Journal of Advanced Research (2016) 7, 1019–1028

Cairo University

Journal of Advanced Research

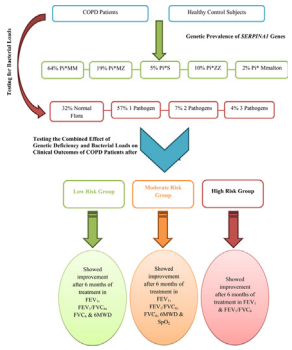
ORIGINAL ARTICLE

The effect of α_1-antitrypsin deficiency combined with increased bacterial loads on chronic obstructive pulmonary disease pharmacotherapy: A prospective, parallel, controlled pilot study

Marwa G. Hennawy [a,1], Noha M. Elhosseiny [b,1], Hussein Sultan [b], Wael Abdelfattah [c], Yousry Akl [d], Nirmeen A. Sabry [a], Ahmed S. Attia [b,*]

[a] Department of Clinical Pharmacy, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
[b] Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
[c] Department of Chest Diseases and Allergy, Faculty of Medicine, Ain Shams University, Cairo 11539, Egypt
[d] Department of Chest Diseases and Allergy, Faculty of Medicine, Cairo University, Cairo 11562, Egypt

GRAPHICAL ABSTRACT



* Corresponding author. Tel.: +20 10 65344060; fax: +20 2 23628246.
E-mail address: ahmed.s.attia@staff.cu.edu.eg (A.S. Attia).
[1] The first two authors contributed equally to this study.

Peer review under responsibility of Cairo University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.jare.2016.05.002

ARTICLE INFO

Article history:
Received 6 February 2016
Received in revised form 5 May 2016
Accepted 6 May 2016
Available online 11 May 2016

Keywords:
AAT deficiency
Chronic obstructive pulmonary disease
Bacteria
Genotyping
Pharmacotherapy

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is caused by α1-antitrypsin deficiency (AATD) genetic susceptibility and exacerbated by infection. The current pilot study aimed at studying the combined effect of AATD and bacterial loads on the efficacy of COPD conventional pharmacotherapy. Fifty-nine subjects (29 controls and 30 COPD patients) were tested for genetic AATD and respiratory function. The bacterial loads were determined to the patients' group who were then given a long acting beta-agonist and corticosteroid inhaler for 6 months. Nineteen percent of the studied group were Pi*MZ (heterozygote deficiency variant), Pi*S (5%) (milder deficiency variant), Pi*ZZ (10%) (the most common deficiency variant), and Pi*Mmalton (2%) (very rare deficiency variant). The patients' sputum contained from 0 to 8×10^8 CFU/mL pathogenic bacteria. The forced vital capacity (FVC_6) values of the AAT non-deficient group significantly improved after 3 and 6 months. Patients lacking AATD and pathogenic bacteria showed significant improvement in forced expiratory volume (FEV_1), FEV_1/FVC_6, FVC_6, and 6 min walk distance (6MWD) after 6 months. However, patients with AATD and pathogenic bacteria showed only significant improvement in FEV_1 and FEV_1/FVC_6. The findings of this pilot study highlight for the first time the role of the combined AATD and pathogenic bacterial loads on the efficacy of COPD treatment.

Introduction

Chronic obstructive pulmonary disease (COPD) is defined as the presence of irreversible or partially reversible airway obstruction associated with chronic bronchitis and/or emphysema [1]. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases [2]. COPD is usually diagnosed in patients who have symptoms of cough, sputum production, abnormal shortness of breath, dyspnea, or increased forced expiratory time which improve by therapy [3,4]. The presence of a post bronchodilator forced expiratory volume (FEV_1) < 80% of the predicted value in combination with forced expiratory volume to forced vital capacity ratio (FEV_1/FVC) < 70% confirms the presence of airflow limitation [3,5]. Emphysematous lung destruction is mainly due to oxidative stress in addition to an imbalance of endogenous proteinases and anti-proteinases in the lung; the imbalance may be due to either genetic factors or inflammatory response [6].

The discovery of the relation between α1-antitrypsin (AAT) deficiency and the early onset of COPD suggested the role of AAT in the pathogenesis of the disease [7]. Alpha 1-antitrypsin inhibits protease enzymes and its deficiency leads to protease/anti-protease imbalance, which breaks down the connective tissue matrix of lung alveoli [8,9]. AAT is a single chain protein consisting of 394 amino acids, where methionine at position 358 acts as the active site [7,10]. Protease inhibitor (Pi) gene, locus found on chromosome 14q32.1, encodes AAT protein [11]. Mutations of Pi locus, now called *SERPINA1*, lead to several variants: Pi*M (wild type), Pi*S, Pi*Z, Pi*Mmalton and Q0Cairo [7,12–16]. The Pi*Z allele results from the substitution of glutamic acid at position 342 by lysine (Glu342Lys) [17] resulting in a severe deficiency in AAT levels. The Pi*S allele results from the substitution of glutamic acid at position 264 by valine (Glu264Val) [13] resulting in a mild to moderate deficiency in AAT levels. However, the Pi*Mmalton is characterized by the deletion of the entire codon encoding the phenylalanine at position 52 (52Phedeleted) [16]. The Q0Cairo is characterized by an A → T transversion resulting in a premature stop codon (Lys259 → Stop259) [12]. Both Pi*Mmalton and Q0Cairo variants are very rare resulting in deficiencies in AAT levels.

Bacterial infections cause exacerbations of COPD, resulting in significant mortality and morbidity [18,19]. The pathogenesis of exacerbations is poorly understood, and the role of bacteria is highly controversial [19]. Beside the nature of the bacterial species, bacterial load may also play an important role in the airway inflammation in COPD patients [20].

COPD is becoming more prevalent in Western populations and is set to explode in several developing countries such as India, Mexico, Cuba, Egypt, South Africa and China [21]. Some recent studies tested the prevalence of COPD in the mentioned countries and found it to be 2–22% in India [22], 20.6% in Mexico [23], 9.6% in Egypt [24], 4.1–24.8% in Sub-Saharan Africa [25] and 8.2% in China [26]. Yet very little information is known about the possible combined impact of AAT genetic deficiency and the bacterial loads on COPD treatment, especially in developing countries such as Egypt. Accordingly, the aim of this pilot study was to determine the genetic prevalence of AATD and its effect on the efficacy of COPD standard pharmacotherapy when combined with the effect of bacterial loads, in a limited well-controlled sample of the Egyptian population.

Subjects and methods

Study subjects

Thirty newly diagnosed COPD patients were recruited from the outpatient clinics of Imbaba Chest Research, Allergy Institute, Kasr El-Aini Teaching Hospital, and El-Demerdash Teaching Hospital within the Greater Cairo area. Informed consent was obtained from all the study subjects. *The study*

protocol and the informed consent form were approved by the Research Ethics Committee, Faculty of Pharmacy, Cairo University (protocol serial number: CL 403). The inclusion criteria were as follows: patients newly diagnosed with COPD, age between 18 and 65 years, and non-smoker or ex-smoker (at least 6 months-smoke free period). Exclusion criteria were the presence of cor pulmonale, stage 4 COPD, frequent COPD exacerbations (>2 per year), any other organ affliction, and active smoking history. Twenty-nine healthy subjects (non-smoker or ex-smoker, age between 18 and 65 years with normal lung functions and no other respiratory conditions) were recruited as matched controls.

Study design

This was a pilot, prospective, parallel, and controlled open-label study that was divided into two phases: (i) identifying the presence and the contribution of bacterial loads and AATD allele in the development of COPD and (ii) monitoring the response of the screened subjects to the COPD therapy.

Clinical assessment and medications

The COPD group received a treatment consisting of Symbicort® 320/9 turbohaler (AstraZeneca, Cairo, Egypt) (320 mcg budesonide and 9 mcg formoterol fumarate dihydrate) to be used twice daily, and Vental® metered dose inhaler (ADCO, Cairo, Egypt) (100 mcg salbutamol/puff) to be used when required for 180 days. All the medications were provided to the patients on a monthly basis.

At baseline, all the subjects were screened for their demographic data, smoking habits, and medical and medication history. The patients' monitoring parameters included the following: respiratory function tests (pre and post bronchodilation), arterial blood gases (ABG), pulse oximetry and six-minute-walk distance test (6MWD). The patients were followed up after 3 and 6 months.

Microbial loads determination

The sputum samples were collected at the beginning of the study from the COPD group, where all the patients were asked to spontaneously expectorate into a sterile plastic collection cup. All of the sputum produced over a 10–15 min period was collected. The sputum samples were obtained during the first 4 h after rising that morning, kept cool, and then processed within an hour of collection [20,27]. The sputum samples were screened for acceptability for microbiological evaluation. Samples were accepted and further processed if they contained less than 10 squamous epithelial cells (SEC) per low-power field (LPF) and more than 25 polymorphonuclear neutrophils (PMNs) per LPF [28]. Sputum samples were homogenized by mixing with an equal volume of 100 μg/mL dithiothreitol (Fisher scientific, Loughborough, UK) [20]. Then, they were serially diluted in a phosphate-buffered saline (prepared in laboratory), plated mainly on chocolate agar, and incubated for 48 h at 37 °C in 5% CO_2 atmosphere [20]. Aliquots were also plated on 5% (v/v) blood agar (Oxoid), and MacConkey agar (Oxoid) and incubated for 48 h at 37 °C in air [20]. Colonies were differentiated based on their morphology; each type was counted and isolated. The isolated colonies were stocked at − 70 °C in 30% (v/v) glycerol (Sigma–Aldrich, St. Louis, Missouri, USA) in brain heart infusion. The bacterial isolates were then identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) [29]. The counts for bacterial species with a pathogenic potential to humans were included in the bacterial loads, while those representing commensals of the oral cavity were excluded.

Genotyping

Genomic DNA was extracted from peripheral blood (5 mL of blood in EDTA anti-coagulant tubes) samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA). For the S variant (Pi*S), the method previously described was used [30]. Exon III, primers (IDT DNA, Coralville, Iowa, USA) P3M (5′-GAGGGGAAACTACAG CACCTCG-3′), and P3P (5′-ACCCTCAGGTTGGGGAAT CACC-3′) were used to produce a 98-bp product that was subsequently digested with TaqI restriction enzyme (NEB, Ipswich, Massachusetts, USA). DNA samples with the Pi*M were cut into 78- and 20-bp bands, but the Pi*S remained as a 98-bp band. While for the Z-variant (Pi*MZ, Pi*ZZ), the previously described Hyp99I/amplified fragment length polymorphism (AFLP) method was adopted [31]. Exon V was amplified using the primer pair (IDT DNA) 5M (5′-GAGCC TTGCTCGAGGCCTGGGATC-3′), and 5P (5′-CAGGAA AACATGGGAGGGATTTAC-′3). The amplicon (372 bp) was digested by Hyp99I (NEB). Two fragments (286 and 86 bp) were obtained in the absence of the Pi*Z variant. However, if a sample was heterozygous for the Z variant (MZ) it would be characterized by three bands (372, 286, and 86 bp), and the presence of a 373 bp undigested would indicate that the sample was homozygous for Pi*Z variant (ZZ). For Pi*Mmalton detection, the mismatched restriction fragment length polymorphism–polymerase chain reaction (RFLP–PCR) assay previously described was used [14]. The primer pair (IDT DNA) Mmalton-RFLP-Fw (5′-ACACCAGTC CAACAGCACCAATAAC-′3), and Mmalton-RFLP-Rv (5′-TCTCCGTGAGGTTGAAATTCAGGCC-′3) were used to yield an amplicon of 134 bp. This product was further digested using MboII restriction enzyme (NEB). The Pi*M allele was expected to yield two bands (115 bp and 19 bp), while the Pi*Mmalton allele remained as 134 bp product. Finally, for the Pi*Q0Cairo, the primer pair (IDT DNA) P3 M (5′-GAG GGGAAACTACAGCACCTCG-3′), and Q0Cairo-Rv (5′-A TGGCTAAGAGGTGTGGGCA-3′) were used to amplify a 374 bp product from exon III. This was followed by direct DNA sequencing [32].

Determination of AAT levels

Plasma was obtained from the collected blood samples as described above. The samples were centrifuged at 2000*g* for 10 min at 4 °C, and the supernatants were used as the plasma sample. They were frozen at −70 °C until further processing. The AAT levels in plasma were determined using the alpha 1 Antitrypsin (*SERPINA1*) Human SimpleStep ELISA™ Kit (ab189579) (Abcam, Cambridge, UK) following the manufacturer's recommendations. Briefly, diluted plasma was

incubated in wells that were pre-coated with an AAT specific antibody. After washing, a biotinylated AAT antibody was added followed by streptavidin–peroxidase conjugate. TMB substrate was then added and the reaction was ended by the addition of the stop solution. The color produced was measured immediately at wavelength 450 nm. The same procedures were applied on standards provided in the kit to construct a calibration curve that was then used to determine the final AAT concentration in the assayed samples. Enzyme inhibitor level $\leqslant$89 mg/dL was noted as severely deficient, 90–140 mg/dL was noted as mildly deficient, and $\geqslant$141 mg/dL was noted as normal [33].

Statistical analysis

Statistical analysis was performed using the SPSS software package version 20. Statistical significance was defined as a P-value < 0.05. For continuous variables and nonparametric independent samples, Mann–Whitney U and Kruskal–Wallis tests were performed, while Wilcoxon Signed Rank test was performed to test the efficacy of therapy after completion of treatment course. Chi square test was performed to test for the difference in the prevalence of the deficient genetic variants between the COPD and the control groups [34].

Results

Genotyping

The AAT genetic variability testing revealed that, 38 (64%) subjects were Pi*MM, 11 (19%) Pi*MZ, 3 (5%) Pi*S, 6 (10%) Pi*ZZ and 1 (2%) subject was Pi*Mmalton. A summary of the genetic variability prevalence among the subjects of the study is presented in Table 1. There was a statistically significant difference in the prevalence of the deficient genetic variants (Pi*MZ, Pi*S, Pi*ZZ and Pi*Mmalton) between the subjects in the COPD group and those in the control group ($P = 0.0295$, Chi Square Test). There were no significant differences in the demographic data and the clinical characteristics of the COPD patients and control subjects on recruitment (Table 2).

The COPD group was further divided according to the presence or absence of AATD into AAT deficient (patients with Pi*Mz and Pi*ZZ phenotypes) group ($N = 15$), and AAT non-deficient (patients without genetic variability i.e. Pi*MM) group ($N = 15$).

AAT levels in subjects' plasma

The distribution of AAT level in the COPD group (148.93 $\pm$ 76.54 mg/dL) was significantly lower than that detected in the control group (204.67 $\pm$ 40.36 mg/dL) ($P = 0.0025$). By comparing the AAT level between the genetically deficient and non-deficient patients, it was found that the AAT level was significantly lower in the deficient group (81.52 $\pm$ 47.57 mg/dL) than that recorded in the non-deficient group (216.35 $\pm$ 11.49 mg/dL) ($P = 0.0001$) (Fig. 1). On further analysis of the AAT deficient group, it was found that the Pi*MZ variant had a significantly higher AAT level (117.78 $\pm$ 15.07 mg/dL) than that found in Pi*ZZ variant (27.12 $\pm$ 7.32 mg/dL) ($P < 0.0001$).

Bacterial species isolated from the COPD patients' sputum

Sputum samples were collected from 28 COPD patients. The bacterial strains were isolated and identified. Nine samples yielded no potentially pathogenic bacteria (or only normal mouth flora), 16 samples resulted in one potentially pathogenic bacterial species, 2 samples yield two potentially pathogenic bacterial species, and only one sample had three potentially pathogenic bacterial species. The isolated microorganisms included *Escherichia coli* (39.1%), *Bacillus cereus*. (17.4%), *Klebsiella pneumoniae* (8.7%), *Haemophilus influenzae* (8.7%), *Acinetobacter baumannii* (8.7%), *Staphylococcus aureus* (8.7%), *Streptococcus pneumoniae* (4.3%), and *Proteus mirabilis* (4.3%). Isolated commensals of the oral cavity included the following: *Streptococcus salivarius*, *Streptococcus parasanguinis*, and *Neisseria macacae*. These species were less likely to be pathogenic and were not included in the counts for bacterial loads. Upon enumeration of the potentially pathogenic bacterial species: 9 samples (32%) had zero bacterial count, 5 samples (18%) had 1.5–8 $\times 10^6$, 9 samples (32%) had 1.2–9 $\times 10^7$, and 5 samples (18%) had 2–8 $\times 10^8$ CFU/mL. Detailed information about the identified isolated bacterial species and their counts is provided in Table S1.

Effect of AAT genetic deficiency on COPD therapeutic outcome

By testing the effect of the genetic variants within the AAT deficient group between the Pi*MZ and Pi*ZZ variants, no significant effect was found on the values of FEV_1 (% predicted), FEV_1/FVC_6 ratio, FVC_6 (% predicted) and 6MWD. But there was a statistically significant improvement in the values of

Table 1 Prevalence of AAT deficiency variants among subjects included in the study.

Variant		Control total $N = 29$ N (%)	COPD total $N = 30$ N (%)	P[b]
S variant	Pi*S	3 (10.7)	0 (0.0)	0.0295[c]
Z variant	Pi*MZ	2 (6.9)	9 (30.0)	
	Pi*ZZ	0 (0.0)	6 (20.0)	
Mmalton	Pi*Mmalton	1 (3.4)	0 (0.0)	
Q0Cairo	Pi*Q0Cairo	ND[a]	0 (0.0)	

[a] ND; not done.
[b] Level of significance at $P < 0.05$.
[c] Chi square test.

Table 2 Demographic and clinical characteristics of study subjects.

Variable	Value ($N = 59$)		P^*
	Patient ($N = 30$) (range) "median"	Control ($N = 29$) (range) "median"	
Gender; No. of males (%)	30 (100%)	29 (100%)	–
No. of subjects with smoking history (%)	22 ex-smokers (73.33%)	20 ex-smokers (69%)	0.711[b]
Age (yr)	(20–62) "52"	(32–65) "44"	0.158[a]
FEV_1 (%)	(14–53) "29.5"	(58–100) "86"	< 0.0001[a]
FEV_1/FVC_6 (%)	(36–70) "59.5"	(78–100) "100"	< 0.0001[a]
FVC_6 (%)	(22–89) "52"	(57–100) "86"	< 0.0001[a]
6MWD (m)	(185–480) "347.5"	(375–420) "400"	< 0.0001[a]
SPO_2 (%)	(90–98) "96"	(92–99) "98"	< 0.0001[a]
AAT level (mg/dL)	(17.224–246.992) "175.56"	(101.824–262.28) "215.89"	0.002[a]

FEV_1: Forced expiratory volume after 1 s.
FVC_6: Forced vital capacity.
6MWD: Six minute walk distance.
SPO_2: Peripheral capillary oxygen saturation.
AAT: Alpha 1-antitrypsin enzyme.
* Level of significance at $P < 0.05$.
[a] Independent samples Mann–Whitney U test.
[b] Chi-Square test.

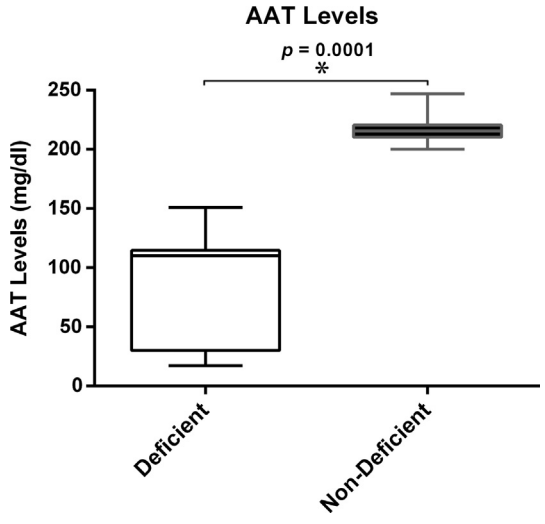


Fig. 1 AAT levels in the plasma of the study subjects. The levels in the plasma of the AAT deficient group (white bar) and AAT non-deficient group (black bar). The * indicates that the difference between the two groups is statistically significant as determined by Independent samples Mann–Whitney U test.

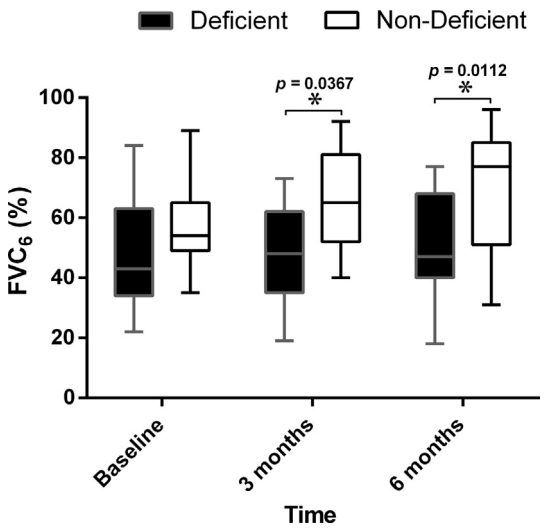


Fig. 2 Distribution of FVC_6 throughout the treatment period in the genetically deficient and non-deficient groups. Forced vital capacity was measured at three occasions: baseline, 3 months, and 6 months post-initiation of therapy. The AAT-non-deficient group (white bars) significantly improved compared to the AAT-deficient group (black bars) at both 3 and 6 months. The * indicates that the difference between the two groups is statistically significant as determined by Independent samples Mann–Whitney U test.

FVC_6 in the AAT non-deficient group after 3 and 6 months of treatment ($P = 0.0367$, $P = 0.0112$ respectively, independent samples Mann–Whitney U test) than the AAT deficient group (Fig. 2) (Table 3). In addition, the SPO_2 values were significantly lower in the AAT-deficient group at baseline ($P = 0.036$).

Effect of AAT levels on COPD therapeutic outcome

The AAT levels had no significant effect on the values of FEV_1, FEV_1/FVC_6 ratio and 6MWD. However, the FVC_6 values were significantly lower in the AAT mildly deficient group at 3 months and 6 months intervals ($P = 0.038$ and $P = 0.039$, respectively) (Fig. 3A). The SPO_2 values varied significantly among the AAT level groups at baseline, 3 and 6 months ($P = 0.043$, $P = 0.043$ and $P = 0.049$, respectively) (Fig. 3B) (Table 4).

Effect of bacterial loads on COPD therapeutic outcome

Upon investigating the effect of the total bacterial counts on treatment, it was found that, there was no significant difference between the 4 groups (0, 10^6, 10^7, and 10^8 CFU/mL) in the clinical outcomes at baseline, 3 and 6 months intervals. Yet, in the 10^6 CFU/mL group, the FEV_1/FVC_6 ratio varied significantly after 6 months of treatment ($P = 0.043$). Also, in the 10^7 CFU/mL group, the 6MWD test differed significantly after 6 months of treatment ($P = 0.025$) (Fig. 4). However, in the 10^8 CFU/mL group there was no significant difference in any of the clinical parameters before and after treatment.

Table 3 Effect of AAT deficiency on FVC_6.

Variable	AAT deficient ($N = 15$) (range) "median"	AAT non-deficient ($N = 15$) (range) "median"	P^*
FVC_6 (%) baseline	35–89 "62"	22–84 "43"	0.098[a]
FVC_6 (%) 3 months	40–92 "65"	19–73 "46"	0.037[a]
FVC_6 (%) 6 months	31–96 "77"	18–77 "47"	0.011[a]

FVC_6: Forced vital capacity.
AAT: Alpha 1-antitrypsin enzyme.
* Level of significance at $P < 0.05$.
[a] Independent samples Mann–Whitney U test.

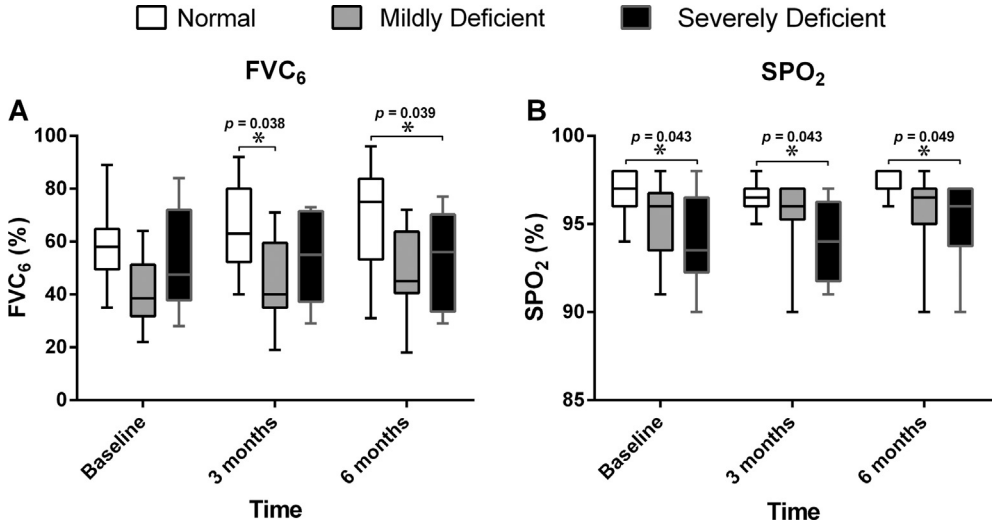


Fig. 3 Distribution of FVC_6 and SPO_2 among AAT enzyme level groups. (A) Forced vital capacity and (B) SPO_2 were measured on three occasions: baseline, 3 and 6 months post-initiation of therapy. The parameters' improvement was monitored in three groups based on the plasma AAT levels: normal (white bars), mildly deficient (grey bars), and severely deficient (black bars). The * indicates that the difference between the three groups is statistically significant as determined by independent samples Kruskal–Wallis test.

Table 4 Effect of AAT levels on therapeutic outcomes of COPD treatment.

Variable	Normal AAT ($N = 16$) (range) "median"	Mildly deficient AAT ($N = 8$) (range) "median"	Severely deficient AAT ($N = 6$) (range) "median"	P^*
FVC_6 (%) Baseline	35–89 "58"	22–64 "38.5"	28–84 "47.5"	0.052[a]
FVC_6 (%) 3 months	40–92 "63"	19–71 "40"	29–73 "55"	0.038[a]
FVC_6 (%) 6 months	31–96 "75"	18–72 "45"	29–77 "56"	0.039[a]
SPO_2 (%) Baseline	94–98 "97"	91–98 "96"	90–98 "93.5"	0.043[a]
SPO_2 (%) 3 months	95–98 "96.5"	90–97 "96"	91–97 "94"	0.043[a]
SPO_2 (%) 6 months	96–98 "97"	90–98 "96.5"	90–97 "96"	0.049[a]

FVC_6: Forced vital capacity.
SPO_2: Peripheral capillary oxygen saturation.
AAT: Alpha 1-antitrypsin enzyme.
* Level of significance at $P < 0.05$.
[a] Independent samples Kruskal–Wallis test.

Combined effect of bacterial loads and AAT levels on COPD therapeutic outcome

The COPD patients were divided according to the AAT deficiency and the presence or absence of bacterial count into 3 groups: group 1 (low risk: non-deficient and zero count, $N = 5$), group 2 (moderate risk: non-deficient and the presence of bacterial count + deficient and zero bacterial count, $N = 12$) and group 3 (high risk: deficient and presence of bacterial count, $N = 11$). A comparison between the

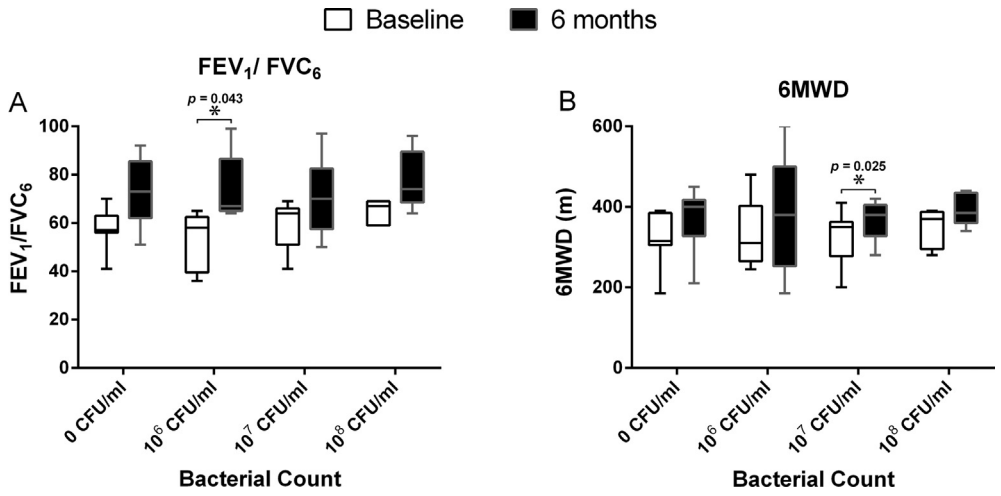


Fig. 4 Effect of bacterial loads on COPD therapeutic outcomes. Respiratory parameters (A) FEV_1/FVC_6, (B) 6MWD were measured on two occasions: baseline (white bars), and 6 months post-initiation of therapy (black bars) among the four groups of bacterial counts. The * indicates that the difference between the two groups is statistically significant as determined by Wilcoxon signed rank test.

Table 5 Demographic and clinical characteristics of the groups studied for the combined effect of AATD and bacterial loads.

Variable		Group #1 (non-AATD + no bacterial load) ($N = 5$)	Group #2 (non-AATD + presence of bacterial load) and (AATD + no bacterial load) ($N = 12$)	Group #3 (AATD + presence of bacterial load) ($N = 11$)	P^*
Gender (no. of males)	*N* (%)	5 (100%)	12 (100%)	11 (100%)	–
Age (yr)	Range	23–60	25–62	44–62	0.265[a]
	Median	40	52.5	55	
FEV_1 (%) baseline	Range	30–37	18–39	14–53	0.315[a]
	Median	35	27.5	29	
FEV_1 (%) 6 months	Range	35–72	22–77	12–67	0.357[a]
	Median	41	39.5	32	
FEV_1/FVC_6 baseline	Range	41–70	36–69	47–69	0.058[a]
	Median	58	56.5	65	
FEV_1/FVC_6 6 months	Range	59–87	50–99	64–96	0.926[a]
	Median	73	74	70	
FVC_6 (%) baseline	Range	47–89	28–78	22–84	0.529[a]
	Median	52	53	43	
FVC_6 (%) 6 months	Range	50–95	31–85	18–77	0.079[a]
	Median	78	65.5	47	
6MWD (m) baseline	Range	185–380	200–480	210–410	0.417[a]
	Median	310	350	345	
6MWD (m) 6 months	Range	210–415	280–600	185–440	0.465[a]
	Median	375	400	380	
SPO_2 (%) baseline	Range	96–98	90–98	91–98	0.051[a]
	Median	98	96	96	
SPO_2 (%) 6 months	Range	97–98	90–98	90–98	0.170[a]
	Median	97	97	96	
AAT level (mg/dL)	Range	200.09–222.82	17.224–246.99	22.728–151.02	0.007[a]
	Median	215.592	212.552	110.12	

FEV_1: Forced expiratory volume after 1 s.
FVC_6: Forced vital capacity, 6MWD: Six minute walk distance.
SPO_2: Peripheral capillary oxygen saturation, AAT: Alpha 1-antitrypsin enzyme.
* Level of significance at $P < 0.05$.
[a] Independent samples Kruskal–Wallis test.

demographic and baseline respiratory parameters is presented in Table 5, where there was a significant difference in AAT level among the 3 groups ($P = 0.007$). Further post hoc analysis using independent samples Mann–Whitney U test revealed a significant difference between groups 1 and 3 ($P = 0.018$) and between groups 2 and 3 ($P = 0.034$).

Overall, there was no significant difference between the three groups in the clinical outcomes at baseline, 3 months

and 6 months intervals. However, group 1 showed a significant improvement after 6 months of treatment in the FEV_1 ($P = 0.043$), FEV_1/FVC_6 ($P = 0.043$), FVC_6 ($P = 0.043$) and 6MWD ($P = 0.043$, Wilcoxon signed rank test). In addition, for group 2, there was a significant improvement after 6 months of treatment in the FEV_1 ($P = 0.004$), FEV_1/FVC_6 ($P = 0.013$), FVC_6 ($P = 0.041$), 6 MWD ($P = 0.003$) and SPO_2 ($P = 0.026$, Wilcoxon signed rank test). On the contrary, for group 3, there were no significant improvements except in the FEV_1 ($P = 0.037$) and FEV_1/FVC_6 ($P = 0.032$, Wilcoxon signed rank test) (Fig. 5A–E).

Discussion

AAT deficiency is a genetic disorder that appears in the form of an early onset pulmonary emphysema, liver cirrhosis and much less frequently skin disease panniculitis [35]. Studies showed that 1.9% of COPD patients have AATD [36]. Respiratory failure was reported to be the cause of death in 50–72% of AATD patients [37]. The risk factors leading to increased death rates include older age, lower education, smoking, lower FEV_1, lung transplantation, and not receiving augmentation therapy [38].

In the present study, the normal serum concentration of AAT ranged between 1.5 and 3.5 g/L (or 20 and 48 μM) [39], with an average serum AAT level in the COPD group to be 1.489 ± 0.765 g/L. More than 30% of the patients were belonging to the AAT deficient group. COPD patients having the Pi*MZ deficiency variant (9/30) were found to be more prevalent than those having the Pi*ZZ variant (6/30).

A comprehensive survey that was conducted in 2012 in 97 countries (not including Egypt) revealed that, 75% of the AATD patients had the Pi*MS, while Pi*MZ and Pi*SS variants were found in 24%. Pi*SZ represented 0.7% and 0.1% had the Pi*ZZ [40]. Rare deficiency variants prevailed in the Mediterranean countries. Pi*Mmmalton variant prevailed over Pi*S and Pi*Z variants in Italy and Central Tunisia [41], while in Jordan Pi*MS was the most prevalent and Pi*S was the least prevalent [40]. A controlled study that was conducted on bronchiectasis Egyptian patients revealed the presence of 1.5% Pi*MZ and 3.5% Pi*SZ deficiency variants [42]. In the current study, Pi*MZ was the most common deficient variant while Pi*S was the least.

Pathogenic bacteria cause a significant proportion of acute exacerbations of COPD and it was found that half COPD exacerbations were attributed to bacterial infections [18,43]. In a study conducted by Wilkinson et al., it was observed that the increase in airway bacterial loads was associated with greater airway inflammation and accelerated decline in FEV_1 [44].

The present study – looking into the effect of the AAT, either genetically or biochemically – indicated that, the non-deficient group showed significant improvement over the

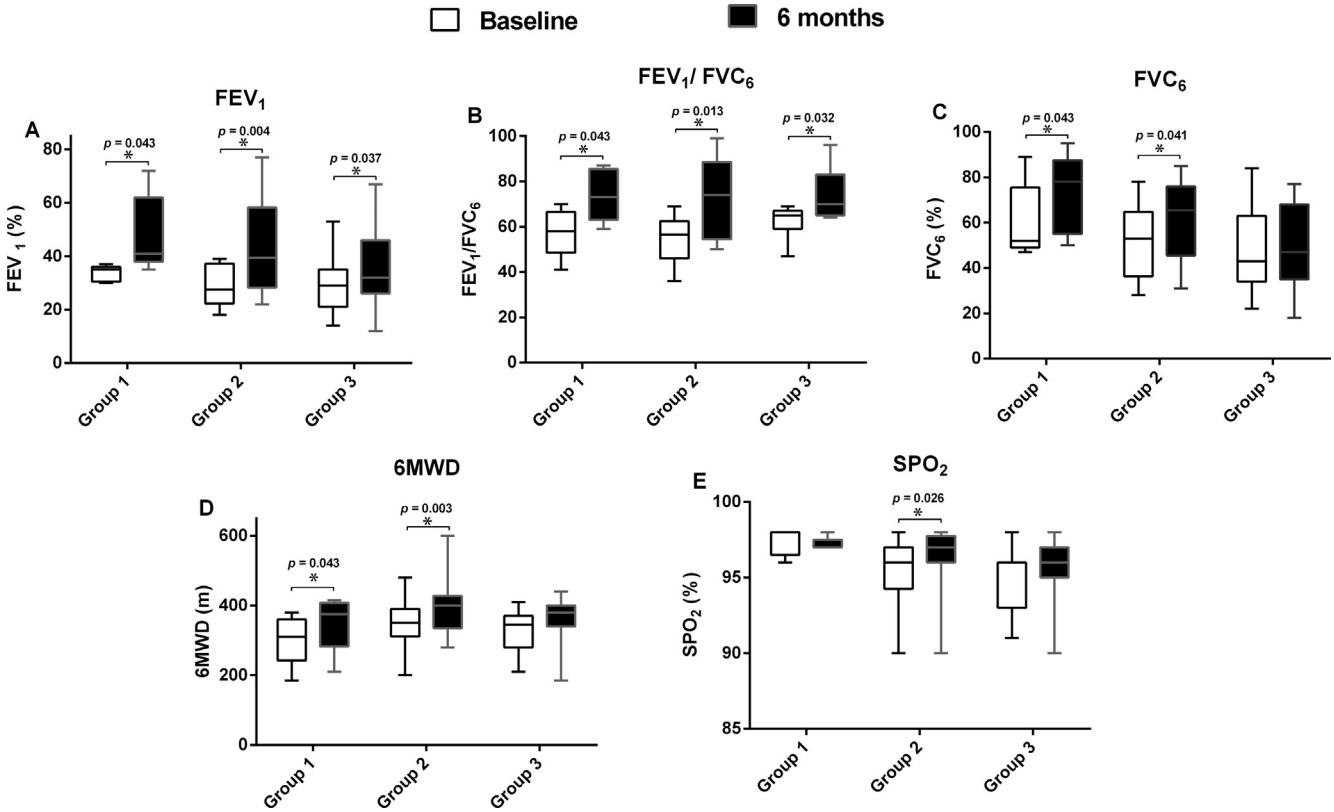


Fig. 5 Distribution of the respiratory parameters among the combination of bacterial loads and AAT enzyme levels groups. Respiratory parameters (A) FEV_1, (B) FEV_1/FVC_6, (C) FVC_6, (D) 6MWD, and (E) SPO_2 were measured on two occasions: baseline (white bars), and 6 months post-initiation of therapy (black bars) among the three groups based on the combination between the bacterial loads and AAT enzyme levels. The * indicates that the difference between the two groups is statistically significant as determined by Wilcoxon signed rank test.

deficient ones in few parameters namely FVC_6 and the SPO_2. On the other hand, when AATD was combined with bacterial loads, a much greater impact was observed in the responsiveness of COPD patients to treatment, where the low-risk group showed a significant improvement in almost all the measured parameters (FEV_1, FEV_1/FVC_6, FVC_6, and 6MWD). However, the high-risk group only showed improvement in the FEV_1 and FEV_1/FVC_6 indicating, for the first time, that the combination of AATD and bacterial loads had a significant impact on the therapeutic outcome of COPD treatment.

Conclusions

The findings of the current pilot study represent a starting point for further investigations of the role of AATD and bacterial load combination on COPD treatment in the Egyptian population. The significance of these findings stems from the opportunity that can be created by decreasing the bacterial loads in the lungs of COPD patients especially those with AAT-deficiency, at the beginning of any pharmacological treatment. Those patients are predicted to respond much better to treatment than those receiving treatment while keeping the bacterial loads high.

Study limitations

The small sample size is the main limitation of this study. This is attributed to the difficulty in recruiting patients with the previously set inclusion and exclusion criteria from Cairo University hospitals, because the treatment in Cairo University hospitals is a completely free service, so priority in the provision of care is always given to critical patients suffering from multiple complications.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

The authors acknowledge the efforts made by outpatient clinics residents and nursing staff, Imbaba Chest Research and Allergy Institute, Kasr el-Aini Teaching Hospital and El--Demerdash Teaching Hospital, Egypt. We also thank Eng. Mahmoud Younis of (Scientific Services Company, Bruker's agent in Egypt) for helping in the bacterial identification using the MALDI-Biotyper. This study was part of an Inter-disciplinary Research Grant (IRG-2011#3), awarded by Faculty of Pharmacy, Cairo University to both NAS and ASA.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jare.2016.05.002.

References

[1] Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, 1986. Am Rev Respir Dis 1987;136 (1):225–44.
[2] Pauwels RA, Buist AS, Ma P, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2001;163:1256–70.
[3] Steinacher R, Parissis JT, Strohmer B, Eichinger J, Rottlaender D, Hoppe UC, et al. Comparison between ATS/ERS age- and gender-adjusted criteria and GOLD criteria for the detection of irreversible airway obstruction in chronic heart failure. Clin Res Cardiol 2012;101(8):637–45.
[4] Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS, Committee GS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) workshop summary. Am J Respir Crit Care Med 2001;163(5):1256–76.
[5] Gupta PP, Sood S, Atreja A, Agarwal D. Assessment of visual evoked potentials in stable COPD patients with no visual impairment. Ann Thorac Med 2010;5(4):222–7.
[6] Repine JE, Bast A, Lankhorst I. Oxidative stress in chronic obstructive pulmonary disease. Oxidative stress study group. Am J Respir Crit Care Med 1997;156(2 Pt 1):341–57.
[7] Wood AM, Stockley RA. Alpha one antitrypsin deficiency: from gene to treatment. Respiration 2007;74(5):481–92.
[8] Stoller JK, Aboussouan LS. Alpha1-antitrypsin deficiency. Lancet 2005;365(9478):2225–36.
[9] Stockley RA. Neutrophils and protease/antiprotease imbalance. Am J Respir Crit Care Med 1999;160(5 Pt 2):S49–52.
[10] Crystal RG. Alpha 1-antitrypsin deficiency, emphysema, and liver disease. Genetic basis and strategies for therapy. J Clin Invest 1990;85(5):1343–52.
[11] Cox DW, Woo SL, Mansfield T. DNA restriction fragments associated with alpha 1-antitrypsin indicate a single origin for deficiency allele PI Z. Nature 1985;316(6023):79–81.
[12] Zorzetto M, Ferrarotti I, Campo I, Balestrino A, Nava S, Gorrini M, et al. Identification of a novel alpha1-antitrypsin null variant (Q0Cairo). Diagn Mol Pathol 2005;14(2):121–4.
[13] Mahadeva R, Chang WS, Dafforn TR, Oakley DJ, Foreman RC, Calvin J, et al. Heteropolymerization of S, I, and Z alpha1-antitrypsin and liver cirrhosis. J Clin Invest 1999;103 (7):999–1006.
[14] Denden S, Lakhdar R, Keskes NB, Hamdaoui MH, Chibani JB, Khelil AH. PCR-based screening for the most prevalent alpha 1 antitrypsin deficiency mutations (PI S, Z, and Mmalton) in COPD patients from Eastern Tunisia. Biochem Genet 2013;51 (9–10):677–85.
[15] Mornex JF, Chytil-Weir A, Martinet Y, Courtney M, LeCocq JP, Crystal RG. Expression of the alpha-1-antitrypsin gene in mononuclear phagocytes of normal and alpha-1-antitrypsin-deficient individuals. J Clin Invest 1986;77(6):1952–61.
[16] Ferrarotti I, Baccheschi J, Zorzetto M, Tinelli C, Corda L, Balbi B, et al. Prevalence and phenotype of subjects carrying rare variants in the Italian registry for alpha1-antitrypsin deficiency. J Med Genet 2005;42(3):282–7.
[17] Elliott PR, Lomas DA, Carrell RW, Abrahams JP. Inhibitory conformation of the reactive loop of alpha 1-antitrypsin. Nat Struct Biol 1996;3(8):676–81.
[18] Iyer Parameswaran G, Murphy TF. Chronic obstructive pulmonary disease: role of bacteria and updated guide to antibacterial selection in the older patient. Drugs Aging 2009;26 (12):985–95.

[19] Veeramachaneni SB, Sethi S. Pathogenesis of bacterial exacerbations of COPD. COPD 2006;3(2):109–15.
[20] Hill AT, Campbell EJ, Hill SL, Bayley DL, Stockley RA. Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. Am J Med 2000;109(4):288–95, Epub 2000/09/21.
[21] Lomas DA, Silverman EK. The genetics of chronic obstructive pulmonary disease. Respir Res 2001;2(1):20–6, Epub 2001/11/01.
[22] Koul PA. Chronic obstructive pulmonary disease: Indian guidelines and the road ahead. Lung India 2013;30(3):175.
[23] Laniado-Laborin R, Rendon A, Bauerle O. Chronic obstructive pulmonary disease case finding in Mexico in an at-risk population. Int J Tuberc Lung Dis 2011;15(6):818–23.
[24] Said AF, Ewis AA, Omran AA, Magdy ME, Saleeb MF. Prevalence and predictors of chronic obstructive pulmonary disease among high-risk Egyptians. Egypt J Bronchol 2015;9 (1):27.
[25] Salvi S. The silent epidemic of COPD in Africa. Lancet Glob Health 2015;3(1):e6–7.
[26] Gao J, Prasad N. Chronic obstructive pulmonary disease in China: the potential role of indacaterol. J Thorac Dis 2013;5 (4):549.
[27] Henig N, Tonelli M, Pier M, Burns J, Aitken M. Sputum induction as a research tool for sampling the airways of subjects with cystic fibrosis. Thorax 2001;56(4):306–11.
[28] Geckler RW, Gremillion DH, McAllister CK, Ellenbogen C. Microscopic and bacteriological comparison of paired sputa and transtracheal aspirates. J Clin Microbiol 1977;6(4):396–9.
[29] Khot PD, Couturier MR, Wilson A, Croft A, Fisher MA. Optimization of matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis for bacterial identification. J Clin Microbiol 2012;50(12):3845–52.
[30] Sandford AJ, Weir TD, Spinelli JJ, Pare PD. Z and S mutations of the alpha1-antitrypsin gene and the risk of chronic obstructive pulmonary disease. Am J Respir Cell Mol Biol 1999;20(2):287–91, Epub 1999/01/28.
[31] Denden S, Khelil AH, Knani J, Lakhdar R, Perrin P, Lefranc G, et al. Alpha-1 antitrypsin gene polymorphism in Chronic Obstructive Pulmonary Disease (COPD). Genet Mol Biol 2010;33(1):23–6, Epub 2010/01/01.
[32] Zorzetto M, Ferrarotti I, Campo I, Balestrino A, Nava S, Gorrini M, et al. Identification of a novel alpha1-antitrypsin null variant (Q0Cairo). Diagn Mol Pathol 2005;14(2):121–4, Epub 2005/05/21.
[33] Serra HG, Bertuzzo CS, Pereira MC, Rossi CL, Pinto Junior W, Paschoal IA. Determination of alpha 1-antitrypsin levels and of the presence of S and Z alleles in a population of patients with chronic respiratory symptoms. J Bras Pneumol 2008;34 (12):1019–25.
[34] Marusteri M, Bacarea V. Comparing groups for statistical differences: how to choose the right statistical test? Biochem Med 2010;20(1):15–32.
[35] Fregonese L, Stolk J. Hereditary alpha-1-antitrypsin deficiency and its clinical consequences. Orphanet J Rare Dis 2008;3(16). http://dx.doi.org/10.1186/1750-1172-3-16.
[36] Lieberman J, Winter B, Sastre A. Alpha 1-antitrypsin Pi-types in 965 COPD patients. Chest 1986;89(3):370–3.
[37] Dawkins PA, Dowson LJ, Guest PJ, Stockley RA. Predictors of mortality in alpha1-antitrypsin deficiency. Thorax 2003;58 (12):1020–6.
[38] Survival and FEV1 decline in individuals with severe deficiency of alpha1-antitrypsin. The alpha-1-antitrypsin deficiency registry study group. Am J Respir Crit Care Med 1998;158(1):49–59.
[39] Fagerhol MK, Laurell CB. The polymorphism of "prealbumins" and alpha-1-antitrypsin in human sera. Clin Chim Acta 1967;16(2):199–203.
[40] de Serres FJ, Blanco I. Prevalence of alpha1-antitrypsin deficiency alleles PI*S and PI*Z worldwide and effective screening for each of the five phenotypic classes PI*MS, PI*MZ, PI*SS, PI*SZ, and PI*ZZ: a comprehensive review. Ther Adv Respir Dis 2012;6(5):277–95.
[41] Denden S, Lakhdar R, Leban N, Ben Chibani J, Haj Khelil A. Rapid genotyping of alpha 1 antitrypsin deletion mutation (PI*Mmalton) using bi-directional PCR allele-specific amplification. Mol Biotechnol 2010;45(2):111–5.
[42] Badawya MS, El Qarnb AF, Mohamadeen HA. Alpha 1 antitrypsin deficiency in non cystic fibrosis bronchiectasis. Egypt J Chest Dis Tuberc 2013;62(2):311–7.
[43] Wilson R. Bacteria, antibiotics and COPD. Eur Respir J 2001;17 (5):995–1007.
[44] Wilkinson TM, Patel IS, Wilks M, Donaldson GC, Wedzicha JA. Airway bacterial load and FEV1 decline in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2003;167(8):1090–5.