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Serum eosinophilic cationic protein and lactoferrin related to smoking history and lung function

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Serum eosinophilic cationic protein and lactoferrin related to smoking history and lung function. E.J. Jensen, B. Pedersen, E. Schmidt, P. Venge, R. Dahl. ©ERS Journals Ltd 1994.

ABSTRACT: Some smokers have an accelerated loss of lung function, possibly due to a chronic bronchial inflammation in which granulocytes are involved. Eosinophil cationic protein (ECP) and lactoferrin (LF) are granule proteins in the eosinophil and neutrophil granulocyte, respectively. We wanted to investigate the relationship of serum (s) ECP and LF concentrations to smoking history and lung function alteration.

This partly cross-sectional and partly prospective study included 98 nonatopic smokers and 31 lifetime nonsmokers. As participants in a smoking cessation programme, 50 of the 98 smokers ceased smoking for ≥1 year. Smoking history, lung function and blood samples were obtained at the start of the study, and smokers and ex-smokers also gave blood samples 3, 6 and 12 months later.

s-ECP and s-LF were elevated in smokers compared to people who had never smoked. s-ECP was linearly associated with daily cigarette consumption and forced expiratory volume in one second (FEV_1) residuals. In a multiple linear regression analysis, low s-ECP and high s-LF were associated with decreased FEV_1 residuals. s-ECP and s-LF together accounted for 10.2% of the variation in FEV_1 residuals. After smoking cessation, s-ECP and s-LF decreased within 6 months.

s-ECP and s-LF are raised in smokers, and may serve as indicators of granulocyte activation. We speculate that they might contribute to prediction of accelerated lung function loss in smokers, but this question needs further investigation in a prospective study.

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Smoking causes decrease in lung function [1–6], but only a minority of smokers develop severe respiratory impairment [7]. Reasons for this difference in susceptibility is not fully understood. Blood eosinophil count is elevated in nonatopic smokers compared to nonsmokers but without associations to lung function [8–10]. Eosinophil cationic protein (ECP) is a granule protein in eosinophil granulocytes [11]. ECP has cytotoxic effects on guinea-pig tracheal epithelium [12], and may be involved in the lung damage in adult respiratory distress syndrome (ARDS) [13], and idiopathic pulmonary fibrosis [14].

The blood leucocyte count is elevated in smokers compared to nonsmokers [10, 15, 16], and the level has been associated with an increased annual fall in forced expiratory volume in one second (FEV_1) [16]. This has been attributed mainly to an increased number of neutrophil granulocytes [17–19]. Lactoferrin (LF) is a protein contained in granules of neutrophils, and may protect the respiratory epithelium from oxygen radical damage by acting as an iron scavenger [20]. LF may inhibit the formation of neutrophils [21], and has antimicrobial activity [20]. In a recent publication, it was suggested that LF has pro-oxidant activity [22].

Serum-ECP (s-ECP) and serum-LF (s-LF) may be activation markers for the eosinophil and neutrophil granulocytes. Their concentrations in serum may reflect the involvement of each granulocyte in the smoking-induced bronchial inflammation and in the pathogenesis of smoking-induced lung damage.

We have studied s-ECP and s-LF in nonatopic smokers and nonsmokers and have looked for associations to smoking history and lung function. The short-term variations in s-ECP and s-LF after recent smoking cessation were followed for one year after smoking cessation.

Material and methods

Material

Four hundred and ninety six smokers volunteered to participate in a smoking cessation programme and were

Table 1. – Demographic details of smokers, divided into female, male, quitters and relapsers

	Overall	Females	Males	Quitters	Relapsers
Number of subjects	98	53	45	50	48
Females/males %	55/45			54/46	55/45
Smoking duration yrs	26.7±6.5	25.2±6.5	27.8±6.2	26.1±6.4	27.3±6.5
Daily tobacco consumption=	23.3±8.9	20.6±8.0	25.4±9.1*	22.5±8.1	24.1±9.8
Pack-years consumption=	31.6±15.6	26.3±12.3	35.6±16.6**	29.8±14.2	33.5±16.8
Age yrs	50±10	50±10	51±11	51±10	50±10
Cigarette smokers	74	39	35	39	35
Weighted daily cigarette consumption=	22.5±9.0	18.9±7.0	26.6±9.3***	23.9±9.0	21.5±9.1
Weighted pack-years consumption=	29.8±14.1	24.2±11.3	36.5±14.3***	32.3±13.8	28.0±14.8

Data are presented as mean±SD. †: see text for definitions. NS: nonsignificant. *, **, ***: p=0.01, 0.005, 0.0001 male v female.

followed for one year. The smoking population was recruited through the press. The nonsmoking control group (n=163) was recruited through requests to local industrial plants and municipal offices, chosen at random. Ninety six percent of the smoking population had professional jobs and the distribution of industrial and office workers was similar for smokers and nonsmokers.

Participants: selection and grouping

98 smokers and 31 lifetime nonsmokers, without signs of atopy, asthma or other diseases, were included in the present study and were chosen randomly from participants who fulfilled the inclusion criteria.

The group of smokers consisted of 50 persons that remained tobacco-abstinent for one year (quitters), and 48 persons with smoking relapse within 6 weeks from the start of the trial (relapsers). Mean age±SD in the lifetime nonsmoking and smoking groups was 48±10 and 51±10 yrs, respectively, (NS); percentage of females was 54 and 61%, respectively, (NS).

Male smokers had higher daily tobacco consumption (DC), weighted daily cigarette consumption (WDC) and weighted pack-years consumption (WPY), compared to female smokers (table 1).

Quitters and relapsers had comparable data with respect to age, gender and smoking history (table 1).

Lung function measurements

Lung function, expressed as FEV_1, and forced vital capacity (FVC), was measured with a dry bellow spirometer (Vitalograph Ltd, Buckingham, UK) before the start of the trial and 3, 6 and 12 months after smoking cessation. Participants were carefully instructed in the performance of the forced expiratory manoeuvre and the best out of three reproducible measurements with less than 5% variation was recorded. Normal values for FEV_1 and FVC were from the European working party on standardization of lung function tests [23]. The 95% confidence limits of FEV_1, FVC, and FEV_1/FVC ratio in the Danish population given as percentages of expected values are 78–129, 77–130, and 84–119%, respectively, [24].

Smoking history

The smoking history was recorded with respect to the actual daily tobacco consumption (DC), duration of smoking (yrs), kind of tobacco smoked (pipe-tobacco, cigarettes, small cigars and cigars), and nicotine content of the brands smoked. The amount of pipe-tobacco, small cigars and cigars were converted into an equivalent number of cigarettes: with 1 g of pipe-tobacco=1 cigarette; 1 small cigar=3 cigarettes; and 1 cigar=4 cigarettes. For cigarette smokers, weighted daily cigarette consumption (WDC) was defined as the number of cigarettes smoked per day multiplied by a coefficient reflecting the relative nicotine content of the brand smoked. The coefficient was 0.66 if nicotine content in one cigarette was 0.8–1.2 mg; 1 if nicotine content was 1.2–1.6 mg; and 1.33 if nicotine content was >1.6 mg. Weighted pack-years consumption (WPC) was WDC times years of smoking divided by 20. Calculations concerning WDC and WPC only applied to cigarette smokers. Pack-years consumption (PY) was defined for all smokers as duration of smoking multiplied by the number of cigarettes smoked per day and divided by 20.

Tobacco abstinence was controlled by measuring the concentration of carbon monoxide in expired air (Ecolyzer CO-monitor, Hawthorne, New York, USA) [25].

Blood samples and allergological examinations

A serum sample was obtained from all participants before smoking cessation, and after 3, 6 and 12 months from quitters and persons in the smoking reference group. Serum was sampled after double centrifugation and stored at -20°C until analysed.

s-ECP and s-LF were determined using methods described previously. Sensitivity of both methods was 1 $\mu g \cdot l^{-1}$. Median (range) between-assay variation (reproducibility)

for s-ECP was 3.8 (2–8)% and for s-LF 4.1 (3–7)% [26, 27].

Blood leucocyte counts were measured with a Coulter counter S (Coulter Electronics, Hialeah, FL, USA), and blood differential counts were determined by counting 200 cells in a smear stained with May-Grünwald Giemsa solution.

All participants had an allergological examination, which included skin prick tests (SPT) (Soluprick, ALK-Laboratories, Copenhagen, Denmark) to: birch, timothy and mugwort pollen, and horse, cat, dog and guinea-pig dander, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* (all 10 histamine equivalent prick (HEP)), *Alternaria tenuis* (1:20 w/v), *Cladosporium herbarum* (1:20 w/v) and three storage mites (*Acarus siro*, *Lepidoglyphus destructer* and *Tyrophagus putrescentia*, all in concentration 10,000 NE·ml^{-1}). Histamine 10 mg·ml^{-1} was used as positive and the solvent as negative control. A positive reaction was defined as a wheal of at least half the size of the histamine reaction. For all reactions with size greater or equal to the negative control, a test for allergen specific serum immunoglobulin E was performed (Phadebas radio allergosorbent test (RAST), Pharmacia Diagnostic AB, Uppsala, Sweden). Persons with a positive skin test or with a RAST ≥0.35 kU·l^{-1} were excluded. All participants filled out questionnaires concerning allergic, asthmatic, and respiratory symptoms, such as occurrence of wheeze, cough and expectoration. Symptoms were graded as none, moderate or severe.

Lifetime nonsmokers

Lifetime nonsmokers underwent clinical examinations and answered questionnaires similar to those of the smokers, with the exception of smoking related questions.

Ethics

All participants gave their informed consent. The study had the approval of the Ethics Committee of Aarhus county.

Statistics

The BMDP statistical software package [28] was employed in all calculations. Comparisons of demographic details between groups were performed with Pearson's Chi-squared and Student's t-test. Student's t-test was used for comparisons of group means, and the paired t-test along with tests for repeated measures were used to evaluate variations in s-ECP and s-LF after smoking cessation. The technique of standardized residuals of FEV_1 and FVC was used to evaluate lung functions [29]. The standardized residual is derived by subtracting the predicted from the observed value of a specific lung function test and dividing the result by the residual standard deviation. Expressions of lung function data as standardized residuals are unbiased from age, height and sex, and superior to other expressions of lung function regarding statistical analyses.

A normal distribution of s-ECP and s-LF was obtained by employing natural logarithms to their value. In the text, geometric mean values and 25% (Q1) and 75% (Q3) quartiles are presented.

Results

Eosinophil cationic protein (ECP)

Geometric mean (Q1, Q3) s-ECP in smokers was 15.0 (8.3, 27.3) µg·l^{-1} compared to 7.2 (4.2, 16.0) µg·l^{-1} in lifetime nonsmokers ($p<0.0005$) (fig. 1). In smokers generally, the level of s-ECP was independent of smo-king history. In cigarette smokers with WDC ≤25 (n=52), geometric mean s-ECP was 13.0 (8.3, 26.0) µg·l^{-1}, compared to 22.6 (8.1, 48.5) µg·l^{-1} in cigarette smokers with greater consumption ($p<0.05$) (fig. 2). The association between WDC and s-ECP was linear ($r=0.24$; $p<0.05$). In female smokers (n=44) geometric mean s-ECP was 12.9 (6.4, 21.8) µg·l^{-1}, compared to 20.1 (8.3, 33.5) µg·l^{-1} in male smokers ($p<0.005$). Considering the influence of gender and WDC on s-ECP the influence from gender was lost and the influence of WDC was strengthened ($p<0.005$).

In 28 smokers with FEV_1 residuals decreased more than 1 SD, geometric mean s-ECP was 10.9 (6.5, 20.0) µg·l^{-1}. This was significantly lower ($p<0.05$) compared to 70 smokers with FEV_1 residuals within the 95% confidence limits who had s-ECP of 22.6 (8.1, 48.5) µg·l^{-1}. Ln(s-ECP) had a linear relationship with FEV_1 residuals ($r=0.24$; $p<0.01$) and accounted for 6.2% of the variation in FEV_1 residuals (fig. 3). s-ECP had no association with airway symptom scores. Eosinophil count and total number of blood eosinophils had no association with FEV_1 residuals. Ln(s-ECP) showed a linear association with the eosinophil count ($r=0.34$; $p<0.001$). Mean age, smoking history and sex distribution were comparable for smokers with decreased and normal FEV_1 residuals.

Lactoferrin

Geometric mean (Q1, Q3) s-LF in smokers and lifetime nonsmokers was 445.8 (293.8, 670.5) µg·l^{-1} and 290.9 (230.2, 435.3) µg·l^{-1}, respectively, ($p<0.0001$) (fig. 1). S-LF was independent of smoking history (fig. 2), age and gender and had no association with FEV_1 residuals and airway symptoms. s-LF was linearly related to the total number of neutrophils ($p<0.0001$). The total number of neutrophils showed a trend towards a negative association with FEV_1 residuals ($0.05<p<0.1$).

s-ECP, s-LF and lung function

s-ECP and s-LF correlated with each other both in smokers ($r=0.56$; $p<0.0001$) and in lifetime nonsmokers

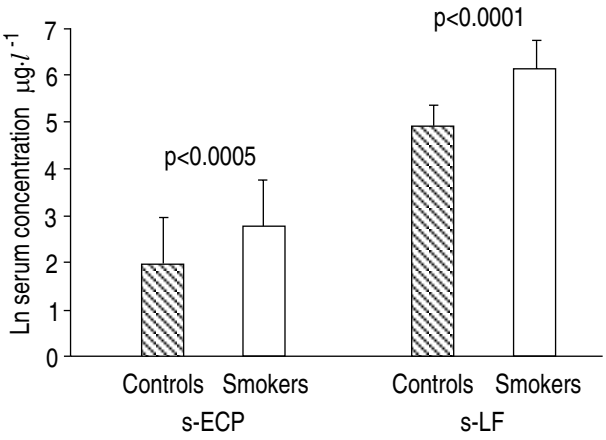

Fig. 1. – Natural logarithm (ln) of serum eosinophil cationic protein (s-ECP) and serum lactoferrin (s-LF) for 31 nonsmokers (controls) and 98 smokers. All participants were nonatopic, had no signs or history of asthma, and were otherwise healthy. Columns represent means and bar lines represent 1 SEM. p is the significance of the difference between groups.

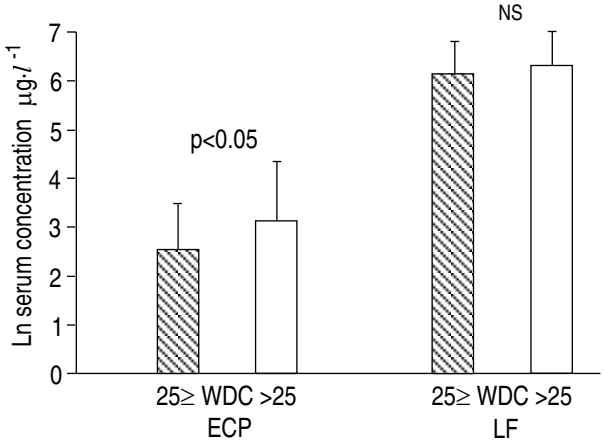

Fig. 2. – Natural logarithm (ln) of serum eosinophil cationic protein (s-ECP) and serum lactoferrin (s-LF) in 98 smokers grouped according to weighted daily cigarette consumption (WDC) ≤25 and >25. For definition of WDC see text. All participants were nonatopic, had no signs or history of asthma, and were otherwise healthy. Columns represent means and bar lines represent 1 SEM. p is the significance of the difference between groups.

(r=0.51; p<0.0001). The correlation among lifetime nonsmokers, smokers with decreased FEV_1 residuals, and smokers with normal FEV_1 residuals was similar.

In a multiple regression analysis, both s-ECP (p<0.001) and s-LF (p<0.05) contributed to prediction of the regression equation for FEV_1 residuals. The coefficient±SD was 0.7±0.2 for s-ECP and -0.6±0.3 for s-LF.

S-ECP and s-LF together accounted for 10.2% of the variation in FEV_1 residuals.

s-ECP and s-LF in smokers compared to lifetime nonsmokers

For each smoker, d-LF and d-ECP was defined as the percentage increase from the geometric means of s-LF and s-ECP in lifetime nonsmokers. These figures did not follow a Gaussian distribution.

The overall median (Q1, Q3) d-ECP was 123 (13.5, 280.0)% and d-LF was 38.5 (10.0, 130.5)% (p<0.0001). In smokers with decreased FEV_1 residuals the median d-ECP and d-LF were 63.5 (-9.2, 179.3)% and 38.9 (0.9, 144.3)%, respectively, (NS). The corresponding figures in smokers with normal FEV_1 residuals were 161.2 (15.2, 367.9)% and 37.7 (0.0, 133.1)%, respectively, (p<0.001). d-ECP was different in smokers with normal and decreased FEV_1 residuals (p<0.01). d-LF was similar in the two groups.

Blood leucocytes in smokers and lifetime nonsmokers

d-EOS, d-NEU and d-LEU were defined for each smoker in the same way as d-ECP and d-LF for the total eosinophil count, total neutrophil count and total leucocyte count, respectively. The medians (Q1, Q3) for d-EOS, d-NEU, and d-LEU, respectively, were 11.7

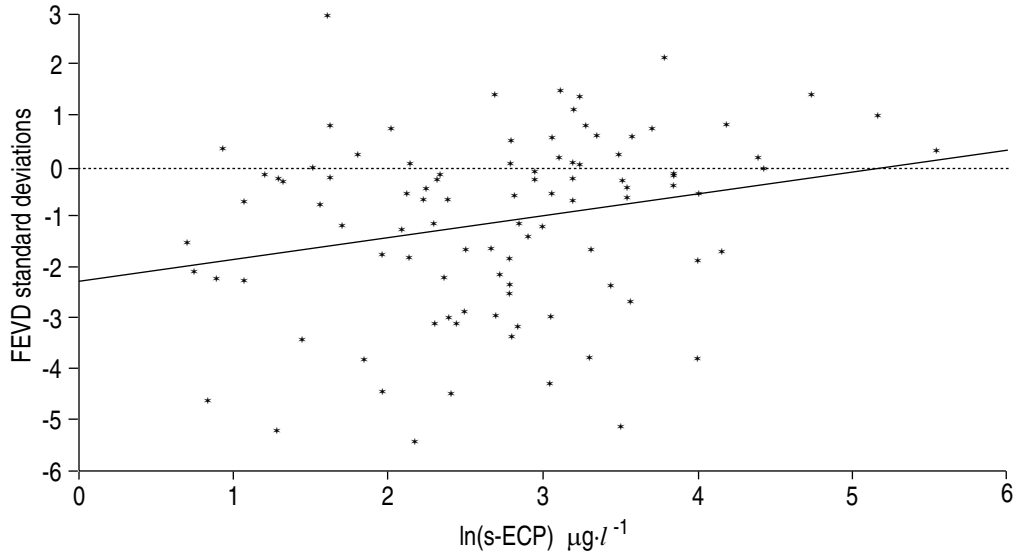

Fig. 3. – Linear regression analysis of the natural logarithm of serum eosinophil cationic protein (ln(s-ECP)) and standardized FEV_1 residuals (FEVD) in smokers. The regression equation was: FEVD=-2.3+0.44×(ln(s-ECP)) (p<0.01). FEV_1: forced expiratory volume in one second.

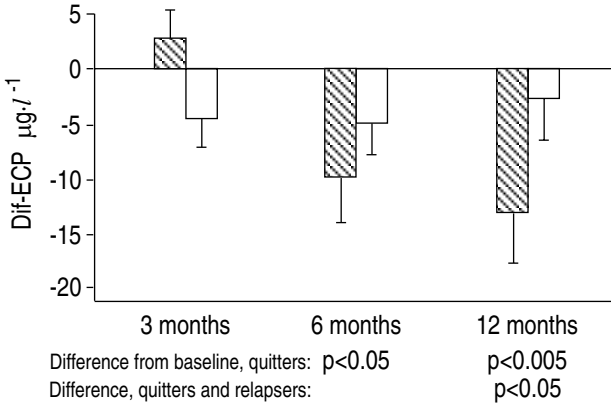

Fig. 4. – Dif-ECP is the difference in serum eosinophil cationic protein (s-ECP) from baseline at different time-points as indicated by month. Columns represent means and the bar lines represent 1 SEM. Fifty persons who remained tobacco abstinent throughout 1 yr (quitters) are represented by hatched bars and 48 persons with smoking relapse within 6 weeks after the start of the trial (relapsers) are represented by open columns. Dif-ECP was significant at 6 ($p<0.05$) and 12 ($p<0.005$) months in quitters, and was different in quitters compared to relapsers at 12 months ($p<0.005$).

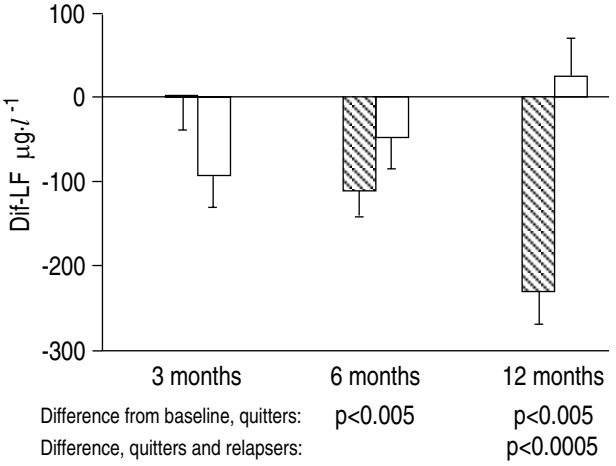

Fig. 5. – Dif-LF is the difference in serum lactoferrin (s-LF) from baseline at different time-points as indicated by month. Columns represent means and the bar lines represent 1 SEM. Fifty persons who remained tobacco abstinent throughout 1 year (quitters) are represented by hatched columns, and 48 persons with smoking relapse within 6 weeks after the start of the trial (relapsers) are represented by open columns. Dif-LF was significant at 6 ($p<0.005$) and 12 months ($p<0.0001$) in quitters, and was different between quitters and relapsers at 12 months ($p<0.0005$).

(-21.9, 68.1)%, 32.0 (3.6, 68.4)% and 26.0 (5.0, 54.0), and were of similar magnitude. d-EOS was independent of FEV_1 residuals. d-NEU showed a trend towards an inverse association to FEV_1 residuals ($0.05<p<0.1$). d-ECP ($p<0.005$) and d-LF ($p<0.01$) were larger than d-EOS and d-NEU, respectively.

Variation in s-ECP and s-LF during 1 yr after smoking cessation

To evaluate the variation in s-ECP and s-LF after cessation of smoking, we defined dif-ECP and dif-LF as the difference in s-ECP and s-LF from baseline at 3, 6 and 12 months. These figures followed a Gaussian distribution.

The mean±SEM dif-ECP was significant in 50 quitters at 6 ($p<0.05$) and at 12 months ($p<0.005$); the level of s-ECP remained unchanged throughout one year in 48 relapsers (fig. 4). Dif-ECP at 1 yr in quitters was -13.1±4.5 $\mu g \cdot l^{-1}$, compared to -2.7±3.9 $\mu g \cdot l^{-1}$ in relap- sers ($p<0.005$, χ^2-test). The variation in s-ECP after smoking cessation was independent of smoking his- tory, FEV_1 residuals, airway symptom scores, age and gender.

In the 50 quitters, dif-LF was significant at 6 months ($p<0.005$) and 1 yr ($p<0.00001$), whereas, no significant change in s-LF was observed in the 48 relapsers during the observation year (fig. 5). In quitters and relapsers, respectively, dif-LF at 1 yr was -230.7±35.5 $\mu g \cdot l^{-1}$ and 25.2±58.7 $\mu g \cdot l^{-1}$ ($p<0.0005$) (fig. 5). The variation in s-LF after the cessation of smoking was not associated to smoking history, age, gender or FEV_1 residuals.

Discussion

The present study evaluated the influence of smoking on s-ECP and s-LF, the relationship between s-ECP, s-LF, lung function and airway symptoms in smokers, and the variation in s-ECP and s-LF after smoking cessation.

Serum concentrations of ECP and LF were higher in smokers compared to lifetime nonsmokers, and s-ECP correlated to the daily consumption of cigarettes in a linear fashion. There was a weak, inverse, linear correlation between s-ECP and FEV_1 residuals. The combined expression of s-ECP and s-LF could explain 10% of the variation in FEV_1 residuals, which seems biologically important. After smoking cessation, s-ECP and s-LF decreased significantly within 6 months.

We confirmed that the number of eosinophils in peripheral blood was not associated to decreased lung function in smokers [8–10, 30]. The previously reported association between the blood neutrophil count and a decreased FEV_1 [17] was supported, although this correlation did not reach statistical significance in the present study. The size of the study population may have influenced our results concerning blood leucocyte counts and lung function. Both measures present large variations, and evaluation requires a large study population. The correlation between s-ECP and the blood eosinophil count was in accordance with previous studies [26]. In the present study, we found a close correlation between the blood neutrophil count and s-LF. Our study confirmed an influence of comparable size of smoking on the blood leucocyte, neutrophil and eosinophil counts [9]. The magnitude of the smoking-induced increase in neutrophils and eosinophils was similar to results reported previously [31].

The magnitude of the increase in s-LF and, especially, in s-ECP in smokers compared to lifetime nonsmokers was larger than the increases in neutrophil and eosinophil counts. This was probably the result of the combination of an increase in the number of these cells and an enhanced secretory activity of the granulocytes in

smokers compared to lifetime nonsmokers, and an expression of an activation of the cells. A chronic inflammation in the lower airways occurs in most smokers [32]. s-ECP and s-LF may be an indirect measure of substances enhancing granulocyte production and activation, and may better reflect a chronic inflammation than the leucocyte counts *per se*. The serum concentration of the granule proteins may represent a functional measure of granulocyte activity involving an enhanced secretory response. The activated granulocytes may eventually gather in the lungs of smokers [18, 33], and an increased secretory response is not reflected in the peripheral leucocyte counts.

Eosinophil granulocytes may be activated in adult respiratory distress syndrome and the increase in ECP in bronchoalveolar lavage (BAL) fluid and serum relate to indices of lung damage [13]. Our results concerning a relationship between high levels of s-ECP and high FEV_1 residuals were, therefore, unexpected. An imbalance in eosinophil and neutrophil counts and activities may occur in some smokers. The difference in the percentage increase in s-ECP and s-LF resulted mostly from a relatively small increase in s-ECP in smokers with decreased FEV_1 residuals. The rather modest correlation between s-ECP and s-LF, with about 30% of the variation of one variable explained by the other, indicated an advantage of evaluating the influence on lung function of s-ECP and s-LF simultaneously rather than in isolation. The better prediction of FEV_1 residuals obtained in this way indicated that a low s-ECP and a high s-LF was correlated to a low FEV_1 residual. This suggests a protective role of the eosinophil towards lung damaging products from neutrophils [34, 35] or other sources.

There is, to our knowledge, no direct support in the literature for a theory of opposing effects of neutrophil and eosinophil granulocytes in smokers. Our results, however, were not contradictory to earlier studies. The blood eosinophil count has been related to an increased lung function deterioration in nonsmokers but not in smokers [8, 9]. This may be caused by a stimulus dependent secretory response [36], or by a modulatory role of the eosinophils, as seen in some hypersensitivity and inflammatory reactions in the lungs [6, 37–39, 40]. Asthma-like bronchitis or chronic airflow limitation with some degree of reversibility has been related to elevated levels of blood eosinophils [8], and bronchial hyperreactivity and reversibility of bronchial obstruction have independent influences on the course of lung function [41]. A prospective study that followed 117 smokers [42, 43] showed a lower annual decline in FEV_1 in smokers with an asthma-like bronchitis, compared to smokers with an emphysematous form of bronchitis. The former group also had higher blood eosinophil counts. It is possible that the eosinophil can act not only as a potential tissue damager but may also, as in the case of smokers, have protective or modulating functions. The influence of eosinophils on pathophysiological processes seem dependent on the type of inflammation and network of cells present.

The levels of s-ECP correlated with the daily tobacco consumption but not to duration of smoking or pack-years consumption. This indicates that the smoking-induced increase in s-ECP is an acute or subacute response. The relationship between s-ECP and daily cigarette consumption adjusted for nicotine content of the brand smoked could be caused by a direct influence of nicotine and tar constituents on the inflammatory regulator cells, and may reflect a dose-related response. s-LF was not associated with FEV_1 residuals, smoking history, or demographic data. s-LF correlated closely with neutrophil counts and was raised in smokers. The reason for the independency of s-LF to other variables may have been a great variance.

After smoking cessation, no association was seen between changes in s-ECP or s-LF and FEV_1 residuals, but this was probably caused by a large variation in both s-ECP, s-LF and FEV_1 after smoking cessation. The decrease in serum concentrations of ECP and LF in recent ex-smokers supported the fact that smoking was responsible for the increased levels of the proteins in smokers.

Our study population was comprised of nonatopic, otherwise healthy adults who had no evidence of present or recent infection and used no medication. Parasitic diseases are extremely rare in Denmark and common causes for elevated blood eosinophil and neutrophil counts were negligible. The group of lifetime nonsmokers was small, which limited an evaluation of the influence of s-ECP and s-LF on lung function. Smokers with decreased and normal FEV_1 residuals were comparable with respect to age, gender and smoking history. It was, therefore, not necessary to control the influence of s-ECP and s-LF on the lung function for an influence from smoking history. Furthermore, high tobacco consumption was associated to high s-ECP and decreased lung function to low s-ECP.

s-ECP and s-LF may reflect tobacco-induced bronchial inflammation and represent an alternative method of monitoring bronchial inflammation in smokers. We speculate that s-ECP and s-LF may contribute to the prediction of lung function deterioration in smokers, as they accounted for about 10% of the variation in FEV_1 residuals with a combination of low s-ECP and high s-LF associated to decreased FEV_1 residuals.

The relationships between s-ECP, s-LF and lung function in this study were weak and the study population was small. The risk that our results were due to chance cannot, therefore, be overlooked. To establish the proposed relationships, these should be reinvestigated in a large prospective study.

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