TREBALL DE FI DE GRAU MEDICINA

SERUM ANTI-ELASTASE ACTIVITY AND ALPHA-1 ANTITRYPSIN LEVELS IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE AND ALPHA-1 ANTITRYPSIN DEFICIENCY

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ABSTRACT

Context: El Dèficit alfa-1 antitripsina (DAAT) és una malaltia autosòmica codominant que causa una disminució dels nivells sèrics d'alfa-1 antitripsina (AAT), provocant un desequilibri entre l'activitat elastasa i anti-elastasa (AEA) i danys pulmonars, especialment en el fenotip Pi*ZZ.

Objectiu: Determinar la independència de la AEA respecte als nivells de AAT i la seva associació amb la gravetat de la malaltia pulmonar. A més, avaluar la utilitat de la AEA com a indicador de l'evolució clínica.

Mètodes: Estudi transversal que va incloure pacients amb Malaltia Pulmonar Obstructiva Crònica (MPOC) sense DAAT, pacients amb DAAT amb fenotip Pi*ZZ i un grup control d'individus sans. Es van recopilar dades clíniques, sociodemogràfiques i de funció pulmonar, i es van mesurar els nivells sèrics de AAT i AEA.

Resultats: Es van reclutar 38 pacients MPOC sense DAAT, 19 pacients Pi*ZZ (8 sense tractament, 11 amb tractament substitutiu) i 30 individus sans. Els pacients Pi*ZZ van presentar nivells disminuïts d'AAT i AEA, i es va observar una correlació positiva i significativa en els pacients MPOC sense DAAT i Pi*ZZ. No es va observar una correlació significativa entre la AEA i el FEV_1% ni entre la AAT i el FEV_1% en cap dels grups estudiats.

Conclusions: El nostre estudi va determinar que la AEA es correlaciona amb la AAT en pacients Pi*ZZ i MPOC, però no es relaciona amb la gravetat de la malaltia pulmonar. La mesura de la AEA podria ser útil per avaluar la funció de la AAT, però són necessaris estudis longitudinals per determinar si podria ser un indicador de l'evolució de la malaltia pulmonar.

ABSTRACT

Contexto: El Déficit de alfa-1 antitripsina (DAAT) es una enfermedad autosómica codominante que causa una disminución de los niveles séricos de alfa-1 antitripsina (AAT), provocando un desequilibrio entre la actividad elastasa y anti-elastasa (AEA) y daño pulmonar, especialmente el fenotipo Pi*ZZ.

Objetivo: Determinar la independencia de la AEA respecto a los niveles de AAT y su asociación con la gravedad de la enfermedad pulmonar. Además, evaluar la utilidad de la AEA como indicador de la evolución clínica.

Métodos: Estudio transversal que incluyó pacientes con Enfermedad Pulmonar Obstructiva Crónica (EPOC) sin DAAT, pacientes con DAAT con fenotipo Pi*ZZ y un grupo control de individuos sanos. Se recopilaron datos clínicos, sociodemográficos y de función pulmonar, y se midieron los niveles séricos de AAT y AEA.

Resultados: Se reclutaron 38 pacientes EPOC sin DAAT, 19 pacientes Pi*ZZ (8 sin tratamiento, 11 con tratamiento sustitutivo) y 30 individuos sanos. Los pacientes Pi*ZZ obtuvieron niveles disminuidos de AAT y AEA, y se observó una correlación positiva y significativa en los pacientes EPOC sin DAAT y Pi*ZZ. No se observó una correlación significativa entre la AEA y el FEV_1% ni entre la AAT y el FEV_1% en ninguno de los grupos estudiados.

Conclusiones: Nuestro estudio determinó que la AEA se correlaciona con la AAT en pacientes Pi*ZZ y EPOC, pero no se relaciona con la gravedad de la enfermedad pulmonar. La medición de la AEA podría ser útil para evaluar la función de la AAT, pero se necesitan estudios longitudinales para determinar si podría ser un indicador de la evolución de la enfermedad pulmonar.

ABSTRACT

Context: Alpha-1 antitrypsin deficiency (AATD) is an autosomal codominant disease that leads to decreased serum levels of alpha-1 antitrypsin (AAT), resulting in an imbalance between elastase and anti-elastase activity (AEA) and pulmonary damage, particularly in the Pi*ZZ phenotype.

Objective: Determine the independence of AEA from AAT levels and their association with the severity of pulmonary disease. Additionally, to evaluate the utility of AEA as an indicator of clinical progression.

Methods: This cross-sectional study included patients with chronic obstructive pulmonary disease (COPD) without AATD, AATD patients with the Pi*ZZ phenotype, and a control group of healthy individuals. Clinical, sociodemographic, and pulmonary function data were collected, and serum levels of AAT and AEA were measured.

Results: A total of 38 COPD patients without AATD, 19 Pi*ZZ patients (8 untreated, 11 receiving replacement therapy), and 30 healthy individuals were recruited. Pi*ZZ patients had decreased levels of AAT and AEA, and a positive and significant correlation was observed between AEA and AAT in patients COPD without AATD and Pi*ZZ patients. No significant correlation was found between AEA and FEV_1% and neither between AAT and FEV_1% in any of the studied groups.

Conclusions: Our study determined that AEA correlates with AAT in Pi*ZZ and COPD patients but does not correlate with the severity of pulmonary disease. Measurement of AEA may be useful for evaluating AAT function, but longitudinal studies are needed to determine if it could be an indicator of the evolution of the lung disease.

INTRODUCTION:

Chronic obstructive pulmonary disease (COPD) is a respiratory disorder that affects millions of people worldwide. This entity is characterized by an irreversible obstruction of airflow to the lungs, with smoking being its main risk factor (1). This disease has different phenotypes described, one of which is pulmonary emphysema, that can have a genetic predisposition known as alpha-1 antitrypsin deficiency (AATD). It affects men and women equally, with a median age of diagnosis around 40-45 (2).

In normal situation, alpha-1 antitrypsin (AAT) is a protein synthesized in the liver and goes to the lungs through passive diffusion (3). Its main function includes inhibiting neutrophil elastase as well as anti-inflammatory effects, among others. Therefore, as in the AATD there are low levels of the protein, the inhibitory capacity of neutrophil elastase is lost, producing an imbalance in the axis of elastase and anti-elastase activity. This leads to destruction of elastic fibers, collagen, and other connective tissue elements in the lungs. Therefore, it increases the capacity of distensibility, which over time and by losing the elastic retraction, ends up causing air trapping and impairing gas exchange in the distal alveoli (4). This process results in pulmonary emphysema, whose main symptom is dyspnea. Other reported symptoms include wheezing and coughing, although other studies have found individuals with a history of asthma (5). Additionally, in the context of AATD, the mutated AAT protein undergoes a conformational change that leads to self-folding and deposition in hepatocytes. This accumulation of abnormal protein predisposes individuals to develop liver disease in cases of severe AATD (6). Furthermore, there is evidence of AAT association with other diseases such as systemic vasculitis and necrotizing panniculitis (7-9).

Genetic studies of AAT show an autosomal codominant pattern, where each allele corresponds to one of the parents. In cases of deficiency, allelic mutations of the SERPINA1 gene are observed on chromosome 14, specifically at the Pi (*protease inhibitor*) locus. To diagnose AATD, it is recommended to determine AAT concentrations through nephelometry, followed by identifying the phenotype based on the electrophoretic migration speed in a magnetic field (10). Among the variants known until today, only 30 have clinical significance, ranging from mild to severe: MM > ZZ > NullNull (11-13).

The phenotype without AATD corresponds to Pi*MM, where serum AAT levels are normal (>110 mg/dL). In the case of AATD, the Pi*ZZ phenotype shows a severely decreased serum AAT levels[1] (<50 mg/dL). The null phenotype, although being rare, patients have undetectable levels of AAT and is associated with a high risk of emphysema but not hepatopathy due to the absence of AAT accumulation in the liver (14).

The frequency of the Pi*ZZ phenotype in the general population varies in different regions of Europe. It is estimated to affect around 1 individual per 2,000 to 5,000 people (15). The highest prevalence of severe AATD has been observed in coastal regions of northwestern Europe, gradually decreasing towards the east. In contrast, the presence of this deficiency is almost non-existent in Asia (16). Additionally, the Iberian Peninsula has shown a higher predominance of the S allele and a lower presence of the Z allele (5).

It is important to highlight that there is a problem of underdiagnosing the disease, making it recommended for all patients diagnosed with COPD to undergo an examination to measure their AAT level at least once in their lifetime (16).

Given the significant role of AATD as the primary genetic factor in the development of lung disease, several techniques have been developed in recent years to not only assess AAT concentrations but also evaluate the functionality of the existing protein (17). In previous studies, the anti-elastase activity (AEA) has been evaluated in bronchoalveolar lavage to determine the effectiveness of AAT replacement therapy in these patients (18). However, there are currently limited studies evaluating serum concentration of AEA and its correlation with AAT and the severity of lung disease.

Consequently, the primary objective of this study was to investigate whether there is a relationship between serum levels of AEA and AAT in patients with the Pi*ZZ phenotype and COPD. Additionally, a secondary objective was to demonstrate if serum levels of AEA are associated with parameters that reflect the severity of lung disease.

[1]Based on the electrophoretic migration velocity in a magnetic field, we can classify Pi as follows: M for medium migration velocity, F for fast migration velocity, S for slow migration velocity, and Z as the lowest migration velocity being the last letter of the alphabet.

METHODS:

Patients:

This cross-sectional descriptive study was conducted at the AATD reference center of Vall d'Hebron Hospital in Barcelona, Spain. The participants of the study were consecutively recruited from the outpatient clinics of the Pneumology Department at the hospital and were divided into different groups:

- Control Group: It included adults who were never smokers or ex-smokers for more than six months, with Pi*MM phenotype and normal AAT levels (> 110 mg/dL).
- COPD patients without AATD: The inclusion criteria were ex-smoker patients with a cumulative history of more than 10 pack-years, diagnosed with COPD based on spirometry: post-bronchodilator FEV_1/FVC ratio < 0.7 (19), and having Pi*MM phenotype and normal AAT levels.
- COPD patients with Pi*ZZ phenotype: It included patients diagnosed with Pi*ZZ AATD and fulfilling the previously described diagnostic criteria for COPD.

The study was conducted in accordance with the principles of the Helsinki Declaration and the current regulations for research involving human subjects. To ensure data confidentiality, compliance with the Data Protection Law 2016/679 was followed. In addition, the study was approved by the Ethics and Clinical Research Committee of Vall d'Hebron University Hospital II (Barcelona, Spain) with approval number PR (AG) 156/2016, and all participants provided their written informed consent.

Variables:

Sociodemographic data such as age, sex, body mass index (BMI) and smoking status were collected, along with clinical characteristics including cough, wheezing, and dyspnea. In addition, spirometry was performed, and the values of forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC) and the FEV_1/FVC were recorded.

Furthermore, data from the following questionnaires were collected: Charlson comorbidity Index (20), the BODEX prognostic index (*Body mass index*, *Airflow obstruction, Dyspnea, Exercise capacity*, *Exacerbations)* (21), and the CAT test *(COPD Assessment test*) (22) to measure the impact and quality of life of COPD patients.

Analysis of serum AAT and AEA:

Peripheral blood samples were taken from all participants. For patients with Pi*ZZ phenotype who were receiving replacement therapy with exogenous AAT, samples were obtained before administration to avoid a potential interference with the results. Plasma samples were collected and stored at -80 °C until analysis. To assess the total amount of AAT protein in the plasma samples, immunonephelometry measurement was performed on a BNII instrument (Siemens Healthcare, Malvern, PA, USA).

For the serum measurement of AEA, an additional analysis was conducted using the same plasma samples employing a porcine elastase inhibition assay (17). The assay is based on the hydrolytic activity of porcine pancreatic elastase, which is inhibited by AAT in a chromogenic substrate, and it is determined using a kinetic spectrophotometric method. Plasma samples were diluted with tris buffer with human serum albumin (HSA), and 100 µl was added to a microplate, followed by the addition of 50 µl of porcine pancreatic elastase. After incubation for 10 minutes at room temperature, AAT in the samples formed an irreversible complex with a portion of elastase. Subsequently, 50 µl of a specific chromogenic substrate (N-succinyl Ala-Ala-Ala p-nitroanilide, Sigma Aldrich) was added and incubated for 3 minutes. The remaining uncomplexed elastase hydrolyzed the elastase substrate into a yellow compound that absorbs at 405 nm. The reaction was stopped with 50% acetic acid, and the plate was read after 2 minutes. The concentration of functionally active AAT was determined by interpolation from a calibration curve constructed with an AAT standard provided by Grifols®.

The buffer used for sample dilution was used as a negative control (without elastase inhibition, maximum absorption), and a set of serum samples with a known AEA of AAT (provided by Grifols) was used as a positive control (elastase inhibition, low absorption). This technique was adapted and carried out on the Triturus ELISA instrument (Grifols), a fully automated and open ELISA analyzer. The instrument performed all steps, including dilutions, dispensing, reading, and result calculation, except for standard dilutions and sample predilution, which were performed manually.

All samples were analyzed in triplicate and AEA was expressed as the concentration of functionally active AAT (mg/ml). Specific activity, which is the ratio of functional AAT to total protein, was calculated as the ratio of functionally active AAT concentration to the total protein amount determined by immunonephelometry.

Statistical Analysis:

Qualitative variables were described using absolute frequencies and percentages. For quantitative variables, mean, standard deviation (SD), median and quartiles were used for description. The normality of distributions was assessed using the Kolmogorov-Smirnov test.

To analyze quantitative variables, ANOVA tests were performed. Sociodemographic, clinical variables, and levels of AEA and AAT were compared according to the study groups. Furthermore, linear relationships between AEA, clinical characteristics, AAT levels and lung function which were evaluated by the Chi-square test (with the Fisher test for frequencies <5) and the Pearson correlation coefficient, respectively.

Statistical significance was considered for p-values < 0.05 in all tests. The analyses were conducted using R Studio software (V2.5.1).

RESULTS:

Characteristics of the participants:

A total of 57 patients were included: 38 COPD without AATD, 8 Pi*ZZ without treatment and 11 Pi*ZZ with treatment. The control group consisted of 30 individuals.

The mean age was 64.1 (12.3) years, and of all participants, 49.0 (56.3%) were male.

Among the three groups of patients, 48.0 (56.5%) were ex-smokers with an average smoking history of 22.9 (23.9) pack-years. The mean of Body mass index (BMI) was 26.8 (4.9) kg/m^2. The mean Charlson Index was 4.6 (1.9) and the mean BODEX score was 3.2 (1.9). The CAT questionnaire obtained a mean score of 9.1 (8.5).

The mean exacerbations in the last year were 0.35 (0.6) in COPD patients, 0.38 (0.5) in Pi*ZZ patients without treatment and 0.36 (0.5) in treated Pi*ZZ patients (Table 1).

Spirometry interpretation: COPD, Pi*ZZ phenotype with and without treatment:

Spirometry tests showed an overall mean FEV_1 (%) of 48.5 (20.7)%. When comparing the groups, a lower FEV_1 (%) was observed in Pi*ZZ patients on substitution therapy compared to COPD without AATD and Pi*ZZ without treatment (31.1 (13.0) % vs. 52.4 (21.0) % vs. 58.2 (11.6) %; $p<0.01$) (Table 1).

Table 1. Sociodemographic and clinical characteristics of the total population*

Variables	Total (n= 87)	COPD without AATD (n= 38)	Pi*ZZ without treatment (n= 8)	Pi*ZZ with treatment (n= 11)	p value
Age	64.1 (12.3)	71.2 (10.2)	68.6 (10.0)	62.0 (11.7)	0.001
Sex (male)	49.0 (56.3%)	30.0 (78.9%)	2.0 (25%)	5.0 (45.5%)	0.002
BMI (Kg/m^2)	26.8 (4.9)	28.2 (5.0)	23.5 (3.7)	24.0 (2.3)	0.005
Smoking consumption (pack/years)	22.9 (23.9)	43.9 (19.4)	9.4 (6.8)	22.1 (9.3)	< 0.001
- Nosmoker	31.0 (36.5%)	0 (0%)	2.0 (25%)	0 (0%)	< 0.001
- Ex smoker	48.0 (56.5%)	34.0 (91.9%)	3.0 (37.5%)	11.0 (100%)	< 0.001
Charlson index	4.6 (1.9)	5.4 (1.8)	3.5 (1.1)	3.1 (1.3)	< 0.001
BODEX	3.2 (1.9)	3.2 (2.0)	1.8 (1.2)	4.1 (1.6)	0.020
CAT	9.1 (8.5)	13.6 (5.7)	12.3 (9.4)	17.0 (5.9)	<0.001
FVC (post) L	2.5 (1.0)	2.6 (0.9)	2.3 (1.0)	2.1 (1.4)	0.188
FEV_1 (post) L	1.3 (0.6)	1.4 (0.6)	1.2 (0.3)	0.9 (0.7)	0.012
FEV_1 (post) %	48.5 (20.7)	52.4 (21.0)	58.2 (11.6)	31.1 (13.0)	0.001
Exacerbations	0.36 (0.6)	0.35 (0.6)	0.38 (0.5)	0.36 (0.5)	0.874

**Values are expressed as mean, mean standard deviation (SD) and percentage (%).*

BODEX: *Body Mass Index, Airflow Obstruction, Dyspnea and Exercise Capacity, Exacerbations.*
CAT: *COPD Assessment Questionnaire.*
FVC: *Forced Vital Capacity.*
FEV_1: *Forced expiratory volume in the first second.*

Interpretation of serum AAT and AEA levels:

The mean AAT (mg/dL) levels of all participants was 124.5 (46.2) mg/dl. In the group of COPD patients without AATD, the mean AAT level was 145.9 (31.4) mg/dl, while in patients with untreated Pi*ZZ it was 24.4 (5.9) mg/dL, and in those with specific treatment was 111.7 (60.9) mg/dl (Table 2).

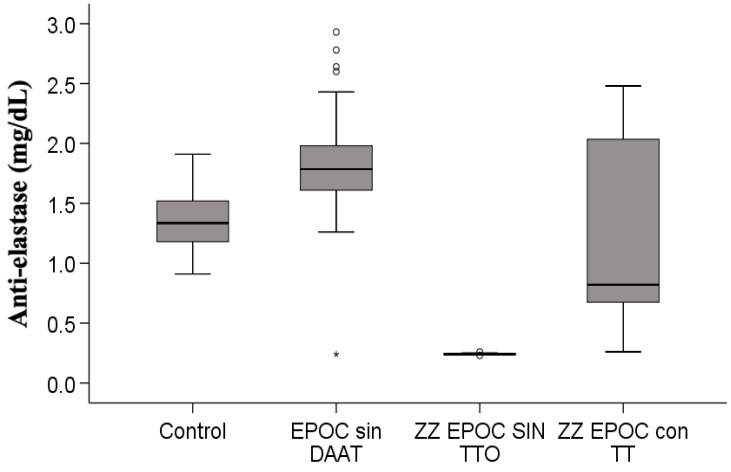
The mean absolute AEA (mg/mL) for all participants was 1.4 (0.63) mg/mL. Higher levels of AEA were observed in patients with COPD without AATD, followed by Pi*ZZ patients with treatment, and finally untreated Pi*ZZ (1.8 (0.48) mg/mL vs. 1.3 (0.81) mg/mL vs. 0.2 (0.01) mg/mL, p<0.001) (Figure 1).

Table 2. Serum AAT and AEA concentrations in the total population*

Variables	Total (n= 87)	COPD without AATD (n= 38)	Pi*ZZ without treatment (n= 8)	Pi*ZZ with treatment (n= 11)	p value
AAT levels (mg/dL)	124.5 (46.2)	145.9 (31.4)	24.4 (6.0)	111.7 (60.9)	<0.001
AEA Absolute (mg/mL)	1.4 (0.6)	1.8 (0.5)	0.2 (0.0)	1.3 (0.8)	<0.001

**Values are expressed as mean, standard deviation of the mean (SD) and percentage (%).*

Figure 1. Elastase of the total population



Correlation between serum AEA and AAT levels:

The relationship between AEA levels and AAT concentrations was examined in all groups. In patients with COPD without AATD, a moderate and statistically significant association was observed between AAT levels and AEA ($r = 0.610$; $p < 0.001$) (Figure 2.1). For untreated patients with Pi*ZZ phenotype, a strong positive and significant correlation was found between AEA and AAT levels ($r = 0.850$; $p<0.007$) (Figure 2.2). Finally, in the group of Pi*ZZ patients with replacement therapy, a strong positive and statistically significant correlation was observed between AEA and AAT concentrations ($r = 0.890$; $p < 0.001$) (Figure 2.3).

Figure 2. Correlation between AEA and AAT levels in patients

Figure 2.1 COPD without AATD (n = 38)

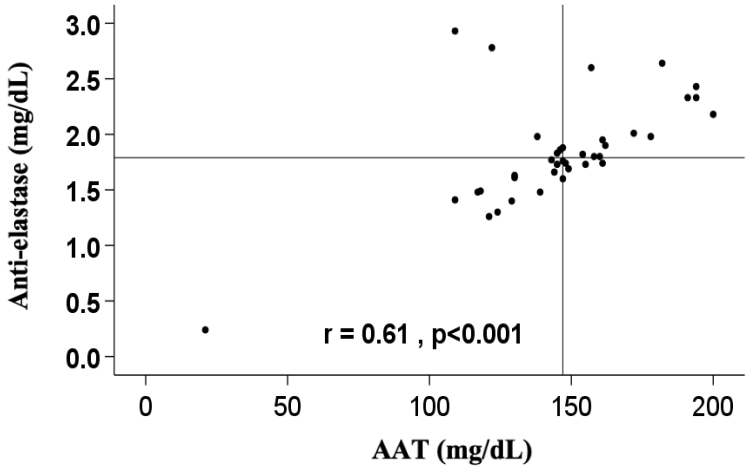


Figure 2.2 Pi*ZZ without treatment (n = 8).

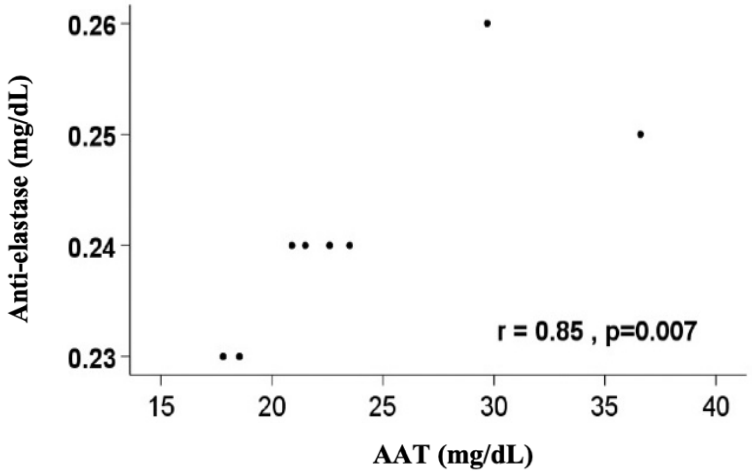
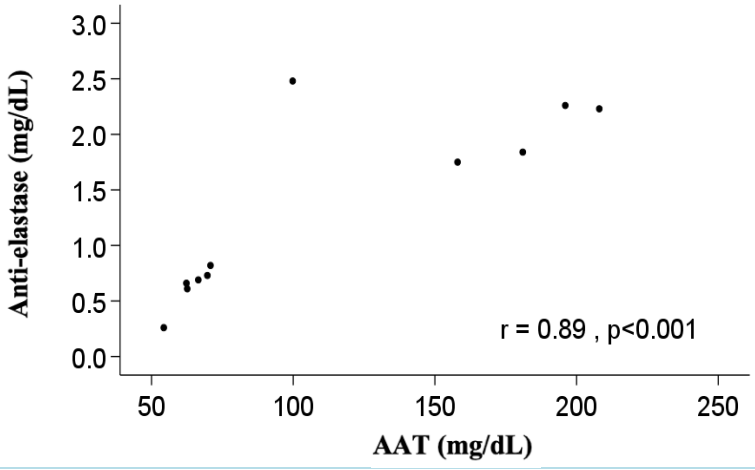


Figure 2.3 Pi*ZZ with treatment (n = 11)



Correlation between AEA levels and lung function measured by FEV_1:

In addition, the association between the levels of AEA and the degree of obstruction measured by FEV_1 (%) was determined. In patients with COPD without AATD, no significant correlation was observed (r = -0.063; p = 0.709) (Figure 3.1).

Regarding patients with Pi*ZZ phenotype, no correlation was found between AEA and FEV_1(%) in either patients without (r = 0.110; p = 0.801) or with augmentation therapy (r= 0.340; p = 0.331) (Figure 3.2 and 3.3).

Figure 3. FEV_1 & Antielastase activity

Figure 3.1 COPD without AATD (n= 38)

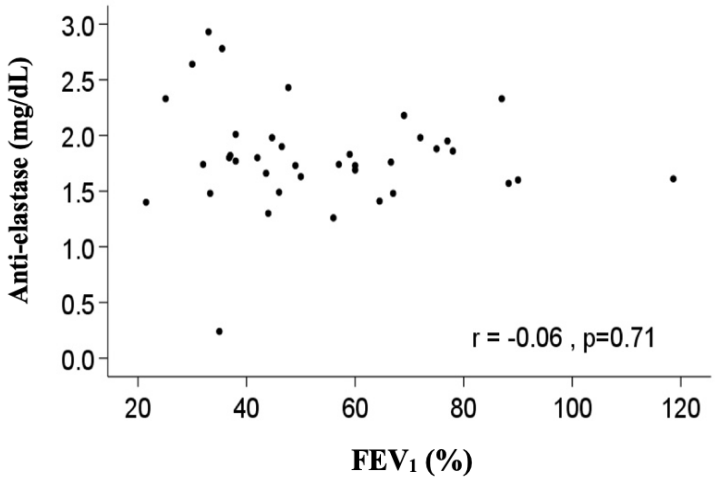


Figure 3.2 Pi*ZZ without treatment (n= 8)

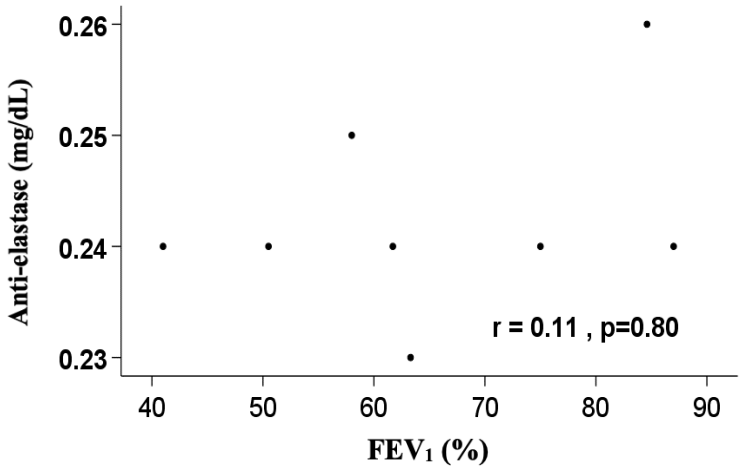
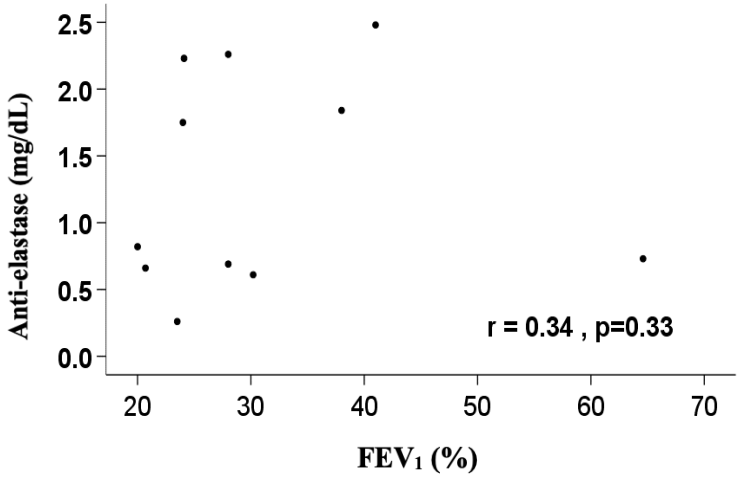


Figure 3.3 Pi*ZZ with treatment (n= 11)



DISCUSSION:

In our study, we observed that patients with COPD without AATD had higher levels of AEA and AAT, followed by patients with AATD Pi*ZZ on treatment, and finally without treatment. Regarding the severity of lung disease, we observed that Pi*ZZ patients on replacement therapy had a higher obstruction measured by FEV_1% compared to Pi*ZZ patients without treatment and COPD without AATD. In addition, we investigate the relationship between serum concentrations of AEA and AAT in all groups, finding a positive and significant correlation in both groups, COPD without AATD and Pi*ZZ patients with and without treatment. Finally, we also examined the potential relationship between AEA and lung function, but no significant correlation was observed in any of the groups.

Initially, AEA of AAT was measured at the bronchoalveolar level mainly to determine whether replacement therapy in patients with AATD was effective not only increasing serum AAT concentrations but also in determining if it increased their inhibitory capacity against neutrophil elastase. In the study conducted by Hubbard et al., the authors observed that after AAT replacement therapy, Pi*ZZ patients doubled the AEA concentrations measured in bronchoalveolar lavage compared to untreated Pi*ZZ patients. Despite receiving treatment, AEA levels in bronchoalveolar lavage did not reach the same values as those in the healthy population (18).

On the other hand, other studies have determined a correlation between AEA concentrations in bronchoalveolar lavage and serum AAT concentrations. In two studies conducted in patients with AATD receiving exogenous AAT therapy, the authors found a significant correlation between serum AAT levels and AEA measured in bronchoalveolar lavage (23). Similarly, Hubbard et al. observed a similar peak in serum levels of AAT and AEA measured in bronchoalveolar lavage at the same time of taking medication, followed by a parallel decrease over the next 24 hours in patients with AATD receiving aerosolized AAT therapy (24).

Unlike previous research, in our study the AEA was measured in serum. We observed a strong correlation between serum AEA and AAT levels in both patients with COPD without AATD and Pi*ZZ AATD group. Therefore, the technique we have used appears to accurately assess AAT activity in a less invasive way compared to the previously used bronchoalveolar lavage method.

Moreover, our study aimed to demonstrate a possible relationship between serum AEA and the severity of lung disease, but we did not find a significant correlation in any of the groups. This could be explained by the small sample size, which did not allow us to establish any relationship among these two variables. As far as we know, this is the first study that evaluates the potential impact of serum AEA on lung disease.

Our study has presented several limitations, including: 1) its cross-sectional design, not allowing us to establish a relationship between serum AEA and the progression of lung function; 2) due to AATD being a rare disease, we were unable to obtain a large sample size. Therefore, longitudinal studies are needed to establish causal associations between the variables studied, as well as the use of international registries to include a larger sample of patients (25).

Finally, we believe that the use of serum AEA in clinical practice is important because serum AAT measurement using nephelometry does not reflect the amount of functional protein, or the possible contribution of other antiproteases and total AEA. The analysis of serum AEA can be useful in cases where normal levels of AAT are detected but the protein is dysfunctional.

For this reason, and as a strength of our study, we believe that measuring serum AEA in clinical practice could improve the diagnosis of the disease, avoiding invasive procedures such as bronchoscopy. Furthermore, serum AEA would also benefit a larger group of patients by having a more accessible diagnostic test and receiving the treatment earlier.

CONCLUSION:

In conclusion, our study determined that serum AEA correlates with AAT in Pi*ZZ patients with COPD, but it does not correlate with the severity of lung disease. Therefore, longitudinal studies and the use of international AATD registries are needed to establish the utility of AEA as an indicator of lung disease severity.

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Not only that, but this team is specialized in AATD, which has allowed me to acquire more knowledge in a common genetic disease, but despite this, under-diagnosed.

On the other hand, I would also like to acknowledge Marc's team, with whom I have had the privilege to work closely, directly, and instructively since the first day. I want to particularly thank Alexa Nuñez, a pulmonologist from the same department, for being an exceptional mentor. It has been an honor to learn from her knowledge and experience. Also, my thanks go to Gina, Cristina and Gerard, for their support and their great capacity for teamwork.

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