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Sex hormone alterations and systemic inflammation in a group of male COPD smokers and their correlation with the +138 insA/delA Endothelin-1 gene polymorphism. A case-control study

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Abstract. – *Background:* **Chronic obstructive pulmonary disease (COPD) is characterized by the presence of a low-grade systemic inflammation that is implicated in the pathogenesis of numerous extrapulmonary manifestations, such as hypogonadism. Endothelin-1 (ET-1) is a molecule that demonstrates pro-inflammatory properties and can augment the airway and systemic inflammation. Single nucleotide polymorphisms (SNPs) of the ET-1 gene that increase ET-1 serum levels are an important area of investigation. We examined the alterations in inflammatory markers [C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)] and in the levels of testosterone, free testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) in a group of male COPD smokers when compared to their age-matched controls and how these alterations relate to the presence of a functional ET-1 SNP, the adenine insertion SNP +138 insA/delA.**

Materials and Methods: **In this case control study, 80 male control smokers and 82 male COPD smokers were recruited for comparison. Among the male COPD smokers, 37 were carriers of the +138 insA/delA SNP. Two COPD subgroups according to genotype were formed: (1) A group of 45 males homozygous for the wild type allele (3A/3A) and (2) a group of 37 males heterozygous for the mutant allele (3A/4A).**

Results: **Levels of testosterone and free testosterone were lower in the COPD group and even lower in the 3A/4A COPD group. CRP and ESR levels were higher in both COPD groups, but their elevation was statistically significant only for the 3A/4A COPD group. Testosterone and free testosterone levels correlated positively with PaO_2 for both COPD groups. An inverse correlation between testosterone and CRP was demonstrated for the 3A/4A COPD subgroup.**

Conclusions: **Levels of testosterone correlated to FEV_1, hypoxemia and weakly to CRP. The synchronous presence of the +138 insA/delA SNP resulted in even greater sex hormone level decline probably due to the presence of a more intense systemic inflammation.**

Key Words:

Endothelin-1, Testosterone, COPD, CRP.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic airflow limitation that progresses slowly over time and, by definition, the airflow obstruction is largely irreversible[1]. Although at present the current medical treatment is predominantly focused on the primary organ impairment, is has become obvious that COPD is accompanied by a low-grade systemic inflammation that intensifies as the disease progresses and even more so during its acute exacerbations[2,3]. This systemic inflammation is implicated with the important extrapulmonary manifestations of COPD such as osteoporosis, weight loss (especially free fat mass) and peripheral muscle wasting, reduced exercise tolerance, cardiovascular disease, depression, hypogonadism and reduced health status in general[4,5].

Elevated levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and of circulating proinflammatory cytokines, such as IL-6 and TNF-α have been described in COPD that could result in a shift towards catabolism, muscle wasting and cachexia by activating proteolysis[3,6]. CRP has a proinflammatory role of its own and can induce the release of cytokines in monocytes[7,8]. Moreover, an inverse relationship

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between higher levels of circulating inflammation-sensitive proteins such as CRP and lower spirometric values has been described in several samples of middle-aged to older general population[9-11], and with an increased risk of developing COPD in a population-based sample of smokers[12]. CRP levels have also been correlated with the functional impairment in COPD and with the general quality of life as it is determined by the St. George Questionnaire[13,14].

There appears to be a regulatory loop between proinflammatory cytokines and anabolic steroids. Increased levels of proinflammatory cytokines reduce testosterone secretion by interfering with Leydig-cell function[15]. On the other hand, anabolic hormones tend to downregulate the expression of cytokines such as IL-6[16]. Moreover, low anabolic hormone level may synergize the catabolic effects of proinflammatory cytokines, or, conversely, high anabolic hormone level may protect against negative effects of cytokines[16].

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide which can also act as a proinflammatory molecule. Acting through its specific endothelin receptors ET_A and ET_B, it can induce the synthesis of cytokines such as IL-6 and TNF-α from blood mononuclear cells, endothelial cells, vascular smooth muscle cells and airway epithelium[17-21]. Its production is augmented from the aforementioned cytokines and, therefore, an inflammatory loop develops that can sustain the airway inflammation in COPD even when the inciting stimulus [i.e. cigarette smoke] has abated[22]. ET-1 is also capable of stimulating rat vascular smooth muscle cells to produce CRP both in protein and in mRNA levels *in vitro* and *in vivo* via its ET_A receptor[23]. A functional ET-1 gene polymorphism that involves an adenine insertion 138 bp downstream of the transcription start site in the 5'-UTR (Untranslated Region), in exon 1 (+138/ex1ins/delA) has been associated with both increased plasma ET-1 levels and accelerated lung function decline, thus increasing predisposition to COPD development[24-27]. It is worth noting that this allele has a prevalence of 28% in the Caucasian population[24].

The aim of this study was to evaluate the effect of the functional ET-1 gene polymorphism +138/ex1ins/delA on markers of systemic inflammation such as ESR and CRP and its effect on the serum levels of sex hormones to determine if it is associated with any change in the systemic inflammation and hypothalamic-pituitary-gonadal axis function in men, respectively.

Materials and Methods

Subjects

A total of 162 consecutive male smokers (80 male non-COPD smokers marked as controls and 82 male COPD smokers) were recruited from the general Caucasian population of our community who were selected during their visit as outpatients. All subjects had a full physical examination, had their height and weight measured and their BMI (**B**ody **M**ass **I**ndex) calculated (in kg/m^2). 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. 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. Patients with a history of tuberculosis, cystic fibrosis, bronchiectasis, former COPD or asthma diagnosis and, therefore, currently receiving inhaled β_2-agonists and corticosteroids, pulmonary fibrosis, a1-antitrypsin deficiency or other major comorbidity (such as congestive heart failure, cirrhosis, renal failure, cancer, diabetes mellitus etc.) were appropriately excluded from the study. 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.

The COPD group was subsequently divided into two sub groups according to their genotype: (1) A group comprising 45 males homozygous for the wild type allele (designated 3A/3A) and (2) a group comprising 37 males heterozygous for the mutant allele (designated 3A/4A).

Pulmonary Function Tests

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

v.401, Morgan Scientific Inc., Haverhill, MA, USA). 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 FEV_1 and FEV_1/FVC in two technically satisfactory tracings. At least three and up to five to six forced expiratory volume in 1 second (FEV_1) maneuvers were performed by a nurse who had been trained to carry out spirometry. The two largest FEV_1 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 FEV_1 of at least 200 ml and 12% over the prebronchodilator value) was examined as a covariate. Those found to have over 12% reversibility in FEV_1 were excluded from the study. After staging, the appropriate therapy was prescribed to them according to the GOLD guidelines [GOLD Report, 2006]. As already stated, we selected only subjects that weren't on any kind of inhalational therapy (bronchodilators, corticosteroids or both).

Sex Hormones

Serum levels of sex hormones [testosterone, free testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH)] were measured using electrochemiluminescence immunoassay (Elecsys 2010; Roche Diagnostics, Laval, Quebec, Canada). Sex hormone levels were measured in both groups at the time of their enrollment in the study.

CRP and ESR

Venous blood samples were collected into empty tubes and serum was obtained by centrifuging it at 3,000 rpm for 15 min. The samples were then stored at –70°C until analyzed. Serum high sensitive CRP was measured by the high sensitivity nephelometric method (Beckman Coulter Inc., Brea, CA, USA). The results were given in mg/dL with a normal CRP value <0.8 mg/dL. The ESR was measured according to the Westergreen method.

Blood Gas Analysis

The radial artery and less commonly the brachial artery were punctured for the collection of approximately 2 ml of blood for gas analysis, which was tested immediately under anaerobic conditions. The arterial blood sample kit was manufactured by Marquest (Marquest Quick ABG syringe, Englewood, CO, USA). The blood gas analyzer used was a Radiometer ABL 5 (Radiometer Medical Aps, Copenhagen, Denmark).

Statistical Analysis

A statistical software package (SPSS, version 17.0; SPSS Inc; Chicago, IL, USA) was used for all statistical analyses. All results and data were presented as mean value±SD of the mean (95% confidence interval) for variables that were normally distributed. All variables were tested for normality according to the Kolmogorov-Smirnov test. Differences between the groups were analyzed by using the student's unpaired *t*-test or Mann-Whitney U test according to the variables mode of distribution. A two-sided level of significance was set at <0.05. Correlations between parameters were evaluated using Pearson's rank correlation analysis.

Results

Baseline Characteristics, Pulmonary Function Tests and Blood Gas Analysis

The demographic characteristics of the groups studied are presented in Table I along with the results of spirometry and blood gas analysis. It can be seen that the group of male smokers who were heterozygous for the mutant allele had worst pulmonary function tests than the male COPD smokers who were homozygous for the wild type allele.

Sex Hormones and Systemic Inflammation

The sex hormones serum levels, CRP levels, ESR values and p values for all groups studied are presented in Table II.

Comparison between the control group and the 3A/3A COPD group: Testosterone and free testosterone were lower in the 3A/3A COPD group when compared with controls (p=0.000). Both FSH and LH levels were increased in the 3A/3A COPD group, although only the increase in LH serum levels was remarkable (p=0.004). Although both markers of systemic inflammation (CRP and ESR) were higher in the COPD group, this result did not reach statistical significance (p=0.102 and p=0.465 respectively).

Table I. Demographic characteristics, pulmonary function tests and blood gas analysis among the groups studied.

	Controls	COPD smokers (3A/3A)	COPD smokers (3A/4A)	*p*-value*
Subjects (n)	80	45	37	–
Age (yrs)	59.32 (± 11.31)	63.41 (± 8.23)	64.15 (± 7.86)	NS
Smoking habits (pack/yrs)	65.86 (± 17.23)	70.15 (± 21.25)	68.44 (± 15.76)	NS
BMI	28.63 (± 5.54)	27.95 (± 4.81)	28.53 (± 4.34)	NS
FVC	98.09 (± 12.33)	68.64 (± 10.56)	62.41 (± 11.95)	0.006
FEV_1	2.96 (± 0.65)	1. 45 (± 0.55)	1.37 (± 0.61)	0.003
FEV_1 (% predicted)	101.23 (± 11.67)	53.81 (± 10.57)	46.63 (± 9.41)	0.005
FEV1/FVC %	80.63 (± 12.90)	58.51 (± 10.65)	55.72 (± 11.39)	NS
PaO_2	97.24 (± 3.43)	70 (± 2.4)	67.8 (2.9)	NS
$PaCO_2$	39.26 (± 3.67)	41.32 (± 2.33)	42 (± 1.48)	NS
pH	7.41 (± 0.05)	7.40 (± 0.02)	7.39 (± 0.03)	NS

**p*-value was calculated for the two groups of COPD smokers that differed in their genotype (3A/3A and 3A/4A respectively). It can be seen that there was a statistically significant difference in the parameters of their pulmonary function, namely Forced Vital Capacity (FVC), Forced Expiratory volume in 1 sec (FEV_1) and Forced Expiratory volume in 1 sec (FEV_1 % predicted). NS = non-significant.

Comparison between the control group and the 3A/4A COPD group: Testosterone and free testosterone were both significantly lower in the COPD group (p=0.000). FSH and LH serum levels were increased in the COPD group when compared with controls (p=0.024 and p=0.001 respectively). Systemic inflammation was more intense in the COPD group as it is demonstrated by the statistically significant increase in the levels of CRP (p=0.000) and ESR (p=0.035).

Comparison between the 3A/3A COPD group and the 3A/4A COPD group: Testosterone and free testosterone levels were much lower in the 3A/4A COPD group (p=0.003 and p=0.000 respectively). The latter group showed also a more significant increase in the levels of LH (p=0.040). Finally, systemic inflammation was greater in the 3A/4A COPD group as it is revealed by the greater rise in the levels of CRP (p=0.015) and ESR (p=0.022) when compared with the 3A/3A COPD group.

Correlation tests

In the group of COPD smokers with the 3A/3A genotype:

- *Positive correlations*: (1) Testosterone with FEV_1 (r=0.343, p=0.021) and PaO_2 (r=0.351, p=0.018). (2) Free testosterone with FEV_1 (r=0.338, p=0.023) and PaO_2 (r=0.371, p=0.012).
- *Inverse correlations*: (1) LH was inversely correlated with FEV_1 (r=–0.315, p=0.035).

Table II. Sex hormone, crp and esr levels among the groups studied.

	Controls	COPD smokers (3A/3A)	COPD smokers (3A/4A)	*p**	*p***	*p****
Subjects (n)	80	45	37	–	–	–
Testosterone (ng/mL)	5.30 (± 1.3)	3.22 (± 1.26)	2.39 (± 1.22)	0.000	0.000	0.003
Free Testosterone (ng/dL)	20.86 (± 7.23)	3.11 (± 0.18)	1.95 (± 0.12)	0.000	0.000	0.000
FSH (IU/L)	7.62 (± 3.54)	9.73 (± 4.83)	11.95 (± 5.44)	0.065	0.024	0.214
LH (IU/L)	6.23 (± 2.33)	10.64 (± 2.56)	12.41 (± 4.95)	0.004	0.001	0.040
CRP (mg/dL)	0.84 (± 0.53)	2.00 (± 0.91)	2.64 (± 1.22)	0.000	0.000	0.007
ESR (mm/h)	11.71 (± 2.54)	13.77 (± 3.22)	15.12 (± 2.87)	0.465	0.035	0.022

*p**: statistical value obtained by comparison of controls and COPD smokers homozygous for the wild type allele (3A/3A), *p***: statistical value obtained by comparison of controls and COPD smokers heterozygous for the mutant allele (3A/4A) and *p****: statistical value obtained by comparison of COPD smokers homozygous for the wild type allele (3A/3A) and heterozygous for the mutant allele (3A/4A).

In the group of COPD smokers with the 3A/4A genotype:

- *Positive correlations*: (1) There was an even stronger correlation between the testosterone level and FEV_1 (r=0.468, p=0.003) and (2) free testosterone and FEV_1 (r=0.513, p=0.001) than among the 3A/3A group (3) Testosterone with PaO_2 (r=0.392, p=0.016).
- *Inverse correlations*: (1) Between testosterone and CRP, albeit weak (r=–0.372, p=0.023), (2) between CRP and FEV_1 (r=–0.355, p=0.031), (3) LH and FSH with FEV_1 [LH and FEV_1 (r=–0.468, p=0.003), FSH and FEV_1 (r=–0.408, p=0.012)]

Discussion

COPD should no longer be considered a local airway inflammation. Rather, it should be seen as a complex chronic systemic inflammatory syndrome, due to the presence of numerous cytokines into the circulation, which are implicated in the pathogenesis of extrapulmonary disorders such as hypogonadism.

Among the molecules incriminated, ET-1 has received much attention due to the multiplicity of actions with which it is associated. Apart from being a strong vasoconstrictor and bronchoconstrictor, it can also act as a proinflammatory cytokine and it has been shown that it is among the first molecules synthesized during inflammation[28-30]. Through its specific endothelin receptors, it can induce the production of cytokines such as IL-6 and IL-8, from blood mononuclear cells[17-21]. Cytokines such as TNF-α, IL-1, IL-6 and transforming growth factor-β (TGF-β) can induce its synthesis and its release, thus sustaining and augmenting the airway and systemic inflammation of COPD[22]. Studies have already shown that the levels of ET-1 are consistently elevated in sputum, bronchoalveolar lavage and urine sampled from COPD patients[31-33]. In a prospective study conducted in COPD patients, the serum levels of ET-1 were inversely correlated with FEV_1 both during the stable state and during the course of an exacerbation[34]. Hence, those with more severe COPD (lower FEV_1) had higher ET-1 serum levels. In one of our own studies we have examined the effect of functional polymorphisms of the ET-1 gene known to cause increased serum levels of ET-1. We concluded that the mutant allele +138/ex1ins/delA is responsible for an accelerated loss of lung function observed even among heterozygotes and hence with an increased risk for COPD development[27]. The increased levels of ET-1 produced under the control of the mutant allele eventuate in an even more intense inflammation both locally (airways) and in the circulation. One can conclude, therefore, that ET-1 and the functional polymorphisms of its gene can affect the progress of COPD. In the present study, the 3A/4A COPD group had worse pulmonary function tests than the 3A/3A COPD group as depicted by the worse FEV_1 values (p=0.003). Both groups were adjusted for sex, smoking habits, BMI and age.

Among the cytokines whose release is induced by ET-1, IL-6 and IL-8 can stimulate the production of CRP in the liver[35]. Both ET-1 and CRP have been found to be elevated in the serum of patients with COPD as well as in other pulmonary diseases[36-38]. ET-1 has been shown both *in vitro* and *in vivo* to induce the release of CRP in rat vascular smooth muscle cells[23]. CRP has also some proinflammatory actions of its own and both ET-1 and CRP can act synergistically and augment the inflammatory process. A study that was conducted in stable patients with COPD showed a weak correlation between CRP and FEV_1[39]. In a population-based study (EPISCAN), CRP and cytokine levels were also shown to be elevated in COPD patients when compared to controls[40]. Another recent study found a significant correlation between the serum levels of ET-1 and CRP in patients with COPD, supporting the presence of a link between CRP levels and ET-1[41]. In our study, a weak inverse correlation among CRP and FEV_1 was demonstrated for the 3A/4A COPD group (r=–0.355, p=0.031). No correlation was found between FEV_1 and ESR. It is worth noting that both CRP and ESR were higher in the 3A/4A group of COPD patients when compared with their 3A/3A counterparts, a finding consistent with a more intense systemic inflammation in the 3A/4A COPD patient group (Figure 1).

COPD has been related to a dysfunctional hypothalamic-pituitary-gonadal axis and a decreased response to the administration of gonadotrophin-releasing hormone (GnRH) has been found in hypoxemic patients with chronic airway disease[42-45]. Although low gonadotrophin levels have been reported as the cause of hypogonadism in COPD patients, other studies have shown significantly higher gonadotrophin levels than eugonadal patients[42-44]. FSH and LH provide the necessary feedback in the biosynthesis of testos-

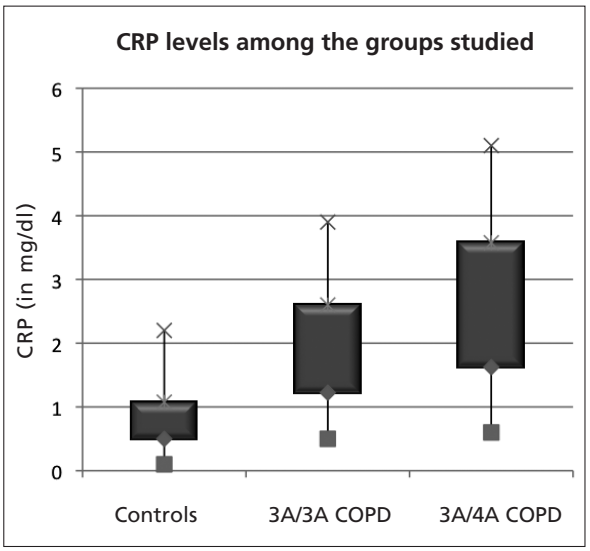

Figure 1. CRP levels between controls and COPD smokers. The increase in CRP was more pronounced in the group of COPD smokers bearing the mutant allele +138 insA/delA.

terone. Therefore, hypergonadotrophism represents a compensatory mechanism of the hypothalamic–pituitary axis to correct for the low circulating testosterone concentrations in patients with COPD. Male controls as well as male COPD patients show this expected rise in gonadotrophins every time their testosterone levels fall[42,46]. The hypergonadotrophism observed in COPD can be appointed to a primary testicular dysfunction as a result of the systemic inflammation that accompanies the primary airway disease although a hypofunctioning hypothalamic-pituitary-gonadal axis cannot be ruled out[47]. Hypoxemia, systemic inflammation, smoking and the use of oral corticosteroids have been implicated as the culprits of hypogonadism in COPD[44,47].

Low testosterone levels have been found in acutely ill, hospitalized COPD patients with concurrent hypoxemia with the degree of testosterone suppression being proportional to the severity of hypoxemia and hypercapnia[47]. The stage of COPD has been shown to affect the level of sex hormones, with testosterone levels being lower and FSH and LH levels compensatively higher in patients with more severe disease[48]. In our study, levels of testosterone and free testosterone were found to be lower in both COPD groups when compared with their age-matched controls (Figure 2). They correlated positively with the value of FEV_1. Their levels were even lower in the 3A/4A COPD group when compared to those of the 3A/3A COPD group (p=0.003) where their correlation with FEV_1 was found to be stronger [(r=0.468, p=0.003) and (r=0.513, p=0.001) respectively]. LH levels were found to be higher in the 3A/3A COPD group when compared to the control group (p=0.004) with an even higher value in the 3A/4A COPD group (p=0.001). FSH levels showed a statistically significant elevation after comparing the 3A/4A COPD group with their age-matched controls (p=0.024). FSH and LH levels were both inversely related to FEV_1 for the 3A/4A COPD patient group [LH and FEV_1 (r=–0.468, p=0.003), FSH and FEV_1 (r=–0.408, p=0.012)]. A recent study[49] demonstrated low testosterone and high FSH and LH levels in a group of COPD patients when they were compared to their age matched controls. These alterations in the sex hormone levels were even more marked during an exacerbation. They also demonstrated a positive correlation between testosterone and FEV_1 (r=0.226, p=0.040).

Systemic inflammation has been suggested as a possible cause of low circulating testosterone concentrations in male patients with COPD, with a weak but significant inverse relationship found between the circulating levels of IL-6 and bioavailable testosterone (r=–0.33)[16]. Also, weak inverse relationships were found between circulating levels of testosterone and free testosterone with acute-phase C-reactive protein[16]. In our study, there was a similar finding, with the level of

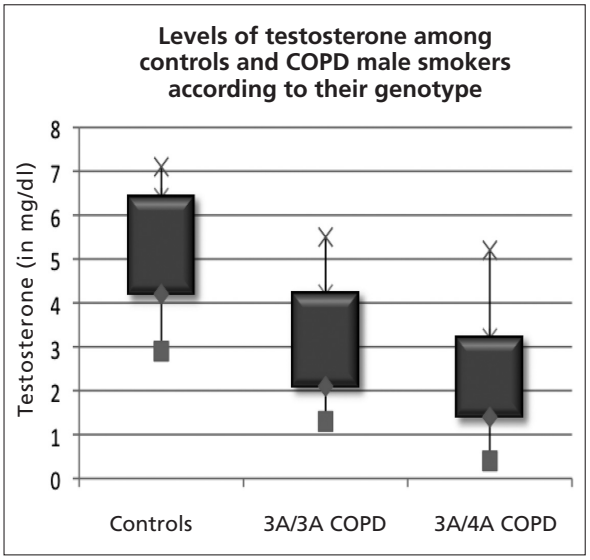

Figure 2. Testosterone levels among controls and COPD male smokers. It can be seen that the decline in testosterone levels was more marked in the 3A/4A COPD group.

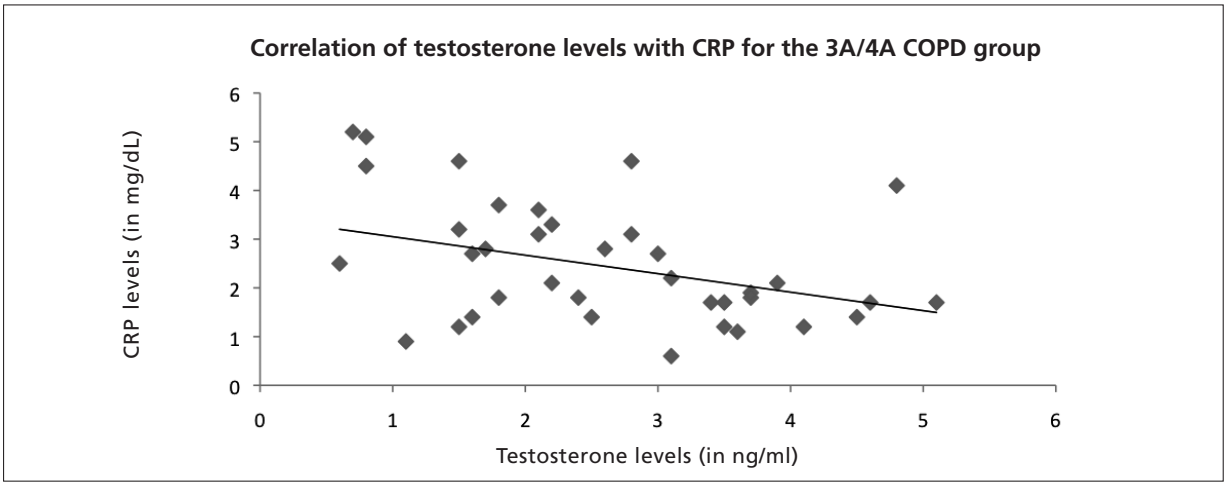

Figure 3. Testosterone levels among controls and COPD male smokers. It can be seen that the decline in testosterone levels was more marked in the 3A/4A COPD group.

testosterone showing a weak inverse relationship with the serum levels of CRP in the 3A/4A COPD patient group (r=–0.372, p=0.023) (Figure 3).

Smoking has been associated with the development of hypogonadism. The results have been contradictory, with several studies supporting this finding[50,51] while others failing to show such a causal relationship[52]. In particular, fewer Leydig cells and degeneration of the remaining cells were found in the testes of men with longstanding chronic bronchitis and emphysema without changes in the circulating gonadotrophin concentrations[53]. In our study, we did not find any correlations between testosterone levels and degree of smoking (represented by pack-years) between the COPD and the control group, which is in concordance with the results provided by another study[49]. Therefore, the effect smoking has on testosterone levels is still a matter of debate.

The present paper presents the effects of a functional ET-1 gene polymorphism on markers of systemic inflammation and on sex hormone levels in a population of COPD smokers. A number of limitations might as well be reported. Although only heterozygotes for the mutant allele were included in the study, due to the rather small number of homozygotes that were available, it has been clearly demonstrated that the presence of a single copy of the mutant allele is associated with a number of discernible events: (1) Even lower levels of testosterone and (2) more intense inflammation, as judged by the levels of CRP and ESR. Testosterone levels are, of course, influenced by the levels of the sex hormone binding globulin (SHBG) which wasn't measured in our study. Another issue is that we didn't measure ET-1 serum levels, which of course is the missing link between the genotype and the effects already presented.

In conclusion, the hypogonadism related with COPD development and progression is a matter with important clinical consequences. Apart from the loss of the anabolic effects of testosterone on muscle (muscle wasting, weakness, sedentary life), reduced libido and erectile dysfunction are also problems that have a negative impact on the quality of life of male COPD patients. Among patients on chronic therapy with supplemental oxygen, 67% of them present with impotence[54]. Hormone replacement therapy resulted in improved erectile function and overall sexual quality of life[55]. Therefore, apart from the maintenance bronchodilator and corticosteroid therapy and the effective treatment of exacerbations, hormone replacement therapy might be indicated in the later stages of COPD in order to maintain anabolic actions and improve muscle function that it is known to deteriorate during the disease progression and is one of the key factors responsible for the quality of life deterioration.

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