

# Human Neutrophil Peptides sputum levels in symptomatic smokers and COPD patients

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**Abstract. – Background and Objectives:** Human Neutrophil Peptides (HNP) are major neutrophils' products which may contribute to the airway inflammation and lung remodelling during chronic obstructive pulmonary disease (COPD). We aimed to assess whether HNP sputum concentrations could be used as indicators of airway inflammation and progression towards pulmonary functional impairment, and correlate with the degree of airways obstruction.

**Materials and Methods:** We measured, by ELISA tests, HNP concentrations from 37 symptomatic smokers and 34 COPD patients. All participants underwent pulmonary function tests. Sputum samples were collected at the enrolment, and 6 months after smoking cessation. Differences between groups and correlation coefficients between variables were determined using non parametric tests.

**Results:** Sputum HNP concentrations were higher in COPD patients as compared to symptomatic smokers ( $14 \pm 1.5 \mu\text{g/ml}$  vs  $1.6 \pm 0.4 \mu\text{g/ml}$ ;  $p < 0.0001$ ). Among COPD patients HNP concentrations were higher in individuals with severe obstruction than in patients with mild to moderate COPD ( $19.9 \pm 2.3 \mu\text{g/ml}$  vs  $10.3 \pm 0.8 \mu\text{g/ml}$ ,  $p = 0.003$ ). A negative correlation was observed between HNP levels and FEV<sub>1</sub> ( $\rho = -0.38$ ,  $p = 0.02$ ), and FEV<sub>1</sub>/FVC ( $\rho = -0.42$ ,  $p = 0.01$ ). No differences were found in HNP levels before and after 6 months of smoking withdrawal ( $1.1 \mu\text{g/ml} \pm 0.3$  vs  $1.1 \mu\text{g/ml} \pm 0.3$  for symptomatic smokers,  $p = 0.9$ , and  $14.4 \mu\text{g/ml} \pm 1$  vs  $16 \mu\text{g/ml} \pm 1.1$  for COPD,  $p = 0.6$ ).

**Discussion:** Sputum levels of HNP may represent a marker of severity of functional impairment in COPD. Our data support the hypothesis that HNP may have a role in smoking- and COPD-related lung inflammation.

## Key Words:

Airway inflammation, Chronic obstructive pulmonary disease, Human neutrophil peptides, Smoking, Sputum.

## Abbreviations

$\alpha$ 1-AT =  $\alpha$ 1-antitrypsin

BAL = Bronchoalveolar Lavage

COPD = Chronic Obstructive Pulmonary Disease

ECP = Eosinophilic-Cationic Protein

FEV<sub>1</sub> = Forced Expired Volume in one second

FEV<sub>1</sub>/FVC = FEV<sub>1</sub> expressed as a percentage of FVC

HNP = Human Neutrophil Peptides

IL-6 = Interleukin-6

IL-8 = Interleukin-8

IL-10 = Interleukin-10

MMPs = Matrix Metalloproteinases

MPO = Myeloperoxidase

StcO<sub>2</sub> = Transcutaneous oxygen saturation

TIMPs = Tissue Inhibitors of Metalloproteinases

TNF- $\alpha$  = Tumor Necrosis Factor- $\alpha$

## Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a major cause of chronic morbidity and mortality throughout the world, and remains an impor-

tant public health problem. It is a complex and heterogeneous respiratory disease characterized by chronic airflow limitation generally not fully reversible and progressive over time.

It is widely recognized that the prolonged exposure to harmful factors, such as cigarette smoke, induces a sustained inflammatory response which contributes to the airway damage and remodelling leading to the airflow obstruction characteristic of the disease<sup>1,2</sup>. The degree of this inflammatory response correlates with the grade of airways obstruction<sup>3,4</sup> and with the prognosis of patients with COPD<sup>5</sup>.

Cigarette smoking has been reported to be associated with an increased number of neutrophils in the lung, suggesting their involvement in smoking-related lung inflammation<sup>6</sup>.

Defensins are small (29-47 amino acids) multi-functional cationic peptides that form part of both the innate and adaptive immune responses. Human Neutrophil Peptides (HNP) are major constituents of neutrophil azurophilic granules (30-50% of the total protein content) and in addition to antimicrobial and cytotoxic activities, modulate cell proliferation, induce cytokine production, and are chemotactic for T lymphocytes<sup>7,8</sup>.

Nearly absent in the lung of healthy individuals, HNP have been found increased in airways of patients with cystic fibrosis<sup>9</sup>,  $\alpha$ 1-antitrypsin ( $\alpha$ 1-AT) deficiency<sup>10</sup>, and other neutrophil-related inflammatory lung diseases<sup>11,12</sup>. Recently the presence of HNP has been demonstrated in the respiratory tract of current smokers<sup>13</sup>.

Aim of our study was to assess whether the levels of HNP in sputum of smokers with COPD could be used as markers of airway inflammation and could be correlated with the degree of airways obstruction. In addition, in order to investigate the effect of smoking cessation on airway inflammation, we collected sputum samples from the subjects who quit smoking.

## Patients and Methods

### Patients

A population of current smokers with and without COPD (according to the Global Initiative for Chronic Obstructive Lung Disease, GOLD, guidelines<sup>1</sup>) was referred to our smoking cessation centre or underwent pulmonary function tests for respiratory symptoms. Inclusion criteria were: age >18, a smoking habit of  $\geq$ 10 cigarettes

per day for at least 2 years, a self-assessed intention to quit smoking, no sign of atopy and no respiratory tract infection for at least 30 days prior to the study. A total of 71 subjects were included in the study and were stratified in 2 groups: (1) symptomatic smokers, subjects with chronic respiratory symptoms, *i.e.* chronic cough and sputum production, for at least 3 months for 2 successive years, and a FEV<sub>1</sub>/FVC post-bronchodilator  $\geq$ 0.7, with a FEV<sub>1</sub>% predicted  $\geq$ 80%; (2) COPD patients having FEV<sub>1</sub>/FVC post-bronchodilator <0.7.

According to GOLD guidelines, the severity of COPD was based on FEV<sub>1</sub>% predicted post-bronchodilator: (1) stage I, mild COPD, with a FEV<sub>1</sub> >80%; (2) stage II, moderate COPD, with 50%  $\leq$  FEV<sub>1</sub> <80%; (3) stage III, severe COPD, with 30%  $\leq$  FEV<sub>1</sub> <50%; (4) stage IV, very severe COPD, FEV<sub>1</sub> <30%, or 30%  $\leq$  FEV<sub>1</sub> <50% plus chronic respiratory failure.

During the study subjects only used long-acting or short-acting  $\beta$ <sub>2</sub>-agonists or anticholinergics; corticosteroids were not allowed and patients who during the study needed to use oral or inhaled corticosteroids were excluded from further evaluation.

A complete smoking history, including smoking habits and number of cigarettes per day was recorded.

All individuals underwent an identical smoking cessation intervention and received face to face behavioural counselling with or without a specific pharmacological treatment. Behavioural counselling sessions were performed by trained physicians and experienced nurses on a weekly basis during a three month period. Pharmacological treatment, dosage and delivery systems were based on daily cigarette consumption and on nicotine dependence.

The Ethical Committee approved the protocol (no. 584/CE), and all the participants agreed by signing the informed consent.

### Follow-up

Current smoking status was investigated weekly during counselling. After the treatment phase, participants were evaluated monthly in order to reach a 6 month analysis period. Self-assessed smoking cessation was validated by measuring carbon monoxide concentration in expired air. Sustained quitters were defined as participants whose self-assessed continuous smoking cessation, at each evaluation, was validated by carbon monoxide (CO) concentration in exhaled air  $\leq$ 10 ppm.

Current smokers were considered individuals who admitted smoking and/or whose exhaled CO levels were  $>10$  ppm<sup>14</sup>.

Individuals who did not attend follow-up visits or evaluations were considered as treatment failures and as assumed smokers.

### **Pulmonary Function Tests and Transcutaneous Oxygen-Haemoglobin Saturation**

Spirometric measurements (Quark PFT Cosmed, Pavona, Italy) were performed by experienced personnel following the American Thoracic Society and European Respiratory Society recommendations and the reference values were those of the European Community for Coal and Steel approved by the European Respiratory Society<sup>15</sup>. Bronchodilator responsiveness to 400 mcg salbutamol was measured and post-bronchodilator values were used<sup>1</sup>.

Transcutaneous oxygen saturation (StcO<sub>2</sub>) was measured by a pulse oximeter (Respironics, Marietta, GA, USA).

### **Sputum Processing and HNP Measurement**

Sputum samples were obtained with permission from all the individuals at the enrolment, and 6 months after smoking cessation from the subjects who quit smoking.

Samples with less than 70% of squamous epithelial cells were considered adequate and accepted for further assessments<sup>16</sup>.

Samples were diluted with two volumes of phosphate-buffered saline (PBS), vortexed, and then centrifuged at  $8,000 \times g$  for 20 minutes; supernatants were stored at  $-40^{\circ}\text{C}$  until protein quantification assays were performed.

Supernatants were thawed, diluted 1:1000 and then sputum HNP concentrations were quantified by using a commercially available ELISA kit with a sensitivity less than 41 pg/ml, following manufacturer instructions (Cell Sciences, Canton, MA, USA).

### **Statistical Analysis**

Data are shown as means  $\pm$  standard error of mean. Differences in sputum HNP values between groups were analysed using the Mann Whitney U test. In the group of individuals who successfully quit smoking, differences in HNP levels at baseline and after 6 months were analysed by the Wilcoxon signed-rank test.

Correlation coefficients between variables were determined by the Spearman's rank correlation analysis. Values of  $p < 0.05$  were considered significant.

Statistical analyses were performed by using SPSS 15 software (Statistical Package for the Social Sciences Inc, Chicago, IL, USA).

## **Results**

### **Patients' Characteristics**

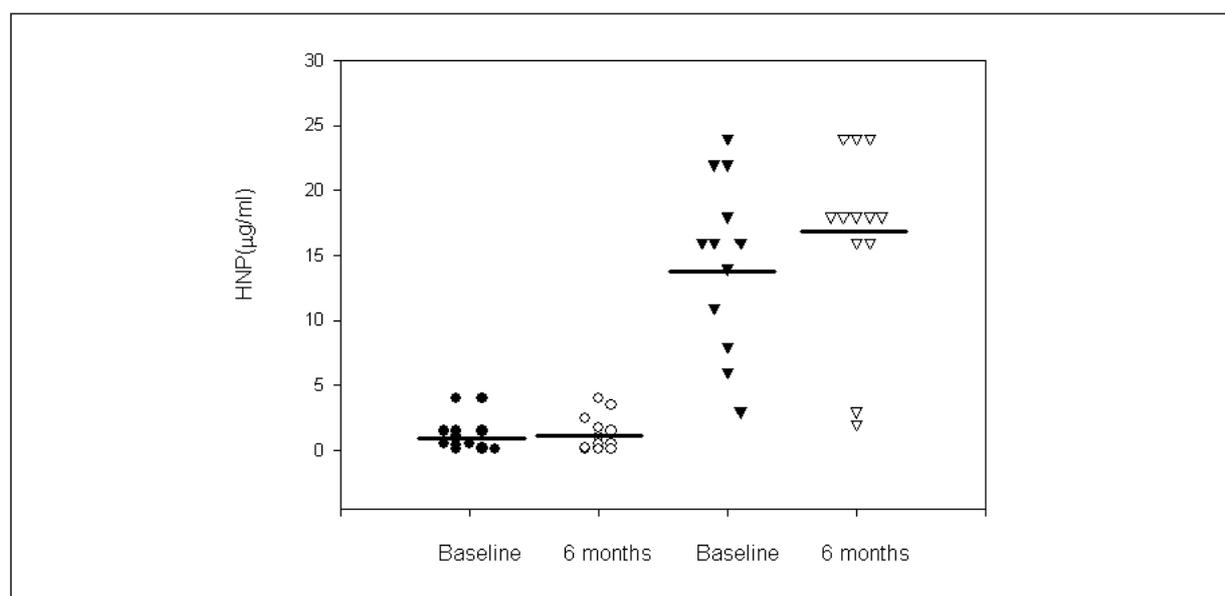
We enrolled in the study 71 smokers, 37 were symptomatic smokers with no airway obstruction, 21 individuals had mild to moderate functional impairment and 13 subjects had severe COPD. None of the patients fulfilled the criteria for GOLD stage IV. Baseline characteristics of individuals enrolled in the study are shown in Table I.

**Table I.** Baseline characteristics.

Variables	Symptomatic smokers (n = 37)	COPD (n = 34)	Significance
Gender (M/F)	22/15	20/14	0.93
Age (Y)	50.3 $\pm$ 1.9	55.3 $\pm$ 2.5	0.1
Cigarette/d	26.3 $\pm$ 2.3	29 $\pm$ 2.6	0.4
Pack-years	38.7 $\pm$ 5.2	46.7 $\pm$ 5.7	0.3
Years of smoking	27.9 $\pm$ 1.9	31.8 $\pm$ 2.4	0.2
FEV <sub>1</sub> (% of predicted)	88.2 $\pm$ 1.8	57 $\pm$ 3.6	< 0.0001
FEV <sub>1</sub> /FVC (%)	86.4 $\pm$ 1.2	59 $\pm$ 2	< 0.0001
StcO <sub>2</sub>	97.7 $\pm$ 1.1	94.1 $\pm$ 1.1	< 0.0001

Values are expressed as mean  $\pm$  SEM. FEV<sub>1</sub> = Forced Expired Volume in one second; FEV<sub>1</sub>/FVC = FEV<sub>1</sub> expressed as a percentage of Forced Vital Capacity; COPD = Chronic Obstructive Pulmonary Disease; StcO<sub>2</sub> = Transcutaneous oxygen-haemoglobin saturation at rest.





**Figure 2.** Effects of smoking cessation on Human Neutrophil Peptides sputum concentrations: Symptomatic smokers at baseline (●) and after 6 months (○) and COPD patients at baseline (▼) and after 6 months (◄).

(TNF)- $\alpha$ <sup>2,22,30</sup>, and Eosinophilic-Cationic Protein (ECP)<sup>20,21,23</sup> have been evaluated, with the intent to assess whether their levels, in sputum and/or bronchoalveolar lavage (BAL) fluid, are increased in smokers and COPD patients, and correlate with the degree of airflow obstruction.

HNP are small molecular weight polypeptides which are abundant in neutrophils. Although most of HNP activity takes place in neutrophil vacuoles, activated neutrophils may release up to 10% of their HNP content in the extracellular environment<sup>34</sup>. In a previous report we found increased levels of HNP in BAL from smokers<sup>13</sup>, but no correlation analysis between HNP levels and functional impairment was performed. Cigarette smoking has been reported to be associated with an increased number of neutrophils in the lung; defensins may contribute to the pathogenesis of COPD by amplifying the smoking-induced airway inflammation which contributes to lung injury.

In the present study sputum HNP levels were significantly higher in COPD patients with severe airway obstruction as compared to individuals with mild to moderate functional impairment and with symptomatic smokers.

This may suggest that HNP could be involved in the development of the functional impairment or may reflect the airway obstruction.

The HNP levels we found in COPD smokers sputum may contribute directly or indirectly to lung damage. Indeed, the levels measured in sputum from severe COPD patients exceed the concentrations reported to be toxic for airway epithelial cells, pulmonary fibroblasts and alveolar macrophages<sup>10,13,35</sup>. Although *in vitro* HNP levels found in COPD sputum appear to be cytotoxic, their activity *in vivo* remains to be determined, because it is possible that some or even most of their effects may be partially mitigated by the binding to  $\alpha$ 1-AT, mucopolysaccharides and nucleic acids.

Defensins may also promote airway damage by means of indirect mechanisms. The average HNP levels we observed in symptomatic smokers and in mild COPD may contribute to neutrophils recruitment by stimulating alveolar macrophage's release of chemotactic molecules (e.g. LTB<sub>4</sub> and IL-8)<sup>10</sup> or may exacerbate the imbalance between  $\alpha$ 1-AT and neutrophil elastase by inactivating  $\alpha$ 1-AT<sup>36</sup>.

In our study we observed a significant correlation between HNP levels and functional impairment, while previous reports found similar results for IL-8 and HNE<sup>2,4,17-23,37</sup>.

The observation that the concentrations of IL-8, a potent neutrophil chemoattractant, and HNE and HNP, major products of neutrophil activation

and degranulation, correlate with functional impairment, may be a further evidence of the pivotal role of PMN in the lung damage associated with cigarette smoke and with COPD.

Finally, with the aim to identify new molecules that could trace the effect of smoking cessation on airway inflammation in individuals with and without COPD, we organized a smoking cessation programme and designed a longitudinal study to analyse whether smoking withdrawal may reduce HNP concentration, as an effect of the decreased inflammatory status.

According to what previously showed by Willemse et al<sup>21</sup>, who studied IL-8 and ECP concentrations in a similar number of patients, we found no decrease of HNP levels in sputum samples of smokers who successfully quit smoking. The explanation for this finding may be not univocal. It may reflect the persistence of lung inflammation after smoking cessation or it may mirror a healing of defence mechanisms after recovering from the deleterious effects of smoking.

In conclusion, to the best of our knowledge, this is the first report investigating the role of defensins in the chronic inflammation and pulmonary functional impairment that occur during smoking and COPD and also the first longitudinal study designed to analyse the effects of smoking cessation on HNP sputum levels. We believe that our data support the hypothesis that HNP, a major product of neutrophils, may play a role in smoking- and COPD-related lung inflammation. Our study may prompt further investigations on the potential ability of sputum biomarkers to monitor disease progression in patients with COPD.

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