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CHARACTERIZATION OF ETV6-RELATED THROMBOCYTOPENIA (ETV6-RT): A NEW FORM OF THROMBOCYTOPENIA ASSOCIATED WITH A RISK OF DEVELOPING HEMATOLOGIC NEOPLASMS

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ABSTRACT

ETV6-Related Thrombocytopenia (*ETV6*-RT) is a rare form of inherited autosomal dominant thrombocytopenia first identified in 2015 that, together with *ANKRD26*-Related Thrombocytopenia *(ANKRD26*-RT) and Familial Platelet Disorder (FDP/AML), belongs to the category of "myeloid neoplasms with germline predisposition and preexisting platelet disorders" according to the 2016 World Health Organization classification.

ETV6-RT is caused by germline mutations in *ETV6* gene that encodes a transcriptional repressor known for its role in hematopoiesis and megakaryopoiesis. *ETV6* was initially identified as tumor suppressor frequently involved in somatic translocations responsible for leukemia development. Despite its role in tumor process is well characterized, *ETV6* germline mutations and their involvement in megakaryopoiesis are still poorly described.

Therefore, the main aim of this doctoral research was to give insights into the molecular characterization of novel *ETV6* variants together with their patogenicity mechanism.

Thanks to the consolidated collaboration with IRCCS S. Matteo (Pavia), all the three years have been constantly characterized by the screening on the *ETV6* gene in probands belonging to a large cohort of probands affected by Inherited Thrombocytopenia (IT) but lacking of a molecular diagnosis. The screening highlighted so far a total of 7 new *ETV6* missense variants in 11 families. After the *in silico* prediction, we set up and performed a functional study workflow (western blot, reporter assay and immunofluorescence) in order to evaluate the pathogenic role of each variant. Luciferase (reporter) assay showed the loss of repressive function in 5 out of 7 ETV6 variants and the immunofluorescence clarified that the pathogenic mechanism consists in an abnormal retention of mutated proteins in the cytoplasm and not to a defect in DNA binding, as expected by the position in which the mutations occurs. Moreover, we hypothesized that ETV6, like RUNX1, could control the expression level of ANKRD26 during megakaryopoiesis, severily impairing platelet production and promoting the neoplastic evolution under defective conditions.

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1. INTRODUCTION

1.1 Platelets biogenesis and the importance of the genes encoding hematopoietic trancription factors

Platelets are small, anucleate, discoid cells roughly 1-3 μm in diameter that play, along with the coagulation factors, a pivotal role in hemostasis and wound healing repairing vascular injuries thus preventing excessive bleeding. They are generated through a complex process called megakaryopoiesis that is finely regulated by a concert of factors that control every step of the migration of precursors cells from the hematopoietic niche to the sinusoidal blood vessels where mature platelets are ultimately released. During the hematopoietic differentiation the Hematopoietic Stem Cell (HSC) gives rise to progressively committed progenitors of blood cells including the Megakaryocyte Erythroid Progenitors (MEP) that are bipotential precursors of both megakaryocytic and erythroid lineages. Under the regulation of Thrombopoietin (TPO) early megakaryocytes begin to increase their ploidy by a process called endomitosis that is a physiological way to increase the cytoplasm volume. After this stage, megakaryocytes undergo cytoplasmatic maturation that involves the formation of alpha (α) and delta (δ) granules in addition to other mRNA, organelles and surface receptors fundamental for the normal function of platelets such as the adhesiveness and the hemostasis. The following step is the migration of the megakaryocytes to the vascular niche of the bone marrow where the formation and elongation of pseudopodia permit the extension of proplatelets into the sinusoidal blood vessels through a microtubule remodelling process driven by apoptotic events producing mature platelets (1).

As shown in the schematic representation in Figure 1, multiple transcription factors and their co-activators take part to the complex network. Mutations affecting their coding genes disrupt the correct differentiation and maturation of megakaryocytes or the release of mature platelets into the blood stream giving rise to several forms of thrombocytopenia. These factors act by binding regulatory elements located upstream of the promoter of specific target genes activating or repressing their transcription and regulating lineage specific gene expression during the hematopoiesis. More recently, the identification of a growing number of patients with defects in transcription factor genes resulting in unbalanced platelets' count and function has provided detailed knowledge about the role of these factors in platelet biogenesis. Thus, germline mutations in hematopoietic factors (e.g. RUNX1, GATA-1, FLI1, GFI1b and ETV6) have been documented to result in hereditary platelet dysfunction, thrombocytopenia and variable bleeding symptoms (2). Therefore, the increasing number of genes and mutations that are supposed to be

responsible of different forms of thrombocytopenia and even associated to other clinical manifestations become fundamental for the advance in knowledge about Inherited Thrombocytopenias (ITs).

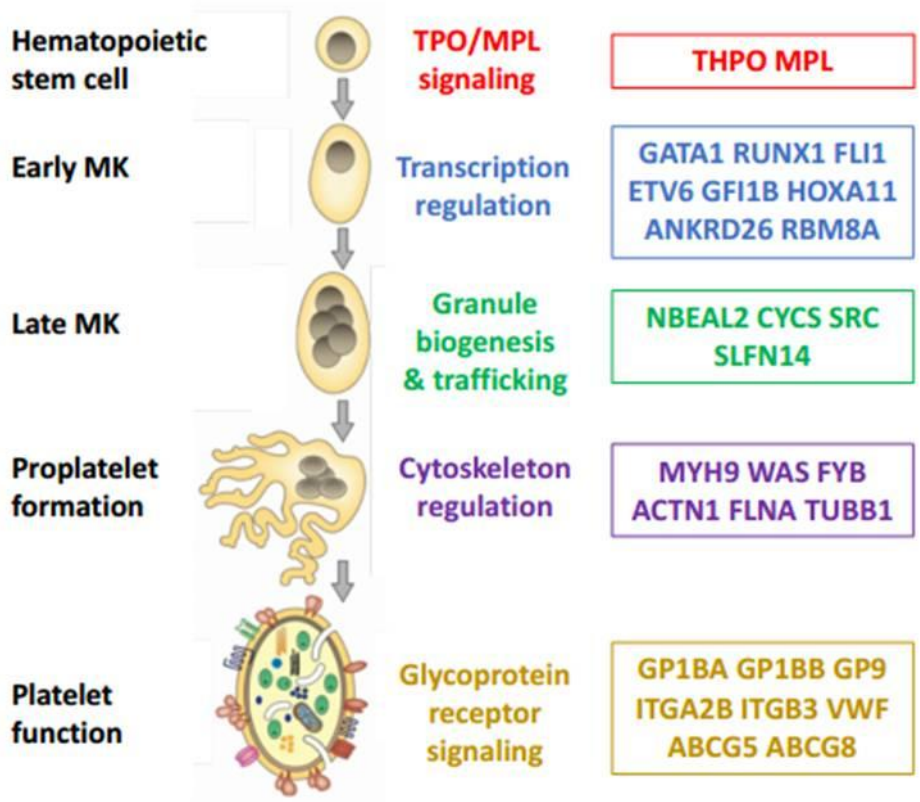

Figure 1. Representation of megakaryopoiesis process and platelets formation. Stages of the platelets biogenesis and the main factors involved in each phase: from the hematopoietic stem cell to the mature platelet production. Each factor, if mutated can give rise to different form of ITs. Abbreviation: MK,megakaryocytes. Adapted from *Lentaigne et al, Blood 2016*(3).

1.2 Inherited thrombocytopenias (ITs) and classification

Congenital and familial thrombocytopenia syndromes, also called Inherited Thrombocytopenias (ITs) are a group of rare disorders characterized by a reduced platelet count (<150 * 10^9 platelets/L) with Autosomal Dominant (AD), Recessive (AR) or X-Linked (XL) pattern of inheritance. They are characterized by a variable expressivity of bleeding tendency, ranging from recurrent spontaneous bleeding in several cases to milder episodes only after surgery, trauma or delivery and the cause can be due to the low number of the platelets but even to their disturbed function.

Given the high grade of heterogeneity of the ITs, over the years various attemps for an efficient classification have been made, including parameters like the mode of inheritance, platelets size or the presence of additional clinical findings other than thrombocytopenia.

The traditional diagnostic algorithm used for the differential diagnosis of IT examined patients medical history and physical features to distinguish syndromic forms, whereas the evaluation of platelet size and peripheral blood smear guided to the diagnostic process in non-syndromic forms of ITs (4).This approach helped to find at least 30 genes as cause of ITs so far, although these genes account only for approximately 50% of the total ITs suggesting that the diseases are still underdiagnosed and novel forms have yet to be identified.

In addition to the growing number of genes as cause of ITs thanks to the massive NGS sequencing, recent improvements in clinical characterization revealed that most of ITs were characterized by mild bleeding tendency or even no bleeding at all, but exposing patients to acquire additional clinical manifestations that can be even fatal or strongly compromise the quality of life. The clinical picture is expected to become increasingly complex, therefore a more effective classification has been proposed.

As summarized in Table 1, ITs have been divided into 3 categories according to their clinical features: 1) forms characterized only by thrombocytopenia, 2) disorders in which the platelet phenotype associates with additional congenital defects (syndromic forms) and 3) forms characterized by increased risk of acquiring additional relevant diseases during life (5).

Among the group of ITs with only thrombocytopenia some patients can present no bleeding at all, whereas others may have spontaneous bleeding or hemorrhages only during hemostatic challenges but without carrying associated syndromes. The two most representative forms of this group are the Bernard-Soulier Syndrome (BSS) and the Actinin-Related Thrombocytopenia (ACTN-RT) which represent a large amount of IT patients, more or less the 20%.

Among the ITs syndromic forms, the molecular defects in genes responsible for thrombocytopenia can induce complex phenotypes. The spectrum of abnormalities can include cognitive impairments, malformations of the central nervous or cardiovascular system, immunodeficiencies and skeletal deformities as in the case of TAR syndrome (Thrombocytopenia-Absent Radius syndrome).

The last group includes patients characterized by a strong predisposition of acquiring additional illnesses such as bone marrow aplasia, juvenile myelofibrosis, or end-stage renal disease that can be far more dangerous for the patient's life than only hemorrhages episodes. A group of ITs characterized by an increased risk of developing myeloid neoplasms is included in this cluster; they have been classified by the "2016 revision of the World Health Organization classification of myeloid neoplasms and acute leukemia" as a new category named "Myeloid neoplasms with germline predisposition and pre-existing platelet disorders" that includes myeloid malignancies arising from germline mutations in *ANKRD26*, *RUNX1*, and *ETV6* genes (6).

Table 1.
Classification of Inherited Thrombocytopenias (ITs) according to their clinical features.

Disease (abbreviation, OMIM entry)		Frequency*	Inheritance	Gene	Locus
Forms with only thrombocytopenia					
Bernard-Soulier syndrome	Biallelic	++++	AR	GP1BA	17p13
(BSS, 231200/153670)	Monoallelic	+++	AD	GPIBB	22q11
				GP9	3q21
Gray platelet syndrome (GPS, 139090)		++	AR	NBEAL2	3p21
ACTN1-related thrombocytopenia (ACTN1-RT, 615193)		++	AD	ACTN1	14q24
ITGA2B/ITGB3-related thrombocytopenia (ITGA2B/ITGB3-RT, 187800)		+	AD	ITGA2B	17q21
				ITGB3	17q21
TUBB1-related thrombocytopenia (TUBB1-RT, 613112)		+	AD	TUBB1	20q13
CYCS-related thrombocytopenia (CYCS-RT or THC4, 612004)		+	AD	CYCS	7p15
GFI1b-related thrombocytopenia (GFI1b-RT, 187900)		+	AD	GFI1B	9q34
FYB-related thrombocytopenia (FYB-RT or THC3, 273900)		+	AR	FYB	5p13.1
SLFN14-related thrombocytopenia (SLFN14-RT or BDPLT20, 616913)		+	AD	SLFN14	17q12
FLI1-related thrombocytopenia (FLI1-RT or BDPLT21, 617443)		+	AR	FLI1	11q24.3
Inherited thrombocytopenia from monoallelic THPO mutation (THPO-RD, NA)		+	AD	THPO	3q27.1
Von Willebrand disease types 2B (VWD2B, 613554)		++	AD	VWF	12p13
Forms with additional clinically relevant congenital defects/syndromic forms					
Thrombocytopenia-absent radius syndrome (TAR, 2740009)		+++	AR	RBM8A	1q21
Wiskott-Aldrich syndrome (WAS, 301000) X-linked thrombocytopenia (XLT, 313900)		++++	XL	WAS	Xp11
FLNA-related thrombocytopenia (FLNA-RT, NA)		+	XL	FLNA	Xq28
GATA1-related disease (GATA1-RD) (Dyserythropoietic anemia with thrombocytopenia - NA, 300367 – X-linked thrombocytopenia with thalassemia – XLTT, 314050)		++	XL	GATA1	Xp11
Thrombocytopenia associated with sitosterolemia (STSL, 210250)		+	AR	ABCG5 ABCG8	2p21
Forms with increased risk of acquiring additional illnesses/predisposing forms					
Congenital amegakaryocytic thrombocytopenia (CAMT, 604498)		++	AR	MPL	1p34.2
MYH9-related disease (MYH9-RD, 155100)		++++	AD	MYH9	22q12
Familial platelet disorder with propensity to acute myelogenous leukemia (FPD/AML, 601399)		++	AD	RUNX1	21q22
ANKRD26-related thrombocytopenia (ANKRD26-RT or THC2, 188000)		++	AD	ANKRD26	10p12
ETV6-related thrombocytopenia (ETV6-RT or THC5, 616216)		+	AD	ETV6	12p13
Radioulnar synostosis with amegakaryocytic thrombocytopenia (RUSAT, 605432)		+	AR	HOXA11	7p15
Thrombocytopenia 6 (THC6, 616937)		+	AD	SRC	20q12

1.2.1 ITs with predisposition to hematological malignancies

ANKRD26- Related Thrombocytopenia (*ANKRD26*-RT), Familial Platelet Disorder with propensity to Acute Myelogenous Leukemia (FDP/AML) and *ETV6*-Related Thrombocytopenia (*ETV6*-RT) are three rare Autosomal Dominant (AD) inherited thrombocytopenias caused by mutations in the *ANKRD26*, *RUNX1* and *ETV6* genes respectively, with a different prevalence among ITs (18% for *ANKRD26*-RT, 3% for FDP/AML and 5% for *ETV6*-RT) (5). The grade of thrombocytopenia that is usually mild to moderate, the normal platelet size and the morphology of blood cells shared by the three forms make them clinically indistinguishible. However the most relevant shared feature is the association with an increased risk of developing hematological neoplasms such as Myelo Dysplastic Syndrome (MDS), Acute Myeloid Leukemia (AML) and Acute Lymphoblastic Leukemia (ALL) that account for the 8%, 40% and 20% of *ANKRD26*-RT, FDP/AML and *ETV6*-RT patients, respectively (7).

Due to the rarity of these diseases, knowledge about their clinical and molecular characterization is still poor, in particular for *ETV6*-RT that is the most recently identified form. Therefore extensive genetic screening and the identification of novel pathogenic variants combined with collection of clinical data at the diagnosis and during the follow up, would provide fundamental insights on this emerging group of ITs. These studies are absolutely crucial for the development of an accurate genetic couseling and clinical interventions for individuals with a dangerous hereditable predisposition to malignancies (7).

1.2.2 *ETV6*- Related Thrombocytopenia (*ETV6*-RT)

Four independent studies in 2015 described germline defects in *ETV6* gene to be responsible for the autosomal dominant form of thrombocytopenia named *ETV6*-Related Thrombocytopenia (*ETV6*-RT, OMIM: 616216), also known as Thrombocytopenia 5 (THC5) without any syndromic association. However, affected individuals have an increased predisposition to develop hematologic neoplasms suggesting the hereditary pattern both for the thrombocytopenic condition and the neoplasm susceptibility (8–11).

The *ETV6* gene (*ETS* variant 6), previously known as *TEL* (Translocation ETS Leukemia) encodes a master hematopoietic transcriptional repressor and it is located on chromosome 12 (12q13.1) with a lenght of 5989 bp structured in 8 exons (Ensembl: ENSG00000139083). ETV6 transcription factor is a 57kD protein composed by 452 aminoacids structured in three functional domains: the

N-terminal pointed (PNT), the regulatory domain (or central regulatory domain CRD) and C-terminal DNA-binding (ETS) (Figure 2). The high conserved ETS domain is involved in the binding of promoter regions of the target genes containing ETS motifs whereas the PNT domain, that is required for the oligomerization with itself and other factors, explets the transcriptional repression together with the CRD (12,13). The dimerization of ETV6 is fundamental for a high affinity DNA-binding in the repression of the transcription of genes that are involved in cell growth and differentiation (14,15).

Figure 2. **Schematic representation of ETV6 protein**. ETV6 is composed by PNT, Regulatory and ETS domains . The domains involved in DNA binding and in repression of transcription are indicated by horizontal black bars (Adapted from Daly et al., 2017).

ETV6 is an important player in hematopoiesis, in the mainteinance of the vascular network development and in the survival of the stem cells staminal niche. Although ETV6 binds to the promoter of many genes considered important for the erythropoiesis and the platelets production, data concerning its involvement in human megakaryocytopoiesis remain poorly described (16–18).

Since its original description, *ETV6* gene has been identified as a tumor suppressor, widely espressed in all tissues but well known for its role of somatic hot spot in chromosomal rearrangements associated with childhood leucemia (19). It is one of the most commonly rearranged genes in human acute leukemia since the fusion with *RUNX1* gene occurs in 22% of children with B cell Acute Lymphoblastic Leukemia (B cell-ALL), but somatic mutations are also reported to be associated with MDS and T-cell leukemia (17,20,21).

Despite the well defined role of *ETV6* in somatic tumor episodes, only germinal mutations have been reported in families in the context of hereditary thrombocytopenia, in which the incidence of hematological malignancies in *ETV6* germinal carriers is higher compared to that in the general population. In fact, the hereditary predisposition involves acute leukemia that is the most common form among pediatric cancer but, although compromising both lymphoid and myeloid lineage, *ETV6*-RT predisposes to ALL for the majority of the cases (20).

From the first description of *ETV6*-RT, several pathogenic germinal *ETV6* variants have been identified so far in unrelated thrombocytopenic families with an autosomal dominant pattern of transmission. The contribution of whole genome and exome sequencing approach, easily highlighted mutations in *ETV6* gene that included non-sense, frameshift, premature stop codon, splicing and missense variants. Whereas there is no doubt on the deleterious effects of nonsense and frameshift alterations, the pathogenic role of Variants of Uncertain Significance (VUS) as missense alterations that are the most frequently detected in patients, is not always obvious. Therefore, although bioinformatic prediction tools can give a first indication about the effect of each variant identified, functional studies are specific and easy-developing experiments needed to confirm or exclude the pathogenicity. In order to demonstrate it, reporter assays have been performed on promoters which harbour multiple ETS binding sites such as stromelysin-3 (MMP3) and platelet factor-4 (PF4) that are physiological ETV6 targets (11).

The majority of *ETV6* germinal variants emerged so far in the thrombocytopenia onset show that they are mostly private and clustered within the ETS DNA-Binding Domain, except for the p.Pro214Leu missense encoded by the c.641C>T substitution that hits the central inhibitory domain and it is the most represented (10).

For instance, Zhang and collaborators identified two missense variants located in the ETS binding domain resulting in the aminoacid change p.R399C, p.R369Q, for which a loss of repression of the targets has been documented. Moreover, the addition of the wild-type ETV6 was unable to restore the transcriptional repression suggesting a dominant negative mechanism of action (8).

The absence of any relevant clinical feature that can be used to raise the suspiscion of *ETV6*-RT from the routine diagnostic workup has led to underestimate the incidence of the disease, in fact, except for the presence of a mild thrombocytopenia (44,000–132,000 platelets/μL) and an elevated red cell mean corpuscular volume (MCV) (88-101.2 fL), patients exhibited hematological parameters like Mean Platelet Diameter (MPD) and Mean Platelet Volume (MPV) overall within the normal range. Moreover, any consistent defect of *in vitro* platelet aggregation, activation, adhesion assays or on the major platelet glycoproteins expression have been detected. Only a minority of patients showed a red blood cell macrocytosis and the platelets spreading on fibrinogen has been found reduced in a not significative number of individuals investigated so far (21).

These data suggest that hematologic parameters and assays in patients' platelets are not completely informative, at least for the identification of *ETV6*-RT. Therefore, further investigations are strongly required in order to provide effective genetic counseling and appropriate follow-up .

2. AIM OF THE STUDY

Although knowledge about Inherited Thrombocytopenias (ITs) has significantly progressed in the last ten years, around 50% of these diseases still lack of a molecular characterization. This project aims to expand the knowledge about the characterization of *ETV6*-Related Thrombocytopenia (*ETV6*-RT) mostly through the identification of novel ETV6 missense variants in patients.

Identification of *ETV6* mutations in probands and their family members together with the collection of their clinical data are critical to improve knowledge about this form of IT, particularly interesting for its association with an high risk of developing hematological malignancies. Due to the rarity and the absence of any relevant clinical feature that can address the routine diagnostic workup, the identification of *ETV6*-RT patients is still difficult. Although a great improvement in diagnosis was obtained thanks to the Next Generation Sequencing (NGS) methodologies that allow the identification of variants more quickly and accurately, the detection of *ETV6* variants is not always sufficient to ascertain their pathogenicity especially as regard missense variants that are the most frequent variants detected in patients. Indeed, if the pathogenic role of nonsense or frameshift variants is usually recognized without any further investigation, the pathogenic role of missense alterations is not obvious (VUS, Variants of Uncertain Significance). Therefore targeted *in vitro* functional studies for these variants are needed to support the bioinformatic prediction and segregation analysis.

The detection of *ETV6* germline variants coupled to an efficient and rapid functional assay to assess the pathogenicity is essential because even if the only thrombocytopenic condition doesn't compromise the patients' quality of life, the high risk of developing hematological neoplasms has a deleterious potential.

Although the scientific community has conflicting opinions regarding the importance to diagnose the IT forms associated with an increased risk of hematological malignancies in terms of genetic counseling and management of patients (22), this aspect is of fundamental importance from a scientific research point of view. In our opinion, the better characterization of these diseases and the discovery of the mechanism that underlies them, could lead to the identification of innovative and targeted therapies for these patients who cannot take advantage of standard thrombomimetic based therapies because of their predisposition to hematological tumors.

3. MATERIALS AND METHODS

3.1 Prediction of Pathogenicity

The potential pathogenic effect of the *ETV6* missense variants on the protein function has been evaluated using the bioinformatic softwares PolyPhen-2 (Polymorphism Phenotyping v2), PROVEAN (Protein Variation Effect Analyzer), Mutation Assessor and CADD (Combined Annotation Dependent Depletion).

- PolyPhen-2 (Polymorphism Phenotyping v2) is a tool that predicts the possible impact of an amino acid substitution on the structure and the function of a human protein using straightforward physical and comparative considerations.
  (http://genetics.bwh.harvard.edu/pph2/).
- PROVEAN (Protein Variation Effect Analyzer) predicts whether an amino acid substitution or indel has an impact on the biological function of a protein.
  (http://provean.jcvi.org/index.php).
- Mutation Assessor predicts the functional impact of amino-acid substitutions in proteins, such as mutations discovered in cancer or missense polymorphisms. The functional impact is assessed based on evolutionary conservation of the affected amino acid in protein homologs. (http://mutationassessor.org/r3/).
- CADD instead is a numberbased tool for the prediction of deleteriousness of each variant that is assigned a CADD score. Although developers suggest a wide range between 10 and 20 for considering the potential damaging effect, we set a threshold of 15.

We took in account the concordance between these four bioinformatic tools for the prioritization of variant of uncertain significance that led to further investigations as purpose of this thesis.

3.2 Plasmids and Mutagenesis

In order to perform functional *in vitro* studies we first generated expression vectors; the wild type (wt) full-lenght *ETV6* sequence was amplified from normal platelet cDNA and inserted into a pcDNA3.1 (+) expression vector with a 5'Myc Tag sequence and containing an ampicillin resistance cassette (Invitrogen).

ETV6 mutants were generated by PCR site-directed mutagenesis by using primers designed with Quick Change Primer Design (Agilent) software.

(https://www.chem.agilent.com/store/primerDesignProgram.jsp).

The parental methylated DNA was eliminated by digesting with DpnI restriction enzyme and the construct obtained was transformed in TOP10 competent cells and grown up with ampicillin selection.

All the sequences cloned have been checked by sequencing to confirm the correct frame and the orientation within the vector and stored at 80° C and 20° for long or short term usage, respectively.

The same technique was used to clone the stromelysine-3 (MMP3) promoter region upstream the luciferase gene in the PGL3 basic vector (Promega) with the difference that the amplification started from genomic DNA since it is a regulatory region. The control reporter vector expressing Renilla Luciferase under the control of a constitutive promoter CMV (pRL-CMV), have been purchased from Addgene (www.addgene.org) now distributed by Promega. Detailed technical features of the vectors are avalilable on Promega website (www.promega.com).

3.3 Immunoblotting

HEK293T cells were mantained in DMEM: Dulbecco's Modified MEdium (Euroclone) with 10% FBS. Cells were plated onto 6 cm dishes at a density of $3.5*10^5$ cells/well and transfected after 24 hours with 2.5µg of the corresponding ETV6 (wt or mutations) construct by calcium-phosphate standard method**.** At 36 hours post transfection, cells were harvested and the total protein lysate was obtained using M-Per Mammalian Protein extraction reagent (Thermo Fisher Scientific) then separated by SDS-PAGE and transferred onto a nitrocellulose membrane followed by incubation with mouse monoclonal anti-c-myc antibody (9E10) and mouse monoclonal anti-HSP90 (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse antibody were used for secondary incubation and specific binding was detected by chemiluminescence (Chemidoc Bio-Rad).

3.4 Luciferase Reporter Assay

HEK293T cells were plated on a 24 wells plate ($5*10^4$ cells/well); after 24 hours 1.5 µg of each ETV6 cDNA expression vector (wt or mutations) was transiently transfected by calcium-phosphate method together with 300 ng of the MMP3-PGL3 (LucF). Each sample was additionally transfected with 30ng of pRL-CMV (LucR) to perform the normalization of data output. After 30 hours cells were lysed with 100µL of Passive Lysis Buffer 1X and the assay for the transactivation activity was

performed using Dual Luciferase Reporter (DLR) assay system according to the manufacturer's instructions (Promega). The results were espressed as ratio of firefly to renilla (LucF/LucR) graphically displayed by a histogram that represents the mean value of at least three independent experiments with standard deviations. Statistical analysis was done using an unpaired t-test.

3.5 Immunofluorescence Assay and Confocal Microscopy

HeLa cells were plated onto 1.7 cm^2 chamber culture slides (Falcon) with a density of 1.5 * 10^5 cells/well and transfected after 24 hours with 1µg of (wt and variants) ETV6 cDNA expression vectors with Lipofectamin 3000 following standard protocol (Thermo Fisher Scientific). After 24 hours from the transfection cells were fixed with 4% paraformaldehyde for 20 minutes and then permeabilized with 0.1% Triton X-100 and then treated for at least 30 minutes with Blocking Buffer (BSA 0,2% + FBS 1% + 0.1% Triton X-100, in PBS). For detection of ETV6 primary mouse monoclonal antibody anti c-myc tag was used (9E10, 1:50, Santa Cruz Biotechnology) followed by anti mouse FITC as secondary antibody (F0479,1:1000, Dako) whereas nuclei were stained by incubating the chamberslide with Hoechst staining solution for 5 minutes. After washing in PBS buffer (Euroclone) the chamberslide was covered with a cover slip and a drop of liquid mountant (ProLong, Thermo Fisher Scientific).

Images were obtained with a Nikon C1si confocal microscope, containing a 488nm argon laser line and 561 nm diode laser. Light was delivered to the sample with an 80/20 reflector. Electronic zoom was kept at minimum values for measurements to reduce potential bleaching. The images were acquired using a 60X Plan Apo objectives (with a corresponding NA of 1.4), collecting series of optical images at 1 µm z resolution step size. The corresponding voxel size was 100x100x1000 nm (X×Y×Z). Images were processed for z-projection (maximum intensity), brightness and contrast regulation using ImageJ 1.45 (NIH, Bethesda, USA).

4. RESULTS AND DISCUSSION

4.1 Selection criteria for the design of the study

The results of this thesis work well reflect the situation of the Italian cohort of *ETV6*-RT patients. Our laboratory has collected a wide range of patient cases thanks to the consolidated collaboration with IRCCS San Matteo (Pavia), which is the clinical recruitment center of IT patients. This cohort is composed by about 500 patients affected by inherited thrombocytopenia but lacking for a definitive molecular diagnosis.

The long collaboration has allowed us to provide the diagnosis for about the 50% of the patients of this cohort thanks to a diagnostic algorithm refined over the years that exploits patients medical history for a differential diagnosis of ITs (4). In the last ten years, thanks to the advent of Next Generation Sequencing (NGS), the way of studying genetic diseases, including inherited platelet disorders has deeply changed.

Currently, all the patients with suspected inherited thrombocytopenia are screened for 28 IT genes included in a diagnostic panel based on IonTorrent PGM^{TM} Platform developed in our laboratory.

This approach allowed us to increase the number of *ETV6*-RT patients analyzed and to recruit a total of 11 families with *ETV6* missense alterations that have been studied during my 3-year PhD; a group of 7 patients have been analyzed thanks to the classical diagnostic algorithm above described, whereas the remaining families emerged from the NGS diagnosis service.

All the informations about the families and the *ETV6* variants considered in this study will be discuss in the following paragraphs.

4.2 Clinical features of ETV6-RT patients

Since the patients included in the present study were recruited in different ways, a detailed clinical design is available only for patients identified by the classical approach. These patients are related to families F1÷F6 and have been clinically analyzed at IRCCS San Matteo in terms of blood cell and megakaryocytes analysis, platelets activation, adhesion and spreading assays in addition to haematologic informations such as platelets number, meanplatelet volume (MPV) and bleeding tendency. The results of this clinical analysis are described in Melazzini et al. (21).

However, the hematologic parameters are available for all the *ETV6*-RT patients enrolled in this study and they show a platelet amount that ranges between about 60 to 110 $*10^9$/L (moderate thrombocytopenia), the MPV that is within the normal range (8-13 fL) and the bleeding tendency that is mild to moderate.

As already described, since *ETV6*-RT patients show a mild thrombocytopenic phenotype without recurrent hematological or non-hematological abnormalities, it becomes difficult to distinguish *ETV6*-RT among the spectrum of mild ITs of different origin. Therefore, given the potential deleterious susceptibility to malignancies, the molecular study of the *ETV6* variants becomes essential.

The segregation of the *ETV6* variants for all the families enrolled was consistent with the dominant transmission pattern when relatives informations were available except for the variants found in F7 proband (R396G) that wasn't detected in parents suggesting a de novo or non paternity case (Figure 3).

Before the availability of the functional analysis, the segregation within a family, was considered a strong clinical evidence for the pathogenic role of a variant. For this reason, since we didn't dispose of the segregation informations for families F8 (Q347P), F9 (R399H) and F10 (S22N), the functional analysis of the variants became essential to understand whether they could be causative for the thrombocytopenic condition.

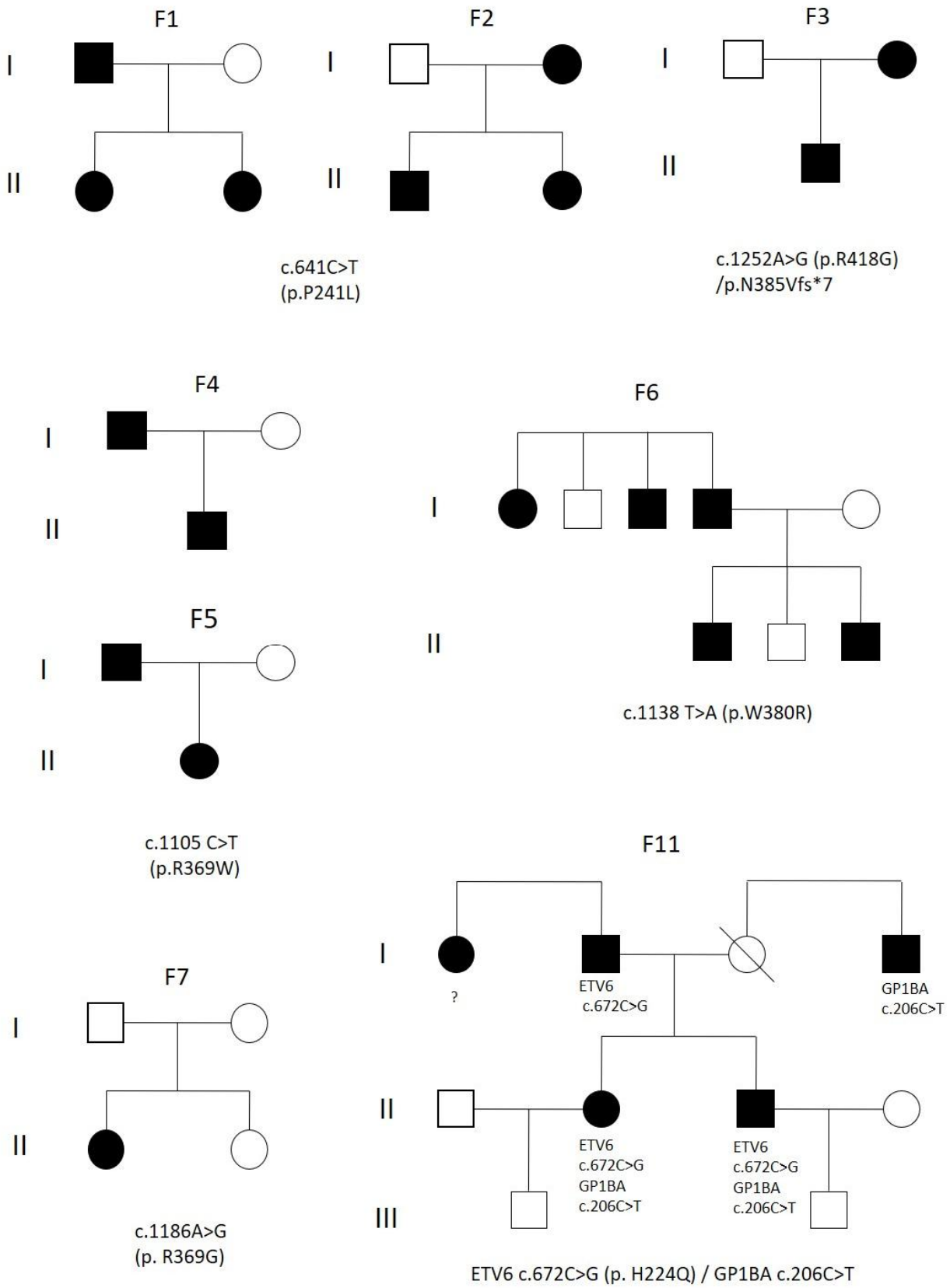

Figure 3. Pedigrees of families. Pedigrees were available for F1, F2, F3, F4, F5, F6, F7 and F11 families enrolled in this study. Different ETV6 variants carried by each family are indicated.

4.3 *ETV6* variants description and in silico prediction

The collection of data allowed us to include in our study overall 7 novel *ETV6* germline missense variants emerged from the screening of probands belonging to a total of 8 families (F4÷F11, Table 2). F1, F2 and F3 families carried the two known *ETV6* pathogenic mutations c.641C>T (p.P214L) and c.1252A>G (p.R418G and p. N385Vfs*7) so they have been included as positive controls (Table 2) (10,21). Moreover, although the three variants c.1138T>A c.1105C>T and c.1186A>G had been already described in previous studies, they lacked functional studies that demonstrated the pathogenicity therefore they have been incuded among our cases (21,23).

The nucleotide substitutions together with the encoded aminoacidic changes of all the variants are summarized in Table 2. All of the missense variants are clustered within the ETS binding domain of the *ETV6* gene except for the S22N that hits a non functional site at the N-terminal and the H224Q that is located in the regulatory domain (RD) as the control mutation P214L.

Family	Nucleotide	Protein	Domain	Type of mutation	Reference
F1 and F2	c.641C>T	p.P214L	RD	Missense	Noetzli et al.,2015; Melazzini et al.,2016
F3	1252A>G	p. R418G / p.N385Vfs*7	ETS	Missense	Noetzli et al.,2015; Melazzini et al.,2016
F4 and F5	c.1105 C>T	p.R369W	ETS	Missense	Melazzini et al.,2016
F6	c.1138T>A	p.W380R	ETS	Missense	Melazzini et al.,2016
F7	c.1186A>G	p.R396G	ETS	Missense	Poggi et al.,2017
F8	c.1040A>C	p.Q347P	ETS	Missense	Never described
F9	c. 1196G>A	p.R399H	ETS	Missense	Never described
F10	c.65G>A	p.S22N	N-term	Missense	Never described
F11	c. 672C>G	p.H224Q	RD	Missense	Never described

Table 2. Germline variants identified in *ETV6* gene: Nucleotide A of the ATG translation initiation start site of the ETV6 gene cDNA in GenBank sequence NM_ 001987.4. 1 is indicated as nucleotide +1.

The ETS domain, where the majority of validated germline *ETV6* mutations have been detected so far, is a critical site for its role in binding the DNA, suggesting that its impairment can lead to a loss or an alteration in transcriptional repression (Figure 4).

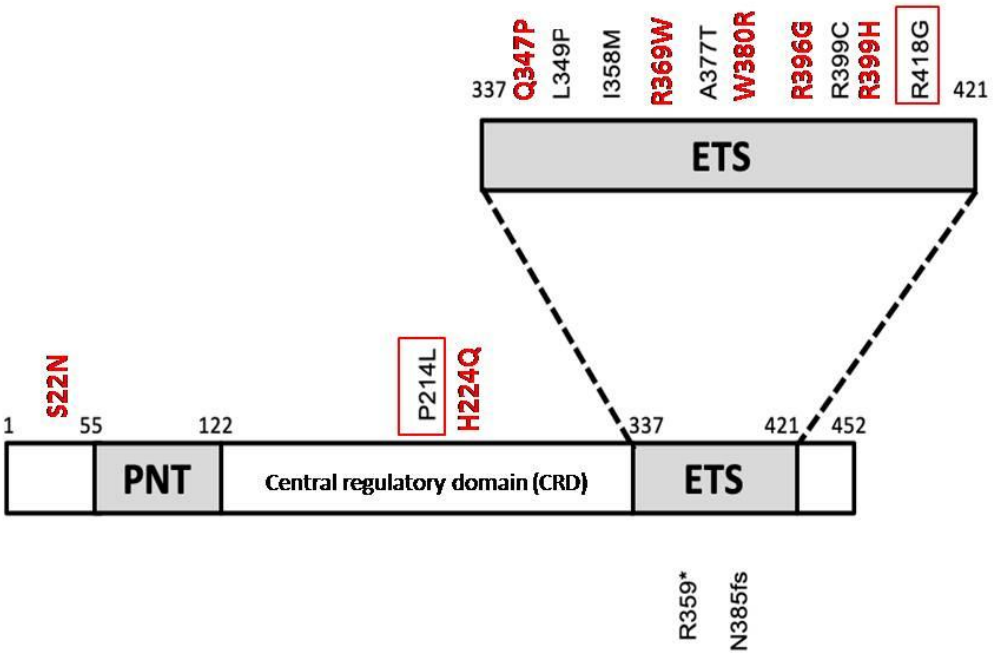

Figure 4. Functional domains of ETV6 gene and validated germline variants identified so far. In red the novel variants analyzed in the present study; in red boxes the two mutations used as controls. Adapted from *Savoia et al.,2017* (7).

Moreover, the Combined Annotation Dependent Depletion (CADD) scores were high for the five variants that reside in the ETS domain (range 26.7- 35) and consistently with this, other bioinformatic tools predicted a higher impact on the proteins function naming the aminoacidic substitutions as “deleterious” (D), “medium” (M) or “high”. (H). Instead, the CADD scores for H224Q and S22N were lower predicting a likely non pathogenic (“benign” or “tolerated”) role for the encoded products (Table 3).

H224Q was a curious case since the *ETV6* variant co-segregates within the family F11 with the c.206C>T variant in the *GP1BA* gene, known to be responsible for another form of thrombocytopenia called Bernard Soulier Syndrome (BSS). The proband carrying both variants showed a phenotype consistent both with *ETV6*-RT thrombocytopenia as well as the heterozigous form of BSS; therefore it has been included in the study in order to investigate if it could be a

poligenic case or alternatively, if *ETV6* could be the sole responsible for the onset of the thrombocytopenic condition since the defect is close to the P214L pathogenic control mutation.

Despite the bioinformatic evidences such as the absence or the presence in public annotation databases (dbSNP taken as representative) that strongly support or exclude the pathogenicity for the variants considered in this study, a functional characterization is needed since the *in silico* prediction, turned out to be wrong in many cases (24).

Nucleotide	Protein	dbSNP	PROVEAN (ex SIFT)	Polyphen-2	Mutation Assessor	CADD
c.641C>T	p.P214L	rs724159947	N	Possibly damaging	M	22.7
1252A>G	p.R418G + p.N385Vfs*7	rs786205226	D	Probably damaging	M	29.3
c.1105 C>T	p.R369W	n.r.	D	Probably damaging	M	35
c.1138T>A	p.W380R	n.r.	D	Probably damaging	H	31
c.1186A>G	p.R396G	n.r.	D	Probably damaging	H	26.7
c.1040A>C	p.Q347P	n.r.	D	Probably damaging	M	27.4
c. 1196G>A	p.R399H	n.r.	D	Probably damaging	M	34
c.65G>A	p.S22N	n.r.	T	Benign	L	15.37
c. 672C>G	p.H224Q	rs781065435	N	Benign	N	12.46

Table 3: Bioinformatic analysis for predicting the functional effect of *ETV6* variants : Effect of variations was evaluated using three pathogenicity prediction programs: Polyphen-2 , PROVEAN (ex SIFT) ,Mutation Assessor and the CADD score.
PROVEAN legend: N: neutral, D: deleterious, T: tollerate.
Mutation Assessor: L: Low, M: medium, H: high.
CADD score: threshold for deleteriousness >15.

4.4 ETV6 mutations are no longer able to repress its targets

After the generation of the expression vectors and the assessment of the correct protein expression of the wt and mutant forms of ETV6 by immunoblotting (western blot), we performed a reporter assay in order to evaluate the functional consequences of the proteins, specifically measuring the luciferase expression induced by the promoter of the known ETV6 target Stromelysin-3 (MMP3) (15). ETV6 functions as a trancriptional repressor of promoters harboring ETS binding sites therefore luciferase reporter assay is an appropriate tool for the study of gene expression at transcriptional level and in addition to being a unexpensive tool to obtain rapid quantitative measurements.

As shown in Figure 3, we observed that wt ETV6 repressed the luciferase expression by approximately 50% compared to the empty vector, whereas Q347P, R369W, W380R and R396G showed to loose the repression ability as observed for the two mutations used as control (P214L and R418G). The four mutations tested in our assay bind the ETS binding domain so it is easily conceivable that the loss of the repressive ability can be due to an affected DNA binding although the same loss of repression has been observed for P214L that hits the CRD, expected to be non functional for the repressive activity. Our results recapitulate those of Zhang et al. in which two missense variants that hit the ETS domain have been analyzed considering P214L as control. In light of these evidences they have proposed a dominant negative (DN) effect demonstrating by a reporter assay that ETV6 mutants antagonized the repression mediated by the wt in a dose dependent manner. It is possible that, instead of a dysfunction in DNA binding, missense mutants could inhibit transrepression of the targets by forming dysfunctional wt-mutants complexes since the PNT domain mediates the homodimerization of the functional ETV6 transcription factor (8). In fact, it would be interesting to clarify whether the DN underlying mechanism could be an impaired cellular localization of the wt ETV6 in the presence of its mutant forms.

On the contrary, the repressive activity was very clear for S22N and H224Q that is comparable to the wt, demonstrating that the two variants don't have any effect on repression of the target. Even if the result about S22N was partially expected due to the bioinformatic prediction and its location that was both out of the functional domains and the CRD, the functional analysis was recommended.

Although the H224Q change was predicted benign by the bioinformatic tools, the repressive effect couldn't be taken for granted since the variant affected an aminoacid very close to the control

mutation P214L, in the CRD. Nevertheless our functional study definitely led to exclude *ETV6* as responsible for the thrombocytopenia shifting the focus on the possible pathogenic role of *GP1BA* variant, for which further functional studies will be carried out.

Another surprising case was about the variant R399H that showed a regular marked repressive activity recapitulating a wt behaviour. This result was in contrast with the bioinformatic prediction that considered R399H as "damaging" coupled with a high 34 CADD score. Moreover, in addition to being located in the ETS domain, R399H affects the same aminoacidic residue of a known pathogenic mutation, that hits the preceding nucleotide (c. 1195C>T, p.R399C).

Although impressive, an opposite functional impact of two aminoacid changes on the same residue had already been demonstrated at least for another gene (*ACTN1*) responsible for the onset of another form of IT (*ACTN1*-RT). Once again, this result supports the importance of the functional evaluation of the novel variants identified in the context of ITs (24).

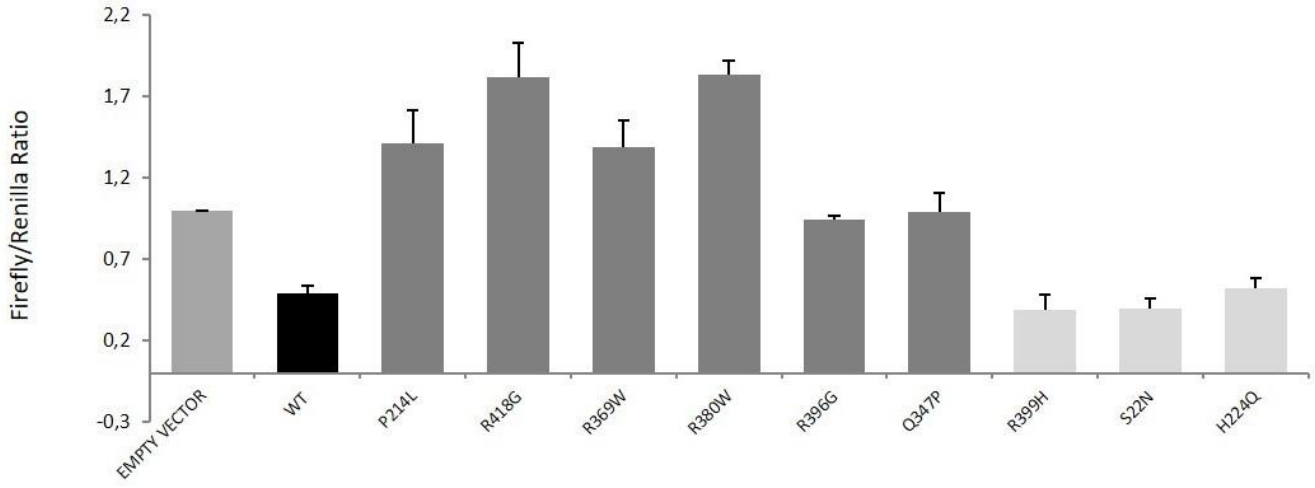

Figure 5. Effect of ETV6 mutations on transcription. Luciferase activity under the control of the MMP3 promoter after transfection of the wild type (ETV6 WT in black), the mutated forms of ETV6 (grey) and the neutral variants (light grey) in HEK cells. Histograms represent the means of three replicates for each condition and repeated three times. Statistical analysis was done using an unpaired t-test, the error bars show the standard deviations. *=p<0,05; **=p<0,01; ***=p<0,001.

4.5 ETV6 mutations fail to enter into the nucleus

The immunofluorescence assay was set up in order to understand the mechanism by which the mutated forms of ETV6 prevent the repression of the target genes. All but one of the mutations that reside in the ETS domain have shown to be responsible of a defect in the transcription of target genes through the reporter assay. Given the position of the mutations, an impairment in DNA binding that leads to a loss of function had been logically hypothesized.

The pathogenic mechanism that underlies the control mutation P214L (CRD) is a disruption of the cellular localization because the mutant protein is retained in the cytoplasm being unable to enter the nucleus, that would be its physiological location since the nature of transcription factor. The same mechanism was surprisingly demonstated also for R418G that affects the ETS domain, suggesting that a defect compromises the cellular localization instead of the expected binding to DNA (10). However, the c.1252A>G transition induces also an alternative splicing and exon skipping product (50%), therefore it is not a suitable model for testing the functional effect of the missense variants on ETS domain. For this reason we included two positive controls in our study.

In order to better clarify this point we examined the cellular distribution of the proteins encoded by novel mutations using an immunofluorescence assay.

Staining with myc tag in cells transfected with Q347P, R369W, W380R and R396G mutant constructs showed an abnormal subcellular localization confirming that, like the control mutations, the loss of repressive activity of ETV6 mutant proteins is due to their retention in the cytoplasm. On the contrary S22N, H224Q are perfectly able to enter the nucleus as well as the wt form (Figure 6). Unfortunately, immunofluorescence of R399H is not available, due to its recent identification.

These evidences led us to hypothesize that some changes in the protein inhibit the nuclear localization that could explain a dominant negative effect. ETV6 is a transcriptional suppressor that is active in a dimeric form therefore in a context of heterozygous variations the product of the mutated allele (50%) could interact with the wt (50%) preventing its migration into the nucleus and in turn generating a condition of loss of function.

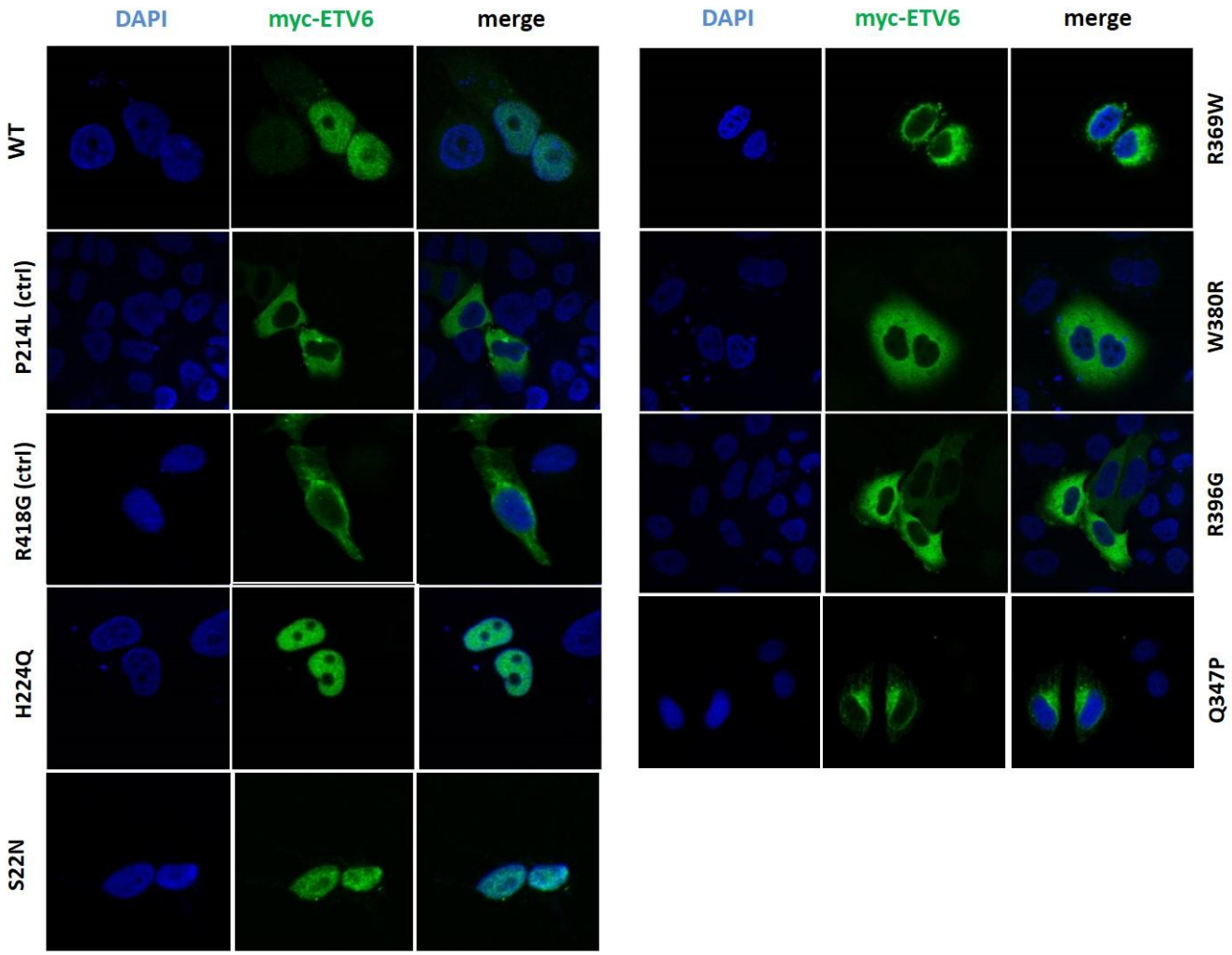

Figure 6. Immunofluorescence assay in HeLa cell line. Cellular localization of the wt and the mutant forms of ETV6. WT, H224Q and S22N are completely located in the nucleus (blue DAPI) whereas the controls (P214L and R418G) and the mutations R369W, W380R, R396G and Q347P are retained in the cytoplasm.

5. ETV6 is involved in ANKRD26 expression and regulation of some key leukemic factors: preliminary data

As previuosly reported in "Background", *ANKRD26*-related thrombocytopenia (*ANKRD26*-RT), Familial Platelet Disorder with propensity to Acute Myelogenous Leukemia (FPD/AML) and *ETV6*-related thrombocytopenia (*ETV6*-RT) are three rare autosomal dominant inherited thrombocytopenias characterized by a moderate thrombocytopenia with platelets of normal size and mild bleeding tendency, associated with an increased risk of developing hematological neoplasms. The pathological mechanisms responsible for *ANKRD26*-RT, FPD/AML and *ETV6*-RT are not completely understood, but the extreme similarity in the clinical phenotype that makes these three diseases clinically indistinguishable, suggests that the genes mutated in these ITs could interact between them.

Indeed, mutations of *ANKRD26* are all clustered in a short specific stretch in the 5' untranslated region (5'UTR) containing the consensus sequence for the binding of the transcription factor RUNX1. RUNX1 inhibits *ANKRD26* transcription during megakaryopoiesis leading to almost complete absence of mRNA in platelets. If either the 5'UTR of *ANKRD26* or *RUNX1* are mutated, *ANKRD26* expression is not downregulated, presumably because of defective binding of RUNX1 to DNA. Data demonstrated that ANKRD26 downregulation inhibits the MAPK/ERK1/2 pathway; instead, the persistent expression of ANKRD26 maintains the MAPK/ERK1/2 pathway active, causing a severe impairment in proplatelet formation (25). Recently, it has been observed that in human glioblastoma *RUNX1* regulates metastatic invasion through the MAPK pathway (26).

Considering that FPD/AML and *ANKRD26*-RT are interconnected, and that *ETV6*-RT is clinically indistinguishable from the other two diseases, we hypothesized that even *ETV6* belongs to the same pathway controlling directly or indirectly the transcription of *ANKRD26* gene during hematopoiesis.

In order to investigate if ETV6, like RUNX1, can control the expression level of ANKRD26, we performed reporter assay experiments in HEK cells testing the activity of the ANKRD26 and MMP3 promoters cloned upstream of the luciferase gene after transfection of *ETV6* cDNA. Similarly to data obtained with the MMP3 promoter, expression of ETV6 generates a significant decrease in luciferase activity when the reporter gene is under the control of the ANKRD26 promoter (Figure 7).

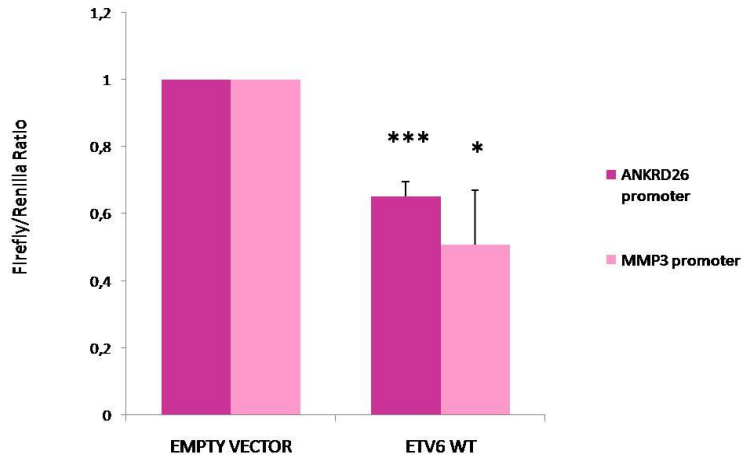

Figure 7. **ETV6 regulates ANKRD26 expression.** Comparison of gene reporter assay between the luciferase gene controlled by ANKRD26 and MMP3 promoters after transfection of wild type ETV6 in HEK cells. *=p<0,05; ***=p<0,001.

To further explore the effects of *ETV6* we have preliminarly evaluated its role on the regulation of the two oncosuppressors EGR1 and TRAF1 known to be downregulated in leukemic context (27,28). The expression levels were evaluated by qPCR with the use of validated primers (11) and the experiment was carried out in HEK cells transfected with ETV6 wt and the control mutation P214L. Consistent with previous studies (11) levels of EGR1 and TRAF1 are upregulated in the presence of ETV6 wt whereas there is a clear down regulation when the cells are transfected with the P214L control mutation (Figure 8). Despite preliminary findings, our data provide a first indication about the role of ETV6 in the regulation of key leukemic factors that opens up new perspectives for further investigations.

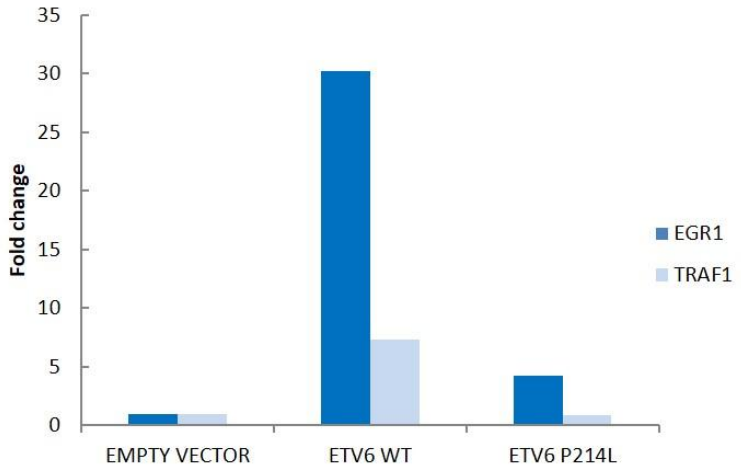

Figure 8. ETV6 regulates key leukemic factors: preliminary data. qPCR evaluation of the endogenous expression level of EGR1 and TRAF1 oncosuppressors after tranfection with wt ETV6 and the P214L mutation in HEK cell line.

6. CONCLUSIONS

Inherited Thrombocytopenias (ITs) are a group of rare genetic disorders characterized by a high grade of clinical and genetic heterogeneity except for the syndromic forms that are characterized by severe and spontaneous hemorragies. Although most of the ITs show a mild thrombocytopenia that doesn't compromise the quality of life, some of these forms expose patients to acquire additional clinical manifestations or associated illnesses that can be far more dangerous than only hemorrhages episodes.

Within these mild forms a group of ITs characterized by an increased risk of developing myeloid neoplasms including *ANKRD26*-RT, FDP/AML and *ETV6*-RT has been recently identified.

Whereas *ANKRD26*-RT and FDP/AML are well characterized at least from a genetic point of view being known for many years, *ETV6*-RT has been described only in 2015.

For this reason my thesis is mainly focused on the genetic and clinical characterization of this latest form of inherited thrombocytopenia.

A critical aspect for the correct characterization of the disease was the setting up of a functional assay aimed to establish the pathogenic effect of *ETV6* variants identified in patients through the mutational screening.

Despite the advent of NGS techniques has extremely accelerated the screening of mutation of IT patients, it has not solved the interpretation of the functional effect of variants missense that are the most represented type of genetic variations found in patients.

The functional assays setted up during my PhD project have provided a tool able to discriminate *ETV6* pathogenetic variants from those with neutral effect as well as giving important elements for understanding the pathogenetic mechanism leading to *ETV6*-RT.

Indeed mutations of *ETV6* gene, responsible for the disease, show an aberrant subcellular localization that prevents their entrance into the nucleus therefore inhibiting the repressive physiological function.

This study highlights the fundamental importance of combining mutational screening with specific assays aimed to establish the functional effect of variants identified. These achievements have important implications both in provide a proper diagnosis and a better characterization of *ETV6*-RT from clinical and molecular point of view.

Although the scientific community has conflicting opinions regarding the importance to diagnose the IT forms associated with an increase risk of hematological malignancies in terms of genetic counseling and management of patients (22), this aspect is of fundamental importance for the

scientific research purposes. For this reason, another important topic addressed during this PhD project was focused on the study of interaction between ANKRD26, RUNX1 and ETV6.

The downregulation of ANKRD26 expression by RUNX1 was previously described in literature and we demonstrated that also ETV6 is involved in this process.

The evidence that *ANKRD26*, *RUNX1* and *ETV6* genes interact between them and the indication that ETV6 could have a role in the regulation of leukemic factors open up an important scenario regarding the knowledge of these three diseases and the mechanisms underlying their increased risk of leukemia. Confirming the hypothesis that these three forms of IT share a common defective pathway will allow to identify a common therapeutic pathway and a targeted treatment for the patients who cannot take advantage of the administration of thrombopoietin mimetic drugs successfully used in other forms of ITs.

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