Human neutrophil lipocalin, a highly specific marker for acute exacerbation in cystic fibrosis

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ABSTRACT: Cystic fibrosis (CF) is characterized by the production of abnormally thick secretions in the airways, chronic bacterial endobronchial infections and a chronic, predominantly neutrophilic inflammatory response. Therefore, myeloperoxidase (MPO) and lactoferrin are frequently used as inflammatory markers. Recently, a new protein in the neutrophil granules, human neutrophil lipocalin (HNL) has been discovered. The aim of the present study was to investigate HNL in sera of patients with CF and its relation to MPO and lactoferrin as well as to acute pulmonary exacerbation.

Serum concentrations of HNL, MPO and lactoferrin were determined in 42 patients with CF and in 25 healthy subjects. Patients with CF were divided into groups with and without acute pulmonary exacerbation (APE) and also with and without colonization with *Pseudomonas aeruginosa* (Pa).

Median serum levels of HNL ($200.5 \mu\text{g}\cdot\text{L}^{-1}$), MPO ($595 \mu\text{g}\cdot\text{L}^{-1}$) and lactoferrin ($1,356.5 \mu\text{g}\cdot\text{L}^{-1}$) were significantly increased in patients with CF compared to control subjects (57.7 , 178 and $478 \mu\text{g}\cdot\text{L}^{-1}$, respectively; $p<0.0001$). CF patients with APE had significantly increased serum concentrations of HNL (321 versus $97.7 \mu\text{g}\cdot\text{L}^{-1}$; $p<0.0001$), MPO ($1,125$ versus $300 \mu\text{g}\cdot\text{L}^{-1}$; $p<0.005$) and lactoferrin ($4,936$ versus $980 \mu\text{g}\cdot\text{L}^{-1}$; $p<0.001$) compared with patients in stable clinical condition. Similarly, patients colonized with Pa had significantly higher concentrations of HNL, MPO and lactoferrin than Pa negative patients.

These results indicate that in patients with cystic fibrosis, serum concentrations of human neutrophil lipocalin are markedly increased with a strong relationship to myeloperoxidase and lactoferrin. Thus, determination of serum human neutrophil lipocalin concentrations may be another useful diagnostic tool to monitor neutrophil inflammation in cystic fibrosis. The more marked difference in human neutrophil lipocalin compared with myeloperoxidase concentrations with no overlap between patients with acute pulmonary exacerbation and those in stable condition even suggests that human neutrophil lipocalin may be a more sensitive and specific discriminator.

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Cystic fibrosis (CF) is characterized by its genetically determined abnormalities of mucus secretion, airway electrolytes and impaired mucociliary clearance resulting in an increased susceptibility to pulmonary colonization with *Pseudomonas aeruginosa* (Pa) and other pathogens [1]. These infections induce a chronic inflammatory response and are associated with progressive loss of pulmonary function [2]. In the interaction between bacteria and host defense mechanisms, polymorphonuclear cells release cytotoxic granule proteins. Their proteolytic activity in combination with oxidant stress results in progressive damage of the endobronchial matrix [2, 3].

Serum myeloperoxidase (MPO) concentrations used as a marker of neutrophil activity was found to be strongly associated with the degree of tissue destruction and airflow obstruction [2, 4, 5]. Recently, a new protein of the secondary granules, human neutrophil lipocalin (HNL), was discovered [6]. Increased concentrations of HNL

have been demonstrated in the sera of patients with acute bacterial infections, and HNL appears to be more specific and sensitive than C-reactive protein (CRP) in the distinction between viral and bacterial infections in non-CF subjects [7]. Further studies have shown that HNL was secreted more readily from the neutrophils than any other specific marker protein. [8] Since pulmonary inflammation, neutrophil activity and tissue destruction were found to directly correlate in patients with CF, a sensitive and specific marker for the degree of pulmonary inflammation, which can also be used as a therapeutic monitoring tool, seems warranted. In addition, the ongoing chronic inflammatory process within the lungs of patients with CF makes it difficult to accurately diagnose an acute pulmonary exacerbation. Therefore, the capacity of HNL to be used as a sensitive and specific diagnostic tool to overcome these difficulties was evaluated in comparison with other neutrophil proteins such as MPO and lactoferrin.

Materials and methods

Patients and control subjects

Forty-two patients (24 male, 18 female children) with CF from the authors' University CF Care Centre were investigated. The diagnosis of CF was confirmed by repeated sweat testing and genetic analysis. The median age was 14.5 yrs (range 8–17 yrs). Depending on their sputum cultures they were grouped as follows: 1) 16 patients with no evidence of Pa colonization, and 2) 26 patients with Pa alone or in combination with *Staphylococcus aureus* and/or *Haemophilus influenzae*.

Twenty-five healthy subjects without evidence of infection were used as a control group (14 male, 11 female children), with a median age of 13.4 yrs (range 3–16 yrs) as previously described [9]. Acute pulmonary exacerbation was defined as a significant increase of CRP, increase of cough and purulent sputum production, fever, new lung infiltrates on chest radiography and deterioration of oxygen saturation and of lung function.

Parental agreement was obtained in all cases and drawing of blood was only performed in routine sampling for clinical examination.

Assessment of serum human neutrophil lipocalin, myeloperoxidase and lactoferrin

Blood sampling, handling and storing were performed according to the manufacturer's instructions. HNL, in serum was analysed by a fluoroenzyme-immunoassay technique (Pharmacia CAP System HNL FEIA; Pharmacia, Uppsala, Sweden), a prototype assay, which is not commercially available. In brief, anti-HNL is coupled to ImmunoCAP. Standards and samples are then added and incubated at room temperature for 30 min. After washing, β -galactosidase-conjugated anti-HNL is added and incubated at room temperature for 2.5 h. After washing, 4-methylumbelliferyl β -D-galactoside is added to form a fluorescent product which can be detected after 10 min. The measuring range of this test is 20–600 $\mu\text{g}\cdot\text{L}^{-1}$. The inter-assay coefficient of variation ranged 4.6–6% for HNL concentrations 53–462 $\mu\text{g}\cdot\text{L}^{-1}$.

The concentration of MPO in serum was determined by a sensitive and specific double antibody radio immunoassay (Pharmacia Diagnostics AB, Uppsala, Sweden). The interassay coefficient of variation was <8% [10, 11].

Lactoferrin in serum was assessed using an enzyme-linked immunoassay of the sandwich type (Lactof-EIA; Bioxytech SA, Marseilles, France) according to the manufacturer's instructions. In brief, the samples were incubated in a microtitre plate, which was coated with a primary monoclonal antibody against lactoferrin. The resulting complex was then detected on a biotin-avidin coupling in which avidin was covalently linked to horse radish peroxidase. The amount of lactoferrin was measured enzymatically. The interassay coefficient of variation was <5%.

Assessment of pulmonary function and clinical conditions

Forced vital capacity (FVC), forced expiratory volume in one second (FEV₁) and maximum expiratory flow at

50% of vital capacity (MEF₅₀%) were recorded in the form of a maximum flow volume curve according to the American Thoracic Society standard (Masterlab, Jäger, Würzburg, Germany) [12]. The best of three efforts was used for calculation. Results are presented as percent predicted based on accepted reference standards [13].

Disease severity was assessed by the Shwachmar-Kulczycki-Score [14], without reviewing the chest radiograph, resulting in a score range of 0–75 points [11]. In addition, serum concentrations of immunoglobuline (Ig)G were determined owing to their prognostic role in CF [15].

Statistical analysis

Results are expressed as median (quartile 1–quartile 3) unless otherwise stated. Correlation coefficients were obtained using the Kendall Tau B Test. Nonparametric statistical tests were used for comparative analysis. If more than two groups were compared the Kruskal-Wallis procedure was applied first and in the case of significance. If only two groups were compared, each pairing was examined by the Mann-Whitney U-test. A p-value <0.05 was considered statistically significant.

Results

Clinical characteristics of patients

At the time of examination, 19 CF patients had an acute pulmonary exacerbation requiring hospitalization and 23 patients were in a stable condition. Pulmonary function testing demonstrated significant differences for FVC% (69.4 (41.0–84.2)% versus 87.8 (79.6–102.3)%; $p<0.001$), for FEV₁% (66.7 (41.8–74.8)% versus 81.2 (72.4–98.4)% $p<0.002$), and for MEF₅₀% (34.2 (21.2–44.8) versus 68.2 (42.4–89.5)% $p<0.001$) between patients with and without acute pulmonary exacerbation, respectively.

Neutrophil counts, human neutrophil lipocalin, myeloperoxidase, lactoferrin, and C-reactive protein

In CF patients, neutrophil counts, as well as serum HNL, MPO, lactoferrin and CRP concentrations were significantly increased compared with the values in the control

Table 1. – Serum concentrations of human neutrophil lipocalin (HNL), myeloperoxidase (MPO), lactoferrin and C-reactive protein (CRP) as well as neutrophil counts in patients with cystic fibrosis (n=42) and 25 healthy control subjects

	Cystic fibrosis	Control subjects	p-value
HNL $\mu\text{g}\cdot\text{L}^{-1}$	200.5 (86.9–320)	57.7 (37.3–63.6)	<0.0001
MPO $\mu\text{g}\cdot\text{L}^{-1}$	595 (245–1875)	178 (160–214)	<0.0001
Lactoferrin $\mu\text{g}\cdot\text{L}^{-1}$	1357 (928–5420)	478 (398–578)	<0.0001
CRP $\text{mg}\cdot\text{L}^{-1}$	16.0 (2.0–48.0)	0 (0–8.0)	<0.0003
Neutrophil cells μL^{-1}	5600 (1800–13400)	4160 (3200–4600)	<0.01

Results are expressed as median (quartile 1–quartile 3); p-values indicate statistical differences between the two groups.

group (table 1). In patients with CF, serum HNL significantly correlated with CRP ($r=0.726$; $p<0.0001$), with MPO ($r=0.506$; $p<0.0001$), with lactoferrin ($r=0.490$; $p<0.0001$) and with neutrophil counts ($r=0.339$; $p<0.005$). In addition, there was a weak, albeit significant correlation of HNL, serum concentration with IgG serum concentrations ($r=0.289$; $p<0.01$).

Correlation coefficients of HNL serum concentrations with pulmonary function were $r=-0.27$, $p<0.01$ for FVC%, $r=-0.25$, $p<0.02$ for FEV1%; and $r=-0.25$, $p<0.02$ for MEF50%. No significant correlation was detected between neutrophil counts, MPO and lactoferrin concentrations with pulmonary function parameters. In addition, there was a significant albeit weak correlation between the Shwachman-Kulczycki Score and serum concentrations of CRP ($r=-0.24$, $p<0.03$) and of HNL ($r=-0.24$, $p<0.03$) but not with neutrophil counts, MPO and lactoferrin concentrations.

Dividing CF patients according to their pulmonary function data into patients with a FEV1 $>70\%$ ($n=26$) and into those with a FEV1 $<70\%$ ($n=16$), HNL serum concentration was significantly increased in the latter group (table 2). No significant differences were found for MPO and lactoferrin serum concentrations (table 2).

Influence of acute pulmonary exacerbation

Comparing CF patients with ($n=19$) and without ($n=23$) acute pulmonary exacerbation, the neutrophil counts, CRP, HNL, MPO and lactoferrin serum concentrations were significantly increased in patients with acute pulmonary exacerbation (fig. 1).

The difference in HNL concentrations between patients with acute pulmonary exacerbation and those in a stable clinical condition was more marked ($p<0.0001$) with almost no overlap between these two groups than the difference in MPO or lactoferrin concentrations (fig. 1).

In patients with acute pulmonary exacerbation, HNL correlated significantly with CRP but weakly with MPO (CRP: $r=0.544$, $p<0.002$; MPO: $r=0.380$, $p<0.03$), but not with lactoferrin and neutrophil counts ($r=-0.17$, $p=0.31$; $r=0.275$; $p=0.1$, respectively).

Sensitivity and specificity of these neutrophil markers in discriminating acute pulmonary exacerbation in patients with CF are presented in table 3.

Table 2. – Serum concentrations of human neutrophil lipocalin (HNL), myeloperoxidase (MPO), and lactoferrin depending on the forced expiratory volume in one second (FEV1) in patients with cystic fibrosis

	FEV1% $>70\%$ ($n=26$)	FEV1% $<70\%$ ($n=16$)	p-value
HNL $\mu\text{g}\cdot\text{L}^{-1}$	128.0 (86.4–161.6)	306.0 (182.5–419.5)	<0.02
MPO $\mu\text{g}\cdot\text{L}^{-1}$	595 (250–1560)	618 (212–3125)	NS
Lactoferrin $\mu\text{g}\cdot\text{L}^{-1}$	1250 (8928–5420)	1468 (812–7458)	NS

Results are expressed as median (quartile 1–quartile 3); p-values indicate statistical differences between the two groups. NS: not significant.

Fig. 1. – Influence of acute pulmonary exacerbation (APE) on serum concentrations of a) human neutrophil lipocalin (HNL), b) myeloperoxidase (MPO) and c) lactoferrin in comparison with patients in stable clinical condition (Non-APE). Data are presented as the median value and the quartile 1–quartile 3 range and individual values.

Influence of bacterial colonization

Patients were divided into two different groups according to their sputum culture results: 1) 16 subjects in whom no *Pa* colonization was detected, and 2) 26 subjects with chronic *Pa* infection/colonization. *Pa*-positive patients had significantly higher concentrations of HNL, MPO and lactoferrin compared to patients without *Pa* colonization (table 4). In patients with and without *Pa* colonization, HNL correlated significantly with CRP ($r=0.593$,

Table 3. – Sensitivity and specificity of human neutrophil lipocalin (HNL), myeloperoxidase (MPO), lactoferrin and C-reactive protein (CRP) to discriminate patients with and without acute pulmonary exacerbation (APE *versus* Non-APE)

	APE		Non-APE	
	n	%	n	%
HNL >200 µg·L ⁻¹	19	100	2	8.7
HNL <200 µg·L ⁻¹	0	0	21	91.3
Sensitivity/specificity		100%		91%
MPO >500 µg·L ⁻¹	15	79	6	26
MPO <500 µg·L ⁻¹	4	21	17	74
Sensitivity/specificity		79%		74%
Lactoferrin >1000 µg·L ⁻¹	17	89.5	10	43.5
Lactoferrin <1000 µg·L ⁻¹	2	10.5	13	56.5
Sensitivity/specificity		90%		56%
CRP >10 mg·L ⁻¹	18	94.7	5	21.7
CRP <10 mg·L ⁻¹	1	5.3	18	78.3
Sensitivity/specificity		95%		78%

$p < 0.001$ and $r = 0.660$, $p < 0.004$, respectively), with MPO ($r = 0.735$, $p < 0.0001$ and $r = 0.623$, $p < 0.005$, respectively), with lactoferrin ($r = 0.731$, $p < 0.0001$ and $r = 0.627$, $p < 0.005$, respectively) as well as with neutrophil counts ($r = 0.504$, $p < 0.003$ and $r = 0.415$, $p = 0.057$, respectively). Pulmonary function parameters also differed significantly between the groups (table 4).

Discussion

The present study examined the role of HNL, a recently isolated protein from the secondary granules of neutrophils, and found it to be a highly specific inflammatory marker for acute exacerbation in patients with CF.

Table 4. – Influence of chronic endobronchial colonization with *Pseudomonas aeruginosa* (Pa) on human neutrophil lipocalin (HNL), myeloperoxidase (MPO), lactoferrin, neutrophil counts and pulmonary function in 42 patients with cystic fibrosis

	Pa-positive (n=26)	Pa-negative (n=16)	p-value
HNL µg·L ⁻¹	291.5 (134.0–334.0)	98.9 (68.4–125.0)	<0.01
MPO µg·L ⁻¹	963 (250–2500)	253 (225–360)	<0.04
Lactoferrin µg·L ⁻¹	1979 (980–8106)	943 (862–1345)	<0.03
Neutrophil cells·µL ⁻¹	6150 (4900–7200)	4350 (3800–5600)	NS
FVC %	76.3 (49.6–88.6)	90.0 (83.6–102.3)	<0.02
FEV1%	70.0 (43.7–86.0)	85.6 (77.2–98.6)	<0.02
MEF50%	36.8 (27.2–68.6)	64.7 (48.4–88.7)	<0.04

Data are presented as median (quartile 1–quartile 3); p-values indicate statistical differences between the two groups. FVC: forced vital capacity; FEV1: forced expiratory volume in one second; MEF50%: maximum expiratory flow at 50% of vital capacity; NS: not significant.

Pulmonary morbidity in CF includes chronic inflammation of the airways, with neutrophils as the predominant inflammatory cells. Bronchoalveolar lavage (BAL) studies in CF have demonstrated neutrophils to be enlarged and activated, containing many phagocytic vacuoles. Neutrophils contribute to progressive lung destruction through damage by highly reactive metabolites of molecular oxygen on the one hand and potent digestive enzymes and proteases present in their granules on the other [3]. Serum levels of some neutrophil-derived enzymes, such as MPO from the primary granules or lactoferrin from the secondary (specific) granules directly correlate with the degree of airway obstruction and lung bacterial burden in patients with CF [5, 16, 17]. Recently, a new protein, HNL, purified from the secondary granules of neutrophils, was shown to be secreted to a much higher degree from neutrophils than MPO or lactoferrin [6, 8, 18, 19]. Since the chronic inflammatory process within the lungs of patients with CF makes it difficult sometimes to accurately diagnose an acute pulmonary exacerbation, it was speculated as to whether HNL might be a sensitive and specific diagnostic tool to overcome these difficulties. These data, showing significantly increased serum levels of CRP, MPO, lactoferrin and HNL in CF patients with a high correlation of HNL, with CRP and MPO, the most commonly used inflammatory markers, demonstrate HNL, to be an equally well suited diagnostic tool. In addition, by dividing the CF study population into patients with and without acute pulmonary exacerbation, a more marked difference in HNL concentrations with almost no overlap between these two groups compared with the difference in MPO and lactoferrin concentrations was found. These data indicate that HNL, might be a better discriminator and thus a more specific marker of neutrophilic inflammation in CF than MPO. Interestingly, only recently, HNL, was found to be the better discriminator compared to MPO for asthma and chronic obstructive pulmonary disease (COPD), a chronic disease characterized by airflow limitation and bronchial neutrophilic inflammation [20]. These findings are corroborated by the current data, showing HNL serum concentrations but not MPO concentrations to be significantly increased in patients with more marked airflow limitation.

Endobronchial colonization with Pa is a frequent and characteristic complication in patients with CF. Ineffective phagocytosis by macrophages resulting in the persistence of Pa infection drives neutrophil recruitment, activation and degranulation of neutrophils with associated progression of lung disease [21]. It has been shown that levels of neutrophil-derived enzymes, such as MPO, correlate with the degree of airway obstruction and lung bacterial burden [2, 17, 22]. In agreement with these data, this study also demonstrated that patients chronically colonized with Pa had markedly increased concentrations of the neutrophil granule proteins MPO, lactoferrin and HNL. The difference between Pa-colonized and noncolonized patients was most striking for HNL concentrations compared with MPO and lactoferrin concentrations. These findings support data from previous release studies, showing HNL to be more readily secreted from neutrophils upon stimulation than MPO and lactoferrin [18].

However, noncolonized patients with CF also had significantly increased serum concentrations of neutrophil granule proteins compared with the control subjects, thus

supporting previous findings of neutrophil inflammation in patients with CF even in absence of any endobronchial infection [23].

In conclusion, this study found high serum concentrations of human neutrophil lipocalin in cystic fibrosis patients with a strong correlation to the other widely used neutrophil proteins, myeloperoxidase and lactoferrin. Human neutrophil lipocalin, however, appears to be a greater discriminator between acute pulmonary exacerbation and chronic stable inflammatory conditions. In the light of these findings, the measurement of human neutrophil lipocalin may therefore be a more sensitive and specific marker for acute neutrophil inflammation than myeloperoxidase and thus be a valuable diagnostic tool for clinical monitoring and for the assessment of disease severity in cystic fibrosis.

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