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Rapid detection of Mmalton α_1-antitrypsin deficiency allele by real-time PCR and melting curves in whole blood, serum and dried blood spot samples

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Abstract

Background: α_1-Antitrypsin deficiency (AATD) is an autosomal codominant disorder associated with a high risk of developing lung and liver disease. The most common deficient alleles are known as Z and S. However, another deficient variant, called Mmalton, which causes a deficiency similar to variant Z, is considered to be the second cause of severe AATD in Spain. Nevertheless, the Mmalton allele is not recognizable by usual diagnostic techniques and therefore, its real prevalence is underestimated. We describe a rapid real-time PCR and melting curves assay designed for the detection of Mmalton AATD.
Methods: We tested the applicability of this new technique for the identification of the Mmalton allele in AATD screening using whole blood, dried blood spot (DBS) and serum samples. Mmalton heterozygote and homozygote samples and samples without this allele were included in the study.
Results: This new assay is able to detect homozygous and heterozygous genotypes in the same reaction and in a single step, giving matching results with those obtained by *SERPINA1* gene sequencing.
Conclusions: This technology is optimal for working with small amounts of DNA, such as in DBS and even with residual DNA present in serum samples, allowing improvement in routine algorithms of AATD diagnosis or large-scale screening. This method will be useful for obtaining more in depth knowledge of the real incidence of the Mmalton variant.

Keywords: α_1-antitrypsin deficiency; dried blood spot; FRET probes; melting curves; Mmalton variant.

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Introduction

Human α_1-antitrypsin (AAT) is a member of the Serpin (Serin Proteases Inhibitor) superfamily and is a secretory protein mainly produced by the liver, being the most abundant circulating serum antiprotease [1]. AAT, also known as α_1 protease inhibitor (α_1-PI), shows a broad spectrum of antiserine-protease activities, including the inhibition of free elastase from neutrophils, but also has antioxidant, anti-inflammatory, anti-infectious and immunomodulation effects [2, 3]. AAT is encoded by the *SERPINA1* gene (PI locus: 14q32.1) which is organized into three non-encoding exons (1A, 1B and 1C) and four encoding exons (2–5) [4]. Despite its small size (394 amino-acid), AAT is a highly polymorphic protein with over 120 variants, including at least 60 deficiency alleles [5]. α-1-antitrypsin deficiency (AATD) is a hereditary autosomal disorder characterized by low circulating

AAT levels 35% (or 0.5 g/L) below the normal value as the consequence of mutations in the *SERPINA1* gene. These mutations are associated with the misfolding of AAT molecules promoting the production of intrahepatic AAT polymers which can cause cell stress and liver damage [6]. Moreover, as a result of polymerization/retention into hepatocytes, the concentration of AAT in blood and tissues is reduced, being associated with lung tissue damage and emphysema due to uncontrolled elastase activity [5].

Normal alleles are most commonly M subtypes, which account for 95% of the gene variants. The most common deficient alleles are PI S (rs17580: c.863A>T; p.Glu264Val), with a prevalence of 5%–10% in Caucasians and PI Z (rs28929474; c.1096G>A; p.Glu342Lys), with a prevalence of 1%–3% [7]. PI S and PI Z alleles are associated with reduced serum levels of AAT of up to 40% and 10%–20%, respectively. Therefore, the PI Z allele is related to severe deficiency and is the phenotype most often associated with lung and liver disease [8]. More than 95% of individuals with severe deficiency have the PI ZZ genotype. Non-S and non-Z deficient variants are called "rare" because of their low frequency. In a retrospective review of AATD studies performed in our laboratory, a remarkably high frequency of the deficient PI I and PI Mmalton alleles was observed, together accounting for 54% of all rare AAT variants in Spain (34% for PI I and 20% for PI Mmalton) [9]. While the PI I variant causes moderate AATD (60%–70% of normal level), from a clinical point of view, the PI Mmalton variant mimics the PI Z clinical phenotype [10]. This last mutation has been detected in many countries [9]. For example, in Central and Southern Italy (particularly in Sardinia), the PI Mmalton and the PI Mprocida (Leu41Pro) variants are more prevalent than PI Z [10]. The PI Mmalton variant consists of the deletion of an entire TTC codon in exon II, and subsequent deletion of the Phe51 or Phe52 residue of the mature protein [11].

The current approach to laboratory diagnosis of AATD involves the use of a combination of serum AAT measurement and phenotype characterization by isoelectric focusing (IEF) or the usual allele-specific genotyping assay included in large-scale screening programs for detecting PI S and PI Z alleles [12]. Nevertheless, most rare variants are difficult or even impossible to detect with the above methods, which may have contributed to a misclassification of many of these cases, with the subsequent underestimation of their true frequency. Most of these alleles can only be detected by molecular biology techniques, such as exonic sequencing of *SERPINA1* gene, which is not available in all routine laboratories [13]. In this sense, PI Mmalton is characterized by a normal isoelectrophoretic pattern (M-Like variant) [14]. Thus, the characterization of this variant as part of routine laboratory algorithms for AATD diagnosis is not possible using an isoelectrophoretic pattern, making application of molecular methods for the identification of this variant, such as Southern blot hybridization [11], denaturing gradient gel electrophoresis [15], DNA haplotypes [16] and *SERPINA1* sequencing mandatory. Nonetheless, this methodology is time consuming and requires multiple manual steps and is not available in most routine laboratories. Therefore, a more suitable, rapid, and sensitive method is necessary to obtain in depth knowledge of the real incidence and impact of this variant in AATD.

In this paper we describe the design of specific fluorescence resonance energy transfer (FRET) probes for the detection of the PI Mmalton variant by real-time PCR and melting curves analysis using the LightCycler analyzer. This technique allows DNA amplification and simultaneous detection and documentation of genetic polymorphisms. We tested its applicability for the identification of the PI Mmalton allele in AATD screening with dried blood samples (DBS). In addition, we determined its possible utility in the molecular detection of this variant in residual DNA of serum samples used for AAT levels quantification and the IEF technique, which would allow its direct implementation in routine AATD studies.

Materials and methods

Clinical samples

Individuals with severe AATD were recruited from the departments of Biochemistry and Pneumology of the Vall d'Hebron University Hospital (Barcelona, Spain). The study was approved by the Ethical Committee Board and informed consent was obtained from all patients for the genetic analyses. The study was conducted in accordance with the principles of the Declaration of Helsinki, Guidelines for Good Clinical Practice and in full conformity with relevant regulations.

For the design of the technique, EDTA (ethylenediaminetetraacetate) whole blood and DBS samples were obtained from three related individuals characterized by IEF as MM, MS and MZ, but with clearly low serum AAT levels: 0.75, 0.45 and 0.32 g/L, respectively. In the three cases the determination of the AAT genotype was carried out with an exonic sequencing of *SERPINA1* gene in DNA isolated from whole blood as described below.

DNA sequence analysis showed that three individuals were heterozygous for the PI Mmalton allele (Mmalton/M, Mmalton/S and Mmalton/Z, respectively), which had previously been wrongly characterized as a normal M allele.

In addition, we used 10 DBS and whole blood samples from individuals with an AAT genotype with alleles different from the PI Mmalton variant (M/M, M/S, M/Z, S/Z, S/S, Z/Z, M/Null Mattawa, M/I, M/Plowel and M/Mvalld'hebron) as specificity controls of the new technique.

All DBS samples processed in this study were spotted from whole blood samples obtained by venopuncture. The drops of blood were applied to the AlphaKit/DBS filter paper (Grifols, Barcelona, Spain) which were then left to dry at room temperature.

DNA extraction

DNA extraction from EDTA whole blood and serum samples were performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and DNA from DBS was obtained using a previously described protocol [17].

Exonic sequencing of *SERPINA1* gene

This method consists of the amplification of DNA extracted by PCR followed by Sanger automated sequencing of the PCR products. This determination requires a complete study of the DNA sequences of the four encoding exons of the *SERPINA1* gene. We used the same primers for both PCR and sequencing methods and these are shown in Table 1 (GeneBank accession n.K02212).

Obtaining homozygous PI Mmalton

To obtain a positive control for genotyping with real-time PCR and melting curves, molecular cloning was performed from a sample of EDTA whole blood corresponding to a Mmalton/M heterozygote individual, previously characterized by *SERPINA1* sequencing. A fragment of 450 bp of the second AAT exon (nucleotide 7251-7700) was amplified by PCR and cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). The clones obtained were sequenced following the same procedure as that used with clinical samples. The nucleotide numbering corresponds to the human AAT gene sequence obtained from the Gene Bank (Accession no. K02212).

Design of the primers and FRET probes

SERPINA1 genotyping was performed in the LightCycler 2.0 analyzer (Roche Diagnostic, Mannheim, Germany), which achieves rapid real-time PCR. This thermal cycler detects mutations by analysis of the melting point of one of the two adjacent fluorescently labeled probes.

A schematic representation of oligonucleotide primers, FRET probes and the sequence used for genotyping the PI Mmalton allele is shown in Figure 1. Primers were designed to flank a region of 171 bp of the second *SERPINA1* gene exon: Serpina 1S: 5′-TTCAACAAGATCACCCCCAACC-3′ (nucleotide 7454-7475) and Serpina 1A: 5′-GCCCTCCAGGATTTCATCGTG-3′ (nucleotide 7604-7624). FRET probes

Table 1: Primers sequence, position and annealing temperature for PCR and exonic sequencing of *SERPINA1* gene.

Exon	Sequence	Position	Annealing temperature, °C
2	18Fw: 5′ GATCACTGGGAGTCATCATGTGC 3′	7251-7273	54
	18Rv: 5′ GGTTGAGGGTACGGAGGAGT 3′	7681-7700	
	19Fw: 5′CCAAGGCTGACACTCACGAT 3′	7590-7609	
	19Rv: 5′AGGAGAGTTCAAGAACTGATGGTT 3′	8021-8044	
3	3Fw: 5′ TTCCAAACCTTCACTCACCCCTGGT 3′	9362-9386	60
	3Rv: 5′ CGAGACCTTTACCTCCTCACCCTGG 3′	9889-9913	
4	4Fw: 5′ CCCAGAAGAACAAGAGGAATGCTGT 3′	10882-10906	54
	4Rv: 5′ CATTCTTCCCTACAGATACCATGGT 3′	11114-11138	
5	5Fw: 5′ TGTCCACGTGAGCCTTGCTCGAGGC 3′	11841-11865	54
	5Rv: 5′ GACCAGCTCAACCCTTCTTTAATGT 3′	12155-12179	

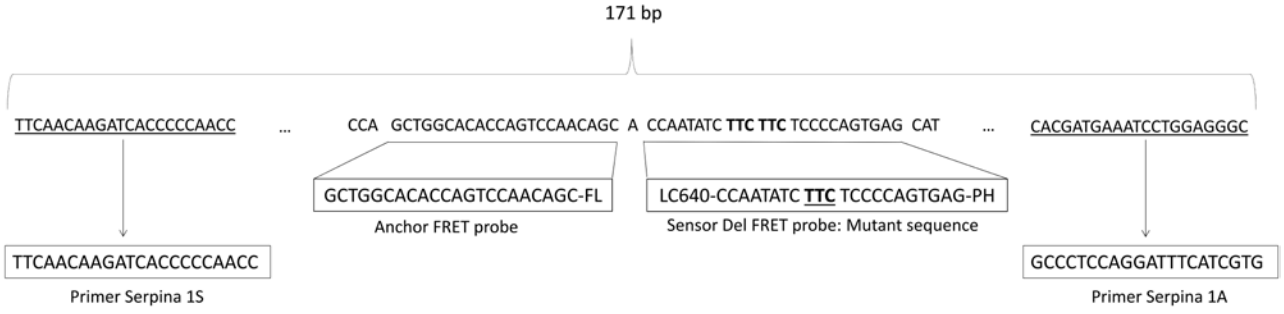

Figure 1: Representation of sequence, primers and FRET probes employed in this study.

hybridize very close together and were: Anchor: 5′-AGCTGGCACAC-CAGTCCAACAGC-3′ (nucleotide 7506-7528) labeled with fluorescein at the 3′ end and Sensor Del: 5′-CCAATATCTTCTCCCCAGTGAG-3′ (nucleotide 7530-7554) labeled at the 5′ end with red fluorophore LC640. This last probe hybridizes over the mutation position and matches perfectly with the mutated sequence (deletion of an entire TTC codon). The theoretical melting temperatures of the primers and probes (Tms), possible oligonucleotide dimers formation, and self-complementarity, were calculated using the Oligo Analyzer 3.1 (Integrated DNA Technologies).

Real-time PCR conditions

The reaction was conducted at a final volume of 20 µL containing 2 mM $MgCl_2$, 0.5 µM of each primer, 0.2 µM of each FRET probe, 2 µL of LightCycler Fast Start DNA Master Hybridization probe mix (Roche Diagnostics, Mannheim, Germany) and 5 µL of genomic DNA. The PCR program was run as follows: an initial denaturation step of 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 5 s, annealing at 54 °C for 10 s and extension at 72 °C for 15 s. After amplification, melting curves were generated by denaturation at 95 °C for 20 s, maintaining the sample at 40 °C for 20 s and slow heating the sample at 85 °C. Fluorescent measurements were recorded during each annealing step and continuously during the heating of melting step. Fluorescence was measured at a wavelength of 640 nm.

LightCycler software converts melting curves into melting peaks which allows both normal and mutated alleles to be easily distinguished by melting temperatures.

New diagnostic algorithm

Following its design and evaluation, this technology was included in the diagnostic algorithm of AATD (Figure 2), as follows: AAT levels were determined, and in cases with concentrations <1.2 g/L, the phenotype was determined by IEF. If AAT values were in accordance with the phenotype observed, the laboratory results were considered definitive [13]. However, if AAT levels did not correspond with the phenotype, the AAT genotype was determined by an allele-specific genotyping assay for the S and Z variants, the new real-time PCR technique for detection of the PI Mmalton allele and exonic sequencing of *SERPINA1*.

During a period of 6 months, 12 patients with a normal (MM) phenotype but with low AAT levels were analyzed by *SERPINA1* gene sequencing (DNA samples obtained from EDTA whole blood) and the new real-time PCR technique (DNA samples obtained from serum, DBS and EDTA whole blood).

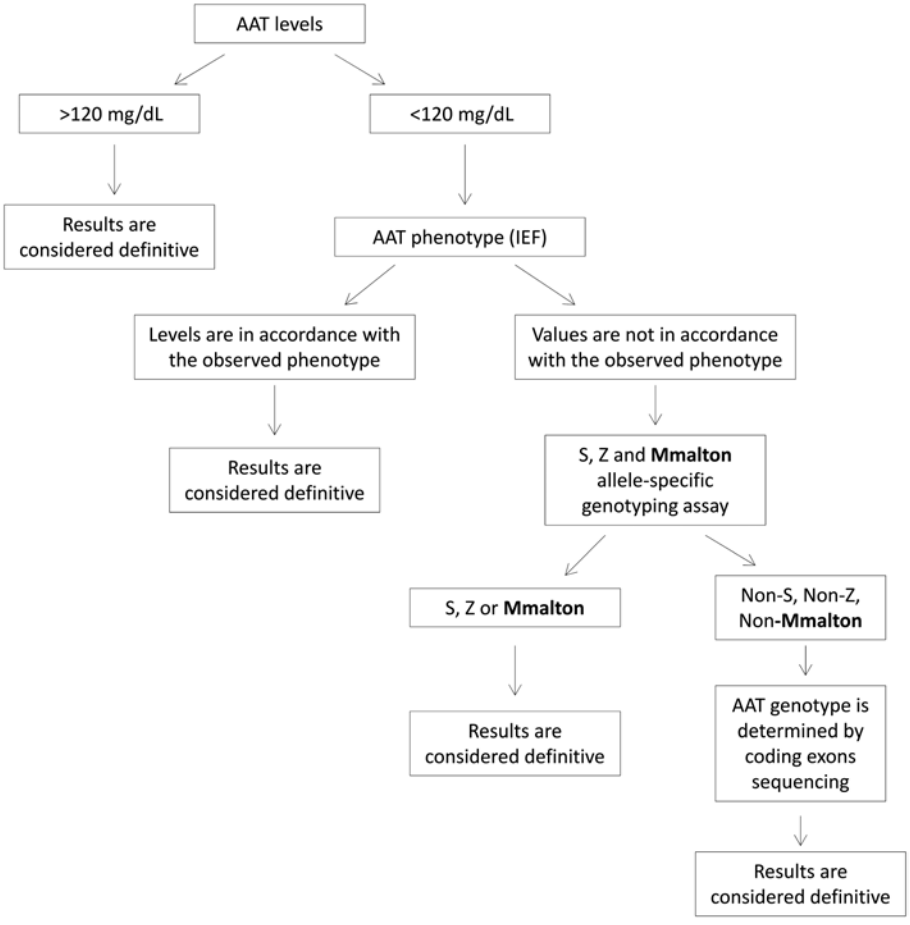

Figure 2: Proposed AATD diagnostic algorithm.

Results

Processing of clinical samples

In the case of the three related individuals with a PI Mmalton allele (Mmalton/M, Mmalton/S and Mmalton/Z), comparative sequencing analysis showed total concordance between genotyping results obtained with this new real-time PCR assay and the encoding exons sequencing method using DBS and EDTA whole blood samples. In the melting curves analysis, the three individuals showed both 50 °C and 62 °C melting peaks (all samples were Mmalton heterozygous). There was also complete concordance between the results of both *SERPINA1* gene sequencing and real-time PCR using 10 DBS and whole blood control samples without the PI Mmalton allele. All samples showed a single melting peak at 50 °C.

Molecular cloning

We obtained 20 clones which were characterized by exonic sequencing of *SERPINA1* gene: seven clones were PI Mmalton homozygous and 13 clones did not have this variant (normal variant). All the clones were analyzed by the new real-time PCR and melting curves technique. After melting curve analysis, the 13 clones with normal alleles showed a melting peak at 50 °C, while the seven clones with a mutated PI Mmalton allele showed a melting peak at 62 °C. The original sample genotyping (heterozygous) showed peaks at 50 °C and 62 °C (Figure 3). These three patterns were confirmed by sequencing (Figure 4). Hence, the peak at 62 °C indicates the presence of the PI Mmalton allele and the peak at 50 °C indicates the absence of this allele.

Implementation of the diagnostics algorithm

Real-time PCR and melting curves methodology for detecting the PI Mmalton variant was included in the diagnostic algorithm of AATD (Figure 2). During the 6-month study period, we detected 12 patients with the MM phenotype and low AAT levels. Samples obtained from serum, DBS and EDTA whole blood were analyzed by the new real-time PCR technique, and we detected five Mmalton heterozygous individuals (showing peaks at both 50 °C and 62 °C). The genotypes of the remaining seven samples were M/M, M/Mvalld'hebron and M/Null Mattawa. The results were identical for the three types of samples, with total concordance between this new technique and exonic sequencing method. Patients' AAT levels, phenotype, sequencing analysis and melting curves output of all the patients are shown in Table 2.

Discussion

Our results have shown that the new technique for the detection of the PI Mmalton variant is rapid, specific, easy to

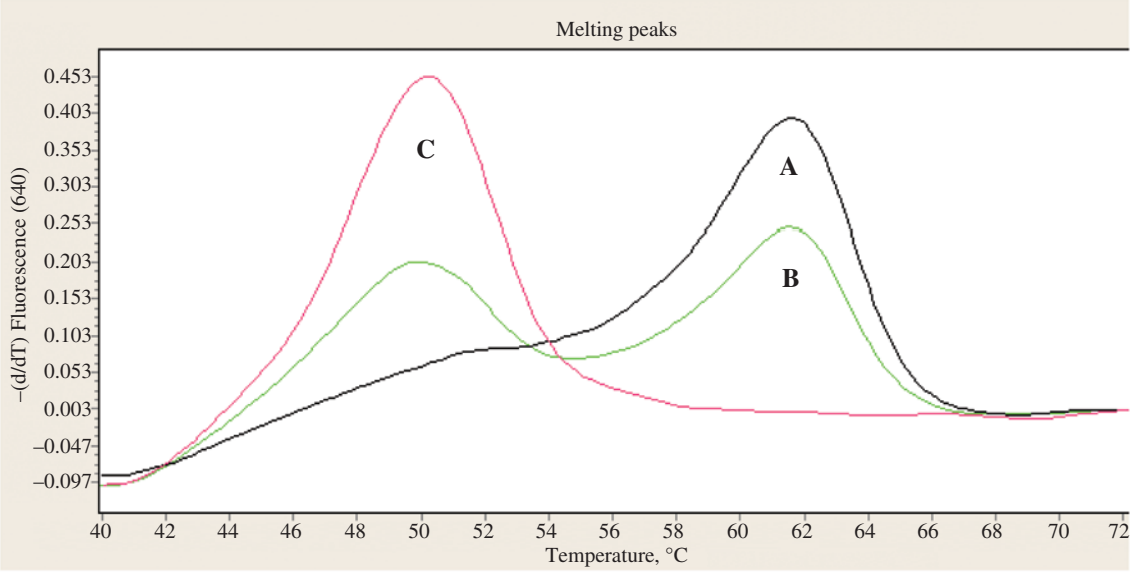

Figure 3: Melting peaks obtained by real-time PCR containing the different PI Mmalton genotype profiles.
(A) Homozygous mutated sample, (B) heterozygous, (C) wild type sample.

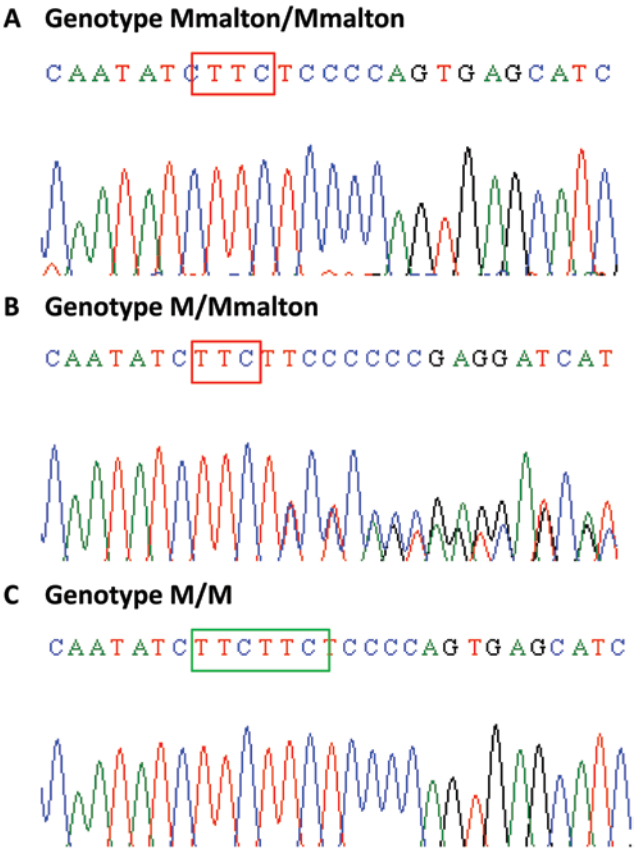

Figure 4: Results of exonic sequencing of *SERPINA1* gene. (A) Homozygous mutated sample, (B) heterozygous, (C) wild type sample.

Table 2: Patients' AAT levels, phenotype, sequencing analysis and melting curves results.

Case no.	AAT levels, g/L	Phenotype	Sequence analysis	Melting curves
1	1.00	M/M	M/M	N
2	0.86	M/M	M/M	N
3	0.77	M/M	M/M	N
4	0.72	M/M	M/Null Mattawa	N
5	0.71	M/M	M/Mmalton	H
6	0.86	M/M	M/M	N
7	0.89	M/M	M/Mmalton	H
8	0.87	M/M	M/Mmalton	H
9	0.94	M/M	M/Mvalld'hebron	N
10	0.64	M/M	M/M	N
11	0.72	M/M	M/Mmalton	H
12	0.60	M/M	M/Mmalton	H

Melting curves results: H, heterozygous Mmalton; N, no Mmalton allele.

interpret and reproducible. Therefore, the implementation of this method in our diagnostic algorithm may contribute to the detection of new cases of this important deficient allele.

Little is known about the epidemiology of the non-S and non-Z deficient AAT variants, due to their low prevalence. A retrospective study performed in Barcelona showed that among these variants, PI Mmalton represented 20% of cases [9]. In the present study, we found five PI Mmalton alleles among 12 samples with the MM phenotype and low AAT levels. Hence, this allele may not be as rare as previously thought. The methodological difficulties associated with routine assays are a common problem in the study of rare alleles [18]. For this reason, it would be of interest to develop standardized studies to obtain conclusive data on the prevalence of rare AAT variants, such as real-time PCR melting curves, currently used in screening programs for the detection of S and Z AAT variants. Moreover, this method could be used for the study of the most prevalent rare variants in each region.

The use of PCR plus DNA sequencing has become an essential tool in research and diagnostic laboratories. Nevertheless, this method requires a relatively large amount of DNA and involves multiple manual steps, making it labor intensive. Moreover, an additional whole blood sample is required for exonic sequencing of *SERPINA1* gene when there is discordance between AAT levels and the IEF phenotype pattern. This last fact represents an additional invasive blood extraction and a significant delay in AATD diagnosis. Therefore, simpler, faster and more automated genotyping methods are needed for routine use and population screening. These methods must be able to perform AATD diagnosis in DBS and in serum samples used by the routine AAT level determination and phenotyping.

Real-time PCR is now more rapid, sensitive and reproducible and has a reduced risk of carry-over contamination [19]. The combination of PCR technology using real-time fluorescence and analysis of differences in the melting temperatures of specific probes allows polymorphism to be easily detected [20]. Among the different types of probes available, the FRET probes are increasingly used in many applications involving nucleic acid mutation analysis. This technique is highly sensitive allowing the use of DBS specimens as described previously by our group in a study of the PI S and PI Z AAT alleles [17]. This approach has been reported to be particularly useful for large-scale population studies, in which samples from different areas are sent to a central laboratory [21]. Blood collection on filter paper requires a small amount of sample, is minimally invasive and produces a specimen that is inexpensive to ship and needs no special storage conditions. The extremely high sensitivity of this technology, which is able to study a very low amount of DNA recovered from DBS samples, could potentially allow similar studies in

residual DNA which remains in standard clinical serum samples [22].

With this objective in mind, the present study reports the development of a new qualitative fluorescent real-time PCR assay to detect the PI Mmalton AATD allele using specific FRET probes and DBS specimens and also standard serum samples. Homozygous and heterozygous Mmalton genotypes can be detected at the same time.

Therefore, this real-time PCR for PI Mmalton genotyping, together with real-time PCR for the PI S and PI Z variants, may contribute to the detection of a more complete range of AATD variants and determine the real frequency of the PI Mmalton variant in different populations [23]. The utility of this new technology in serum samples is also a very interesting result of our study. In this sense, it should be kept in mind that routine AATD studies (AAT levels and IEF phenotyping) are performed in serum samples. In the case of rare AATD variants not previously correctly characterized by these standards studies, further molecular characterization of these variants requires new EDTA whole blood samples and their genotyping by sequencing, with the subsequent significant delay in the obtainment of results. The results of our study using standard serum samples have demonstrated the utility of this new methodology in the molecular characterization of the rare deficient PI Mmalton variant practically within the same time as that of standard studies. This represents an important improvement of the algorithm for in vitro AATD studies at clinical level. In this sense, Snyder et al. [24] reported an improved diagnostic algorithm that initially involves AAT quantification and allele-specific genotyping, with IEF used as a reflex test. In our proposed algorithm, the IEF assay is used as the initial technique and allele-specific genotyping acts as the reflex technology. Use of IEF before genotyping enables detection of some rare AATD alleles without the need to design costly allele-specific genotyping assays. For example, in the Spanish population, there is a high frequency of PI I, PI Mmalton, and PI Plowel rare AAT variants [9]. The PI I and PI Plowel variants can be detected by phenotyping, which is more cost-effective than genotyping.

Widespread use of this real-time PCR technology in DBS screening and routine AATD studies could demonstrate the real prevalence of this rare variant, and may even allow the clinical characterization of PI Mmalton individuals who, to date, are not well characterized.

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