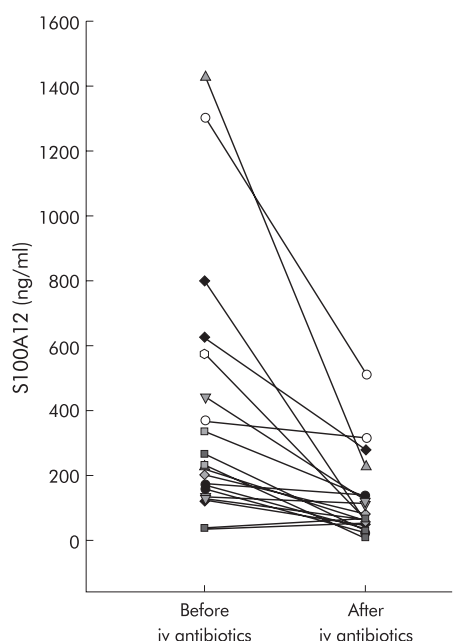
#### Purification of S100A12 protein and antibodies

S100A12 was isolated from human granulocytes as described previously,<sup>6,13</sup> and polyclonal affinity purified rabbit antisera against human S100A12 (a-S100A12) were prepared as reported by Vogl *et al.*<sup>6</sup> Monospecificity of rabbit anti-human S100A12 antibody was analysed by immunoreactivity against purified human and recombinant S100A12 and western blot analysis of granulocyte lysates.

#### Determination of S100A12 concentrations by sandwich ELISA

Concentrations of S100A12 in the serum or sputum of CF patients were determined by a double sandwich enzyme linked immunosorbent assay (ELISA) system established in

our laboratory. Flat bottom 96-well microtitre plates (Maxisorp; Nunc, Wiesbaden, Germany) were coated with 20 ng/well anti-S100A12 in 0.05 M sodium carbonate buffer (pH 9.5), incubated for 16 hours at 4°C; washed three times with phosphate buffered saline and 0.1% Tween 20, pH 7.4 (wash buffer), and blocked with wash buffer containing 0.25% bovine serum albumin (block buffer) for 1 hour at 37°C. The plates were washed once with wash buffer and 50  $\mu\text{l}$  samples with varying dilutions in block buffer were added for 2 hours at room temperature. The ELISA was calibrated with purified S100A12 in concentrations ranging from 0.016 to 125 ng/ml. The assay has a linear range of 1–20 ng/ml and a sensitivity of <0.5 ng/ml. After three washes 20 ng/well biotinylated rabbit anti-human S100A12 was added and incubated for 30 minutes at 37°C. The plates were washed and incubated with streptavidin-horseradish peroxidase conjugate (1:5000 dilution; Pierce, Rockford, Illinois, USA) for 30 minutes at 37°C. After washing three times the plates were incubated with 2,2'-azino bis(3-ethylbenzthiazoline sulfonic acid) (ABTS; Roche Diagnostics, Mannheim, Germany) and H<sub>2</sub>O<sub>2</sub> in 0.05 M citrate buffer, pH 4.0, for 20 minutes at room temperature.



**Figure 2** S100A12 serum levels in 21 CF patients with acute infectious exacerbations. Almost all the patients showed a significant decrease after treatment with intravenous antibiotics for 2 weeks.

Absorbency at 405 nm was measured with an ELISA reader (MRX Microplate Reader, Dynatech Laboratories, Denckendorf, Germany).

**Statistical analysis**

To determine differences in S100A12 expression the Mann-Whitney U test was used for unpaired variables and the Wilcoxon test for paired variables. Data are expressed as medians

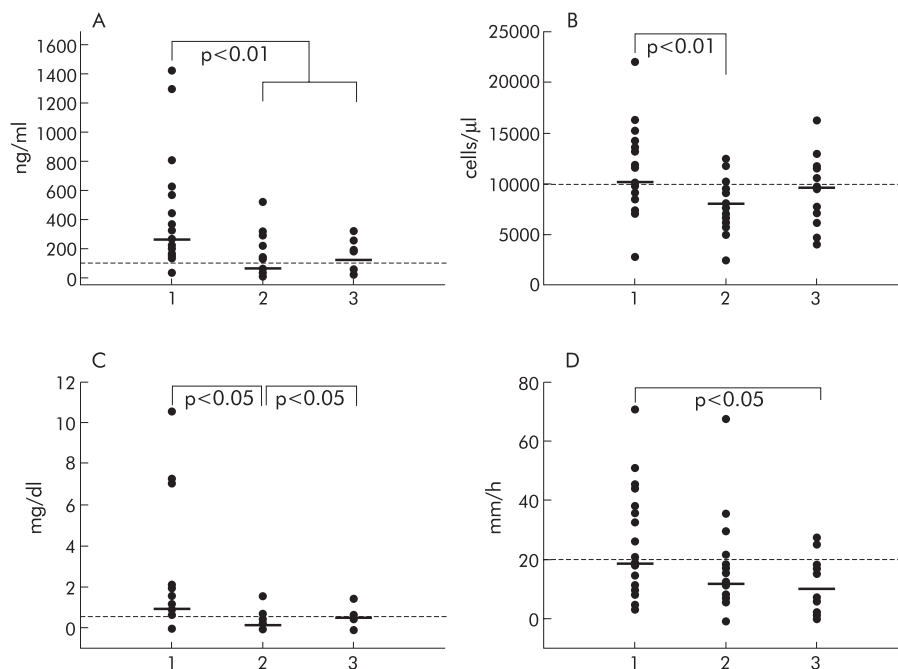
with interquartile ranges except where otherwise stated. p values <0.05 were considered significant.

**RESULTS**

Immunohistochemical studies of CF lung biopsy specimens revealed a high expression of S100A12 in lung tissue with a distinct pattern of subepithelial distribution. Single S100A12 positive granulocytes were found adherent to small lung vessels and invading the interstitial connective tissue. Staining with CD15 antibody revealed a pattern of expression resembling the distribution of S100A12. Double labelling experiments showed co-expression of S100A12 and CD15 (fig 1).

The median level of S100A12 in the sputum of CF patients with acute infectious exacerbations was 4200 ng/ml (range 2700–10 600). Blood samples were collected from 17 inpatients with CF (nine boys) who received intravenous antibiotics on 21 occasions. The median age of the inpatients at the time of entry to the study was 21 years (range 10–35) and the mean duration of hospitalisation for the actual treatment was 2 weeks. Fourteen patients were positive for *P aeruginosa*. The main reasons for admission to hospital were global deterioration of well being, excessive production of viscous sputum, and an increase in productive coughing. Eighteen CF outpatients without acute infectious exacerbations (10 boys) who were seen in our clinic on 20 occasions were investigated for the detection of S100A12. Their median age was 19 years (range 8–31) and 13 were positive for *P aeruginosa*.

Mean serum levels of S100A12 were 64 (95% CI ±15) ng/ml in healthy adult controls and 50 (95% CI ±15) ng/ml in the healthy children. The overall median S100A12 level in healthy controls was 46 ng/ml (range 11–135). Patients with acute infectious exacerbations had significantly higher serum levels of S100A12 (median 225 ng/ml, range 40–1430; p<0.001). In 18 of 21 cases (86%) serum levels of S100A12 were above the normal mean (±2SD) level. After 2 weeks of treatment with an intravenous antibiotic the median S100A12 level in these



**Figure 3** Inflammatory markers in different populations of patients with CF. (A) S100A12, (B) leucocyte count, (C) C reactive protein, and (D) erythrocyte sedimentation rate were determined in different subgroups of CF patients: (1) those with acute infectious exacerbations before antibiotic therapy (n=21); (2) after 2 weeks of antibiotic treatment for acute infectious exacerbations (n=21); and (3) outpatients without an exacerbation (n=20). Data are expressed as individual point plots; horizontal lines indicate median for analysed groups. Upper limits of normal range are shown.



patients decreased significantly to 76 ng/ml (range 17–525;  $p < 0.01$ ). The individual courses of S100A12 levels in 21 cases with acute infectious exacerbations before and after antibiotic treatment are shown in fig 2. There was a significant difference between S100A12 values of patients with acute infectious exacerbations before and after treatment. The median S100A12 level in CF outpatients without exacerbations was 105 ng/ml (range 35–320;  $p < 0.01$ ).

CRP levels were increased in 13 of 21 patients with acute infectious exacerbations (62%) before starting antibiotic therapy. There was a significant difference between median CRP levels in patients with acute infectious exacerbations before (0.9 (1.4) mg/dl, range 0–10.6) and after antibiotic treatment (0.0 mg/dl, range 0–1.6;  $p < 0.05$ ). However, the difference between those with acute infectious exacerbations and outpatients without acute infection (median 0.5 mg/dl, range 0–1.5) was not significant. ESR was above the normal range in 10 of the 21 patients (48%). A significant difference in median ESR was found between patients with acute infectious exacerbations (19 mm/h, range 4–51) and outpatients (12 mm/h, range 1–28;  $p < 0.05$ ). The ESR in patients with acute infectious exacerbations before and after antibiotic treatment (median 13 mm/h, range 6–36) did not differ significantly. In 12 cases (56%) the leucocyte counts were above 10 000/ $\mu$ l. Leucocyte counts were significantly higher in patients with acute infectious exacerbations before (median 10 150/ $\mu$ l, range 2900–22 100) than after antibiotic treatment (median 7750/ $\mu$ l, range 2500–12 500;  $p < 0.01$ ), but no such difference was found between patients with acute infectious exacerbations before treatment and outpatients (median 9850, range 4300–16 500). These data are summarised in fig 3.

## DISCUSSION

One of the main tasks for physicians in CF is adjusting treatment to acute pulmonary complications of chronic inflammation. Identifying acute infectious exacerbations is usually based on clinical experience and depends on subjective impressions, measurements of pulmonary function, and some acute phase markers of inflammation. Lung function tests or sputum cultures are of limited value in diagnosing acute exacerbations in patients with CF. Conventional markers of inflammation such as CRP or ESR have been found generally to be unreliable in CF exacerbations.<sup>14</sup> More sophisticated markers such as interleukins or tumour necrosis factor are not considered to be useful tools by all investigators.<sup>15–18</sup> Adhesion molecules<sup>19</sup> and vascular endothelial growth factor<sup>20</sup> have been detected in CF exacerbations. Eichler *et al*<sup>21</sup> proposed human neutrophilic lipocalin as a marker for CF exacerbations. Sputum levels of various cytokines are detectable, but measuring markers of inflammation in sputum is problematic.<sup>15 22 23</sup> Reliable examination often requires bronchoalveolar lavage.<sup>4</sup> Exhaled nitric oxide is of limited usefulness in CF.<sup>24</sup>

We present the first data on S100A12 in human lung disease. Our immunohistochemical studies demonstrated expression of S100A12 in CF lungs. Neutrophils adhere to the endothelium of small pulmonary vessels, consecutively invading the interstitial tissue and forming subepithelial infiltrates. Granulocytes are found in the bronchial lumen. Secretion of S100A12 by activated neutrophils has been shown previously.<sup>25</sup> Sputum levels of S100A12 in patients with CF were about 10 times higher than serum levels. We can only speculate that S100A12 is secreted by infiltrating cells at the site of inflammation, hence serum levels might directly reflect local bronchial inflammation and not a systemic response as with acute phase proteins such as CRP or ESR.

The present study shows that S100A12 is potentially useful as a marker for the need for intravenous antibiotics. Serum levels of S100A12 were significantly higher in CF patients with

exacerbations than in healthy controls, and serum levels correlated with disease activity in individual patients. S100A12 levels decreased during antibiotic treatment in all patients, including four with initial levels inside the normal range, indicating that personal profiles might be more useful than single serum tests.

The exact role of S100A12 in the pathogenesis of bronchial inflammation in exacerbations of CF remains unclear. Two other S100 proteins (S100A8 and S100A9) accumulate at sites of inflammation and are found in high levels in inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and cystic fibrosis.<sup>12 26–29</sup> Overexpression of murine S100A8 was detected in a mouse model of CF,<sup>30</sup> and direct association of S100A8 with abnormal regulation of the immune system in CF has been proposed. No murine S100A12 has been described to date, making analysis of S100A12 expression in CF mice impossible. Cole *et al*<sup>31</sup> described a fragment of S100A12 which might be an important antimicrobial effector for airway protection in CF. This newly described antimicrobial peptide named calcitermin consists of the 15 C-terminal residual amino acids of S100A12. Calcitermin acts primarily against Gram negative bacteria in the presence of zinc, which is found in high concentrations in the nasal fluid of patients with CF.<sup>31</sup> In addition, several proinflammatory properties of S100A12 via interaction with RAGE have been described.<sup>10 11 32</sup>

Promising therapeutic effects of agents blocking RAGE activation have been reported in mouse models of delayed type hypersensitivity, collagen induced arthritis, experimental autoimmune encephalitis, and chronic colitis.<sup>7 32</sup> Soluble (s)RAGE, the extracellular ligand binding domain of the receptor, is able to restore efficient wound healing<sup>33</sup> and to suppress atherosclerosis in diabetic mice.<sup>34</sup> The most profound suppression of inflammation is achieved by combined treatment with blocking agents against S100 proteins and RAGE.<sup>32</sup>

S100A12 is extensively expressed in CF. This study suggests that it may have some value as an additional serum marker for acute infectious exacerbations. Its functional role as a proinflammatory protein with a receptor mediated signalling pathway might be a target for future interventions with blocking agents.

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