Alpha-1 antitrypsin Null mutations and severity of emphysema

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Pulmonary emphysema; Null mutations; SERPINA1; Alpha-1 antitrypsin

Summary
Background: Alpha-1 antitrypsin (AAT) deficiency is an autosomal-codominant disorder, caused by mutations in the SERPINA1 gene on chromosome 14. Individuals affected by the most common mutations, SZ and ZZ, have serum AAT concentrations of 25% and 15% of normal levels, and present a higher risk of emphysema. Mutations causing total absence of serum AAT (Null mutations) were suggested to be associated with very early onset emphysema but their clinical phenotype is poorly known.

Hypothesis: Absence of AAT in Null mutations results in more severe emphysema as compared to ZZ and SZ.

Methods: We genotyped all known Dutch subjects (n = 12) with absent serum AAT, and compared their lung function values (FEV1 and KCO) with those of individuals with ZZ and SZ genotype, matched for age and smoking history.

Results: All subjects with absent serum AAT presented homozygous Null mutations. In three subjects, a new mutation in exon 2 of the SERPINA1 gene was found. Subjects with Null mutations showed significantly lower lung function values than SZ and ZZ individuals (p = 0.000 and 0.001 for FEV1 and KCO, respectively). In all groups, there was a positive correlation between serum AAT and lung function values (p = 0.025 and 0.014 for FEV1 and KCO, respectively).

Conclusions: Serum levels of AAT are correlated with the severity of pulmonary phenotype. Subjects with Null mutations should be considered a subgroup at particularly high risk of emphysema within AAT deficiency (AATD). Early detection of carriers of this genotype would be important for preventive and therapeutic interventions.

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Introduction

Alpha-1 antitrypsin (AAT) is the most prevalent serine protease inhibitor in serum; it is synthesized by hepatocytes and belongs to the family of serpins (serine protease inhibitors). Its main role is that of inhibiting neutrophil elastase (NE), an enzyme that degrades several components of the extracellular matrix in the lungs. Insufficient inhibition of NE in AAT deficiency (AATD) can cause severe, early age onset pulmonary emphysema, resulting in high incidence of lung transplantation and reduced life expectancy.

AATD is one of the most common autosomal-codominant genetic disorders in the Caucasian population. To date, about 100 genetic variants of AAT have been identified. The normal AAT genotype is Pi MM, (94–96% of the white population), characterized by protein serum levels of 150–350 mg/100 mL (20–48 µM). The S variant, originated in the Iberian Peninsula, and the Z variant, arisen in the Viking population, account for more than 95% of the mutations in patients with severe AATD. Both variants are missense mutations in the serpina1 gene (SERPINA1) on chromosome 14q32.1. In contrast to the S, the Z phenotype is characterized by polymerization of AAT, which can result in liver cirrhosis. Individuals with SS, SZ, and ZZ genotypes have serum AAT concentrations of approximately 85%, 25%, and 15% of normal levels, respectively. Homozygosis for S mutation has not been associated with disease. A very limited number of studies investigated the different risk of developing emphysema between ZZ and SZ genotypes. Most studies concluded that the SZ genotype is less important than the ZZ in the development of emphysema and SZ patients develop emphysema at an older age than ZZ patients. However, it is not known whether the different risk is linked to the different AAT serum levels between the two populations or to other, unknown, factors.

Among the variants within the SERPINA1, several mutations have been described leading to total absence of AAT production (Null mutations). Although the number of these mutations is large, they are rare within the population, thus very little is known about their clinical phenotype. In 1988, Cox and Levison showed that lung disease associated with Null homozygosis (Null Mattawa: Q0mat) was more severe than that associated with ZZ genotype. The very small sample size of that study (three homozygous sisters) prevents generalizability of the findings; however, this study and other sparse clinical reports seem to point to a higher risk and severity of emphysema in subjects with absence of AAT in serum. If this would be the case, early detection of the Null homozygous genotype should be promoted and should lead to stronger educational intervention (e.g. smoking avoidance) and to a possible use of the replacement therapy as preventive treatment. Recently, Ferrarotti et al. reported lower values of FEV₁ in subjects with rare variants of AAT as compared to ZZ; however, in their study ZZ and rare variants had similar AAT plasma levels and the lung function results were confounded by smoking. Thus, the authors could not discriminate between the importance of genetic factors (type of mutation), protein serum levels, and environmental factors (smoking) in determining the severity of the disease.

Aim of our study was to analyze whether homozygous Null genotype is related to severity of emphysema in AATD and whether protein serum levels are the major determinants of severity. To this aim, we characterized the SERPINA1 mutations in the Dutch Null population, we compared lung function of Null subjects with that of ZZ and SZ subjects matched for age and smoking history, and correlated the functional data with AAT serum levels.

Methods

Subjects

All subjects included in the study were recruited from the Alpha-1 International Registry database (AIR, www.aatregistry.org). AIR is the largest international database of individuals with AATD, containing lung function data, clinical history, and AATD phenotype of more than 2600 individuals with severe deficiency from 21 different countries, collected in the years 1997–2006. A review with a detailed description of the AIR database development and methodology has been recently published. The Dutch part of the AIR database includes a total of 290 subjects, and one of the largest Null populations ever detected, composed by 12 subjects from 7 families. All 12 Null subjects were included in our analysis.

Study design

The study had a matched-paired design. For each Null case, a subject with ZZ and a subject with SZ genotype were selected from the AIR Registry, matched on the basis of age (±5 years) and smoking (±7 pack-years). Due to the young age of the Null subjects, only one-to-one matches with complete lung function data were found within the ZZ and SZ population. The study was approved by the Ethics Committee of the Leiden University Medical Centre and patients gave their written informed consent.

Lung function

Lung function tests to determine the severity of emphysema were performed according to the European Respiratory Society guidelines. All tests were performed after nebulization of 5 mg of salbutamol and 500 mcg of ipratropium bromide. Among the lung function measurements, forced expiratory volume as percentage of predicted (ppFEV₁) and the coefficient of diffusion of carbon monoxide as percentage of predicted (ppKCO) were included in the analysis, since they are currently considered as the most relevant for determining emphysema severity in AATD.

Serum AAT levels

Serum AAT levels were measured using a completely automated immunoassay, as previously described. The lower limit of detection of the assay is 10 nM. As confirmation of absence of an AAT band observed in the gel of isoelectrophoresis of serum samples, all subjects with AAT serum values lower than 1.5 µM were considered as having
no serum AAT. Values of AAT are presented as micromolar and gram per liter.

Statistical analysis

The software package SPSS 11.5 (SPSS inc., Chicago, USA) was used. Due to their young age, for two Null/Null subjects it was not possible to find in the database matched ZZ and SZ subjects with KCO data. Therefore, only 10 subjects per group were included in the analysis of KCO. Data are expressed as mean (S.D.) when normally distributed, as median (range) otherwise. For normally distributed data, differences within the means of the three groups were analyzed by the analysis of variance (ANOVA) test. Differences between two groups at one time were established using as post-hoc the Student's t-test. When data were not normally distributed, Kruskal–Wallis test was used for comparisons within the three groups and Mann–Whitney test was applied post-hoc to compare two groups at a time. Correlations between lung function parameters and AAT serum levels were tested using the non-parametric Spearman’s correlation coefficient. The results were considered statistically significant for p-values below 5%.

Genotype and haplotype analysis

Genotype analysis

Genomic DNA was isolated from 10 ml of peripheral blood using standard procedures.24 Genotypic analysis was performed in the Null/Null subjects by direct sequencing after PCR amplification of the all exons (1a, 1b, 1c, and 2–5) of SERPINA1. Primer sequences (obtained from K. Morgan, Nottingham, UK) and annealing temperatures used for PCR amplification are shown in Table 1. PCR products were purified using the Qiagen PCR purification kit (Qiagen Benelux, Venlo, the Netherlands). Sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Arrington, UK) and products were analyzed on the ABI 3730 sequencer. Total RNA was extracted from fresh blood using the RNA Insta-Pure extraction kit (Eurogentec, Maastricht, the Netherlands). RNA was obtained from control individuals without A1ATD and one A1ATD subject to determine the expression of a novel Null mutation in blood monocytes. RNA from controls and a homozygous Null patient was sequenced after RT-PCR amplification using the forward primer of exon 4 and the reverse primer of exon 5 to determine the allelic expression.

Haplotype analysis

Polymorphic markers (D14S1054, D14S299, D14S1142, and D14S749) flanking SERPINA1 were used to determine the haplotype inheritance within a family with a compound heterozygous Null mutation (family E). Table 2 shows the primers sequences and conditions for PCR amplification. The forward primers of D14S1054 and D14S1142 were labeled with FAM fluorophores; the forward primers of D14S299 and D14S749 were labeled with HEX. PCR fragments were analyzed on ABI3700 sequencer.

Results

Subjects

Characteristics of Null subjects

The characteristics of Null subjects are presented in Table 3. Genotypes of the Null subjects are presented in Table 4. Seventy-five percent of the subjects were current or...
ex-smokers. The reason of assessment was lung disease in 9 out of 12 subjects (75%), while the other 3 (25%) were ascertained because of family screening. Similar results for ascertainment were found in the SZ subjects (75% lung disease vs. 25% family screening) while in the ZZ group reason for ascertainment was lung disease in 10 subjects (83%) and family screening in 2 (17%). In the Null population, lung function could vary between siblings from the same family, even when the subjects had comparable age and smoking history. In family A, sibling 2 (S2) and sibling 3 (S3) had similar age and were both non-smokers, nevertheless S3 had almost double ppFEV1 than S2. Interestingly, these two subjects had similar KCO impairment. Similarly, FEV1 was different between S1 and S2 in family D, with much higher

<table>
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<tr>
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<th>Member</th>
<th>Age</th>
<th>Sex</th>
<th>Smoking Pack-years</th>
<th>Reason assessment</th>
<th>FEV1 (% predicted)</th>
<th>KCO (% predicted)</th>
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Table 4 Genomic mutations in individuals with absent serum AAT.

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<tr>
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<th>Nucleotide change</th>
<th>Mutated exons</th>
<th>Amino-acid changes</th>
<th>Name mutation</th>
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<td>PiNull Bellingham</td>
</tr>
<tr>
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<td>K217X/Normal</td>
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<tr>
<td>A</td>
<td>Sibling 1 (Null)</td>
<td>A737T/A737T*</td>
<td>3/3</td>
<td>K217X/K217X'</td>
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<td>K217X/K217X</td>
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</tr>
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</tr>
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<td>PiM Heerlen</td>
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<tr>
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<td>5/5</td>
<td>P369L/P369L</td>
<td></td>
</tr>
<tr>
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<td>–</td>
<td>–</td>
<td>Normal/Normal</td>
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</tr>
<tr>
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<td>–</td>
<td>–</td>
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<tr>
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<tr>
<td>G</td>
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<td>5/5</td>
<td>P369L/P369L</td>
<td></td>
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</table>

*A nucleotides are numbered from the translation start site in exon 2.

*Amino-acids are numbered from the signal peptide cleavage site (the first methionine of A1AT is located at position 24).*
FEV_1 in S2. Discordant impairment of ppFEV_1 and ppKCO was noted in individuals A-S2, B-S2, and G-S1.

Genotype of Null subjects
Genomic mutations of SERPINA1 on chromosome 14 were characterized in all 12 subjects with absent serum AAT. All subjects were shown to be homozygous for Null mutations (Null/Null). Whenever possible, genotypic analysis was performed also in the first’s degree relatives of the Null/Null subjects. The results are shown in Table 2. In five families (A, B, D, F, and G), previously published mutations were identified (PiNull Bellingham and PiM Heerlen). Interestingly, a new mutation was found in three individuals from two different families (C and E). A nucleotide substitution of a C to G in exon 2 at position 548 (counted from the translation start site of SERPINA1) resulted in a premature stop codon (Y160X). Both siblings of family C were homozygous carriers of the novel Null mutation, while the subject in family E was shown to be a heterozygous carrier. Haplotype analysis of family E is shown in Figure 1. One of the haplotypes (gray) of the proband with the Null/Null genotype (sibling 1: S1) is shared by his father (F) and brother; the other haplotype (white) is shared by his mother and sister. Sequence analysis showed that the white haplotype is associated with the PiM Heerlen mutation in exon V of SERPINA1 and the gray haplotype is associated with the novel Null mutation in exon 2. The novel mutation is named PiNull Bredevoort after the place of residence of the oldest known carrier (F).

We analyzed the expression of both alleles of the proband S1 (containing the PiM Heerlen and the PiNull Bredevoort mutation) in blood monocytes. Part of the DNA and cDNA (RNA) sequences of exon 5 of SERPINA1 for the proband and for a control are shown in Figure 2. The proband is heterozygous for the T nucleotide of the PiM Heerlen mutation, showing both alleles at DNA level (Figure 2a). At cDNA level only the T nucleotide of the PiM Heerlen mutation is observed, while the C-allele associated with the PiNull Bredevoort mutation is missing (Figure 2b). Control sequences show only the normal C-allele at both DNA and cDNA level (Figure 2c and d). Sequence analysis of exon 2 from father and brother of the proband confirmed the presence of a heterozygous PiNull Bredevoort mutation (Figure 2e and f).

Characteristics of Null/Null, ZZ, and SZ subjects
The demographic characteristics of all subjects, their lung function values and protein serum levels are shown in Table 5. The mean age difference between groups was 0.5 years (2.7) and the mean difference in smoking history 0.8 pack-years (4.1). Being the groups matched for age and smoking history, no significant differences were observed in these parameters ($p > 0.5$ for all comparisons).

Severity of emphysema
The comparisons of lung function measurements are presented in Figure 3. Significant differences among the groups were detected for both ppFEV_1 ($p = 0.000$) and ppKCO ($p = 0.001$). Post-hoc analysis showed lower values of ppFEV_1 in Null/Null as compared to ZZ ($p = 0.002$), and to SZ subjects ($p = 0.000$). Interestingly, a trend but not a statistically significant difference in FEV_1 was observed between the ZZ and SZ group ($p = 0.062$). Similar results were obtained from the comparison of ppKCO in the three groups, with lower values in Null/Null as compared to ZZ ($p = 0.000$) and to SZ ($p = 0.007$). No significant difference was observed between ZZ and SZ ($p = 0.16$).

Correlation with AAT serum levels
Considering ZZ and SZ as a combined group, we found a positive correlation between severity of emphysema, represented by ppFEV_1 and ppKCO, and serum levels of AAT (Figure 4). The levels of AAT in the serum of Null/Null subjects were all so low (<0.25 mM) that, although even in this group there was a positive correlation between lung function and levels, the authors doubted whether inter-subject differences in this group could be considered biologically relevant.

Discussion
In this study, we showed that subjects with absent serum AAT due to homozygous Null mutations present more severe pulmonary emphysema than subjects with ZZ or SZ mutations. This finding, and the correlation between AAT levels and lung function in SZ and ZZ that were matched for age.

Figure 1 Segregation in family E of four polymorphic markers flanking SERPINA1, and of four intragenic single nucleotide polymorphisms. The G in exon 2 of SERPINA1 causes the novel PiNull Bredevoort mutation (gray haplotype); the T in exon 5 causes the PiM Heerlen mutation (white haplotype).
and smoking history, suggest that the severity of emphysema in AATD is strongly determined by the serum levels of the protein. Furthermore, we characterized and genotyped the largest population of Null/Null subjects ever described, and found a new Null mutation at position 548 in exon 2 of SERPINA1 (Pi Null Bredevoort).

Until now only a very limited number of Null/Null subjects had been studied and even though they appeared to have worse lung function than other phenotypes, the limited number of cases and the confounding by smoking did not allow drawing a clear conclusion. The population of our study is larger, matched for age and smoking history with ZZ and SZ, and all lung function values were obtained post-bronchodilator. Thus, on the basis of our results we conclude that subjects carrying Null homozygous mutations should be considered a subgroup at particularly high risk of emphysema within AATD. It is important to raise more awareness about this rare but very severe condition within the medical community, for early detection of carriers of this genotype. Furthermore, an important implication of our study is that Null/Null subjects could possibly benefit from early prophylactic treatment with replacement therapy.

The correlation between severity of the disease and AAT serum levels is an interesting matter for discussion. A wide range of AAT concentrations and lung function values are represented in our ZZ and SZ populations, thus a positive correlation between AAT serum levels and lung function values contains important information. Even though the association between variants characterized by very low levels of AAT (ZZ, rare variants, and Null/Null) and emphysema severity has been reported in several publications since the discovery of AATD (reviewed in Ref. 1), a direct correlation between lung function and protein serum levels has, to the knowledge of the authors, never been reported. Possible reasons include lack of adequate number of cases (Null and rare variants), possible other mechanisms of lung damage (ZZ mutations), confounding by smoking, and lack of adequately standardized lung function measurements. Furthermore, ascertainment bias could have led to overestimate the risk in referred ZZ populations, and AAT replacement therapy gave controversial results on lung function outcomes in clinical trials, thereby not always supporting a direct relationship between AAT concentrations and severity/progression of emphysema. The possibility of smoking as a confounder in our study is run out by the matching criteria. Ascertainment is always a critical point in studies concerning rare diseases. For most rare diseases, including AATD, neonatal screening is not recommended;
therefore the subjects known to have the genetic defect are referred because they present the disease or because of family screening. In a recently published review of the methodology of the AIR Registry, ascertainment was due to lung/liver disease in 68.8% and to family screening in 19.2% of the subjects, similarly to what described in the National Heart, Lung and Blood Institute registry (72.3 and 19.8, respectively). The Null subjects of our study were ascertained because of lung disease in 75% of cases and for family screening in the remaining 25%. Similar percentages were found for ascertainment reasons in the ZZ and SZ subjects of our study. Therefore, we consider our study population representative of the general, known, AATD population.

In ZZ subjects, the presence of polymerized AAT in the lungs is thought to contribute to the pathogenesis of emphysema and may account for the difference in lung disease reported in the literature between ZZ and SZ patients. Polymers of AAT are chemotactic to neutrophils in vitro and co-localize with neutrophils in the alveoli, where they can work as a proinflammatory stimulus, exacerbating the lung disease of ZZ subjects. The magnitude of this effect can now be put into perspective against what is seen in our Null/Null subjects. There is no polymer formation in these individuals, yet the lung function of this population is significantly worse than that of ZZ, suggesting that low serum levels of AAT could be more important in the development of emphysema than the combination of low serum level and Z-AAT polymer formation.

We described a new mutation in the coding regions of SERPINA1 in two different families. This new mutation, called PiNull Bredevoort, is a nucleotide substitution mutation causing a premature stop codon in exon 2 and was identified in homozygous state in two sibs, and in heterozygous state in three members of another family. The Bredevoort mutation occurs at the same position as the Granite Falls mutation. In the Pi Null Granite Falls, deletion of a C-nucleotide causes a frameshift and a direct premature stop codon in the same position (Y160X) where a G-nucleotide substitutes a C-nucleotide in the PiNull Bredevoort mutation. Studies into the intra-cellular mechanism using Northern blot analysis showed no detectable levels of AAT mRNA in alveolar macrophages of homozygous PiNull Bredevoort mutation. Thus, the same mechanism is probably involved in causing total absence of serum protein in the PiNull Bredevoort mutation.

A possible limitation of our study is the small size of the study population. AATD is a rare disease, according to the definition of rare diseases as those with a prevalence equal or lower than 1 in 2000. The Null variants, which exact prevalence is not known, represent an extremely rare condition within AATD. Indeed, only three other Null/Null subjects have been registered in the whole AIR database besides the subjects of our study. For these reasons, our study population can be considered as representative of the general Null/Null populations.

In conclusion, our study showed that serum levels of AAT are correlated with the severity of emphysema. Subjects with Null mutations should be considered a subgroup at particularly high risk of emphysema within AATD. Early detection of carriers of this genotype would be important for preventive and therapeutic interventions.

### Table 5: Characteristics of Null/Null, ZZ, and SZ subjects.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age* (y)</th>
<th>Pack-years</th>
<th>FEV₁ (l)*</th>
<th>FEV₁ (% predicted)*</th>
<th>KCO (% predicted)</th>
<th>Kₜ₁ (mmol/min/KPa/l)*</th>
<th>(N = 10)</th>
<th>A1AT serum levels (µM)</th>
<th>(N = 10)</th>
<th>A1AT serum levels (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null/Null</td>
<td>12</td>
<td>44 (32)</td>
<td>6 (25)</td>
<td>1.48 (0.69)</td>
<td>39.02 (14.9)</td>
<td>0.8 (2.1)</td>
<td>50.8 (12.1)</td>
<td>75/25</td>
<td>1.7 (2.4)</td>
<td>0.23 (0.32)</td>
<td></td>
</tr>
<tr>
<td>ZZ</td>
<td>12</td>
<td>44 (32)</td>
<td>6.3 (25)</td>
<td>2.86 (0.97)</td>
<td>78.98 (31.1)</td>
<td>1.2 (0.4)</td>
<td>73 (19.6)</td>
<td>83/17</td>
<td>45.8 (43.5)</td>
<td>6.3 (15.1)</td>
<td></td>
</tr>
<tr>
<td>SZ</td>
<td>12</td>
<td>42.9 (32)</td>
<td>8 (22.8)</td>
<td>3.67 (1.12)</td>
<td>102.3 (19.1)</td>
<td>1.4 (0.4)</td>
<td>85.9 (19.6)</td>
<td>75/25</td>
<td>111 (57)</td>
<td>14.8 (7.6)</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as (*) mean (S.D.) or (µM) median (range).
Conflict of interest statement

All authors declare that they have no competing interests. They all disclose any financial and personal relationship with people or organizations that could inappropriately influence their work.

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References