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Improving the Laboratory Diagnosis of M-like Variants Related to Alpha1-Antitrypsin Deficiency

Valentina Barzon [1], Stefania Ottaviani [2,*], Alice Maria Balderacchi [2], Alessandra Corino [2], Davide Piloni [2], Giulia Accordino [2], Manuela Coretti [2], Francesca Mariani [2], Angelo Guido Corsico [1,2] and Ilaria Ferrarotti [2]

[1] Centre for Diagnosis of Inherited Alpha-1 Antitrypsin Deficiency, Department of Internal Medicine and Therapeutics, University of Pavia, 27100 Pavia, Italy

[2] Centre for Diagnosis of Inherited Alpha-1 Antitrypsin Deficiency, Laboratory of Biochemistry and Genetics, Pulmonology Unit, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy

* Correspondence: s.ottaviani@smatteo.pv.it; Tel.: +39-0382501015

Abstract: Alpha1-antitrypsin (AAT) is a serine protease inhibitor that is encoded by the highly polymorphic *SERPINA1* gene. Mutations in this gene can lead to AAT deficiency (AATD), which is associated with an increased risk of lung and/or liver disease. On the basis of electrophoretic migration, AAT variants are named with capital letters; M (medium) signifies the normal protein. Among pathological variants, the M-like ones represent a heterogeneous group of rare allelic variants that exhibit the same electrophoretic pattern as the M wild-type protein, which makes them difficult to detect with routine methods. In order to avoid their misdiagnosis, the present study defines and validates effective methods for the detection of two pathogenic M-like variants, $M_{wurzburg}$ and $M_{whitstable}$. Comparison of protein phenotypes using isoelectric focusing of samples that presented the $M_{wurzburg}$ variant, as revealed by exons 5 sequencing, identified a particular electrophoretic pattern amenable to the $M_{wurzburg}$ protein. The specific phenotyping pattern was retrospectively validated, thus enabling the detection of 16 patients with $M_{wurzburg}$ variant among the subjects already tested but not sequenced according to our diagnostic algorithm. The $M_{whitstable}$ allele was detected by intron 4 sequencing of SERPINA1 gene. $M_{wurzburg}$ and $M_{whitstable}$ are often misdiagnosed and the introduction of diagnostic improvements can help the clinical management, especially in patients with established lung disease without any other reported risk factors.

Keywords: alpha1-antitrypsin; *SERPINA1* gene; rare pathogenic variants; $M_{wurzburg}$; $M_{whitstable}$; laboratory diagnosis

1. Introduction

Alpha1-antitrypsin (AAT) is a serine protease inhibitor mainly produced in the liver and, to a minor extent, by neutrophils, monocytes, and epithelial cells in the lung and gut. The key function of AAT is the regulation of the proteolytic effects of neutrophil elastase (NE) in the lungs [1]. AAT is encoded by the highly polymorphic *SERPINA1* gene (or Protease Inhibitor (PI) system) located on the long arm of chromosome 14 (14q32.13). The *SERPINA1* gene is organized into four coding and three non-coding exons and spans over 12.2 kb. The encoded protein includes 394 amino acids, with the active site of the enzyme inhibitor at methionine 358. Low AAT serum levels can lead to Alpha1-antitrypsin deficiency (AATD) (OMIM #613490; https://www.ncbi.nlm.nih.gov/omim/; accessed on 3 August 2022), a disorder caused by variations in the *SERPINA1* gene, inherited in an autosomal recessive pattern with co-dominant expression of alleles. AATD is related to an increased risk of developing early-onset emphysema and/or chronic liver disease [2].

Over 120 variants of AAT have been described [3]. The wild-type allele is referred to as the M allele; the Z (p.E366K c.1096G>A rs28929474) and S (p.E288V c.863A>T rs17580) alleles are the most frequent variations that lead to AATD, but several other pathogenic

variants, usually referred to as "rare", have been identified [4]. An updated list of allelic variants was recently published [3]. Due to the different molecular mechanisms linked to each variant, wide variations are seen in the incidence and severity of lung and liver disease.

Rare variants are usually thought to have a low prevalence, but many of them are actually rarely investigated and under-diagnosed [5]. One reason could be the difficulty in identifying them using routine analysis. Many rare variants can only be detected by molecular methods, such as PCR sequencing, that are not available in all diagnostic laboratories. To date, there is no universally-established algorithm used by laboratories for the detection of AATD patients. The algorithms applied in different countries depend on diverse criteria based on the availability of diagnostic facilities and expertise, country-specific indications, and the occurrence of variants [6].

The "classic" nomenclature of AAT variants was established based on electrophoretic migration of AAT serum before the encoding SERPINA1 gene was identified [7]. AAT variants were initially named on the basis of their migration velocity in starch-gel electrophoresis as M (medium), S (slow), F (fast), or Z (very slow). Subsequently, the other alleles were designated with a letter A–L or N–Z depending on their proximal or distal location, respectively, to the M protein band. When a new variant is identified, the birthplace of the index case is added to the letter that corresponds to the IEF migration.

M-like variants (M_{malton}, $M_{procida}$, $M_{heerlen}$) are a heterogeneous group of rare AAT mutations that display the same electrophoretic pattern as the M wild-type protein.

This paper focuses on two M-like variants, namely $M_{wurzburg}$ [8] and $M_{whitstable}$ [9], and the diagnostic procedures aimed at their identification. Both alleles are classified as M-like variants because their isoelectric focusing (IEF) pattern is easily confused with the normal M protein. Previously documented $M_{wurzburg}$ electrophoretic patterns [10] are not replicable. The $M_{wurzburg}$ protein (p.P393S c.1177C>T rs61761869; https://www.ncbi.nlm.nih.gov/snp/; accessed on 3 August 2022) is an M-like variant forming ordered polymers that are retained as inclusions within the endoplasmic reticulum (ER) of hepatocytes. The $M_{wurzburg}$ variant was first identified in a heterozygous individual with serum AAT deficiency but no evidence of lung or liver disease [8]. The $M_{whitstable}$ variant consists of a 26-bp deletion with an associated insertion of two deoxyguanosine residues in intron 4. This variant usually arises on the normal M2 allele. The $M_{whitstable}$ mutation was first detected in two related Z heterozygous subjects [9].

2. Results

2.1. $M_{wurzburg}$

We carried out a retrospective review of our diagnostic phenotyping results from March 2014 to January 2019 in all samples carrying the $M_{wurzburg}$ allele (76 out of 5916) as revealed by exon 5 sequencing of the SERPINA1 gene, which was performed according to the recently described diagnostic algorithm [6]. Direct comparison identified a particular electrophoretic pattern amenable to the $M_{wurzburg}$ protein. Interestingly, the specific IEF pattern was detectable in both serum and dried blood spot (DBS) samples. The specific IEF bands amenable to the $M_{wurzburg}$ protein were clearly visible at the top of the gel, as shown by the arrows in Figure 1.

Conversely, M-like variants such as M_{malton} and $M_{procida}$ are not associated with a particular IEF pattern, but are very similar to the normal M protein pattern (Figure 1, Lanes 2 and 5).

Subsequently, we selected 18 patients displaying the $M_{wurzburg,}$ IEF pattern from all samples in our archives that had not been sequenced using the current diagnostic algorithm [6], mainly because AAT concentrations were above the decisional cut-off values. We then checked them for the presence of the $M_{wurzburg}$ allele by sequencing exon 5 of the SERPINA1 gene (Figure 2).
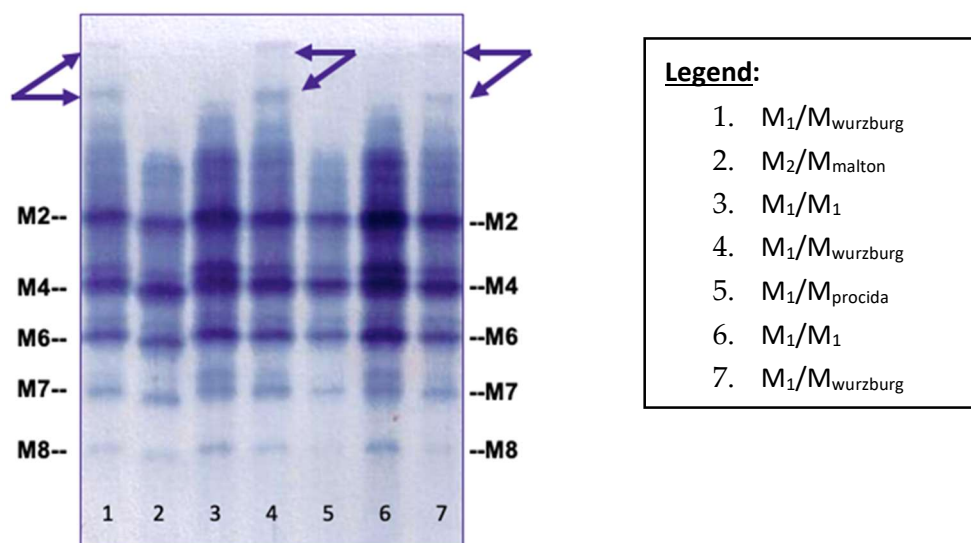
Figure 1. IEF pattern of M-like variants obtained by Sebia Hydrasys® System on DBS samples. M2, M4, M6, M7, and M8 represent the different M isoforms [11,12]. (Arrows show the specific pattern of $M_{wurzburg}$).

Sixteen of the eighteen subjects (88.9%) were found to carry the $M_{wurzburg}$ variant (Table 1).

Table 1. Clinical data of selected and re-examined patients with the $M_{wurzburg}$ IEF pattern.

| Sample	Genotype	AAT (mg/dL)	CRP (mg/dL)	Age at Diagnosis	Smoking Habits (Pack/Year)	Disease
1	$SM_{wurzburg}$	86	Not reported	63	Current (31)	Healthy
2	$MM_{wurzburg}$	122.5	0.11	67	Not reported	Emphysema
3	$MM_{wurzburg}$	123	0.11	67	Current (47)	Emphysema
4	$MM_{wurzburg}$	123.2	0.34	25	Not reported	Not reported
5	$MM_{wurzburg}$	124	0.32	64	Former (30)	Asthma
6	$MM_{wurzburg}$	125	0.15	61	Former (16)	Bronchiectasis
7	$MM_{wurzburg}$	125.2	0.28	27	Not reported	Not reported
8	$MM_{wurzburg}$	126	0.05	23	Not reported	Not reported
9	$MM_{wurzburg}$	126	0.14	68	Never	Not reported
10	$MM_{wurzburg}$	131.6	0.06	77	Not reported	Not reported
11	$MM_{wurzburg}$	146.3	0.62	74	Not reported	Healthy
12	$MM_{wurzburg}$	177.2	0.2	55	Never	Asthma, bronchiectasis, dyspnea—hepatitis
13	$MM_{wurzburg}$	180.5	0.11	45	Not reported	Not reported
14	MM	182	0.25	78	Never	Bronchiectasis
15	MM	184	Not reported	48	Not reported	Healthy
16	$MM_{wurzburg}$	197.4	0.05	55	Not reported	Emphysema
17	$MM_{wurzburg}$	206	3.27	78	Not reported	Not reported
18	$MM_{wurzburg}$	315.1	0.09	64	Former (30)	Bronchitis
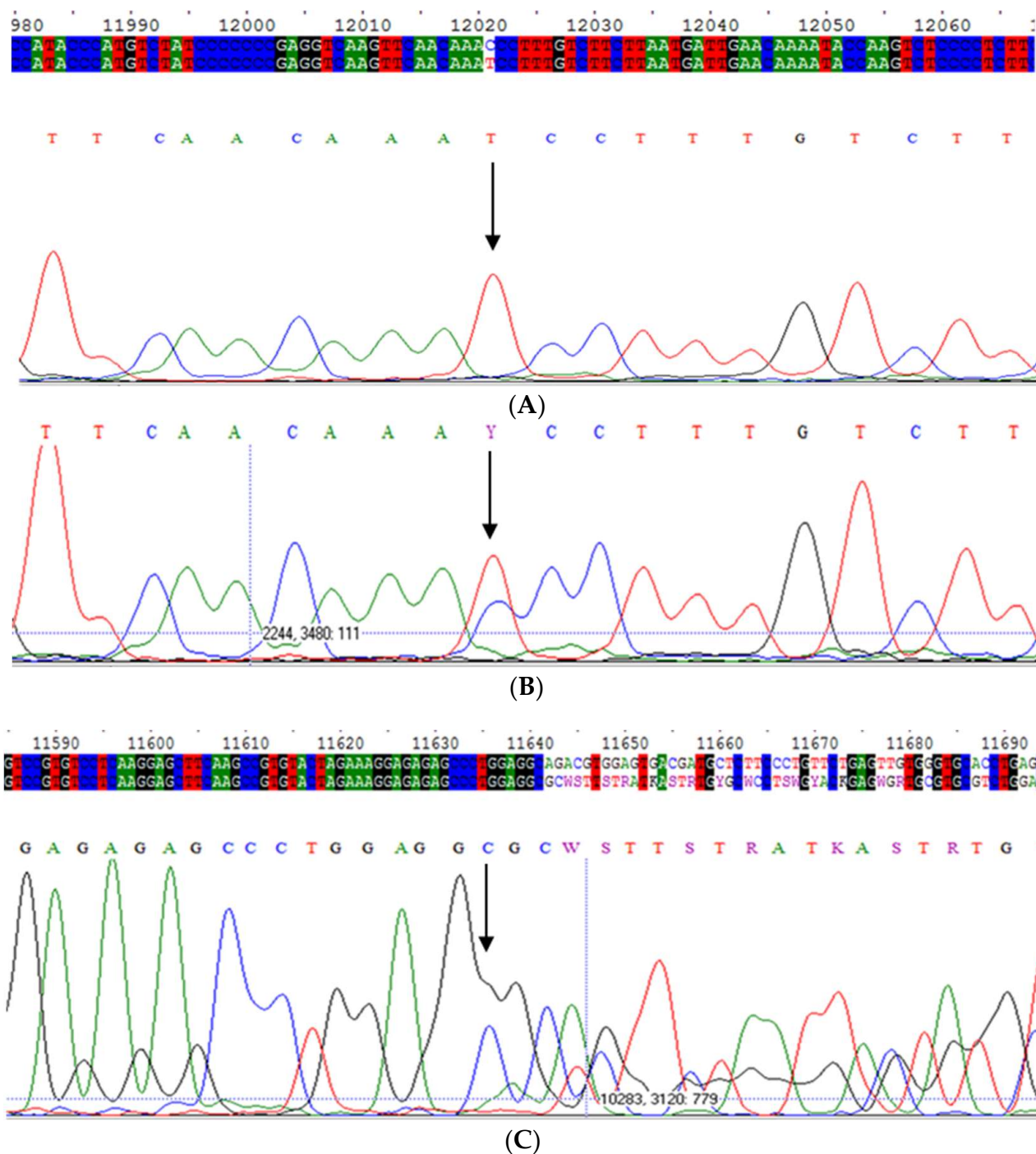
Figure 2. (**A**) Electropherogram of M$_{wurzuburg}$ variant in homozygosis; (**B**) Electropherogram of M$_{wurzuburg}$ variant in heterozygosis; (**C**) Electropherogram of M$_{whitstable}$ variant in heterozygosis.

Interestingly, one subject had already proven to be S heterozygous and thus had a final PI*SM$_{wurzburg}$ genotype. All other subjects had the PI*MM$_{wurzburg}$ genotype.

Seven of the sixteen reported lung disease patients (43.75%) and one subject (6.25%) also had liver disease.

2.2. M$_{whitstable}$

The M$_{whitstable}$ allele consists of: (a) g.11640del26bp, insGG in Sanger annotation [9]; (b) c.1066-262_1066-246delAGTGACGATGCTCTTCC rs553862825 and c.1066-271_1066-

265delCAGACGT rs535461370 in Next Generation Sequencing (NGS) annotation (BaseSpace Annotation; Engine 3.6.2.0; Variant Caller: GATK 1.6, Illumina, San Diego, CA, USA).

During routine diagnoses [6] of AATD at Centre for Diagnosis of Inherited Alpha-1 Antitrypsin Deficiency in Pavia, Italy (http://www.alfa1antitripsina.it; accessed on 3 August 2022) from January 2015 to December 2019, we identified the $M_{whitstable}$ allele by intron 4 sequencing in 36 out of 7885 subjects (0.46%), 30 of whom were $PI*MM_{whitstable}$, 5 $PI*ZM_{whitstable}$, and 1 $PI*SM_{whitstable}$ (Table 2).

Table 2. Clinical data of patients carrying the $M_{whitstable}$ rare allele.

Sample	Genotype	AAT (mg/dL)	CRP (mg/dL)	Age at Diagnosis	Smoking Habits (Pack/Year)	Disease
1	$ZM_{whitstable}$	48	0.1	64	Never	Not reported
2	$M_2M_{whitstable}$	55.7	0.1	60	Not reported	Not reported
3	$M_2M_{whitstable}$	56	0.1	70	Never	Emphysema
4	$ZM_{whitstable}$	56.2	0.11	58	Never	Hepatitis
5	$ZM_{whitstable}$	59.9	0.03	25	Never	Healthy
6	$ZM_{whitstable}$	60.6	0.01	35	Never	Asthma
7	$ZM_{whitstable}$	62	1.4	85	Former (18)	Not reported
8	$M_2M_{whitstable}$	66	0.1	55	Former (10)	Bronchitis,
9	$M_1M_{whitstable}$	66.3	0.28	76	Former (6.3)	Asthma, bronchitis, bronchiectasis
10	$M_1M_{whitstable}$	68.4	0.6	73	Former (18)	Bronchitis
11	$M_1M_{whitstable}$	70.7	0.2	64	Never	Dyspnea-hepatitis
12	$M_2M_{whitstable}$	72.4	0.2	46	Never	Emphysema, pneumothorax
13	$M_1M_{whitstable}$	74	Not reported	Not reported	Not reported	Healthy
14	$M_1M_{withstable}$	77.1	0.1	38	Not reported	Chronic bronchitis, allergic asthma
15	$SM_{whitstable}$	78	0	15	Never	Not reported
16	$M_1M_{whitstable}$	78.3	0.06	55	Former (38)	Asthma
17	$M_1M_{withstable}$	82.1	0.01	47	Not reported	Not reported
18	$M_1M_{whitstable}$	84.5	0.23	53	Never	Asthma
19	$M_2M_{whitstable}$	86	0.1	10 months	Never	Bronchitis
20	$M_1M_{whitstable}$	87.5	0.1	24	Never	Bronchiectasis
21	$M_3M_{whitstable}$	88	0.1	67	Current (35)	Bronchiectasis, dyspnea, emphysema, bronchitis-hepatitis
22	$MM_{whitstable}$	89.3	0.4	61	Current (45)	Dyspnea
23	$M_3M_{whitstable}$	89.8	0.13	71	Former	Asthma
24	$M_1M_{whitstable}$	89.9	0.004	64	Current (31)	Emphysema-hepatitis
25	$M_1M_{whitstable}$	90	0.13	47	Never	Asthma, emphysema
26	$M_1M_{whitstable}$	90.7	0.1	66	Never	Bronchiectasis
27	$M_1M_{whitstable}$	91	Not reported	62	Former (40)	Emphysema
28	$M_2M_{whitstable}$	91.7	0.01	54	Former (Not reported)	Bronchitis
29	$M_1M_{whitstable}$	92.9	0	37	Not reported	Not reported

Table 2. *Cont.*

Sample	Genotype	AAT (mg/dL)	CRP (mg/dL)	Age at Diagnosis	Smoking Habits (Pack/Year)	Disease
30	$M_1M_{whitstable}$	93.3	0.1	73	Not reported	Emphysema, pulmonary fibrosis
31	$M_3M_{whitstable}$	93.7	0.03	3	Never	Healthy
32	$M_2M_{whitstable}$	94.6	0.12	50	Current (12)	Emphysema
33	$M_2M_{whitstable}$	96	0.2	55	Current (40)	Emphysema
34	$MM_{whitstable}$	99.6	0.13	27	Current (7.4)	Healthy
35	$M_1M_{withstable}$	103.9	0.83	72	Not reported	COPD, emphysema
36	$M_3M_{whitstable}$	104	1	64	Former (Not reported)	Emphysema
37	$M_1M_{whitstable}$	111.4	0.9	72	Former (0.25)	Asthma, bronchiectasis, dyspnea
38	$M_2M_{whitstable}$	125	0.05	56	Current (45)	Bronchitis, emphysema, dyspnea
39	$MM_{whitstable}$	128.1	0.12	31	Current (14)	Healthy

We retrospectively selected 31 samples analyzed at Centre for Diagnosis of Inherited Alpha-1 Antitrypsin Deficiency in Pavia, Italy, in January 2015, prior the introduction of the intron sequencing into the diagnostic algorithm [6]. The samples had the following features: (a) serum levels equal to or below 120 mg/dL and the absence of pathogenic variants by exon sequencing and the presence of the M2 allele; (b) serum levels higher than 120 mg/dL, C reactive protein (CRP) equal to or higher than 0.8 mg/dL, and the absence of pathogenic variants by sequencing the *SERPINA1* gene coding regions and the presence of the M2 allele. The $M_{whitstable}$ allele was identified by intron 4 sequencing of the AAT gene (Figure 2).

The retrospective analysis of the 31 samples detected the $M_{whitstable}$ variant in 3 subjects (Table 2).

The mean value of AAT serum levels found in patients with the rare $M_{whitstable}$ allele was 87.5 mg/dL, which corresponds to an intermediate AAT deficiency. However, two $PI*MM_{whitstable}$ patients (patients # 2 and #3 in Table 2) displayed lower than expected AAT serum levels and Sanger sequencing did not detect other variants aside from $M_{whitstable}$. Further studies with Next Generation Sequencing are needed for these samples.

Twenty-seven of the thirty-nine subjects carrying the $M_{whitstable}$ allele reported lung diseases (69.2%). Of these, nine were non-smokers (33.3%), nine were former smokers (33.3%), and six were current smokers (22.2%); no information about smoking habits was available for three patients.

3. Discussion

$M_{wurzburg}$ and $M_{whitstable}$ are two M-like pathological variants of AAT, which are hard to diagnose using routine laboratory techniques. IEF phenotyping is not usually capable of identifying AAT M-like variants, and the recent rapid diagnostic method A1AT Genotyping Test (Progenika, Biopharma Derio, Biscay, Spain) [13,14] does not include these variants in its panel. Therefore, only sequence analysis enables their diagnosis. The importance of an accurate identification of $M_{wurzburg}$ and $M_{whitstable}$ variants in samples with suspected AATD is evident.

$M_{wurzburg}$ is an AAT pathogenic variant with a tendency to form polymers. Polymerization is the consequence of a transport block between the ER and Golgi complex and it is usually associated with neonatal hepatitis, cirrhosis, and hepatocellular carcinoma. The intracellular polymerization of the $M_{wurzburg}$ protein has been demonstrated in cell

models [15,16]. The clinical impact of this variant is not yet clear, since clinical case reports of AATD with the $M_{wurzburg}$ variant are infrequent and controversial [8,17,18]. Nevertheless, in our experience, its presence is rare but not negligible. Accordingly, the allele frequency of $M_{wurzburg}$ in all subjects analyzed in our lab over the last 16 years (for a total of 10,660 samples) is 0.006 (Figure 3).
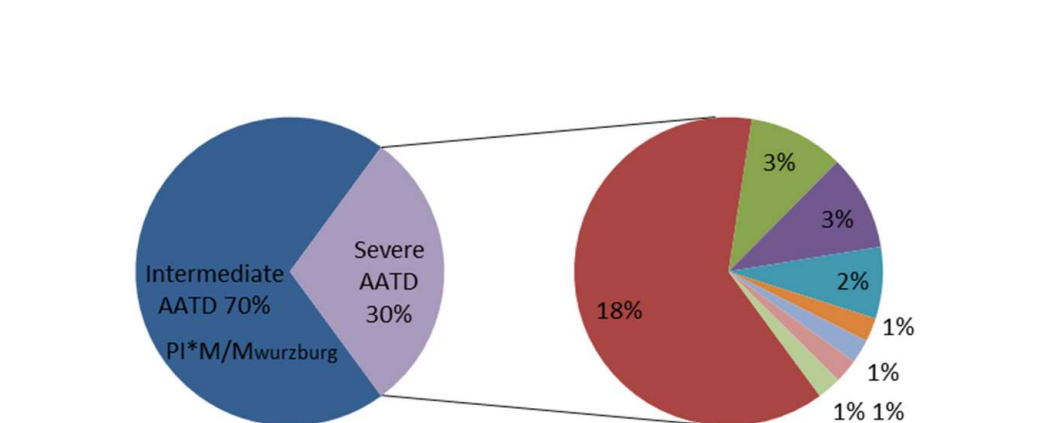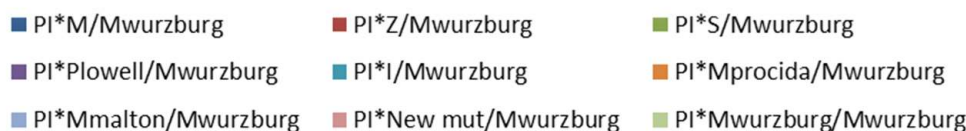

Figure 3. Frequencies of genotypes carrying the $M_{wurzburg}$ allele analysed in the Centre for Diagnosis of Inherited Alpha-1 Antitrypsin Deficiency in Pavia in the last 16 years.

This value is non-neglectable if we compare it to the frequencies reported in the global (0.000594) and European (0.000642) populations [19].

As shown in Table 1, all samples that present the $M_{wurzburg}$ allele after re-analysis had AAT serum levels above 120 mg/dL, which are normally considered non-pathological concentrations [6]. In our experience, the $M_{wurzburg}$ allele is related to a medium–low AAT concentration, with AAT values lower than the established decisional cut-off (110 mg/dL) [6]. We evaluated a mean AAT of 105.6 mg/dL in the cohort of 94 PI*M/$M_{wurzburg}$ samples diagnosed at Centre for Diagnosis of Inherited Alpha-1 Antitrypsin Deficiency in Pavia. Moreover, the mean AAT value in 25 PI*Z/$M_{wurzburg}$ samples diagnosed at our facility was 53.7 mg/dL, which was in the lower limits of the protective threshold. We should also note that AAT values are occasionally raised not only in individuals with active inflammatory/infectious processes, but also in the event of dehydration, during pregnancy, and in women taking contraceptives [20]. Thus, considering the great importance of an accurate AATD diagnosis related to rare *SERPINA1* variants, the identification of a specific IEF pattern for the $M_{wurzburg}$ variant could prevent the misclassification of samples and make it possible to improve early and accurate AATD detection.

$M_{whitstable}$ is a poorly-studied and pathogenic intronic variant which is not reported in databases. The only available paper in the literature estimated its frequency at 0.0125 in the United Kingdom [9]. Most diagnostic algorithms for the identification of AATD patients include sequence analysis of coding exons by Sanger or Next Generation Sequencing, thus losing the rare variants located within the introns.

According to our series, the variant was evenly distributed in Italy, with a diagnostic rate of 0.002. Most of the PI*M/$M_{whitstable}$ patients that we identified had lung diseases (69.2%) and 82% of subjects reported lung symptoms when questioned on their health status. To underline the role of the $M_{whitstable}$ allele in the lack of lung protection from elastase, we noticed that when smoke was not a co-factor, lung symptoms were reported

by 9 out of 11 adult subjects. Interestingly, patients with the $M_{whitstable}$ variant have lower levels of AAT, similar to the MZ population, and thus require a higher level of attention in the diagnostic process.

From a clinical point of view, several patients in our $M_{whitstable}$ cohort were reported to have varying degrees of lung disease, ranging from healthy subjects to those with bronchitis, COPD, or emphysema, regardless of their smoking history in most cases.

The accurate diagnosis of these variants can help the diagnostic process and clinical management, especially in patients with established lung disease without any other reported risk factors. Therefore, it is crucial to refer those patients/samples to expert laboratories, since the IEF or rapid diagnostic tools alone are not usually able to detect those variants. In order to avoid the risk of not identifying patients carrying rare AAT variants, continuous updating of diagnostic algorithms is of chief importance. In this paper, we focused our attention on the identification of two rare M-like AAT variants that are often misdiagnosed. Moreover, for the first time, we have identified the clinical profiles of patients with AATD caused by the $M_{whitstable}$ allele in a fairly large cohort.

4. Materials and Methods

4.1. Samples

Samples submitted to the Italian Reference laboratory were tested for AATD, following the recently reported diagnostic algorithm [6]. Blood samples from patients were collected in ethylenediamine tetraacetic acid (EDTA) or by using DBS samples (Schleicher & Schuell Grade 903, Keene, New Hampshire, USA) [21].

Informed consent for genetic testing was obtained from all patients according to the institution's ethical recommendations.

4.2. Isoelectrophocusing (IEF)

The phenotype of the $M_{wurzburg}$ variant was obtained by using the semiautomatic Sebia Hydrasys® System and the Hydragel 18 A1AT Isofocusing® kit (Sebia, Diagnostic Department, Evry, France). The procedure consists of sample runs on ready-to-use 0.1% agarose gels that contain ampholytes with a pH gradient ranging from 4.2 to 4.9. After sample migration, the detection of AAT was performed with specific AAT antiserum labelled with peroxidase. The gel was then washed and dried automatically [11,12].

Serum samples and controls were diluted 1:10 in a specific diluent, according to the manufacturer's instructions. Dried blood spots were cut into 6mm-diameter circles and eluted in 30 μL of water overnight at 4°C. Subsequently, a 1:8 dilution was applied.

4.3. Sequencing

We confirmed the presence of the $M_{wurzburg}$ allele by sequencing exon 5 of the SER-PINA1 gene. We sequenced the intron 4 region to search for the $M_{whitstable}$ variant.

Polymerase chain reactions (PCR) were performed with the AccuPrimeTM Taq DNA Polymerase System kit (Invitrogen by Thermo Fisher Scientific, Waltham, Massachusetts, USA) and both reaction conditions were as follows: 100 ng of DNA, 2.5 μL of Buffer II, 0.25 μL of MgCl2, 0.5 μM of each primer, and 0.5 μL of Taq DNA Polymerase. Primers used in the amplification and sequence reactions are listed in Table S1. Amplification and sequence reactions were carried out in an I-cycler Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). Then, the reaction products were purified with a commercial kit based on magnetic beads (Ampure XP, Inc., Brea CA, USA). Sequence reactions were performed as indicated by the manufacturer, and sequence products were purified by a commercial kit based on magnetic beads (Agencourt CleanSeq, Inc., Brea, CA, USA).

Sequencing was performed by the CEQ 8800 genetic analysis System (Beckman Coulter, Pasadena, California, USA).

The NCBI Reference Sequence which we refer to is the following: NM_001002235.2.

5. Conclusions

The identification of a specific IEF pattern amenable to the $M_{wurzburg}$ variant could help detect this pathogenic variant during routine analysis based on phenotyping. Conversely, the sequencing of intron 4 of *SERPINA1* gene enables the identification of $M_{whitstable}$ allele, whose frequency is not neglectable. Since patients carrying the $M_{wurzburg}$ or $M_{whitstable}$ alleles have a higher risk of developing lung and liver diseases or only lung diseases, respectively, an early and precise detection would improve the clinical management of these patients.

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