Progression of Atherosclerosis Is Associated With Variation in the $\alpha_1$-Antitrypsin Gene


Objective—$\alpha_1$-Antitrypsin (AAT) protects elastic tissue and may play a role in atherogenesis. The association of atherosclerosis progression with common AAT variants was considered in 2 clinical trials.

Methods and Results—We examined the association of AAT V213A, S and Z deficiency alleles, and the functional 3’ UTR 11478G>A with change in minimal luminal diameter, a measure of focal disease, in the Lopid Coronary Angiography Trial gemfibrozil study of post-bypass men. S or Z carriers (n=14) showed strong progression of disease on placebo (11.5%) but responded well to treatment (3% regression). 11478A carriers treated with placebo or gemfibrozil showed significantly more disease progression (n=8, −14.5% and n=16, −4.0%, respectively) than 11478GG men (n=107, −7.0% and n=108, −1.4%, respectively; overall, $P=0.003$). VV213 men treated with gemfibrozil (n=68) showed −4.8% progression, whereas A213 carriers (n=55) showed +1.4% regression of disease ($P=0.001$). No V213A effect was seen on placebo ($P=0.11$). In the Diabetes Atherosclerosis Intervention Study fenofibrate trial of angiographic progression in type 2 diabetes, the association of 11478A with increased disease progression was confirmed in the treatment group, but not the gemfibrozil-treated A213 association with regression, suggesting a pharmacogenetic difference.

Conclusions—Disease progression is associated with variation in AAT, and low AAT levels promote atherogenesis. (Arterioscler Thromb Vasc Biol. 2003;23:644-649.)

Key Words: $\alpha_1$-antitrypsin • gene variants • atherosclerosis progression • fibrates

$\alpha_1$-Antitrypsin (AAT) is an acute-phase reactant, serine-protease inhibitor with a major role in protecting vulnerable elastic tissue in the lungs, gut, and vasculature from degradation by neutrophil elastase. AAT is synthesized primarily in the liver as well as by neutrophils and monocyte/macrophages. Because elastin is a major component of vessel wall elastic lamina, and degradation of elastic fibers may be important in the loss of vessel tone and the development of atherosclerosis, this implicates AAT in the atherogenic process. However, there are no reports of an increased incidence of atherosclerosis in AAT-deficient subjects (see http://www.ncbi.nlm.nih.gov:80/entrez/dispomim.cgi?id=107400), although this has not been fully examined.

If AAT plays a role in atherosclerosis, it would have to be available to inhibit local elastase activity. The suggested route of entry of AAT into the plaque comes from studies that demonstrated direct interaction between AAT and apolipoprotein B on LDL particles. Furthermore, an AAT-LDL complex has been detected in human atherosclerotic lesions in coronary arteries. The anti-elastase properties and involvement in lipid accumulation thus provide opposing mechanisms by which AAT could modulate atherogenesis. The AAT locus is highly polymorphic, with >75 reported common and rare variants. The rare mutations, including the more common S and Z variants, have been primarily associated with emphysema or liver disease and display reduced activity and mass even in the heterozygous state. The common M variants, which introduce amino acid substitutions, eg, M1V213A, are not associated with altered disease states or differences in AAT activity and mass. A common functional, noncoding variant, 11478G>A, alters a 3’ enhancer sequence, with the 11478A allele disrupting an Oct-1 binding site that has been shown to act cooperatively with the tissue-specific nuclear factor NF-II6 as part of the acute-
phase response.\textsuperscript{6} Thus, under acute-phase conditions, the 11478A variant would lead to reduced levels of AAT gene expression and thus relative AAT deficiency, although this is unlikely to be of the same magnitude as S and Z variants.

We have determined the relationship between angiographically defined progression of coronary artery disease and common AAT variants, 11478G>A, V213A, and the deficiency alleles S and Z, in 2 clinical trials. In the Lipid Coronary Angiography Trial (LOCAT) of post–coronary bypass men,\textsuperscript{7} compared with placebo, gemfibrozil significantly retarded the progression of focal disease.\textsuperscript{8} A second study, the Diabetes Atherosclerosis Intervention Study (DAIS), examined the effect of fenofibrate on progression of disease in patients with type 2 diabetes. Fenofibrate had significant lipid-lowering effects and retarded the focal disease progression in the treatment versus placebo groups.\textsuperscript{9} We hypothesized that if AAT protected against atherosclerosis progression, this would be evident in carriers of the deficiency alleles S, Z, and 11478A.

**Methods**

**Patients and Drug Treatment**

**LOCAT**

Details of the entry criteria and the screening process were described previously.\textsuperscript{1} Three hundred ninety-five men who had undergone coronary bypass surgery were randomly assigned to receive slow-release gemfibrozil (Lopid SR) 1200 mg once daily or placebo. Native coronary arteries and bypass grafts were imaged at baseline and after 2.5 years.\textsuperscript{7}

**DAIS**

The entry criteria, protocol, and results have been previously reported.\textsuperscript{10,11} Four hundred eighteen men and women with type 2 diabetes, recruited in Finland, Sweden, and Canada, were randomly assigned to receive micronized fenofibrate (200 mg/d) or placebo. Clinical coronary disease was assessed by quantitative angiography at the start and end of the study. Each participant had at least 1 minimal lesion, but clinical coronary disease was not necessarily present. There was no heterogeneity of effect of sex, and therefore data from men and women were assessed together.

**Quantitative Coronary Angiography**

The quantitative coronary angiography was determined for both LOCAT and DAIS using the identical analytical system (QCA-CMS, Medis).

**DNA Genotyping**

Details of the polymerase chain reaction genotyping protocols for 11478 G>A (TaqI), V213A: T>C, S variant (E264V: A>T), and Z variant (E342K: G>A) are presented as supplementary data (see the online data supplement, available at http://atvb.ahajournals.org).

**Biochemical Measures**

Measurement of AAT mass was determined by immunoturbidimetry using anti-human AAT (Code Q0363) (Dako) and calibrated using IMPRO/NORDIC-calibrator (Labquality). The reference range was 0.96 to 1.78 g/L, and the coefficient of variation was 3.7% for LOCAT samples taken, on average, 16 months after baseline.

**End Point and Statistical Analysis**

Observed numbers of each genotype were compared with those expected if the sample were in Hardy-Weinberg (H-W) equilibrium using $\chi^2$ analysis. Allele frequencies in different groups were compared by gene counting and $\chi^2$ analysis. Allelic association was estimated using the $\Delta$ calculation.\textsuperscript{12} The per-patient changes in minimal luminal diameter (MLD) from baseline to follow up were entered into the models as dependent variables.\textsuperscript{13} The analyses were carried out in the placebo and gemfibrozil groups separately. $P<0.05$ was considered statistically significant.

**Results**

The baseline characteristics of the participants in LOCAT and DAIS are presented in Table 1. In LOCAT, all participants had a history of CHD, whereas in DAIS, all of participants had type 2 diabetes but only 48% had clinical CHD. Other characteristics were similar; however, as expected for diabetic subjects, the DAIS participants had higher body mass index and baseline TG levels than the LOCAT subjects.

**LOCAT**

**Allele and Genotype Frequencies**

Genotype distributions were in H-W equilibrium. Rare allele frequencies for the common variants, 11478G>A and V213A, are presented in Table 2. The rare allele frequency for the S and Z alleles were 0.007 (95% CI, 0.00 to 0.01) and 0.012 (95% CI, 0.00 to 0.02), respectively. There was no significant lipid-lowering effects and retarded the focal disease progression in the treatment versus placebo groups.\textsuperscript{9} We hypothesized that if AAT protected against atherosclerosis progression, this would be evident in carriers of the deficiency alleles S, Z, and 11478A.

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| TABLE 1. Baseline Characteristics [mean (SD)] of Participants in the LOCAT and DAIS Trials |
|----------------------------------|----------------------------------|
| LOCAT (Syvanne et al)\textsuperscript{7} | DAIS (Steiner et al.)\textsuperscript{11} |
| No. of participants | 395 | 418 |
| % Men | 100 | 72.9 |
| Mean age, y | 59.1 (6.8) | 56.8 (5.9) |
| BMI, kg/m\textsuperscript{2} | 26.4 (2.2) | 28.7 (3.2) |
| History of CHD, % | 100 | 48 |
| History of type 2 diabetes, % | 0 | 100 |
| Duration of disease, y | CHD 4 (range, 1 to 20) Type 2 DM 8.6 (2.28) |
| Triglycerides, mmol/L | 1.64 (0.64) | 2.42 (0.92) |
| Cholesterol, mmol/L | 5.17 (0.64) | 5.57 (0.72) |
| HDL-C, mmol/L | 0.82 (0.14) | 1.04 (0.20) |
| LDL-C, mmol/L | 3.61 (0.53) | 3.43 (0.69) |

**TABLE 2. Comparison of the Rare Allele Frequencies (95%CI) of the V213A and 11478G>A in LOCAT (Finnish) and in DAIS (Finnish, Canadian, and Swedish) Studies**

<table>
<thead>
<tr>
<th>V213A Rare Allele Frequency\textsuperscript{*†}</th>
<th>11478G&gt;A Rare Allele Frequency\textsuperscript{‡}</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOCAT</td>
<td>Finland 0.28 (0.24–0.31), n=295</td>
</tr>
<tr>
<td>DAIS</td>
<td>Finland 0.31 (0.26–0.36), n=195</td>
</tr>
<tr>
<td>Sweden</td>
<td>0.098 (0.03–0.16), n=41</td>
</tr>
<tr>
<td>Canada</td>
<td>0.13 (0.10–0.17), n=163</td>
</tr>
</tbody>
</table>

\* Comparison of the V213A rare allele frequency in LOCAT with Finnish centres of DAIS by $\chi^2$, $P=0.58$

\†Comparison by $\chi^2$ within DAIS of the V213A rare allele frequency in Finnish centers with Swedish center ($P<0.005$) and with Canadian centers ($P<0.0005$).

\‡Comparison of the frequency of the rare allele of the 11478G>A between LOCAT and Finnish centres in DAIS and within DAIS by in all centre, $P=0.32$ by $\chi^2$. 

**Results**

The baseline characteristics of the participants in LOCAT and DAIS are presented in Table 1. In LOCAT, all participants had a history of CHD, whereas in DAIS, all of participants had type 2 diabetes but only 48% had clinical CHD. Other characteristics were similar; however, as expected for diabetic subjects, the DAIS participants had higher body mass index and baseline TG levels than the LOCAT subjects.
TABLE 3. Mean (SD) of AAT Mass According to AAT Genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>AAT Mass, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>W+*† n=97</td>
<td>1.14 (0.12)</td>
</tr>
<tr>
<td>VA+AA+† n=106</td>
<td>1.12 (0.12)</td>
</tr>
<tr>
<td>S, n=5</td>
<td>0.92 (0.19)</td>
</tr>
<tr>
<td>Z, n=9</td>
<td>0.73 (0.08)</td>
</tr>
<tr>
<td>11478GG, n=217</td>
<td>1.13 (0.12)</td>
</tr>
<tr>
<td>11478A, n=11</td>
<td>1.12 (0.11)</td>
</tr>
</tbody>
</table>

Normal range 0.96 to 1.78 g/L.
*From ANOVA, comparing mass values of V213, A213, S and Z, P<0.001.
†From ANOVA, comparing mass values of VA+AA, P=0.22.
‡From ANOVA, comparing mass values of 11478GG vs 11478A, P=0.04.

statistically significant allelic association between any of these sites (Table 1) (see the online data supplement, available at http://atvb.ahajournals.org).

Effect of AAT Genotype on AAT Mass
The association between genotypes and AAT mass is presented in Table 3. As expected, carriers of either the S or Z deficiency alleles had 17% and 36%, respectively, lower AAT mass than all other genotype groups (ANOVA, P<0.001). Comparing 11478G>A and V213A variants, there was no statistically significant difference in AAT mass (P=0.84 and 0.22, respectively) by genotype.

Effects of AAT S and Z Variants on Progression of Disease
Because atherosclerosis is a focal disease, we concentrated on effects on the focal progression of disease (change in MLD) between genotype groups. A decrease in MLD change (negative value) denotes progression of disease, whereas a positive change results if the lumen has increased in diameter, indicating regression of disease. Considering the deficiency variants, although numbers were small, S and Z carriers showed strong progression of disease on placebo (n=8), with a change in MLD of -0.192 (±0.060) mm representing 11.5% disease progression. Treated with gemfibrozil, these men (n=6) showed a 3% regression of disease, with a change in MLD of +0.048 (±0.061) mm (P=0.05, for difference).

3’ UTR 11478G>A and Disease Progression
In both the placebo and gemfibrozil groups, 11478A carriers (excluding the S and Z carriers) showed significantly more progression of disease than 11478GG men (overall P=0.003) (Figures 1A and 1B). In the placebo group, 11478GG men showed 4.0% progression of disease, whereas men who carried the 11478A allele showed a 3.6-fold greater disease progression (14.5%, P=0.013). In the gemfibrozil group, where progression was reduced overall, a similar significant 5-fold difference between 11478GG and 11478A carriers was seen (1.4% and 7.0%, respectively, P=0.03), representing an ≈50% decrease in MLD in the gemfibrozil group compared with the placebo group for each genotype. In multivariate analysis, after adjustment for baseline measures and time between angiograms, the effect of genotype was highly statistically significant (P=0.002) and that of treatment was of borderline significance (P=0.056), with no significant interaction between genotype and treatment (P=0.50).

V213A and Disease Progression
The effects of the V213A variant on the change in MLD in the placebo and gemfibrozil groups (excluding S and Z carriers) are presented in Figures 2A and 2B. In the placebo group, A213 carriers showed less progression of disease (3.1%) than VV213 men (6.7%), although this difference was not statistically significant (P=0.11). In the gemfibrozil-treated group, VV213 men showed a 4.8% decrease in MLD, a modest effect of treatment compared with VV213 men in the placebo group. However, carriers of the A213 allele responded well to gemfibrozil and showed regression of disease of 1.4%. This difference by genotype was highly significant in the gemfibrozil-treated group (P<0.001). In multivariate analysis of the whole cohort, controlling for baseline MLD and time between angiograms, both V213A genotype (P=0.001) and treatment group (P=0.011) were independently predictive of change in MLD, without evidence of interaction between the 2 factors (P=0.36).

DAIS
To validate the associations of disease progression with AAT genotype seen in LOCAT, the main genotypic effects were examined in a second study, DAIS. Genotype distribution was in H-W equilibrium. Although the allele frequency of the 11478G>A was similar in all 3 recruitment countries and in LOCAT, the frequency of the A213 allele was significantly higher in the Finnish centers (in agreement with LOCAT) compared with both the Canadian (P<0.0005) and Swedish (P<0.005) recruitment centers (Table 2).
The association of 11478G/H11022A and V213A with change in MLD is presented in Figures 2A and 2B. Confirming the results from LOCAT, 11478GG individuals showed less progression of disease than 11478A carriers, and in the fenofibrate group this difference reached statistical significance ($P = 0.03$). There was no association with change in MLD according to V213A in either the treatment ($P = 0.66$) or placebo groups ($P = 0.89$).

Structural Modification Made by V213A Change and the Potential for Differential Interaction With Gemfibrozil

The effects on progression of disease in LOCAT and DAIS by V213A genotype were obviously different. To explore whether this difference could be attributable to the treatment drug (gemfibrozil versus fenofibrate) and interactions with amino acid residue 213, molecular structural studies were undertaken. Examination of the crystal structure of AAT predicted by x-ray crystallography\textsuperscript{14,15} shows that residue 213 is exposed on the surface (Figures 3A and 3B). The protein surface of AAT was examined to see if it contained a region to permit binding of a moderate-sized hydrophobic ligand such as gemfibrozil, which is a nonhalogenated phenoxypentanoic acid with a hydrophobic structure not dissimilar from a fatty acid. The GRID program\textsuperscript{16} was used with a hydrophobic probe to find regions of the protein where hydrophobic atoms of a ligand might preferentially bind. There is a large cleft region, adjacent to the loop containing V213, that could bind a medium-sized hydrophobic molecule, ie, a putative site for gemfibrozil binding. Protein-ligand docking was carried out with a flexible model of gemfibrozil in the protein cleft of the uncleaved AAT using the program Q-fit\textsuperscript{17}. Figure 3A shows the conformation of gemfibrozil in the predicted binding mode. The dimethyl-benzyl group of gemfibrozil is able to fit into the hydrophobic cleft, but larger or more conformationally restricted molecules, such as fenofibrate, would have difficulty fitting into the pocket (Figure 3B).

Discussion

The analysis in LOCAT, using 2 common, genetically independent AAT variants, provides, for the first time, strong evidence that AAT participates in atherosclerosis progression. This was confirmed for the 11478G>A variant in DAIS.

AAT and Atherogenesis

There is a large body of work that suggests that AAT could play both a proatherogenic and antiatherogenic role. Elastin is the main component of elastic fibers, and loss of elastin fibers causes loss of elasticity of the arterial wall, which is a factor in the progression of arteriosclerosis and heart insufficiency in elderly patients.\textsuperscript{18} Increased elastase activity attributable to AAT deficiency would therefore be expected to accelerate hardening of arterial walls and arteriosclerosis.

The interaction of AAT with elastase and other serine proteinases results in the suicide cleavage of AAT. During this process, the C-terminal 36 amino acids (C-36) are cleaved off yet remain attached to the complex.\textsuperscript{19} This C-36 cleaved peptide is biologically active and increases LDL binding and internalization, upregulates the LDL-receptor, induces cytokine and glutathione production, and upregulates AAT synthesis itself.\textsuperscript{20} Thus, there is strong suggestive evidence that C-36 participates in processes involved in atherogenesis.
Effect of Low AAT Levels on Atherogenesis

As expected, AAT levels in S and Z carriers were significantly lower than all other genotypes combined, although not as low as those reported in ZZ homozygotes.5 Z variant homozygosity is associated with emphysema6 and increased hepatic AAT aggregation attributable to loop-sheet polymerization, causing reduced secretion and liver disease.21 The S variant causes AAT deficiency in compound heterozygosity with other recessive mutations22,23 and is also prone to polymerization and intracellular degradation.24 In LOCAT, S and Z carriers taking placebo showed greater progression of disease, suggesting that low AAT increases atherosclerosis progression in these men. S and Z carriers taking gemfibrozil showed overall regression of disease, but numbers were small. The novel aspect of this study is that this is the first report of the association of these AAT variants with atherosclerosis progression. We are unaware of any studies in the literature that have examined the incidence of CHD in AAT-deficient individuals to support our findings.

11478G>A and Disease Progression

Atherosclerosis is an inflammatory process,25 and under such conditions plasma levels of acute-phase proteins such as fibrinogen, C-reactive protein, and AAT26 are elevated. Because 11478A results in a lack of acute-phase upregulation of AAT compared with the G11478 allele,27 11478A carriers would be expected to have lower AAT levels when in an inflammatory situation. In this study, there was no statistically significant difference in AAT mass by 11478G>A genotype. However, these samples were taken, on average, 16 months from baseline, by which time these patients were in a stable condition and unlikely to be in an acute inflammatory situation. Because the AAT deficiency, seen in S and Z carriers, was associated with progression of disease, we predicted that the impaired acute-phase response of 11478A carriers would also be associated with increased progression of disease. In LOCAT, in both the placebo and gemfibrozil groups, 11478A carriers displayed significantly greater progression of disease than 11478G homozygotes, confirming this prediction, and the on-treatment effect on disease progression by 11478G>A was mirrored in DAIS.

V213A and Disease Progression

In LOCAT, the effect of V213A on disease progression in the placebo group was not statistically significant. Treated with gemfibrozil, VV homozygotes showed only a slight reduction of disease progression compared with VV men taking placebo (−4.8% compared with −6.7% with placebo; 28% change) and thus a poor response to the drug. By comparison, A213 carriers responded well to the drug, with +1.4% regression of disease compared with −3.1% progression with placebo, representing a 68% change. The effect of V213A on gemfibrozil treatment suggests a possible pharmacogenetic interaction between V213-AAT and the hydrophobic drug gemfibrozil.

Comparison of LOCAT and DAIS Studies

The association between 11478G>A genotype and CHD progression, identified in LOCAT, was confirmed in DAIS, but the V213A effect was not. This raises a question about the validity of the V213A effect, but although there are similarities in study design and to some extent in patient characteristics between the 2 studies, there are also differences, which reduces the chance that modest genotypic effects would be reproduced exactly. Both studies examined angiographically quantified progression of disease and used the same analytical system. Overall, the atherosclerosis in LOCAT men was likely to be more severe than in DAIS, with all of LOCAT men having had bypass surgery, whereas only 48% of DAIS patients had CHD. In LOCAT, gemfibrozil had a highly significant effect on disease progression and reduced MLD by 2.25-fold, whereas in DAIS the response to fenofibrate treatment on disease progression was more modest at 1.6-fold. Thus, DAIS had a lower power to detect modest effects associated with genotypic factors. In addition, in DAIS, all patients had type 2 diabetes, and this may itself have an impact on AAT variants, because diabetes has a distinct influence on atherosclerosis due to protein glycation.28 Finally, in DAIS, subjects were recruited from Finland, Sweden, and Canada, and although there is no reason to expect that in white subjects from different recruitment centers the effect of genotype would be different, the lower frequency of the 213A allele in Canadian and Swedish subjects would also reduce the power of DAIS to detect a significant V213A effect. However, the primary reason for the difference in the V213A effect in the 2 studies could be attributable to the specific fibrate used in the trial.

AAT and Fibrates

Gemfibrozil and fenofibrate are both members of the fibrate class of lipid-lowering drugs, which are ligands for peroxisome proliferator-activated receptor α (PPARα).29 We postulate that the presence of a hydrophobic groove adjacent to the exposed residue 213 allows AAT to interact with gemfibrozil, which is then entrapped in the groove, thereby compromising its ability to activate PPARα. The lower levels of gemfibrozil would lead to a reduced impact on the disease process and progression of disease. The reduced PPARα activation, predicted by this model, would be expected to result in a poorer lipid-lowering response, but this was not observed in V213 carriers (data not shown). This suggests that the effect of AAT on disease progression is local rather than systemic. We previously examined the effect of functional PPARα variants on disease progression in LOCAT but also found no association of PPARα variants with lipid levels.33 Thus, the fibrate effect, at the level of the plaque and systemically, may be different.

This entrapment hypothesis is supported by a study that demonstrated that sera from patients (of undetermined AAT genotype) undergoing gemfibrozil treatment had reduced antielastase activity compared with native AAT.30 This loss of antielastase activity was also mimicked when gemfibrozil was added to normal sera or pure AAT in vitro.30 Because in LOCAT the A213 variant is associated with a greater difference in the change in MLD in response to gemfibrozil, this suggests that the residue change may affect gemfibrozil binding, possibly by altering the loop conformation and the binding-site volume of this region. The valine to alanine...
change is conservative, and additional work will be necessary to clarify if the amino acid change reduces interaction of gemfibrozil with AAT. The results, however, suggest that with less AAT present, in the case of S and Z variants, less gemfibrozil is inactivated and more is available for therapeutic action via PPARα activation. Furthermore, the structural analysis suggests that whereas gemfibrozil could be accommodated into the hydrophobic groove, fenofibrate with its 2 benzene rings would be sterically hindered. This is a plausible explanation for the lack of effect of V213A genotype in DAIS treatment group. However, without confirmation from additional angiographic studies, where the comparison of gemfibrozil and fenofibrate can be evaluated, we cannot rule out that this could be a chance finding or context-dependent, i.e., modulated by environmental factors.

Thus, our data from 2 independent studies strongly support a protective role for AAT in the progression of established coronary artery atherogenesis. This does need to be confirmed in other studies. At this stage, we cannot determine whether the AAT is liver-derived or produced locally by monocyte or macrophages and also whether its action is through its role in preventing excessive matrix remodelling or in the vessel wall via binding to LDL. Additional work is necessary to clarify these issues.

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References


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Genotyping methodology

11478 G>A (TaqI)
Forward 5’-ACTGTCTAACCACACTCAGTC
Reverse 5’-ACTGAATCATGTAGAATTGCT giving a PCR product of 114bp, the G allele digesting to 70 and 44bp with TaqI.

V213A: T>C
Forward 5’-AGTCAAGGACACCGAGGAAG
Reverse 5’-GGTGAGTTCAATTCCAGG giving a PCR product of 209bp, the T allele digesting to 169 and 40bp with BstEII.

R101H: G>A
Forward 5’-GACCAAGGCTGACACTACAGA
Reverse 5’-CTGTCTGGTGTTGAGGAG giving a PCR product of 123bp, the G allele digesting to 103 and 23bp with Csp6I or RsaI.

E264V: A>T
Forward 5’-GCTGGGTGCTGCTGATGAAAT
Reverse 5’-CCTCAGTCCCAACATGGCTAAGA giving a PCR product of 201bp, the T allele digesting to 125 and 76bp with SexAI.

E342K: G>A
Forward 5’-ATAAGGCTGCTGCTGACCAGTCTCG (T forced from A)
Reverse 5’-TTGGGTGGGATTCACCACTTTTC giving a PCR product of 179bp, the G allele digesting to 157 and 22bp with TaqI.

E376D: A>C
Forward 5’-AACCCTTTGTCTTCTTAATGATTGT (T forced from A)
Reverse 5’ –AGGCAGGGACCAGCTCAA giving a PCR product of 168bp, the A allele digesting to 143 and 25bp with Csp6I or RsaI.

The PCR conditions were as follows:

**E264V, E342K and E376D**: 96°C for 5 minutes, followed by 30 cycles of 96°C for 1 minute, 56°C for 1 minute, 72°C for 45 seconds; then 72°C for 5 minutes; 16°C for 20 minutes.

**11478G>A**: 95°C for 5 minutes, followed by 30 cycles of 96°C for 1 minute, 53°C for 1 minute, 72°C for 1 minute; then 72°C for 5 minutes; 16°C for 10 minutes.

**V213A**: 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute; then 72°C for 10 minutes.

**R101H**: 96°C for 5 minutes, followed by 30 cycles of 96°C for 1 minute, 55°C for 1 minute, 72°C for 45 seconds; then 72°C for 5 minutes; 16°C for 20 minutes.

All PCR products were separated using microarray diagonal gel electrophoresis (MADGE) {Day, Humphries, et al. 1995 79 /id}
Table I  Genotypes and rare allele frequencies for the AAT variants studied in LOCAT

<table>
<thead>
<tr>
<th>Genotype numbers</th>
<th>11478G&gt;A</th>
<th>V213A</th>
<th>E264V (S)</th>
<th>E342K (Z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>282</td>
<td>VV 150</td>
<td>EE 283</td>
<td>EE 280</td>
</tr>
<tr>
<td>GA</td>
<td>32</td>
<td>VA 128</td>
<td>EV 4</td>
<td>EK 7</td>
</tr>
<tr>
<td>AA</td>
<td>0</td>
<td>AA 17</td>
<td>VV 0</td>
<td>KK 0</td>
</tr>
<tr>
<td>Rare allele frequency (95%CI)</td>
<td>0.012 (0.03-0.07)</td>
<td>0.28 (0.24-0.31)</td>
<td>0.007 (0.00-0.01)</td>
<td>0.012 (0.00-0.02)</td>
</tr>
</tbody>
</table>