Setting up of High Resolution Melting technique with LIGHTCYCLER 480, ROCHE® in haematological diagnosis and its comparative with HAIN, LIFESCIENCES®.

Final Degree Project

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Academic Year: 2020-2021, Tarragona.

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ACKNOWLEDGEMENTS

This work has been carried out in the molecular biology laboratory of the haematology service of the University Clinical Hospital of the Valencian Community in partnership with the health research institute INCLIVA, specifically through collaboration with the Haematopoietic Transplantation Research Group.

In the first place, I would like to express my appreciation to Dr. Carlos Solano Vercet and the Dr. Juan Carlos Hernández Boluda for giving me the opportunity to collaborate with their Research Group.

I would also like to express my deep gratitude to Dra. Blanca Ferrer Lores who has allowed me to take part of her work group for carrying out the research on which my final degree project has been based. As well as she has given me the chance to participate in different Haematology meetings and conferences. These experiences have enabled me to evolve both personally and professionally.

In the same way, I would like to express my very great appreciation to the biotechnologist Alicia Serrano Alcalá for her constant support, her teachings and the confidence transmitted during all this project.

I would also like to extend my thanks to the technicians of the molecular biology unit for the treatment and support I have received during my internship period.

I would like to acknowledge to Dr. Juan Bautista Fernández Larrea, my academic tutor who has guided me in carrying out this work.

Finally, I wish to thank my family specifically to my parents and my sister and to my friends who have supported and trusted me throughout this period of my life.

Work carried out according to the results obtained in the external practices conducted in the Research Foundation of the Clinical Hospital of the Valencian Community (INCLIVA Foundation) directed by the Dra. Blanca Ferrer Lores and the biotechnologist Alicia Serrano Alcalá.

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ABSTRACT (English)

Currently, medical genetics is essential in the study of human variability and heredity. Genotyping studies have allowed the determination of mutation associated with different pathologies. Although new methodologies are used to detect these variations, there are still difficulties, particularly in the determination of single nucleotide polymorphisms, which are more frequent and whose clinical implication is less defined.

Therefore, the hypothesis of this research pursues the optimization of genotyping studies, aimed at haematological diagnosis. The main objective for achieving it, was the setting up of the High Resolution Melting technique, with *LightCycler480®, ROCHE* previously applied with *HAIN®, Lifesciences.* To validate the new system, a comparative analysis was performed between the two platforms, for the identification of variations c.845G>A and c.187C>G in Hereditary Hemochromatosis and c.1691G>A and c.20210G>A in Venous Thrombosis, from genomic DNA samples of 18 and 93 patients respectively.

As a result, for Hereditary Hemochromatosis 7 patients presented a p.H63D wild type genotype by HAIN® and 6 by LightCycler480®, 7 heterozygotes by HAIN® and 6 by LightCycler480® and 4 homozygotes by both. For the p.C282Y variant, 16 patients were wild type, 2 heterozygotes and none homozygotes on both platforms.

In Venous Thrombosis, 86 patients had a c.1691G>A (Leiden Factor V) wild genotype by HAIN® and 84 by LightCycler480® and 7 heterozygous by both. While for c.20210G>A (Factor II mutation), 87 were wild type and 6 heterozygous for both platforms. Under no circumstances, homozygous were observed.

Therefore, the results were homogeneous in both systems, so that enabling the setting up of LightCycler480®, the only discrepancies found were due to the detection of new variants using the LightCycler480®.

In conclusion, the development of the High Resolution Melting technique in the LightCycler480® allowed the optimization of genotyping studies in haematological diagnosis.

ABSTRACT (Spanish)

Actualmente, la genética médica es fundamental en el estudio de la variabilidad y herencia humana. Los estudios de genotipado han permitido la determinación de mutaciones asociadas a distintas patologías. Aunque se emplean nuevas metodologías que posibilitan la detección de dichas variaciones, todavía existen dificultades, especialmente en la determinación de polimorfismos de un solo nucleótido, más frecuentes y cuya implicación clínica no está tan definida.

La hipótesis de esta investigación persigue por tanto la optimización de los estudios de genotipado, dirigidos al diagnóstico hematológico. Para ello el objetivo principal fue la puesta a punto de la técnica High Resolution Melting (HRM), con el *LightCycler480®, ROCHE*, anteriormente aplicada con el *HAIN®,Lifesciences*. Para validar el nuevo sistema, se realizó un análisis comparativo entre ambas plataformas, para la identificación de las variaciones c.845G>A y c.187C>G en la Hemocromatosis Hereditaria (HH) y c.1691G>A y c.20210G>A en la Trombosis Venosa (TV), a partir de muestras de ADN genómico de 18 y 93 pacientes respectivamente.

Como resultado, para la HH, 7 pacientes presentaron un genotipo p.H63D *wild type* por *HAIN®* y 6 por *LightCycler480®*, 7 heterocigotos por *HAIN®* y 6 por *LighCycler480®* y 4 homocigotos por ambas. Para la variante p.C282Y, 16 pacientes fueron *wild type,* y *2* heterocigotos*,* sin detectar homocigotos por ambas plataformas.

En la TV, 86 pacientes presentaron un genotipo c.1691G>A, *wild type* por *HAIN®* y 84 por *LightCycler480®* y 7 heterocigotos por ambas. Mientras que para c.20210G>A, 87 eran *wild type,* y 6 heterocigotos, por ambas plataformas. En ningún caso, se observaron homocigotos.

Los resultados fueron homogéneos en ambos sistemas, las únicas discrepancias encontradas fueron debidas a la detección de nuevas variantes mediante el *LightCycler480®*.

En conclusión, la puesta a punto de la técnica en el *LightCycler480®* permitió la optimización de los estudios de genotipado en el diagnóstico hematológico.

ABSTRACT (Catalan)

Actualment, la genètica mèdica és fonamental en l'estudi de la variabilitat i herència humana. Els estudis de genotipat han permès la determinació de mutacions associades a diferents patologies. Tot i que s'empren noves metodologies per la detecció d'aquestes variacions, encara existeixen dificultats, especialment en la determinació de polimorfismes d'un sol nucleòtid, més freqüents i la implicació clínica dels quals no està tan definida.

La hipòtesi d'aquesta investigació persegueix per tant l'optimització dels estudis de genotipat, dirigits al diagnòstic hematològic. Per tal d'assolir-la, l'objectiu principal va ser la posada a punt de la tècnica *High Resolution Melting*, amb el *LightCycler480®, ROCHE*, anteriorment aplicada amb el *HAIN®, Lifesciences.* Per validar el nou sistema, es va realitzar una anàlisi comparativa entre totes dues plataformes, per a la identificació de les variacions c.845G>A i c.187C>G en la Hemocromatosis Hereditària i c.1691G>A i c.20210G>A en la Trombosi Venosa, a partir de mostres d'ADN genòmic de 18 i 93 pacients respectivament.

Com a resultat, per a la Hemocromatosis Hereditària, 7 pacients van presentar un genotip p.H63D *wild type* per *HAIN®* i 6 per *LightCycler480®*, 7 heterozigots per *HAIN®* i 6 per *LightCycler480®* i 4 homozigots per ambdues. Per a la variant p.C282Y, 16 pacients van ser *wild type*, i 2 heterozigots, sense detectar homozigots per totes dues plataformes.

En la Trombosi Venosa, 86 pacients van presentar un genotip c.1691G>A (Mutació Factor V) *wild type* per *HAIN®* i 84 per *LightCycler480®* i 7 heterozigots per ambdues. Mentre que per a c.20210G>A (Mutació Factor II), 87 eren *wild type* i 6 heterozigots, per totes dues plataformes. En cap cas, es van observar homozigots.

Els resultats van ser homogenis en tots dos sistemes, les úniques discrepàncies trobades van ser degudes a la detecció de noves variants mitjançant el LightCycler480®.

En conclusió, la posada a punt de la tècnica High Resolution Melting en el *LightCycler480®* va permetre l'optimització dels estudis de genotipat en el diagnòstic hematològic.

LIST OF ABREVIATIONS

- **A:** Adenine.
- **aa:** amino acids.
- **Arg:** Arginine.
- **APC:** Activated Protein C.
- **bp:** Base Pair.
- **C:** Cysteine.
- **C:** Cytosine.
- **cM**: Centimorgan.
- **Cp:** Crossing point.
- **D:** Acid aspartic.
- **Da:** Dalton.
- **dbSNP:** SNP database.
- **dNTPs:** Deoxyribonucleotides triphosphate.
- **ddNTPs:** Dideoxyribonucleotides triphosphate.
- **DNA:** Deoxyribonucleic acid.
- **dsDNA:** Double strand DNA.
- **dL:** Decilitre.
- **FII:** Prothrombin Factor II.
- **FIIa:** Thrombin.
- **FVa:** Activated Factor V.
- **FV:** Factor V.
- **G:** Guanine.
- **Gln**: Glutamine.
- **H:** Histidine.
- **HH:** Hereditary Hemochromatosis.
- **HRM:** High Resolution Melting.
- **K:** Lysine.
- **Kb:** Kilobase.
- **LC480®:** Light Cycler 480®.
- **LIS:** Laboratory Information System.
- **mL:** Millilitre.
- **nn:** Nanometres.
- **PE:** Pulmonary Embolism.
- **PB:** Peripheral Blood.
- **PCR:** Polymerase Chain Reaction.
- **PS:** Protein S.
- **R:** Arginine.
- **RNA:** Ribonucleic acid.
- **rs:** Reference SNP.
- **S.** Serine.
- **ssDNA:** Single strand DNA.
- **SNPs:** Single Nucleotide Polymorphisms.
- **T:** Thymine.
- **Tf:** Transferrina.
- **Tfr:** Receptor de la transferrin.
- **Tm:** Melting Temperature.
- **TS:** Iron-bound transferrin saturation.
- **VTD:** Venous Thromboembolic Disease.
- **VT:** Venous Thrombosis.
- **WHO:** World Health Organisation.
- **Y:** Tyrosine.
- **µL:** Microliter.
- **µM:** Micromoles.
- **µg:** microgram

KEY WORDS

High Resolution Melting, Hereditary Hemochromatosis, Venous Thromboembolic Disease, p.H63D. Mutation, p.C282Y. Mutation, Leiden FV, Prothrombin FII, HAIN®, LC480®, Homoduplex, Heteroduplex, setting up and SNP.

1 Introduction.

Currently, medical genetics plays a crucial role in the study of variability and human heredity, while it has allowed the development of methodologies that provide new approaches to many pathologies, including those linked to the field of haematology.[8]

These new points of view have made possible to afford, both at individual level and at the level of society, the necessary tools for the knowledge and prevention of diseases.[8]

In reference to genetic factors, specifically congenital factors, that are involved in different pathologies, the genetic variations play an essential role in the appearance and development of the same. Such variations involve both, mutations which tend to occur more scarcely, and polymorphisms. Single Nucleotide Polymorphisms (SNPs) are variations in the Deoxyribonucleic acid (DNA) that occur when only one nucleotide is altered, unlike mutations, SNPs are more frequent and their clinical consequences are not so clear, therefore its research is necessary in order to define its clinical implication in different pathologies, which may be key both for early diagnosis and for the application of new treatments or interventions that prevent the outbreak of the disease or minimize its severity.[9]

In addition, it should keep in mind that, although most genetic diseases appear during childhood, it does not imply that they may not appear in later stages, for example as a result of the accumulation of toxic metabolites. Therefore early diagnosis is of utmost importance. [9]

Nowadays, there are different types of genetic tests depending on the anomaly to be determined, they are classified into: cytogenetic, biochemical and molecular.[9]

Cytogenetic tests involve the evaluation of all chromosomes in search of possible irregularities, through the previous culture of the cells under study. Biochemical tests are based on techniques that analyse proteins in order to detect possible alterations. Regarding, molecular testing focuses on DNA analysis, making it a more effective method, specially if protein function is unknown.[9]

The problem lies in the fact that genetic diseases can be associated with a large number of different mutations, so it is great challenge to examine the entire gene sequence to identify the causative mutation. Thus, it implies an important limitation for carrying out genetic studies, so research in this area should be increased given its relevance in preventing the development of serious pathologies. [9]

In connection with that, the hypothesis of this work is based on the optimization of genotyping studies particularly aimed at haematological diagnosis. To achieve this hypothesis, the main objective of this research is the setting up of High Resolution Melting (HRM) technique in the *LightCycler480®; ROCHE (LC480®)* system, for the

determination of mutations associated with hereditary hemochromatosis (HH) and venous thromboembolic diseases (VTD).

In order to evaluate the implementation of this new system in haematological diagnosis, this study was carried out in the molecular biology laboratory of the haematology department of the University Clinical Hospital of the Valencian Community in partnership with the health research institute INCLIVA, specifically through collaboration with the Haematopoietic Transplantation Research Group of that institute.

This study was based on the analysis by HRM technique, of nucleic acid sequences of 18 patients for HH and 93 patients for VTD, with the purpose of identifying specific SNPs, associated with the previously mentioned pathologies, detected so far using the HAIN® system.

1.1 Hereditary Hemochromatosis.

1.1.1 Concept.

Hereditary Hemochromatosis (HH) is an autosomal recessive congenital disorder of iron metabolism, characterized by greater gastrointestinal absorption of iron and consequently its accumulation in different organs, causing its damage and dysfunction, may evolve into serious pathologies.[10,11,12]

Although its geographical distribution is worldwide, it is most frequently observed in populations of northern European origin, particularly of Nordic or Celtic descent. [1]

Therefore, it represents one of the most common hereditary diseases among Caucasians, affecting approximately 1 out of every 400 individuals, which leads to a frequency in carriers of between 1 in every 10 people.[10,7] It is more frequent in men than women, at a rate of 2.2 per 1 respectively, this fact may be due to the physiological blood loss that involves menstruation.[10,7]

In patients with advanced disease, iron deposition may increase from 1g in normal individuals to 20-40g in individuals with HH. Such accumulation in the cells of parenchyma of different tissues, can produce different effects: in the liver can cause fibrosis or cirrhosis as well as eventual hepatocellular carcinoma; in the pancreas diabetes mellitus; arthropathy in the joints; cardiomyopathy in the heart; hypogonadism may also occur and in later stages cutaneous hyperpigmentation.[13]

In the early stages of the disease, HH is asymptomatic, so severe effects usually do not appear until decades after of progressive accumulation of iron.[10,12]

Therefore, early diagnosis and treatment can prevent the disease from leading to the various irreversible lesions mentioned above.

As for its diagnosis, it is based on the identification of an increase in iron reserves 150 μg/dL). [14] Thus, the initial approach is through indirect markers of iron resrves[1], such as an increase in saturation of iron-bound transferrin (TS), an early reliable indicator of the risk of iron overload. Obtaining two measures higher than 60% in men and 55% in women, imply a predisposition to HH. It also allows the early detection of the disease as it is positive at a fairly early stage. [15,16]

On the other hand, serum ferritin concentration increases progressively over time in individuals with untreated HH[16], in turn its levels reflect an increase in hepatic iron, where levels >400μg/L are HH suspected.[14] Although it does not allow for early detection, its analysis is important, considering that it allows to determine the status of the patient and the convenience of performing a liver biopsy, a more invasive method that enables to diagnose with certainty the disease, based on the concentration of hepatic iron that must be greater than 1.9 to confirm the diagnosis.[15]

It can also be diagnosed by molecular genotyping studies of different point mutations associated with an increased risk of HH development, such as mutation in the HFE gene, p.C282Y and p.H63D. These techniques allow both a definitive diagnosis and a family study.[15]

The treatment is based on the induction of periodic phlebotomies of about 400-500mL, which remove 200-250mg of iron, thus allowing to reduce the concentration of iron in the blood and consequently, avoiding the accumulation of iron and the future complications. These treatment should be followed for life.[15]

1.1.2 Types.

As progress has been made in the study of this disease, it has been discovered which genes are affected by it, so that they can be distinguished, mainly: HFE Hemochromatosis, Juvenile Hemochromatosis (HJV), Hepcidin (HAMP), Hemochromatosis associated with TfR2 and ferroportin disease, shown in **Table.1.** In particular, this study was based on the analysis of hemochromatosis associated with the HFE gene.[17]

Clasificación	Gen	Posición cromosómica	Algunas Mutaciones	Patrón de herencia	OMIM
Tipo 1 o clásica	*HFE*	6p21.3	p.Cys282Tyr p.His63Asp p.Ser65Cys	AR	235200
Tipo 2A o juvenil	*HJV*	1q21.1	p.Gly320Val p.Arg326Ter p.Ile222Asn	AR	602390
Tipo 2B	*HAMP*	19q13.12	c.95delG p.Arg56Ter c.148_150+1delATGG	AR	613313
Tipo 3	*TFR2*	7q22.1	p.Tyr250Ter c.88dupC p.Met172Lys p.Arg455Gln	AR	604250
Tipo 4	*SLC40A1*	2q32.2	p.Asn144His p.Ala77Asp c.485_487delTTG p.Val162del p.Asp157Gly	AD	606069
Sobrecarga de Hierro	*FTH1*	11q12.3	p.A49T	AD	134770
Aceruloplasminemia	*CP*	3q24-q25	c.395-1G>A c.2511dupT p.Trp877Ter c.2389delG	AR	604290
Atransferrinemia	*TF*	3q22.1	p.Cys156Tyr p.Gly296Asp p.Gly521Cys	AR	209300

Table.1. *Different classifications for Hereditary Hemochromatosis. Online Mendelian Inheritance in Man (OMIM); AR: Autosomal Recessive; AD: Autosomal Dominant* [7]

1.1.3 HFE gene's characteristics.

The first connection between mutations in a gene and hereditary hemochromatosis was discovered in 1996, thanks to investigations conducted by Feder et al, where the HFE gene was studied. [18]

The HFE gene is located on the short arm of chromosome 6 (6p), near the HLA locus, specifically positioned at 1-2 centiMorgans (cM) of the HLA-A gene, so it was originally called HLA-H.[10,18,19]

This gene encodes a membrane protein that has a structure similar to class I MHC molecules.[18] It has a low expression in most tissues, except in the liver and small intestines, organs that are involved in iron metabolism.[19]

The correct expression of the HFE protein requires interaction with the β2-microglobulin protein, as shown in **Figure.1.** This complex interacts with the Transferrin receptor (TfR), so that it decreases the binding affinity of the Transferrin (Tf), in charge of transporting the iron, with its receptor. Consequently, the HFE protein is involved in the regulation of iron, reducing the intracellular absorption and accumulation of iron in presence of HFE.[19]

Feder et al, discovered two missense point mutations, which were found repeatedly in patients with HH.[18]

The first consists of the substitution of a guanine (G) by an adenine (A) at position 845 of the HFE gene, specifically located in exon 4.[20] It causes a change from a cysteine (C) to a tyrosine (Y) in the amino acid 282 of the protein (C282Y mutation). [18]

The second mutation is caused by a replacement of a cytosine (C) by a guanine (G) at the 187 position of the gen, located in exon 2[20], and consequently a change of a histidine (H) by an aspartic acid (D) at the position 63 of the protein (H63D mutation).[18]

Later in 1999, a new missense mutation was found at the position 193 of the HFE gene, where an adenine (A) is replaced by a thymine (T), leading to the change of a serine (S) to cysteine (C) at position 65 of the protein (S65C Mutation). This mutation is located in exon 2[20], very close to the H63D mutation, and appears to be associated with mild forms of HH.[18]

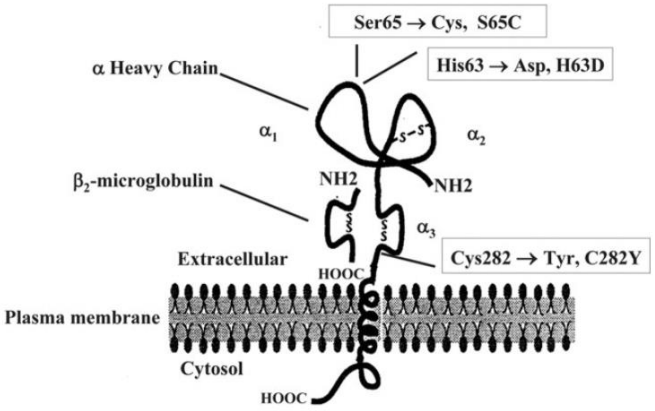


Figure.1. *Representation of the protein product of HFE gene. Most of the protein is extracellular, with three alpha loops and a short cytoplasmatic tail. Mutations are identified .*[1]

1.1.4 Variants, incidence and pathogenicity.

In numerous studies, the association of HH with the described mutations has been proven, so that its incidence and pathogenicity can be determined:

- Homozygous for the C282Y mutation account for 80-90% of patients in northern Europe with HH, therefore, it has been confirmed that this mutation is the most prevalent in HH.[11,18,21]
- Composite heterozygotes (C282Y/H63D) are also associated with patients with high iron loads, however, they represent only between 0.5-2% of people who may develop clinical signs of iron overload. So it can be considered as a risk factor to develop iron overload if not treated.[11,7]

- Similarly, compound heterozygotes (C282Y/S65C) may confer a slight increase in disease risk, thus contributing to a mild disease phenotype, however their penetrance is very low, occurring in 1.5% of the European population.[18]
- In people with a single copy of either of the two mutations (p.C282Y heterozygous or p.H63D heterozygous), they have been shown to have no higher risk of hemochromatosis than the normal population.[18]
- In homozygotes both for (H63D/H63D) and for (S65C/S65C), although biochemical abnormalities may occur, clinical manifestations rarely take place, being their penetrance even lower than for the genotype (C282Y/H63D).[7,18] Moreover, for the H63D/H63D mutation it is not clear that it alone can cause iron overload, being present in 15-40% of healthy European individuals.[7,21]
- As for heterozygotes (H63D/S65C), the risk of iron overload is not significantly increased compared to heterozygosity S65C alone which can lead to mild to moderate hepatic iron overload but in none of the patients carrying this mutation, clinically manifest hemochromatosis was found nor extensive hepatic fibrosis associated with iron.[19]

  S65C carriers have a risk of developing an iron overload, but not to the same extent as C282Y homozygotes or C282Y / H63D.19 heterozygotes.[19]

Regarding the relationship between the mutations and the pathology, the C282Y mutation interrupts the non-covalent interaction with β2-microglobulin, so that the mutated HFE protein is not expressed on the membrane surface, thus allowing iron-bound Tf to interact with Tfr and consequently increasing iron absorption. In contrast, the mutation in H63D does not modify the expression of the protein, but its affinity for TfR is diminished compared to the HFE- wild type, therefore, the Tf will be able to interact more with its receptor.[19]

It should be noted that due to the proximity in the HFE gene between the C282Y and H63D mutations, the two mutations have never been found simultaneously on a single chromosome 6. That is, patients who are homozygous for the C282Y mutation are always negative for the H63D mutation and vice versa.[18]

Therefore, the HFE gene is currently considered to be the gene responsible for HH, due to the high percentage of patients with HH who are homozygous for the C282Y mutation.[18]

The remaining forms of HH that are not produced by mutations in the HFE gene, may be related to mutations in genes encoding for protein involved in the regulation of iron such as hepcidin, hemojuvelin, transferrin receptor 2 and ferroportin, among others, known as non-HFE genes.[1]

1.2 Thrombotic diseases.

1.2.1 Concept.

Venous thromboembolic disease (VTD) refers to serious and life-threatening pathological processes characterized by the formation of a thrombus caused by platelet and fibrin clotting within the venous system, being more frequent in the veins of the lower extremities, so that the blood flow is completely or partially obstructed.[22,23]

There are different manifestations of this pathology, mainly Venous Thrombosis (VT) and Pulmonary Embolism (PE).[22]

On the one hand, VT is characterized by the formation of a thrombus within a vein accompanied by an inflammatory response. While PE, involves the generation of a thrombus inside a vein and its subsequent fragmentation and displacement in the direction of blood flow reaching and obstructing the pulmonary artery.[22]

VTD represents a major health problem specially in western societies, with an incidence of approximately 1 of every 1000 individuals and increasing, being more frequent in elderly people.[24]

The death toll is around 50,000 people a year in the United States and represents the fifth cause of hospital death in Europe. VTD mortality is 2.5% in patients diagnosed quickly and appropriately, so that the importance of early diagnosis and treatment is evident, in order to reduce the number of deaths related to this pathology.[23]

Regarding the diagnosis, most thrombus do not show clinical manifestations, so that their presence can only be demonstrated in half of the patients in whom there is a suspicion. Both invasive and non-invasive diagnostic techniques are used, including computerized axial tomography, nuclear magnetic resonance imaging, which is useful in differentiating an acute from a chronic obstruction, therefore it is an effective technique for the detection of recurrences and biological methods (D-dimers).[22]

Both VT and PE are based on anticoagulant treatment, comprising acute treatment with heparin parenterally for about 5-10 days, followed by secondary prophylaxis with oral anticoagulants or an alternative method, for at least six months.[22]

Blood clotting is a complex, multifactorial and dynamic process, regulated by the equilibrium between procoagulant substances, which stimulate blood clotting and anticoagulant substances, which inhibit it. [2]

Specifically, the plasma coagulation process is based on the transformation of fibrinogen (soluble) to fibrin (insoluble), by means of the proteolytic enzyme, thrombin formed thanks to the activation of prothrombin.[2] It consists of three key points:

- Formation of the prothrombin activator complex "prothrombinase" in response to injury.
- Activated prothrombin catalyses the conversion of prothrombin to thrombin.
- Thrombin transforms fibrinogen into fibrin, which allows clot formation.[2]

Therefore, the state of hypercoagulability is defined as the predominance of procoagulant factors over anticoagulants, leading to a pathological state such as the development of venous thrombosis in which this study is focused on.[2]

The pathogenesis of this disease involves both genetic factors as a consequence of genome variations that alter the sequences of the factors involved. As well as circumstantial or acquired factors, such as: smoking, male sex, advanced age, malignant neoplasia, pregnancy, obesity, diabetes, surgery, immobilization, erythrocytosis, dyslipidemia, use of oral contraceptives and inflammatory conditions.[22,24]

In particular, this study is aimed at VT and its linkage with two genes: Factor V (FV) and prothrombin Factor II (FII).

1.2.2 Leiden Factor V mutation.

1.2.2.1 Factor V gene.

Factor V is encoded by a long gene, more than 80kb (Kilobases), located in the q21.q25 region of the chromosome 1. It consists of 25 exons of different sizes, between 72-2820bp (base pair) and 24 introns plus the 5' and 3' ends not translated (**Figure.2.**)[2]

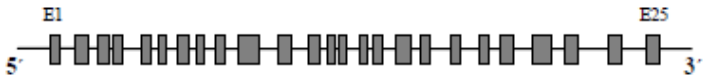


Figure.2. *Structure of Factor V gene.*[2]

1.2.2.2 Factor V function.

The FV gene codes for a plasma glycoprotein, involved in the coagulation cascade, which maintains the anticoagulant and procoagulant balance of the blood, thus acting in one way or another depending on the situation.[2]

The FV acts as a procofactor, so that, when is activated, either by Factor Xa and/or thrombin, it passes to FVa (Activated FVa).[2]

FVa, together with FXa, in the presence of calcium and in contact with membrane surfaces, form the prothrombinase complex, responsible for transforming prothrombin into thrombin, and consequently, allows the passage from fibrinogen to fibrin, that is, it allows coagulation to take place as shown in **Figure.3.**[2]

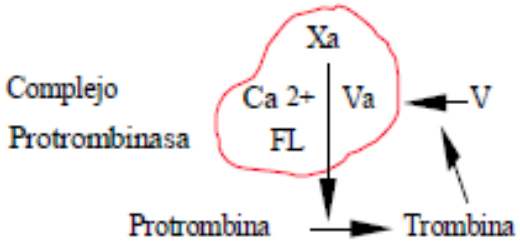


Figura.3. *Factors involved in blood clotting.*[2]

On the other hand, activated protein C (APC) degrades proteolytically the factors Va and VIIIa, thus allowing the control of procoagulant mechanisms.[2]

In situations where FV is not activated, it can act as an anticoagulant, enhancing the anticoagulant activity of Protein S (PS), so that these two proteins act as cofactors of PCA, to degrade VIIIa and inhibit coagulation.[2]

Recently, it has been described that FVa also acts as a cofactor for APC in the process of inactivation of FVIIIa, involved in the coagulation cascade.[2]

Therefore, FV has a fundamental role in the control of haemostasis of blood clotting. [2]

1.2.2.3 FVa structure.

As stated above, the FV is a procofactor, it is formed by a simple chain with a molecular weight of 330,000 Daltons (Da). When activated by FXa and/or thrombin, the FV is activated becoming FVa.[2]

The FVa is composed of a heavy chain, with a molecular weight of 105,000 Da, which keeps the terminal NH2 end, and covers residues 1-709 of protein (domains A1-A2). And for a light chain, with a molecular weight of 74,000 Da, which includes the residues of 1546-2196 (domains A3-C2-C2), as detailed in **Figure.4.** These two chains are not covalently bound.[2]

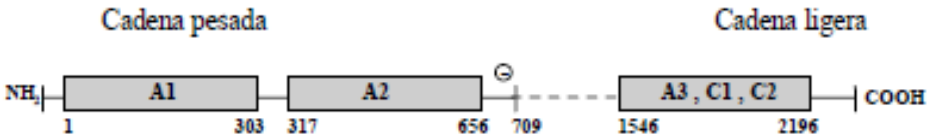


Figure.4. *FVa Structure.*[2]

APC has a proteolytic function that allows the inactivation of the FVa at the level of its heavy chain. So, it breaks the links Arg506, Arg306 and Arg679, sequentially, obtaining three fragments at the end of the process. Thus, breakage in Arg506 is necessary for optimal exposure of the remaining Arg306 and Arg679 target amino acids. (**Figure.5**)[2]

1.2.2.4 Factor V of Leiden.

Leiden FV mutation is based on the substitution of a Guanine(G) by an Adenine(A) at position 1691, located at exon 10 of the coding gene.[2]

Such mutation causes a change of the normal triplet CGA, encoding for an arginine (Arg), by the new triplet CAA that codifies for a glutamine (Gln) in the amino acid 506.[2]

This change causes that APC does not recognize this new cut-off point in amino acid 506, so it continues looking for the other two remaining points. So that, it makes the rate of inactivation of FV by APC, slower, thus allowing the coagulation pathway to be activated for a longer time and thereby producing a higher level of thrombin. (**Figure.5**).[2]

That is, the Gln506 is not insensitive to APC but is 10 times less sensitive than Arg506 of FVa wild type. Therefore, although the proteolysis in Arg306 allows to inactivate the FVa, it is not efficient because it is 10 times slower without the previous break of Arg506. This process is known as resistance to APC.[2]

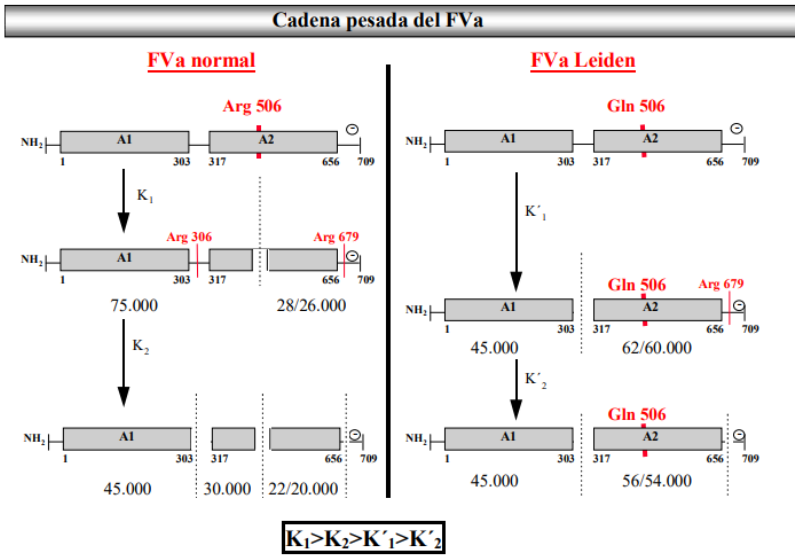


Figure.5. *Comparation of FVa Inactivation between Fva normal gene and FVa Leiden."K" refers to Inactivation speed.*[2]

1.2.2.5 Leiden's Factor V, incidence and pathogenicity.

Leiden FV prevalence varies according to ethnic group and geographic area studied. [2]

Leiden FV is present in 20-50% of patients who develop venous thrombosis. In normal individuals it presents a frequency of 5% among the Caucasians, and is found in less frequency in the populations of the United States, Asia, Africa and Latin America.[2,25]

In heterozygous carriers of Leiden FV, the risk of suffering, a thrombotic event increases 3-7 times, while in homozygous the risk is 80 times higher.[25] So this mutation appears to be the most prevalent genetic defect so far, in association with thrombotic diseases.[2]

However, it should be noted that if the mutation is not associated with other defects, either genetic such as APC, PS or antithrombin deficiencies, or circumstantial defects, thrombotic effects may not occur until old age or may never occur.[2]

1.2.3 Factor II muation.

1.2.3.1 Prothrombin gene.

The coding gene for prothrombin (Factor II), is located on chromosome 11, with 21 kb long and consists of 14 exons. (**Figure.6.**). Instead, the resulting mRNA has 2.1 kb, which includes an untranslated 5' region, a reading sequence, and an untranslated 3' region of 97 bp.[2]

Figure.6. *Structure of Prothrombin gene.*[2]

Prothrombin is a vitamin k-dependent protein, formed by a single polypeptide chain. Structurally, it can be divided into two parts, the amino-terminal fragment, called fragment(F1+2), which has 273 amino acids(aa) and a molecular weight of 35,000Da and the carboxyterminal fragment, of 306 aa and with a molecular weight of 38,000Da, said fragment is the precursor of thrombin.[2]

1.2.3.2 Factor II function.

Prothrombin has no coagulant activity in its zymogen form, so it must be transformed into thrombin (FIIa). The conversion of prothrombin to thrombin is mediated by the prothrombinase complex mentioned above.[2]

Thrombin functions may vary in the coagulant and anticoagulant systems:

- It acts as a coagulant, being the enzyme responsible of transforming fibrinogen into fibrin, as well as FV to FVa, FVIII to FVIIIa and FVXIII to FVXIIIa, involved in the blood clotting process.[2]
- It acts as an anticoagulant, considering that it participates in the activation of protein C, involved in the inhibition of FVa and VIIIa, which have coagulant action.[2]

1.2.3.3 Factor II mutation.

The point mutation of Factor II, consists in the change of a guanine (G) by an adenine (A) in the position 20210 of the gene, which belongs to the region 3' not translated from the same.[2]

It should be noted that the coagulating protein, prothrombin of an individual affected by such SNP is structurally and functionally identical to that of an unaffected individual.[2]

However, Poort et al, found that this polymorphism was increased in patients with VTD compared to the normal population. Significant differences were also found between plasma levels of prothrombin in individuals with wild type genotype (GG) and mutated individuals (GA or AA), who had higher levels of this protein.[2]

Although, it is not yet known with certainty, why this polymorphism increases prothrombin levels, it appears that G/A substitution in the untranslated region 3', implies a greater efficiency in the transcription process or a greater stability of the transcribed mRNA, thus favouring the increase in the synthesis of said protein.[2]

1.2.3.4 Factor II mutation, incidence and pathogenicity.

Similarly to Leiden FV mutation, the prevalence of FII mutation depends on ethnic group and geographic distribution studied.[2]

Its prevalence has been studied in different populations, being more common in the south (2.3%-6.5%) than the north (1.2%-2.6%) of Europe, while it is rarely found in Asia or in the African population.[25,2]

This polymorphism constitutes a thrombotic risk factor, it is found in 15% of individuals with VTD, family history of VTD or with an inherited alteration associated with thrombophilia.[2]

Specifically, heterozygous individuals have a risk between 2 to 4 times more of suffering a thrombotic event compared to unaffected individuals. In terms of homozygous individuals, they are associated with elevated levels of plasma prothrombin[25], however Poort et al, estimated that the expected prevalence was 0.014% ,so conducting studies in this population is very complicated.[2]

Table.2. shows all the variants under study and their main characteristics. In addition is included the variant p.S65C, the study of which will be explained later.

PATHOLOGY	DNA MUTATION	PROTEIN MUTATION	VARIANT	LOCATION
HEREDITARY HEMOCHROMATOSIS	c.845G>A	p.C282Y	rs1800562	Exon 4
	c.187C>G	p.H63D	rs1799945	Exon 2
	c.193A>T	p.S65C	rs1800730	Exon 2
VENOUS THROMBOSIS	c.1691G>A	p.R506Q	rs6025	Exon 10
	c.20210G>A	Polyadenylation region	rs1799963	Nontranslated 3' Region

Table.2. *Summary of the different variants studied.*

1.3 Relevance of High Resolution Melting (HRM) technique in haematological diagnosis.

1.3.1 HRM's concept.

The High Resolution Melting (HRM) technique is a simple, fast and low-cost method that allows the effectively identification of genetic variations in nucleic acid sequences such as SNPs, mutations, methylations, etc…[26]

It is based on the characterization of PCR (Polymerase Chain Reaction) products according to the dissociation mode of the strands. In this way, the variations in the sequences are detected both by a change in the melting temperature (Tm) and by a change in the shape of the melting curve with an exceptional specificity and sensitivity and with a high sample utilisation.[26,4]

1.3.2 Stages of the technique.

The HRM technique consists of four stages: Pre-incubation, Amplification (PCR), Melting curve and Cooling

First, a pre-incubation cycle is carried out where the temperature is raised to 94-96ºC, denaturation temperature of the DNA strands, in order that the unique strands in the PCR reaction are accessible for the enzyme. [27]

Then the target region is amplified by a PCR reaction, which is usually based on a series of 20-35 repeated temperature changes, called cycles. The temperatures used and the time applied in each cycle depend on a wide variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs (deoxyribonucleotides) in the reaction, and the binding temperature of primers, as well as the length of DNA to be amplified.[27]

Once the fragment of interest has been amplified, the melting stage takes place. It is at this phase where homoduplex (perfectly matched hybrids, that is, A/T and G/C) and heteroduplex (disparate hybrids, or A/G, A/C, T/C and T/G) are generated, obtained after the amplification of a heterozygous and/or homozygous case. [4]

Finally, during the last stage, Cooling, the temperature is reduced to about 40ºC.[27]

1.3.3 HRM using HAIN, Lifesciences®.

The system *HAIN®* carries out a fluorescence-based PCR using the *Fluorotype®, Lifescience Kit.* This is a specific and unique commercial kit for each genetic determination, composed of hybridization probes marked with fluorophores specifically designed to hybridize in a complementary way with the target sequence of the amplicon under study.[28]

Specifically, this system uses two probes, one is specific of the region with the variant and another that binds to the wild type sequence. After this binding, the fluorophores are excited, so that an increase in fluorescence can be detected.[28]

In the subsequent analysis of the melting curve the probes are separated from the amplicon at their specific temperature leading to an immediate decrease of the fluorescence.[28]

Subsequently, the data are analysed and processed with the *software FluoroCycler®,* so that a profile of the characteristic melting curve of each amplicon is obtained which allows to determine if the patient is homozygous, heterozygous or wild type for the mutation under study.[28] (**Figure.7.**).

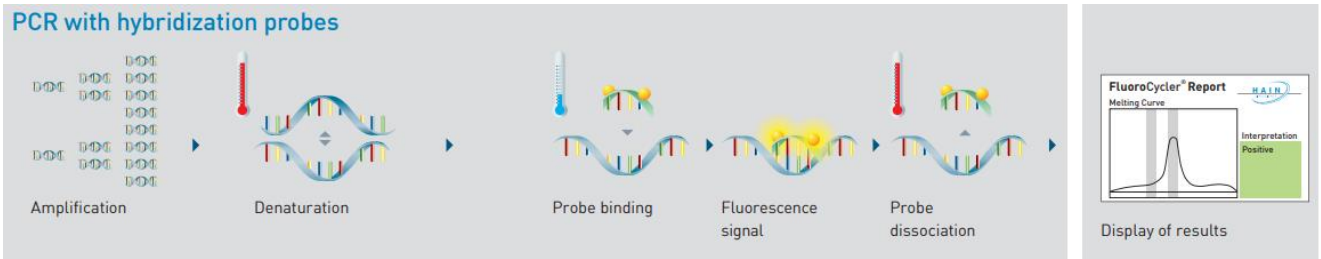


Figure.7. *HRM process with HAIN®, Lifesciences platform.* [3]

1.3.4 HRM using LightCycler 480, ROCHE®.

The *LC480®* platform is based on the same principle of amplification PCR, but in the presence of a fluorescent pigment, in this case the *LightCycler480® HRM Master, ROCHE* specific to double strand DNA (dsDNA). This pigment is inserted into the amplified fragments as the PCR advances, without interfering with the amplification reaction (**Figure.8.**).[4]

This pigment has a high fluorescence when bound to dsDNA, while this fluorescence decreases in single strand DNA (ssDNA).[29]

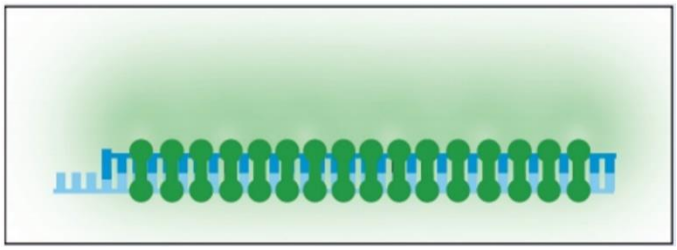


Figure.8. *Binding of the LightCycler480® HRM Master to dsDNA.*[4]

Then in the Melting stage, the amplified fragments are denatured from their original sister chains and re-hybridize randomly, so that Homoduplex is generated, if both chains hybridize perfectly, or Heteroduplex when there is a mismatched.[4] (**Figure.9.**).

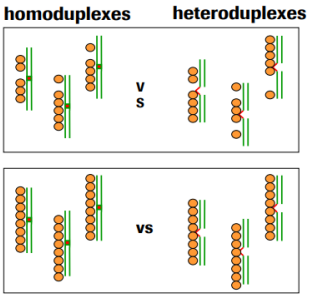


Figure.9. *Homoduplex and heteroduplex formation process.* [5]

Subsequently, the temperature is gradually raised again, so that the fragment is denatured and consequently the fluorescence decreases.

This change in fluorescence makes it possible to measure the degree of dissociation of the DNA sample. At low temperatures the pigment will be strongly bound to dsDNA, instead, as the temperature increases, this intercalant fluorophore, is released, causing the fluorescence variation.[29]

The rate of fluorescence release is generally higher near the Tm of the PCR product. This Tm is defined as the temperature at which half of the fragment is in the form of dsDNA and the other half in ssDNA. Each Tm is characteristic of the PCR product as it depends on the GC content (Tm is higher in GC-rich products) and the length of the fragment.[29]

Therefore, this fluorescence change is not only useful to determine the Tm of the amplicon, but also to make an absolute quantification of the product obtained during the PCR.[4]

Finally, the data are analysed with *LightCycler 480® SW 1.5.1* and through comparison with both, positive and negative controls, it is determined if the patient is homozygous, heterozygous or wild type for the mutation.

In short, due to a sequence mismatch, heteroduplex DNA melts at a different temperature than homoduplex DNA, therefore this temperature difference is detected enabling samples differentiation[4]

If it is a HOMOZYGOUS sample for the mutation, perfectly matched hybrids (homoduplex) will form but including the mutation in its sequence. Similarly, for a WILD TYPE sample, homoduplex will also be formed but in the absence of the mutation.[4] (**Figure.10**.).

In the case that the sample is HETEROZYGOUS, it will form, on the one hand perfectly matched hybrids (homoduplex), which do not contain the variant, and on the other hand not matched hybrids (uneven) (heteroduplex) with the variant.[4] (**Figure.10.**).

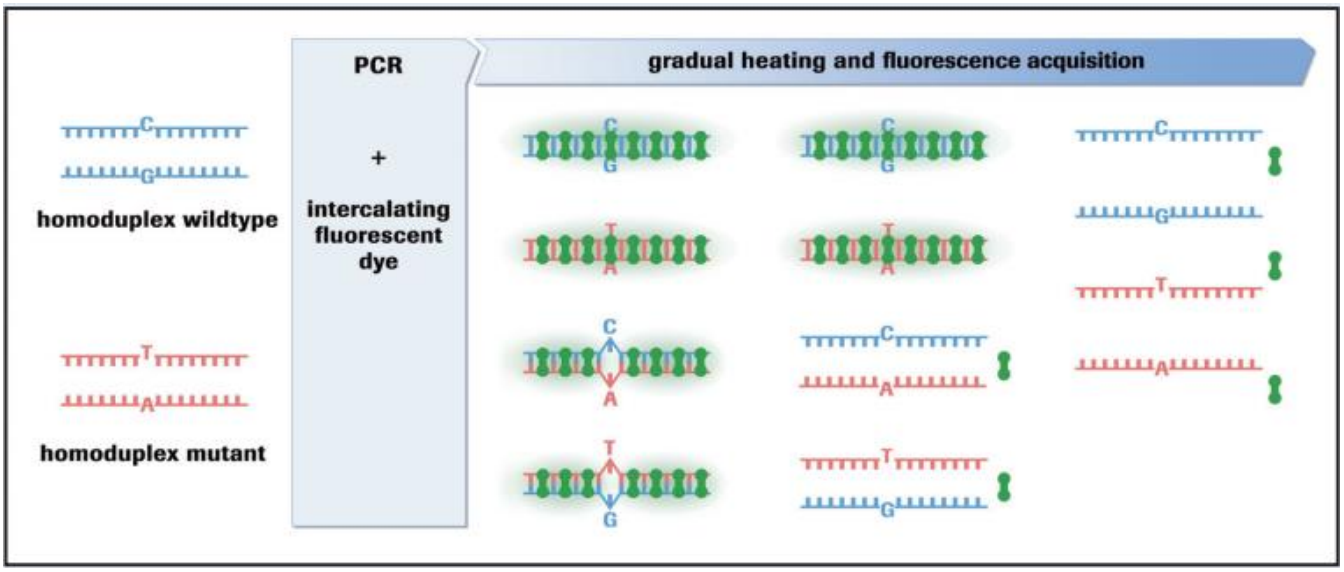


Figure.10. *HRM process with LC480®, ROCHE platform, for different types of samples.*[4]

Thus, the melting curves obtained with homozygous and heterozygous samples are significantly different. In fact, the HRM technique is so sensitive that, in most cases, it can even detect variations of a single base between homozygous samples.[4]

Unlike the *HAIN®,* this system requires the previous design of the forward and reverse primers for each of the variants under study. In addition, it is necessary to search for negative and positive controls, both homozygous and heterozygous, that allow comparison with the samples under study and consequently the determination of a result.

2 Objectives.

2.1 Main objective.

The main objective of this study is the setting up of the High Resolution Melting technique using *LightCycler480®, ROCHE* Real Time PCR platform, for genotyping study and consequently the detection of point mutations in the following pathologies: Hereditary Hemochromatosis and Thrombotic diseases.

2.2 Secondary objectives.

The secondary objectives to reach are:

- To identificate new variants in nucleic acid sequences associated with these pathologies.
- Compare by High Resolution Melting, which of the systems: *LightCycler480®, ROCHE* or *HAIN®, Lifesciences* is better for haematological diagnosis.

3 Materials and Methods.

As previously stated, the present work has been carried out in the molecular biology unit of the haematology service of the University Clinical Hospital of the Valencian Community in partnership with the health research institute INCLIVA.

The methodology of this study was divided into different stages: First, genetic material was extracted from peripheral blood samples from patients diagnosed or suspected of HH or VTD. Then it was quantified and its quality assessed. For the application of the HRM technique on the *LC480®,* it was necessary to carry out a prior search for positive and negative controls. The technique was then designed and optimized for each of the determinations of the two pathologies under study. This section also includes the protocol followed for the application of HRM in *HAIN®.* Finally, the Sanger sequencing process is explained for samples with discordant results by the HRM technique.

Each of the above sections is explained in more detail below:

3.1 Patient Selection.

Patients consecutively diagnosed of Hereditary Hemochromatosis (HH) or Venous Thromboembolic Disease (VTD) at the University Clinical Hospital of Valencia, from January 2020 to April 2021. The sample size is 18 for HH and 93 for VTD.

The retrospective and prospective patient selection will be addressed by the clinician´s group at the University ClinicalHospital of Valencia, according to the following common criteria:

1) Suspected or diagnosed patients with HH or VTD, based on the World Health Organization (WHO) 2016 criteria.

2) Provide written informed consent for the bio bank collection and use of samples for research purposes according to the Spanish regulatory authority.

3.2 DNA Extraction.

For the extraction of DNA from whole peripheral blood (PB) samples in 3-12mL EDTA tubes, was used the blood kit in automatic nucleic acid extractor, *Maxwell® DNA LEV Blood Kit, Promega.*

The protocol used was the following:

1) From 250-300µL of frozen or fresh blood.

2) Mix the samples at room temperature for 5 min.

3) Add 300µL of Proteinase K Solution that comes in the DNA kit (mix for pipetting). Protein K helps to break the membranes and nucleus, so together with the lysis buffer allow extract the DNA.

4) Add 300µL of Lysis Buffer that comes in the DNA kit.

5) Vortex for 10 seconds and incubate at 56ºC for 20 min.

6) Place the cartridges on the rack and uncover them.

7) Place the plunger in well 8 and the elution tube with 100 µL elution buffer of the kit (30-50 µL). (**Figure.11 and 12**).

8) Add the lysate to well 1 (closest to the cartridge label).

9) Select the DNA/blood protocol.

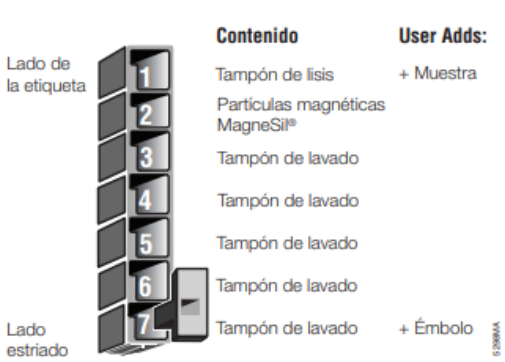


Figure.11. *Preparation of Maxwell®16 cartridges for DNA extraction.*[6]

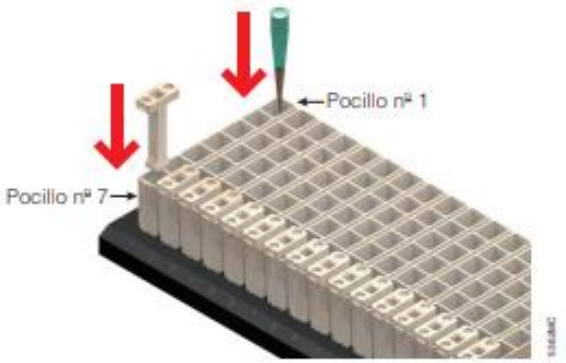


Figure.12. *Distribution of the different wells.*[6]

This stage is crucial to achieving a successful analysis, since a low quality DNA can produce non-specific PCR products that impair the subsequent analysis of HRM and consequently result in inconclusive or low resolution data.[29] In the Annex are explained other types of extraction performed in the biology unite of the haematology service.

3.3 Quantification and quality evaluation.

After the extraction of DNA, the quantification and evaluation of its quality by spectrophotometry was carried out with the *Nanodrop®, ThermoFisher scientific* system. To do this, absorbance was measured at two different wavelengths: 260nm (nanometres) and 280nm. With the absorbance at 260nm is obtained the value of DNA concentration, while the ratio between 260 nm and 280 nm represents the purity of it, which must be a value between 1.8-2.

This stage made it possible to verify that the genetic material used was of quality and therefore that it allowed the obtaining of optimal results. In addition, it is necessary to know its concentration since throughout the setting up, the concentration of the samples under study and the controls used were adjusted.

3.4 Control search.

The controls allow to group the samples under analysis with the behaviour of the melting curves of the control samples and thus be able to conclude a diagnosis.

In order to obtain both positive and negative controls, a retrospective sample search was carried out in the laboratory information system (SIL) *Gestlab,* so that a minimum of two/three homozygous, heterozygous and wild type controls were selected, for each determination.

These controls were previously characterized by the *FluoroCycler® 12, HAIN, Lifescience* platform.

3.5 Design and optimization.

Many factors can affect to the design and optimization of PCR by HRM, such as the quality of the extracted DNA, the length of the amplicon under study, the design of primers, the selection of pigments and reagents involved in the PCR process.[29]

3.5.1 Length amplicon.

While amplicons larger than 500 bp show a multiphase melting behaviour, and very small amplicons produce lower fluorescence signals due to less incorporation of the fluorescent pigment, the ideal amplicon design measure is 150-200bp.[4,29]

The variation of a single base affects more to the melting behaviour of a 100bp amplicon than a larger amplicon, therefore small amplicons are more likely to manifest small variations in the sequence.[4]

In this study, the measurement of amplicons designed for the determination of variants in both HH and VTD ranges from 175bp-288bp, in **Table.3.** is indicated in more detail.

PATHOLOGY	MUTATION	AMPLICON LENGTH
HEREDITARY HEMOCHROMATOSIS	p.C282Y	198 bp
	p.H63D	175bp
VENOUS THROMBOSIS	Leiden FV	288 bp
	Prothrombin FII	195 bp

Table.3. *Length of the amplicons analysed in the study.*

3.5.2 Primer's design for each determination.

The design and selection of the primers is a key stage for the execution and optimization of the PCR. Primers must be robust and region-specific in order to achieve optimal analysis.

This study was focused on the analysis of SNPs c.845G>A and c.187C>G for HH and c.1691G>A and c.20210G>A for VTD, therefore, it was necessary the previous design of primers that covered these positions in the genome.

For the design of the primers the *Primer3*[4] software was used. There are several strategies and points to take into account, to obtain optimal primers, among them are:

- Design of primers with an Annealing temperature above 60ºC.

- Percentage of G/C in the sequence of the primer.

- Avoid sequences likely to form dimers or non-specific products.

- Check by BLAST that the primer sequences are species- and gene-specific.

- Use of low primer concentrations, thus avoiding the formation of non-specific products. [4]

The procedure is the same, for the different determinations:

First, using the *ENSMBL* platform, we searched for the genes under study, specifically the HFE gene for the determination of p.C282Y and p.H63D, and the F5 and F2 genes for the determination of Leiden FV and FII mutation, respectively, belonging to the human species. Then, in the sequence section, the transcript that presents the NM was chosen. Then, in the exons section, the complete sequence subject of study was obtained.

Over each region, each of the coded variants was selected as (rs, reference SNP). This is a reference value associated with the variant specific SNP database (dbSNP). This

code provides information about the SNP subject of study, such as the MAF value, which indicates the frequency of the SNP in the normal population. As well as, its clinical significance. For further details in this area, the section *"see in dbSNP"* allows access to the *ClinVar* database, where the effects of these SNPs appear in different pathologies. Thus, it was possible to determine the clinical implication of each of the SNPs in the diseases under study, all of them being pathogenic in association with their pathologies. It should be noted that, these reference SNP are those indicated in the previous **Table.3.**

In the **Figures.34-38.** situated in the Annex are shown the sequences that cover each of the SNPs, as well as the selected variants (rs) and their clinical significance according to different interpretations in the ClinVar database.

Once the regions containing each of the variations were obtained, they were introduced on the *Primer.3* platform. Then, different parameters were adjusted to obtain quality primers, such as its size that should be about 20 nucleotides, its melting temperature of about 60ºC and the percentage of GC of 20%.

It was necessary to design a pair of primers for the determination of each of the variants, since although the variants of the HH are in the same gene, they are located in different exons, so the use of a single pair of primers would not be possible because the amplicon would be very large and the results would not be conclusive.

The **Table.4.** collects the characteristics of the designed primers, their sequence, their Tm, as well as the primer starting concentration and the amount of H_20 that must be added to achieve a concentration of 100 µM.

PRIMERS	SECUENCE	Melting Temperature	Amount of Oligo	Add for 100µL
H63D Forward	5'- TCT TCA TGG GTG CCT CAG AG-3'	56.6ºC	25.7 nmoles = 0.16 mg	227 µL
H63D Reverse	5'- AGA CTC TGA CTC AGC TGC AG-3'	56.4ºC	29.8 nmoles = 0.18 mg	298 µL
C282Y Forward	5'- TAC CCC CAG AAC ATC ACC AT-3'	55.7ºC	24.8 nmoles = 0.15 mg	248 µL
C282 Reverse	5'-GAT CAC AAT GAG GGG CTG AT-3'	54.7ºC	22.7 nmoles = 0.14 mg	227µL
FII Forward	5'- GAA CCA ATC CCG TGA AAG AA-3'	52.9ºC	107.3 nmoles = 0.66 mg	1.073 mL
FIIP Reverse	5'-CGA GTG CTC GGA CTA CCA G-3'	57.3ºC	96.4 nmoles = 0.56 mg	963 µL
FV Forward	5'- GGA ACA ACA CCA TGA TCA GAG CA-3'	57.4ºC	118.7nmoles = 0.84 mg	1.187mL
FV Reverse	5'- TAC CCA GGA GAC CTA ACA TGT TC-3'	55.8ºC	108.3 nmoles = 0.76 mg	1.083mL

Table.4. *Final design and characteristics of the primers used.*

The sequences to be amplified with the design of primers performed for each of the determinations subject to study are shown below. The nucleotides that vary in each analysis are also pointed out.

STUDY VARIANT **H63D NEW VARIANT** **FV NEW VARIANT** **PRIMERS**

Factor V (288bp)

Forward: 5'-GGAACAACACCATGATCAGAGCA-3'
Reverse: 5'-TACCCAGGAGACCTAACATGTTC-3'

GGAACAACACCATGATCAGAGCAGTTCAACCAGGGGAAACCTATACTTATAAGTGGAACATCT
TAGAGTTTGATGAACCCACAGAAAATGATGCCCAGTGCTTAACAAGACCATACTACAGTGACG
TGGACATCATGAGAGACATCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTAAGAGCAGAT
CCCTGGACAGGCGAGGAATACAGGTATTTTGTCCTTGAAGTAACCTTTCAGAAATTCTGAGAA
TTTCTTCTGGCTAGAACATGTTAGGTCTCCTGGCTA

Factor II (195bp)

Forward: 5'-GAACCAATCCCGTGAAAGAA-3'
Reverse: 5'-CGAGTGCTCGGACTACCAG-3'

GAACCAATCCCGTGAAAGAATTATTTTTGTGTTTCTAAAACTATGGTTCCCAATAAAAGTGACT
CTCAGCGAGCCTCAATGCTCCCAGTGCTATTCATGGGCAGCTCTCTGGGCTCAGGAAGAGCCA
GTAATACTACTGGATAAAGAAGACTTAAGAATCCACCACCTGGTGCACGCTGGTAGTCCGAGC
ACTCG

C282Y (198bp)

Forward: 5'-TAC CCC CAG AAC ATC ACC AT -3'
Reverse: 5'-GAT CAC AAT GAG GGG CTG AT-3'

TACCCCCAGAACATCACCATGAAGTGGCTGAAGGATAAGCAGCCAATGGATGCCAAGGAGTT
CGAACCTAAAGACGTATTGCCCAATGGGGATGGGACCTACCAGGGCTGGATAACCTTGGCTG
TACCCCCTGGGGAAGAGCAGAGATATACGTGCCAGGTGGAGCACCCAGGCCTGGATCAGCCC
CTCATTGTGATC

H63D (175bp)

Forward: 5'-TCT TCA TGG GTG CCT CAG AG-3'
Reverse: 5'-AGA CTC TGA CTC AGC TGC AG-3'

TCTTCATGGGTGCCTCAGAGCAGGACCTTGGTCTTTCCTTGTTTGAAGCTTTGGGCTACGTGGA
TGACCAGCTGTTCGTGTTCTATGATCATGAGAGTCGCCGTGTGGAGCCCCGAACTCCATGGGT
TTCCAGTAGAATTTCAAGCCAGATGTGGCTGCAGCTGAGTCAGAGTCT

3.5.3 Primer's reconstitution.

The designed primers were obtained through *Integrated Dna Technologies*. To optimize their conservation and avoid contamination are lyophilized, therefore, they had to be reconstituted. For this purpose, the amount of sterilized water indicated in **Table.4.** was added, to achieve a concentration of each of the primers at 100 µM.

This process was carried out in laminar flow cabinet, to avoid any kind of contamination. In addition, before opening the vial, it is necessary to vortex and give a small centrifugation, in order to recover the content.

3.5.3.1 Concentration adjustment.

Once reconstituted, the concentration of each of the primers was adjusted to avoid the formation of possible dimers as well as non-specific products that could significantly reduce the efficiency of the analysis.

As the experiments were carried out, the required concentration of primers was adjusted.

3.5.4 Standardize sample and the reaction mixture preparation.

The HRM technique compares amplicons of independent PCR reactions, therefore, it is of utmost importance to minimize variability between reactions.

A crucial phase in this process is the standardization of sample preparation, this can be achieved for example by performing the same extraction procedure for all samples or by using highly reproducible techniques.[4] For this purpose all samples were extracted with the *Maxwell® DNA LEV Blood Kit, Promega*, and quantified with Nanodrop®, *ThermoFisher scientific*. In addition, all samples, controls and samples were adjusted to the same concentration and the same amount was used to carry out the reaction.

Another determining factor is the optimization of the reaction mixture.

The *LightCycler480® High Resolution Melting Master Mix, ROCHE* is a reaction mixture designed to produce optimal HRM results.

In this kit are included all the necessary reagents for amplification, except the primers that as already indicated, were previously designed and obtained. Therefore, each kit includes:

1. Master Mix, 2x concentrated which includes the high-resolution fluorescent fusion pigment, specifically in this study the *LightCycler480® HRM Master, ROCHE* was used. In addition, it also includes the enzyme DNA polymerase and the dNTPs for the reaction. It should be noted that the fluorescence pigment is photosensitive, therefore it is important to protect it from light when it is not in use.

2. $MgCl_2$, Stock Solution, 25mM.

3. H_20, PCR.

In this study different tests were carried out, adjusting each of the elements of the PCR reaction, until the quantities and concentrations that allowed the obtaining of quality results were determined, thus ensuring that the test was robust and specific.

Table.5. shows the setting up of different components in both pathologies:

	HEREDITARY HEMOCHROMATOSIS and VENOUS THROMBOSIS					
	TRIAL 1		TRIAL 2		TRIAL 3	
	QUANTITY (x1)	**CONCENTRATION**	**QUANTITY (x1)**	**CONCENTRATION**	**QUANTITY (x1)**	**CONCENTRATION**
DNA	1 µL	50 ng/ µL	1 µL	50 ng/ µL	1 µL	25 ng/ µL
MasterMix	5 µL	OPTIMISED CONCENTRATION Kit	5 µL	OPTIMISED CONCENTRATION Kit	5 µL	OPTIMISED CONCENTRATION Kit
$MgCl_2$	1,20 µL		1,50 µL		1,50 µL	
H_20	2,20 µL		2,50 µL		1,50 µL	
Primer Forward	0,30 µL	[5µM]	0,50 µL	[5µM]	0,50 µL	[5µM]
Primer Reverse	0,30 µL	[5µM]	0,50 µL	[5µM]	0,50 µL	[5µM]

Table.5. *HH and VT setting up of the different components involved in the PCR reaction. The final values are those indicated in Trial 3.*

Therefore, the final protocol used to carry out the PCR with the *LC480®* platform for the determination of the different variants in HH and VT is as follows:

- Use in each round of analysis, a negative control for the detection of possible contamination, as well as negative (Wild type) and positive (Homozygous and Heterozygous) controls to confirm the results obtained.

- Dilutions at [25ng/µL] are prepared for both the controls and the samples to be analysed. Duplicate each of them.

- Preparation of the reaction mixture with the established quantities and concentrations of reagents once set up. (**Table.5.**). (Vortex and centrifuge the reagents before use).

- Addition of 9µL of the reaction mixture in each of the samples to be determined and 1µL of the DNA of the sample, being the final volume of 10 µL in each well. During the whole process is worked in cold by using coolers.

- Centrifuge the plate.

- Load into the *LightCycler480®, ROCHE* and select the corresponding protocol.

3.5.5 Optimize the PCR and Melting programs.

As mentioned above, the HRM technique consists of 4 phases, of which PCR amplification and melting stage are the key phases of the technique. Therefore, these programmes should be precisely designed and optimised to enable quality results to be achieved.

In the same way that the setting up of the components of the PCR was carried out, the setting up of the programs to perform the HRM technique was conducted in the two pathologies under study.

In **Table.6.** and **Table.7.** the different tests carried out are collected. Each trial consists of varying the temperatures of the different programs, until the technique is implemented for the detection of the variants in the HH and in the VT, respectively.

PROGRAM		HEREDITARY HEMOCHROMATOSIS								
		TRIAL 1			TRIAL 2			TRIAL 3		
		T (ºC)	Hold (hh:mm:ss)	Rampe rate (ºC/s)	T (ºC)	Hold (hh:mm:ss)	Rampe rate (ºC/s)	T (ºC)	Hold (hh:mm:ss)	Rampe rate (ºC/s)
Pre-incubation (x1 cycle)		95	00:10:00	4,4	95	00:10:00	4,4	95	00:10:00	4,4
PCR (x45 Cycles)	Denaturation	95	00:00:10	4,4	95	00:00:10	4,4	95	00:00:10	4,4
	Annealing	**56**	00:00:15	2,2	**56**	00:00:15	2,2	**56**	00:00:15	2,2
	Extend	72	00:00:20	4,4	72	00:00:20	4,4	72	00:00:20	4,4
Melting (x1 cycle)	Denaturation	95	00:01:00	4,4	95	00:01:00	4,4	95	00:01:00	4,4
	Cooling	40	00:01:00	2,2	40	00:01:00	2,2	40	00:01:00	1,5
	Melting	**60**	00:00:01	4,4	**75**	00:00:01	4,4	**75**	00:00:01	1
	Heating	95	continuous	0,02	90	continuous	0,02	90	continuous	0,03
Cooling		45	00:00:10	2,2	45	00:00:10	2,2	40	00:00:10	2,2

Table.6. *Setting up of the HRM programming for HH. The final values are those indicated in Trial 3. Annealing and Melting programs are key stages of the setting up.*

PROGRAM		VENOUS THROMBOSIS								
		TRIAL 1			TRIAL 2			TRIAL 3		
		T (ºC)	Hold (hh:mm:ss)	Rampe rate (ºC/s)	T (ºC)	Hold (hh:mm:ss)	Rampe rate (ºC/s)	T (ºC)	Hold (hh:mm:ss)	Rampe rate (ºC/s)
Pre-incubation (x1 cycle)		95	00:10:00	4,4	95	00:10:00	4,4	95	00:10:00	4,4
PCR (x45 Cycles)	Denaturation	95	00:00:15	4,4	95	00:00:15	4,4	95	00:00:15	4,4
	Annealing	**56**	00:00:15	2,2	**56**	00:00:15	2,2	**56**	00:00:15	2,2
	Extend	72	00:0015	4,4	72	00:00:15	4,4	72	00:00:15	4,4

Melting (x1 cycle)	Denaturation	95	00:01:00	4,4	95	00:01:00	4,4	95	00:01:00	4,4
	Cooling	40	00:01:00	2,2	40	00:01:00	2,2	40	00:01:00	1,5
	Melting	**65**	00:00:05	4,4	**65**	00:00:05	4,4	**75**	00:00:05	1
	Heating	95	continuous	0,02	95	continuous	0,02	95	continuous	0,03
Cooling		45	00:00:30	2,2	45	00:00:30	2,2	40	00:00:30	2,2

Table.7. *Setting up of the HRM programming for VT. The final values are those indicated in Trial 3. Annealing and Melting programs are key stages of the setting up.*

3.6 HAIN®.

For the determination of the variants using the HRM technique on the *HAIN®, Lifesciences* platform, it was used the *Fluorotype® kit, Lifesciences* which is specific and unique to each genetic determination. The protocol followed is detailed below:

Components	Quantities
DNA	3 µL
AM-A (red tube)	1,5 µL
AM-B (blue tube)	3,5 µL
Final Volume	8 µL

Table.8. *Components and quantities for the reaction mixture preparation with HAIN® system.*

3.7 Sanger sequencing.

The data analysis with *LightCycler® 480 SW 1.5.1* software allowed the collection of discordant samples that were not associated with the controls. Therefore, these samples were sequenced by Sanger in order to conclude a diagnosis. The Sanger Sequencing process consists of different stages:

• A conventional PCR of the fragment subject to study was first performed from DNA in order to amplify the target sequence. Then, by means of gel electrophoresis with the system *QIAxcel Advanced System, QIAGEN* it was verified that the desired product had been amplified, as well as possible contamination in the previous stage was visualized.

• Then the purification process took place, that is, the PCR product is required to be a clean product without excess dNTPs, primers dimers and non-specific hybridizations, in order to obtain quality nucleotide sequences. Specifically, an enzymatic enrichment was carried out using the *ExoSAP-IT™ PCR Product Cleanut Reagent Kit, TermoFisher Scientific.*

• Once purified, the sequencing PCR was performed with the *BrigthDye Terminator Cycle Sequencing Kit, MCLAB.* At this stage, the previous fragment was further amplified, but with each of the primers separately and using ddNTPs,

didesoxirribonucleotides (ddATP, ddTP, ddCTP, ddGTP), each marked with a different fluorescent pigment. In this way, the sequencing was carried out in both directions, in order to obtain two different readings of the region subject to analysis.

• Then, the sequencing PCR product was further purified by centrifugation using *Optima ™ DTR, Edge Bio* purification columns.

• Finally, the Sanger Sequencing was carried out by capillary electrophoresis, with the product purified by the *ABI PRISM 3130 Genetic Analyzer* sequencer and *ABI PRISM 3730 Genetic Analyzer, AppliedBioystems, USA.*

Once the nucleotide sequence of the discordant samples was obtained, it was visualized with the *Chromas* program in order to detect the possible mutation. In addition, the *MUSCLE* tool was also used to make an alignment of the problem sequence with the original sequence or wild type, to detect the discrepant nucleotide.

The detected variants were consulted in the *MutationTaster* program and in the *ClinVar* database, in order to know their clinical implication and their prognostic value in the haematological disease under study.

The methodology followed in this study is outlined in **Figure.13.**

WORKFLOW

Patient selection

DNA Extraction

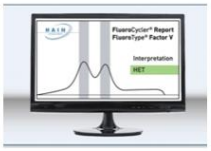
Quantification and DNA evaluation

Control search

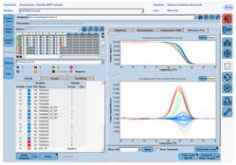
Design and optimization

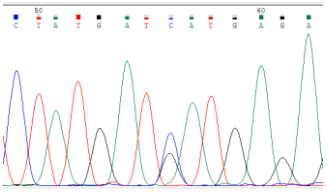
- **Length amplicon**
- **Primer's design from each determination**
- **Primer's reconstitution and concentration adjustment.**
- **Standardize sample and reaction mixture.**
- **Optimize PCR and Melting Programs**

HAIN®



LC480®





PCR assembly with kits	Incorporation into the system	Data Analysis	Sanger Sequencing for discordant results
- Kit FluoroType® *- Kit Light Cycler480® HRM Master*	*- HAIN®, Lifesciences* *- LightCycler480®, ROCHE*		

Figure.13. *Workflow diagram for the application of HRM technique, both with LC480® and with HAIN®.*

4 Results and Discussion.

For the analysis of the data obtained by the HRM technique, the *Software LightCycler480® SW 1.5.1*. was used. This instrument has a data acquisition rate, which allows to generate a melting curve for each of the samples under study.[4]

Based on these values, the modules used were:

• **Quantification absorbance**: the amplification curve must reach a stabilization level at a point >30 relative fluorescence units. In addition, the value of the Crossing point (Cp) must be less than 30 cycles and this value must not vary more than 5 units of Cp between samples.[4]

• **Tm Calling**: allows to identify what is the melting temperature of each amplicon, which allows to visualize in a more general way, the results that will be obtained, since depending on whether a sample is wild type, homozygous or heterozygous for mutation will be obtained some Tm or others.[4]

• **Gene Scanning**: is based on three stages.

- **Normalisation** of raw melting curve data by adjusting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples.[4]
- **Temperature shift,** displace the curves already normalized, along the temperature axis, in order to equalize the point at which the dsDNA of each of the samples becomes completely denatured.[4]
- **Difference plot**, for this we subtract the normalized and displaced curves from a reference curve, also known as "base curve". This action makes it possible to obtain a graph that clearly visualizes the differences in the shape of the melting curve of the samples, so that these samples can be automatically clustered into different groups, according to the similarity of their dissociation curves.[4]

The different stages of the Gene Scanning module can be seen in **Figure.14.**

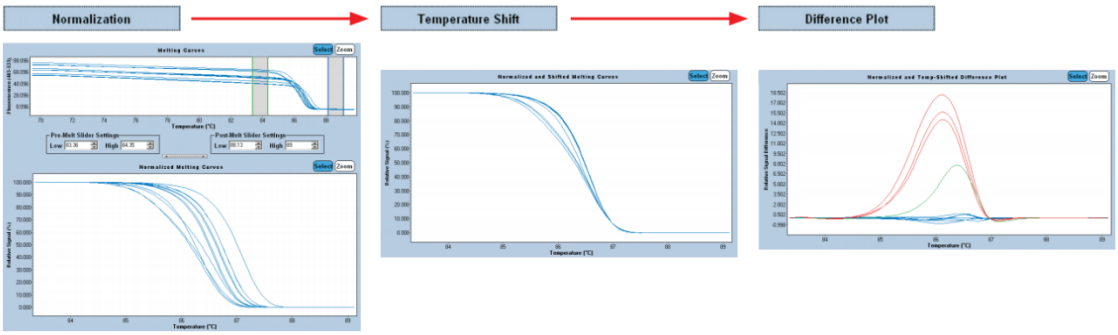


Figure.14.*HRM data analysis by using Gene Scanning Analysis Software Module.*[4]

4.1 Hereditary Hemochromatosis.

4.1.1 Tm calling, Quantification absorbance and Gene Scanning of H63D Mutation.

Figure.15.*HRM data analysis by using Quantification absorbance and Tm calling Module for p.H63D detection.*

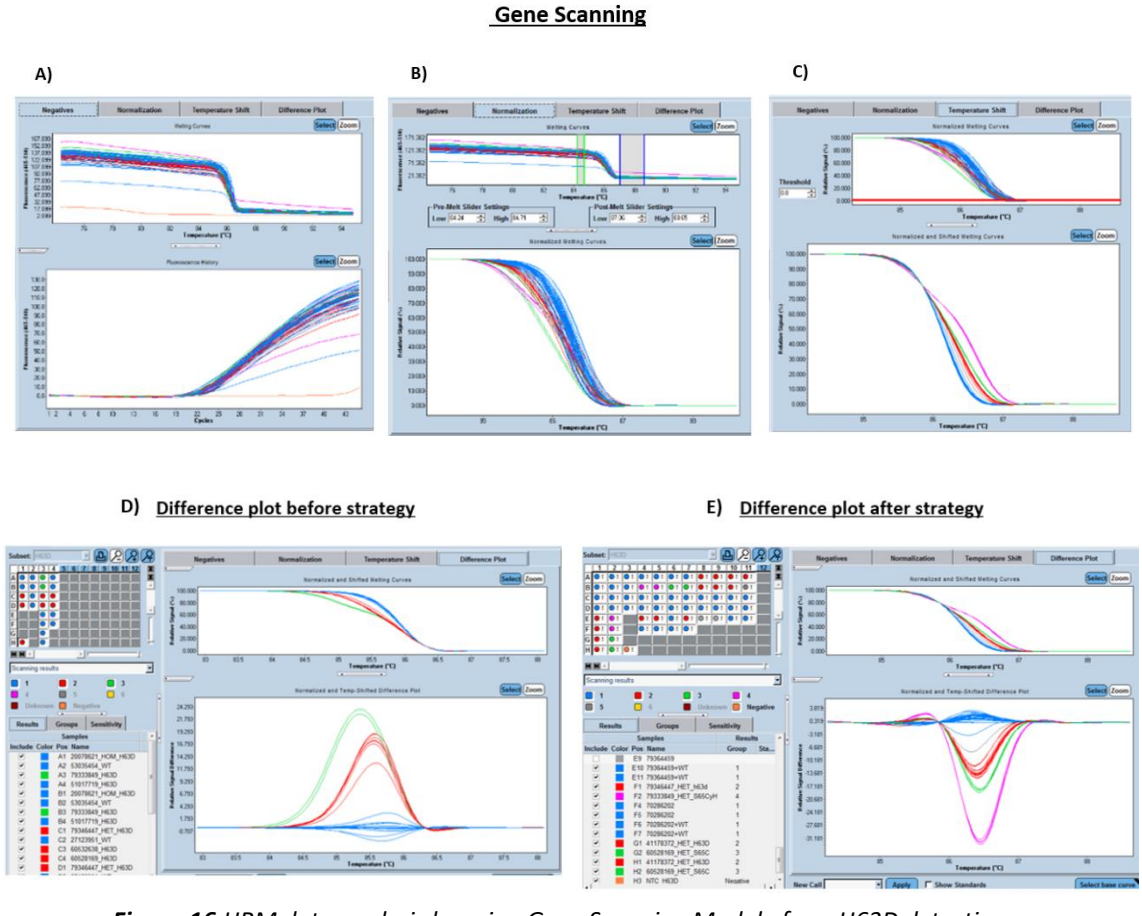


Figure.16.*HRM data analysis by using Gene Scanning Module for p.H63D detection.*

The analysis by Quantification absorbance (**Figure.15.A**) for p.H63D, determined that the amplification was effective since the values obtained met the criteria indicated above. Also, most of the samples presented a similar amplification curve, which indicates that the results were homogeneous and therefore that the technique is reproducible.

The Tm for the p.H63D variant is about 86ºC. (**Figure.15.B**). As can be seen, a wrong sample was detected, which had a different Tm than the rest, namely 80.71ºC and 81.15ºC for each of the replicas, this may be due to some possible contamination.

Using the Gene Scanning module (**Figure.16**), the parameters were adjusted, allowing the differentiation of the samples.

The fluorescence emitted decreases as the temperature increases, because the fluorescent pigment is specific to dsDNA so that when the laser has an impact on it, fluorescence is emitted but as the temperature increases, the amplicon is denatured and consequently the pigment is released and the fluorescence decreases. This leads to a fluorescence change which is most clearly perceived in the Tm zone of the fragment, as this is where half of the sequence is in the form of dsDNA with the pigment joined and the other half is in the form of ssDNA without the pigment.

Thus, by displacing the pre-melt and post-melt bars in the fragment's Tm zone (**Figure.16.B**), this value could be adjusted more precisely for each of the samples and consequently allowed their differentiation which was later analysed using the Difference plot. (**Figure.16.D** and **Figure.16.E**).

In particular, in the determination of the p.H63D variant, it was difficult to differentiate wild type samples from homozygotes for p.H63D. As shown in **Figure.16.D.** In the initial stages of the setting up, both homozygotes and wild types were all grouped in the same group, both of which are represented in blue. While heterozygous, represented in red, could differ from the rest.

In reference to the problem of differentiating homozygous and wild types in association with the p.H63D variant, the possible reason for this finding was studied more closely.

As mentioned above, the p.H63D mutation consists of replacing a cytosine (C) with a guanine(G) at position 187 in the genome. First, in the HRM technique, the fragment in question is amplified by PCR. Then in the melting stage, the chains are dissociated from their original sisters and immediately are randomly re-joined, it allows the generation of homoduplex and heteroduplex. In the **Table.9.** the possible combinations of the affected base pairs, which may result from the formation of homoduplex and heteroduplex, are collected. In green the original nucleotide is highlighted, while in orange is pointed out the mutated nucleotide.

RESULT	ALLELE 1	ALLELE 2	POSSIBLE COMBINATIONS			
HOMOZYGOUS: Homoduplex for the mutation.	5' – T**G**A – 3' 3' – A**C**T – 5'	5' – T**G**A – 3' 3' – A**C**T – 5'	5' – T**G**A – 3' 3' – A**C**T – 5'			
WILDTYPE: Homoduplex without the muation.	5' – T**C**A– 3' 3' – A**G**T – 5'	5' – T**C**A – 3' 3' – A**G**T – 5'	5' – T**C**A – 3' 3' – A**G**T – 5'			
HETEROZYGOUS: Homoduplex and Heteroduplex with the variant.	5' – T**C**A – 3' 3' – A**G**T – 5'	5' – T**G**A – 3' 3' – A**C**T – 5'	5' – T**C**A – 3' 3' – A**G**T – 5'	5' – T**C**A – 3' 3' – ACT – 5'	5' – T**G**A – 3' 3' – A**C**T – 5'	5' – T**G**A – 3' 3' – AGT – 5'

Table.9. *Representation of possible combinations that can be obtained as a result of Homoduplex and Heteroduplex formation in detection of p.H63D.*

As indicated in **Table.9.** analysis of the H63D mutation implies that the affected base pair is the same (G/C) in both Homozygous and Wild type patients, therefore they cannot be differentiated, and in the plot difference they all appear in the same group (**Figure.16.D**).

To solve this problem, at the time of preparation of the PCR, the samples to be studied and the homozygous controls on the PCR plate were duplicated, so that 1 µL of a wild type sample was added to a duplicate of the homozygous controls, having therefore a replica with normal homozygotes and another replica with homozygotes plus the wild type. The same procedure was followed for the samples under study.

Thus, those samples that were homozygous for the mutation, when mixed with the wild type, became heterozygous, being able to form the different combinations of homoduplex and heteroduplex previously indicated (**Table.9**). Consequently, these samples were grouped with the heterozygous control in the difference plot.

As shown in **Figure.16.E.** wild type samples were associated with the wild type control, which are those that appear in blue. Samples of heterozygous individuals were grouped with their control and were represented in red. In contrast, homozygous individuals were grouped in both groups. On the one hand, samples of homozygous individuals that had not been mixed with wildtype were grouped with wild types. However, homozygous samples mixed with the wild type were grouped with heterozygous.

Thus, it was possible to differentiate whether the patient under study was wild type, homozygous or heterozygous for the p.H633D mutation.

In addition, samples which presented melting curves different from the other controls were detected, and therefore were not grouped with any of them, so that a valid diagnosis could not be concluded. As is the case of the samples shown in **Figure.16.D** and **Figure.16.E** with green and pink curves. In the next section we will look at it in more detail.

4.1.1.1 S65C Mutation detection.

4.1.1.1.1 Discordant Result.

In order to setting up the HRM in the *LC480®,* the analysis of the samples was carried out with both the *HAIN®* and the *LC480®* in order to validate the new technique.

In this validation, results for the analysis of p.H63D. from two samples that did not coincide between the techniques were obtained.

On the one hand, the results obtained with the *HAIN®* are shown in **Figure.17,** as we can observe patient 1 was heterozygous for p.H63D, while patient 2 was wild type for p.H63D.

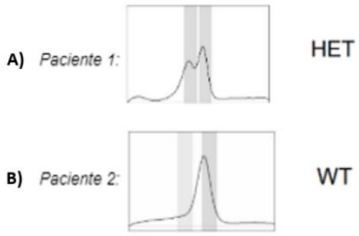


Figure.17. *Melting curve analysis for p.H63D determination by HAIN® in Patient 1 and 2.*

In the analysis of the same samples with *LC480®* for p.H63D. the melting curves of those samples were found to be different from the other controls. In **Figure.18** it can be observed that the sample of patient 1 (pink curve) and patient 2 (green curve) had a different Tm and melting curve and therefore could not be grouped with any of the controls. Unlike *HAIN®* which determined that individuals were heterozygous and wild type respectively for p.H63D, with *LC480®* a valid diagnosis could not be concluded. Therefore, such samples were sequenced by Sanger to detect which anomaly made them different from the others.

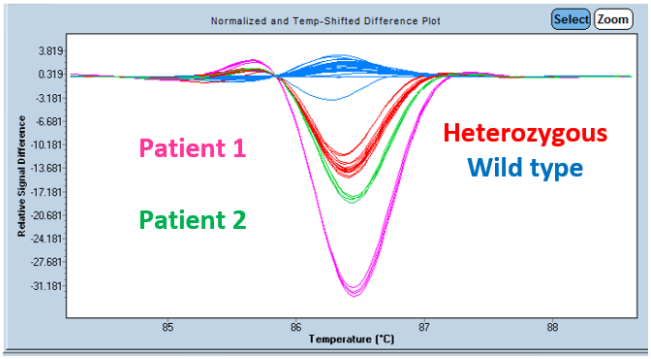


Figure.18. *Analysis of a different melting curve from Patient 1 and Patient 2 for p.H63D determination by using LC480®.*

4.1.1.1.2 Sanger's Electropherogram. following results:

These two samples that presented discordant results and therefore could not determine a result, were sequenced by Sanger and analysed with the programs *Chromas* and *MUSCLE*, obtaining the following results:

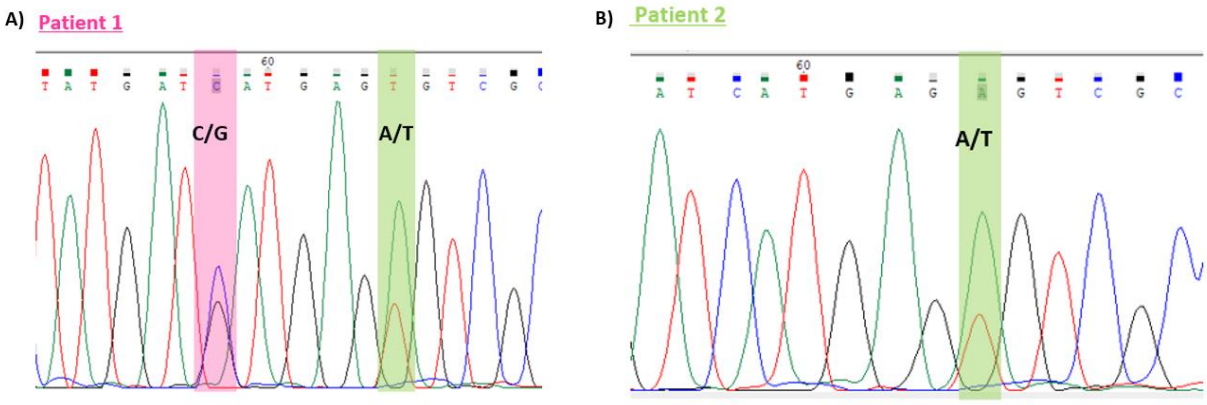


Figure.19. *Nucleotide sequence analysis for Patient 1 and Patient 2, obtained by Sanger sequencing.*

The analysis of the nucleotide sequences obtained by Sanger Sequencing allowed to conclude the definitive diagnosis of these patients. On the one hand, in **Figure.19.A** Patient 1 is represented. As indicated his analysis by the HAIN®, it was heterozygous for p.H63D, but also presented another variation of an A by a T, in the 193 position of the sequence. This variation corresponds to another mutation in the HFE gene associated with HH, concretely p.S65C that involves the substitution of a Serine (S) by a Cysteine (C) in amino acid 65 of the protein, as seen in the introduction of this study.

On the other hand, patient 2, represented in **Figure.19.B** was indeed wild type for p.H63D as evidenced by *HAIN®* however, the variant p.S65C was also detected, so this patient was heterozygous for p.S65C.

Thus, patient 1 was heterozygous for p.H63D and p.S65C, while patient 2 was only heterozygous for p.S65C. Regarding the clinical implication of these variations, as indicated above, in heterozygotes (H63D/S65C), the risk of iron overload is not significant compared to heterozygosity S65C alone, which may cause mild to moderate hepatic iron overload, but none of the carriers of this mutation, participants in the study, found clinically manifest HH or hepatic fibrosis. So carriers of S65C have a risk of developing an iron overload, but not to the same extent as homozygotes C282Y or heterozygotes C282Y / H63D, therefore, it is a milder variant.[19]

In conclusion, the implementation of HRM technique in the *LC480®* for the determination of the p.H63D mutation allowed the detection of other variants, concretely p.S65C, which was not possible to detect in the analysis by *HAIN®.* This is due to the fact that, unlike the *HAIN®* that uses specific probes for the mutation under study, the *LC480®* allows to cover the sequence for which the primers have been

designed, in this case, as the mutation p.H63D and p.S65C are very close, specifically in positions 187 and 193 respectively, it allows that the design of primers performed can determine both variants in a single analysis.

4.1.1.1.3 H63D Retrospective.

Based on these findings, a retrospective analysis was performed using the HRM technique with the *LC480®* platform, of 70 patients, who had already been analysed for the p.H63D mutation but with *HAIN®* system. Therefore, the detection of possible patients with the p.S65C variant could have been omitted. Both the heterozygous sample for p.H63D and p.S65C and the heterozygous sample for p.S65C were used as controls for the detection of p.S65C. **Table 10.** shows the results obtained:

	ANALYSIS	HAIN®	LC480®
HEREDITARY HEMOCHROMATOSIS	**p.H63D_WILDTYPE**	41	40
	p.H63D_HETEROZYGOUS	23	22
	p.H63D_HOMOZYGOUS	6	6
	p.H63D+p.S65C:HETEROZYGOUS	0	1
	p.S65C_HETEROZYGOUS	0	1

Table.10. *Results of the retrospective of p.H63D.*

As detailed in **Table.10** a heterozygous patient was detected for both p.H63D and p.S65C mutations of the HFE gene and another heterozygous patient was detected for p.S65C.

Their *LC480®* analyses and their subsequent sequencing by Sanger are shown in **Figure.20** and **Figure.21** respectively.

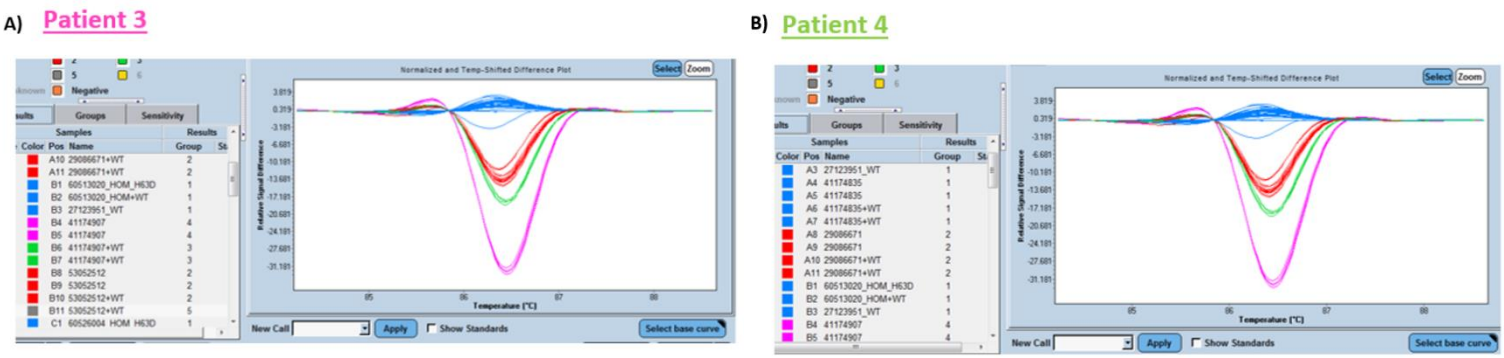


Figure.20. *Analysis by LC480® from Patient 3 which is heterozygous for both variants and Patient 4 which is heterozygous for p.S65C.*

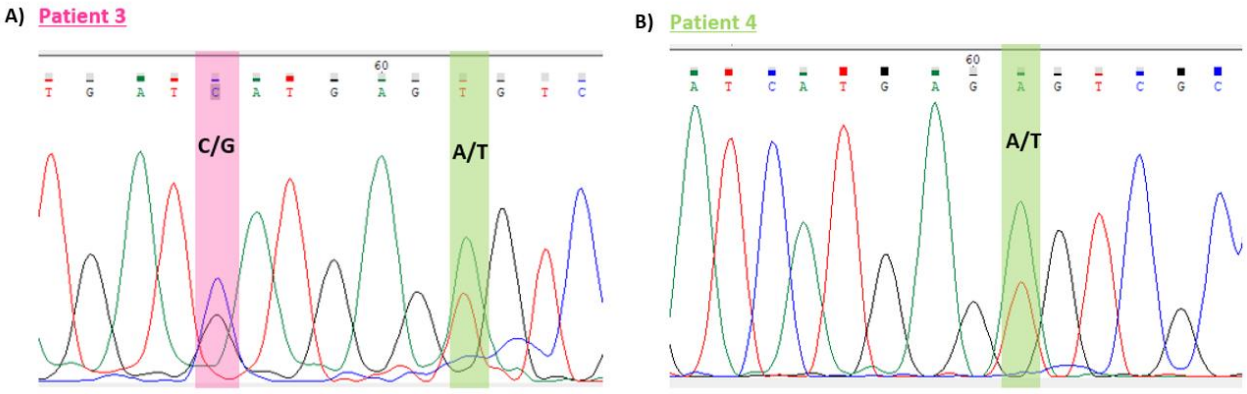


Figura.21. *Nucleotide sequence analysis for Patient 3 and Patient 4, obtained by Sanger sequencing.*

On the one hand, in **Figure.20.A** it can be observed that patient 3 presented a melting curve that was associated with the heterozygous control for p.H63D and p.S65C.(pink curve). In the sequencing by Sanger, represented in **Figure.21.A** it was confirmed that this patient presented both variants.

As for patient 4, on the one hand, its analysis by *LC480®,* showed that its melting curve (pink curve) was associated with the heterozygous control for p.S65C. (**Figure.20.B**). It was also evidenced in its nucleotide sequence (**Figure.21.B**), which showed the variation of one A by one T in the corresponding position.

4.1.2 Tm calling, Quantification absorbance and Gene Scanning of C282Y Mutation.

Figure.22.*HRM data analysis by using Quantification absorbance and Tm calling Module for p.C282Y detection.*

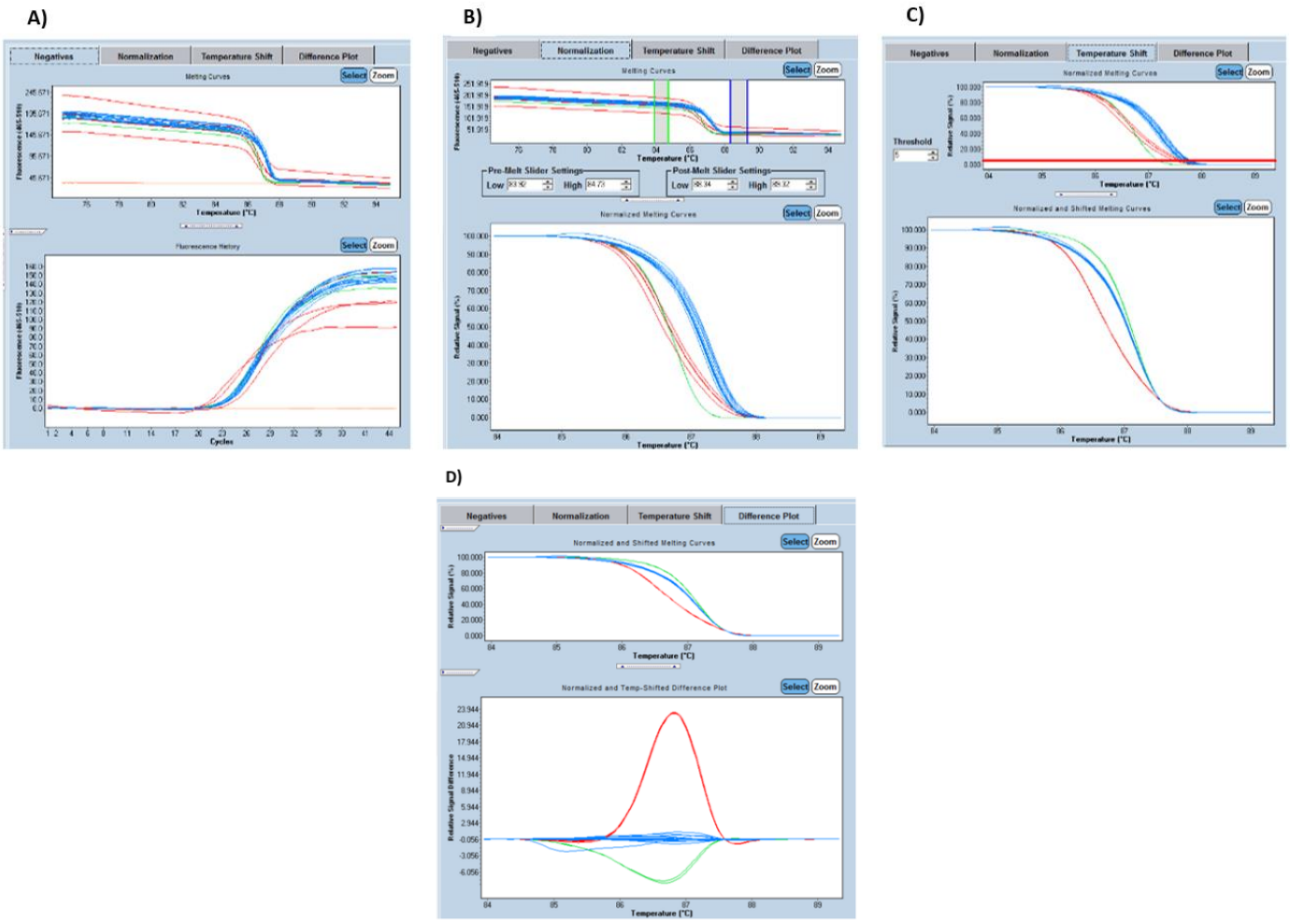


Figure.23. *HRM data analysis by using Gene Scanning Module for p.C282Y detection.*

For the analysis of p.C282Y. the same methodology was followed as for p.H63D. As can be seen, in **Figure.22.A** the amplification was correct as it met the established values. As for the homogeneity between the samples, although some of them differed more from the rest, they were generally quite similar to each other.

The Tm calling module, represented in **Figure.22.B** indicated that p.C282Y had a Tm of about 86.7-87.3ºC. Two rather differentiated melting curves can be observed, this may be due to the different Tm that can be given according to the patients studied are homozygous, heterozygous or wild types for the mutation. The grouping of the samples was defined in the Difference Plot, (**Figure.23.D**) distinguishing the homozygous represented in green, heterozygous in red or wild type in blue for p.C282Y.

In the analysis for p.C282Y no discordant samples were found that will not be associated with controls.

4.2 Thrombotic diseases.

4.2.1 Tm calling, Quantification absorbance and Gene Scanning of Leiden Factor V.

Figure.24. *HRM data analysis by using Quantification absorbance and Tm calling Module, for Leiden FV detection.*

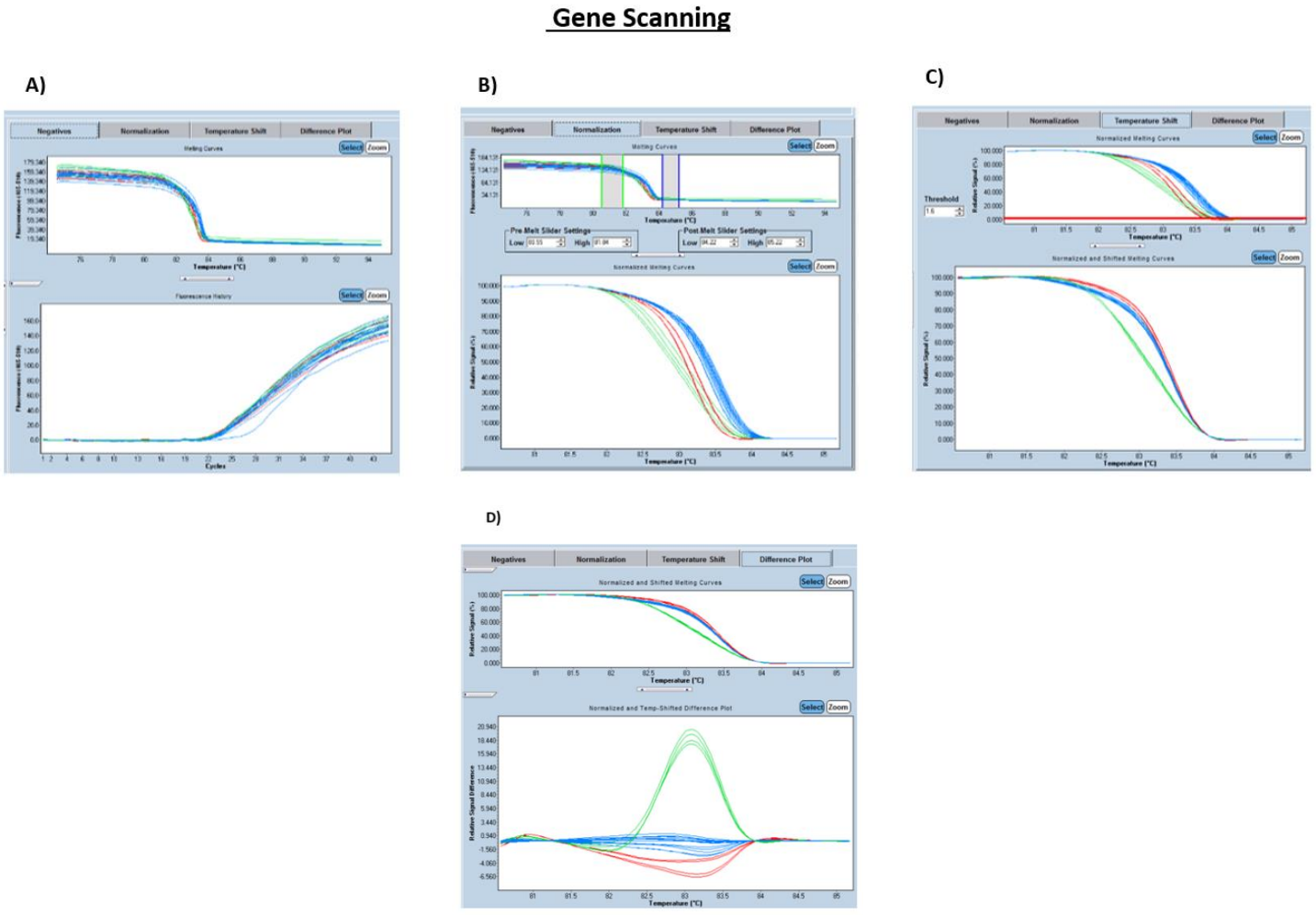


Figure.25. *HRM data analysis by using Gene Scanning Module for Leiden FV detection.*

In the setting up analysis of the determination of Leiden FV mutation, the same procedure as for the HH was followed.

It was found that the amplification had been performed effectively, by analysing their curves in the Quantification absorbance module (**Figure.24.A**). As for the homogeneity of the samples, some of them differed more from the rest in the level of stabilization of the curves, but as indicated, all exceeded the minimum value.

The Tm was about 82-83ºC, as shown in **Figure.24.B** based on the analysis between the control samples, slight differences could be observed between the samples, specifically the homozygotes had a higher Tm of approximately 82.8ºC while the wild types had a Tm closer to 82.3ºC. The Tm of heterozygous samples was around 82.7ºC. These small differences were adjusted by subsequent analysis by Gene Scanning (**Figure.25**) allowing the differentiation of the samples, in which three groups were obtained: the homozygotes represented in red, heterozygous that appear in green and wild types in blue.

4.2.1.1 New Variant detection:

In the same way as for the analysis of the HH, in the setting up of the VTD using the *LC480®* system, a validation of all the samples to be studied was carried out using the two systems: *HAIN®, Lifesciences* and *LC480®, ROCHE.*

In this validation, discordant results were obtained for two samples, which did not coincide between the two techniques. Then, they will be analysed in detail.

4.2.1.1.1 Discordant Result.

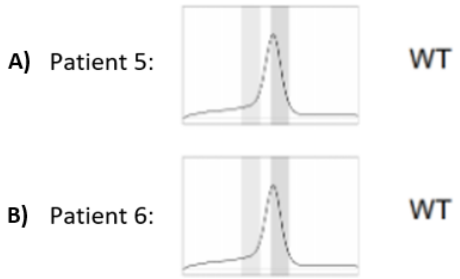


Figure.26. *Melting curve analysis for Leiden FV determination by HAIN® in Patient 5 and 6.*

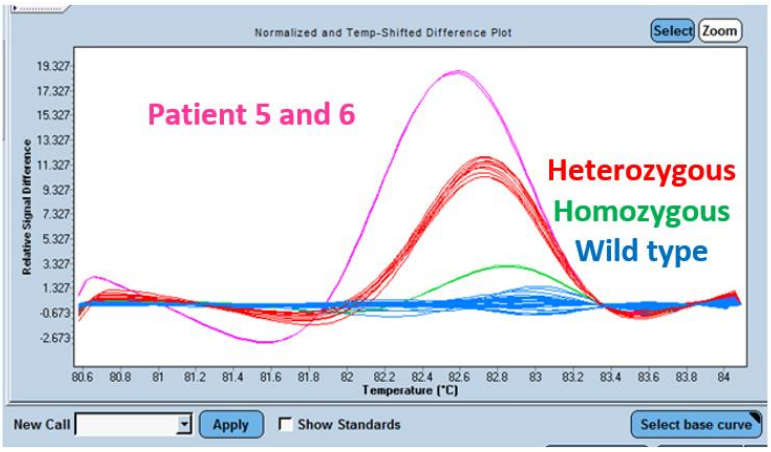


Figure.27. *Analysis of a different melting curve from Patient 5 and Patient 6 for Leiden FV determination by using LC480®.*

On the one hand, the analysis of these two samples using the *HAIN®* indicated that they were wild type for the Leiden FV mutation (**Figure.26**) On the other hand, in the analysis of the same samples by *LC480®* (**Figure.27**), it was observed that these samples had different melting curves (pink curves) that did not allow their association with any of the controls established, so that a conclusive diagnosis could not be determined.

Therefore, we proceeded to sequencing by Sanger, with the objective of being able to determine the factor that made them differentiate themselves from the rest.

4.2.1.1.2 Sanger's Electropherogram.

The same way it was done for p.H63D. those discordant samples for Leiden FV in their analysis by *LC480®,* were sequenced by Sanger and analysed by the programs *Chromas* and *MUSCLE*, obtaining the following results:

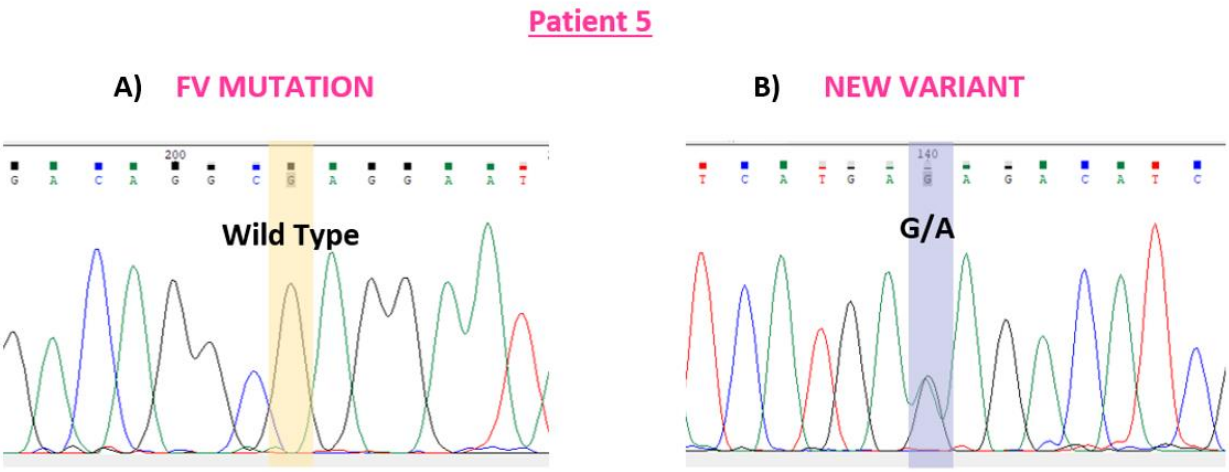


Figure.28. *Nucleotide sequence analysis for Patient 5 by Sanger Sequencing.*

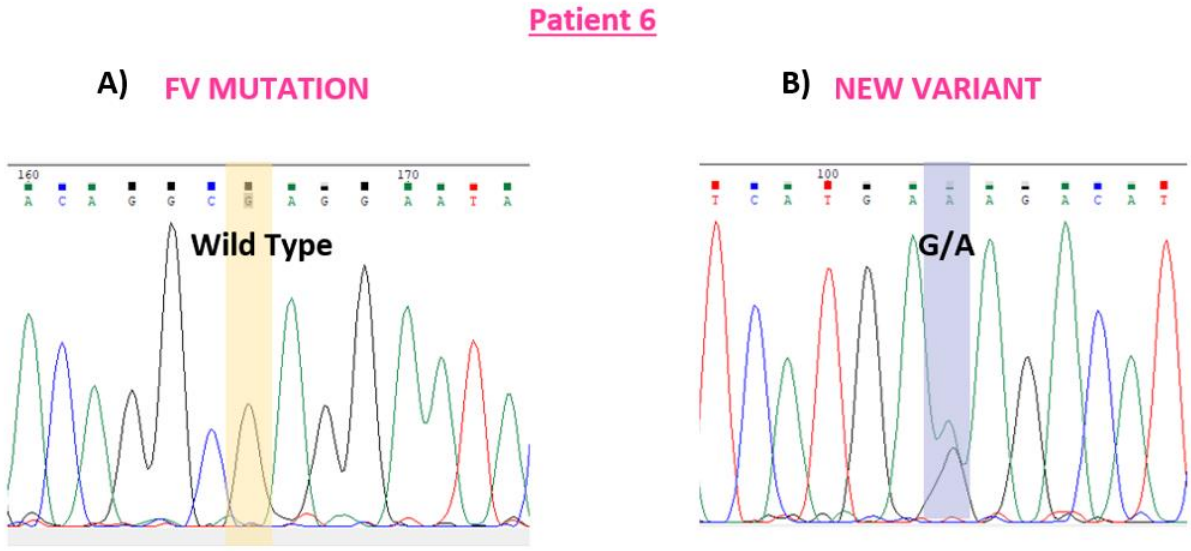


Figure.29. *Nucleotide sequence analysis for Patient 6 by Sanger Sequencing.*

The analysis of the nucleotide sequences **Figure.28** and **Figure.29**, allowed to determine the definitive diagnosis of these two patients.

Both Patient 5 and Patient 6 had not Leiden FV mutation. As can be seen pointed out in yellow only the wild type nucleotide curve appears in the position of FV mutation, in this case a G, therefore, both patients were wild type as indicated by their analysis by HAIN®. (**Figure.28.A** and **Figure.29.A**).

However, the rest of the sequence was analysed in order to detect what was the factor that differentiated the melting curve of these two samples from the other controls. Based on this analysis, a substitution of one Guanine (G) by one Adenine (A) was discovered. (**Figure.28.B** and **Figure.29.B**).

When examining this SNP by means of the *Mutation taster* programme, an application which allows to evaluate the potential of causing disease from different alterations in DNA sequences, it was determined that said SNP is based on the substitution of a G by an A at 1538 position of the DNA sequence (c.1538G>A). This implies the substitution of an Arginine(R) by a Lysine(K) in the amino acid 513 of the protein (p.R513K). It also provided a known variant associated with this SNP, namely rs6020. (Figure.30.).

The study of this variant in the dbSNP, as well as its subsequent evaluation according to different interpretations in the Clinvar database, allowed to determine that said SNP appears to be benign in association with venous thrombosis, as shown in **Figure.30** and **Figure.31.**

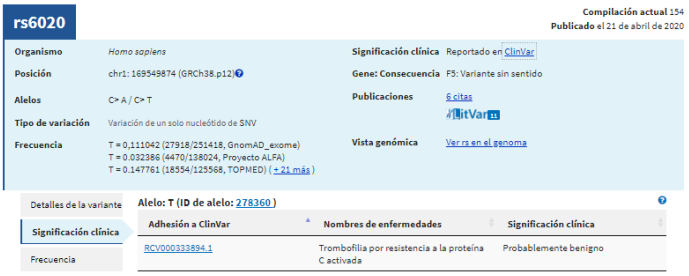


Figure.30. *Variant associated with the SNP c.1538G>A in dbSNP.*

Figure.31. *Different interpretations of the SNP c.1538 G>A in Clinvar.*

In short, the setting up of VTD through its analysis by *LC480®,* allowed the identification of another variant, located in the sequence covered by the primers designed for the determination of the Leiden FV. However, this variant appears to be benign in relation to the disease being studied.

4.2.2 Tm calling, Quantification absorbance and Gene Scanning of Factor II mutation.

Figure.32. *HRM data analysis by using Quantification absorbance and Tm calling Module, for FII Mutation detection.*

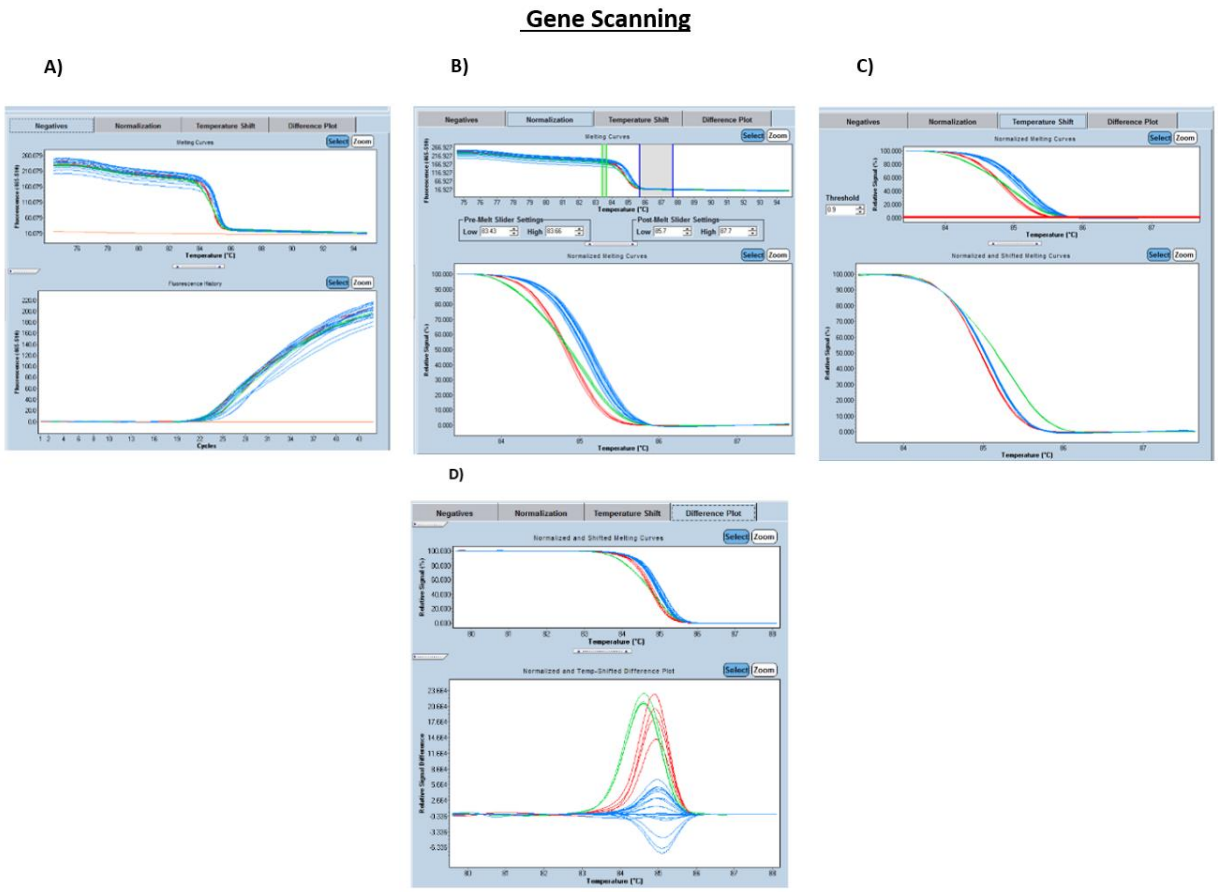


Figure.33.*HRM data analysis by using Gene Scanning Module for FII mutation detection.*

The Quantification absorbance module (**Figure.32.A**) noted that the amplification process had been carried out effectively, based on the same criteria followed previously (Cp >30, free of contaminants, stabilisation>30 fluorescence signals and homogeneity between samples).

The analysis by Tm calling (**Figure.32.B**) indicated that the Tm for the FII mutation is about 84.7-85.10ºC. Being closer to 85ºC in wild type controls, while for homozygotes it was approximately 84.8ºC. As mentioned above, this allowed a first grouping of samples, based on their similarity with the Tm of the different controls. The Gene Scanning module was used to precisely group the samples, allowing a clear grouping of the different samples to be studied (**Figue.33**).

In the case of the analysis for the FII mutation by *LC480®,* no other variants associated with this pathology were detected, in the zone amplified by the primers designed.

4.3 Comparative with HAIN®'s Results.

		HEREDITARY HEMOCHROMATOSIS				
		HAIN®		LC480®		
	GESTLAB	**p.H63D**	**p.C282Y**	**p.H63D**	**p.C282Y**	**p.S65C**
1	79333849	HET	WT	Different curve	WT	HET
2	60532638	HET	WT	HET	WT	
3	26068430	HOMO	WT	HOMO	WT	
4	35192707	WT	WT	WT	WT	
5	51017719	WT	HET	WT	HET	
6	60528169	WT	WT	Different curve	WT	HET
7	79333877	WT	WT	WT	WT	
8	79342250	WT	WT	WT	WT	
9	53039849	HET	WT	HET	WT	
10	36216418	HOMO	WT	HOMO	WT	
11	41180455	WT	WT	WT	WT	
12	70302178	WT	WT	WT	WT	
13	41181858	HET	HET	HET	HET	
14	60558534	HET	WT	HET	WT	
15	60387315	HOM	WT	HOM	WT	
16	33046088	WT	WT	WT	WT	
17	36214420	WT	WT	WT	WT	
18	70309285	HET	WT	HET	WT	
Melting Temperature				**86ºC**	**86,7-87,3ºC**	

Table.11. *Results of HH setting up by using HAIN® and the LightCycler480®.*

		VENOUS THROMBOSIS				
		HAIN®		LC480®		
	GESTLAB	FV	FII	FV	FII	NEW VARIANT
1	70288297	WT	WT	WT	WT	
2	70305374	WT	WT	WT	WT	
3	79360648	WT	HET	WT	HET	
4	79328584	WT	WT	WT	WT	
5	79332199	WT	WT	WT	WT	
6	79360665	WT	WT	WT	WT	
7	70302499	WT	WT	WT	WT	
8	36211256	WT	WT	WT	WT	
9	37058739	WT	WT	WT	WT	
10	38261397	WT	WT	WT	WT	
11	70305272	HET	WT	HET	WT	
12	79334421	WT	WT	WT	WT	
13	79338774	WT	WT	WT	WT	
14	79360931	WT	WT	WT	WT	
15	79360933	WT	WT	WT	WT	
16	79362298	WT	HET	WT	HET	
17	70296906	WT	WT	WT	WT	
18	79362322	WT	WT	WT	WT	
19	79362334	WT	WT	WT	WT	
20	79362336	WT	WT	WT	WT	
21	70286554	WT	WT	WT	WT	
22	70288307	WT	WT	WT	WT	
23	70305323	WT	WT	WT	WT	
24	79362796	WT	WT	WT	WT	
25	31225924	WT	WT	WT	WT	
26	41180737	WT	WT	WT	WT	
27	60532693	WT	WT	WT	WT	
28	70296921	WT	WT	WT	WT	
29	70304136	WT	WT	WT	WT	
30	79362967	WT	WT	WT	WT	
31	79362972	WT	WT	WT	WT	
32	79361530	WT	WT	WT	WT	
33	60540028	WT	WT	WT	WT	
34	70305392	WT	WT	WT	WT	
35	79362702	HET	WT	HET	WT	
36	79363034	WT	WT	WT	WT	
37	79363177	HET	WT	HET	WT	
38	79363179	WT	WT	WT	WT	
39	79363180	WT	HET	WT	HET	
40	31211100	WT	WT	WT	WT	

41	79363215	WT	WT	WT	WT	
42	79363216	WT	WT	WT	WT	
43	29085140	WT	WT	WT	WT	
44	38262883	WT	WT	WT	WT	
45	338262894	WT	WT	WT	WT	
46	55164788	WT	WT	WT	WT	
47	55936763	WT	WT	WT	WT	
48	79362952	WT	WT	WT	WT	
49	79362953	WT	WT	WT	WT	
50	55936764	WT	WT	WT	WT	
51	55936765	WT	WT	WT	WT	
52	79363257	WT	WT	WT	WT	
53	70286676	WT	WT	WT	WT	
54	27121836	WT	WT	WT	WT	
55	70305457	WT	WT	WT	WT	
56	79353695	WT	WT	WT	WT	
57	79353696	WT	WT	WT	WT	
58	79353697	WT	WT	WT	WT	
59	60532399	WT	WT	Different curve	WT	New Variant c.1538G>A
60	70305466	WT	WT	WT	WT	
61	70305463	WT	WT	WT	WT	
62	79353731	WT	WT	WT	WT	
63	79363408	WT	WT	WT	WT	
64	41181909	WT	WT	WT	WT	
65	79363438	HET	WT	HET	WT	
66	45146937	WT	WT	WT	WT	
67	79353806	WT	WT	WT	WT	
68	79353807	WT	WT	WT	WT	
69	38263364	WT	WT	WT	WT	
70	60526085	HET	WT	HET	WT	
71	79354213	WT	WT	WT	WT	
72	79354219	WT	WT	WT	WT	
73	79354224	WT	WT	WT	WT	
74	79353695	WT	WT	WT	WT	
75	79354225	WT	WT	WT	WT	
76	37059095	WT	WT	WT	WT	
77	70305749	WT	WT	WT	WT	
78	79354244	WT	WT	WT	WT	
79	79354246	WT	WT	WT	WT	
80	79354247	WT	WT	Different curve	WT	New Variant c.1538G>A
81	79354248	HET	HET	HET	HET	
82	79354249	WT	HET	WT	HET	
83	79354250	WT	WT	WT	WT	

84	79354251	WT	WT	WT	WT	
85	79354413	WT	WT	WT	WT	
86	79354414	HET	WT	HET	WT	
87	79354421	WT	WT	WT	WT	
88	79353906	WT	WT	WT	WT	
89	79354599	HET	WT	HET	WT	
90	79354598	WT	HET	WT	HET	
91	79354608	WT	WT	WT	WT	
92	79354609	WT	WT	WT	WT	
93	37059077	WT	WT	WT	WT	
Melting Temperature				**82-83ºC**	**84.7-85,10ºC**	

Table.12. *Results of VT setting up by using HAIN® and the LightCycler480®.*

PATHOLOGY	ANALYSIS	HAIN®	LC480®
HEREDITARY HEMOCHROMATOSIS 18 PATIENTS	p.H63D_WILDTYPE	7	6
	p.H63D_HETROZYGOUS	7	6
	p.H63D_HOMOZYGOUS	4	4
	Patient 1 (heterozygous_p.H63D_p.S65C)	0	1
	Patient 2 (heterozygous_p.S65C)	0	1
	p.C282Y_WILDTYPE	16	16
	p.C282Y_HETTEROZYGOUS	2	2
	p.C282Y_HOMOZYGOUS	0	0
VENOUS THROMBOSIS 93 PATIENTS	FV_WILDTYPE	86	84
	FV_HETEROZYGOUS	7	7
	FV_HOMOZYGOUS	0	0
	Patients 5 y 6 (New variant)	0	2
	FII_WILDTYPE	87	87
	FII_HETEROZYGOUS	6	6
	FII_HOMOZYGOUS	0	0

Table.13. *Comparison of the existing homogeneity between the results obtained by HAIN® and the LC480® for both determinations.*

Table.11 and **Table.12** show a comparison of the results obtained with both *HAIN®* and *LC480®*, for the determination of each of the variants of the two pathologies studied HH and VTD.

In this way, the homogeneity existing between the results obtained is evident, in order to validate the setting up of HRM technique with the *LC480®* system in the haematological diagnosis.

In the HH analysis, the sample size was 18 patients. On the one hand, in the determination of p.H63D, 4 patients presented a homozygous genotype for this

mutation in both techniques, 7 patients were wild type and another 7 were heterozygous for p.H63D for the *HAIN®,* while in the determination by *LC480®,* 6 wild type patients and 6 heterozygous patients were found for p.H63D. (**Table.11** and **Table.13**.) This difference was due to the detection of the p.S65C variant by the *LC480®* system, that was omitted by *HAIN®.* Thus, the discordance in the patient that was wild type by the *HAIN®* corresponds to patient 2, so that in its analysis by the *LC480®* its melting curve was not grouped with any of the controls and therefore could not be concluded a diagnosis, so sequencing determined that it was wild type for p.H63D and heterozygous for p.S65C. Similarly, the discordance in the patient that was heterozygous for p.H63D for the *HAIN®,* corresponds to patient 1, the analysis of which by *LC480®* and its subsequent sequencing by Sanger determined that it was a heterozygous individual for both p.H63D and p.S65C.

On the other hand, in the determination of p.C282Y. 16 patients were determined wild types for this mutation by both techniques, 2 heterozygous patients by both techniques, and no homozygous individuals. (**Table.11** and **Table.13**).

In this study, no homozygous patient was found for p.C282Y, in contrast to the rest of the literature consulted, which indicates that this homozygous mutation is the predominant in 85-90% of patients with HH.[11,18,21] In contrast, 4 patients were found homozygous for p.H63D, a mutation that according to different studies has a very low prevalence and it is not entirely clear that it can cause an iron overload, even if biochemical abnormalities are present.[7,18,21]

Regarding heterozygous individuals for both mutations (p.H63D/p.C282Y), it t stands out the discovery of one patient, specifically corresponds to 41181858, highlighted in **Table.11.** As indicated above, heterozygous composed of these two variants present a risk of high iron overload and therefore of HH development, although they represent only 0.5-2% of affected people.[11,7]

For simple heterozygous for either of the two study variants, it has been determined that they do not present a higher risk of hemochromatosis compared to the normal population.[18]

Finally, with reference to the heterozygous patient for p.H63D and p.S65C and the heterozygous patient for p.S65C, as indicated above, in individuals with p.H63D/p.S65C heterozygous, iron overload has not been significantly increased compared to individuals with only p.S65C heterozygous, which may be associated with hepatic iron overload, although HH was not found in any of the patients studied, carriers of this mutation, so these individuals present a greater risk of iron overload, but not to the same extent that it occurs in homozygotes for p.C282Y and heterozygotes compounds p.H63D/p.S65C.[19]

It should be noted that the sample size for the analysis of HH has been very low, only 18 patients, this has been due to the fact that the study has been conducted in a correlative way to the number of samples that were admitted during the period of performance of the same.

In reference to the analysis for VT, the sample size was 93 patients. On the one hand, in the determination for the Leiden FV mutation, no homozygous patient was detected by any of the techniques, 7 heterozygous patients were detected by both the *HAIN®* and the *LC480®.* As for wild type individuals, 86 patients were found for *HAIN®* while 84 were found for *LC480®* (**Table.12** and **Table.13**).

This discordance was due to the detection of the new variant using the *LC480®* system. Therefore, these two patients diagnosed as wild type for Leiden FV by HAIN®, were not associated with any of the controls in their analysis by the *LC480®* and consequently their sequencing allowed to determine that indeed they did not present the mutation of the Leiden FV, but instead presented a substitution of a G by an A at the 1538 position of the DNA. However, the analysis of this SNP determined that it was benign in association with this pathology and therefore its presence did not imply any risk of developing venous thrombosis.

In the analysis of the FII mutation, no homozygous patient was found by any of the techniques, 6 heterozygous patients were detected by both techniques and 87 wild type patients by the two techniques. (**Table.12** and **Table.13**)

Based on the results obtained in this study, the prevalence of homozygous individuals for both Leiden FV and Prothrombin FII is very low, none has been found, a fact that corresponds with the literature consulted.[2]

However, heterozygous patients were found, who present a higher risk of suffering a thrombotic event compared to wild type individuals, as mentioned above, FV is present in the 20-50% of patients who develop venous thrombosis while FII has a prevalence of 15%. As for compound heterozygous, only one individual was found, corresponding to the number 79354248 in **Table.12**, the risk of which will also be increased.[2,25]

However, it should be noted that thrombosis seems to have a multigene origin, so a single mutation is not always sufficient to set off the thrombotic process although it increases the risk.[2]

Compared to HH, the sample size for VT was higher, but it is still a fairly small value. As already indicated, the number of samples analysed was correlated to the samples that were admitting during the period of the study.

In short, the results obtained for the analysis of the variants p.H63D and p.C282Y for the HH, and the Leiden FV mutation and FII mutation for the VT, both by the *HAIN®*

and by the *LC480®,* showed uniform results between both techniques. The only discrepancies found were due to the detection of the new variants by using the *LC480®* platform. On the one hand, the p.S65C in the HH that involves being able to develop an iron overload and instead the variant c.1538G>A that turns out to be benign in association with venous thrombosis.

In this way, the results obtained by the *LC480®* were validated with those obtained by the *HAIN®,* so that the setting up of the HRM technique in *LC480®* system, aimed at haematological diagnosis, was achieved.

4.4 Benefits of LightCycler 480® versus HAIN®.

VARIABLES	HAIN®	LC480®
Price / patient	10.83	7.55
Speed of analysis	1h 40 min	1h 11 min
Number of samples	12	96
Detection of other variants	NO	YES
Other uses	NO	YES

Table.14. *Comparison of the benefits provided by LC480® in contrast to HAIN®.*

Finally, a comparison between the two techniques was carried out which reflects the benefits provided by the *LC480®* system for the performance of genotyping studies in contrast to the *HAIN®* system used so far by the molecular biology unit in haematological diagnosis.

As detailed in **Table.14**, at an economic level, the price of the sample analysis is lower in the *LC480®* although the difference is not very large this can be seen in a clearer way in the individual price of the kits that goes from 1000€ in the *HAIN®* to 501€ in the *LC480®,* which means a halving in the price of the kits.

In addition, the analysis time is fewer in the *LC480®* approximately 30 minutes less.

On the other hand, the *LC480®* provides an analysis capacity of up to 96 samples compared to the 12 that *HAIN®* allows in a single round, this factor makes a big difference between both devices, so that the *LC480®* greatly speeds up the pace of laboratory work.

Likewise, the *HAIN®* system, by using probes specifically designed for the mutation under study, does not allow the detection of other variants with possible association with the pathologies of interest, a fact that is possible with the *LC480®* depending on the design made for the primers. Finally, the system *LC480®* has the advantage that it can be used for other determinations because unlike the *HAIN®* whose *FluoroType®, Lifesciences kit* presents all the reagents mixed, the *Light Cycler480® HRM Master Mix, ROCHE kit* presents its reagents separately, as well as primers that are designed and added independently according to the analysis to be performed.

This study is based on the application of the HRM technique in haematological diagnosis through the use of two real-time PCR platforms, the *HAIN®, Lifesciences* and the *LightCycler480®, ROCHE.*

The results that have been discussed throughout this section show that the objectives set at the beginning of this investigation have been achieved.

First, the *LC480®* system has been implemented for the determination of genome variations by applying the HRM technique. Specifically, it allows the effective detection of p.H63D mutations and p.C282Y in HH and Leiden FV mutations and Prothrombin FII mutations in VTD. Therefore, the main objective of this study has been achieved.

In the second place, the implementation of the HRM technique by means of the *LC480®,* has allowed to identify other variations associated with the pathologies under study, which with *HAIN®* was not possible to detect. This has been thanks to the design of primers, so that the sequence covered by said primers, in addition to the possible mutations under study, also includes the nucleotides susceptible of variation that are affected in the new variants found.

Finally, the comparative analysis between both techniques, evidences the benefits provided by the *LC480®* system in contrast to the *HAIN®,* so that it allows the optimization of the HRM technique, thus obtaining more precise results at a lower cost and time. It also takes advantage of the laboratory space and accelerates the pace of work in the same, which has a direct impact on the benefit of the patient.

As for future perspectives, the molecular biology unit of the haematology service of the University Clinical Hospital of Valencia, may be based on the methodology followed in this study for the application of the HRM technique in other analyses of genetic variations in association with haematology, carried out so far with the *HAIN®* system. Therefore, it can be dispensed with this device and consequently increase the quality and efficiency of genotyping studies carried out in that centre, the initial hypothesis on which this research is based.

5 Conclusions.

As stated above, this study was carried out in the molecular biology laboratory of the haematology service of the University Clinical Hospital of Valencia in collaboration with the INCLIVA Foundation.

This fact implies the integration of the different stages that take place in a haematology laboratory at hospital level in association with the study, from the selection of patients diagnosed or suspected of the pathologies under study until the determination of a conclusive diagnosis for them.

The initial establishment of a structured and concise methodology has allowed the investigation to be carried out in an orderly manner, and in the case of detecting errors

such as contamination errors in the preparation of reagents or errors in the application of protocol, has made it possible to locate the possible error committed and to assess the possible solution to the problem. Likewise, the process of primers design has been key, both for the detection of the variants initially defined and for the identification of new polymorphisms.

It should be noted that the main limitation of this study has been the sample size, which has been very small, to be able to establish conclusions regarding the prevalence of the analysed variants.

Summing up the results presented here, and those previously published, we may conclude that:

1. The system *LC480®* has been implemented in the haematologic diagnosis specifically for the determination of SNPs c.845G>A and c.187C>G in the HH and c.1691G>A and c.20210G>A in the VTD, by applying the HRM technique.

2. In addition to the proposed study variants, the use of the *LC480®* allows covering different nearby SNPs and identifying other allelic variants that were omitted with the *HAIN® system.*

3. The analysis between the two techniques demonstrates the advantages of the *LC480®* system over the *HAIN®,* so that its implementation allows an optimization of both the work in the laboratory and the haematological diagnosis.

In conclusion, the results obtained in this research, have allowed to optimize genotyping studies aimed at haematological diagnosis, in order to identify the disease early, as well as enabling a precociously treatment directed towards minimizing the serious effects of these pathologies.

6 Bibliography.

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7 Annex.

7.1 Other extraction protocols.

7.1.1 Paraffin DNA extraction with Maxwell RSC DNA FFPE KIT.

Sometimes the biological material is conserved as tissue embedded in paraffin (PET), this constitutes a great source of information in retrospective investigations. For the extraction and obtaining of nucleic acids and consequently be able to analyse them, there are different techniques, one of them is by using the *Maxwell RSC DNA FFPE KIT, Promega* by means of the *Maxwell RSCiii, Promega*. The protocol is as follows:

1. Centrifuge each paraffin at maximum speed for 15 seconds.

2. Turn on the thermoblock at 80ºC, 2 minutes.

3. Add 300 µL of mineral oil into each tube. Vortex for 10 seconds.

4. Place the samples in the thermoblock at 80ºC for 2 minutes. After time, leave at room temperature. While preparing the master mix. (Reagent).

Reagent	Amount/Reaction	Reactions (number to be run +2)	Total
Lysis Buffer	224 µL	n + 2	224 x (n+2) µL
Proteinase K	25 µL	n + 2	25 x (n+2) µL
Blue Dye	1 µL	n + 2	1 x (n+2) µL

Table. 15. *Components and quantities for the production of the DNA extraction mix.*

- Master mix should not be stored after use.
- n + 2: refers to the number of samples, plus 2 for pipetting error.
- It should be noted that the 56ºC allows the lysis buffer to work better and thus makes it easier for the K protein to break the membranes, so that nucleic acids can leave. It may be that after 30 minutes the K protein is denatured due to temperature, however, sufficient time has elapsed for it to have performed its function.

5. Adjust the thermoblock to 56ºC.

6. Add 250 µL of the master mix to each sample and vortex for 5 seconds.

7. Centrifuge each tube at 10000g x 20 seconds to separate the layers.

8. Place the samples in the thermoblock at 56ºC for 30 minutes.

9. Transfer the samples back to the thermoblock at 80ºC and incubate for 4 hours.

10. Take the samples from the thermoblock and leave them at room tempearture 5 minutes.

11. Add 10 µL RNAse A to the bluish aqueous layer of each sample. Mix by pipetting, in this way you remove the RNA, which does not interest.

12. Incubate the samples at room temperature for 5 minutes. In this waiting time, prepare the Maxwell. (As indicated in the section on DNA extraction).

- Place the plunger in the last well and the elution tube with 50/60 µL of Nuclease Free Water. In the second well the magnetic balls allow to unite the DNA since it presents negative charge and the balls positive charge, in this way the rest of components that do not interest are eliminated and finally in the elution tube, a "magnetic field" releases the particles of DNA and water by presenting positive charge allows to unite the DNA. In addition, it is free of nucleases to prevent it from degrading.

13. Centrifuge samples at maximum speed for 5 minutes.

14. Transfer the bluish aqueous layer to well 1 of the cartridge.

15. Select the FFPE DNA protocol.

7.1.2 RNA extraction of blood in EDTA with Maxwell® Simply RNA Blood Kit.

For the extraction of RNA from blood samples stored with EDTA, is used *Maxwell® Simply RNA Blood Kit, Promega*. The protocol is the following:

1. Prepare the amount of 1-thioglycerol/Homogenization Solution required: Add 20 µL of 1-thioglycerol per mL of Homogenization Solution.

*1-thioglycerol should be stored at 4ºC.

2. Prepare the DNAse: Add 275 µL of kit water and 5 µL of Blue Dye to the lyophilised DNAse.

*Reconstituted DNAse is stored at -20ºC and can be frozen/thawed 3 times.

3. Transfer 2.5-5 mL of fresh blood with EDTA into a 15 mL sterile tube.

4. Add 7.5-15 mL of Cell Lysis Solution (**Table.16**).

Volumen de sangre (mL)	Cell Lysis Solution (mL)	1-tioglicerol/Homogenization Solution (µL)	Lysis Buffer (µL)
2,5-3	7,5	200	200
4-5	15	300	300

Table.16. *Volumes of different components for RNA extraction.*

5. Mix 5-6 times to lyse the red cells.

6. Incubate 10 min at room temperature, investing 2 times in incubation.

7. Centrifuge 10 min to 2000 revolutions per minute.

8. Discard the supernatant using a pipette.

9. Add 200-300 µL 1-thioglycerol/Homogenization Solution in cold to the pellet and mix well by pipetting and vortex.

10. Add 200-300 µL Lysis Buffer and 25-50 µL Proteinase K, vortex 20 seconds.

11. Incubate for 10 minutes at room temperature.

12. Place the cartridges on the rack and uncover them.

13. Place plunger and elution tube with 50-80 µL of kit water.

14. Add 5 µL of DNAse included in the kit in well 4.

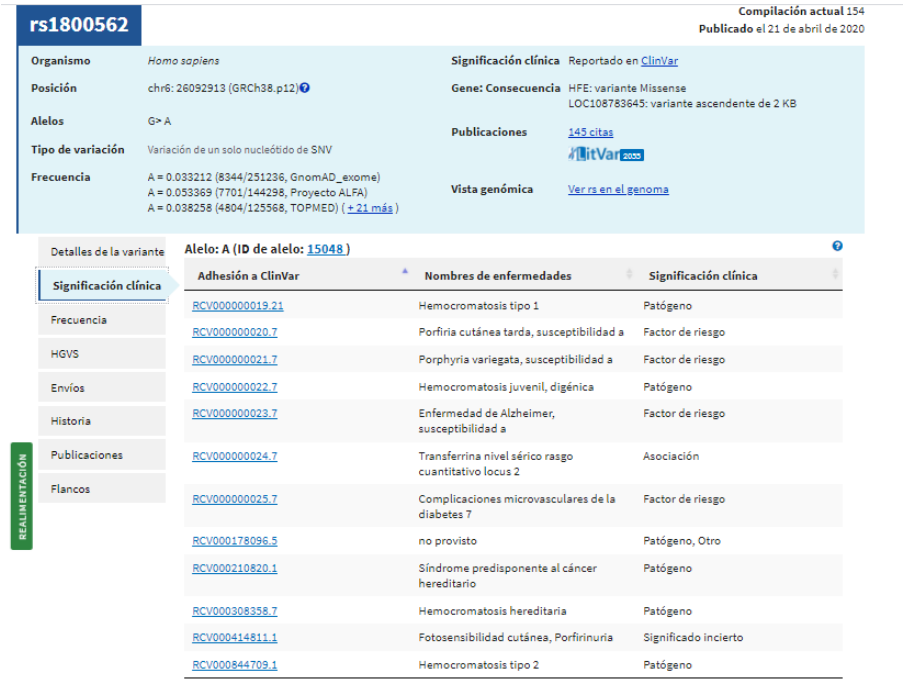
15. Add the lysate to the well 1

16. Select the protocol "SimplyRNA Blood"

17. Load the rack and press "START".

7.2 Comprehensive sequences of SNPs, selected variants and their clinical significance.

Figura.34. *Searching for the sequence that includes the p.C282Y and p.H63D mutation and the selected variant.*



rs1800562

Compilación actual 154
Publicado el 21 de abril de 2020

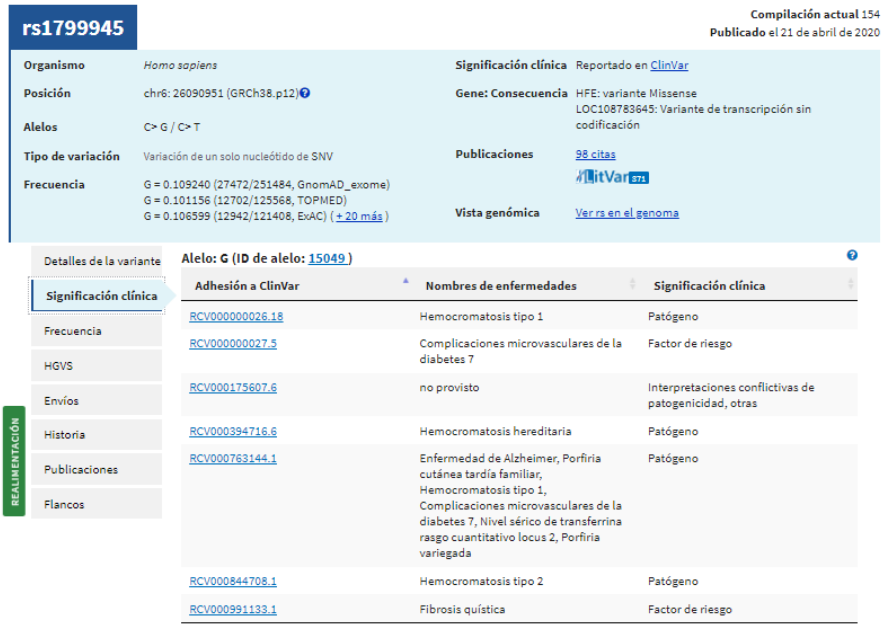
Organismo	*Homo sapiens*	Significación clínica	Reportado en ClinVar
Posición	chr6: 26092913 (GRCh38.p12)	Gene: Consecuencia	HFE: variante Missense LOC108783645: variante ascendente de 2 KB
Alelos	G> A	Publicaciones	145 citas LitVar 2055
Tipo de variación	Variación de un solo nucleótido de SNV		
Frecuencia	A = 0.033212 (8344/251236, GnomAD_exome) A = 0.053369 (7701/144298, Proyecto ALFA) A = 0.038258 (4804/125568, TOPMED) (+ 21 más)	Vista genómica	Ver rs en el genoma

Detalles de la variante
Significación clínica
Frecuencia
HGVS
Envíos
Historia
Publicaciones
Flancos
REALIMENTACIÓN

Alelo: A (ID de alelo: 15048)

Adhesión a ClinVar	Nombres de enfermedades	Significación clínica
RCV000000019.21	Hemocromatosis tipo 1	Patógeno
RCV000000020.7	Porfiria cutánea tarda, susceptibilidad a	Factor de riesgo
RCV000000021.7	Porphyria variegata, susceptibilidad a	Factor de riesgo
RCV000000022.7	Hemocromatosis juvenil, digénica	Patógeno
RCV000000023.7	Enfermedad de Alzheimer, susceptibilidad a	Factor de riesgo
RCV000000024.7	Transferrina nivel sérico rasgo cuantitativo locus 2	Asociación
RCV000000025.7	Complicaciones microvasculares de la diabetes 7	Factor de riesgo
RCV000178096.5	no provisto	Patógeno, Otro
RCV000210820.1	Síndrome predisponente al cáncer hereditario	Patógeno
RCV000308358.7	Hemocromatosis hereditaria	Patógeno
RCV000414811.1	Fotosensibilidad cutánea, Porfirinuria	Significado incierto
RCV000844709.1	Hemocromatosis tipo 2	Patógeno

Figura.35. *Clinical significance of p.C282Y mutation in HH.*



rs1799945

Compilación actual 154
Publicado el 21 de abril de 2020

Organismo	*Homo sapiens*	Significación clínica	Reportado en ClinVar
Posición	chr6: 26090951 (GRCh38.p12)	Gene: Consecuencia	HFE: variante Missense LOC108783645: Variante de transcripción sin codificación
Alelos	C> G / C> T	Publicaciones	98 citas LitVar 571
Tipo de variación	Variación de un solo nucleótido de SNV		
Frecuencia	G = 0.109240 (27472/251484, GnomAD_exome) G = 0.101156 (12702/125568, TOPMED) G = 0.106599 (12942/121408, ExAC) (+ 20 más)	Vista genómica	Ver rs en el genoma

Detalles de la variante
Significación clínica
Frecuencia
HGVS
Envíos
Historia
Publicaciones
Flancos
REALIMENTACIÓN

Alelo: G (ID de alelo: 15049)

Adhesión a ClinVar	Nombres de enfermedades	Significación clínica
RCV000000026.18	Hemocromatosis tipo 1	Patógeno
RCV000000027.5	Complicaciones microvasculares de la diabetes 7	Factor de riesgo
RCV000175607.6	no provisto	Interpretaciones conflictivas de patogenicidad, otras
RCV000394716.6	Hemocromatosis hereditaria	Patógeno
RCV000763144.1	Enfermedad de Alzheimer, Porfiria cutánea tardía familiar, Hemocromatosis tipo 1, Complicaciones microvasculares de la diabetes 7, Nivel sérico de transferrina rasgo cuantitativo locus 2, Porfiria variegada	Patógeno
RCV000844708.1	Hemocromatosis tipo 2	Patógeno
RCV000991133.1	Fibrosis quística	Factor de riesgo

Figura.36. *Clinical significance of p.H63D mutation in HH.*

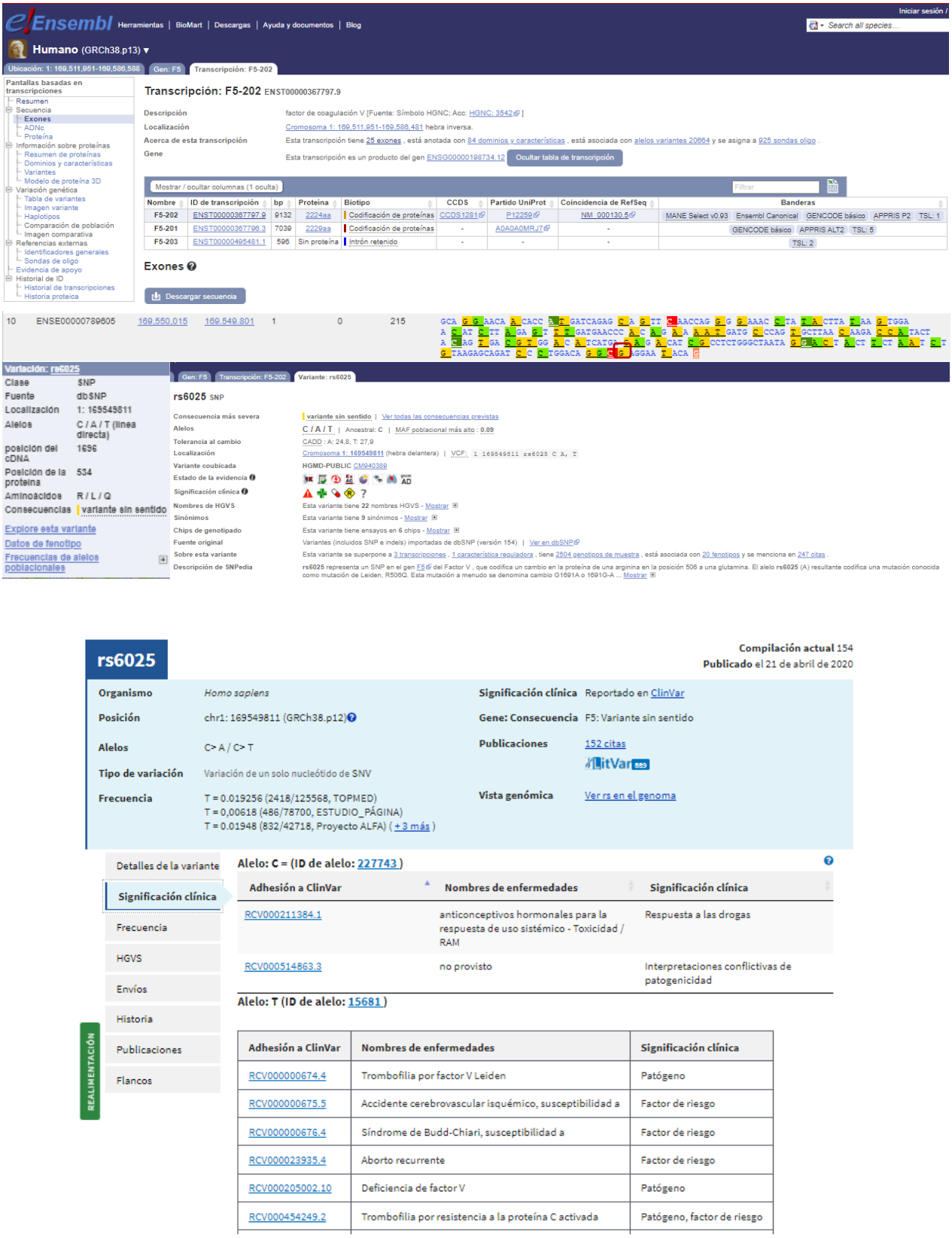


Figura.37. *Searching for the sequence that includes the Leiden FV mutation, the selected variant and its clinical significance.*

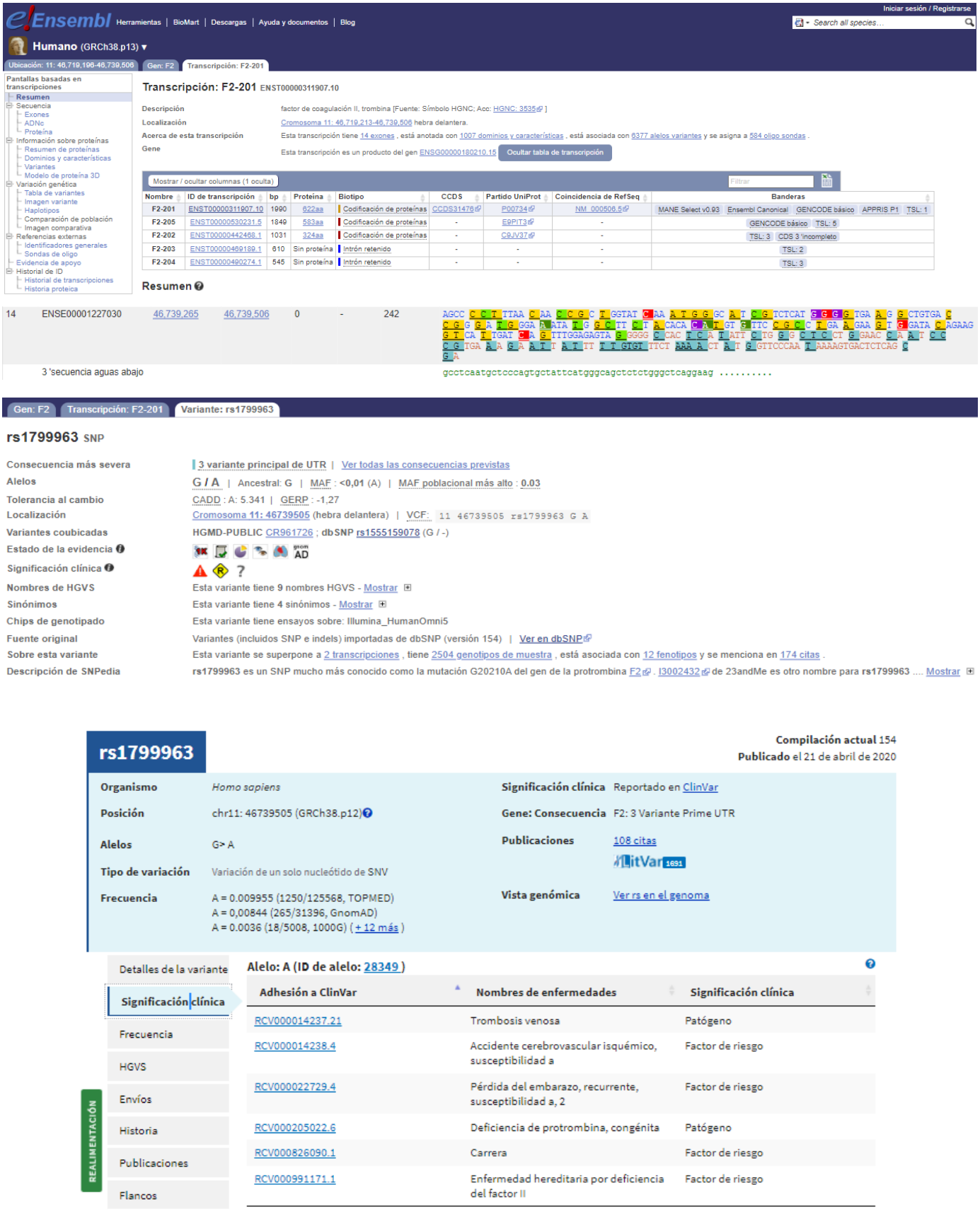


Figura.38. *Searching for the sequence that includes the Prothrombin FII mutation, the selected variant and its clinical significance.*