Abstract. — Background: Endothelin-1 (ET-1) is a potent vasoconstrictor and bronchoconstrictor but it has been shown to have also proinflammatory properties. Its ability to attract inflammatory cells in its site of production, upregulates the synthesis of adhesion molecules and stimulates the release of cytokines. The fact that cytokines have the ability to induce its synthesis and release, creates a dynamic loop for self-preservation and augmentation of the airway inflammation in Chronic Obstructive Pulmonary Disease (COPD), even after the ceasing of the noxious stimulus i.e. cigarette smoke. Therefore, functional polymorphisms that may lead to increased levels of ET-1 may also cause an increased susceptibility to COPD development.

Materials and Methods: We analyzed the longitudinal effect on lung function of two ET-1 gene polymorphisms in a population of 190 smokers (95 non-COPD and 95 COPD smokers). The two polymorphisms involved an insertion polymorphism (+138 adenine insertion 3A/4A, 138bp downstream from the transcription start site, exon 1) and a single nucleotide transversion polymorphism on exon 5 (G/T, Lys198Asn). A total of 190 subjects were enrolled in the study for each polymorphism and were followed for 3 years by annual spirometry sessions.

Results: The adjusted annual decline of forced expiratory volume in 1 second (dFEV₁) was greater for those having at least one copy of the mutated gene ins/delA compared to those with the wild type allele both in the non-COPD smokers group (mean difference in dFEV₁, of 19.4 ml/year, \( p = 0.004 \)) and COPD smokers (mean difference in dFEV₁, of 11.15 ml/year, \( p = 0.003 \)). On the contrary, those heterozygous for the Lys198Asn polymorphism were found to have a slower decline in FEV₁ compared to those homozygous for the wild type allele. The non-COPD smokers group had a gain-in-loss of 11.24 ml/year (\( p < 0.001 \)) while the COPD-smokers group had a slower decline of 11.42 ml/year (\( p = 0.002 \)). Those homozygous for the polymorphisms examined show an even greater deviation from those with the wild type allele but due to the small number comprising their group, the results don’t have enough statistical power. Though, they still show the trend of the effect the polymorphisms have on annual FEV₁ decline.

Conclusions: The present data shows that ET-1 and its functional polymorphisms may be implicated in COPD phenotype and severity.

Key Words: Endothelin-1, Polymorphisms, COPD, FEV₁, FEV₁ decline

Abbreviations

COPD = Chronic Obstructive Pulmonary Disease
SNP = Single Nucleotide Polymorphism,
ET-1 = Endothelin-1,
ppET-1 = Preproendothelin-1

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a major and increasing health problem. It is predicted by the World Health Organization to become the third most common cause of death and the fifth most common cause of disability in the world by 2020¹.

Although many inflammatory mediators have been identified in asthma², there is much less information about the production and role of mediators in COPD. COPD is a complex inflammatory disease that involves many different types of inflammatory and structural cells, all of which
have the capacity to release multiple inflammatory mediators. Among them, the endothelins, have attracted the interest of many scientific groups first in the area of cardiovascular pathology but there has also been a significant accumulation of data indicating the mediator role of endothelins in a variety of lung disorders. Endothelin, which is synthesized from precursors known as proendothelin (ppET), comprising 212 amino acid residues. The large precursors undergo an intermediate cleavage by endopeptidases to form the 38-amino acid biologically inactive proendothelins, also called big endothelins. Endothelin-converting enzymes are membrane-bound metalloendopeptidases that further cleave proendothelins. The biologically active endothelins are 21-amino acid peptides, with two disulfide bridges joining cysteins in positions 1-15 and 3-11 in the N-terminal half, and a cluster of hydrophobic amino acid residues at the C-terminal end of the structure. The structure of the N-terminal domain determines the affinity to the receptor, while the C-terminal domain contains the binding site of the peptide to the receptor. ET-1 is secreted by endothelial cells, epithelial cells, alveolar macrophages, polymorphonuclear leukocytes, and fibroblasts. Release of endothelins is regulated at the level of gene expression and peptide synthesis because cells do not store endothelins. The expression of the gene is induced by a variety of factors including thrombin, angiotensin II, adrenaline, cytokines, and growth factors. Most data gathered involve endothelin-1 (ET-1), originally isolated from cultured endothelial cells, which has a half-life of approximately 4 to 7 min in the blood because of quick binding to tissues and rapid metabolization by a specific endothelin-degrading enzyme. It is a potent vasoconstrictor, bronchoconstrictor and may stimulate mucus production and edema formation in airways. There are also indications that ET-1 participates in inflammatory reactions in the airways by initiating the influx of inflammatory cells to the airway lumen.

Another potential role of ET-1 is in the perpetuation of the inflammation in COPD. It has been shown that the inflammatory response in COPD can sustain itself, even when the injurious agent, most notably cigarette smoke, has been withdrawn. Therefore, even if one quits smoking, airway inflammation continues to persist. There is an increased concentration of ET-1 in induced sputum and bronchoalveolar lavage fluid of patients with COPD, particularly during exacerbations. Plasma ET-1 concentrations are also elevated in COPD patients, particularly in patients who develop nocturnal hypoxemia during the night.

Individuals with accelerated loss of lung function may be at increased risk for developing clinically significant lung disease, including COPD. The definition of rapid or accelerated rate of decline in lung function varies by Author but has included a rate of decline in forced expiratory volume in 1 second (FEV1) of ≥60 mL/year compared with expected rate of decline of 20 to 30 mL/year in the general population of never smokers without respiratory symptoms or conditions. With sufficiently rapid rates of annual decline in FEV1, some degree of respiratory impairment could potentially occur within 10 to 15 years even in the absence of an obvious symptomatic chronic disease state. Since ET-1 is a factor augmenting inflammation in lung tissue, single nucleotide polymorphisms (SNPs) leading to increased levels of active ET-1 may therefore predispose to a more rapid decline in lung function.

The 2026-bp preproendothelin (ppET) mRNA is encoded by the human 6836-bp ET-1 gene, localized on chromosome 6p24–23, which contains five exons and four intervening sequences. Exon 1 contains the whole 5-untranslated region (268 bp) and the coding sequence for the first 21 amino acid residues of ppET-1. An adenine insertion polymorphism (SNP) is located 138 bp downstream of the transcription start site in the 5′-UTR (Untranslated Region), in exon 1 (+138/ex1ins/delA). Transfection studies using reporter constructs exhibited that this insertion could be responsible for increased ET-1 levels, probably due to increased mRNA stability. A single-nucleotide polymorphism in exon 5, Lys198Asn (G to T transversion, codon 198, position +5665), has also been associated with increased ET-1 blood levels. Therefore, genetic alterations that could potentially increase the levels of the active ET-1 (21-aminoacid peptide) may predispose to COPD.

To test this hypothesis we evaluated whether SNPs of ET-1 are more common amongst COPD patients, compared to normal controls and we also did a follow-up of the same group of patients for a total time of 3 years to see the longitudinal effect of the above mentioned functional polymorphisms on pulmonary function. Therefore, we conducted a prospective study to examine the effects of these polymorphisms on lung function loss and their association with COPD in a Caucaisan Greek population.
Materials and Methods

Subjects

Data were collected in the Pulmonary Department of the Patras University Hospital, Greece. A total of 190 subjects were selected from the general Caucasian population of our community from a total of 850 subjects examined during their visit as outpatients and their respiratory function was assessed during a 3-year follow up period (2005 to 2008) by performing spirometry. By utilizing a longitudinal study and a community population, possible selection bias is minimized. Standardized questionnaires were utilized for recording of medical data and smoking habits. At the start of the surveillance, all subjects had a physical examination. Questions as to general health and history were asked, with specific attention to respiratory problems and smoking habits. Subsequently, each subject was asked to complete a standardized questionnaire when undergoing annual spirometry. Detailed questions were asked in regard to smoking, including the age at which the subject started smoking, how many cigarettes or cigars he smoked, if he was a pipe smoker, and his weekly consumption of tobacco. If the subject had stopped smoking, he was asked when he had stopped and for how many years he had smoked. Each subject was weighed and had his height measured annually. All subjects had no major co-morbidities such as congestive heart failure, renal dysfunction, malignancy or severe arterial hypertension. Patients with poorly reversible airflow limitation associated with bronchiectasis, cystic fibrosis and fibrosis due to tuberculosis were also excluded. They all were current or former smokers with a history of at least 20 pack/years smoking. Current smokers were those who reported smoking at least one cigarette per day. The cumulative cigarette dose (pack-year) was calculated using the following formula: pack-year = (packs per day) × (years of smoking). By definition, former smokers had stopped smoking for at least 1 year. Therefore, former smokers who had stopped for less than 1 year were classified as either moderate or heavy smokers according to their previous smoking habits. The questionnaires were checked routinely and smoking histories were compared with prior statements. We assumed that the smoking history obtained from the first questionnaire was the most reliable.

Spirometry, including a bronchodilation test, was performed in all subjects by the same authorized personnel, using a computerized system (Pulmolab 435 Morgan Data Acquisition System 401). The instrument was calibrated on each occasion it was used. Every subject was categorized to the analogous stage according to the Global Initiative for Obstructive Lung Disease criteria, after obtaining values for FEV₁ and FEV₁/FVC in two technically satisfactory tracings. At least three and up to five to six forced expiratory volume maneuvers were performed by a nurse who had been trained to carry out spirometry. The two largest FEV₁ measurements and the forced vital capacity (FVC) had to be within 100 mL of each other to be accepted as valid. Also, every spirometry session had to conform to the American Thoracic Society recommendations. Additionally, bronchodilator responsiveness, coded as whether the subject ever versus never had a bronchodilator response after inhalation of 200 µg salbutamol (defined as postbronchodilator increase in FEV₁ of at least 200 ml and 12% over the prebronchodilator value) at any visit, was examined as a covariate. Those found to have over 12% reversibility in FEV₁ were excluded from the study. After staging, the appropriate therapy was prescribed to them according to the GOLD guidelines (GOLD Report, 2006). We selected only subjects that weren’t on any kind of inhalational therapy (bronchodilators, corticosteroids or both).

Written informed consent was obtained from each subject before inclusion and the protocol was approved by the Ethics Committee and the Scientific Committee of the University Hospital of Patras.

FEV₁ measurements were obtained at least one year apart after subjects were contacted (visit or phone).

Ninety five non-COPD smokers (75 males and 20 females) and ninety five COPD smokers (77 males and 18 females) were recruited for the study of the +138 3A/4A functional polymorphism. From the COPD smokers group, 2 subjects (2.1%) dropped out and another 2 (2.1%) died. The longitudinal effect of the studied SNP on pulmonary function was examined in both population of smokers, after genotyping them in three distinct classes: (1) those with the wild type alleles (3A/3A); (2) those found to carry only one copy of the mutant allele (3A/4A, heterozygotes) and (3) those carrying two copies of the mutant allele (4A/4A, homozygotes).

The relative frequencies and the general characteristics of the subjects enrolled in the study are presented in Tables I and II.
Regarding the Lys198Asn (G to T transversion), our follow up study included the same group of 95 non-COPD smokers and 95 COPD smokers. As was mentioned in the preceding paragraph, from the COPD smokers group, 2 patients (2.1%) dropped out and another 2 (2.1%) died.

As a group, those carrying this polymorphism were more obese from those with the wild type allele, consistent with the findings of other studies. The longitudinal effect of the studied SNP on pulmonary function was examined in both population of smokers, after genotyping them in three distinct classes: (1) those with the wild type alleles (GG); (2) those found to carry only one copy of the mutant allele (GT, heterozygotes) and (3) those carrying two copies of the mutant allele (TT, homozygotes).

The relative frequencies and the general characteristics of the subjects enrolled in the study are presented in Tables III and IV.

### Blood Collection and Genotyping

DNA was isolated from 3 ml whole blood, using QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany). The ET1 gene polymorphisms Lys198Asn (C198 GT) in exon 5 and the +138 3A/4A in the 5’ untranslated region were genotyped in 95 patients and 95 controls. Genotyping was performed using real time PCR and the MX3000p (Stratagene, La Jolla, CA, USA). The primers and MGB Taqman probes used for the Lys198Asn (C198 GT) polymorphism were as previously reported. For the +138 3A/4A polymorphism the primers 5’- TTC TCT CCT GGC AGG -3’ and 5’- ATC TCA AAG CGA TCC TTC -3’ were used in conjunction with the LNA Taqman probes 5’-(6-Fam) AG+TGCC+C+T+T+AAACGG (BHQ1)-3’ (for 3A allele) and 5’-(Hex) AGT GCC +C+T+T+TAA+CG +GG (BHQ1)-3’ (for 4A), where a “+” before the base indicates an LNA (Locked Nucleic Acid) base. Primers were synthesized by Metabion International (Martinsried, Germany), MGB probes by Applied Biosystems (Foster City, CA, USA) and LNA probes by Sigma-ProLogi (The Woodlands, TX, USA). Reactions were performed using Brilliant QPCR Master Mix (Stratagene, La Jolla, CA, USA).

### Table I. General characteristics of the non-COPD smokers group, according to genotype.

<table>
<thead>
<tr>
<th>Genotypes Variables</th>
<th>3A/3A</th>
<th>3A/4A</th>
<th>4A/4A</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (±SD)</td>
<td>56.57 (12.12)</td>
<td>54.25 (9.44)</td>
<td>58.83 (10.08)</td>
<td>95</td>
</tr>
<tr>
<td>BMI (±SD)</td>
<td>28.63 (5.35)</td>
<td>27.95 (5.52)</td>
<td>60 (4.8)</td>
<td>95</td>
</tr>
<tr>
<td>Smoking habits (pack/years) (±SD)</td>
<td>60.69 (29.8)</td>
<td>63.86 (34.8)</td>
<td>60 (10)</td>
<td>95</td>
</tr>
<tr>
<td>Initial FEV1, l/s (±SD)</td>
<td>3 (0.68)</td>
<td>2.97 (± 0.66)</td>
<td>2.43 (0.1)</td>
<td>95</td>
</tr>
<tr>
<td>FEV1/FVC (±SD)</td>
<td>78.63 (12.9)</td>
<td>81.24 (5.71)</td>
<td>83 (1)</td>
<td>95</td>
</tr>
<tr>
<td>Genotypic distribution</td>
<td>52.6%</td>
<td>44.2%</td>
<td>3.2%</td>
<td>100%</td>
</tr>
</tbody>
</table>

### Table II. General characteristics of the COPD-smokers group, according to genotype.

<table>
<thead>
<tr>
<th>Genotypes Variables</th>
<th>3A/3A</th>
<th>3A/4A</th>
<th>4A/4A</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (±SD)</td>
<td>67.59 (11.59)</td>
<td>64.41 (7.71)</td>
<td>69.5 (7.77)</td>
<td>91</td>
</tr>
<tr>
<td>BMI (±SD)</td>
<td>28.86 (7.25)</td>
<td>28.23 (4.34)</td>
<td>29.73 (1.16)</td>
<td>91</td>
</tr>
<tr>
<td>Smoking habits (pack/years) (±SD)</td>
<td>70 (15.19)</td>
<td>68.14 (19)</td>
<td>75 (7.07)</td>
<td>91</td>
</tr>
<tr>
<td>Initial FEV1, l/s (±SD)</td>
<td>1.3 (0.55)</td>
<td>1.41 (0.68)</td>
<td>1.92 (0.19)</td>
<td>91</td>
</tr>
<tr>
<td>FEV1/FVC (±SD)</td>
<td>53.64 (18.57)</td>
<td>47.63 (18.96)</td>
<td>64 (13.43)</td>
<td>91</td>
</tr>
<tr>
<td>Genotypic distribution</td>
<td>29.67%</td>
<td>68.1%</td>
<td>2.19%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Statistical Analysis
Follow-up analysis lasted for 3 years. The subjects were matched with regard to sex, body mass index (BMI), age, smoking habits, forced expiratory volume in one sec (FEV₁) and forced expiratory capacity in one sec/forced expiratory capacity (FEV₁/FVC) during their enrollment. We present only the comparison paired t-tests between subjects with the wild type allele and those heterozygotes for the mutant allele due to the limited number of subjects found homozygous for the polymorphisms studied. All p values were two sided and any values less than 0.05 were considered statistically significant. Tests of normality showed all variables (age, sex, BMI, pack/years, initial FEV₁, FEV₁/FVC ratio, dFEV₁) to have a normal distribution, according to the Kolmogorov-Smirnov test. Additionally, all SNPs tested were in Hardy-Weinberg equilibrium in the population studied, according to the Pearson’s goodness-of-fit chi-square test. All data were expressed as means ±SD (95% confidence interval). All statistical analyses were performed using the SPSS v.15 statistical software. Paired t-test was used to compare the difference in FEV₁ annual decline between groups. The null hypothesis is that the average annual decline of FEV₁ between groups carrying a different allele of the endothelin gene is no different.

Also, all subjects enrolled in the study were evaluated during each annual visit regarding their smoking habits which, according to the data collected from the questionnaires, did not differ much during the period of the study. Mean annual FEV₁ decline for each subject was calculated separately by using simple linear regression analysis. By using the mean annual values for the FEV₁ for each group, the average annual decline of FEV₁ was calculated from the slope of the best-fit line across the values.

Results
We tested the effect of the adenine insertion-deletion (+138 3A/4A) to a population of non-COPD and COPD smokers. Collectively, non-

Table III. General characteristics of the non-COPD smokers group in the Lys198Asn transversion mutation study.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Non-COPD smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG 53</td>
<td>GT 32</td>
</tr>
<tr>
<td>Age, years (±SD)</td>
<td>67.59 (11.59)</td>
</tr>
<tr>
<td>BMI (±SD)</td>
<td>28.86 (7.25)</td>
</tr>
<tr>
<td>Smoking habits (pack/years) (±SD)</td>
<td>70 (15.19)</td>
</tr>
<tr>
<td>Initial FEV₁, l/s (±SD)</td>
<td>1.3 (0.55)</td>
</tr>
<tr>
<td>Initial FEV₁, % predicted (±SD)</td>
<td>53.64 (18.57)</td>
</tr>
<tr>
<td>FEV₁/FVC (±SD)</td>
<td>58.35 (10.65)</td>
</tr>
<tr>
<td>Genotypic distribution</td>
<td>29.67%</td>
</tr>
</tbody>
</table>

Table IV. General characteristics of the COPD smokers group in the Lys198Asn transversion mutation study.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Non-COPD smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG 59</td>
<td>GT 30</td>
</tr>
<tr>
<td>Age, years (±SD)</td>
<td>64.86 (10.53)</td>
</tr>
<tr>
<td>BMI (±SD)</td>
<td>28.11 (4.67)</td>
</tr>
<tr>
<td>Smoking habits (pack/years) (±SD)</td>
<td>67.20 (17.35)</td>
</tr>
<tr>
<td>Initial FEV₁, l/s (±SD)</td>
<td>1.44 (0.61)</td>
</tr>
<tr>
<td>Initial FEV₁, % predicted (±SD)</td>
<td>51.20 (17.03)</td>
</tr>
<tr>
<td>FEV₁/FVC (±SD)</td>
<td>64.50 (13.46)</td>
</tr>
<tr>
<td>Genotypic distribution</td>
<td>64.80%</td>
</tr>
</tbody>
</table>
COPD smokers had remarkably different genetic distribution compared to COPD smokers. Statistical analysis revealed that the frequencies of the +138ins/delA genotype were significantly different between patients and controls \( (p=0.017, \chi^2=8.178) \). Subjects carrying the 3A4A and the 4A4A genotypes had increased risk of COPD development \( (OR=1.427, 95\% \text{ CI}=1.089-1.871 \text{ and } OR=2.622, 95\% \text{ CI}=0.842-8.165 \text{ respectively, Reference genotype: 3A3A}) \) (Sampsonas F et al study, under press, Respir Med 2009).

Calculating the slope of the FEV\(_1\) regression line, the estimated annual decline was 56.73 \( (SD\pm 14.78) \text{ ml/year for those with the wild type genotype and 76.13 \( (SD\pm 17.04) \text{ ml/year for those who are heterozygotes for the mutant allele and 78.03 \( (SD\pm 6.10) \text{ ml/year for those homozygous for the mutant allele (Figure 1) The statistical analysis of the above findings clearly showed that those who had at least one copy of the mutant allele demonstrated a significant excess decline compared to those with the wild type allele of 19.4 ml/year \( (p=0.004) \), while those with two copies of the mutant allele showed an even more accelerated decline of respiratory function of 21.3 ml/year \( (p=0.001) \). As for the group of COPD smokers, those with the wild type allele 3A/3A demonstrated an annual decline of FEV\(_1\) of 71.98 \( \pm 11.35 \text{ ml/year, while those heterozygous for the +138 3A/4A insertion/deletion showed an annual decline of 83.13 \( \pm 13.12 \text{ ml/year (Figure 2). Thus, the heterozygotes for the mutant allele showed an excess decline of respiratory function of 11.15 ml/year \( (p=0.003) \). The COPD patients homozygous for the mutant allele exhibited an annual decline of FEV\(_1\) of 86.15 \( \pm 6.83 \text{ ml/year, showing an excess decline in respiratory function of 14.17 ml/yr \( (p<0.001) \). The second allele that was analyzed was the Lys198Asn (G to T transversion). The distribution of these genotype was significantly different between patients and controls \( (p=0.047, \chi^2=6.109) \). Statistical analysis revealed that subjects carrying the GG genotype were in increased risk of COPD development \( (OR=1.241, 95\% \text{ CI}=0.982-1.568, \text{ Reference genotype: GT/TT}) \) (Sampsonas F et al study, Respir Med 2010; 104: 114-120). Again, we analyzed the annual decline in FEV\(_1\) in both non-COPD and COPD smokers of the population studied. In the non-COPD smokers group, the mean annual FEV\(_1\) decline \( (dFEV\(_1\)) \) was 70.19 \( \pm 15.49 \text{ ml/year for those with the wild type allele and 58.95 \( \pm 13.83 \) ml/year for those heterozygous for the mutant allele (Figure 3). This translates into a slower decline in pulmonary function of 11.24 ml/year \( (p=0.002) \). Those homozygous for the mutant allele exhibited an annual decline of FEV\(_1\) of 54.74 \( \pm 6.88 \text{ ml/year, which again shows a decelerated decline of 15.45 ml/year \( (p=0.001) \) (Figure 3).

Finally, we will refer to the results obtained from the follow-up of the COPD smokers group and the effects of the Lys198Asn conversion. Those with the wild type allele had an annual decline of 76.91 \( \pm 14.98 \text{ ml/year, while those with one copy of the mutant allele showed an annual decline of 65.49 \( \pm 12.67 \text{ ml/year. Thus, they had a gain of 11.42 \text{ ml/year \( (p=0.001) \) (Figure 4). We only had two COPD patients that were found to be homozygous for the mutant allele and they exhibited an annual decline of 64.95 \( \pm 5.65 \text{ ml/year. Although the gain-in-loss of pulmonary function between this group and the patients with the wild type allele did reach statistical significance \( (11.96 \text{ ml/year, } p<0.001), the group was comprised of only two patients and therefore we acknowledge that a study with a bigger number of patients is needed to support this, although based on the comparison with the heterozygotes group, the allele is indeed associated with a protective effect that cannot be ignored.

The above findings of our study are summarized in Table V, which includes all genotypes and the difference (gain or loss) in annual decline of FEV\(_1\) \( (dFEV\(_1)\). Also, on Figure 5 the slopes for the average annual FEV\(_1\) decline for each group studied are presented.

**Discussion**

ET-1 is involved both in the pathogenesis of airway inflammation in COPD and also in COPD exacerbations\(^{17,18} \). With its proinflammatory and chemoattractant properties\(^{15} \) as well as with its ability to enhance mucus production\(^{13} \) and cause bronchoconstriction\(^{12} \), apart from its potent vasoconstrictor properties\(^{11} \) (which are implicated in pulmonary hypertension pathogenesis), it plays an important role in the preservation and augmentation of the airway inflammation in COPD. It is noteworthy that under normal conditions, the low vascular tone of the pulmonary vasculature is mediated through the production of vasodilatory molecules such as prostacyclin (PGI2) and nitric oxide (NO)\(^{35} \). Alteration of this
Figure 1. Average annual FEV₁ decline (dFEV₁) for the total duration of the study, according to the genotype group, based on regression estimates for each individual of the non-COPD smokers group (+138 insA/delA). The smokers carrying the delA polymorphism show a greater loss of pulmonary function compared to those carrying the wild type allele.
Figure 2. Average annual FEV₁ decline (dFEV₁) for the total duration of the study, according to the genotype group, based on regression estimates for each individual of the COPD-smokers group (+138 insA/delA). The excess loss of pulmonary function is evident among those carrying the delA polymorphism.
Figure 3. Average annual FEV₁ decline (dFEV₁) for the total duration of the study, according to the genotype group, based on regression estimates for each individual of the non-COPD smokers group (Lys198Asn SNP). The smokers carrying the T allele show slower decline of pulmonary function compared to those carrying the wild type allele.
Figure 4. Average annual FEV₁ decline (dFEV₁) for the total duration of the study, according to the genotype group, based on regression estimates for each individual of the COPD-smokers group (Lys198Asn SNP). Again, the smokers carrying the T allele show a slower decline of pulmonary function compared to those carrying the wild type allele.
Table V. Decline in FEV\textsubscript{1} according to genotype and the accompanying statistical significance.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Decline in FEV\textsubscript{1} (ml/year)</th>
<th>Decline in FEV\textsubscript{1} (ml/year)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>non-COPD smokers</td>
<td>(\Delta)FEV\textsubscript{1}</td>
</tr>
<tr>
<td>ET-1 +138 insertion polymorphism</td>
<td>3A/3A</td>
<td>56.73</td>
</tr>
<tr>
<td></td>
<td>3A/4A</td>
<td>76.13</td>
</tr>
<tr>
<td></td>
<td>4A/4A</td>
<td>78.03</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>70.19</td>
</tr>
<tr>
<td>Lys198Asn</td>
<td>GT</td>
<td>58.95</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>54.74</td>
</tr>
</tbody>
</table>
delicate balance and its importance thereof between these endothelium-derived molecules can be seen even during the neonatal age, by causing persistent pulmonary hypertension of the newborn (PPHN). The hyper-endothelinemia that is observed in COPD antagonizes their effects and, along with the chronic hypoxia during the late stages of the disease, is responsible for the pulmonary hypertension manifested in patients with advanced COPD. Even the production of NO seems impaired in such cases. Trials to inverse the vasoconstricting effect of endothelin by administration of inhaled NO have shown promising results, although only some seem to respond. Therefore, one can conclude that polymorphisms of ET-1, like the ones studied in our paper, could even lead to a greater incidence of pulmonary hypertension. Performing a cardiac ultrasound study or, as a gold standard, measuring pulmonary artery pressure invasively could

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**Figure 5.** Model of FEV₁ decline for all groups of our study, during the 3 years of follow-up. The slopes of the regression lines represent the mean annual decline of FEV₁ for each group.
be a next step in such cohorts of patients to
demonstrate the validity of this assumption.

On a cellular level, ET-1 acts in both an au-
tocrine and paracrine manner in the surrounding
interstitium and smooth muscle. Through its spe-
cific endothelin receptors, it can induce the pro-
duction of cytokines such as IL-6, IL-8, granulo-
cyte-macrophage colony-stimulating factor (GM-
CSF) as well as monocyte chemoattractant pro-
tein-1 (MCP-1) from blood mononuclear cells. It
can upregulate the synthesis of adhesion mole-
cules such as inter-cellular adhesion molecule 1
(ICAM-1) and vascular-cellular adhesion mole-
cule 1 (VCAM-1) and also induce the synthesis
of transforming growth factor (TNF)-alpha by
human alveolar macrophages in a dose-depen-
dent and ETA receptor selective mechanism. It
can also mimic the action of TNF-alpha by caus-
ing the influx of inflammatory cells and upregu-
late adhesion markers in both leukocytes and en-
dotheilium causing thus sequestration and trans-
migration of inflammatory cells in its site of pro-
duction\textsuperscript{27}. On the other hand, cytokines such as
TNF-alpha, IL-1a, IL-1b, IL-6 and TGF-beta can
induce prepro-ET-1 and the production and re-
lease of ET-1 from the basolateral side of epithe-

dial cells\textsuperscript{34}. One can conclude that there is an in-
flammatiory loop created by the cooperation of
cells, cytokines and ET-1 that could both sustain
and augment the airway inflammation of COPD.
Therefore, endothelin-1 behaves like a potent in-
flammatory molecule and SNPs that can cause
increased endothelin-1 levels may be associated
with an increased susceptibility to COPD de-
velopment.

In our prospective study, we examined the ef-
fect of two functional polymorphisms of the ET-1
gene in both non-COPD and COPD smokers, af-
ger genotyping them in wild type allele carriers,
heterozygotes for the mutant allele and homozy-
gotes for the mutant allele groups. We demon-
strated that the presence of the adenine insertion
SNP 138 bp downstream of the active transcrip-
tion site is associated with an accelerated loss of
lung function in both populations (non-COPD
and COPD subjects) studied, compared with
those of the wild type phenotype. Taking into ac-
count the finding that mRNA, as well as protein
expression, was increased in homozygous carri-
ers of the insertion variant, it is possible that the
+138 Ins polymorphism modulates the transcrip-
tion rate or mRNA stability rather than transla-
tion efficacy. Regulatory elements in the 5'-UTR
are known to affect promoter activity\textsuperscript{26}.

On the follow-up analysis of the C198 G/T
transversion SNP, we demonstrated a protective
effect on the overall loss of pulmonary function
in both populations studied (non-COPD and
COPD smokers). Although the TT genotype
has been shown to cause increased blood levels of
ET-1, no study has yet verified its role in COPD
implication and the effects of this SNP in the air-
ways. Moreover, the amino acid substitution
might decrease the activity of ET-1 (stability and
protein-protein interaction) or interfere with the
cleavage of the peptide. Lysine and asparagine
belong to different amino acid groups (with di-
verse polarity and charge). Therefore, substitu-
tion of lysine by asparagine may alter the tertiary
structure of the protein. Moreover, even though
the C198 G/T SNP is responsible for amino acid
change (Lys198Asn), the locus of this SNP is not
in proximity with the regulatory loci of the trans-
scriptional sites\textsuperscript{32}. Thus, it is rather improbable to
modulate the precursor ET-1 molecules. This
does not exclude the possibility that the SNP is
in linkage disequilibrium with an actual protect-
ing genetic locus, since it is in proximity with the
HLA group of antigens and TNF group of genes
that are implicated in inflammatory process and

tissue damage. Moreover, the amino acid substitu-
tion might decrease the activity of ET-1 (stabi-
lity and protein-protein interaction) or interfere
with the cleavage of the peptide.

As already pointed out, simple regression
analyses were used to provide individual estima-
ed of FEV\textsubscript{i} decline for each of the groups stud-
ied. Intra-individual variability in spirometric
measures tend to be large over short periods of
time and accurate estimates of individual FEV\textsubscript{i}
decline require monitoring over long periods of
time, preferably over at least five years. Due to
the rapid decline of FEV\textsubscript{i} of some individuals
within the groups carrying the SNPs and due to
the small sample of patients, the follow up would
introduce bias if followed for over 3 years due
mainly to the “healthy survivor” effect, i.e. the
result would probably underestimate the true
FEV\textsubscript{i} decline of the group as a whole, as those
with more severe disease and rapid FEV\textsubscript{i} decline
were more likely to have been removed from the
study due to death or major disability and thus
 excluded from analysis.

Since the first reference for peptidergic activi-

ty produced in endothelial cells\textsuperscript{33}, ET-1 was put
forward as a promising molecule implicated in
numerous pathogenic pathways. This study is,
according to our knowledge, the first to implicate
two ET-1 polymorphisms in COPD phenotype. Our data indicate that the +138 3A/4A and G198T G/T SNPs are possibly involved in COPD pathogenesis and might identify subjects at increased risk of COPD development. Additionally, the +138 3A/4A SNP could be associated with small airways disease progression and G198T G/T SNP might be correlated with disease severity. However, further studies are needed to verify our findings since a bigger sample and a longer duration of follow-up would be needed to establish a true association and not just causality (as is the case with our pilot study).

In conclusion, our results indicate that the +138 3A/4A and C198 G/T SNPs could be involved in COPD pathogenesis, as a risk and protective factors respectively, or are in linkage disequilibrium with the actual SNPs that can modulate the inflammatory response to cigarette smoke.

References

“FEV₁ decline related to two SNPs of endothelin”


