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Diagnostic flow chart for targeted detection of Alpha1-antitrypsin deficiency ☆

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Summary
Background: Alpha1-antitrypsin (AAT) deficiency is under-recognized, probably because many individuals affected show no clinical impairment. The targeted detection is a tool to increase its recognition.
Methods: We prospectively submitted to AAT serum levels determination, phenotyping and, if doubtful, genotyping: (i) patients with the early onset of emphysema, emphysema in absence of recognized risk or pneumothorax (path P), antineutrophil cytoplasm antibodies (ANCA) positive vasculitis (path V), cervical artery dissection (path A), Periodic acid-Schiff (PAS) positive bodies in the liver cell or unexplained abnormal transaminase level (Path L) [index cases: IC] and (ii) subjects with low-serum alpha$_1$-globulin (path e) and close relatives of patients with AAT deficiency (path r) [non index cases: NIC]. We determined and compared gender, age, AAT serum levels values, the ratio between AAT deficiency subjects identified and all subjects examined (identified/examined). Receiver operating characteristic (ROC) curve was plotted to find the best threshold for AAT serum levels.
Results: Two hundred and eighty-five individuals were examined and 211 with AAT deficiency identified: 66 were IC and 145 NIC. The ratio identified/examined resulted 0.74. A serum level of 120 mg/dL was able to identify AAT deficiency with a specificity of 73% and a sensitivity of 97%. IC showed male prevalence ($P = 0.005$), more advanced age ($P = 0.02$), lower AAT serum levels ($P = 0.008$).

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Conclusions: Our protocol is effective to detect AAT deficiency in a selected population. About 120 mg/dL (nephelometric method) is a reliable AAT serum level cut-off for selecting subjects/patients to submit to phenotype or genotype; as compared to NIC, IC are older, mostly male and with lower AAT serum levels.

Introduction

Alpha1-antitrypsin (AAT) is a glycoprotein produced mainly by hepatocytes. Its essential action is to inhibit neutrophil elastase. It is coded by a polymorphic gene located on chromosome 14q32.1, where at least 75 alleles have been identified. These alleles may be classified as either normal (M) or deficient (Z, S, null, Mlike, M_{malton}, I.). The two parental alleles are codominant and both contribute to AAT serum concentration. With the nephelometric method of dosage the normal range of AAT serum levels is reported as being between 200/220 and 83/120 mg/dL.[1,2] AAT deficiency is an inherited disorder characterized by a low AAT serum level.[3] Since AAT deficiency was recognized, there have been advances in our knowledge of this disorder. Genetics, molecular basis and some clinical manifestations are now better understood and described, while augmentation therapy for patients affected by pulmonary emphysema and COPD is now available. There is a clear relationship between the ZZ and nullnull phenotype and the risk of early emphysema, liver disease or, more rarely, skin disease. The SZ phenotype is not recognized as a risk factor, even if among SZ smokers the risk of airflow obstruction seems to be similar to that of ZZ individuals.[4] However, in spite of recent extensive efforts to identify cases with AAT deficiency, the disorder is largely under-recognized, most probably due to the fact that many individuals with AAT deficiency do not have significant clinical impairment.[5]

In 1996, a program for detecting AAT deficiency in Italy was started in order to improve knowledge of this condition and to create a registry of AAT deficient subjects.[6]

The main purpose of the present study was to develop and assess a targeted detection protocol conceived to identify as many AAT deficient subjects/patients as possible. The secondary aims were to identify a cut-off value for AAT serum level below which it might be useful to ascertain the phenotype or the genotype and to study the difference between index cases (IC) (symptomatic patients detected because of a related disease) and non-index cases (NIC) (asymptomatic subjects detected as relatives of IC or because of low $alpha_1$ globulin level found with electrophoresis).

Methods

After approval by the local Ethical Committee, in the period from January 1996 to December 2004, we prospectively searched for subjects or patients affected by AAT deficiency following the protocol shown in Fig. 1. Accordingly, we submitted hospital patients with high suspicion of AAT deficiency to AAT serum level determinations (nephelometric method), during stable clinical conditions and with the certainty that they were not taking drugs like progesterone or estrogens. These patients were affected by: (1) the early onset of emphysema (at the age of 45 or less), emphysema in the absence of recognized risk factors (i.e. smoking, occupational dust exposure) or spontaneous pneumothorax (we named this diagnostic approach path "P"); (2) cervical artery dissection (CAD) (path "A"); (3) PAS (periodic acid-Schiff) positive bodies in the liver cell cytoplasm, in specimens from liver biopsy, suggesting AAT pathological accumulation (path "L"); (4) an unexplained and isolated abnormal level of transaminases (path "L"); (5) ANCA (antineutrophil cytoplasm antibodies) positive vasculitis (path "V"); (6) low $alpha_1$ globulin level at

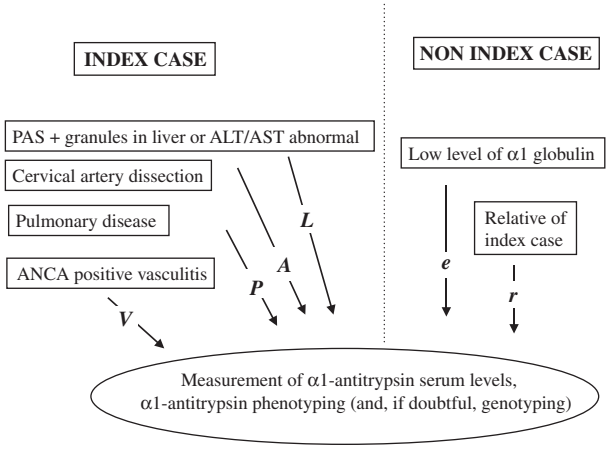

Figure 1 The flow chart adopted to identify patients (IC) or subjects (NIC) affected by AAT deficiency. (PAS: periodic acid-Schiff; ALT: alanine transferase; AST: aspartate transaminase; P = pulmonary diseases; A = cervical artery dissection; L = liver disease; V = vasculitis; e = low $alpha_1$-band at electrophoresis; r = relative of an IC).

electrophoresis according to our laboratory reference values (<200 mg/dL) (path "e"). AAT phenotyping in dried blood specimens absorbed on special paper was performed[7] to prove AAT deficiency. If the phenotyping of the pathological alleles was uncertain, genotyping was performed with a polymerase chain reaction (PCR) method and the direct sequencing of AAT exons II–V using amplification primers and sequencing primers.[8] The patients examined by paths P, A, L and V were considered IC, while the subjects examined by path "e" were considered NIC. In addition, we considered NIC (path "r") the parents, offspring or siblings of a patient or subject examined in an out-hospital population (with regard to this, we advise testing of all close relatives of any subjects identified, both homozygous and heterozygous). All IC patients and NIC (path e) subjects were referred to us from different divisions of our hospital (Internal Medicine, Nephrology, Neurology, Pathology). The NIC subjects (path "r") were approached by their IC relative and they referred to us with the logistic support of the Italian Association of Patients affected by AAT deficiency.

Based on the phenotype or genotype we defined, the patients or subjects affected by AAT deficiency as: (1) homozygous deficient if both alleles were pathological and identical (i.e. ZZ); (2) double heterozygous deficient if the alleles were pathological but of a different kind (i.e. SZ, ZM_{malton}); (3) heterozygous deficient if only one allele was pathological (i.e. MZ, MS, MM_{malton}). We divided all the subjects studied, both IC and NIC, into two groups, those with AAT deficiency and those without. We evaluated, in all subjects with identified AAT deficiency and again in both groups, the distribution of the paths of identification and the ratio between the subjects with identified AAT deficiency and all the subjects examined (identified/examined). Following which, after grouping the patients or subjects identified as deficient according to their pathological phenotype or genotype, we determined the distribution of gender, the male/female ratio, the values of AAT serum levels and their age at the time of diagnosis.

Since a low limit of AAT serum level would be warranted in order to identify as many subjects as possible for phenotyping or genotyping, we tried to detect the best cut-off value for AAT serum levels from the values of all the subjects studied.

Statistic analysis

Each value is shown as mean $\pm$ SD. The male/female ratios of different groups were compared by χ^2 test. The variables of interest were compared in each group using the unpaired Student's *t*-test. A *P*-value of less than 0.05 was considered significant. We plotted the receiver operating characteristic (ROC) curve to find the best threshold for AAT serum levels, for which we calculated the specificity, the sensitivity, the positive and negative predictive values and the likelihood of a positive ratio.

Results

Over 9 years 285 individuals were examined, and 211 (66 IC, 145 NIC) patients or subjects affected by AAT deficiency were identified. The remaining 74 were identified as MM (that are not affected by AAT deficiency).

Out of the 211 subjects with AAT deficiency, 108 (51%) were identified by path r, 37 (18%) by path e, 33 (15%) by path P, 29 (14%) by path L, three (1.5%) by path A, one patient (0.5%) by path V. The ratio between the subjects with identified AAT deficiency and all other subjects examined (identified/examined) was 0.74, while for each path this ratio was 0.85 for path P, 1 for path A, 0.91 for path L, 1 for path V, 0.93 for path e, 0.64 for path r, respectively (Table 1).

Seventy-four subjects had a MM phenotype; six of them were affected by pulmonary disease, three by liver disease while 62 were examined following path r and three path e (Table 1).

A genotype was needed to define the rare AAT-deficient variant after an uncertain phenotype in 14 subjects (one ZI, one ZM_{malton}, one M3Z, four M2Z, two M2S, five MM_{malton}).

Forty-five out of 211 subjects (21%) had homozygous or double heterozygous AAT deficiency. Out of these 26 (12% of all cases) had a homozygous ZZ phenotype; 15 (7%) were IC (12 were affected by pulmonary emphysema, two by liver disease and one by CAD) and 11 (5%) were NIC. Nineteen (9%) were double heterozygous, 17 of which (8%) SZ; four (2%) were IC (three affected by pulmonary emphysema and one by liver disease) and 13 (6%) NIC. Two male patients needed genotype confirmation: one was ZI, the other ZM_{malton}. Both were affected by pulmonary emphysema and thus considered as IC.

One hundred and sixty-six subjects/patients out of 211 (79%) had heterozygous AAT deficiency. Out of these 131 (62%) were MZ; 31 (15%) were IC (20 with liver disease, 10 with pulmonary emphysema, one with Wegener granulomatosis) and 100 (47%) NIC. Twenty-nine (14%) had MS phenotype; nine of them (3%) were IC (three with liver disease, two

Table 1 Distribution of each identification path according to phenotype or genotype and ratio identified/examined for all subjects studied and for each path (P = pulmonary diseases, A = cervical artery dissection, L = liver disease, V = vasculitis, e = low alpha1-band at electrophoresis, r = relative of an Index Case, AATD = Alpha1-antitrypsin deficiency subjects).

Subjects/patients	Path of identification						
	All paths	P	A	L	V	e	r
ZZ	26	12	1	2	0	3	8
SZ	17	3	0	1	0	6	7
ZM_{malton}	1	1	0	0	0	0	0
ZI	1	1	0	0	0	0	0
MZ	131	10	0	20	1	20	80
MS	29	4	2	3	0	7	13
MM_{malton}	6	2	0	3	0	1	0
Total AATD identified	211	33	3	29	1	37	108
MM	74	6	0	3	0	3	62
Total examined	285	39	3	32	1	40	170
Identified/examined	0.74	0.85	1	0.91	1	0.93	0.64

with spontaneous pneumothorax, two with CAD, two with pulmonary emphysema) and 20 (9%) NIC. Six out of 211 (3%) were MM_{malton}; all but one were IC (three with liver disease and two with pulmonary emphysema).

A different gender distribution with a male prevalence was found between IC and NIC in the groups ZZ ($P = 0.008$), SZ ($P = 0.02$), MS ($P = 0.0003$) and in all subjects with AAT deficiency ($P = 0.005$). The IC were older in the group ZZ ($P = 0.008$), and in all subjects with AAT deficiency ($P = 0.02$) as compared to NIC. A significant difference of AAT plasma level between IC and NIC was found in all subjects with AAT deficiency ($P = 0.008$) and in MS ($P = 0.03$) and ZZ ($P = 0.04$) groups (Table 2).

The ROC curve identified a level of 120 mg/dL as the best cut-off value for selecting patients suspected of AAT deficiency to submit to phenotype or genotype testing. The sensitivity and specificity of this cut-off value was 97% and 73%, respectively (Fig. 2). Its positive predictive value was 92% and its negative predictive value was 90%, with a positive likelihood ratio of 3.6.

Discussion

The following information emerged from this study: (i) the current protocol is effective at detecting AAT deficient subjects/patients; (ii) the AAT serum level of 120 mg/dl has good sensitivity and specificity and represents a valuable cut-off value to search for AAT deficiency; (iii) IC are older, mostly male, with a lower AAT serum level, as compared to NIC.

Although the knowledge of the genetics underlying this disease, and the technology for screening and detection are rapidly advancing, the most appropriate screening and detection methodologies in terms of a target population and yield of positive tests have not yet been well defined.[9] Clearly, the best way to identify as many subjects affected by AAT deficiency as possible would be a widespread screening of all patients with airflow obstruction, as recommended by WHO,[10] but from a cost-effective point of view a targeted screening would appear to be more appropriate. For this purpose, we feel our protocol represents an effective procedure for discovering subjects or patients with AAT deficiency in a selected population. In fact, the ratio between subjects identified and subjects examined (identified/examined), considered by us as a reliable index of effectiveness, ranges from 1 (paths A and V) to 0.64 (path r) with a value of 0.74 for all cases (Table 1). This algorithm provides some suggestions about which subjects should be examined and how, along with the inclusion criteria for targeted detection. The ATS/ERS Statement[2] suggests a prompt concern about AAT deficiency for: (i) the early onset of emphysema, (ii) basilar emphysema in the absence of a recognized risk, (iii) individuals with unexplained liver disease, (iv) adults with necrotizing panniculitis, (v) ANCA positive vasculitis, (vi) a family history of emphysema, bronchiectasis, liver disease or panniculitis, (vii) bronchiectasis without evident aetiology, (viii) siblings of an individual with AAT deficiency. In line with the Statement, our protocol also provides two

Table 2 Characteristics (number, gender, ratio male/female [m/f], Alpha1-antitrypsin [AAT] serum level and age at the time of diagnosis) of different groups of subjects studied, according to their AAT phenotype or genotype (pts = patients, sbs = subjects. N.S. = not significant. AATD = Alpha1-antitrypsin deficiency).

	Number (pts/sbs)	M	F	Ratio (m/f)	*P*	AAT (mg/dL)	*P*	Age	*P*
Examined	285	145	140	1.1		92±36		46±17	
Index	75	49	26	1.9		77±34		49±15	
					0.0001		0.0002		N.S.
Non-index	210	96	114	0.8		98±35		46±17	
With AATD	211	110	101	1.1		78±28		46±17	
Index	66	40	26	1.5		70±30		49±15	
					0.005		0.008		0.02
Non-index	145	70	75	0.9		81±27		44±17	
Homozygous									
ZZ	26	11	15	0.7		24±7		46±15	
Index	15	9	6	1.5		26±8		53±14	
					0.008		0.04		0.008
Non-index	11	2	9	0.3		20±14		35±11	
Double heterozygous									
SZ	17	8	9	0.9		58±10		48±19	
Index	4	3	1	3		61±1		49±21	
					0.02		N.S.		N.S.
Non-index	13	5	8	0.6		57±10		48±11	
ZM_{malton} (Index)	1	1	0	—		20		32	
ZI (Index)	1	1	0	—		58		55	
Heterozygous									
MZ	131	68	63	1.1		85±16		45±17	
Index	31	17	14	1.3		87±17		48±17	
					N.S.		N.S.		N.S.
Non-index	100	51	49	1.0		85±17		45±17	
MS	29	17	12	1.4		106±20		49±14	
Index	9	7	2	3.5		94±14		49±17	
					0.0003		0.03		N.S.
Non-index	20	11	9	1.2		111±21		49±15	
MM_{malton}	6	3	3	1		77±14		57±16	N.S.
Index	5	2	3	0.7		75±15		53±14	
Non-index	1	1	0	—		81		79	
Without AATD (MM)	74	35	39	0.9		133±21		47±17	
Index	9	9	0	—		129±19		43±15	
							N.S.		N.S.
Non-index	65	26	39	0.7		134±22		48±18	

categories: the diagnostic category (which refers to individuals with signs and/or symptoms consistent with AAT deficiency) and the predispositional category (which refers to asymptomatic individuals with a high risk of AAT deficiency). The present algorithm differs little from this Statement, the differences being the inclusion of patients with cervical arterial dissection or with spontaneous pneumothorax along with a more important role attributed to plasma protein electrophoresis.

For these reasons, some aspects of the identification paths we followed deserve comment.

In only two MS patients with spontaneous pneumothorax, were subpleural blebs detected by high-resolution computed tomography. Our findings support the studies showing that spontaneous pneumothorax is not associated with a higher prevalence of AAT deficiency and therefore should not be searched routinely for in these patients.[11]

We found 29 patients affected by liver disease (two ZZ, one SZ, 20 MZ, three MS, three M_{malton}). In the presence of the common deficient variant (Z) and rare variants (i.e. M_{malton}, S_{iiyama} and others) AAT accumulates within the liver cells, inducing

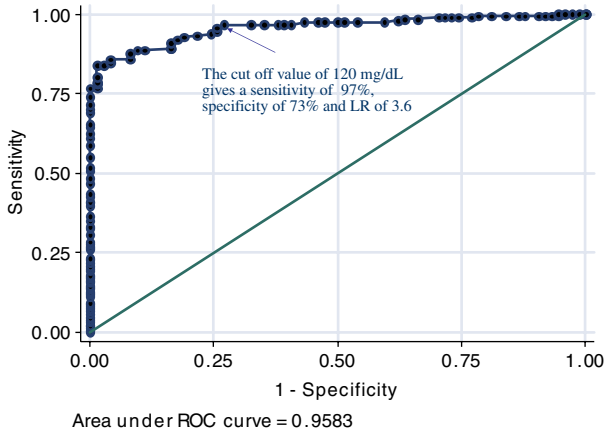

Figure 2 The ROC curve plotted with all values of subjects examined. The area under the curve is 0.9583.

hepatocellular damage which in most individuals is a pathological finding that remains largely subclinical. Patients with heterozygous AAT deficiency and one Z allele also bear an increased risk of various chronic liver diseases including cirrhosis.[12] Only one MZ patient was identified by path "V". A high incidence of the Z variant, either in the homozygous (ZZ) or in the heterozygous (MZ) state, had previously been found in patients with systemic vasculitis.[13–15] AAT reacts specifically against proteinase-3 (PR3), which in turn represents the target antigen for anti-PR3 antibodies. Hence, the Z gene carrier state could be either an aetiological risk factor for the initiation of systemic vasculitis or a determinant of the disease course once the pathological process has started. In accordance with the small number of patients identified by path "V", the latter possibility is the most likely.[16]

We identified three patients affected by CAD (one ZZ, two MS). By disturbing the balance between elastase and antielastase AAT deficiency might favour the degradation of the extracellular matrix, contributing to the arterial damage process. AAT deficiency has been suggested as a potential risk factor for aortic aneurysm[17] and for CAD[18]; in addition, the distensibility of the aorta is increased in men with AAT deficiency.[19] AAT has been found attached to endothelial cells in a polymerized form and the loss of antiprotease serpins could predispose the arteries to vascular injury.[20] The magnitude of these potential AAT-dependent effects might be higher when transient triggering mechanisms predisposing to CAD, such as recent infections are operant[21] and in vessels such as carotid arteries, in which the elastic component is greater.[22]

It is useful to underline the need to perform AAT dosage in stable clinical conditions, as carried out by our team, because no single MZ individual can be detected without phenotype or genotype during the acute-phase reaction.[23]

With our protocol, a number of rare AAT mutations were detected by DNA analysis performed with mutagenic PCR and gene sequencing, since the isoelectric focusing method cannot be conclusive with regard to the rare variants. Indeed, variants such as M_{malton} are of clinical relevance because five out of six subjects identified with this allele, either double heterozygous or heterozygous, were IC.

We found a relevant number (45 patients) of heterozygous IC with either pulmonary or other diseases. In a longitudinal study, it was argued that 28 MZ subjects were at higher risk of developing pulmonary emphysema than 28 matched normal subjects without AAT deficiency.[24] Furthermore, heterozygotes with phenotype MZ have an increased risk of hospital admission because of COPD if they are siblings or close relatives of ZZ IC.[25] Therefore, both homozygous and heterozygous is a condition that must be ascertained in every subject with a high suspicion of AAT deficiency. In addition, a recent meta-analysis suggested that the risk of COPD in MZ heterozygotes is uncertain,[26] thus an effort to identify a large number of heterozygotes might be useful to definitely ascertain the presence and magnitude of the risk of COPD or other diseases in these individuals. It could be argued that the term "AAT deficiency" is debatable when addressing patients with MS or even MZ phenotypes/genotypes. In these patients, the AAT serum levels are above the threshold value associated, for instance, with the development of pulmonary emphysema, but the real risk of these subjects is not currently known. For this reason the reference values should have a lower limit of 120 mg/dl instead of 80 mg/dL. Out of the subjects or patients we identified, 112 of them had an AAT serum level ranging between 80 and 120 mg/dL: by considering 80 as the low normal limit, we would have misdiagnosed them.

The ROC curve revealed an optimal sensitivity and good specificity for 120 mg/dL, with a positive and negative predictive value adequate for identifying subjects to test with phenotyping or genotyping. Accordingly, phenotype/genotype should only be performed if AAT serum levels are <120 mg/dL. This cut-off value cannot be generalized because there are differences in reference values according to each technique adopted (rocket immunoelectrophoresis and radial immunodiffusion besides nephelometry). A cut-off value with a widely

accepted quantitative technique might represent a useful approach for the targeted detection of AAT deficiency in the future. Finally, a targeted detection program would be preferable based on a relatively high AAT plasma level of 120 mg/dL in order to identify also heterozygotes.

Although the main purpose of this study was to develop and assess a targeted detection protocol, from the epidemiological point of view it could be considered an incidence study; consequently, the subgroup analyses and subsequent conclusions need to be interpreted with some caution. The IC group was mostly male and older than the NIC group. If this difference is related to lifestyle or genetic factors it remains to be elucidated. Moreover, IC, as a whole group, showed a significantly lower AAT serum level. It is difficult to explain this difference but, to a large extent, it is due to the MS and ZZ subgroups (Table 1). It was observed that in subjects with severe AAT deficiency survival was lesser in patients identified by pulmonary impairment (IC)[27] than in subjects identified by family studies (NIC), regardless of whether they smoked or not; in addition, survival in the NIC non-smoker was similar to that of the general population.[28] Thus, the likelihood of severe AAT deficiency-related respiratory disease is probably less than previously believed and, although smoking is a major risk factor, the development of pulmonary emphysema in subjects with AAT deficiency might be multifactorial, due either to unknown genetic factors, environmental influence or both.

In conclusion, the results obtained show the current protocol as effective for finding subjects or patients with AAT deficiency in a selected population. In addition, the serum level cut-off we identified, might be used in a very large non-selected population, avoiding excessive phenotyping or genotyping. IC were older, mostly male and characterized by lower AAT serum levels as compared to NIC; IC could be heterozygous and affected also by diseases other than lung and liver illnesses, although such disorders were the most common. In our experience, 120 mg/dL (nephelometric method) was shown to be a reliable cut-off value of AAT serum level for selecting subjects/patients to submit to phenotyping or genotyping.

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