PATHOLOGIC HEMATOPOIESIS: CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II, CONGENITAL ERYTHROCYTOSIS AND THROMBOCYTOPENIAS

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Pathologische hematopoiese: congenitale dyserythropoietische anemie type II, congenitale erythrocytose en trombocytopenieën

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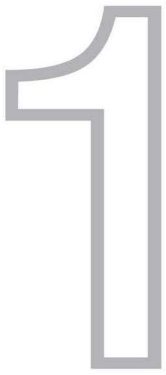

Introduction

The blood contains several different types of cells. Each of these cell types is quite distinct in appearance, and each has a specific biological function. Despite their extreme structural and functional differences, blood cells are the progeny of a single type of cell: the hematopoietic stem cell (HSC). The processes involved in the production of the various cell types of the blood from the HSCs are collectively called hematopoiesis (1).

Hematopoiesis includes HSC self-renewal, HSC commitment to specific lineages, and maturation of lineage-committed progenitors into functional blood cells. Self-renewal may occur by symmetric HSC division, such as expansion of the HSC pool during fetal life or post-HSC transplantation. Other possible fates of HSC divisions include apoptosis or mobilization to the peripheral circulation following stress such as growth factor stimulation or depletion of marrow cells by irradiation or chemotherapy. During normal steady state conditions, HSCs reside mainly in the marrow cavity, but under certain stress conditions HSCs can migrate and colonize other organs like liver and spleen in a process termed extramedullary hematopoiesis. Hematopoiesis begins early during embryogenesis and undergoes many changes during fetal and neonatal development. Unlike some organ systems that form in early life and are not continually replaced, turnover and replenishment of the hematopoietic system continue throughout life (2).

Hematopoiesis

The cells of the blood have finite life spans, which vary depending on the cell type. In humans, granulocytes and platelets have life spans of only a few days, whereas some lymphocytes can exist for many months. Cells are replaced as the older cells are removed and the newly formed, mature cells are added. The numbers of the various cell types in the blood are normally kept in relatively constant ranges. In particular, variations in the erythrocyte number are normally minimal, and values 30% above or below the norm for the population have significant health effects. Although the numbers of other blood cell types are not as constant as the number of erythrocytes, the production of all blood cells is highly regulated. The regulation of hematopoiesis is complex. Some regulatory factors influence overall hematopoiesis by affecting very early progenitor cells: the HSCs and/or their progeny that have not undergone commitment to a single cell lineage. Also, specific regulatory growth factors play key roles in fostering the production of cells in each lineage. Lineage-specific regulation is necessary because of the widely varying life spans and widely varying functions of the different mature blood cell types. Several cell types are derived from the HSCs but are not found in the blood; the final steps of differentiation of these last types of cells occur in the tissues in which they reside. Such cells include dendritic cells of the lymphoid tissues and the skin (Langerhans cells), specialized macrophages of all types, and mast cells. Figure 1 illustrates the cell types associated with hematopoiesis and their distribution among bone marrow, blood, and lymphatic tissues (2).

During prenatal development, the sites of hematopoiesis change several times (3-6). In humans and other vertebrates, the first hematopoietic cells arise during late gastrulation in the extra embryonic yolk sac in structures known as blood islands. This initial hematopoiesis is named primitive hematopoiesis and serves a supportive role to very quickly produce erythroid cells as the circulatory system is being formed. Primitive hematopoiesis is transient, occurring on embryonic days (E) 19 through week 8 in humans. Primitive hematopoiesis provides erythrocytes and macrophages, but not lymphocytes or granulocytes; evidence also exists for primitive platelets (7-11).The primitive erythrocytes are large nucleated cells that have reduced erythropoietin (EPO) requirements during their development compared to definitive erythroid cells (12) that develop later. Definitive hematopoietic tissue arises from the mesodermal tissue located in the anterior portion of the aorta-gonad-mesonephros (AGM) region.

Definitive hematopoiesis begins approximately 1 to 2 days later than primitive hematopoiesis, with colony-forming unit–spleen (CFU-S) cells. CFU-S cells are the first lineage of multipotent hematopoietic stem cells and arise in much smaller numbers in the yolk sac, also on day 9 in mice (13). Long-term repopulating hematopoietic stem cells (LTR-HSCs) are the adult-type definitive stem cells that are able to produce all lineages of hematopoietic cells over the entire life span of an animal, mouse or human. Once definitive hematopoiesis begins, lymphocytes, monocytes, granulocytes, and platelets are formed as well as definitive erythrocytes. Definitive erythrocytes differ from primitive erythrocytes in that they are smaller, enucleate prior to entry into the circulation, require erythropoietin to survive, and contain fetal and adult hemoglobins instead of embryonic globin chains. One day after appearing in the AGM, LTR-HSCs are found in very small numbers in the yolk sac, and 2 days later they are also found in the mouse liver. It is thus believed that LTR-HSCs arise in the AGM and seed the liver, the site of fetal hematopoiesis. Yolk sac–derived hematopoiesis becomes unnecessary at this point and disappears. Eventually, LTR-HSCs migrate from the fetal liver to the bone marrow in the circulation, and the bone marrow becomes the primary site of hematopoiesis, with a very small reserve of stem cells remaining in the liver. Under stress conditions of anemia or hypoxia, certain grow factors are induced and they lead to proliferation of erythroid cells (8, 14-17).

Erythroid Cells

Commitment of Stem Cell Progeny to Erythroid Differentiation

The work of Till and McCulloch (18) has provided experimental evidence for the presence in the bone marrow of cells capable of both self-renewal and production of progenies with potential for differentiation into red cells, granulocytes, and megakaryocytes. The cell type that gives rise to these colonies was termed multipotent hematopoietic stem cell.

These stem cells can restore the hematopoiesis of the recipient mice (19). Factors that affect commitment of stem cell progeny into a specific differentiation pathway are poorly understood and generally undefined (20, 21). Although expression of lineage-

specific transcription factors is the earliest molecular event associated with commitment toward a specific line, the stimuli responsible for their expression remain undefined. It is generally accepted that commitment and differentiation are irreversible events. A differentiated cell cannot regress to an undifferentiated stage or change into another differentiation pathway. Under normal conditions, once commitment occurs, differentiation proceeds fully to the stage of mature cell, which, in the case of blood cells, has a limited life-span. Thus, differentiation is a process that leads to cell death. These concepts are generally well proven for the mature, morphologically recognizable hematopoietic cells and their progenitors, such as the marrow erythroblasts and their erythroid progenitors, but whether they apply equally to more immature cells is less well known. There are three major theories that address the process of commitment of stem cell progeny into a specific differentiation pathway (22). According to the stochastic theory, commitment is a random event that progressively restricts the potential for differentiation (18). This theory allows for regulatory factors to act only at later stages of hematopoiesis. This model derives its experimental support from the non normal (non-Poisson) distribution of lineage-specific cells in CFU-S–derived colonies (18) and in colonies derived from multilineage progenitor cells (23), as well as from the identification in vitro of progenitor cells with bipotent differentiation potential such as erythroid/megakaryocytic, erythroid/eosinophil, or neutrophil/erythroid (24-26). The second theory that of the hemopoietic-inductive micro environment, proposes that commitment of stem cell progeny to a specific pathway depends on the environment that surrounds each hematopoietic stem cell (27). This model is based on the sequential analysis of colony type in spleen colonies (CFU-S) and received further support by recent experiments with purified stem cells (19). The third theory proposes that commitment depends on humoral factors that compete among themselves at the stem cell progeny level in promoting differentiation toward one specific pathway (28, 29).

Erythroid Progenitors

Erythroblasts in the bone marrow are generated from proliferating and differentiating earlier, more immature erythroid cells termed erythroid progenitors. These progenitor cells cannot be identified morphologically, but they are detectable functionally by their ability to form in vitro colonies of erythroblasts (30). The development of tissue culture techniques for cloning hematopoietic progenitor cells in semisolid culture media in vitro has led to the recognition in the human and murine bone marrow of at least two erythroid progenitors, the colony-forming unit–erythroid (CFU-E) and the burst-forming unit–erythroid (BFU-E). Under the influence of EPO, these progenitors can grow in semisolid culture media and give rise to colonies of well hemoglobinized erythroblasts (31, 32).

Erythrocytes

The anuclear mature human erythrocyte is one of the most highly specialized of cells. Lacking such cytoplasmic organelles as nucleus, mitochondria, or ribosomes, the red

cell is unable to synthesize new protein, carry out the oxidative reactions associated with mitochondria, or undergo mitosis. More than 95% of the cytoplasmic protein is hemoglobin. The remainder includes those enzymes required for energy production and for the maintenance of hemoglobin in a functional reduced state. However, the erythrocyte expresses a surprising number of proteins that serve functions associate with other cells, including a variety of transport proteins, adhesion molecules, receptors, and signaling pathways. Thus, the red cell is now recognized as performing a number of crucial and complex functions in the human body (1).

The normal human erythrocyte has the shape of a flattened, bilaterally indented sphere, a shape often referred to as a biconcave disc (Fig. 2). In fixed stained blood smears, only the flattened surfaces are observed; hence, on fixed blood films the erythrocyte appears circular, with a diameter of about 7 to 8 micron and an area of central pallor corresponding to the indented regions (1-3). Average values for the mean cellular volume in normal subjects range from 85 to 91fl, depending on the combination of methods used. The variation in cell size can be documented by means of a frequency distribution curve of red cell volumes generated from the output of a Coulter counter. Ninety-five percent of normal red cells are between about 60 and 120 fl in volume (1, 6). However, some workers have challenged these values using transmission electron microscopy and stereology. They estimate that the true volume of mature red cells is only 44 fl, and that only 51% of the volume of the red cell column observed in a hematocrit tube is occupied by erythrocytes. Total hemoglobin content and red cell volume vary considerably more than does hemoglobin concentration. It has been proposed that mature red cell size and hemoglobin content are primarily dependent on erythroid precursor cell size at the last cell division during erythropoiesis (30). Reticulocytes are 24% to 35% larger than mature red cells, although they have similar total hemoglobin content (and thus a lower hemoglobin concentration). The disc shape is well suited to erythrocyte function. The ratio of surface to volume approaches the maximum possible value in such a shape, thereby facilitating both gas transfer and deformability as the red cell traverses the microcirculation. The erythrocyte can pass through a vessel of about 4 micro m in maximum diameter. Erythrocyte shape may also vary between large and small vessels and under conditions of high or low shear stress (1).

Megakaryocytes

Platelet production begins in the yolk sac (33) and, like the remainder of hematopoiesis, shifts to the fetal liver and then to the marrow at the time of gestation. Platelets have a circulatory half-life of 10 days. The platelet count varies among the healthy population but remains within a narrow range in any individual. In times of increased demand, platelet production can raise 10-fold or more. It is hoped that a thorough understanding of the processes through which megakaryocytes develop and generate thousands of platelets that after 10 days undergo programmed cell death (PCD) will clarify the underlying mechanisms responsible for their pathologic disruption

and aid in devising better strategies to intervene in those conditions associated with reduced, enhanced, or disordered megakaryocyte or platelet function, or both (34).

Megakaryopoiesis

The basic concepts of a hierarchic organization of stem and progenitor cells leading to mature blood cell production were formulated by Till and McCulloch in the early 1960s (35), although the concept of a common "mother cell" of all blood elements in the adult dates to Danchakoff in 1916 (36). The capacity to transplant marrow cells and reconstitute all of hematopoiesis in lethally irradiated recipients provided an in vivo assay for the hematopoietic stem cell (HSC), but it was not until the development of clonal in vitro assays of hematopoietic progenitors that a coherent model of blood cell production emerged. The pioneering work of Pluznikand Sachs (37) and of Bradley and Metcalf (38) provided a convenient method to enumerate and characterize marrow cells committed to various hematopoietic lineages. These investigators independently developed culture conditions that allowed colonies of cells to develop from single progenitors. However, the first hematopoietic colonies were composed of leukocytes. Due to the more difficult conditions required for megakaryopoiesis in vitro, the description of clonal megakaryocyte growth did not occur for another decade or more (39-42). Recent work using density fractionation, cell sorting, and fluorescent dye exclusion methods has yielded purified populations of stem cells (43-47), common myeloid (48) and lymphoid (49) progenitors, and lineage-restricted hematopoietic progenitors (50, 51); these methods have greatly advanced understanding of the cell and the molecular biology of megakaryocyte development. Culture conditions that support the proliferation of megakaryocytic progenitors have been described using methylcellulose, agar, or a plasma clot assay. Multiple investigators have demonstrated two colony morphologies that exclusively contain megakaryocytes. The colony-forming unit–megakaryocyte (CFU-MK) is a cell that develops into a simple colony containing 3 to 50 mature megakaryocytes (39-41). Larger, more complex colonies that include satellite collections of megakaryocytes and contain up to several hundred cells are derived from the burst-forming unit–megakaryocyte (BFU-MK) (42, 43). Such lineage-restricted colonies have been described using marrow cells from both human and murine sources. Because of the difference in their proliferative potential and by analogy to erythroid progenitors, BFUs-MK and CFUs-MK are thought to represent primitive and mature progenitor cells restricted to the megakaryocyte lineage. Human marrow BFUs-MK are CD34+ and develop into multifocal collections of at least 100 megakaryocytes within approximately 21 days in culture (42, 43, 53, 53). CFU-MK–derived colonies are morphologically simpler, containing as few as three megakaryocytes and developing in 10 to 12 days (39-42, 54, 55). Recently, the close relationship of the erythroid and megakaryocyte lineages was reinforced by the identification of a mixed erythroid-megakaryocyte progenitor (56-60). It is unlikely that all CFUs-MK and BFUs-MK arise from colony-forming unit–erythroid-megakaryocytes, although definitive evidence has not been presented yet. More recently, fractionation

methods have been devised that yield purified populations of CFUs-MK for functional and biochemical analysis (51).

Platelets

Structure and Function

Light microscopy of Wright-stained smears (Fig. 3) reveals platelets as small, anucleate fragments with occasional reddish granules, measuring approximately 2 micron diameter with a volume of approximately 8 fl (34) and exhibiting considerable variation in size and shape. Platelets released from the marrow under "conditions of stress" such as thrombocytopenia and termed stress platelets are large and often beaded in shape, whereas young platelets, recently released from the marrow, are termed reticulated in reference to their RNA content and in analogy to young red cell reticulocytes (61).

Platelets exist in two distinct forms, resting and activated, with the resting state marked by baseline metabolic activity and the activated form resulting from agonist stimulation (i.e. response to thrombin). By scanning electron microscopy, circulating resting blood platelets appear as flat discs with smooth contours, rare spiny filopodia, and random openings of a channel system, the surface-connected canalicular system (SCCS), which invaginates throughout the platelet and is the conduit by which granule contents exocytose after stimulation (34, 61). Although the platelet is anucleate, transmission electron microscopy reveals a complex surface and a cytoplasm packed with a number of different sub platelet structures and organelles that are essential to the maintenance of normal hemostasis. Platelet structure is classified into four general areas: the platelet surface, membranous structures, cytoskeleton (solgel zone), and granules (34).

Another important platelet structure is a layer of lipids, sugars, and proteins, 15 to 20 nm thick that coats the outside surface of the platelet plasma membrane, including the SCCS, and interacts with both the plasma and the cellular components of the blood and blood vessels. This platelet structure is termed glycocalyx.

The layer provides a transfer point for plasma proteins such as fibrinogen as they are taken up into secretory granules by endocytosis (39). The glycocalyx contains glycoproteins, glycolipids, mucopolysaccharides, and adsorbed plasma proteins (61) and produces a negatively charged net on the surface, mainly because of the presence of sialic acid residues on certain proteins such as GpIb (40). This charge is thought to minimize attachment of circulating platelets to each other and to vessels. Being rich in the extra cellular regions of adhesive glycoproteins and agonist receptors, the glycocalyx is a fundamental participant in all aspects of platelet function (41).

Pathologic Hematopoiesis

The process of hematopoiesis previously described in short, is complex and tightly regulated, many things can go wrong during this process, and this will result in a variety of diseases that we can group in 3 main branches: diseases affecting red cell production, affecting white cell production or affecting platelets.

Among the diseases affecting red cell production there are many types of anemias. They can be genetically determined or acquired, and caused by reduced red blood cell number, altered function or shortened cell survival. In addition, increased red cell number leads to erythrocytosis which can be acquired or due to a genetic cause.

Among the diseases affecting the platelet production, there are the thrombocitopenias, which can be due to a genetic cause or or not (acquired). Thrombocytopenias, similar to anemias can be due to shorten cell survival, reduced cell number or altered cell function.

Here we discuss 3 genetic diseases that are caused by congenital defects in the normal hematopoiesis process and manifest during paediatric age: Congenital Dyserythropoietic Anemia Type 2, Congenital Erythrocytosis (due to mutations affecting the Oxigen Sensing Pathway) and Thrombocytopenias: Autosomal Dominant Thrombocytopenia (THC2) and Idiopatic Thrombocytopenia Purpura.

CDAs (Congenital Dyserythropoietic Anemias)

The group of congenital dyserythropoietic anemias consists of three entities:

CDAI, is characterized by megaloblastic changes, ineffective erythropoiesis and nuclear abnormalities of erythroblasts on electron microscopy. Hemoglobin levels are variable with mean hemoglobin levels between 8–11 g/dl. The disorder follows an autosomal recessive inheritance. About 10% of CDA I patients present skeletal abnormalities associated with the disorder (62). After linkage analysis (63) the gene defect in CDA I could be characterized as codanin-1 (CDAN1) (64); the function of the gene product is unknown. Several patients show a response to alpha-interferon therapy with increased blood hemoglobin levels and decreased iron overload. More than 150 patients suffering from CDA I are known (65).

CDAIII, is a more heterogeneous disorder consisting of subtypes with an autosomal recessive inheritance or sporadic occurrence. Most data are derived from a large Swedish family. Patients show dyserythropoiesis with giant multinucleated erythroblasts. In most cases anemia is mild and does not require transfusions. In contrast to other types of CDA patients present no relevant iron overload. Only a few patients with CDA III have been described. The genetic defect is unknown although mapping analysis located the CDA III gene to a 4.5 cM interval at chromosome 15p23 (66).

CDAII (Congenital Dyseritropoietic Anemia type 2)

Congenital dyserythropoietic anemia type II (CDA II) is the most frequent member of the congenital dyserythropoietic anemia family (OMIM 224100). The main European Registries (German, Italian and French) have counted 367 patients (67). CDAII patients show characteristic biochemical and bone marrow changes that can be used for diagnostic purposes. A common finding in all typical CDA II patients is an impaired glycosylation of erythrocyte membrane proteins (68).

Clinically CDAII patients present mild to severe anemia, jaundice and splenomegaly. Red cell size is mostly normocytic and the mean hemoglobin concentration is 9.1–9.8 g/dl (69). Most CDAII patients do not require regular transfusions. However, up to 15% of the patients show transfusion dependent anemia and some patients were treated by bone marrow transplantation (70). Splenomegaly is present in 50–60% of CDAII patients. Beyond the age of 20 most patients develop iron overload and some patients develop liver cirrhosis, diabetes and heart failure (69, 71).

Bone marrow samples show characteristic changes: distinct hypercellularity due to erythroid hyperplasia with 45–90% erythroid precursors. In CDAII, 10 to 45 percent of all erythroblasts are bi- and multinucleated (72).

CDAII Band 3 (anion exchange protein 1) and band 4.5 (glucosetransporter 1), two abundant erythrocyte membrane proteins, show a sharper band and faster migration on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) due to decreased glycosylation (73, 74). The main erythrocyte membrane protein represents band 3 comprising 25–30% of total erythrocyte membrane protein (75).

In 1972 was described for the first time a phenomenon named “Double membranes” in late erythroblasts and some erythrocytes from CDAII patients (76). In 1996, Alloisio et al. proved the inner membrane fragments to be endoplasmic reticulum using western blot and electron microscopic studies (77). The elimination of organelles is a unique feature of erythroid cell maturation. As described before, late in erythropoiesis the erythroblast loses its nucleus and becomes a reticulocyte which further degrades internal organelles in order to become a mature erythrocyte (78). In addition to the nuclear abnormalities unique to CDAII erythroblasts, the retention of organelle residues suggests that CDAII might be a maturation disorder of erythroblasts indicating that the hypoglycosylation of membrane proteins could be a secondary phenomenon (79).

The diagnosis of CDAII is typically derived from a bone marrow cytology showing the characteristic changes of erythroblasts described above. Due to the early description of increased hemolysis of CDAII erythrocytes under acidified conditions and after the addition of selected donor sera, hemolysis induced by established donor sera is used to verify the diagnosis in specialized laboratories (80). However, the test has a great variability in different laboratories and the sensitivity as well as specificity of the test is rather poor. Some laboratories use antibodies for the i-antigen in order to prove the diagnosis of CDAII. It is well established, that there is an increased amount of i antigen on erythrocytes of CDAII patients, resulting in an increased aggregation after addition of anti-i-antibodies. Sensitivity seems to be good in a series of 45 CDAII patients (69).

The main pitfall may be that increased presentation of the i-antigen is also present in other disorders going along with increased erythrocyte turnover that might be a differential diagnosis to CDAII (81). An apparent doubling of the cell membrane of some erythrocytes of CDAII patients was found as a typical feature in CDAII. As mentioned above, the doubling is due to residual membranes of endoplasmic reticulum that normally is eliminated during erythropoiesis (77). Thus, electron microscopy studies of CDAII erythrocytes might be helpful for the diagnosis of CDAII. However, sensitivity and specificity of this phenomenon are unknown, especially with respect to other disorders affecting erythropoiesis. Another test for CDAII is based on the detection of endoplasmic reticulum proteins in membrane preparations of erythrocytes using SDS-PAGE. Already in the first years after the first description of CDAII as a distinct entity, an altered mobility of band 3 (anion exchange protein 1) and band 4.5 (glucose transporter 1) on SDS-PAGE was described (73). This increased mobility of band 3 as well as the decreased band width on SDS-PAGE is used as a diagnostic criterion. This criterion is a reliable, indirect hint for decreased glycosylation of band 3 although the quantification is subjective and sometimes ambiguous, so a positive result alone is not sufficient for the diagnosis. Heimpel et al. proposed that the diagnosis of CDAII requires evidence of congenital anemia, ineffective erythropoiesis and typical bone marrow findings and at least one of the biochemical criteria: positive acidified serum test, typical abnormalities in SDS-PAGE of erythrocyte membranes or double membrane of late erythroblasts (69).

For over a period of 30 years biochemical studies did not clearly reveal a candidate gene, in 1997 Gasparini et al. performed a genome-wide linkage analysis of twelve Italian families and one French family suffering from CDAII with the typical clinical and biochemical picture of the disorder. A candidate region on chromosome 20q11.2, termed CDAN2 locus with a maximum two-point LOD score of 5.4, spanning 5 cM, was indicated as containing the CDAII gene (82). Sequencing of candidate genes within this area performed by the same group did not show any mutation (83). Later on, families of Italian and other origin were investigated and did not show linkage to 20q11.2 (84, 85, 86, 87). At least 10% of CDAII patients did not show linkage to the 20q11.2 area and the authors suggested that CDAII would be a heterogeneous disorder (71). In 2003, Paw et al described dyserythropoiesis in zebrafish as a sequel of mutations in the gene slc4a1, the band 3 analogue in zebrafish (88). The fish showed anemia and double membranes of some erythrocytes comparable to CDAII patients. Afterwards, Perrotta et al. performed haplotype analysis of the gene locus coding for band 3 (17q21–q22) and analysis of band 3 protein on SDS-PAGE to address the question whether band 3 alterations may cause CDAII in patients not associated with the 20q11.2 locus, but did not find genetic linkage nor abnormalities of the band 3 protein (86). A major breakthrough in CDAII research was achieved in 2009, when after a genome-wide SNP analysis Schwarz et al. and Bianchi et al. found mutations of the *SEC23B* gene in patients with CDAII. *SEC23B* is located in a region on chromosome 20, (20p11.23-20p12.1) (89, 90). Sec23B protein is an essential

component of coat protein complex II (COPII), coated vesicles that transport secretory proteins from the ER to the Golgi complex (91). So far, *SEC23B* changes have been identified mainly by direct genomic sequencing of the coding region of the gene (89, 90, 92-94). Since the initial identification of *SEC23B* mutations in CDAII patients, 59 mutations have been identified (67). Two missense mutations have been repeatedly identified in a large proportion of patients: p.Glu109Lys andp.Arg14Trp (32 % and 19 %, respectively). To date, these missense mutations account for approximately 50% of the mutant alleles in CDAII patients. Notably, evidence of a founder effect has been described for p.Glu109Lys among Israeli Moroccan Jewish patients (92). Concerning mutation type, missense (52%) and nonsense (21%) mutations are the most commonly observed followed by deletions or insertions that lead to frameshifts in the nucleotide sequence. Splicing mutations are rare, with only six mutations reported. The *SEC23B* gene appears to play a pivotal and probably unique function in erythroid precursors (89, 90, 92-96). Although detailed genetic analyses have been conducted, the effects of the mutations on mRNA content in erythroblast cells have not been documented. Moreover, no data were available about the effects of mutations on Sec23B protein content in red cell precursors. In ***Chapter 2*** we describe the finding of 4 new *SEC23B* mutations and we demonstrated the reduced *SEC23B* gene expression in lymphocytes mRNA and erythroid precursors mRNA.

Congenital Erythrocytosis

We refer to erythrocytosis when the red cell mass is raised and the haematocrit is elevated above normal range. The causes of an absolute erythrocytosis can be primary where there is an intrinsic problem in the bone marrow and secondary where there is an event outside the bone marrow causing erythropoiesis. This can further be divided into congenital and acquired causes. There is also an unexplained group of erythrocytosis classified for this reason as “Idiopathic”. The erythropoietin level provides some guidance as to the direction in which to proceed and the order and extent of investigation necessary in an individual patient. An elevated hemoglobin or hematocrit (Hct) raises the possibility of an erythrocytosis. The Hct reflects whole blood viscosity most accurately (97) but it can be underestimated by some analysers in the presence of iron deficiency (98). In this thesis we are going to discuss the Congenital Erythrocytosis.

In the Congenital types of erythrocytosis a genetic abnormality has been identified and the disease usually is present since paediatric age because of course, the mutation has been present from birth. There will often be a family history as the genetic defect is inherited although the possibility of de-novo mutation exists. The patients who appear to have a congenital cause fall into one of two sets, those with Erythropoietin (EPO) levels below the normal range that can be assumed to have defects of the EPO signalling pathway while those with inappropriately normal for the haemoglobin level or elevated EPO levels may have defects of the oxygen sensing pathway.

EPO attaches to its receptor and initiates a series of events which leads to gene transcription and ultimately production of more red cells. This process is switched off whenever sufficient red cells have been produced by binding of SHP-1. Truncation of the EPO receptor results in failure of attachment of SHP-1, on going production of red cells and thus erythrocytosis. Another cause of erythrocytosis might be the presence of high oxygen-affinity haemoglobin. During oxygenation haeme iron moves into the plane of the porphyrin ring and this results in change in the shape of the globin chains. A change in one globin chain will facilitate the uptake of oxygen by the other chains. The capacity of a haemoglobin to deliver oxygen to the tissues is expressed by the shape of the haemoglobin-oxygen dissociation curve and shift of the curve to the left indicates an abnormal haemoglobin which has increased affinity for oxygen (so called high oxygen affinity haemoglobin) (99). Haemoglobin variants can be detected by electrophoresis but 20–25% are electrophorectically silent. The globin genes can also be fully sequenced (100).

Here we focus on congenital erythrocytosis (congenital polycythemia) that are due to mutations in the genes in the oxygen sensing pathway. One of them is the Chuvash erythrocytosis (*VHL* gene mutation).

A large group of individuals were identified in the Chuvash region of Russia all of whom had erythrocytosis. Genetic research identified that affected individuals were all homozygous for a single mutation C598T, leading to an amino acid 200 arginine changed to tryptophan in the von Hippel Lindau (*VHL*) gene (101). The *VHL* gene has an important role in hypoxia sensing, where cells sense a decrease in oxygen and allow the organism to adapt. The protein hypoxia inducible factor (HIF) has a central role in this process. It exists in three isoforms HIF-1α, HIF-2 α and HIF-3 α. Oxygen activates prolyl hydroxylase which then hydroxylates HIFα. This leads to the binding of VHL protein and ubiquitination of HIF and destruction of the proteins. In contrast, in hypoxia, HIF-α associates with its beta subunit; the complex binds to hypoxia responsive elements within the genome and this event lead to the activation of downstream genes and increased production of proteins such as EPO. The Chuvash variant of familial polycythemia was first described in more than 100 individuals from about 80 families living in the mid-Volga River region of European Russia (102). The disease is characterized by a high hemoglobin level, increased plasma erythropoietin (Epo) level, varicose veins, pulmonary hypertension, vertebral hemangiomas, low blood pressure, and an elevated serum concentration of vascular endothelial growth factor (VEGF). Patients affected by Chuvash polycythemia die early, mainly as a result of cerebral vascular events or peripheral thrombosis. These injuries seem to be linked to mechanisms other than blood hyperviscosity or serum Epo content (103). Indeed, the prevalence of low blood pressure in patients with Chuvash polycythemia contrasts with the hypertension frequently associated with polycythemia vera and other familial polycythemias resulting from excess of Epo. We identified another large cluster with the same mutation in the island of Ischia, Italy (104) (Chapter 2 of this Thesis). It appears that all these subjects are descendents from a common founder (105).

Patients with erythrocytosis have also been described who are compound heterozygotes for the Chuvash mutation and other *VHL* mutations (106). Interestingly there are also a number of individual who are heterozygous for one *VHL* mutation, the other allele is active and erythrocytosis is present. Mechanisms leading to disease in these heterozygous patients are unknown and are under investigation.

A number of different genes in the oxygen sensing pathway have been shown to be mutated and cause erythrocytosis, among them are the prolyl hydroxlases (PHD) genes. These genes, in conditions of normoxia, hydroxlate the α-subunits of HIF which is the first step in the degradation of HIF. A mutation in one of the *PHD*s interferes with this process and drives HIF down the hypoxia pathway and lead to increased EPO and erythrocytosis. A family with a very mild erythrocytosis has been described with a mutation in the *PHD2* gene resulting in a proline to arginine change at codon 317 (107). Another family with a *PHD2* mutation resulting in an arginine to histidine change at position 317 shows gain of function of the PHD2 activity in vitro (108).

Also mutations in *HIF-α* have been found to be a genetic cause of congenital Erythrocytosis. The transcription factor *HIF α* is hydroxylated by the PHDs and then degraded by VHL. In hypoxic conditions hydroxylation is inhibited. A family with erythrocytosis and elevated EPO levels in three generations was found to have a mutation of the oxygen degradation domain of HIF-2α resulting in a change of glycine to tryptophan at amino acid 537. In vitro studies showed that this mutation would result in major change in function of the protein (109). Further mutations in HIF-2α have been described. (***Chapter 3*** of this Thesis)

Thrombocytopenias

Genetic defects of the megakaryocyte lineage give rise to bleeding syndromes of varying severity. Blood platelets are unable to fulfil their hemostatic function of preventing blood loss on vessel injury because of an altered function or because of a reduced number. Here we describe 2 diseases due to reduced platelet number (peripheral blood platelet count $<150X10^9$/L), named thrombocytopenias.

The more frequent cause of thrombocytopenia is Idiopathic thrombocytopenic purpura (ITP). This is an autoimmune disorder characterized by thrombocytopenia due to autoantibodies binding to platelet antigen(s) causing their premature destruction by the reticulo endothelial system, particularly in the spleen (110). ITP diagnosis is based on low platelet number in the absence of other hematologic abnormalities or other causes of thrombocytopenia (111). The annual incidence of pediatric ITP is about 4 to 6 cases per 100,000. About 50% of childhood ITP cases show an acute onset following a viral or bacterial infection that commonly resolves within weeks to months without treatment. Nevertheless, about one fourth of these patients go on to develop a chronic disease, defined by a platelet count less than $150X10^9$/L at six months after diagnosis (112, 113). Although the immunopathogenesis of ITP is autoantibody mediated, the exact mechanism of immune dysfunction is not known. However, there is substantial

evidence to suggest that T cells and their cytokines play a pivotal role in the control of anti platelet autoantibodies (114, 115). A number of T-cell abnormalities have been demonstrated in patients with ITP and three main mechanisms have been hypothesized: i) a T-helper (Th)1 bias compared with Th2, particularly in chronic ITP; ii) the release of cytokines that interfere with megakaryocyte maturation and/or platelet release; and iii) a direct cytotoxic effect of T cells (116). T cells, as well as all other cellular components of the immune system, express cannabinoid receptors type 1 and 2 (CB1 and CB2). The endocannabinoid system is also involved in immune regulation by suppressing cell activation, modulating Th1 and Th2 balance, and inhibiting pro-inflammatory cytokine production (117-119). CB2 is encoded by the CNR2 gene, mapping on 1p36.11 (GeneID 1269; GenBank: NM_001841.2). Genome scan studies revealed a key role of the 1p36 region in different autoimmune diseases (120-122). In ***Chapter 6*** we address the question whether the *CNR2* gene variation rs35761398 (Q63R) is associated with childhood chronic ITP.

More rare causes of Thrombocytopenias are the ones due to a single gene mutation, also called Inherited Thrombocytopenias.

Inherited Thrombocytopenias comprise a variety of rare disorders that result from defects of platelet production or shortened platelet survival. Although many forms have been characterized and a diagnostic algorithm has been proposed and validated to facilitate their diagnosis, many patients with familial thrombocytopenia do not fall into the category of any defined disease (123). Approximately 50% of patients remain without a definite diagnosis, which suggests that they are affected with novel forms of these disorders (124). Most of these patients manifest a non-syndromic, isolated thrombocytopenia without any apparent abnormality of platelet morphology or function. This kind of Thrombocytopenia is usually associated with autosomal dominant inheritance (125). It might be very difficult to distinguish them from subjects with Idiopathic Thrombocytopenic Purpura or even impossible when no other family members are affected and no previous blood count demonstrates that their thrombocytopenia was present since birth. Thus, patients with indefinite genetic thrombocytopenias are at risk of misdiagnosis and unnecessary therapies (126). The clinical and molecular characterization of any new forms of Inherited Thrombocytopenia is an important achievement since it allows differential diagnosis between inherited and acquired forms and facilitates treatment. For example, Congenital Amegakaryocytic Thrombocytopenia is always presents with severe thrombocytopenia at birth and rapidly progresses to trilineage bone marrow failure that benefits from bone marrow transplantation (127). Severe thrombocytopenia at birth is also present in “Thrombocytopenia with absent radii,” but platelet count improves over the first year of life and eventually approaches normal levels in adult life (128). Thus, only supportive treatment is usually required. Recently, thrombopoietin (TPO) mimetics were shown to increase platelet count in *MYH9*-related disorders (129), opening up new therapeutic possibilities for these illnesses. Some Inherited Thrombocytopenias, such as familial platelet disorder with propensity for myeloid malignancy, significantly

increase the risk of leukemia (130), whereas others, such as MYH9-related disorders (131), expose the patients to the risk of extra hematologic defects that may benefit from early recognition and appropriate treatment (132). But as mentioned above making a definite diagnosis is not possible in several patients because their disorders have never been described. An analysis of a series of 46 consecutive patients revealed that these "new" illnesses affect nearly 40% of patients (124). Because prognosis and treatment remain poorly defined in a large portion of cases, the identification and characterization of "new" forms are important objectives in present research.

In the last years great advances have been made in the identification and characterization of an autosomal dominant form of Thrombocytopenia defined as Thrombocytopenia 2 (THC2, MIM 188000). This form presents mild thrombocytopenia was originally described in only 2 families, one from United States and the other from Europe (133, 134). Most affected family members reported increased bruising but did not appear to have significant bleeding issues related to surgery or child birth. Many patients from these families were often erroneously diagnosed with immune thrombocytopenic purpura (ITP), with some undergoing unnecessary splenectomy (134). Affected individuals had platelet counts reduced to 18 to 27% of their unaffected relatives. Their mean platelet volume was identical to that of their unaffected family members, and the platelet function assays, such as aggregation studies, indicated normal platelet function. Affected individuals had a statistically significant increased level of plasma thrombopoietin (THPO) as opposed to unaffected family members and normal controls (133, 134). Both families demonstrated a reduction in numbers of mature, polyploid megakaryocytes in the bone marrow, suggesting the thrombocytopenia might be due to a defect in megakaryocyte cell maturation. In one family, the bone marrow cells exhibited an increased capability to proliferate in megakaryocyte colony forming units (CFU-MK) assays, but failed to mature to polyploidy megakaryocytes in liquid culture, further indicating a failure to complete the maturation process in these patients. An increase in white blood cell counts (mainly dependent on elevated neutrophils) was also observed in several thrombocytopenic family members although there was no indication of infection. Affected individuals do not appear to suffer any other hematopoietic syndromes such as leukemia or myelodysplasia (133-135).

The THC2 locus (OMIM188000) was mapped on the short arm of chromosome10p11.1-p12 in both families (134, 135). Thereafter, Gandhi et al. (136) indicated the microtubule associate serine-threonine kinase like (*MASTL*) gene as a possible genetic cause of thrombocytopenia in a family linked to the THC2 locus. Afterwards in a zebrafish model, they described a deficiency in circulating thrombocytes after transient knockdown of *mastl* (137). Despite this finding, the members of the Italian family in which the THC2 locus was initially described did not carry *MASTL* gene mutations. In addition, we were unable to ascertain whether this gene was variably expressed in our patients as *MASTL* is not expressed in blood. No

mutations in *MASTL* were found in any of our cohort of 54 index cases, of whom 10 belong to Italian families with autosomal dominant thrombocytopenia. In ***Chapter 4 and 5*** we identify the THC2 responsible gene: *ANKRD26*, and we addressed the question about the role of *ANKRD26* in embryonic development and Megakaryocytes development knocking down its expression in zebrafish.

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Figures:

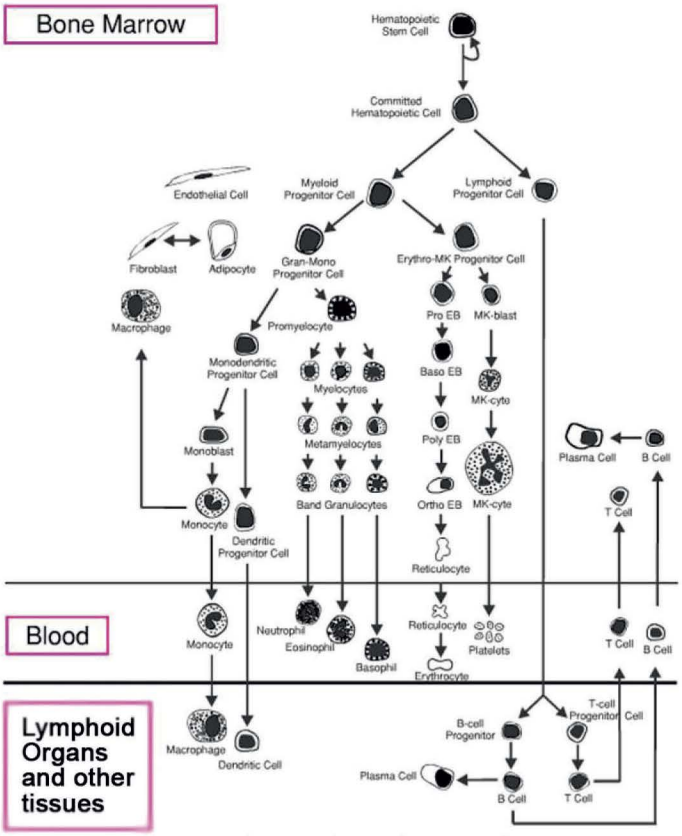

Figure 1 - The cell types associated with hematopoiesis and their distribution among bone marrow, blood, and other tissues

Figure adapted from the web

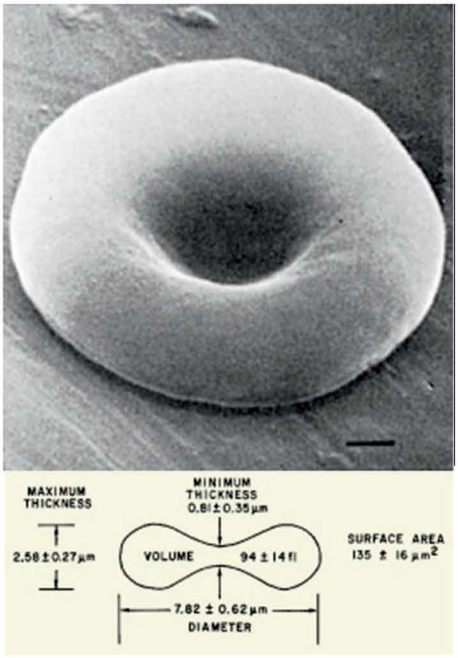

Figure 2 - Normal, mature red blood cell visualized by electron microscopy and a scheme of its dimensions - Figure adapted from the web

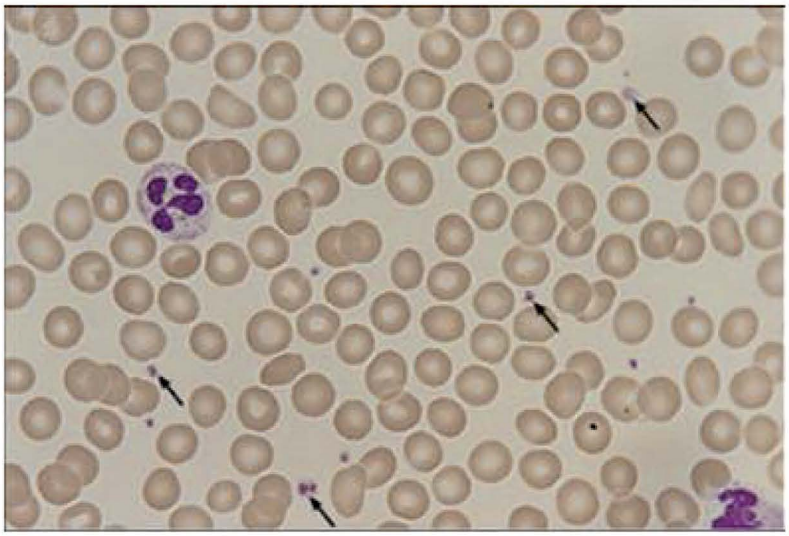

Figure 3 - Human peripheral blood smear stained with Wright-Giemsa by light microscopy. Platelets are indicated by arrows - Figure adapted from the web

Scope of the Thesis

Haematopoiesis is the process through which blood cells are produced in the bone marrow and released in the blood stream. All blood cells derive from the Hematopoietic Stem Cell undergoing a process of cell division and differentiation. Genetic factors and oxygen concentration are essential for blood cell generation. In this thesis we studied 3 rare disorders that originate due to abnormal haematopoiesis of a genetic cause and alterations in oxygen sensing pathway.

In **Chapter 1** we give an overview on normal hematopoiesis and describe the process of productions of the cells that, when present in defective or excessive number, cause the diseases described in this thesis. These cells are:

a) Erythrocytes: anucleate, biconcave discoid cells filled with haemoglobin, the major protein that binds oxygen. The erythrocytes transport the respiratory gases oxygen and carbon dioxide.

b) Platelets: very small, anucleate cells that contain molecules required for haemostasis. In addition, platelets provide haemostasis through their abilities to adhere, aggregate, and provide a surface for coagulation reactions.

In **Chapter 2** we describe mutations on the *SEC23B* gene, that cause defect of red cell differentiation, resulting in reduced red cell production and lead to Congenital Dyserythropoyetic Anemia Type II. We also showed how these genetic mutations cause the reduction of SEC23B transcript and protein, resulting in a defect of cell division and post transductional modifications (presence in patient's bone marrow of multinuclear erythroblasts and deficit of Band 3 protein glycosylation).

In **Chapter 3** we investigated how mutations in "mediators" of oxygen sensing pathway (VHL and HIF2) can cause a different type of defect in haematopoiesis, in this case due to increased red cell production: Congenital Erythrocytosis.

Chapter 4 deals with a defect of haematopoiesis that involves platelet production. A reduced number of platelets cause thrombocytopenia (platelet count<$150x10^9$/L). After joined efforts we identified *ANKRD26*, the gene responsible for Autosomal Dominant Thromobocytopenia (THC2) and in **Chapter 5** we attempted to understand the function of *ANKRD26*, the gene mutated in THC2 Thrombocytopenia using the zebrafish as an animal model.

In **Chapter 6,** to underline how genetic factors can influence also the phenotype of an acquired haematopoiesis defect, we studied the effect of common genetic variant (SNPs) within the *CNR2* gene, which is known to influence autoimmune response. We show how the presence of this variant in patients, influence the clinical course of the Idiopathic Thrombocytopenia.

Chapter 7 discusses the findings described in the previous Chapters and hypothesizes which might be the next steps in the genetic and possibly, treatment of rare hematologic diseases.

2

Congenital Dyserythropoietic Anemia Type II:

molecular analysis and expression of the SEC23B Gene

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Background: Congenital dyserythropoietic anemia type II (CDAII), the most common form of CDA, is an autosomal recessive condition. CDAII diagnosis is based on invasive, expensive, and time consuming tests that are available only in specialized laboratories. The recent identification of *SEC23B* mutations as the cause of CDAII opens new possibilities for the molecular diagnosis of the disease. The aim of this study was to characterize molecular genomic *SEC23B* defects in 16 unrelated patients affected by CDAII and correlate the identified genetic alterations with *SEC23B* transcript and protein levels in erythroid precursors.

Methods: *SEC23B* was sequenced in 16 patients, their relatives and 100 control participants. *SEC23B* transcript levels were studied by quantitative PCR (qPCR) in peripheral erythroid precursors and lymphocytes from the patients and healthy control participants. Sec23B protein content was analyzed by immunoblotting in samples of erythroblast cells from CDAII patients and healthy controls.

Results: All of the investigated cases carried *SEC23B* mutations on both alleles, with the exception of two patients in which a single heterozygous mutation was found. We identified 15 different *SEC23B* mutations, of which four represent novel mutations: p.Gln214Stop, p.Thr485Ala, p.Val637Gly, and p.Ser727Phe. The CDAII patients exhibited a 40-60% decrease of *SEC23B* mRNA levels in erythroid precursors when compared with the corresponding cell type from healthy participants. The largest decrease was observed in compound heterozygote patients with missense/nonsense mutations. In three patients, Sec23B protein levels were evaluated in erythroid precursors and found to be strictly correlated with the reduction observed at the transcript level. We also demonstrate that Sec23B mRNA expression levels in lymphocytes and erythroblasts are similar.

Conclusions: In this study, we identified four novel *SEC23B* mutations associated with CDAII disease. We also demonstrate that the genetic alteration results in a significant decrease of *SEC23B* transcript in erythroid precursors. Similar down-regulation was observed in peripheral lymphocytes, suggesting that the use of these cells might be sufficient in the identification of Sec23B gene alterations. Finally, we demonstrate that decreased Sec23B protein levels in erythroid precursors correlate with down-regulation of the *SEC23B* mRNA transcript.

Background

Congenital dyserythropoietic anemias (CDAs) are a group of rare hereditary disorders characterized by ineffective erythropoiesis and distinct morphological abnormalities of the erythroblasts in the bone marrow (1). CDA type II (CDAII, OMIM 224100), which is transmitted as an autosomal recessive condition, is the most frequent; the main European Registries (German, Italian and French) have counted 367 patients (2). The clinical picture is characterized by mild to moderate anemia associated with jaundice, splenomegaly, and iron overload (3,4). In clinical practice, evidence of CDAII is primarily based on bone marrow examination (5,6).

Confirmation of diagnosis is based on at least one of the following biochemical tests, including: a positive acid serum lysis test with ABO-compatible sera; band 3 protein glycosylation defects evidenced by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE); a discontinuous double membrane in mature erythroblasts (visible by electron microscopy), and the presence of endoplasmic reticulum (ER)-specific proteins (5,7-9). However, these tests are expensive, time consuming, and often available in only a few specialized laboratories. For these reasons, the correct diagnosis of CDAII is often delayed or erroneously suspected.

A major breakthrough in CDAII research was achieved in 2009, when Schwarz *et al.* and Bianchi *et al.* found mutations of the *SEC23B* gene in patients with CDAII (10,11). Sec23B protein is an essential component of coat protein complex II (COPII), coated vesicles that transport secretory proteins from the ER to the Golgi complex (12). So far, *SEC23B* changes have been identified mainly by direct genomic sequencing of the coding region of the gene (10,11,13-15); however, the precise effects of the described mutations on the RNA expression level in erythroid cells has not been studied.

Moreover, a reduction of Sec23B protein in CDAII erythroid precursors has not been reported. In this study, we investigated *SEC23B* gene mutations, by both genomic and cDNA direct sequencing, in 16 unrelated Italian CDAII patients from 16 families. In all cases, we identified *SEC23B* mutations, and four of these were novel. We also evaluated the effects of different *SEC23B* mutations on mRNA and protein expression levels.

Methods

Patients

We collected blood samples from 16 unrelated Italian CDAII patients belonging to 16 families and 100 unrelated Italian controls (included in the DNA sequence analyses). The diagnosis of CDAII was made on the basis of clinical features, bone marrow examination, and/or SDS-PAGE. All patients provided their written informed consent for the study, which was approved by the research ethics committee of the Second

University of Naples, Italy. The study was conducted in accordance with the Declaration of Helsinki.

Erythroid precursor cultures

After informed consent has been obtained, peripheral blood from CDA II patients and from 5 healthy control relatives was collected into sterile heparinised tubes. Light-density mononuclear cells obtained by centrifugation on Lymphoprep (Nycomed Pharma) density gradient were enriched for CD34+ cells by positive selection using CD34 microbeads (Miltenyi Biotech) according to the manufacturers' instructions. CD34+ cells were cultured at a density of 105 cells/mL in alpha-minimal essential medium (a-MEM; GIBCO) supplemented with 30% fetal bovine serum (FBS; GIBCO), as previously described (16). To induce cells proliferation and erythroid differentiation, cells were cultured with 20 ng/mL rH stem cell factor (SCF, PeproTech), 10 ng/mL rH interleukin-3 (IL-3, PeproTech) and 3 U/mL recombinant human (rH) erythropoietin (rHuepo, Janssen- Cilag). Cells were incubated at 37°C with an atmosphere of 5% CO2 for 14 days; after 7 days of culture the medium was changed to ensure good cells feeding. Cell samples were collected on days 14 of culture (mature erythroblast stage) for further analysis.

Molecular analysis of the SEC23B gene

Genomic DNA was isolated using the Flexigene DNA extraction kit (Qiagen). All *SEC23B* exons, their flanking splice junctions, and their 5'- and 3'-untranslated regions were amplified with 21 polymerase chain reactions (PCRs). cDNA was prepared, using the iScript cDNA synthesis kit (Bio-Rad), from approximately 100 ng mRNA obtained from lymphocytes (Trizol Reagent Kit - Invitrogen) from all 16 patients and 8 healthy control relatives. cDNA was obtained also from erythroblasts (16) from 8 patients (ID: F1, G2, B3, A4, C5, B11P13, and M15) and 5 of the 8 healthy control relatives mentioned above. The coding region of the *SEC23B* cDNA was covered by six PCR fragments. Sequences of all primers can be found in Table 1. The PCR conditions were: 94°C for 5 min; 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec; and 72° C for 7 min. Amplified DNA and cDNA were purified (Exo-Sap-IT) and sequenced using BDT v3.1 on an ABIPrism 3130XL genetic analyzer. Sequences were analyzed using the SeqScape program, version 2.6 (Applied Biosystems).

Real-time PCR

cDNA was prepared from patients' mRNA from lymphocytes and erythroblasts. Real-time PCR was performed in accordance with manufacturers' instructions. The reactions were run on an ABI 7300 real-time PCR system (Applied Biosystems); the cycling conditions were 10 min at 95°C (initial denaturation) followed by 40 cycles of 15 sec at 94°C (denaturation) and 1 min at 68°C (annealing/extension/data collection). In the first step, we determined the stability of a control gene (β-actin) for the normalization of the real-time PCR products. The linearity and efficiency of this assay

were tested over dilutions of input cDNA spanning five orders of magnitude. Assays were performed in triplicate. We used the $2^{-\Delta\Delta Ct}$ method to analyze the data obtained.

Western blotting

Proteins were extracted from erythroid cultures of patients A4, B3, and C5 using RIPA Lysis Buffer (Millipore) and following the manufacturer's instructions. Sec23B was characterized in total lysates from erythroid cultures by Western blotting. Membranes were incubated overnight at 4°C with rabbit polyclonal anti-Sec23B antibody (1:500 dilution; SAB2102104, Sigma-Aldrich); reactive bands were detected by chemiluminescence (SuperSignal). An anti-β-actin antibody (1:500 dilution; Sigma) was used to check for comparable protein loading and as a housekeeping protein. Images were captured, stored, and analyzed using Quantity One software (BioRad).

Results

Molecular analysis of the SEC23B gene

We identified SEC23B mutations in all 16 patients enrolled in the study. Among the 15 mutations characterized, four are novel: c.640C > T, c.1453A > G, c.1910T > G, and c.2180C > T (Table 2). In total, we identified 10 missense, three nonsense, one in-frame deletion of 3 nucleotides, and one splice-site mutation. The splice-site alteration creates a new donor site after exon 2 of the *SEC23B* gene. Most of the investigated patients were compound heterozygotes. Only four patients had homozygous *SEC23B* mutations; three of these were homozygotes for the c.325G > A variant that leads to the amino acid change p.Glu109Lys (the most frequent *SEC23B* mutation encountered, with a prevalence of 32% among CDAII patients). The fourth patient was homozygous for the c.1254T > G mutation (p. Ile418Met). In two patients, we observed only a single heterozygous mutation (Table 2). Because individuals with a heterozygous *SEC23B* mutation are not affected by CDAII, we investigated in detail the putative occurrence of heterozygous exon deletions/insertions in the alternative allele in these patients. PCR of cDNA using primers located in the 5' and 3' untranslated regions of the gene revealed full-length transcript in both patients and no aberrant products, excluding the presence of heterozygous exon deletions/insertions. cDNA sequence analysis confirmed the heterozygous missense mutation and the presence of two alleles (i.e., wild-type and mutant).

We also sequenced *SEC23B* cDNA from the 16 CDAII patients. In all cases, the mutations identified by genomic analysis were confirmed by cDNA sequencing. Even in the patients carrying nonsense mutations (which most likely lead to RNA decay), it was possible to visualize the genetic change by cDNA sequencing (Figure 1A). None of the novel mutations described in this study was found in 100 unrelated Italian controls. All mutations were absent from the 1094 individuals from the 1000 Genomes project.

Real-time PCR

To directly evaluate the consequences of the genomic *SEC23B* mutations, we investigated *SEC23B* mRNA levels in patients and healthy individuals using quantitative PCR (qPCR). Patients that were compound heterozygotes for a missense and a nonsense mutation exhibited a drastic decrease in mRNA expression levels of approximately 50-60% when normalized to the endogenous control gene β-actin (Table 2 and Figure 1B). Patients with two missense mutations showed a milder reduction in *SEC23B* mRNA levels (approximately 40-45%) (Table 2). The results were similar when using RNA from lymphocytes or mature erythroblasts (Figure 1B and data not shown). The two patients with a single heterozygous mutation on *SEC23B* (C9 and P17) also exhibited a reduction of *SEC23B* transcript (Table 2).

Patient A4 was compound heterozygote for a splicing mutation (c.221+31A > G) and a nonsense change (c.367C > T). In this individual, we suspected very low or no wild-type (WT) transcript. Therefore, we investigated A4 *SEC23B* mRNA more in detail. First, the presence of the +31A > G allele was confirmed on agarose gel by the presence of an additional 31-bp band. In the other allele, the nonsense mutation creates a restriction site for the enzyme HpyCH4III. After enzymatic digestion of the PCR product with HpyCH4III restriction enzyme, we observed four fragments: an upper band for the + 31A < G allele, two lower bands representing the cut allele carrying the nonsense mutation, and a normal- sized band (Figure 2). The occurrence of a normal transcript indicates that at least a small amount of WT *SEC23B* RNA is present. This finding corresponds to the observed 35-40% *SEC23B* mRNA expression level measured by qPCR.

Western blotting

Finally, we investigated the amount of Sec23B protein in the erythroblasts of 3 CDAII patients (C5, B3 and A4) by immunoblotting. The Sec23B content was normalized to β-actin. As depicted in Figure 3A, the Sec23B content of patients C5 and B3 was clearly reduced compared to two different controls. Similar results were obtained in two independent experiments. Moreover, the estimated Sec23B protein level in erythroblasts from patient A4 suggested that it corresponded to approximately 30-35% of that of a healthy individual (Figure 3B). Therefore, our data suggest good correspondence between the transcript amount and protein content, underscoring the usefulness of mRNA evaluation.

Discussion

In this study, we identified four novel *SEC23B* gene mutations by analyzing 16 Italian patients with CDAII (Table 2). We also identified two CDAII patients with only one heterozygous mutation each.

Since the initial identification of *SEC23B* mutations in CDAII patients, 59 mutations have been identified, including the current work (Table 3) (10,11,13-15,17,18). Two missense mutations have been repeatedly identified in a large proportion of patients:

p.Glu109Lys and p.Arg14Trp (32% and 19%, respectively). To date, these missense mutations account for approximately 50% of the mutant alleles in CDAII patients (Table 3). Notably, evidence of a founder effect has been described for p. Glu109Lys among Israeli Moroccan Jewish patients (13). Concerning mutation type, missense (52%) and nonsense (21%) mutations are the most commonly observed followed by deletions or insertions that lead to frameshifts in the nucleotide sequence. Splicing mutations are rare, with only six mutations reported (Table 3). The *SEC23B* gene appears to play a pivotal and probably unique function in erythroid precursors (19,20). Although detailed genetic analyses have been conducted, the effects of the mutations on mRNA content in erythroblast cells have not been documented. Moreover, no data are available about the effects of mutations on Sec23B protein content in red cell precursors. To evaluate the effect of missense and nonsense mutations on *SEC23B* mRNA expression levels, we performed quantitative (qPCR) analysis of *SEC23B* transcripts on all of our patients. In this study, we used both cDNAs prepared from erythroid precursor cultures and peripheral lymphocytes. Although we demonstrate that all patients have a significant reduction of *SEC23B* mRNA, this reduction was more pronounced in patients with missense/nonsense mutations (Table 2). From a diagnostic point of view, it is interesting to note that the results obtained in erythroid precursors and lymphocytes were comparable, suggesting that peripheral lymphocytes not only represent a good source of *SEC23B* transcript, but also replicate the effect of the genetic change observable in the erythroid population. In addition, to search for genotype-phenotype correlation, we grouped patients according to their degree of anemia. We did not observe any correlation between degree of anemia, type of mutation, and relative *SEC23B* mRNA reduction (data not shown). Almost all CDAII patients harbor mutations in both *SEC23B* alleles. In a few cases (10 out of 111 described in the literature, or 9%), only a single heterozygous *SEC23B* mutation has been found. This finding raises the possibility of the occurrence of mutations that have thus far escaped the exon screening technology. In our study, two CDAII patients were identified in whom a mutation was observed in only one allele. In these participants, mRNA analysis (cDNA sequencing and long range PCR on cDNA) confirmed the presence of both wild-type and mutated alleles. However, qPCR analysis revealed a reduction in *SEC23B* mRNA expression of approximately 40% in these patients compared with control participants, similar to the reduction observed in patients with two missense mutations. This finding suggests the possible occurrence of mutations that affect the regulatory regions of the *SEC23B* gene. Alternate mechanisms such as microRNA dysregulation could be responsible for CDAII in these cases where the second heterozygote mutation has not been found. Here we demonstrate that *SEC23B* mutations result in reductions of both the relative transcript and protein content in erythoid precursors. So far, only one study has investigated protein levels of Sec23B in CDAII patients (11). No reduction of Sec23B protein levels was observed, most likely due to the type of cell used in the study (fibroblasts). Our data, although comprising a small number of cases, clearly demonstrate that CDAII erythroblastoid cells show a strong reduction of the protein that parallels the data regarding mRNA levels. Future

investigations are necessary to clarify the effect of protein reduction on the patients' phenotype. Patients lacking Sec23B expression have never been described. We identified a single patient (A4) with a nonsense mutation and a splice site mutation (c.221+31 A > G) that causes a stop codon after exon 1. In our view, this patient could have had a very strong reduction of Sec23B expression. On the basis of this hypothesis, we analyzed the amount of *SEC23B* mRNA and protein in this patient in detail. The results demonstrate that there is still a small amount of WT *SEC23B* mRNA and that the Sec23B protein level in this patient corresponds to 30% of the level observed in healthy participants, suggesting that the absence of *SEC23B* expression may be lethal.

Conclusions

This study reports *SEC23B* gene mutations in all 16 CDAII patients studied, confirming the causative relevance of the gene to the condition. We also demonstrated that the *SEC23B* gene mutations lead to a remarkable reduction of *SEC23B* transcript in erythroid precursors, the cell type altered in the disease. We also demonstrated that quantifying and sequencing *SEC23B* mRNA from peripheral lymphocytes (and not only from erythroid cultures) might facilitate the genetic diagnosis of CDAII. Our data on heterozygote patients suggest (although indirectly) the occurrence of rare mutations is not restricted to its coding regions. Finally, we demonstrate that the relative mRNA reduction directly corresponds to a protein decrease in erythroblastoid cells. Future studies will be devoted to characterizing the effect of Sec23B protein down-regulation on erythropoiesis and clarifying *SEC23B* gene regulation.

List of abbreviations

bp: Base pair; COPII: Coat Protein Complex II; CDAs: Congenital Dyserythropoietic Anemias; CDAII: Congenital Dyserythropoietic Anemia type II; ER: Endoplasmic reticulum; PCR: Polymerase Chain Reaction; qPCR: Quantitative PCR; rH: Recombinant human; SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; WT: Wild-type.

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and functional characterization of mammalian homologues of the COPII component Sec23. Mol Biol Cell 1996, 7:1535-1546.

Figures:

Table 1: Primer sequences for *SEC23B* cDNA amplification

Oligo Name	Oligo Sequence
SEC23B cDNA 1F	ACCTGTCTTGCCCTGTTCC
SEC23B cDNA 1R	TACAGGCCCAAAGTTTTGCT
SEC23B cDNA 2F	AGCAGGCCAACTTGTAAAGC
SEC23B cDNA 2R	CTTGAAGCAAAAGGGTGCTC
SEC23B cDNA 3F	ACAGGATATGTTGGGCCTGA
SEC23B cDNA 3R	TTGCACAACACTTCATCTCCA
SEC23B cDNA 4F	GAACAGCTGCAAATGGTCAC
SEC23B cDNA 4R	CACAGTCGGATGAGTTGTCG
SEC23B cDNA 5F	GACCGACAACTCATCCGACT
SEC23B cDNA 5R	TTTCCTGTCCCCAAGCATAC
SEC23B cDNA 6F	CAGTCAGGCTCGATTCCTTT
SEC23B cDNA 6R	CACCTAAACAAGCTGCCAAA

Table 2: SEC23B mutations in 16 Italian patients - Novel mutations are bold

Patient ID	Allele 1	Allele 2	Protein change 1	Protein change 2	cDNA %
F1	c.953 T > C	**c.1910 T > G**	p.Ile318Thr	p.Val637Gly	55
G2	c.40 C > T	c.1015 C > T	p.Arg14Trp	p.Arg339X	42
B3	c.325 G > A	c.325 G > A	p.Glu109Lys	p.Glu109Lys	60
A4	IVS1 +31 A > G	c.367 C > T	Donor site ins	p.Arg123X	36
C5	c.40 C > T	c.1857-1859delCAT	p.Arg14Trp	p.I619del	54
C6	c.40 C > T	c.2101 C > T	p.Arg14Trp	p.Arg701Cys	62
P7	c.40 C > T	c.1015 C > T	p.Arg14Trp	p.Arg339X	42
C9	c.40 C > T	-	p.Arg14Trp	-	58
D10	c.325 G > A	c.325 G > A	p.Glu109Lys	p.Glu109Lys	60
B11	**c.1453 A > G**	c.1589 G > A	p.Thr485Ala	p.Arg530Gln	55
P13	c.40 C > T	**c.640 C > T**	p.Arg14Trp	p.Gln214X	50
G14	c.325 G > A	c.325 G > A	p.Glu109Lys	p.Glu109Lys	61
M15	c.40 C > T	**c.2180 C > T**	p.Arg14Trp	p.Ser727Phe	55
F16	c.325 G > A	c716 A > G	p.Glu109Lys	p.Asp239Gly	61
P17	c.40 C > T	-	p.Arg14Trp	-	64
E18	c.1254 T > G	c.1254 T > G	p.Ile418Met	p.Ile418Met	53

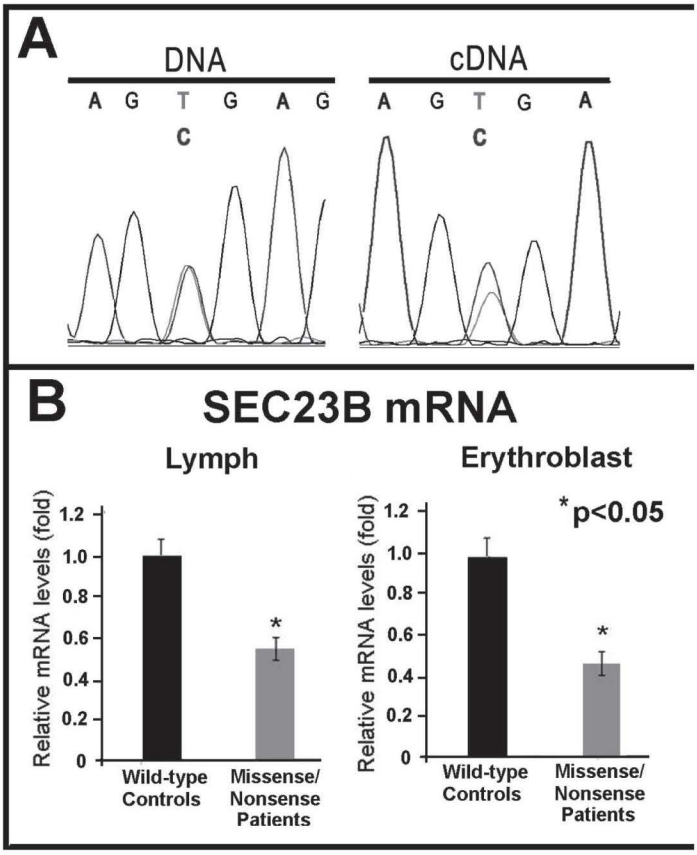

Figure 1: A) Electopherogram depicting the nonsense mutation (c.367C>T) observed after DNA and cDNA sequencing; B) Relative *SEC23B* mRNA expression levels in lymphocytes and erythroblasts from patients with nonsense/missense mutations compared with healthy controls.

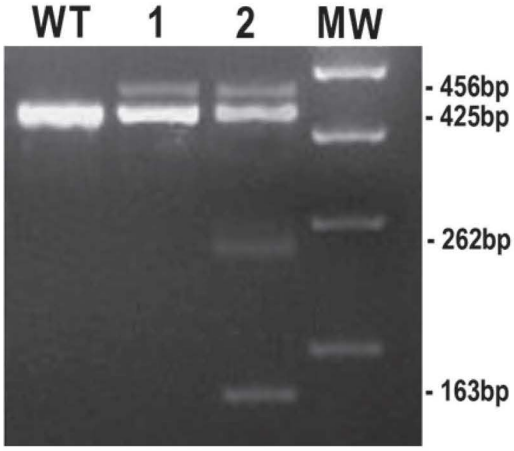

Figure 2: Characterization of *SEC23B* mutations in patient A4. Lymphocytic cDNA was amplified using primers localized in exons 1 and 4 (A4-SEC23B-1F: TTGTACCCCTGGCTTGTCTC and A4-SEC23B-4R: ATGAACCTGCACCATCCTTC) The control shows the 425-bp product (wild-type, W.T.); (1) Patient A4: Two bands (456-bp and 425.bp) were present. The additional 456-bp band is due to the splicing mutation: c.221 +31 A>G; (2) The nonsense mutation (c.367C>T) creates a restriction site for the enzyme *HpyCH4III. SEC23B* PCR product from patient A4 was enzymatically digested. The two resulting bands (262-bp and 163bp) represent the cut allele carrying the nonsense mutation. M.W. = Molecular Weight.

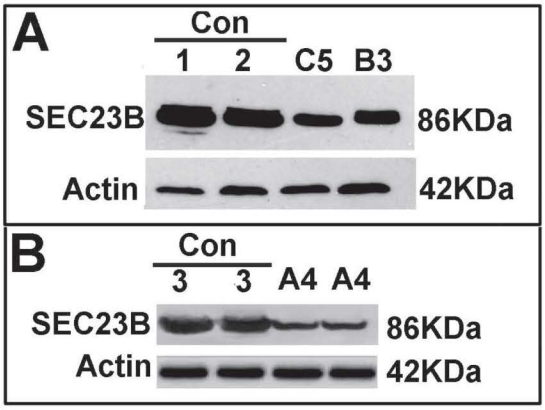

Figure 3: Sec23B protein analysis in erythroid precursors. Western blots from patients C5 and B3 (panel A) and A4 (panel B) demonstrate reduced Sec23B expression compared with control partecipants (Con). Approximately 40 µg of protein were loaded. β-actin was used as a loading control. The experiment was representative of two different experiments.

Table 3: Summary of all *SEC23B* mutations in CDAII patients, predicted effect on protein and allelic frequencies

Exon/Intron	Nucleotide change	Protein change	Type of mutation	Allelic frequency, n (%)	References
2	c.40 C > T	p.Arg14Trp	Missense	48 (19)	[11]
2-3	c.221+31 A > G	-	Splice-site change*	2 (0.8)	[14]
2	c.53 G > A	p.Arg18His	Missense	3 (1.2)	[11]
2	c.197 G > A	p.Cys66Tyr	Missense	1 (0.4)	[17]
3	c.235 C > T	p.Arg79X	Non-sense	3 (1.2)	[11]
3-4	c.279 +3 A > G	-	Splice-site change	1 (0.4)	[14]
3-4	c.222-817_366+4242del	-	Frame-shift	1 (0.4)	[11]
4	c.325 G > A	p.Glu109Lys	Missense	81 (32)	[11]
5	c.367 C > T	p.Arg123X	Non-sense	2 (0.8)	[14]
5	c.387(delG)	p.Leu129LeufsX26	Frame-shift	1 (0.4)	[14]
5	c.428delAinsCG	-	Frame-shift	1 (0.4)	[10]
5	c.568 C > T	p.Arg190X	Non-sense	1 (0.4)	[10]
6	c.640 C > T	p.Gln214X	Non-sense	1 (0.4)	Present study
6	c.649 C > T	p.Arg217X	Non-sense	4 (1.6)	[10]
6-7	c.689+1G > A	-	Splice-site change	4 (1.6)	[10]
7	c.716 A > G	p.Asp239Gly	Missense	3 (1.2)	[11]
7	c.790 C > T	p.Arg264X	Non-sense	3 (1.2)	[11]
8	c.938 G > A	p.Arg313His	Missense	4 (1.6)	[11]
8	c.953 T > C	p.Ile318Thr	Missense	6 (2.4)	[11]
8	c.970 C > T	p.Arg324X	Non-sense	2 (0.8)	[11]
9	c.1015 C > T	p.Arg339X	Non-sense	3 (1.2)	[14]
9	c.1043 A > C	p.Asp348Ala	Missense	1 (0.4)	[10]
9	c.1063delG	-	Frame-shift	1 (0.4)	[11]
9-10	c.1109 +5 G > A	-	Splice-site change	1 (0.4)	[14]
9-10	c.1190 +1 G > A	-	Splice-site change	1 (0.4)	[14]
10	c.1157 A > T	p.Gln353Leu	Missense	1 (0.4)	[11]
10	c.1201 C > T	p.Arg401X	Non-sense	1 (0.4)	[11]
11	c.1254 T > G	p.Ile418Met	Missense	3 (1.2)	[14]
11	c.1276 G > A	p.V426I Poly	Missense	2 (0.8)	[11]
11	c.1307 C > T	p.Ser436Leu	Missense	1 (0.4)	[14]
12	c.1385 A > G	p.Tyr462Cys	Missense	6 (2.4)	[11]
13	c.1453 A > G	p.Thr485Ala	Missense	1 (0.4)	Present study
13	c.1489 C > T	p.Arg497Cys	Missense	9 (3.6)	[10]
13	c.1508 G > A	p.Arg503Gln	Missense	1 (0.4)	[18]
14	c.1571 C > T	p.Ala524Val	Missense	5 (2)	[11]
14	c.1588 C > T	p.Arg530Trp	Missense	1 (0.4)	[11]
14	c.1589 G > A	p.Arg530Gln	Missense	2 (0.8)	[18]
14	c.1603 C > T	p.Arg535X	Non-sense	2 (0.8)	[14]
14	c.1648 C > T	p.Arg550X	Non-sense	4 (1.6)	[18]
14	c.1654 C > T	p.Leu552Phe	Missense	1 (0.4)	[14]
14	c.1660 C > T	p.Arg554X	Non-sense	2 (0.8)	[10]
15	c.1685 A > G	p.Tyr562Cys	Missense	1 (0.4)	[18]
15	c.1733 T > C	p.Leu578Pro	Missense	2 (0.8)	[14]
15	c.1735 T > A	p.Tyr579Asn	Missense	1 (0.4)	[14]
16	c.1808 C > T	p.Ser603Leu	Missense	1 (0.4)	[10]
16	c.1821delT	-	Frame-shift	3 (1.2)	[10]
16	c.1832 G > C	p.Arg611Pro	Missense	1 (0.4)	[14]
16	c.1858 A > G	p.Met620Val	Missense	2 (0.8)	[14]
16	c.1857_1859delCAT	p.Ile619del	In frame deletion**	2 (0.8)	[14]
17	c.1910 T > G	p.Val637Gly	Missense	1 (0.4)	Present study
17	c.1962-64delT	p.Thr654ThrfsX13	Frame-shift	1 (0.4)	[18]

Table 3: Summary of all *SEC23B* mutations in CDAII patients, predicted effect on protein and allelic frequencies (continued)

17	c.1968 T > G	p.Phe656Leu	Missense	1 (0.4)	[18]
18	c.2101 C > T	p.Arg701Cys	Missense	9 (3.6)	[10]
18	c.2129 C > T	p.Thr710Met	Missense	1 (0.4)	[13]
18-19	c.2149 -2 A > G	-	Splice-site change	2 (0.8)	[14]
19	c.2150(delC)	p.Ala717ValfsX7	Frame-shift	1 (0.4)	[14]
19	c.2166 A > C	p.Lys723Gln	Missense	1 (0.4)	[18]
19	c.2180 C > T	p.Ser727Phe	Missense	1 (0.4)	Present study
20	c.2270 A > C	p.His757Pro	Missense	1 (0.4)	[14]

*creation of a new donor site

**1 aminoacid deletion

3

Congenital Erythrocytosis

3.1

Von Hippel-Lindau –dependent polycythemia is endemic on the island of Ischia: identification of a novel cluster

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Chuvash polycythemia (MIM 263400) is an autosomal recessive disorder characterized by a high hemoglobin level, relatively high serum erythropoietin, and early death. It results from a Von Hippel-Lindau *(VHL)* gene mutation (C598T) that causes increased HIF-1α activity and erythrocyte production in the face of normoxia. This polycythemia is endemic in Chuvashia, whereas its worldwide frequency is very low. We investigated the incidence of the Chuvash-type *VHL* mutation in Campania (South Italy) and identified 14 affected subjects (5 families). Twelve live on the island of Ischia (Bay of Naples). From analysis of the mutated allele, we found that the disease was more frequent on Ischia (0.070) than in Chuvashia (0.057). The haplotype of all patients matched that identified in the Chuvash cluster, thereby supporting the single-founder hypothesis. We also found that non affected heterozygotes had increased HIF-1α activity, which might confer a biochemical advantage for mutation maintenance. In conclusion, we have identified the first large cluster of Chuvash erythrocytosis outside Chuvashia, which suggests that this familial polycythemia might be endemic in other regions of the world.

Introduction

The Chuvash variant of familial polycythemia was first described in more than 100 individuals from about 80 families living in the mid-Volga River region of European Russia (1). The disease is characterized by a high hemoglobin level, increased plasma erythropoietin (Epo) level, varicose veins, vertebral hemangiomas, low blood pressure, and an elevated serum concentration of vascular endothelial growth factor (VEGF) (2). Patients affected by Chuvash polycythemia die early, mainly as a result of cerebral vascular events or peripheral thrombosis. These injuries seem to be linked to mechanisms other than blood hyperviscosity or serum Epo content (2). Indeed, the prevalence of low blood pressure in patients with Chuvash polycythemia contrasts with the hypertension frequently associated with polycythemia vera and other familial polycythemias resulting from excess Epo. Genome-wide screening and candidate gene characterization demonstrated that the Arg200Trp mutation (C598T) of the Von Hippel-Lindau *(VHL)* gene causes the Chuvash form of polycythemia (3). Thereafter, the mutation was detected in homozygosity in patients with sporadic or familial congenital erythrocytosis from diverse ethnic groups (4-8). However, 19 homozygotes have been identified among the more than 150 known cases of non-Chuvash familial erythrocytosis (9). Furthermore, 8 other *VHL* mutations (Arg79Cys, Gly104Val, Asp126Tyr, Val130Leu, Gly144Arg, Tyr175Cys, Leu188Val, His191Asp, Pro192Ala) were detected in either homozygotes or compound heterozygotes (4,5,7,8,10). These mutations were detected in a total of 10 cases, which indicates that the C598T transition is the major cause of *VHL*-related erythrocytosis. The C598T mutation likely originated from a single founder event because the *VHL* haplotype in non-Chuvash patients is identical to that in polycythemic patients from Chuvashia (6,11). The *C598T* allele is very rare outside the Chuvash population. In fact, its frequency in Chuvashia is about 0.057,3 whereas the worldwide frequency of the Chuvash-associated haplotype is about 0.001 377 (11). The different haplotype recently identified in a patient of Turkish ancestry probably represents an independent mutational event (7). Together with other proteins (elongin B, C, Rbx1, and Cul2), the VHL protein participates in the hypoxia-sensing pathway, where it binds the proline-hydroxylated form of the hypoxia inducible factor-1α (HIF-1α), thereby committing the transcription factor to polyubiquitination and proteasomal degradation (12-15). Under normoxic conditions, HIF-1α is hydroxylated and rapidly degraded, thereby resulting in down-regulation of the transcription of HIF-1α–regulated genes (13,16). Conversely, the C598T mutation impairs VHL function and causes an increase in the HIF-1 complex, which in turn could cause overexpression of its target genes (3). HIF-1α regulates such important genes as *EPO*, *VEGF*, *SDF1*, *GLUT1*, triosephosphate isomerase 1 *(TP1)* transferrin, and the transferrin receptor (3,17,18). Although an increased serum level of Epo is considered the major cause of polycythemia (1) other HIF-1α–modulated genes might be involved in the pathogenesis of erythrocytosis. In this study, we investigated the frequency of the C598T *VHL* mutation in the Campania Region of South Italy. Unexpectedly, we discovered a cluster of the disease on the island of Ischia (Bay of

Naples), which has a population of about 55 000. This is the first region other than Chuvashia where this congenital polycythemia is endemic.

Patients, materials, and methods

Patients

Twenty-two patients from 13 families with suspected Chuvash-like congenital polycythemia were included in the study. Diagnostic criteria included (a) persistent elevated hemoglobin level (> 180 g/L [18 g/dL] in males, > 165 g/L [16.5 g/dL] in females, or > 2 SD above the mcdian of the sex-and age-specitic normal range in children); (b) absence of splenomegaly; (c) normal leukocyte and platelet counts; (d) normal hemoglobin oxygen affinity; (e) high or inappropriately high serum Epo level (1, 3, 9, 19), and (f) absence of known causes of secondary erythrocytosis. The median age of patients at diagnosis was 19 years (range, 1-34 years). Some patients had undergone sporadic or regular phlebotomy treatment. Eleven patients were members of 2 unrelated families: 8 from family A and 3 from family B. The other 11 subiects reported no affected relatives. All cases were recruited through the Department of Pediatrics (Second University of Naples) and the Division of Hematology (Federico II University of Naples). The study was approved by the Institutional Review Board of the Second University of Naples and performed in accordance with the World Medical Association Declaration of Helsinki of 1975, as revised in 2000. Written informed consent for molecular genetic analysis, data analysis, and publication was obtained from all participants.

Family A. Family A includes 8 polycythemic patients: a mother (P13), her 2 sons (P15 and P16), her brother (P12), her father (P05), her uncle (P04), and 2 cousins (P07 and P08) (Figure 1A). All lived on the island of Ischia. No patient had a history or evidence of thrombotic complications or cancer. There is no record of consanguinity in the family. Erythrocytosis was discovered in the mother when she was 10 years old, at which time the hemoglobin (Hb) was 165 g/L (16.5 g/dL) and packed cell volume (PCV) was .54 (54%). The serum Epo concentration was 35 IU/L (mIU/mL) (normal range, 11-30 IU/L [mIU/mL]) before phlebotomy therapy was initiated. Her husband was not affected by erythrocytosis. Polycythemia was diagnosed in her 2 sons shortly after birth, and they began a therapeutic phlebotomy program to maintain their hematocrit level below .45 (45%). Their Epo levels were 55 and 99 IU/L (mIU/mL) before phlebotomies.

Family B. Family B includes 3 polycythemic subjects. A 9-year-old girl from Ischia (P22) (Figure 1B) was diagnosed with erythrocytosis at the age of 3 months, Hb was 210 g/L (21.0 g/dL) and PCV was .59 (59%), whereas O_2 P50 was normal. The Epo concentration was 41 IU/L (mIU/mL). Polycythemia was diagnosed in her mother (P19) and uncle (P21) when they were 10 and 12 years old, respectively. They had elevated levels of Hb (197 g/L [19.7 g/dL] and 225 g/L [22.5 g/dL], respectively) and Epo (34 IU/L [mIU/mL] and 75 IU/L [mIU/mL], respectively). There is no record of consanguinity

in the family. The patients do not have a history of cerebrovascular complications or cancer.

Detection of gene mutations

Genomic DNA was extracted from peripheral blood leukocytes with the Flexigene DNA Kit (Qiagen GmbH). To search for VHL mutations, we sequenced all 3 *VHL* exons and their intron-exon boundaries. Polymerase chain reaction (PCR) was performed essentially as reported in Ang et al. (3), and the reaction products were purified using a QIAquick Gel Extraction Kit (Quiagen GmbH). The products were sequenced using the ABI 310 DNA Sequencer and the ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems), according to the manufacturer's instructions. The coding regions of the elongin B and elongin C genes and the HIF-1α sequences spanning the oxygen-dependent degradation/pVHL interaction domain (residues 417-698, exons 10-12) were screened for DNA sequence variations by PCR amplification and DNA sequencing using the oligonucleotide primers and PCR conditions described in Clifford et al. (20) DNA sequencing of PCR products was carried out as reported.

Mutation screening for the C598T base change

The C598T mutation abolishes the Fnu4HI restriction endonuclease recognition site. Thus, to screen for the mutation, we digested 14 µL PCR-amplified exon 3 product with 0.5 U Fnu4HI (New England Biolabs) for 3 hours at 37°C. The digested products were visualized by electrophoresis on 2% agarose.

Haplotype analysis

We used 8 single-nucleotide polymorphisms that span the *VHL* gene (rs1056286, rs722509, rs779805 A>G, rs779808, rs1678607, 1149A>G, rs696356, rs378630), which are known to be highly informative in the Chuvash population (11), to characterize polycythemic patients. The PCR products were sequenced as reported in "Detection of gene mutations".

Reverse transcription polymerase chain reaction

We isolated RNA from 5 X 10^6 Epstein-Barr virus (EBV)–transformed lymphoblastoid cells using the Trizol Reagent Kit (Invitrogen). Total RNA was prepared from reticulocytes as described elsewhere (21). cDNA was synthesized using the Superscript II Kit (Invitrogen) with random hexamers and 1.5 µg total RNA. PCR amplification of the whole *VHL* coding region was carried out with primers cVHL1-3F1 5'-CAGCTCCGCCCCGCGTCCGAC-3' (located at the 5'-untranslated region) and cVHL1-3R1 5'-AAGGAAGGAACCAGTCCTGT-3' (located at the 3'-untranslated region). PCR conditions were as reported in Cario et al. (7). The reaction products were analyzed by agarose gel electrophoresis. cDNA was amplified with a primer located in the second *VHL* exon (cVHL2-3F1 5'-CTCTTCAGAGATGCAGGGACAC-3') and the cVHL1-3R1 primer in separate experiments. The reaction product (377 bp)

was digested with 0.5 U Fnu4HI as described in "Mutation screening for the C598Tbase change".

The expression of the *EPO, VEGF, SDF1,* and *TP1* genes was evaluatedby reverse transcriptase (RT)–PCR with cDNAs prepared as described in "Detection of gene mutations." The primers and conditions were as follows: *EPO* (Forward: 5'-CGCGCCCGCTCTGCTCCGACACC-3' and Reverse: 5'-GGAGCGACAGCAGGGACAGGAGA-3' for 32 cycles, each consisting in steps at 95°C for 45 seconds, 56°C for 45 seconds, and 68°C for 45 seconds), *VEGF* (Forward: 5'-TCGGGCCTCCGAAACCATGA-3' and Reverse: 5'-CTCCTCCTTCTGCCATGGGT-3' for 32 cycles, each consisting in steps at 95°C for 45 seconds, 56°C for 45 seconds, and 68°C for 45 seconds), *SDF-1* (Forward: 5'-GTGTCACTGGCGACACGTAG-3' and Reverse: 5'-TCCCATCCCACAGAGAGAAG-3' for 32 cycles, each consisting in steps at 95°C for 45 seconds, 58°C for 45 seconds, and 68°C for 45 seconds), *TP1* (Forward: 5'-GTGAAGGACTGGAGCAAGGT-3' and Reverse: 5'-GGGCTCATTGTTTGGCATTG-3' for 28 cycles, each consisting in steps at 95°C for 45 seconds, 58°C for 45 seconds, and 68°C for 45 seconds). The PCR products were analyzed by electrophoresis on 1.8% agarose gel. Before amplification with each specific primer pair, an aliquot of the cDNA preparation was amplified using primers for β-actin to determine the integrity of the generated cDNA (BD Biosciences Clontech). Moreover, we used 5 different cDNA concentrations to ensure that signals were proportional to input mRNA. Each experiment was performed at least in triplicate and, in several cases, in quadruplicate. The expression of β-actin, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *EPO,* and *SDF-1* was also evaluated by real-time PCR as reported elsewhere (17, 22, 23). Human kidney RNA (BD Biosciences Clontech) served as a positive control for EPO expression.

Preparation of lymphoblastoid cell lines

We established EBV-transformed lymphoblastoid cell lines from the peripheral blood of 8 subjects: 2 homozygotes for the C598T mutation (P12 and P22), 2 polycythemic heterozygotes (P24 and P25), 2 non polycythemic heterozygotes (P09 and P11), and 2 control subjects. The EBV lines were produced with 0.2-nm filtered culture medium of the B-95.8 EBV-producing marmoset line (24) and PHA-M. Cells (10^7) were pelleted and resuspended in 1 mL B-95.8 cell line supernatant. This preparation was incubated in a conical tube at 37°C and occasionally resuspended. After 60 to 90 minutes, the cells were pelleted and resuspended in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 μg/mL), gentamycin (100 μg/mL), and 10% heat-inactivated fetal bovine serum in the presence of 5 μg/mL PHA-M. Half the medium was replaced every 3 to 4 days. Outgrowth of EBV-transformed cells was evident after 4 to 6 weeks.

HIF-1α functional analysis and immunoblotting

HIF-1α transcription factor activity was determined with the TransAM HIF-1α Kit (Active Motif) according to the manufacturer's instructions. Briefly, in this enzyme-linked immunoadsorbent assay (ELISA) method, an oligonucleotide containing the hypoxia-response element is immobilized on a 96-well plate. HIF dimers in nuclear extracts specifically bind to the oligonucleotide and are identified by means of an anti-HIF-1α antibody. The procedures used for immunoblotting are reported else where (25). The antibodies directed against aldolase and GAPDH were from Santa Cruz Biotechnology (Santa Cruz); the antisera against HIF-1α is contained in the kit.

Results

The VHL gene C598T mutation in the Campania Region

We studied 22 patients from 13 families putatively affected by Chuvash-like erythrocytosis. We studied only patients whose hemoglobin-adjusted Epo serum concentration was higher than normal under nonphlebotomized conditions (3, 9). All subjects live in the Campania region of southern Italy, which has a population of about 5 million. All declared that their family had resided in Campania for several generations. No patient is of Chuvash origin. We first evaluated the occurrence of the C598T mutation by sequencing the 3 *VHL* exons (Figure 2A). We verified the results by amplifying the third *VHL* exon and digesting the PCR product with *Fnu4HI* (Figure 2B). Because the mutation abolishes the *Fnu4HI* restriction site, the amplified product containing C598T was not digested (Figure 2B). Identical results were obtained with the 2 approaches, thereby validating the second method in the screening of multiple DNA samples. We identified the mutation in 14 patients (5 families) of the 22 examined (Figure 2B). Twelve patients were homozygotes for the C598T mutation and 2 were heterozygotes. A similar screening of putative Chuvash-like polycythemic patients living in other Italian regions revealed no patient with the C598T mutation (data not shown). All the 12 homozygote patients (3 families) live on Ischia. Although the 2 heterozygotes do not live in Ischia, their parents who carried the *VHL* mutation live on the Naples coastline directly facing the island. No mutations were found in the 3 *VHL* exons of the other 8 polycythemic patients who did not carry the C598T mutation.

VHL mutation in heterozygotes

The 2 patients with a heterozygous C598T mutation were a 4-year-old boy (P24) and a 30-year-old man (P25). Erythrocytosis was discovered at the age of 2 years (P24) and 10 years (P25). Their present hemoglobin levels are 170 g/L (17 g/dL) and 205 g/L (20.5 g/dL), PCV of .54 (54%) and .65 (65%), and serum Epo 33 IU/L (mIU/mL) and 54 IU/L (mIU/mL), respectively. Thus far, they have no hyperviscosity symptoms or thromboembolic complications. These 2 patients (and their parents) declared that no other family member is affected by erythrocytosis. There was no consanguinity in the parents. In both cases, the C598T mutation was inherited from the father, but neither father had any clinical or laboratory signs of polycythemia.

Subjects heterozygous for the C598T mutation do not usually manifest erythrocytosis. However, 2 independent cases of polycythemic patients heterozygous for the C598T mutation have been reported (6, 7, 8, 10). We next carried out a series of analyses to look for other genetic aberrations (eg, mutations, deletions, and silencings) that could affect the apparently wild-type *VHL* allele. First, we sequenced the *VHL* gene promoter and did not find any mutations. However, this finding does not exclude abnormalities that may affect the expression of the wild-type *VHL* allele (eg, epigenetic events) or result in altered transcripts. Therefore, we analyzed the *VHL* transcripts. We retrotranscribed total RNA from EBV-transformed B lymphoblasts of the 2 patients and amplified the cDNA using primers localized in the 5'- and 3'-UTR of the *VHL* mRNA. This experiment revealed 2 full-length *VHL* transcripts, as previously reported (26). One transcript includes all 3 exons, and a smaller transcript results from splicing-induced skipping of the second exon. The 2 amplified products were purified and digested with *Fnu4HI*, and the assay mixtures were separated by electrophoresis on agarose gel. Both the wild-type and the mutated *VHL* allele expressed the 2 transcripts (data not shown). We also amplified the cDNAs using a forward primer localized in the second exon and a reverse primer at the 3'-UTR of the mRNA. In this case, only 1 amplified product was obtained. The PCR product was digested with *Fnu4HI*. Although these results are semiquantitative, they suggest that the 2 alleles are transcribed with a similar efficiency (Figure 3A). Because the 2 heterozygote polycythemic patients have high Epo serum levels, it is conceivable that genetic alterations of components of the oxygen-sensing pathway other than *VHL* may contribute to erythrocytosis. Consequently, we looked for mutations in the *HIF1A* gene and in 2 genes (ie, elongin B and elongin C) that encode other components of the E3 complex that ubiquitinates HIF-1α. No mutations were found (data not shown).

VHL gene mutation is endemic on Ischia

The 12 patients, homozygous for the Chuvash-like mutation, come from 3 families that live on Ischia (Figures 1-2). Greater than 50% of the patients are affected by hypotension and varicose veins. No cancer or other symptoms of Chuvash polycythemia were observed. In addition to families A and B (corresponding to 11 patients), the other homozygous subject (P23) was a 35-year-old man with a long-standing history of erythrocytosis. He is the only member of his family affected by polycythemia, and at present he has a hemoglobin level of 210 g/L (21 g/dL), PCV of .64 (64%), and a serum Epo level of 42 IU/L (mIU/mL). His heterozygous parents have no history of consanguinity. Our 2 affected families included 6 homozygotes (P08, P12, P13, P15, P16, and P22) who had one homozygous parent (P04, P05, P13, and P19) (Figures 1-2). Because the other parent was an obligate heterozygote (P03, P06, P14, and P20) and parental consanguinity was denied, we inferred a high frequency of the C598T mutation in Ischia. In this population, we determined that the mutation occurred with a frequency of 0.0703 (9 heterozygotes in 64 healthy subjects). None of the investigated subjects belonged to families that included homozygote patients. Conversely, we found no *VHL* mutations in 100 healthy subjects (200 chromosomes)

from other Italian areas. The haplotype pattern in our 12 patients (data not shown) was identical to that previously reported in patients with Chuvash polycythemia (11). The data obtained in this novel *VHL*-dependent polycythemic cluster suggest that heterozygotes may have some selective advantages that favor the spread and maintenance of the mutated allele. To address this issue, we determined HIF-1α activity in EBV-transformed B lymphoblasts from healthy subjects, heterozygotes and homozygotes. As shown in Figure 4A, HIF-1α activity was 3-fold higher in homozygotes compared with controls. Surprisingly, activity was also increased in heterozygous subjects. These results were confirmed by a sensitive immunoblotting procedure (Figure 4B). Using RT-PCR, we also measured the expression of the *EPO, VEGF, SDF1* (Figure 5A) and *TP1* (data not shown) genes, which are targets of activated HIF-1α (27). In particular, *SDF1* has recently been demonstrated to be modulated by HIF-1α (17). Because the SDF-1 protein is involved in angiogenesis, it could play a role in lowering blood pressure in homozygous and heterozygous subjects with Chuvash polycythemia. As shown in Figure 5A, *VEGF* expression was increased in homozygotes (by more than 3-fold as assessed by gel scanning). In contrast, *EPO* and *SDF1* (Figure 5A) and *TP1* (data not shown) gene transcription was identical in all samples. We also evaluated the expression of *SDF1, VEGF* and *EPO* in reticulocytes from a healthy subject and from C598T homozygote. Only *VEGF* transcription was up-regulated, whereas *EPO* and *SDF1* expression was unchanged, thereby confirming the findings obtained in lymphoblastoid cells (Figure 5B). Quantitative PCR confirmed the data on *EPO* and *SDF1* expression. Immunoblotting experiments with 2 other HIF-1α targets-aldolase and *GAPDH* revealed no variations (Figure 5C).

Discussion

This study demonstrates that Chuvash polycythemia is frequent in Campania and is endemic on the island of Ischia. This is the only cluster known besides the original Chuvash cluster. Our observation supports the notion that this erythrocytosis variant is spread throughout the world and demonstrates that it might be very frequent in some areas. A study carried out in other Italian regions (data not shown) suggests that the Ischia cluster is unique in Italy. Moreover, the finding that our 12 patients have the same haplotype as the Chuvash patients (11) supports the single-founder hypothesis. Although there is no proof of direct contact between the Chuvash and the inhabitants of Ischia, there is evidence, albeit weak, of a link between the 2 populations that might account for the high incidence of the disease on the island. The Chuvash derive from the Huns, who are thought to derive from the Middle East populations of the Sumerians and Scythians. The Huns also interacted with the Hungars and Vandals (28). Thus, the C598T *VHL* allele may have reached Ischia consequent to (a) the Vandals' invasion of Ischia that started from a Carthaginian harbor at the time of Attila the Hun, (b) the Hungars' conquest of central and south Italy, and (c) the pillaging by Turks of Ischia and surrounding areas (29). Irrespective of the route of transmission, we cannot explain the high incidence of the C598T mutation in Ischia and not in other Italian regions with a similar history. It is conceivable that a founder effect in a small isolated

population within an island under social and environmental conditions that retard outbreeding may have led to the emergence of the mutation. Another possibility is that the altered gene might convey advantages in terms of iron metabolism, erythropoiesis, and embryonic development (2, 3). For instance, improved erythropoiesis could compensate for iron deficiency consequent to a fish-based diet. Moreover, a slight increase of HIF-1α–regulated cytokines might be useful in such conditions as preeclampsia. Our data on HIF target gene transcription in Chuvash polycythemia differ from those of a previous study (3) in 2 aspects. First, *EPO* expression was not up-regulated in our EBV-transformed B- lymphocytes, which might reflect the low expression of *EPO* in these cells. Second, and more intriguing, is the observation that of the 5 genes expressed in lymphoblastoid cells, namely *VEGF, SDF1, TP1,* aldolase, and *GAPDH* (30,31), only *VEGF* appears to be up-regulated in the Chuvash-like polycythemic lymphoid cells. It is probable that, in an identical genetic background, different HIF-1α levels are required to express specific genes or sets of genes, which would explain, at least in part, the distinct phenotypes observed in subjects with different *VHL* mutations. The mechanism underlying *VHL*-dependent polycythemia in patients with only one altered allele is not clear. In this context, it is noteworthy that we also found polycythemic patients who fulfilled the Chuvash-like erythrocytosis criteria (9) and had high serum Epo, but who had no *VHL* mutations (S.P. and F.D.R., manuscript submitted). This raises the possibility of alterations at other steps of the HIF-1α-related pathway. A clinical aspect of this study is that in regions, such as Chuvashia and Ischia, congenital polycythemia should be considered a "frequent" nonbenign hematologic disease. Awareness of this frequency may lead to early diagnosis and hence better patient management. Finally, because it is not strictly confined to Chuvashia and not solely a result of the C598T mutation, we suggest that "*VHL*-dependent polycythemia" would be a more accurate term for this condition.

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Figures:

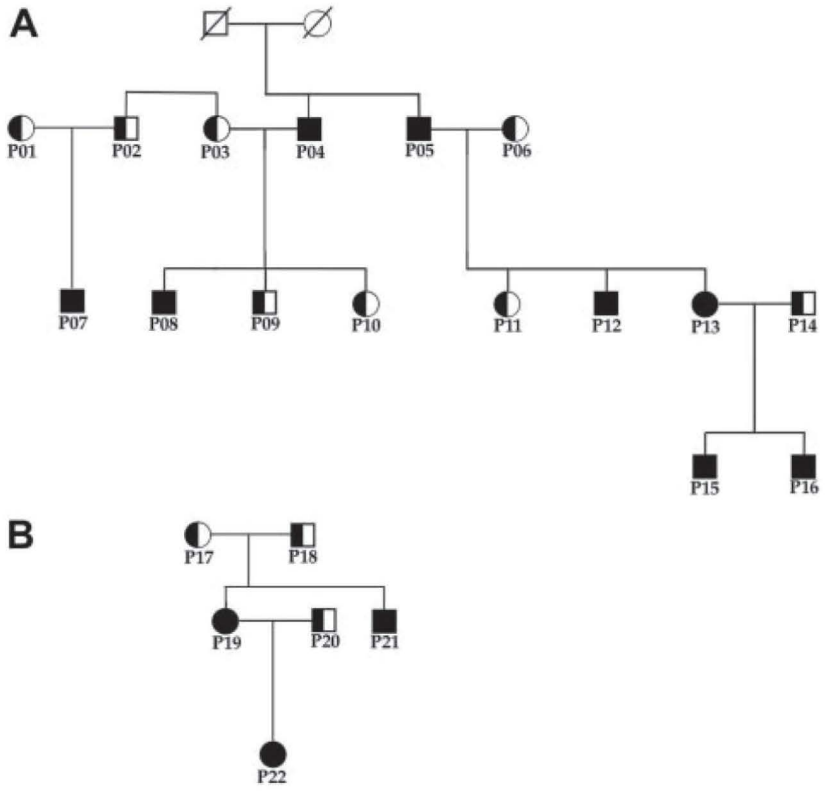

Figure 1: Pedigrees of 2 families affected by *VHL*-dependent polycythemia living on the island of Ischia. The *P* code denotes individuals from whom DNA samples were obtained. Filled symbols denote polycythemic subjects who are homozygous for the C598T mutation; half-filled symbols, heterozygous subjects.

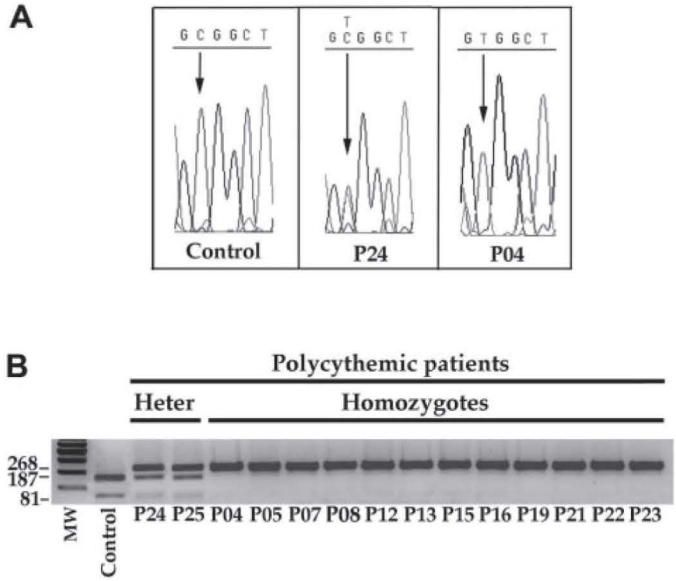

Figure 2: Detection of the *C598T* mutation in DNA samples from 2 heterozygotes and 12 homozygotes. (A) Examples of sequences of the relevant region of the *VHL* gene in a control subject, in a heterozygote (P24), and in a homozygote (P04) patient. (B) *FnU4HI* digested the 268-bp PCR product of the wild-type *VHL* allele into 187- and 81-bp bands. Conversely, the *C598T* mutation resulted in an uncut 268-bp band. Patients P24 and P25 were heterozygotes for the mutation, whereas the other patients are homozygotes. MW indicates molecular weight standards; Heter heterozygotes.

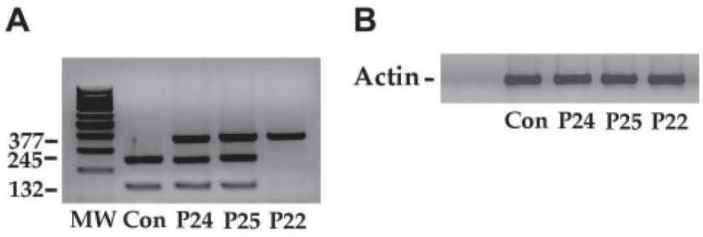

Figure 3: *VHL* expression in lymphoblastoid cells from a control subject (Con), 2 heterozygotes (P24 and P25), and a homozygote (P22). (A) Total RNA from each cell line was retrotranscribed to cDNA, which was amplified as reported in "Patients, materials, and methods," and the product (377 bp) was digested with *FnU4HI*. The normal allele yielded 2 fragments of 245 bp and 132 bp. The *C598T* mutation abolished the restriction site and resulted in an uncut 377-bp band. The 2 heterozygote patients (P24 and P25) expressed the allele in roughly similar amounts. Conversely, the homozygote for the *VHL* mutation shows a single undigested band. (B) The expression of the actin gene served as control.

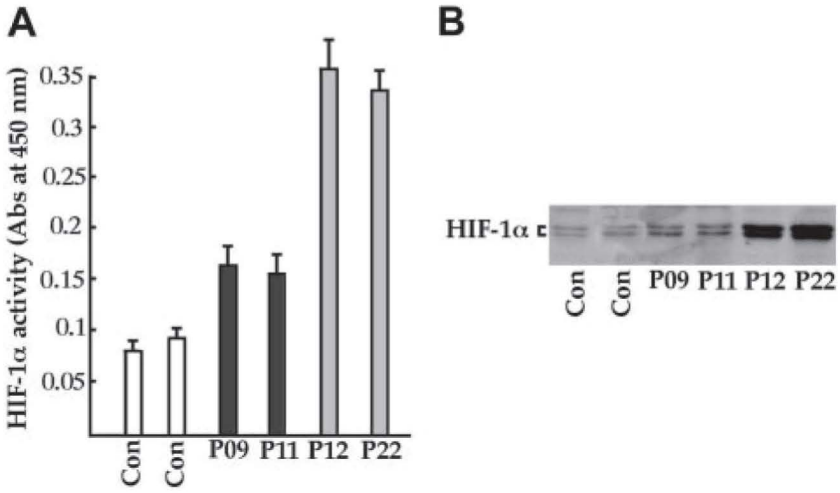

Figure 4: HIF-1α activity in lymphoblastoid cell lines. (A) Samples from 2 control subjects (Con), 2 healthy heterozygotes (P09 and P11), and 2 homozygotes (P12 and P22) were cultured, and nuclear extracts were assayed for HIF-1α activity with the TransAM HIF-1 kit (Active Motif) and (B) with immunoblotting with the antibody in the TransAM HIF-1 kit. Error bars indicate 2 SDs.

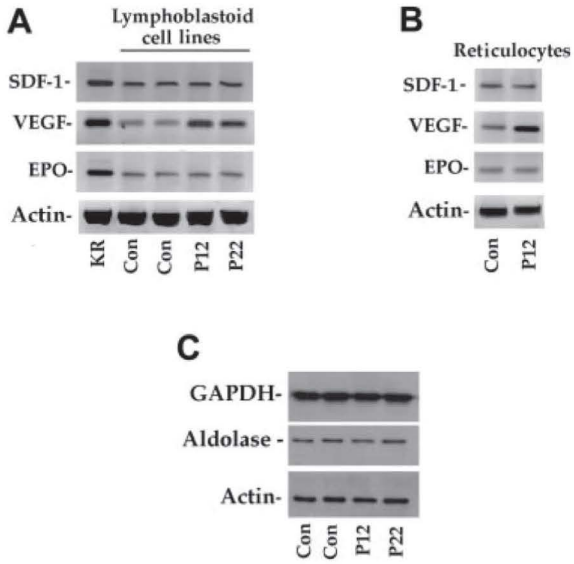

Figure 5: HIF-1α–dependent expression in lymphoblastoid cell lines and reticulocytes. (A) Total RNA from lymphoblastoid cells of 2 control subjects (Con) and 2 homozygote patients (P12 and P22). Kidney total RNA (KR) was used as a positive control. Expression of the actin gene served as a control. (B) Total RNA from the reticulocytes of a control and a homozygote patient (P12) were used as starting material. (C) Immunoblotting analysis of aldolase and GAPDH in cellular extracts of 2 control subjects and 2 homozygotes (P12 and P22). See “Patients, materials, and methods” for further details.

3 - Congenital Erytrocytosis

3.2

Erythrocytosis associated with HIF2A mutations confirms the critical role of the 531-hydroxyl acceptor proline

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Manuscript in preparation

Introduction

The ultimate result of tissue hypoxia is increased erythropoietin (EPO) production driving increased red cell production and, thus, increased oxygen supply to the tissues. This occurs as a result of increased *EPO* gene transcription. Induction of this gene by hypoxia led to the discovery of the transcription factor hypoxia-inducible factor (HIF) and the pathway through which this protein responds to differing oxygen levels.

The three major proteins of this oxygen-sensing pathway regulating the *EPO* gene are the prolyl hydroxylases (mainly the type 2, i.e. PHD2), HIF-2α, and the von Hippel-Lindau (VHL) [Lee, 2011]. PHD2 is a prolyl hydroxylase that site-specifically modifies HIF-2α in an oxygen-dependent manner [Schofield 2004].

The primary site of hydroxylation is Pro-531 of HIF-2α, and this post translational modification allows recognition by VHL, a component of an E3 ubiquitin ligase complex [Majmundar 2010, Kaelin 2008, Semenza 2010]. This complex consists of elongin B elongin C, the cullin scaffold protein Cul2 and the ring-finger protein Rxb1. HIF-2α is then a target for proteasomal proteolysis by the ubiquitin-proteasome pathway [Tanimoto 2000, Maxwell 1999, Ohh 2000]. Under normoxic conditions, VHL targets HIF-2α for constitutive degradation. Under hypoxic conditions, prolyl hydroxylation is decreased, allowing stabilization of HIF-2α.

HIF-2α then, accumulates and associates with the stable HIF-β in the nucleus and forms a transcriptionally active HIF complex. This complex binds to a series of promoters and enhancers and leads to the transcription of a large number of genes and, protein production. These genes include those involved in hormone regulation, energy metabolism, angiogenic signaling, vasomotor regulation, matrix and barrier functions, transport, virus-related genes, transcriptional regulation, growth and apoptosis, and cell migration. The HIF complex controls numerous cell functions, including the *EPO* gene transcription and thus, EPO production, increased red cell formation and hence increased oxygen delivery to tissues [Fandrey 2004, Schofield 2004, Jelkmann 2007]. There is a striking conservation of this system across higher eukaryotes, from nematodes to vertebrates.

Recent studies have identified erythrocytosis-associated mutations in the genes that encode for these three proteins of the oxygen-sensing pathway [lee 2011, Percy Haem 2008, Yoon 2011Wenger 2010, McMullin Exp rev 2010]. These include homozygous or compound heterozygous mutations of *VHL* gene, heterozygous PHD2 gene mutations, and heterozygous mutations of the *HIF2A* gene, [Lee 2011, Gordeaux 2005]. Intriguingly, haplotypes in the *HIF2A* and *PHD2* genes have also been associated with adaptation to high altitudes in Tibetans, highlighting a central role for these genes in hypoxic adaptation [Beall 2010, Simonson 2010, Yi 2010]. Current evidence indicates that the *PHD2* and *VHL* mutations lead to loss of function of the respective proteins, while the *HIF2A* mutations lead to a gain of function of HIF-2α [Lee 2011].

A number of *HIF-2A* mutations have been described (Pro534Leu, Met535Val, Met535Ile, Met535Thr, Gly537Trp, Gly537Arg, Phe540Leu) (Percy Nejm 2008, Percy blood 2008, Martini Haem 2008, gale 2008, Furlow 2009 Percy AJH 2012). Generally, these patients had a dominant erythrocytosis with inappropriately normal or raised EPO levels and, in some cases, a history of thrombosis or pulmonary hypertension.

All of these considerations make the documentation of human mutations in the oxygen-sensing pathway of considerable interest.

In the present report, we identify two *HIF2A* mutations associated with erythrocytosis.

Results and Discussion

Patient **D.I.**, an asymptomatic 15-year-old Italian male, presented with increased hemoglobin (Hb) of 16.9 g/dl, hematocrit (Hct) of 0.57, white cell count of 9.3 x 109/l, and platelet count of 245 x 109/l during routine blood tests at 1 year of age. The oxygen dissociation curve and abdominal ultrasound were both normal. There is no history of thrombosis or pulmonary hypertension and no family history of erythrocytosis. Both parents showed normal Hb and Hct levels. Repeat Hb level was 18.9 g/dl and at this time his serum EPO was 6.8 mU/ml (reference range 5.0–25.0 mU/ml). He remains asymptomatic with Hb at this level.

Patient **E.G.**, a 45-year-old male, presented with headache, dizziness and fatigue. His routine blood picture showed a Hb of 21.4 g/dl, a Hct of 0.66 with a white cell count of 7.9 x 109/l and normal platelet counts. No splenomegaly was detected. He was a smoker with no history of either thromboembolic events or pulmonary hypertension. He did not use any medications. Arterial blood gas analysis showed normal oxygen saturation and p50 values. EPO level was 18.2 mU/ml (reference range 5.0–25.0 mU/ml). The patient has been treated with phlebotomies and acetylsalicylic acid. His father, who had erythrocytosis, died of an acute ischemic cerebral vascular event at the age of 50. His asymptomatic 10-year-old son had also erythrocytosis (Hb of 19.0 g/dl, Hct of 0.61)

No mutations of *JAK2* V617F, *JAK2* exons 12 and 14, *EPOR* exons 7 and 8, *VHL* or *PHD2* were detected in both patients. Sequencing of exon 12 of *HIF2A* revealed two mutations (Figure). Patient D.I. was heterozygous for a c.1609 G>A mutation, which replace a glycine with arginine at aminoacid 537 **(Gly537Arg)** (Figure 1). This mutation has been previously reported by us and others, but this is the first case described where the genetic change appeared as a *de novo* mutation being not present in both of his parents. For this reason, a paternity test was performed. The result confirmed the paternity index of 99%. The *de novo* character of the present mutation strengthens the view that codon 537 is a 'hot spot' for mutations. Importantly, the serum EPO level for the patient D.I. was repeatedly found within the reference range, while in many of the described cases with the same and others *HIF2A* mutations, the EPO level was above the reference range [(Percy Nejm 2008, Percy blood 2008, Martini Haem 2008, gale 2008, Furlow 2009 Percy AJH 2012)].

Patient E.G. was heterozygous for a novel A to G change at c.1597 (c.1597C>G), resulting in Ile533Val mutation. The mutation creates a *BmsFI* restriction site. The change was found in his son confirming the family history of erythrocytosis (Figure 2). To test whether this mutation was a single-nucleotide polymorphism, the control panel of 200 DNA samples was screened by means *BmsFI* digestion (Figure 3). It was found to be negative for the mutation. Finally, the serum EPO levels were also in this case within the reference range in both patients.

Both mutations share two key features with previously described *HIF2A* mutations [Lee 2010]: They are heterozygous and they affect residues that strictly follow the primary site of prolyl hydroxylation in HIF-2α (i.e. Pro-531).

Gly537Arg mutation has been reported to impair in vitro the interaction of HIF-2α with PHD2 [Furlow 2009]. We also tested this effect. However, our findings suggest that the hydroxylation of HIF-2α by all the PHD (PHD1, 2 and 3) was not affected by Gly537Arg (or by Gly537Arg and Gly537Ala mutations) (data not reported) (Figure 4).

Conversely, the in vivo transcriptional activity (and stability) of an HIF-2α peptide was remarkably increased by Gly537Arg mutation (Figure 5).

The HIF-2α Ile533Val mutation affects a residue (Ile533) that has been identified as a target of genetic change causative of hereditary erythrocytosis. To examine the functional consequences of the Ile533Val mutation, we performed the same experiment reported for Gly537Arg mutation: we evaluated whether the mutation alters the capability of the mutant to act as substrate and we investigated the transcriptional activity of HIF-2α Ile533Val. We found that the peptide is hydroxylated by all the PHD isoenzymes as the wild type (Figure 4) and that the mutated protein was clearly more stable than the wild-type counterpart (Figure 5).

We next examined, by Real Time PCR, the expression of the HIF target genes adrenomedullin (*ADM*), N-myc downstream regulated gene 1 (*NDRG1*) and vascular endothelial growth factor (*VEGF*) in the wild type and mutant HIF-2α bearing cells. The occurrence of Gly537Arg and Ile533Ile HIF-2α, induced a significantly increase of mRNA transcript levels of *ADM, NDRG1* and *VEGF* genes when compared to wild type in the circulating erythroid and endothelial progenitors (Figure 6).

Taken together, these observations support the assignment of the Ile533Val *HIF2α* mutation as new cause of erythrocytosis.

The present study adds novel informations in the field of hereditary polycythemia and on the mechanisms by which O2 pressure controls cell physiology.

First of all, both our patients although show a remarkable erythrocytosis, do not present an increased serum erythropoietin. This suggests that different mechanisms, probably specific of the erythopoiesis (including the erythroid precursors or the bone marrow environment) might be affected by the stabilization of HIF-2α protein.

Second, all the changes we investigated do not affect the hydroxylation in vitro (catalyzed by all the PHDs) measured as the binding to VHL/cullin B and cullin C complex. These data also differ from the findings in literature. A possible explanation is difference in the experimental methodology employed. However, further studies are required to clarify whether a reduced hydroxylation is the cause of the HIF-2α protein stabilization.

Third, we identified a novel genetic change, i.e. Ile533Val mutation, as the cause of hereditary polycythemia. Intriguingly, the mutated residue is the closest to Pro-531 in the respect of any other of the erythrocytosis-associated mutations reported so far. The seemingly conservative substitution of a bulky hydrophobic amino acid with another bulky hydrophobic residue, yet it produces significant functional defects. It may be noted that Ile-533 is conserved in HIF-2α proteins from mammalian species, human HIF-1α, and HIF-3α, as well as HIF-2α proteins from chicken, frog, and zebrafish (Figure). The importance of this residue is further highlighted by X-ray crystallographic studies. As a matter of fact, the configuration of a HIF-1α peptide bound to VHL and the cocrystal structure of HIF-1α (556-574):PHD2 have been published [Min 2002, Hon 2002, Chowdhury 2009]. These structures show that Ile-533 of HIF-2α corresponding to Ile-566 in HIF-1α, is localized in one of two regions that are predicted to make essential contacts with VHL and PHD2 (residues 528-533 and residues 539-542).

Fourth, the *de novo* character of the mutation at codon 537 implies that this codon is a 'hot spot' for mutations and also confirms that Gly537 is critical for the conformational stability and functional association with VHL and PHD2.

In conclusion, our results support HIF2A mutations, all of them residing in exon 12, as a cause of erythrocytosis and further substantiate an important role for HIF-2α in the regulation of Epo synthesis in humans.

Materials and Methods

Mutational screening

Peripheral blood samples were collected at diagnosis from all patients and relatives. The study was approved by the local Ethical Committee and all subjects gave their informed consent. Genomic DNA was extracted with Blood Core Kit B (Qiagen, Hilton, Germany) according to the manufacturer's instructions. Amplifications of the entire exon 12 of HIF2A gene were performed under standard conditions by polymerase chain reaction (PCR) using FastStart Taq DNA Polymerase (Roche, New Jersey, USA), in a GeneAmp® PCR system 2700 (Applied Biosystems, Foster City, CA, USA).

The PCR products were purified (ExoSap-IT). Sequencing reactions were carried out using Big Dye® sequencing kit (Applied Biosystems).

Direct sequencing was performed in both directions for all samples on an automated sequencer ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Electropherograms were compared with the HIF2A (NCBI GenBank accession n. NC_000002) wild-type sequences. Mutations were named in accordance with the standard international nomenclature guidelines recommended by the Human Genome Variation Society (HGVS, http://www.hgvs.org/mutnomen/).

Erythroid precursor cultures

Liquid cultures of erythroid precursors from peripheral blood were prepared as previously described (Ronzoni et al.). In particular, we employed the peripheral blood CD34+ cells as a source of progenitors.

Circulating endothelial cell evaluation

Circulating endothelial precursors were measured by six-color flow cytometry as previously described (Bertolini). Primary cultures of circulating endothelial precursors were prepared as previously described (Bertolini).

Binding experiments

Biotinylated mouse HIF-2α-derived peptides were bound to NeutrAvidin-coated 96-well plates (Pierce). Hydroxylase reactions using purified recombinant GST-PHD1-3 enzymes were carried out for 1 h at room temperature. A polycistronic expression vector for His6- and thioredoxin-tagged pVHL/elonginB/elongin C (VBC) complex was kindly provided by S. Tan (Pennsylvania State University, University Park, PA). VBC was expressed in bacteria, purified by nickel affinity chromatography followed by ion exchange chromatography (Amersham Biosciences), and allowed to bind to the hydroxylated peptides. Bound VBC complex was detected by rabbit anti-thioredoxin antibodies and secondary horseradish peroxidase-coupled anti-rabbit antibodies (Sigma) using the 3,3,5,5-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding H2SO4, and absorbance was determined at 450 nm in a microplate reader.

Luciferase Assays

Subconfluent 6-wells of HEK293 cultures were co-transfected with 125 ng of pGRE5xE1bluc, 250 ng of wild-type or mutated pM3-HIF2□ (404-569. wild-type or mutated sequence) and 200 ng of the respective PHD expression construct or empty expression vector. Mastermixes contained 3.5 ng pRL-SV40 to normalize for transfection efficiency 24 hours post transfection, were equally distributed onto 12-well plates and grown for an additional 24 hours to 20% oxygen. Cells were subjected to dual luciferase assay as recommended by the manufacturer (Promega).

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LEGEND TO FIGURES

Figure 1

Electropherogram showing the "*de novo*" Gly537Arg substitution and the pedigree of the patient, with unaffected parents.

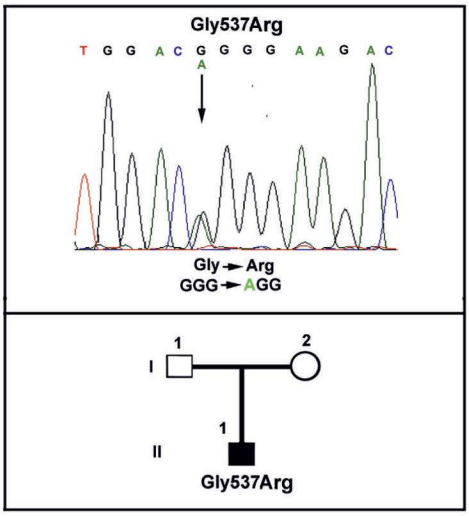


Figure 2

Electropherogram showing the novel variant (Ile533Val) found in our patient, the same variant is present in her father.

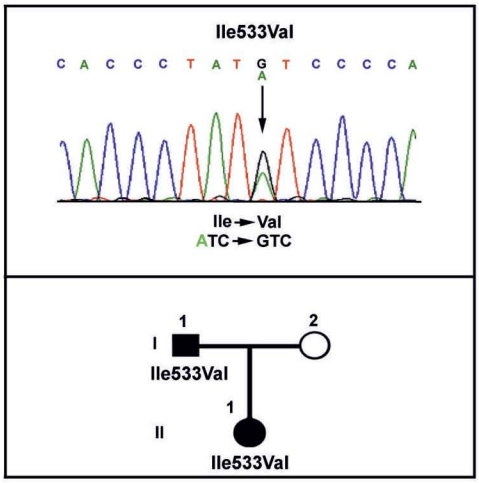


Figure 3

Enzymatic digestion showing the novel heterozygous mutation A1599G, which results in the aminoacid change Ile533Val. The mutation creates an additional restriction site for the enzyme *BmsF*I. The PCR product (868bp) has been digested and loaded on 1,5 agarose gel. Lane 1: Marker, Lane 2: undigested control, Lane 3: Wild type digested control, Lane 4: patient's healthy mother, Lane 5: patient's affected father, Lane 6: Index patient.

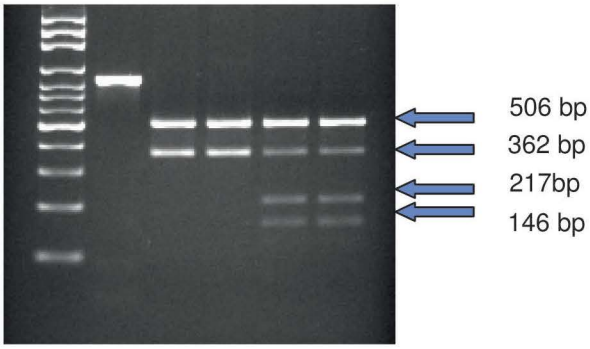


Figure 4: HIF-2a G546R mutation specifically affects hydroxylation by PHD3

Panel A. Mutations in the primary structure of human HIF-2a isoform as tested in panels B and C.

Panel B. HIF-2a peptides as depicted in A containing the indicated point mutations were subjected to VBC binding assay without hydroxylation reaction.

Panel C. Wild-type and mutant (P564A and P531A, respectively) and point-mutated peptides were subjected to in vitro hydroxylation by recombinantly expressed and purified PHD isoforms. While wild-type peptides were efficiently hydroxylated by all PHDs, the HIF-2a G456R mutation showed reduced hydroxylation specifically by PHD3 approximately 40%.

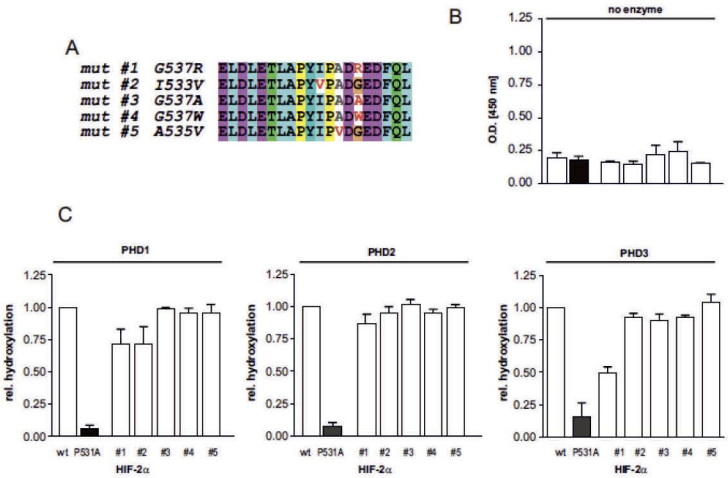
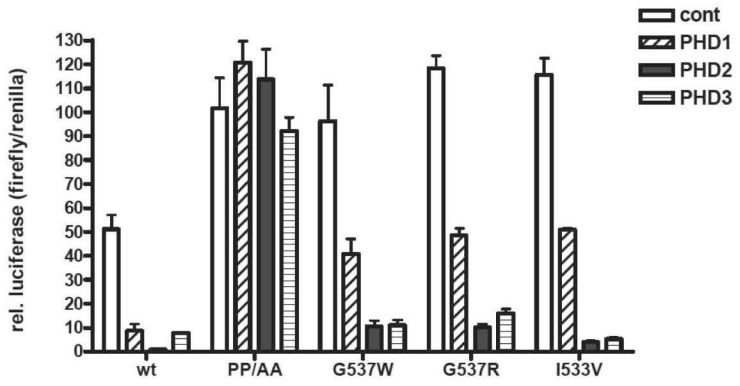


Figure 5: Transcriptional Activity of HIF-2α Ile533Val

Subconfluent 6-wells of Hek293 cultures were co-transfected with 125 ng of PGRE5xE1bluc, 250 ng of pM3-HIF-2 (aa 404-569) and 200ng of the respective PHD expression construct or empty expression vector. Mastermixes contained 3.5 ng of pRL-SV40 to normalize for transfection efficiency. 24 hours post-transfection, cultures were equally distributed into 12-well plates and grown for an additional 24 hours to 20% oxygen. Cells were subjected to dual luciferase assay as recommended by the manufacturer.



Autosomal Dominant Thrombocytopenia

4.1 *A mutation in the acyl-coenzyme A binding domain-containing protein 5 gene (ACBD5) identified in autosomal dominant thrombocytopenia*

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Inherited thrombocytopenias are uncommon and genetically heterogeneous conditions that are characterized by early onset thrombocytopenia and can occur either in isolation or in association with other abnormalities. Although many of these conditions are rare, their frequency is probably underestimated because of the difficultly in diagnosis (1, 2). Non-syndromic thrombocytopenia, with normal platelet morphology and function is usually associated with autosomal dominant inheritance (3).

In 1999, Savoia et al. performed a genome wide search on a large Italian family with autosomal dominant thrombocytopenia and incomplete differentiation of megakaryocytes (Family R.) (4), and in so doing identified the THC2 locus (OMIM 188000) on the short arm of chromosome 10 (10p11.2-12) (1). This locus (10p12-p11.2) was confirmed by Drachman et al. (5) in a second family with autosomal dominant thrombocytopenia. Thereafter, Gandhi et al. (6) indicated the microtubule associate serine-threonine kinase like (*MASTL*) gene as a possible genetic cause of thrombocytopenia in a family linked to the THC2 locus. Recently in a zebrafish model, they described a deficiency in circulating thrombocytes after transient knockdown of *mastl* (7). Despite this finding, the members of Family R. did not carry *MASTL* gene mutations. In addition, we were unable to ascertain whether this gene was variably expressed in our patients as *MASTL* is not expressed in blood. No mutations in *MASTL* were found in any of our cohort of 54 index cases, of whom 10 belong to Italian families with autosomal dominant thrombocytopenia.

We therefore screened the coding regions of all 32 genes that map to the candidate region. All the affected members of Family R. shared a common haplotype. The upper border of the region was defined by a recombination at marker D10S586 in individual 37, while the lower border of the region was defined by a recombination at marker D10S1639 in individual 26. The maximum shared region in all affected individuals is 6 cM, spanning 4.1 Mb on the physical map. The shared region includes 30 protein coding genes, four pseudogenes and two predicted open reading frames according to the NCBI build 37.1.

The only exonic variant that segregated with the disease in the 44 members (19 affected) of the Family R. (Fig. 1A) (1, 4), and the only missense change observed within the 32 genes in the region, was a variant in the gene *ACBD5* (acyl-coenzyme A binding domain-containing protein 5). This mutation (c.22C>T) results in an amino acid substitution of histidine to tyrosine at position 8 (p.His8Tyr).

The p.His8Tyr mutation replaces a charged amino acid with an uncharged polar residue. This residue is the first amino acid of the acyl-coenzyme A binding domain of the protein, which starts at position 8 and ends at position 98. His8 is well conserved between species close to Homo sapiens (e.g. Pongo abelii) or distantly related species such as Gallus gallus, but, interestingly, is not conserved in Rattus norvegicus and Mus musculus (Fig. 1B). However, this is not the first time that a change of an amino acid that is not conserved in these two species has been found to be responsible for a

human disease. One example is the finding of the α-synuclein gene responsible for one form of Parkinson's disease (8).

As suggested by Cotton et al. (9), any DNA variations should fulfil certain criteria before designating it as a disease causing mutation: it should segregate with the disease; it should affect a conserved amino acid; it should occur at a frequency of < 1% in the control population, determined by testing at least 100 chromosomes. In the 32 genes from the THC2 locus that we sequenced, the only variant that fulfils all these criteria is p.His8Tyr in *ACBD5*.

The Acbd5 protein has a predicted molecular weight of 54.7 kDa. We sequenced the 13 exons of *ACBD5* in 54 independent thrombocytopenic patients and found the heterozygous missense variant (p.His8Tyr) in one patient. The patient carrying this variant belongs to a family (Family P.) comprising 10 individuals, of whom four are affected. The family comes from the same geographic area as Family R. All affected individuals of this family carried the same missense variant as that found in the DNA of the affected members of Family R., but it was not present in the unaffected family members.

Patients from both families shared a common disease haplotype, extending from marker D10S586 to D10S611 and comprising the *ACBD5* gene. They come from two small towns close to the city of Sorrento, Italy. Next, we sequenced the exon containing the c.22C>T variant of the *ACBD5* gene in 472 unrelated healthy controls from the same geographic area as Families R. and P. None of these individuals carried the missense variant encoding p.His8Tyr.

To investigate the mRNA levels of *ACBD5* in different blood cell types and between patients and healthy individuals, we performed quantitative real-time PCR (qPCR). RNA from platelets, granulocytes, lymphocytes, reticulocytes was available from healthy individuals, while RNA from total blood was available both from patients and healthy subjects. The qPCR performed on the cDNA of healthy individuals revealed that *ACBD5* is expressed similarly in each of these cell types. The result of the qPCR of total blood mRNA from six patients (four from Family R. and two from Family P.) and four controls (three unaffected relatives of Family R. and one unaffected relative of Family P.) showed an increase in mRNA expression levels in patients of 55% when normalized to endogenous control gene β-actin and 54% to *SDHA* (succinate dehydrogenase complex, subunit A) (10). We consistently obtained the same results across several experiments.

Acbd5 is an acyl-coenzyme A binding protein. This family of proteins plays a role in the sequestration, transport and distribution of long chain acyl-coenzyme As in cells and can also be signaling molecules involved in cell metabolism and gene regulation. They have been studied in relation to hematopoietic cell development (11). The clarification of the involvement of Acbd5 in megakaryocyte differentiation and maturation may in time elucidate the mechanism underlying the molecular defects that result in platelet reduction in patients. Identification of proteins that associate or interact with Acbd5 and

influence its biological activity may reveal more about its function in platelet formation. As well as the amino acid change and its possible effect on the Acbd5 function, we also found a 54% increase in mRNA levels of *ACBD5* in these patients. Further studies are now necessary to reveal how the increase in *ACBD5* expression levels in the cell could lead to the incomplete megakaryocyte differentiation and support the link between Acbd5 and autosomal dominant thrombocytopenia. Although the association between the c.22C>T change and autosomal dominant thrombocytopenia appear very likely, it is conceivable that this mutation might not itself be causative, but rather associated through linkage with another unidentified mutation in *ACBD5* gene or its regulatory regions, or even in another gene within the same locus.

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Figures:

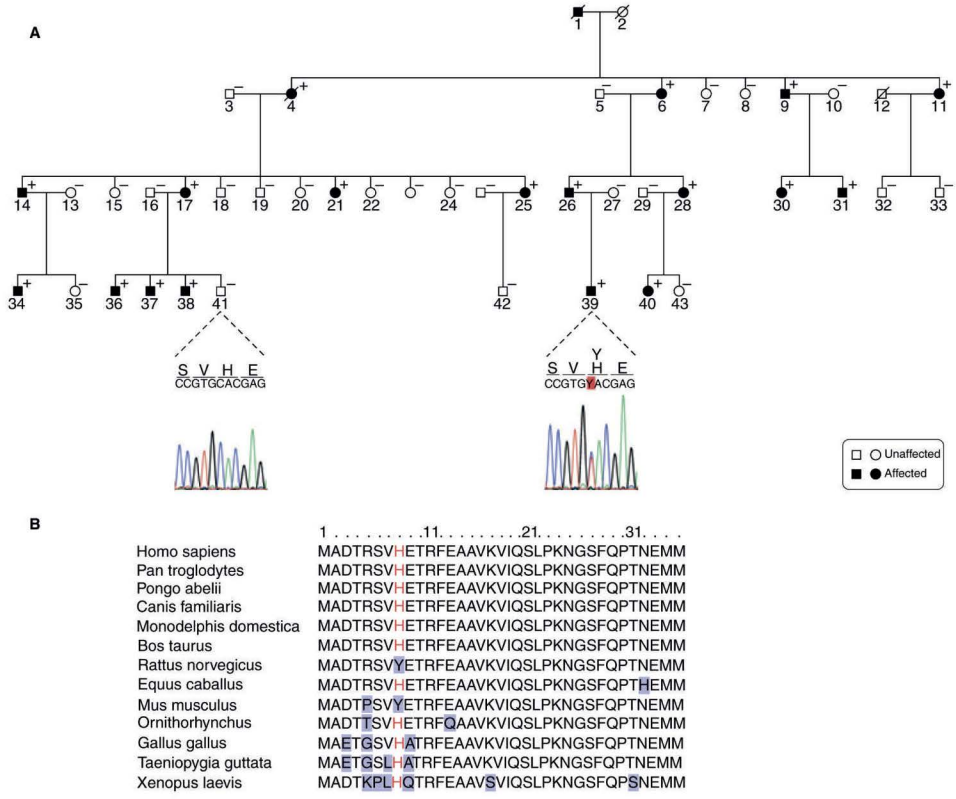

Figure 1: (A) Pedigree of Family R. Plus symbols represent individuals carrying the c.22C>T variant. Minus symbols: not carrying the c.22C>T variant. The wild type and mutant *ACBD5* sequences are shown. (B) Alignment and conservation of the first part of the Acbd5 protein containing the histidine affected by the p.His8Tyr variant. The amino acids not conserved through the species are highlighted in gray.

4.2

Mutations in the 5' UTR of ANKRD26, the Ankirin Repeat Domain 26 Gene, Cause an Autosomal-Dominant Form of Inherited Thrombocytopenia, THC2

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THC2, an autosomal dominant thrombocytopenia described so far in only two families, has been ascribed to mutations in *MASTL* or *ACBD5*. Here, we show that *ANKRD26*, another gene within the THC2 locus, and neither *MASTL* nor *ACBD5*, is mutated in eight unrelated families. *ANKRD26* was also found to be mutated in the family previously reported to have an *ACBD5* mutation. We identified six different *ANKRD26* mutations, which were clustered in a highly conserved 19 bp sequence located in the 5' untranslated region. Mutations were not detected in 500 controls and are absent from the 1000 Genomes database. Available data from an animal model and Dr. Watson's genome give evidence against haploinsufficiency as the pathogenetic mechanism for ANKRD26-mediated thrombocytopenia. The luciferase reporter assay suggests that these 5' UTR mutations might enhance ANKRD26 expression. *ANKRD26* is the ancestor of a family of primate-specific genes termed POTE, which have been recently identified as a family of proapoptotic proteins. Dysregulation of apoptosis might therefore be the pathogenetic mechanism, as demonstrated for another thrombocytopenia, THC4. Further investigation is needed to provide evidence supporting this hypothesis.

Inherited thrombocytopenias are a heterogeneous group of diseases characterized by a reduced number of blood platelets and a bleeding tendency that ranges from very mild to life threatening (1). Fifteen forms of inherited thrombocytopenias are described in OMIM (Online Mendelian Inheritance in Man). For some forms, the genetic defect has been identified in one of the many genes participating in the complex processes of megakaryopoiesis and platelet production, whereas for other forms the gene that is mutated is still unknown (1,2). Moreover, nearly 40% of patients with an inherited form of thrombocytopenia remain without a definite diagnosis because their condition has never been described or was not recognized as pertaining to a known disorder (3).

Thrombocytopenia 2 (THC2 [MIM 188000]) is one of the rarest forms of autosomal-dominant thrombocytopenia. It has so far been reported in only two families, one from Italy and the other from North America (4,5). THC2-affected individuals had a degree of thrombocytopenia ranging from mild to severe and suffered from a mild bleeding diathesis without any major bleeding events. Morphological platelet studies did not identify any relevant defect, and in vitro studies did not reveal any functional abnormality. Thrombocytopenia was attributed to defective platelet production because examination of bone marrow found evident dysmegakaryocytopoietic phenomena in both families. The THC2 locus was mapped to chromosome 10p11.1-p12 through linkage analysis in two independent studies (4,5). Two missense changes in different linked genes were found to be causative of the disease: c.501G was incorrectly named as c.565G (6) of *MASTL* ([MIM 608221], NM_032844.3) in the North American family (6) and c.22C *ACBD5* (NM_001042473.2) in the Italian one (7). Here we report evidence that, at least in the families we studied, THC2 does not derive from defects in either *MASTL* or *ACBD5* but is associated with mutations in a third gene mapping to the same locus.

We studied four pedigrees of Italian ancestry in which 20 individuals showed a clinical phenotype consistent with THC2, in that they had autosomal-dominant, non syndromic thrombocytopenia without any morphological or functional platelet defect. Written informed consent was obtained from all study subjects or their parents or legal guardians. This study was approved by the Institutional Review Board of the IRCCS Policlinico San Matteo Foundation and was conducted according to Declaration of Helsinki principles. When all known forms of autosomal-dominant thrombocytopenia were excluded, linkage to the THC2 locus was investigated. Linkage analysis was performed with Merlin version 1.1.28 under a completely penetrant autosomal-dominant model with disease allele frequency of 0.0001. We selected nine microsatellite markers (D10S586, D10S572, D10S1775, D10S197, D10S111, D10S593, CA repeat1, CA repeat2, and D10S174) across the THC2 locus. All these markers were selected from the Marshfield Genetic Map, except for markers CA repeat1 and CA repeat2, which were identified directly from the genome sequence via the on-line tool Tandem Repeat Finder (Table S1). All available family members were genotyped, and the corresponding haplotypes are represented in Figure 1. Marker allele frequencies were inferred from genotyped individuals, and average male/female

inter-marker cM distances were drawn from the Marshfield Genetic Map. Best haplotypes were estimated with the haplotyping function implemented in Merlin. Of the two larger pedigrees, pedigree 1 exceeded genome-wide significance for linkage at marker CA repeat1 with a pairwise LOD score of 3.31, whereas pedigree 2 showed consistent linkage to THC2 with a pairwise LOD score of 2.35 (Figure 1 and Table 2). In the two smaller pedigrees 3 and 4, a 10p11.1-p12 haplotype was transmitted consistently with disease segregation (Figure 1), but LOD scores were not significant because of the small size of the families (Table 2). Therefore, we searched for mutations in the coding exons and the respective flanking intronic regions of both *MASTL* and *ACBD5* in probands from the four families. The analysis identified a few SNPs present in dbSNP but did not disclose any unreported variants. Because most of the SNPs were detected in the heterozygous state, we could also exclude large intragenic deletions (data not shown).

Recombination events in the pedigrees we analyzed did not refine the THC2 locus, suggesting that a defect in a gene other than *MASTL* or *ACBD5* is responsible for THC2. We therefore analyzed all the other 30 genes in the critical region defined by previous studies. Analysis of the entire coding sequences of all the positional candidate genes detected only known polymorphisms (data not shown). Interestingly, while we were sequencing the 5' and 3' untranslated regions (UTRs), we observed different heterozygous single nucleotide substitutions within the 5' UTR of *ANKRD26* (NM_014915.2). Nucleotide changes c.-118C probands from pedigrees 3, 2, and 1, respectively. We then decided to screen the 5' UTR of *ANKRD26* in 15 additional families, and in the family originally reported to carry a mutation in *ACBD5* (4,7). In seven patients from this last family, as well as in another pedigree, the c.-134G transition was found, whereas in four other families, c.-127A and c.-116C>T, were detected (Table 1; see also Figure S1). These variants, which always segregated with the linked haplotype along the pedigrees, were not found in 500 controls, nor were they reported in the 1000 Genomes database.

In total, six different *ANKRD26* mutations were associated with thrombocytopenia in all the 35 genotyped patients in nine out of 20 independent families, and one of these mutations was found in the original linkage family (4). All of them were located in a stretch of 19 nucleotides of the 5' UTR that is conserved among primates and cattle (Figure 2). Only affected individuals from each family carry the mutation, thus confirming complete penetrance of the trait. These findings indicate that the *ANKRD26* mutations cause thrombocytopenia (Table 1) and support the idea that the p.His8Tyr mutation of *ACBD5* segregating within the family is a private rare variant linked to the THC2 locus, rather than being related to the pathogenesis of thrombocytopenia.

ANKRD26 is the ancestor of a family of primate-specific genes termed POTE (Prostate-, Ovary-, Testis-, and placenta-Expressed genes) whose expression is restricted to a few normal tissues and a larger number of pathological tissues, such as breast cancer and many other cancers (9). With regard to human bone marrow cells,

Macaulay et al. reported that *ANKRD26* is expressed in megakaryocytes, and, to a lesser extent, in erythroid cells (10).

The functional role of ANKRD26 is unknown. Mutant mice with partial inactivation of Ankrd26 develop extreme obesity, insulin resistance, and increased body size, whereas their platelet count is normal (11) (T.K. Bera, personal communication). The recently released DNA sequence of James D. Watson's genome shows that he carries a heterozygous deletion of about 31.5 Kb involving the last six exons of the gene (Database of Genomic Variants, Variation_39047), but clinical signs of thrombocytopenia are not reported (12). Taken together, these data suggest that THC2 is more likely to be due to a gain of function effect rather than a haplo insufficiency of *ANKRD26*.

In order to define the pathogenetic effects of the 5'UTR mutations, we cloned the wild-type and three mutant (c.-127A>T, c.-128G>A, and c.-134G>A) 5' UTR sequences upstream of a reporter luciferase gene. These are the mutations segregating in three families with conclusive LOD scores, including the family in which the locus was originally mapped. We also included the c.-106T>C as a control. The constructs were transiently transfected into two different cell lines: the undifferentiated myeloid K562 cells, which derived from blast crisis of human chronic myelogenous leukemia, and the Dami cells, a human megakaryoblastic cell line whose maturation toward megakaryocytic lineage can be induced by treatment with phorbol 12-myristate 13-acetate (PMA) and thrombopoietin (TPO) (13,14).

To detect differences in expression, we performed a dual-luciferase reporter assay. We first assayed the K562 cells and noticed an average increase in expression from 2.7 to 4.5 times for the c.-134G>A clone with respect to all the other constructs. We then tested the megakaryoblastic Dami cells, either without or with PMA/TPO stimulation to induce megakaryocytic maturation. We performed a one-factor ANOVA to assess the effect of 5'UTR *ANKRD26* mutations on gene reporter expression levels in the two populations of cells and observed that stimulated Dami cells, showed marked differences in expression among mutations ($p<0.001$). Then, we carried out a two-tailed Dunnett's test for multiple comparisons against the c.-106T>C as an internal control in Dami –PMA/TPO and Dami +PMA/TPO. In the first group, the c.-128G<A and c.-134G>A but not the c.-127A>T constructs overexpressed the reporter gene with respect to the control. Finally, when we assayed Dami cells in which stimulation with PMA and TPO had induced megakaryocytic maturation (Figure S2), we observed overexpression for all three mutations ($p=0.016$ for c.-127A>T and $p<0.001$ for c.-128G>A and c.-134G>A; Figure 3).

We then estimated the relative contribution of mutations and cell maturation to the variation in expression with a two-factor ANOVA and found that the largest effect was due to the presence of alterations in the 5' UTR sequence ($p < 0.001$, partial η^2 of 0.9) rather than to PMA-TPO stimulation ($p < 0.001$, partial η^2 of 0.73). This is consistent with a scenario in which the mutation interferes with reporter gene expression, and cell

maturation toward a megakaryocytic lineage then amplifies this effect. On the basis of these results, we can speculate that the mutations observed in THC2 patients interfere with the mechanisms controlling the expression of ANKRD26 and affect megakaryopoiesis and platelet production, possibly by induction of apoptosis. Recently, Liu et al. identified POTE as a new family of proapoptotic proteins (15). Morison et al. demonstrated that a different autosomal-dominant thrombocytopenia (THC4, [MIM 612004]) derives from increased apoptotic activity due to a cytochrome c mutation (16). Their observations suggest that platelet formation is particularly sensitive to changes in the intrinsic apoptotic pathway. Bone marrow examination, performed in two patients with different *ANKRD26* mutations (families 3 and 12), showed that megakaryocytes were present in normal number and that all their maturation stages were represented. This observation, although preliminary, suggests that thrombocytopenia could derive from a defect of platelet release and/or a reduced platelet life span. The preliminary expression data we present support but do not prove that increased expression and subsequent apoptosis in megakaryocytes is a plausible pathogenic mechanism. These arguments will direct further investigation aimed at clarifying the molecular events leading to THC2.

We conclude that mutations in the 5' UTR of *ANKRD26* are implicated in THC2. Analysis of this gene in the North American family previously described (6) will clarify whether THC2 is a genetically heterogeneous disease or the *MASTL*variant is benign.

Web Resources

The URLs for data presented herein are as follows:

1000 genomes, http://browser.1000genomes.org/

Database of Genomic Variants, http://projects.tcag.ca/variation/db

SNP, http://www.ncbi.nlm.nih.gov/projects/SNP/

Marshfield Genetic Map, http://www.bli.uzh.ch/BLI/Projects/genetics/maps/marsh.html

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim

Tandem Repeat Finder, http://tandem.bu.edu/trf/trf.html

Supplemental Data

Supplemental Data include two figures and one table and can be found with this article online at http://www.cell.com/AJHG/.

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Figures:

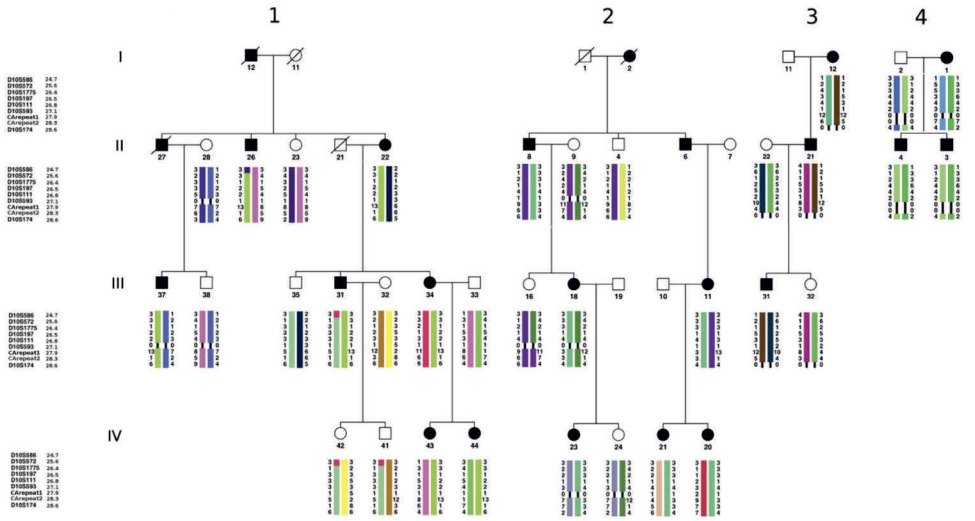

Figure 1: Linkage to Chromosome 10p11.1-p12 in four THC2 Families. Segregation of microsatellite marker haplotypes in the THC2 locus on chromosome 10p11.1-p12 (with the corresponding Mb positions) in the two large THC2-linked families (Family 1 and 2) and in the two smaller families (families 3 and 4). Black symbols indicate affected individuals, and white symbols indicate healthy ones. Slashed symbols mean that those individuals are deceased. Only individuals for whom the corresponding haplotypes are reported were genotyped. Families 1 and 2, which carry the c.-128G>A and c.-127A>T mutations, respectively (Table 1), are consistent with linkage at the THC2 locus. Families 3 and 4 do not provide significant LOD scores, but their 10p11.1-p12 region segregates consistently with the disease. Family 3 carries the c.-118C>T mutation (Table 1). No mutation in *ANKRD26* was found in the affected members of family 4, and therefore segregation of the haplotype is probably not related to the disease in this family. A 0/0 in the haplotype means unsuccessful genotyping for the marker in that individual. Haplotype representation was obtained with Haplopainter version 1.0.(17)

Table 1: *ANKRD26* 5'-UTR Mutations Identified in Nine Families with Autosomal-Dominant Thrombocytopenia and Normal Platelet Size

Family	Mutation	Affected subjects	Healthy subjects	Origin
1 [a]	c.-128G>A	7	5	Italy
2 [a]	c.-127A>T	6	3	Italy
3 [a]	c.-118C>T	3	1	Italy
5	c.-116C>T	3	2	Italy
6	c.-134G>A	2	0	Italy
9	c.-125T>G	2	0	Italy
10	c.-128G>A	3	0	Italy
12	c.-127A>T	2	0	Argentina
Savoia et al.[b]; Punzo et al.[b]	c.-134G>A	7	5	Italy

The number of affected and healthy members of each family tested for *ANKRD26* mutations is reported.

[a] Families studied by linkage analysis (Figure 1).

[b] Family described by Savoia et al.(4) and Punzo et al.(7)

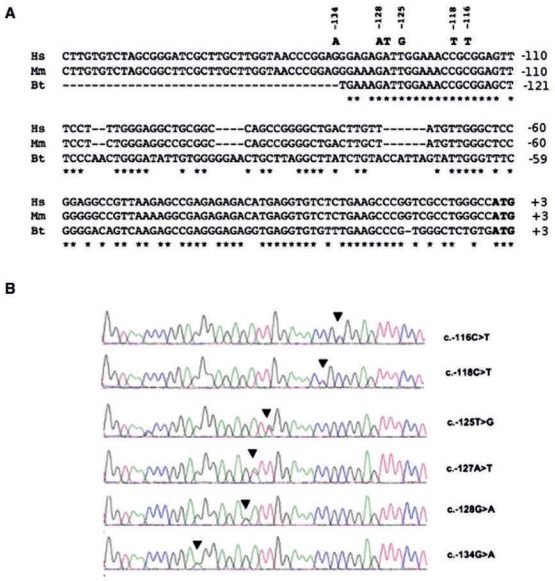

Figure 2: The 5'-UTR-Mutated Sequences of *ANKRD26* (A) Alignment of the 5'-UTRs of orthologs from Homo sapiens (Hs: NM_014915.2), Macaca mulatta (Mm: XM_002808496.1), and Bos taurus (Bt: NM_001113767.1). Nucleotide changes are in bold. Stars indicate matching sites. (B) Electropherograms showing the six different heterozygous mutations identified in THC2 families (Table 1).

Table 2: Single Point and Multi-Point LOD Scores

Family	1	2	3	4
Marker	**Multi-Point LOD Score**			
D10S586	3.1519	2.2887	0.6021	0.2706
D10S572	3.3113	2.3979	0.6021	0.3010
D10S1775	3.3113	2.3979	0.6021	0.3010
D10S197	3.3113	2.3979	0.6021	0.3010
D10S111	3.3113	2.4082	0.6013	0.3010
D10S593	3.3113	2.4082	0.6021	0.3010
CArepeat1	3.3113	2.4082	0.6021	0.3010
CArepeat2	3.3113	2.4082	0.6021	0.3010
D10S174	3.3113	2.4082	0.6021	0.3010
Marker	**Two-Point LOD Score**			
D10S586	0.4700	0.3010	0.6021	0.0000
D10S572	2.7093	1.6428	0.6021	0.3010
D10S1775	1.4313	1.4109	−0.4861	0.3010
D10S197	1.5065	−0.2334	0.6021	0.3010
D10S111	3.2325	0.1087	0.5324	0.0000
D10S593	0.5523	0.5579	0.3010	0.3010
CArepeat1	3.3113	2.3502	0.6021	-
CArepeat2	1.5953	2.3087	0.6021	-
D10S174	2.0967	0.4113	0.6021	0.3

A recombination fraction of 0 was used for single-point scores. A dash stands for "LOD score not calculated."

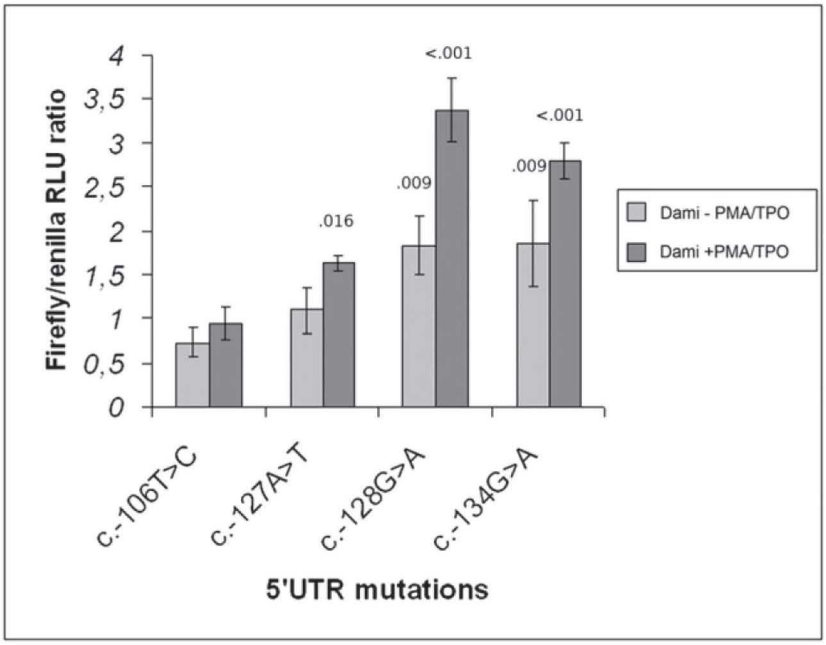

Figure 3: Firefly/Renilla RLU Ratios, Normalized against Wild-Type, for Each of the 5'-UTR Variants in the Functional Study. Scale bars represent means ± standard deviation. Corresponding values of each variant for –PMA/TPO Dami cells (light gray) and +PMA/TPO Dami cells (dark gray) are as follows: c.-106T>C -0.73 ± 0.16, 0.94 ± 0.19; c.-127A>T- 1.09 ± 0.26, 1.63 ± 0.91; c.-128G>A -1.83 ± 0.19, 3.37 ± 0.36; and c.-134G>A -1.84 ± 0.49, 2.79 ± 0.20. The assumption of homogeneity of variances was respected both in - PMA/TPO and in + PMA/TPO Dami (*Levene's* statistic > .13 and .36, respectively). Normality of the distribution was respected for all the samples (Kolmogorov-Smirnov Z Test). ANOVA rejected the null hypothesis of equality of means in both groups. Significant p values at the 5% level for a Dunnett's test against the c.-106T>C control are reported above the corresponding column of the histogram.

4.3

Mutations in ANKRD26 are responsible for a frequent form of inherited thrombocytopenia: analysis of 78 patients from 21 families

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Until recently, thrombocytopenia 2 (THC2) was considered an exceedingly rare form of autosomal dominant thrombocytopenia and only 2 families were known. However, we recently identified mutations in the 5'-untranslated region ofthe *ANKRD26* gene in 9 THC2 families. Here we report on 12 additional pedigrees with *ANKRD26* mutations, 6 of which are new. Because THC2 affected 21 of the 210 families in our database, it has to be considered one of the less rare forms of inherited thrombocytopenia. Analysis of all 21 families with *ANKRD26* mutations identified to date revealed that thrombocytopenia and bleeding tendency were usually mild. Nearly all patients had no platelet macrocytosis, and this characteristic distinguishes THC2 from most other forms of inherited thrombocytopenia. In the majority of cases, platelets were deficient in glycoprotein Ia and a-granules, whereas in vitro platelet aggregation was normal. Bone marrow examination and serum thrombopoietin levels suggested that thrombocytopenia was derived from dysmegakaryopoiesis. Unexplained high values of hemoglobin and leukocytes were observed in a few cases. An unexpected finding that warrants further investigation was a high incidence of acute leukemia. Given the scarcity of distinctive characteristics, the *ANKRD26*-related thrombocytopenia has to be taken into consideration in the differential diagnosis of isolated thrombocytopenias.

Introduction

Making a definitive diagnosis in patients with inherited thrombocytopenia is important because different forms differ with respect to prognosis and treatment. For example, congenital amegakaryocytic thrombocytopenia always presents with severe thrombocytopenia at birth and rapidly progresses to trilineage bone marrow failure that benefits from bone marrow transplantation (1). Thrombocytopenia is severe at birth also in "thrombocytopenia with absent radii," but platelet count improves over the first year of life and eventually approaches normal levels in adult life (2). Thus, only supportive treatment is usually required. Recently, thrombopoietin (TPO) mimetics were shown to increase platelet count in MYH9-related disorders (3), opening up new therapeutic possibilities for these illnesses. Some inherited thrombocytopenias, such as familial platelet disorder with propensity for myeloid malignancy, significantly increase the risk of leukemia (4), whereas others, such as MYH9-related disorders (5), expose to the risk of extrahematologic defects that may benefit from early recognition and appropriate treatment (6). Thus, a careful and targeted follow-up is mandatory for these patients.

Unfortunately, a common experience for all who care for subjects with hereditary thrombocytopenias is that making a definite diagnosis is not possible in several patients because their disorders have never been described. An analysis of a series of 46 consecutive patients revealed that these "new" illnesses affect nearly 40% of patients (7). Because prognosis and treatment remain poorly defined in a large portion of cases, the identification and characterization of "new" forms are important objectives in present research.

Thrombocytopenia 2 (THC2, MIM 188000) is an autosomal dominant form of thrombocytopenia that, until recently, was identified in only 2 families: one in Italy (8) and the other in the United States (9). The THC2 locus was mapped to chromosome 10p11.1-p12 in both families, and 2 missense mutations in the *MASTL* (MIM 608221) and *ACBD5* genes were subsequently identified as the causative defects in the North American family (10) and Italian family (11), respectively. However, we recently showed that 6 different heterozygous single nucleotide substitutions in a short stretch of the 5'-untranslated region (5'-UTR) of *ANKRD26* (MIM 610855), a gene within the THC2 locus, were responsible for thrombocytopenia in 9 pedigrees (12). Notably, the previously identified Italian family with the *ACBD5* substitution also had this type of mutation.

Here we present the results of a systematic screening of 105 unrelated subjects with inherited thrombocytopenias of unknown origin that allowed us to recognize 12 additional families with mutations in the same *ANKRD26* region and to identify 6 novel mutations. We also describe the clinical and laboratory pictures that emerged from the study of all 78 patients with *ANKRD26* mutations identified thus far, and discuss the unexpected finding of high frequency of acute leukemia in affected pedigrees.

Methods

Patients

We enrolled 105 unrelated patients with inherited thrombocytopenias that were being observed at the Istituto di Ricovero e Cura a Carattere Scientitico Policlinico San Matteo Foundation of Pavia and at the Department of Pediatrics of the Second University of Naples. All of them had no definite diagnosis because they did not fit the criteria for any known disorder. Whenever *ANKRD26* mutations were identified, the proband's available relatives were studied. We also included the patients (35 from 9 families) already known to have *ANKRD26* mutations, as recently published (12).

In addition, we investigated 37 patients with autoimmune thrombocytopenia (ITP), This diagnosis was made according to the guidelines recently released by a panel of experts (13) and was confirmed by the evaluation of response to therapy and subsequent clinical evolution.

Bleeding tendency was measured by the World Health Organization bleeding scale: grade 0 indicates no bleeding: grade 1, petechiae: grade 2, mild blood loss; grade 3, gross blood loss; and grade 4, debilitating blood loss. Physicians participating in the study made this evaluation during patient interviews.

The institutional review boards of the Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo Foundation of Pavia and the Department of Pediatrics of the Second University of Naples approved the study. All subjects provided written informed consent in accordance with the Declaration of Helsinki.

Mutation screening

The 5'-UTR of the *ANKRD26* gene was screened for mutations using genomic DNA extracted from peripheral blood. Mutational analysis was performed by polymerase chain reaction (PCR) amplification using oligo 1F (5'-CATGGAGCACACTTGACCAC-3') and 1R (5'-TACTCCAGTGGCACTCAGTC-3'). PCR was carried out in a total volume of 25 μL with 25 ng of genomic DNA, 10μM of each primer, 2.5μM dNTPs, 1.5mM $MgCl_2$, 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 2.5 μL of the corresponding 10X PCR buffer. After an initial denaturation step at 96°C for 12 minutes, amplification was performed for 30 cycles (denaturation at 96°C for 30 seconds, annealing at 62°C for 45 seconds, and extension at 72°C for 50 seconds). PCR products were bidirectionally sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI310 Genetic Analyzer (Applied Biosystems).

Blood cell counts and platelet size

Blood cell counts and mean platelet volume were evaluated by electronic counters in ethylenediaminetetraacetic acid anticoagulated blood samples within 2 hours after sampling. Because electronic instruments are known to be inaccurate in patients with

extreme degrees of platelet macrocytosis or microcytosis (14), in all cases these conditions were excluded by direct examination of peripheral blood films.

Platelet α–granules

According to a previously reported method (15), peripheral blood smears were stained with the P12 antibody (Sigma-Aldrich) against the α-granules secretory proteins thrombospondin-1 (TSP1), and platelets were identified by labeling for F-actin (AlexaFluor-488-conjugated phalloidin, Invitrogen). We evaluated 300 platelets in each specimen and measured the percentage of platelets with less than or equal to 5 $TSP1^+$ granules. This cut-off was chosen to distinguish platelets that, although not completely negative, presented a very marked reduction in α-granules. Results were compared with those obtained in the same sets of experiments for smears from 30 healthy subjects. In control subjects, the percentage of platelets with more than 5 $TSP1^+$ granules ranged from 94% to 99%.

Platelet flow cytometry

Surface expression of platelet glycoproteins (GPs) was investigated in platelet-rich plasma by flow cytometry with an Epics XL flow cytometer (Coulter Corporation) as previously reported (15). The following monoclonal antibodies were used: SZ21 (Immunotech) that recognizes GPIIIa (CD61), P2 (Immunotech) recognizing GPIIb in the intact complex with GPIIIa (CD41), SZ1 and SZ2 (Immunotech) against GPIX (CD42a) when correctly complexed with GPIbα and GPIbα (CD42b), Gi9 (Immunotech) against GPIa (CD49b), and 4B7R (Santa Cruz Biotechnologies) against GPIIa (CD29); MO2 (Coulter Corporation) was used as a negative control. Fluorescein isothiocyanate-conjugated goat antimouse IgG was purchased from Coulter Corporation.

Because platelet expression of GPIa on platelets varies among normal subjects depending on 3 different haplotypes of the GPIa gene (*ITGA2*) defined by 4 polymorphisms (16), the control for each patient was chosen to match his *ITGA2* genotype. To this aim, these polymorphisms were determined in patients and controls as previously described (15).

Platelet aggregation

Blood was collected in 3.8% (weight/volume) sodium citrate (blood-anticoagulant ratio 9:1), and platelet aggregation was evaluated in platelet-rich plasma by the densitometric method of Born as previously reported (15). The analysis was performed only in patients from whom platelet-rich plasma with at least 100 x 10^9 platelets/L were obtained. The following platelet agonists were used: collagen (4 and 20 μg/mL; Mascia Brunelli), and adenosine diphosphate (5 and 20mM) and ristocetin (1.5 mg/mL), both from Sigma-Aldrich. The extent of platelet aggregation was measured 5 minutes after the addition of stimulating agents, and results obtained in patients were compared with the normal ranges in our laboratory.

Serum TPO

Serum TPO levels were determined by a commercially available enzyme-linked immunosorbent assay (Quantikine Human TPO Immunoassay, R&D Systems) according to the manufacturer's instructions. By this assay, TPO serum concentrations can be directly measured when in the range of 7 to 2000 pg/mL. When required by very high TPO concentration, serum was appropriately diluted. For the purposes of this study, a value of 6.9 pg/mL was assigned to the samples with a TPO concentration below the minimum detectable level.

Statistical analysis

Continuous data are presented as mean, SD, and range, categorical variables as counts and percentages. Laboratory parameters were compared with the mean value of the (reference) healthy population by the one-sample Student *t* test, as well as to the upper or lower reference limit (if significantly larger or smaller than the mean reference value). Incidence of hematologic malignancies (per 100 000 person-years) and its Poisson exact 95% confidence intervals are reported. Stata 11 (Stata Corporation) was used for computation.

Results

Mutation screening

Table 1 presents the 11 heterozygous single nucleotide substitutions that have been identified in the 5'-UTR region of the *ANKRD26* gene in 12 of 105 investigated unrelated propositi. Six of the mutations, including c.-127A>G, c.-126T>G, c.-121A>C, c.-119C>A, c.-118C>A, and c.-113A>C, were novel mutations not detected in our previous series of THC2 patients (12). The nucleotide substitutions segregated with the disease along the pedigrees and were not reported in the 1000 genomes (www.browser.1000genomes.org) or other databases. Combining previous and present case series, 12 different mutations, all localized in a short stretch of 22 nucleotides of the 5'-UTR, were identified in 21 THC2 families. Positions -127 and -118 had 2 different substitutions: c.-127A>T or c.-127A>G and c.-118C>T or c.-118C>A. No other substitutions were identified in the 527-bp PCR product analyzed, except for the known polymorphisms, rs41299222 and rs3737056 in the 5'-UTR, and rs7897309, which leads to protein variant p.Q20R.

Of the 21 THC2 families, 17 were from Italy and the remaining from North America, Argentina, Senegal, and Spain, suggesting that THC2 is distributed worldwide.

Clinical picture

To describe the features of *ANKRD26*-related thrombocytopenia, we enrolled 78 affected persons from the 21 families that were genetically characterized. As usual for inherited thrombocytopenias, several patients were initially misdiagnosed with ITP, and 16 of them received immunosuppressive treatments, whereas 2 subjects were

splenectomized. Except for 3 patients who reported a transient increase in the platelet count during prednisone treatment, the others did not respond to these treatments.

Spontaneous bleeding events were usually absent or mild, although a few subjects experienced life-threatening hemorrhages. According to the World Health Organization bleeding scale, 36 of 78 patients had a bleeding score of 0, whereas 17, 17, 5, and 3 subjects had bleeding scores of 1, 2, 3, and 4, respectively. More common bleeding symptoms were petechiae, ecchymosis, gum bleeding, epistaxis, and menorrhagia. One patient had 2 hemorrhagic strokes and one episode of proctorrhagia, and one child had post-traumatic brain hemorrhages. Interestingly, a patient, who previously developed hemothorax during an episode of pneumonia and underwent hysterectomy because of menorrhagia, experienced deep vein thrombosis of the leg despite severe thrombocytopenia (10×10^9 platelets/L) and no predisposing cause except for varicose veins. Twenty-six patients underwent surgery, in most cases without platelet support, and only 3 reported bleeding episodes. Thirteen women gave birth, whether vaginally or through cesarean section, and only in 3 cases there were bleeding complications.

As some forms of inherited thrombocytopenia are syndromic, we searched for diseases possibly associated with thrombocytopenia in THC2 patients. Not to exclude from this study potentially fatal diseases, we sought information on family members who died before our study. Based on clinical records, we identified another 26 family members, first- or second-grade relatives of THC2 patients, who had thrombocytopenia from an early age. Although the diagnosis of THC2 was not confirmed by molecular genetic testing, we considered them as carriers of the *ANKRD26* mutation of their respective family. In this extended series of 105 patients, the only category of illness that was found to affect several subjects was that of hematologic malignancies. Seven subjects from 5 families (families 8, 9, 11, 13, and 20) had acute leukemias (5 myelogenous forms and 2 undefined forms), one patient developed chronic myelogenous leukemia (family 17), and another one chronic lymphocytic leukemia (family 12). Moreover, one patient had myelodysplastic syndrome and subsequently developed chronic lymphocytic leukemia (family 17). Furthermore, 2 other relatives of patients with *ANKRD26* mutations (families 5 and 11) had acute leukemias (one myelogenous and one undefined), but we were unable to ascertain whether they were affected by THC2. Thus, at least 10 THC2 patients had hematologic malignancies and 7 presented acute leukemias. Neither acute leukemias nor other hematologic malignancies have been reported in the 78 relatives without THC2. Considering only the 105 patients with reliable information, the total observation time was 4174 years,and the incidence of all hematologic malignancies was 240 of 100 000 (95% confidence interval, 115 to 441 of 100 000), whereas that of acute leukemia was 167 of 100 000 (95% confidence interval, 67 to 345 of 100 000).

Blood cell counts and peripheral blood film examination

The thrombocytopenia of THC2 patients was moderate in most cases (Tables 1, 2), although 8 of 78 subjects had a platelet count lower than 20×10^9/L and 5 equal or

lower than 10 x 10^9/L. Notably, one patient had a platelet count at the lower limit of the normal range, whereas 4 patients with platelet counts always lower than 50 x 10^9/L had their platelet count transiently increased to more than 150 x 10^9/L during infectious episodes (acute cholecystitis, septic arthritis, urinary tract infection, and bronchiolitis).

Hemoglobin and leukocyte values were within the normal range in the majority of patients, although values higher or lower than normal were recorded for both parameters. Although reduced hemoglobin values are not unexpected in thrombocytopenic patients because of repeated blood loss, the abnormally high levels of hemoglobin observed in 9 cases were surprising and remained without any explanation. Concerning the leukocytosis observed in 14 patients, we cannot exclude that it derived from inflammatory conditions. However, leukocytosis was reported also in the other large thrombocytopenic family linked to 10p11.1-p12 and with amutation in the *MASTL* gene (9).

Platelet size was normal in most cases, but some patients had a reduced mean platelet volume. Importantly, mean platelet volume was slightly increased only in one patient. Thus, THC2 resulting from *ANKRD26* mutations is one of the few inherited thrombocytopenias without platelet macrocytosis.

Examination of May-Grunwald-Giemsa-stained peripheral blood films did not identify any morphologic red cell or leukocyte abnormalities. Instead, platelets appeared “pale” in many cases because of reduced azurophilic granule content (Figure 1). Immunofluorescent labeling of the α-granule protein TSP1 revealed that most patients had α-granule deficiency (Figure 2); the number of $TSP1^+$ granules was lower than that of controls in 32 of 44 (72.7%) investigated cases. This defect did not affect all patients within the same family (data not shown), suggesting that environmental factors interacted with *ANKRD26* mutations to induce the deficiency.

Bone marrow examination

Bone marrow smear and/or biopsy were available for 4 probands (families 4, 8, 11, and 14). Common to all samples was an increased number of megakaryocytes (Mks), which were represented at all stages of maturation, with evidence of dysmegakaryopoiesis. Dystrophic forms consisted mainly of small Mks with hypolobulated nuclei and a small amount of mature eosinophilic cytoplasm. Typical micromegakaryocytes were also present. Representative examples are provided in Figure 3.

Platelet flow cytometry

Flow cytometric evaluation of platelet GPs was performed in 33 patients from 15 families. Components of the GPIb/IX/V and GPIIb/IIIa complexes were similar in patients and controls, whereas GPIa was less than 80% of an appropriate control with the same *ITGA2* genotype in 19 subjects from 14 families (data not shown). As for α-granule deficiency, GPIa was variably expressed in patients of the same family.

Platelet aggregation

Because of the low platelet count, in vitro platelet aggregation was evaluated in only 22 patients from 12 families. No consistent defect in the platelet response to any dose of collagen, ADP, or ristocetin was observed (data not shown).

Serum TPO

Twenty-six of 38 examined patients had serum TPO levels higher than normal range, with a mean TPO concentration 7 times higher in THC2 patients than in control (Table 2). Mean TPO level of THC2 patients was 2.9 times higher than that measured in 37 patients with ITP (38.3 ± 46.0 pg/mL). Because platelet count of ITP patients (51.7 ± 32.6 x 10^9/L) was comparable with those of THC2 patients, differences in TPO levels are expected to correlate with Mk mass ("Discussion").

Discussion

THC2 has long been considered an exceedingly rare form of inherited thrombocytopenia in that only 2 families with this diagnosis were known (8,9). However, we recently identified mutations in the 5'-UTR of *ANKRD26* in 9 THC2 pedigrees (12), and in the present paper we describe 11 additional families, all of them with mutations affecting a short stretch of 22 nucleotides in the same 5'-UTR region. By pooling the data from our previous work and those of the present study, 21 of the 125 families in our case series that remained without a definite diagnosis in the last 10 years actually had *ANKRD26* mutations. To have an idea about the relative frequency of THC2, our database of 210 cases of inherited thrombocytopenias contains, in addition to 21 pedigrees with THC2, 37 families with monoallelic, dominant Bernard-Soulier syndrome resulting from Bolzano mutation, 10 with the classic biallelic, recessive form of Bernard-Soulier, 22 with *MYH9*-related disease, and a few cases of other very rare diseases. Thus, at least in Italy, THC2 represents one of the less rare forms of inherited thrombocytopenia. However, we think that this disorder is present worldwide because 4 of our families were from Spain, Argentina, United States, and Senegal.

The case series of 78 affected family members allowed us to define the clinical picture of THC2. The disorder is transmitted as an autosomal dominant trait in all families with almost complete penetrance, having only one subject with an *ANKRD26* mutation normal platelet count. The degree of thrombocytopenia was variable, and repeated observations revealed that platelet count was variable also in the same subjects. Indeed, we detected transient increases in platelet concentration on 150 x 10^9/L in 4 patients, in all cases during inflammatory events. One possible explanation for this phenomenon may be that the increase in TPO levels that often occurs in reactive conditions (17) benefitted thrombocytopenia by increasing platelet production. The recent demonstration that a TPO mimetic drug, eltrombopag, increased platelet count in patients with *MYH9*-related disease (3) further supports this hypothesis and also offers hope that TPO mimetics may improve thrombocytopenia in THC2.

As the platelet count, also the severity of bleeding diathesis was variable, ranging from severe to mild. Although life-threatening events were reported, we are unaware of any patient dying of hemorrhage.

An alarming finding in our study was the high percentage of patients who developed acute leukemia, in that we identified 7 subjects with myeloid or undefined forms. Moreover, we noticed another 2 relatives of patients with *ANKRD26* mutations who died of acute leukemia, but we were unable to clarify whether they were affected by THC2. Considering only subjects with certain THC2, the prevalence of acute leukemia was 167 of 100 000 (95% confidence interval, 67 to 345 of 100 000), which is much higher than in the general population. Indeed, the prevalence of acute leukemia has been estimated at 3.4 to 6.6 of 100 000, depending on race and sex (National Cancer Institute, www.seer.cancer.gov/statistics). A few THC2 patients also had chronic myelogenous or lymphocytic leukemias, or myelodysplastic syndrome. Two observations from the literature seem to suggest a possible connection between *ANKRD26* and leukemia or, more in general, cancer. One somatic mutation in the coding region of *ANKRD26* was described in one patient with acute myeloid leukemia (18), and a mouse model indicated that the tumor suppressor *RARRES1* regulates *Ankrd26* (19). However, we are far from concluding that *ANKRD26* mutations predispose to acute leukemia or to any other malignancy because the number of investigated patients is still too low. Moreover, the observation that acute leukemia affected only 4 of 21 families argues against this hypothesis. Anyway, we think that this matter deserves further study and that future descriptions of new THC2 patients should include an accurate family history about cancer. Clarifying this point may also be important in the light of the aforementioned potential benefit of treatments with TPO mimetic drugs, which should be used with caution if it is demonstrated that *ANKRD26* mutations facilitate the onset of leukemia.

Concerning diagnosis, the typical patient with mutations inthe 5'-UTR of *ANKRD26* had moderate thrombocytopenia with normal-sized platelets, reduction of platelet α-granules, reduced platelet GPIa, normal platelet aggregation, and increased serum TPO levels. However, several exceptions have been observed, and we suggest that the most constant characteristic of our patients was that the size of their platelets was not enlarged. This feature is very useful for diagnostic purposes, in that the absence of platelet macrocytosis distinguishes THC2 from the majority of other inherited thrombocytopenias.

The observation that platelets from several patients had defective GPIa surface expression and reduced α-granule content deserves comment because, a few years ago, we described 2 families with autosomal dominant thrombocytopenia, normal platelet size, defective platelet expression of GPIa, and reduced platelet α-granules (15). Their clinical and laboratory picture, therefore, was indistinguishable from those of THC2, and we suspect that "autosomal dominant thrombocytopenia with reduced expression of glycoprotein Ia" and THC2 are the same disorder. However, we were

unable to confirm this hypothesis because no affected member of the 2 previous families was available for genetic analysis.

Although the aim of our study was to identify the clinical and laboratory picture of THC2, our data on TPO levels and bone marrow examination in 4 patients allow us to speculate on the pathogenesis of thrombocytopenia in THC2 patients. TPO levels in blood are primarily regulated by platelets and Mks, which internalize and degrade TPO (20,21), and TPO levels are therefore inversely related to total Mk and platelet mass. The mean TPO value in THC2 patients was increased 7-fold compared with controls, indicating that the platelet-Mk mass was reduced. Interestingly, TPO levels in THC2 patients were 2.9 times higher with respect to a case series of 56 patients with ITP that had a completely superimposable platelet count. Considering that subjects with congenital amegakaryocytic thrombocytopenia have TPO levels more than 200 times higher than controls (22), we have to conclude that Mks were well represented in THC2, but to a lesser extent than in ITP, in which the Mk mass is typically expanded and the proportion of very large cells is increased. Therefore, the TPO values of THC2 subjects are consistent with the results of bone marrow examination, which revealed that the number of Mks was increased but also found dysmegakaryopoietic phenomena, such as micromegakaryocytes and Mks with a single nucleus and/or delayed maturation. Quite similar Mk defects were described in the Italian and American THC2 families reported 10 years earlier (8,9). Altogether, these data suggest that *ANKRD26* mutations affect Mk maturation.

In conclusion, THC2 resulting from *ANKRD26* mutations emerged from our study as one of the most frequent forms of inherited thrombocytopenias. Therefore, this diagnosis has to be considered in differential diagnosis of all patients with autosomal dominant forms of thrombocytopenia without platelet macrocytosis. Further investigation is required to ascertain whether the high prevalence of leukemia we observed derived from *ANKRD26* mutations or occurred by chance.

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Figures:

Table 1: Main clinical and laboratory features of patients with *ANKRD26*-related thrombocytopenia

Family/ no. of patients	*ANKRD26* 5′-UTR mutation	Mean age, y (range)	WHO bleeding score (no. of patients)	Mean platelet count, $\times 10^9$/L (range)	Mean MPV, fL (range)	Mean hemoglobin, g/dL (range)	Mean WBC, $\times 10^9$/L (range)
1/3	c.-113A → C	26.6 (14-50)	0, 1 (2)	85 (23-176)	11.43 (11-12.1)	12.53 (11.9-13.2)	3.9 (2.71-5.6)
2*/2	c.-116C → T	21 (6-36)	1, 2	42 (36-48)	10.85 (10.5-11.2)	14.95 (13.9-16)	11.04 (6.08-16)
3/2	c.-118C → T	30.5 (19-42)	1, 1	40 (7-73)	11.5 (11.4-11.6)	13.7 (12.9-14.5)	7.64 (6.12-9.16)
4*/2	c.-118C → T	48.5 (32-65)	0, 2	45 (44-46)	9.9 (9.8-10)	15.15 (15-15.3)	7.43 (5.61-9.26)
5/4	c.-118C → A	75 (58-84)	1, 2 (2), 4	42 (15-79)	8.89* (8.68-9.1)	13.87 (12.3-16.2)	9.87 (7.31-12.6)
6/1	c.-119C → A	10	0	58	6.2	12.2	5.5
7/3	c.-121A → C	55.3 (41-77)	0 (2), 3	63 (28-87)	10.26 (10-10.8)	15.63 (12.8-17)	15.45 (11.6-21.33)
8*/2	c.-125T → G	69.5 (59-80)	0, 1	46.5 (43-50)	6.75 (6.5-7)	16.25 (16-16.5)	10.5 (10-11)
9/2	c.-125T → G	44.5 (37-52)	2, 4	8.5 (7-10)	9.2 (7.6-10.8)	14.65 (12-17.3)	10.4 (7-13.8)
10/3	c.-126T → G	42 (27-69)	2 (3)	44.33 (21-80)	10.6 (10.4-10.9)	15.36 (13.9-16.5)	9.41 (8.55-12.19)
11*/7	c.-127A → T	28.8 (12-65)	0 (6), 3	37.4 (10-56)	11.16 (9.3-14.3)	13.41 (10.5-15.8)	8.76 (7.45-9.9)
12/3	c.-127A → T	23 (9-40)	0 (3)	67.66 (46-94)	NA	ND	8.27 (6.89-9.32)
13*/2	c.-127A → T	35.5 (21-50)	0, 3	31 (23-39)	7.4 (7.3-7.5)	14.5 (13.7-15.3)	8.45 (8.4-8.5)
14/2	c.-127A → G	31.5 (19-44)	1, 1	94 (46-142)	7.04 (6.48-7.6)	16.1 (13.9-18.3)	9.81 (7.93-11.7)
15*/7	c.-128G → A	51.7 (25-83)	0 (3), 2 (4)	30 (14-41)	10.6 (9.8-11.6)	15.35 (12.5-18.4)	9.03 (6.48-10.65)
16/2	c.-128G → A	14.5 (1-28)	0, 1	28.5 (7-50)	9.35 (9.3-9.4)	14.95 (13.6-16.3)	11.1 (10.06-12.57)
17/2	c.-128G → A	35.5 (23-48)	0, 3	52 (35-69)	8.05 (7.8-8.3)	13.25 (13.1-13.4)	6.91 (6.43-7.4)
18*/3	c.-128G → A	25.3 (10-33)	2 (2), 3	33.66 (16-53)	NG	11.83 (10.2-13.6)	7.62 (6.77-8.32)
19/2	c.-134G → A	52 (38-66)	0 (2)	50.5 (44-57)	8.6 (8.4-8.8)	14.4 (13.3-15.5)	9.22 (7.4-11.05)
20*/22	c.-134G → A	41.2 (13-69)	0 (11), 1 (7), 2 (3), 4	53.59 (24-106)	8.53 (7.1-11)	15.18 (10.5-17.8)	9.34 (6.03-16.4)
21*/2	c.-134G → A	26.5 (16-37)	0 (2)	38.5 (30-47)	8.9 (8.8-9)	12.5 (12-13)	10.9 (10.7-11.1)

WHO indicates World Health Organization; NA, not applicable; ND, not done; and NG, data not given by the automatic cell counter.*Mutation in the family has been already reported (12).

Table 2: Hemoglobin concentration, blood cell counts, mean platelet volume, and serum TPO level of patients with *ANKRD26* mutations

Parameter (no. of investigated patients)	Patients, mean (SD), no. of cases with values higher/lower than the normal range	Healthy subjects, mean (normal range)	One sample *t* test vs mean value of healthy subjects, *P* (*P* vs upper/lower reference limit)
Hemoglobin, g/dL			
Male (n = 40)	15.4 (1.9), 4/7	15.7 (14.0-17.5)*	.32
Female (n = 38)	13.5 (1.4), 5/4	13.8 (12.3-15.3)*	.19
WBC, $\times 10^9$/L (n = 78)	9.2 (2.8), 14/2	7.8 (4.4-11.3)*	< .001 (1.000)†
Neutrophils, $\times 10^9$/L (n = 78)	5.51 (2.3), 11/3	4.4 (1.8-7.7)*	< .001 (1.000)†
Platelets, $\times 10^9$/L (n = 78)	47.5 (28.3), 0/77	307 (150-400)*	< .001 (< .001)‡
MPV, fL (n = 71)	9.3 (1.6), 1/15	10.7 (8.0-13.4)*	< .001 (1.000)†
TPO, pg/mL (n = 38)	112.9 (62.5), 29/0	14.6 (6.9-54.4)§	< .001 (< .001)†

WBC indicates white blood cells; MPV, mean platelet volume.*Reference values of hospital central laboratory. †Versus upper reference limit. ‡Versus lower reference limit. §Values obtained in 50 healthy subjects.

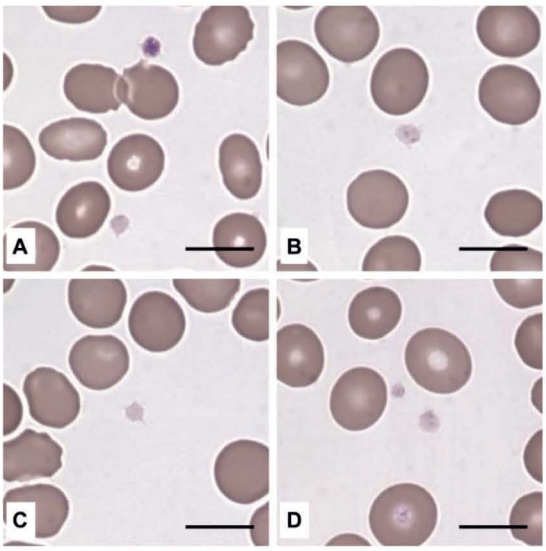

Figure 1: Platelets from THC2 patients have reduced azurophilic granule content. Platelets in peripheral blood smears (May-Grunwald-Giemsa staining) from 4 different probands were normal in size and appeared pale because of reduced content (B,D) or complete absence (A,C) of azurophilic granules. (A) One platelet had a normal granule content. Scale bars represent 10 µm. Images were acquired by an Axioscope 2 Plus microscope (Carl Zeiss) using a 100x/1.30 (oil immersion) Plan Neofluar objective (Carl Zeiss). The Axiocam MRc5camera (Carl Zeiss) and Axiovision 4.6 software (Carl Zeiss) were used to capture and process images.

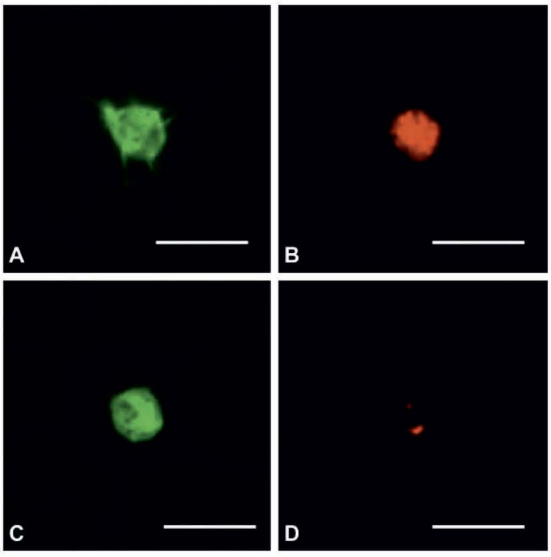

Figure 2: Platelets from THC2 patients have a defect in α-granule content. Peripheral blood smears were stained with the P12 antibody against the α-granule protein TSP1 (red), and platelets were identified by labeling for F-actin (green). (A-B) One platelet from a control subject with normal content in α-granules. (C-D) One representative platelet of aTHC2 patient with 2 $TSP1^{+}$ granules. Scale bars represent 5 µm. Images were acquired as described in Figure 1 legend using a100x/1.30 Plan Neofluar oil-immersion objective from Carl Zeiss.

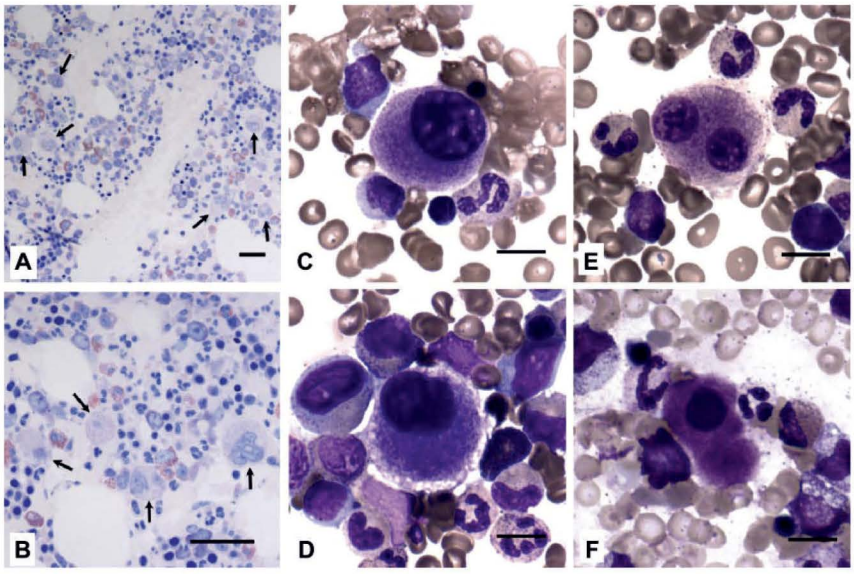

Figure 3: Bone marrow from THC2 patients present an increased number of Mks and dysmegakaryopoiesis. (A-B) Bone marrow biopsy (hematoxylin and eosin staining) of a member of family 4 showed normal cellularity with an increased number of Mks (arrows). Scale bars represent 50 µm. (C-F) Bone marrow smears (May-Grunwald-Giemsa staining) from members of families 8, 11, and 14. Representative examples of small, dystrophic Mks with hypolobulated nuclei and a small amount of mature eosinophilic cytoplasm. Typical micromegakaryocytes (F) were also observed. Scale bars represent 10 µm. Images were acquired as described in Figure 1 legend using a 20x/0.50 Plan Neofluar objective (panels A-B) and a 63x/1.25 Plan Neofluar oil-immersion objective (panels C-F) from Carl Zeiss.

5

Reduction of ANKRD26 protein expression (during development) results in striated muscle phenotype in zebrafish

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Manuscript in preparation

Introduction

Inherited thrombocytopenias are genetically heterogeneous conditions characterized by a reduced number of blood platelets. Thrombocytopenia can occur either isolated or in association with other abnormalities (non-syndromic or syndromic forms) (1-2). Thrombocytopenia 2 (THC2 [MIM 188000]) is an autosomal-dominant form of non-syndromic thrombocytopenia. THC2-affected individuals have a degree of thrombocytopenia ranging from mild to severe and suffer from a mild bleeding diathesis without major bleeding events. Morphological platelet studies did not identify any relevant defect and in vitro studies did not reveal any functional abnormality. Thrombocytopenia was attributed to defective platelet production because patients have evident dysmegakaryocytopoiesis at bone marrow examination with a decreased number of mature polyploidy megakariocytes (MKs), but an increase in colony-forming unit-MKs, as assessed by colony assays. Failure of immature MKs to undergoterminal differentiation suggests that the affected gene blocks MK maturation (3-4).

Through linkage analysis the THC2 locus was mapped to chromosome 10p11.1-p12 in two independent families from US and Europe (4-5). Two missense mutations in the *MASTL* (MIM 608221) and *ACBD5* genes were subsequently identified as the causative defects in the North American family (6) and Italian family (7), respectively. However, mutations in these genes were not identified in other families with a similar clinical phenotype (7). By combined sequencing of positional candidates in several Italian families mapping to the THC2 locus, mutations in a third gene (*ANKRD26*) located very close to *MASTL* and *ACBD5* have been found. Six different heterozygous single nucleotide substitutions in a short stretch of 22bp within the 5' untranslated region (5'UTR) were identified in 9 families (8). Also the previously identified Italian family with the *ACBD5* substitution had a mutation in *ANKRD26*. In total we have described 78 patients with 12 different *ANKRD26* mutations (9). Initial studies on ANKRD26 activity, suggest that the expression of the gene is up-regulated in patients with thrombocytopenia (8), but the biological function of ANKRD26 and the mechanism by which up regulation of the gene lead to THC are still to be unravelled. The high degree of homology at the sequence level between human *ANKRD26* and other species from mammals to fish suggests the biological function of ANKRD26 protein might be conserved from zebrafish to humans. So, since ANKRD26 function is still undefined, even if the gene seems to be up-regulated in patients, we knocked down the gene in zebrafish to explore the role of Ankrd26 in normal zebrafish embryonic development and specifically in relation to platelet formation.

Materials and Methods

Zebrafish strains and care

The zebrafish (Danio rerio, Hamilton 1822) strains used for this work were the transgenic zebrafish carrying a reporter enhanced green fluorescent protein (GFP) construct under regulation of the zebrafish thrombocyte-specific CD41 promoter, developed in the laboratory of Dr. Robert I. Handin at Brigham & Women's Hospital

(Boston, MA, USA) (10). Embryos were all raised at 28.5°C, and adult fish were maintained at 26°C on a 14 h:10 h light:dark cycle. Developmental stages were determined according to Kimmel (11).

Zebrafish Ankrd26 gene

The protein sequences of ANKRD26 from human and zebrafish were taken from the GenBank database of NCBI. The accession numbers used for the alignment are NP_055730.2 and XP_001920876.1 respectively.

Oligos were designed to cover all mRNA sequence (XM_001920841.3), exonic sequences were confirmed by PCR amplification and sequencing of the complete zebrafish A*nkrd*26 mRNA in our zebrafish strain.

Preparation of RNA from zebrafish and polymerase chain reaction

Total RNA was isolated from 50 uninjected and morpholino injected embryos at 1, 2, 3, and 4 days post fertilization (dpf), using RNAbee (Tel-Test.inc). First-strand complementary DNA (cDNA) was prepared using HiScript First-Strand Synthesis System for reverse transcription polymerase chain reaction (Invitrogen) according to manufacturer's instructions.

The following primers were used for PCR amplification:

ankrd26_1F 5'- AGACAGCTGGCCAAAAAGAACG - 3'

ankrd26_3R 5'- TTGGATCGGCCTCGTGTTCC -3'

The primers were designed against the sequence of exon 1 and 3 of the target gene. PCR conditions were as follows: 94°C for 7 minutes, followed by 35 cycles of 94°C, 58°C, and 72°C for 30 seconds each. For analysis of the RT-PCR products, 5 to 10 uL of each PCR product was analyzed by gel electrophoresis.

Morpholino gene silencing in zebrafish

Two non-overlapping morpholino antisense oligonucleotides were designed against different regions in the *Ankrd26* gene. One morpholino was directed against the ATG translational start site in exon 1 (AUG-MO), and the second morpholino was directed against the exon 1 splice acceptor site for the zebrafish *Ankrd26* gene (SB-MO). Both morpholinos were obtained from Gene-Tools (Philomath, OR, USA): AUG-MO=5'-AGTTGAATATCTTCTTCATGGTCGC-3'; Splice Blocking-MO SB-MO=5'-CACTGTACATCCACCATTACCTGTT-3'. Morpholinos were reconstituted in distilled water and further diluted in Danieau solution. A 1% of Phenol Red (Sigma Chemical Co., St Louis, MO, USA) was added to the solution as a tracer to aid in monitoring the injection volume. Injections were carried out using eggs before the two-cell stage, using a pneumatic pico pump (World Precision Instruments, Berlin, Germany). Different concentrations of both Ankrd26 morpholinos were injected to determine the optimum dose (2–10 ng). As the observed phenotype was dosage dependent, most of the experiments were carried out following injection with either 4 ng AUG-MO or 10 ng

SB-MO. Injected embryos were incubated at 28.5°C, and staged at 1, 2, 3, and 4 days post fertilization (dpf).

Co-injection of ANKRD26 MO with p53 morpholino

When performing MO knock down sometimes unspecific phenotypes might be observed (12). The most reproducible of these phenotypes is cell death; this can be visualized by a white fuzziness at the borders of the eyes, brain ventricles, and somites using dark field microscopy at 22 h post fertilization (13). This phenotypic class of off target effects is due to an ectopic up regulation of the p53 apoptosis pathway (14). To evaluate if the phenotypic effects observed were due to the specificity of *Ankrd26* gene knock down, co-injections of Ankrd26-MO and P53-targeted MO (GCGCCATTGCTTTGCAAGAATTG) (14) were performed.

A total of 600 embryos were injected with 4ng P53-MO and 4ng AUG-MO, 4ng P53-MO alone and 4ng AUG-MO alone. Embryos have been staged and observed after injections at 1, 2, 3 and 4 dpf.

Microscopy: fluorescence, histology and whole mount in situ hybridisation

For direct observation of GFP labelled thrombocytes, embryos were raised until 4 dpf, anesthetized with tricaine (3-amino-benzoicethylester; Sigma, 5 g/l) and analyzed *in vivo* under a Leica MZ16FA microscope.

Zebrafish embryos were harvested at 1, 2, 3 dpf, euthanised in tricaine (3-amino-benzoicethylester; Sigma, 25 g/l), fixed with 4% paraformaldehyde and embedded in paraffin. Sections (8 µm) were deparaffinised, rehydrated and stained with hematoxylin/eosine and mounted with Entellan (Merck).

Whole mount in situ hybridisation was performed with digoxigenin (DIG)-labelled RNA probes. Embryos were fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) plus 0.1% Tween 20 (PBT) at 4C°, dehydrated though a series of methanol/PBT solutions (25%, 50%, 75% and 100% methanol), and stored at -22C° until hybridization. Fixed embryos were rehydrated and rinsed twice in PBT. Embryos were treated 10 minutes with proteinase K (10 mg/ml in PBT), then post fixed in 4% paraformaldehyde 1X PBS for 20minutes and then rinsed in PBT 5 times for 5 minutes each. The embryos were prehybridized at least 1 hour at 70°C in hybridization buffer [50% formamide, 5X SSC, 50 mg/ml heparin, 500 mg/ml tRNA, 0.1% Tween 20, 9 mM citric acid]. The hybridization was done in the same buffer containing 50 ng to 100 ng of probe overnight at 70°C. Riboprobes were generated by *in vitro* transcription in the presence of Digoxigenin-UTP (RocheDiagnostics). The antisense and sense probes span 599bp of the coding sequence of *Ankrd26* and were synthesized from linearized Topo plasmid. As positive control we used egr2b probe.

On the second day embryos were washed at 70°C for 10 minutes in [75% hybridization buffer, 25% 2X SSC], 10 minutes in [50% hybridization buffer, 50% 2X SSC], 10 minutes in [25% hybridization mix, 75% 2X SSC], 10 minutes in 2X SSC, 2 times 30

minutes in 0.2X SSC. Further washes were performed at room temperature for 5 minutes in [75% 0.2X SSC, 25% PBT], 5 minutes in [50% 0.2X SSC, 50% PBT], 5 minutes in [25% 0.2X SSC, 75% PBT], 5 minutes in PBT, and then 1 hour in [PBT with 2 mg/ml BSA (bovine serum albumin), 2% sheep serum]. Then the embryos were incubated overnight at 4°C with the preabsorbed alkaline phosphatase-coupled anti-digoxigenin antiserum (described in Boehringer instruction manual) at a 1/5000 dilution in a PBT buffer containing 2 mg/ml BSA, 2% sheep serum. Finally the embryos were washed 6 times for 15 minutes each in PBT at room temperature. Detection was performed in alkaline phosphatase reaction buffer described in the Boehringer instruction manual. When the color was developed, the reaction was stopped in 1X PBS.

Stained embryos were photographed under an Olympus SZX16 microscope.

Results

Identification of zebrafish Ankrd26

The zebrafish Ankrd26 protein was identified (ID: XP_001920876.1) by blasting the human ANKRD26 protein (NP_055730.2) sequence against the zebrafish genome and through the "HomoloGene" database (NCBI). The *ANKRD26* gene is conserved in chimpanzee, cow, mouse, rat, chicken, and zebrafish. The human and zebrafish ankrd26 protein share a maximum of 61% homology. The predicted zebrafish protein was aligned with the human protein as shown in Figure 1. The human ANKRD26 protein has 5 ankirin domans which were also present in the zebrafish protein sequence. The philogenetic analysis of the human, fish and mouse proteins shows 30% homology as shown in Figure 1. This is due to the fact that the zebrafish Ankrd26 is 610 amino-acids longer than the human ANKRD26 protein. Protein blast results show that there are no functional domains within these gaps of non conserved regions. This might indicate that these residues are probably not important for normal ANKRD26 function. Sequence homology is significantly higher when looking at the conserved domains within the protein. The ankirin repeats, located at the C-terminus of the protein, show a homology of ~60% between zebrafish and human ANKRD26, indicating that these domains are important for normal protein function and therefore relatively less variation is observed in these regions. Sequencing of the exonic regions of zf ankrd26 confirmed the predicted sequence (genomic: NC_007136.5, mRNA: XM_001920841.3).

Expression of zebrafish Ankrd26 during embryogenesis and whole mount in situ hybridization

To investigate *Ankrd26* gene expression during early embryogenesis we preformed RT-PCR (reverse transcriptase-PCR). Expression at 1, 2, 3 and 4 dpf was observed (data not shown).

We investigated the spatial expression pattern of *Ankrd26* mRNA at different embryonic developmental stages (1, 2 and 3dpf) by whole mount in-situ hybridization

(WISH). At 1 dpf the expression was diffuse throughout the whole embryo's body with a higher intensity in the head and anterior part of the trunk. At later stages we could not detect any signal. No signal was present when using the sense probe indicating the specificity of the antisese probe signal (Figure 2).

Ankrd26 is required for normal striated muscle development in zebrafish

To gain insights into the role of Ankrd26 in zebrafish development we performed knock down experiments using the ATG blocking morpholino (AUG-MO) and the splice blocking morpholino (SP-MO) located at the exon/intron junction of exon 1- intron 1 (Figure 3a). ATG blocking morpholino is complementary to the translational start site of the *Ankrd26* mRNA and when anneals there, is predicted to inhibit the translation of the mRNA into protein. The splice blocking morpholino binds to the splice site and creates and alternative splice in intron 1. Then intron 1 is partially kept in the mRNA. A new and premature stop codon is introduced 90 nucleotides later, leading to an abnormal and truncated protein. This new protein of only 96 amino acids will be most likely not functionally active.

To investigate if injection of SB-MO leads to a depletion of normal *Ankrd26,* we performed quantitave PCR (Q-PCR) on cDNA obtained from SB-MO injected and uninjected embryos. Q-PCR using oligos designed in the proximity of the SB-MO binding site did not give a product. No optimal primers could be designed in this region. Then, we designed oligos in the second half of the gene. Gene expression was the same in the injected and uninjected embryos (data not shown). This indicates that probably the abnormally spliced mRNA containing a premature STOP codon does not undergo RNA decay and it is detected in the same way as the normally spliced mRNA.

We performed RT-PCR on cDNA obtained from embryos injected with 2, 4 and 8 ng SB-MO. Although RT-PCR is not a quantitative method, PCR products from injected embryos showed less intense PCR bands suggesting a decreased gene expression (Figure 3b).

Zebrafish embryos injected with both AUG-MO and SP-MO showed similar and consistent phenotype although this was more severe when injecting AUG-MO. Injected embryos displayed growth retardation and delayed hatching. Curved bodies with shortened tails or curved tails were observed in 94% of the embryos when injecting SB-MO (n=1292) and in 93% of the embryos injected with AUG-MO (n=790) (Figure 4). These abnormalities were not observed in uninjected embryos (n=1138). In approximately 50% of them also cardiac oedema was observed (SB-MO n=1292, AUG-MO n=790). No differences in mortality were observed between injected and not injected embryos.

Morphological analysis of the morphants showed only a disruption of the striated muscular architecture. Microscopically, the notochord appears intact but the typical bone fish structure of the striated muscle appear disorganized, the cell nuclei are not

aligned anymore and the tail is shortened and thicker than in the control embryos (Figure 5).

To evaluate possible differences in platelet number between the injected and uninjected fish we raised the embryos until 4 dpf, then the platelets expressed GFP. No gross difference was observed in platelets number between the morphants and control embryos. However, using this method, small differences in platelet number can not be assessed.

P53 co-injections

To evaluate if the observed phenotypic effects were specifically due to ankrd26 gene knock down we preformed co-injections of Ankrd26 AUG-MO and P53 MO. In our case co-injection of P53-targeted MO and the AUG-MO showed a phenotype similar to the one observed when injecting the AUG-MO alone: delayed embryonic development, delayed hatching, curved tail and heart oedema. Injecting only p53MO did not produce any abnormal phenotype (Figure 6), indicating the specificity of the phenotypic effects observed in the Ankrd26-MO injected fish.

Discussion

The zebrafish thrombocyte is the functional equivalent of the mammalian platelet, although during thrombocyte formation the nucleus is retained and each cell remains diploid throughout differentiation while platelets have no nucleus (15).

ANKRD26 is the ancestor of a family of primate-specific genes termed POTE (Prostate-, Ovary-, Testis-, and placenta-Expressed genes). Macaulay *et al.* reported that ANKRD26 is expressed in megakaryocytes, and in erythroid cells (16). Also in the database "NextBio Body Atlas" it is reported that ANKRD26 is expressed in all hematopoietic tissues, suggesting that the gene has a crucial role in haematopoiesis. Besides data from gene expression, still very little is known about the functional role of ANKRD26. In mice, partial inactivation of Ankrd26 causes extreme obesity, insulin resistance and increased body size whereas their platelet count is normal (17) while in mouse embryonic fibroblasts and adipose tissues are observed enhanced adipogenesis and diabetes (18-19).

To gain insight about the role of *Ankrd*26 in embryonic development we explored the spatial and temporal expression of the gene in zebrafish. RT-PCR showed that the gene is expressed from 1 to 4 dpf while WISH experiments show an overall expression pattern of the gene in whole embryo body at 1 dpf. We did not observe a specific localization in haematopoietic tissues. This suggests *Ankrd*26 might be a housekeeping gene during early development to become later on a more tissue specific gene.

Next, we use two non-overlapping MO to transiently knock down Ankrd26 expression in zebrafish. Efficiency of the gene knock down could not be confirmed by Q-PCR because an optimal assay targeting the specific gene region could not be designed.

However, the fact that a similar and reproducible phenotype was obtained with two different non-overlapping morpholinos is suggesting a specific gene knock down effect. The lack of phenotype rescue when co-injecting a p53 morpholino with Ankrd26 morpholino supports this specificity. Importantly, we did not see other non-specific features of morpholino use, such as widespread cell death.

Zebrafish embryos consistently showed growth retardation, curved bodies with shortened tails, and occasionally cardiac oedema. Histological analyses revealed a clear striated muscle phenotype, including an abnormal architecture of both heart tissue and skeletal muscle tissue. Morpholino injections did not lead to increased mortality. When we evaluated the platelet number in injected and uninjected embryos no gross differences were observed.

These findings are in line with the *in vitro* studies that suggest that THC2 Thrombocytopenia is caused by a gain of function effect (higher expression) rather than by haploinsufficiency of ANKRD26 (8).

In conclusion we showed A*nkrd*26 is expressed and likely has a role in early zebrafish embryonic development. Our data from two different non-overlapping morpholinos indicate that *Ankrd*26 is important for normal striated muscle development in zebrafish. Probably, other methods for genetic modification (i.e. transgenic animals) will be needed to study the mechanisms by which *ANKRD26* mutations lead to Thrombocytopenia.

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Figures:

1-A

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PREDICTED: Ankyrin repeat domain-containing protein 26 [Danio
rerio]Length=2319
Alignment:
Identities = 260/700 (37%), Positives = 439/700 (63%), Gaps = 42/700 (6%)

Query RPTKKTSNEKNKVKNQIQSMDDVDDLTQSSETASEDCELPHSSYKNFMLLIEQLGMECKD
      R +  +  +K +   +++  DD +DLTQSS+TA+E+ E P S Y+N  LL +QL     D
Sbjct RRSPSSRRKKEQSTGELEMADDFEDLTQSSDTATEELETPVSGYRNASLLFKQLDSSYLD
Query SVSLLKIQDAALSCERLLELKKNHCELLTVKIKKMEDKVNVLQRELSETKEIKSQLEHQK
      SVS++K+Q+     ER ++ +++    L  K  ++E + N L+  L E +  KS LEH K
Sbjct SVSVVKLQNMFHEYERTIQRERDRHSRLADKTSQLEQERNELKMLLDEIRGGKSSLEHLK
Query VEWERELCSLRFSLNQEEEKRRNADTLYEKIREQLRRKEEQYRKEVEVKQQLELSLQTLE
      +E E ++ +L+F L QE+EK ++A  LY K REQL+RKEEQ R E E + + EL +++LE
Sbjct LELETDMNNLKFLLRQEQEKHQSALMLYNKTREQLQRKEEQQRAEAEERHKAELKVRSLE
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Sbjct DARRDMKLSEEALAQTVFQYNGQLSALKAECSVLSAKLEHERQTRQQLEAEAEAGRARLQ
Query AAIHDRDQSETSKRELELAFQRARDECSRLQDKMNFDVSNLKDNNEILSQQLFKTESKLN
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Sbjct AAIQEAERCQASRTEAERSLQRDREEHQRMQEKHIFESGTQRDTIQSLSQKLSKSEARAN
Query SLEIEFHHTRDALREKTLGLERVQKDLSQTQCQMKEMEQKYQNEQVKVNKYIGKQESVEE
      S E E H     L EK + LE + ++  Q   ++KE+E    NE+ + ++   K E+++E
Sbjct SFENECHRNALTLAEKAVLLETLAREKDQALSKLKELEATVLNERDQTSRAGAKHEAMQE
Query RLSQLQSENMLLRQQLDDAHNKADNKEKTVINIQDQFHAIVQKLQAESEKQSLLLEERNK
      RL+Q QSE  LLRQQL++A NK   K+K V ++   F  ++ +L+A+ E++  L+EER++
Sbjct RLAQAQSEAALLRQQLEEALNKGSAKDKAVTDVHQNFAEMLNQLRADGEERVHLVEERSR
Query ELISECNHLKERQYQYENEKAEREVVVRQLQQELADTLKKQSMSEASLEVTSRYRINLED
      EL    + ++E+ Y+ E EKA+RE  +RQLQQELAD LKK SM EASLEV +RYR +LE+
Sbjct ELAKSNSEIREQNYKLEQEKADREASLRQLQQELADCLKKLSMCEASLEVNTRYRNDLEE
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Sbjct EKTRTLKDMDRLKSKLQESEETYVQAERRIAQLKSSLDDKEREACSNAHKLEEALSASAG
Query -----EKMQDHKQKLEKDNAKLKVTVKKQMDKIEELQKNL
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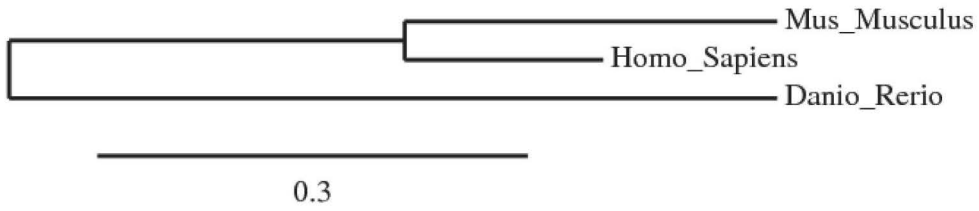

Figure 1: a) Alignment between human ANKRD26 protein and zebrafihs homologous protein showing the conservation of the ankirin domains b) Phylogenetic analysis between mouse, human and zebrafish ankrd26

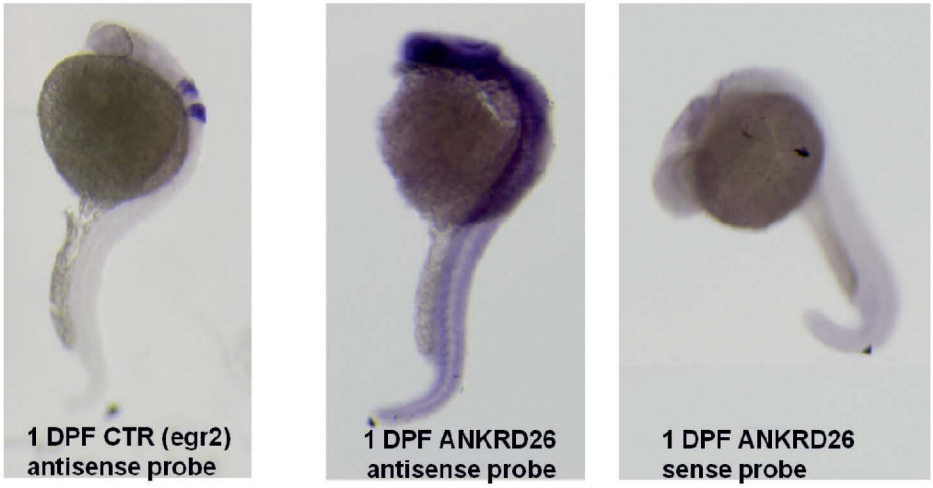

Figure 2 Whole mount in situ hybridization showing 1dpf embryos hybridized with positive control probe egr2, ankrd26 antisense probe showing an overall gene expression and ankrd26 sense probe showing no signal, indicating the specificity of the antisense probe.

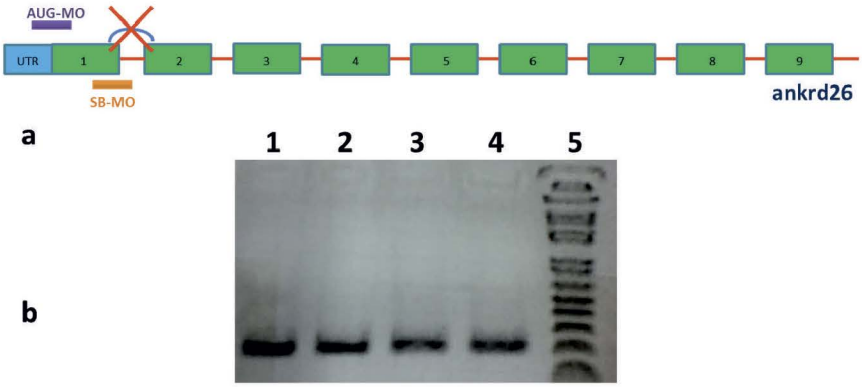

Figure 3 a) Scheme of *ankrd*26 gene (exons in green, UTR in blue, introns in red) showing the localization of the 2 ankrd26-MOs (AUG-MO and SB-MO); b) agarose gel showing RT-PCR product from exon2 to exon3. First lane (1): 2 dpf uninjected embryos cDNA; Second lane (2): 2 dpf embryos cDNA injected with 2 ng SB-MO; third lane (3): 2dpf embryos cDNA injected with 4ng SB-MO; fourth lane (4): 2dpf embryos cDNA injected with 8ng SB-MO; fifth lane (5): 1Kb Ladder

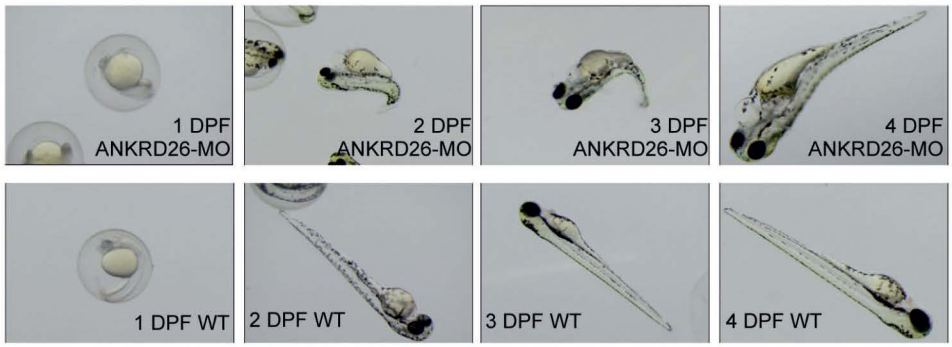

Figure 4 Rapresentative picture illustrating the phenotype of morphant (ankrd26-MO injected) embryos versus control embryos (uninjected) at 1, 2, 3 and 4 dpf, analysed with bright field microscopy

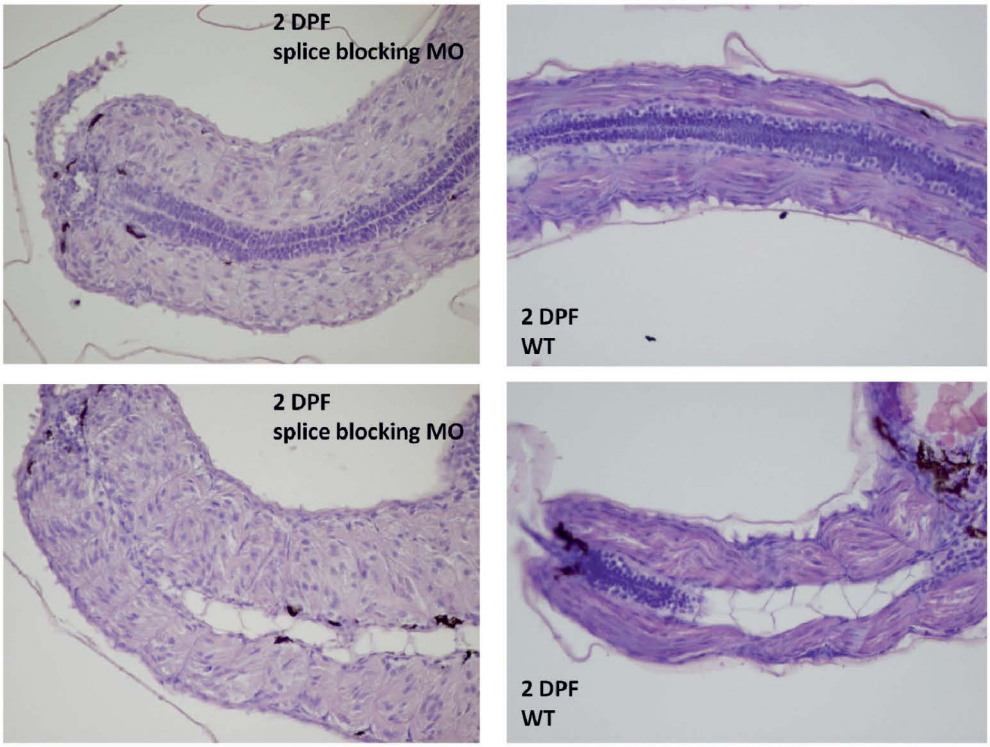

Figure 5 Hematoxilin-Eosine staining of 8um section of 2 dpf embryos fixed in 4%PF and embedded in paraffine, showing the normal muscular structure of uninjected embryos and the disrupted muscular structure of the morphants (ankrd26-MO injected) embryos.

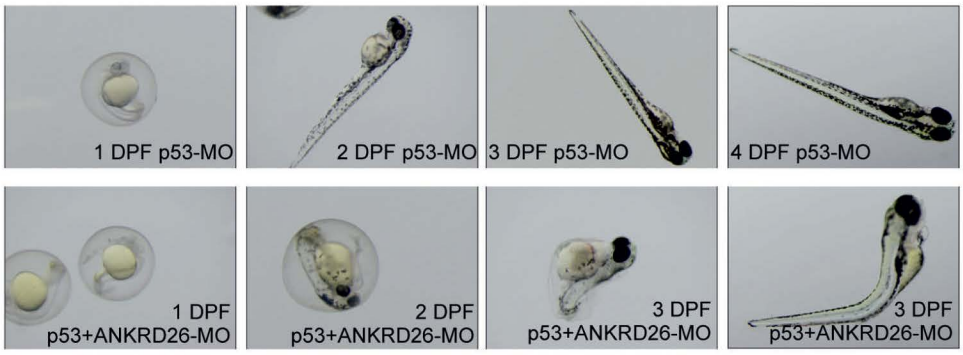

Figure 6 1, 2, 3 and 4 dpf embryos injected with p53-MO only versus 1, 2, 3 and 4 dpf embryos co-injected with ankrd26 AUG-MO and p53-MO. Phenotype was not rescued by p53-MO injections showing the specificity of the observed phenotype

6

Other forms of Thrombocytopenia

6.1

CNR2 functional variant (Q63R) influences childhood immune thrombocytopenic purpura

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Immune thrombocytopenic purpura is an acquired autoimmune disorder that is the most common cause of thrombocytopenia in children. The endocannabinoid system is involved in immune regulation. We evaluated a common missense variant (CAA/CGG; Q63R) of the gene encoding the cannabinoid receptor *type 2* (GeneID 1269) in 190 children with immune thrombocytopenic purpura and 600 healthy controls. The allelic frequencies and genotype distribution of the polymorphism in the patients were significant compared to control samples (P=0.006 and P=0.0001, respectively). Interestingly, when acute and chronic immune thrombocytopenic purpura patients were analyzed separately with respect to controls, a significant overrepresentation of the RR genotype and of the R allele was observed only for the chronic form (P=0.00021 and P=0.011, respectively). The relative odds ratio suggested the risk of developing chronic form was more than double in immune thrombocytopenic purpura children homozygous for the variant (odds ratio=2.349, 95% CI: 1.544-3.573; P<0.001).

Introduction

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by thrombocytopenia (peripheral blood platelet count < 150 x 10^9/L) due to autoantibody binding to platelet antigen(s) causing their premature destruction by the reticuloendothelial system, particularly in the spleen (1). ITP diagnosis is based on low platelet number in the absence of other hematologic abnormalities or other causes of thrombocytopenia (2). The annual incidence of pediatric ITP is about 4 to 6 cases per 100,000. About 50% of childhood ITP cases show an acute onset following a viral or bacterial infection that commonly resolves within weeks to months without treatment. Nevertheless, about one fourth of these patients go on to develop a chronic disease, defined by a platelet count less than 150 x 10^9/L at six months after diagnosis (3,4).

Although the immunopathogenesis of ITP is autoantibody-mediated, the exact mechanism of immune dysfunction is not known. However, there is substantial evidence to suggest that T cells and their cytokines play a pivotal role in the control of antiplatelet autoantibodies (5,6). A number of T-cell abnormalities have been demonstrated in patients with ITP and three main mechanisms have been hypothesized: i) a T-helper (Th)1 bias compared with Th2, particularly in chronic ITP; ii) the release of cytokines that interfere with megakaryocyte maturation and/or platelet release; and iii) a direct cytotoxic effect of Tcells (7).

T cells, as well as all other cellular components of the immune system, express cannabinoid receptors *type 1* and *2* (CB1 and CB2). The endocannabinoid system is also involved in immune regulation by suppressing cell activation, modulating Th1 and Th2 balance, and inhibiting pro-inflammatory cytokine production (8-10). CB2 is encoded by the *CNR2* gene, mapping on 1p36.11 (GeneID 1269; GenBank:#NM_001841.2), and is expressed at 10- to 100- fold greater levels than the CB1 on immune cells, including T lymphocytes, B cells, macrophages and neutrophils (10). Genome scan studies revealed a key role of the 1p36 region in different autoimmune diseases, such as rheumatoid arthritis (11), systemic lupus erythematosus (12), and type 1 diabetes (13).

In this study, we show the *CNR2* gene variation rs35761398 (Q63R) is significantly associated with childhood chronic ITP.

Design and Methods

Patients

The study included 190 (99 females) unrelated Italian children (median age 7 years; range 0.3-15.5) with ITP referred from March 1995 to December 2009 to the Department of Pediatrics of the Second University of Naples. Diagnosis and treatment of ITP were made according to the guidelines of the American Society of Hematology (ASH) (14) and the Italian Association of Pediatric Hematology andOncology (AIEOP) (15,16). Thrombocytopenia that resolved within six months of onset was defined as acute ITP; while persistence of thrombocytopenia for longer than six months was

defined as chronic ITP. Six hundred healthy Italian children (325 female, median age 9.7 years; range 1.8–13.8) served as controls. Controls, matched for age and sex, were recruited from the same geographical areas as ITP patients. These controls did not have a history of hematologic disorders of any kind. Clinical data are summarized in Table 1.

The study was approved by the Medical Ethics Committee of the Second University of Naples and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients' parents prior to participation in the study.

Molecular study

Genomic DNA was extracted from peripheral whole blood. The *CNR2* rs.35761398 polymorphism was studied by polymerase chain reaction (PCR) followed by direct sequencing. The PCR conditions were: 94° C for 4 rnin followed by 31 cycles consisting of 94° C for 30s, 60° C for 30s, and 72° C for 30s. Primers were chosen using *Primer3* software (Forward: 5'-GAGTGGTCCCCAGAAGACAG-3'; Reverse: 5'-CACAGAGGCTGTGAAGGTCA-3'). Amplimers were analyzed by direct sequencing using an ABI PRISM 310 automated sequencer (Applied Biosystem) and the relative genotypes were assigned. The χ^2 test was used to assess differences in genotype and allelic frequencies. The odds ratio for genotype distribudons was calculated using SAS software. *P* values less than 0.05 were considered significant.

Results and Discussion

In total, 190 ITP children were evaluated, divided into acute (n=86, female 45%) and chronic (n=104, female 58%) ITP (Table 1). The number of patients recruited with chronic ITP was greater because the hospital where the study was carried out is the childhood ITP referral center for the Campania region of southern Italy. All patients were genotyped for the *CNR2* rs35761398 variant, which changes the second and third adenosine at codon 63 (CAA) to guanosine (CGG) leading to the missense variant Q63R in the first intracellular signaling loop of the encoded CB2 protein. The allelic frequencies and the genotype distributions in controls and ITP patients are shown in Table 2. Whereas the allele frequencies in the controls were distributed according to Hardy-Weinberg (*P*=0.318) and were comparable to previously reported distributions (17), this was not the case in the ITP patients ($P=7.069\times10^{-3}$). Indeed, there were significant differences in allelic frequencies and genotype distribution in ITP patients compared to control samples (*P*=0.006 and *P*=0.0001, respectively) (Table 2). In addition, the relative odds ratio (OR) suggested a double risk of developing ITP in RR homozygous children with respect to QR heterozygous and QQ homozygous children (OR=2.006 95% CI 1.441-2.795; $P<10^{-3}$) (Table 3).

Interestingly, when acute and chronic ITP patients were analyzed separately in comparison with controls, a significant overrepresentation of the RR genotype and of the R allele was observed only for the chronic form (*P*=0.00021 and *P*=0.011,

respectively) (Table 2). Furthermore, the associated risk of developing chronic ITP increased more than two-fold for RR homozygous children (OR=2.349 95% CI 1.544-3.573; $P<10^{-3}$) (Table 3). The genotype and allele distribution in acute ITP patients was comparable to the control samples (Table 2). Patient's sex, the presence of autoimmune diseases, and platelet-associated antibodies were not significantly influenced by the *CNR2* rs35761398 variant (Table 2).

This case-control association study aimed to explore the molecular determinants that influence the susceptibility to ITP in childhood. For the first time, we showed an association between ITP and a functional variant of the *CNR2* gene, encoding for a protein known to affect immune function. The rationale for our study was based on: i) the linkage between the 1p36 locus, where *CNR2* maps, and several autoimmune diseases; ii) the immunomodulating effect of CB2; and (iii) the evidence of abnormal autoreactive T-cell activation in chronic ITP. Cannabinoid ligands, acting on CB2 receptors expressed by immune cells, can inhibit cytokine production, decrease antigen presentation, modulate cell migration, and mediate suppressive effects on effectors (18,19). Furthermore, immunomodulation by cannabinoids is totally absent in mice lacking the CB2 receptor (20).

The Q63R CB2 variant results in the amino acid substitution of a polar, uncharged, glutamine with a positively charged arginine. This change could affect the CB2 tertiary structure, altering the immunomodulating properties of CB2. It has been shown that human T cells from CB2 R63 homozygotes show a two-fold reduction in endocannabinoid-induced inhibition of proliferation with respect to cells from CB2 Q63 homozygotes (21). Although the immunopathogenic cause of ITP has not yet been clarified, there is overwhelming evidence to suggest that a generalized dysfunction of autoreactive T cells could represent the critical immunopathological factor in chronic ITP.

Data presented in this study confirm the role of CB2 in autoimmunity susceptibility and reveal a significant and previously unknown association between CB2 and childhood ITP. However, other studies are needed before the CB2 receptor, localized on immune effector cells, is considered an eligible molecular target to modulate autoreactive, innate, and adaptive immune responses in the chronic form of ITP.

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Table 1: Clinical findings of 190 ITP children.

	ITP patients (n)	Controls (n)
Subjects	190	600
Female (%)	99 (52)	324 (54)
Median age (years, range)	7 (0.3-15.5)	9.7 (1.8–13.8)
Median age at diagnosis (years, range)	6.16 (0.3-13.5)	-
Acute ITP (%)	86 (45)	-
Acute ITP female (%)	39 (45)	-
Chronic ITP (%)	104 (55)	-
Chronic ITP female (%)	60 (58)	-
Autoimmune diseases^	21	-

^The autoimmune diseases have been reported only in patients with chronic ITP: thyroiditis, celiac disease, rheumatoid arthritis, and systemic lupus erythematosus.

Table 2: Case-control association study of *CB2* Q63R polymorphism in 190 Italian ITP children: allelic frequencies and genotype distribution. Clinical characteristics in ITP patients according to the *CB2* genotype distribution.

	Patients		Controls		
Allelic frequencies (%)	Q	R	Q	R	*P*
ITP *vs.* CTRL	30	70	42	58	**0.006** (χ^2=7.689; df=1)
Acute ITP *vs.* CTRL	34	66			0.197 (χ^2=1.663; df=1)
Chronic ITP *vs.* CTRL	28	72			**0.011** (χ^2=6.442; df=1)

Genotype distribution (n)	QQ	QR	RR	QQ	QR	RR	*P*
ITP *vs.* CTRL (%)	18 (9.5)	79 (41.5)	93 (49)				**0.0001** (χ^2=18.206; df=2)
Acute ITP *vs.* CTRL (%)	9 (10.5)	40 (46.5)	37 (43)	96 (16)	310 (52)	194 (32)	0.108 (χ^2=4.45; df=2)
Chronic ITP *vs.* CTRL (%)	9 (8.5)	40 (38.5)	55 (53)				**0.00021** (χ^2=16.900; df=2)

Demographic and clinical findings (n)	QQ	QR	RR	*P*
Sex				
Female	12	38	49	0.728
Male	8	38	45	(χ^2=0.634; df=2)
Platelet-associated antibodies				
no	3	27	26	0.884
yes	3	25	20	(χ^2=0.246; df=2)
Presence of autoimmune diseases				
no	10	43	60	0.110
yes	2	13	6	(χ^2=0.458; df=2)

CTRL: controls; P *values less than 0.05 (in bold) were considered significant.*

Table 3: Case-control association study of *CB2* Q63R polymorphism in190 Italian ITP children: odds ratios.

ITP *vs.* CTRL	Odds Ratio	95% CI	*P*
RR *vs.* QQ	2.557	1.466-4.455	**0.001**
RR *vs.* (QQ+QR)	2.006	1.441-2.795	**0.000**
(RR+QR) *vs.* QQ	1.820	1.074-3.083	**0.025**
Acute ITP *vs.* CTRL			
RR *vs.* QQ	2.034	0.956-4.319	0.086
RR *vs.* (QQ+QR)	1.580	1.000-2.497	0.052
(RR+QR) *vs.* QQ	1.630	0.800-3.315	0.203
Chronic ITP *vs.* CTRL			
RR *vs.* QQ	3.024	1.453-6.283	**0.002**
RR *vs.* (QQ+QR)	2.349	1.544-3.573	**0.000**
(RR+QR) *vs.* QQ	2.011	0.994-4064	0.053

CTRL: controls; P *values less than 0.05 (in bold) were considered significant.*

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6.2

Absence of CYCS mutations in a large Italian cohort of patients with inherited thrombocytopenias of unknown origin

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Inherited thrombocytopenias comprise a variety of rare disorders that result from defects of platelet production or shortened platelet survival. Although many forms have been characterized and a diagnostic algorithm has been proposed and validated to facilitate their diagnosis, many patients with familial thrombocytopenia do not fall into the category of any defined disease (1). At least in Italy, approximately 50% of patients remain without a definite diagnosis, which suggests that they are affected with novel forms of these disorders (2). Most of these affected individuals manifest a non-syndromic, isolated thrombocytopenia without any apparent abnormality of platelet morphology or function. Therefore, distinguishing them from subjects with idiopathic thrombocytopenic purpura may be very difficult or even impossible whenever no other family members are affected and no previous blood count demonstrates that their thrombocytopenia was present since birth. Thus, patients with indefinite genetic thrombocytopenias are at risk of misdiagnosis and unnecessary therapies (3).

The clinical and molecular characterization of any new forms of inherited thrombocytopenia is an important achievement since it reduces the number of patients with unclassified forms and facilitates differential diagnosis between inherited and acquired forms. In this regards, a missense mutation (Gly41Ser substitution in the amino acid sequence lacking the initiating methionine) in the cytochrome c (*CYCS*) gene has recently been identified as the cause for an autosomal dominant form of non-syndromic thrombocytopenia (THC4, OMIM 612004) in a New Zealand family of English origin (4). Affected individuals had a mean platelet count of 109 x 10^9/L, normal platelet morphology and volume, and mild or no bleeding tendency. The mutation yielded a *CYCS* variant with enhanced activity of the intrinsic apoptosis pathway which is finely regulated during megakaryopoiesis (4). Indeed, patients showed a reduction of platelets due to a dysregulated megakaryopoiesis with premature release of platelets into bone marrow space rather than into sinusoids.

In order to define any potential role of cytochrome c in our thrombocytopenic population, the *CYCS* gene was screened for mutations in 77 patients, who were accurately selected among the 202 unrelated individuals refereed to our centers in the last 10 years and diagnosed to have an inherited thrombocytopenia. In 94 patients of these cases a certain diagnosis was made according to the algorithm proposed by the Italian Platelet Study Group (1). The more frequent disorders were biallelic (no. 10) and monoallelic forms of Bernard-Soulier syndrome (no. 44, 25 of them with the Ala156Val in the gene for GPIbα), *MYH9*-related disease (no. 22), gray platelet syndrome (no. 5) and congenital amegakaryocytic thrombocytopenia (no. 4). In the remaining 108 affected individuals, we failed to reach a diagnosis. Seventy-seven of them had clinical and laboratory features similar to those of patients with the *CYCS* mutation (4). Their mean age was 34 years, and the male/female ratio was 33/44. They had a moderate thrombocytopenia (mean platelet count: 89±39 x 10^9/L) with normal platelet volume (patients 10.6±1.3 fL, controls 10.7±0.9 fL) and normal platelet morphology. In these 77 patients, mutational screening of the *CYCS* gene (coding exons and their flanking intronic regions) did not identify any alterations of the open reading frame. Only one

single nucleotide polymorphism, rs11267038, within the 50 untranslated region was detected in two unrelated individuals.

Due to the large cohort of patients, who were from different geographic areas and accurately selected to match the phenotypic characteristics of those described in the New Zealand family, we concluded that mutational screening of *CYCS* should not be included in the front line diagnostic tests in Italy. We are aware that further investigationis required to characterize the unknown disorders that affect more than half the patients referred to our centers.

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7

General Discussion

In the last decade huge progress has been made in the genetics of hematologic diseases. Many genes responsible for the most common blood diseases have been discovered. This progress in genetic knowledge has facilitated the diagnosis, treatment, and prevention of several hematologic diseases. In addition, new therapeutic approaches have been developed. Excellent examples are the medications that increase foetal haemoglobin in both sickle cell disease and thalassemia, and gene transfer methods in the treatment of children with severe combined immunodeficiency (SCID). However, for many hematological diseases there is no available treatment and still for many of these disease the genetic cause is yet unknown.

In this thesis we studied rare hematologic diseases affecting production of red cells and platelets: Congenital Dyserythropoyetic Anaemia Type II (CDAII), Congenital Erythrocytosis and Thrombocytopenias.

In **Chapter 2**, we screened a group of Italian patients affected with CDAII, which is caused by mutations in *SEC23B* gene. All patients in our cohort presented mutations on *SEC23B,* supporting the hypothesis that CDAII is a genetically homogeneous disease. We described 4 new mutations and we evaluated the *SEC23B* gene expression on lymphocytes mRNA and erythroid precursors mRNA. All patients have a significant reduction of *SEC23B* mRNA and this reduction was more pronounced in patients with missense/nonsense mutations.

The results obtained in erythroid precursors and lymphocytes were comparable, suggesting that peripheral lymphocytes not only represent a good source of *SEC23B* transcript but also replicate the effect of the genetic change observable in the erythroid population. This finding is important from the diagnostic point of view: RNA extraction from lymphocytes is a standard procedure and it is possible to perform it in every laboratory while erythroid cell culturing is a technique available only in few specialized centres. This hopefully will facilitate and shorten the elapsed time from clinical manifestations of the disease to molecular diagnosis.

In a few cases (10 out of 111 described in the literature, or 9%), only a single heterozygous *SEC23B* mutation has been found. This finding raises the possibility of the occurrence of mutations that have thus far escaped the exon screening technology. Other factors (introns, promoter) regulating gene expression and function or another gene involved in the same pathway might be involved and need to be investigated. Furthermore functional studies are needed to clarify the biological role of SEC23B protein and the biochemical mechanisms by which its reduction cause the clinical phenotype.

In **Chapter 3**, we found a cluster for Chuvash polycytemia (Congenital Erithrocytosys) in the island of Ischia (Naples bay-Italy). This is the only cluster known besides the original Chuvash cluster (Russia) (1). We showed that our 12 patients share the same haplotype as the Chuvash patients, supporting the single-founder hypothesis (2). Our findings have several clinical implications. In regions as Chuvashia and Ischia, congenital polycythemia should be considered a “frequent” non-benign hematologic

disease. Making clinicians operating in these areas aware of the high frequency of this disease, will lead to early diagnosis and as consequence better patient management. In fact, in most of the cases these patients are at risk of cardiovascular events that can be prevented with an early and accurate diagnosis.

We also studied another form of Congenital Erythrocytosis due to mutations in *HIF2A.* We confirmed the causative role of *HIF2A* mutations (so far all of them residing in exon 12) in the pathogenesis of erythrocytosis. We described 2 patients with *HIF2α* mutations, one of which is novel, with remarkable erythrocytosis, but without an increased serum erythropoietin. This suggests that different mechanisms, probably specific of the erythopoiesis might be affected by the stabilization of HIF-2α protein. Further studies are necessary to better describe HIF-2α mechanisms of action and interactions with the other elements of the Oxygen Sensing Pathway.

We describe (**Chapter 4**) our efforts to identify the gene responsible for a form of Congenital Thrombocytopenia, with autosomal dominant inheritance and normal platelet size.

After linkage analyses and direct sequencing of all candidate genes in the linked region, we found a novel missense variant in a gene *ACDB5*, that co-segregated with the disease phenotype and was not found in 200 unrelated Italian controls coming from the same geographic area (3). The same variant was found in another Italian family with THC2 Thrombocytopenia. However, no other variants were found in this gene in other cases with this form of Congenital Thrombocytopenia. One year later, when more patients with thrombocytopenia THC2 were ascertained, we found that 6 different heterozygous single nucleotide substitutions in a short stretch of the 5'-untranslated region (5'-UTR) of *ANKRD26*, another gene within the THC2 locus, were responsible for thrombocytopenia in 9 families (4). Our previously identified Italian family with the *ACBD5* substitution also had this type of mutation, indicating that the *ACBD5* variant was most likely only in linkage disequilibrium with the disease gene, and not causative. Subsequently, we performed a systematic screening of 105 unrelated subjects with inherited thrombocytopenia of unknown origin and recognized 12 additional families with mutations in the same *ANKRD26* region (5). The clinical and laboratory pictures that emerged from the study of all 78 patients with *ANKRD26* mutations identified so far is described in Chapter 4.3 where we also discuss the unexpected finding of high frequency of acute leukemia in affected pedigrees.

This finding could have implications in the clinical practice. If confirmed, physicians should carefully monitor these patients for early detection of this malignancy.

In order to better understand its function in vivo, and subsequently clarify its role in the pathogenesis of THC2 Thrombocytopenia we transiently knock down the gene *Ankrd26* in zebrafish (**Chapter 5**). We found disruption of striate muscle structure in injected embryos and in ~50% of the cases heart oedema. We did not observe gross abnormalities in platelet number between injected fish and controls. These findings are in line with previous experiment that showed an up regulation of the mutated

*ANKRD*26 in patients (4). Next step to further investigate the function of ANKRD26 and to enlighten the biochemical mechanisms by which its over expression cause THC2 in patients will be the generation of a transgenic animal.

In **Chapter 6**, we briefly described also other forms of Thrombocytopenias, of other origin. Idiopathic Thrombocytopenia is very frequent in children, it is of autoimmune origin, most of the times the onset follows a viral or bacterial infection. We study a genetic variant (Q63R) within the *CNR2* gene, conferring risk to the occurrence of chronic Idiopathic Thrombocytopenic Purpura. The identification of these genetic factors might significantly improve the risk evaluation and prognosis prediction of this type of Thrombocytopenia. In a near future, the presence or absence of the SNP in *CNR*2 object of this study, will allow personalized and more effective therapy indications in children affected by Idiopathic Thrombocytopenia.

As discussed above, gene identification represents the first step to a better understanding of the physiological role of the underlying protein and disease pathways, which in turn enable molecular diagnosis of patients, gene carriers testing, prenatal diagnosis and serves as starting point for developing therapeutic strategies.

Recent advances in next generation sequencing technologies have dramatically changed the process of disease gene identification. Previously (when the THC2 locus was found (1999) for instance) identification of monogenic disease genes was carried out by performing linkage analysis on large families with many affected individuals and by Sanger sequencing of candidate genes in the linkage region. Several factors where limiting the power of traditional gene-discovering strategies in rare Mendelian phenotypes, for example the availability of only a small number of cases or families to study, reduced penetrance, locus heterogeneity. Even when all these requirements where fulfilled, many years where necessary to individually sequence all candidate genes in the linkage region. This effort costs time and resources. It was not possible to give an answer to the patients about the causes of their illness. It was also difficult to identify the appropriate therapy or perform an adequate genetic counselling.

Exome sequencing (the sequencing of the entire protein coding sequence of the genome) is a powerful and cost-effective new tool for dissecting the genetic basis of diseases. It is allowing discovery of genes responsible of diseases even when only samples from one or two patients are available.

This technique is ideal for identifying genes that underlie Mendelian disorders in circumstances in which conventional approaches are not feasible. Even where conventional approaches are eventually expected to succeed (for example, in homozygosity mapping), exome sequencing provides a means for accelerating discovery. Most alleles that are known to underlie Mendelian disorders disrupt protein-coding sequences and a large fraction of rare, protein-altering variants, such as missense or nonsense single-base substitutions or small insertion– deletions are predicted to have functional consequences. For these reasons, the exome represents

a highly enriched subset of the genome in which to search for variants with large effect sizes.

Over the past 2 years, experimental and analytical approaches relating to exome sequencing have established a rich framework for discovering the genes underlying unsolved Mendelian disorders. It is important to mention that while this approach is very useful for Mendelian diseases it is still not a good approach for complex diseases because it would result in a long list of different mutations but would give no clear indications about the real disease causing/modifier factors. It is estimated that straight forward application of the exome sequencing approaches gives a success rate of 60-80% for Mendelian disorders. It is therefore likely that exome sequencing will become the most commonly used tool for Mendelian disease gene identification in the next years (6).

These considerations, together with the results of our research show that only with technology improvements, joined efforts and dedication it is possible to unravel the causes of rare hematologic diseases. Patients affected with rare diseases deserve the same care and attention that is normally granted to patients affected by common genetic diseases. Hopefully we are going towards a day where the genetic cause of every congenital disease will be unravelled and a treatment will be possible for all patients affected by any kind of genetic disease.

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4. Pippucci T, Savoia A, Perrotta S, Pujol-Moix N, Noris P, Castegnaro G, et al. Mutations in the 5' UTR of ANKRD26, the ankirin repeat domain 26 gene, cause an autosomal-dominant form of inherited thrombocytopenia, THC2. Am J Hum Genet. 2011 Jan 7;88(1):115-20.

5. Noris P, Perrotta S, Seri M, Pecci A, Gnan C, Loffredo G, et al. Mutations in ANKRD26 are responsible for a frequent form of inherited thrombocytopenia: analysis of 78 patients from 21 families. Blood. 2011 Jun 16;117(24):6673-80.

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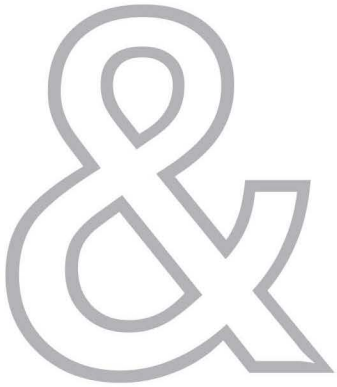

Summary

The blood contains different types of cells each one with a specific biological function. Blood cells all derive from a common progenitor cell, the hematopoietic stem cell (HSC). Haematopoiesis is the process through which blood cells differentiate in the bone marrow and when mature are released in the blood stream. The process of haematopoiesis is complex and tightly regulated and the numbers of the various cell types in the blood are normally kept in relatively constant ranges. Variations in their number have effects on health.

In Chapter 1 I give an introduction about 3 diseases that affect the production of Erythrocytes and Platelets, causing a variation of their normal number, above or under the prescribed values. Among the diseases causing reduced Erythrocytes production there are many types of anemias while increased red cell number leads to erythrocytosis. Among the diseases affecting the platelet production, there are the thrombocitopenias, which can be due to a genetic cause or acquired. Thrombocytopenias, similar to anemias can be due to shorten cell survival, reduced cell number or altered cell function. These 3 genetic diseases are all caused by congenital defects in the normal hematopoiesis process and manifest during paediatric age.

In Chapter 2 I studied Congenital dyserythropoietic anemia type II (CDA II). It affects about 367 patients in Europe. CDAII patients present mild to severe anemia, jaundice and splenomegaly. Red cell size is mostly normocytic. Beyond the age of 20 most patients develop iron overload and some patients develop liver cirrhosis, diabetes and heart failure. Bone marrow samples show characteristic changes: distinct hypercellularity due to erythroid hyperplasia with 45–90% erythroid precursors. In CDAII, 10 to 45% of all erythroblasts are bi- and multinucleated. A common finding in all typical CDA II patients is an impaired glycosylation of erythrocyte membrane proteins: Band 3 (anion exchange protein 1) and band 4.5 (glucose transporter 1) that are the main erythrocyte membrane proteins, show a sharper band and faster migration on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Another typical feature in CDAII is an apparent doubling of the cell membrane of erythrocytes due to residual membranes of endoplasmic reticulum that normally is eliminated during erythropoiesis. The diagnosis of CDAII is typically derived from bone marrow cytology and the detection of erythrocyte proteins using SDS-PAGE but, due to overlapping features among CDAII and other anemias and the rarity of the CDAII condition, in the years this resulted in misdiagnosis and erroneous treatments for patients.

Only in 2009, after a genome-wide SNP analysis Schwarz et al. and Bianchi et al. found mutations of the SEC23B gene in patients with CDAII. So far, SEC23B changes have been identified mainly by direct genomic sequencing of the coding region of the gene. Since the initial identification of SEC23B mutations in CDAII patients, 59 mutations have been identified. We described 4 new mutations in SEC23B gene and for the first time we showed the effects of the mutations on mRNA content in erythroblast cells and in SEC23B mRNA extracted from patient's lymphocytes. We compared the results and concluded that lymphocytes mRNA is a valid starting material to make a molecular diagnosis of CDAII. We also showed the effects of mutations on Sec23B protein content in red cell precursors, demonstrating the reduction of the protein level of the 30% in patients with SEC23B mutations.

In Chapter 3 I describe our findings related to Congenital Erythrocytosis, which is defined by an increase in Erythrocyte number and haematocrit value (Hct). Erythropoietin level gives an indication about the cause of erythrocytosis. Patients with Congenital Erythrocytosis have this condition present since birth and can be divided into one of two sets, those with Erythropoietin (EPO) levels below the normal range that can be assumed to have defects of the EPO signalling pathway while those with normal or elevated EPO levels may have defects of the oxygen sensing pathway. We studied Congenital Erythrocytosis (Congenital Polycythemia) due to mutations in two of the genes of the oxygen sensing pathway: VHL and HIF-2α. Genetic research identified in Chuvashia (Russia) more than 100 individuals from about 80 families, affected with polycythemia. They were all homozygous for a single mutation C598T in the von Hippel Lindau (VHL) gene. Patients have high haemoglobin level, increased plasma erythropoietin (EPO) level, varicose veins, pulmonary hypertension, vertebral hemangiomas, low blood pressure, and an elevated serum concentration of vascular endothelial growth factor (VEGF). Patients affected by Chuvash Polycythemia die early, mainly as a result of cerebral vascular events or peripheral thrombosis. We identified another large cluster with the same mutation in the island of Ischia (Italy) and by comparing the haplotypes of the Russian patients to the Italians, we concluded that all these subjects are descendent from a common founder. Also mutations in HIF-α have been found to be a genetic cause of Congenital Erythrocytosis. We describe 2 HIF-2α mutations in 2 patients with erythrocytosis but interestingly not presenting elevated serum EPO levels. We identified a novel variant: Ile533Val. The other mutation (Gly537Arg) is a de novo variant, suggesting that exon 12 of HIF-2α gene might be a hot spot for mutations and has a crucial role for the normal protein function from the moment that all mutations reported on this gene fall in this exon.

Finally Thrombocytopenias, that we described in Chapters 4, 5 and 6, originate when the number of platelets produced by the bone marrow is reduced below normal values or when the platelets produced have an altered function. The more frequent cause of thrombocytopenia is Idiopathic thrombocytopenic purpura (ITP). This is an autoimmune disorder characterized by thrombocytopenia due to autoantibodies binding to platelet antigen(s) causing their premature destruction. ITP diagnosis is based on low platelet number in the absence of other hematologic abnormalities or other causes of thrombocytopenia. The annual incidence of pediatric ITP is about 4 to 6 cases per 100,000. About 50% of childhood ITP cases show an acute onset following a viral or bacterial infection that commonly resolves within weeks to months without treatment but one fourth of these patients go on to develop a chronic disease. The endocannabinoid system is involved in immune regulation by suppressing cell activation, modulating some signalling molecules and inhibiting pro-inflammatory cytokine production. We demonstrated how the presence of the CNR2 gene variation rs35761398 (Q63R) in homozygosity confers a double risk of developing ITP than in heterozygous children or with wild-type (QQ) haplotype. And in particular this risk is referred to as chronic ITP.

More rare causes of Thrombocytopenias are the ones due to a single gene mutation, also called Inherited Thrombocytopenias. When thrombocytopenia is non-syndromic, isolated and without any apparent abnormality of platelet morphology it might be very difficult to distinguish it from Idiopathic Thrombocytopenic Purpura or even impossible in the absence of other affected family members and/or a previous blood test that demonstrates that thrombocytopenia was present since birth. Thus, patients with

indefinite genetic Thrombocytopenias are at risk of misdiagnosis and unnecessary therapies. The clinical and molecular characterization of any new forms of Inherited Thrombocytopenia is very important because it allows differential diagnosis between inherited and acquired forms and facilitates treatment. In the last years great advances have been made in the identification and characterization of an autosomal dominant form of thrombocytopenia defined as Thrombocytopenia 2 (THC2, MIM 188000). This form was originally described in only 2 families, one from United States and the other from Europe (Italy) where the THC2 locus (OMIM188000) was mapped. Thereafter, Gandhi et al. indicated the microtubule associate serine-threonine kinase like (MASTL) gene as a possible genetic cause of thrombocytopenia in the American family linked to the THC2 locus. We screened this gene in the members of the Italian family but we did not find any MASTL gene mutations. We found instead a mutation in the ACBD5 gene, next to the MASTL gene. This mutation was present in all the affected member of the Italian THC2 family and not present in 200 Italian controls but afterwards we found in a short stretch of 22bp of the 5'UTR of the gene ANKRD26, within the THC2 locus, six different mutations in other Italian pedigrees and also in the family where the locus was originally mapped presented a mutation in this gene. To better understand the role of ANKRD26 in Megakaryocyte development we knocked down the Ankrd26 gene in zebrafish. This resulted in a clear muscular phenotype that indicates Ankrd26 might have a crucial role in early development. Since the gene appears to be up-regulated in thrombocytopenic patients the use of a transgenic animal will be necessary to clarify the pathogenic mechanism by with 5'utr ANKRD26 mutations lead to THC2 Thrombocytopenia.

Samenvatting

Het bloed bevat verschillende soorten cellen, elk met een specifieke biologische functie. Bloedcellen stammen af van één gemeenschappelijke stamcel, de hematopoietische stamcel (HSC). Hematopoëse is het proces waarbij bloedcellen differentiëren en rijpen in het beenmerg waarna ze worden afgegeven in de bloedstroom. Het proces van hematopoëse is complex en stringent gereguleerd en het aantal verschillende celtypen in het bloed is relatief constant. Variaties in het aantal hebben effecten op de gezondheid.

In hoofdstuk 1 geven wij een inleiding over 3 ziekten waarbij er een effect is op de productie van erytrocyten en bloedplaatjes, waardoor waardes ontstaan die boven of onder de erkende waardes liggen. Onder de ziekten die worden veroorzaakt door een verminderde productie van erytrocyten zijn er vele vormen van bloedarmoede, terwijl een verhoogd aantal rode cellen leidt tot erytrocytose. Trombocytopenie is een ziekte waarbij er een effect is op de bloedplaatjesproductie, en kan een genetische of verworven oorzaak hebben. Vergelijkbaar met anemie, kan trombocytopenie ontstaan door een verkorte celoverleving, een verminderd aantal cellen of veranderde celfunctie. Deze 3 genetische ziekten zijn allemaal veroorzaakt door aangeboren afwijkingen van het normale hematopoiese proces en manifesteren zich tijdens de kinder leeftijd.

In hoofdstuk 2 onderzochten we aangeboren dyserytropoietic bloedarmoede type II (CDA II). Het treft ongeveer 367 patiënten in Europa. CDAII patiënten presenteren milde tot ernstige bloedarmoede, geelzucht en splenomegalie. De grootte van de rode bloedcellen is meestal normaal. Na 20-jarige leeftijd ontwikkelen de meeste patiënten een teveel aan ijzer en bij sommige patiënten ontstaan levercirrose, diabetes en hartfalen. Beenmerg monsters vertonen karakteristieke veranderingen: verschillende hypercellulariteit als gevolg van erytroïde hyperplasie met 45-90% erytroïde precursoren. In CDAII zijn 10 tot 45% van alle erytroblasten bi- en meerkernig. Een algemene bevinding in alle CDA II patiënten is een verminderde glycosylering van de membraaneiwitten van de erytrocyten: Band 3 (anionenwisselaar eiwit 1) en band 4.5 (glucose transporter 1) zijn de belangrijkste membraaneiwitten in erytrocyten en geven een scherpere band en snellere migratie te zien op natriumdodecylsulfaat polyacrylamide-gelelektroforese (SDS-PAGE). Een ander typisch kenmerk van CDAII is een schijnbare verdubbeling van het celmembraan van rode bloedcellen als gevolg van resterende membranen van het endoplasmatisch reticulum die normaal worden geëlimineerd gedurende de erytropoiese. De diagnose van CDAII wordt meestal bevestigd door de beenmerg cytologie en de detectie van erytrocyt-eiwitten met behulp van SDS-PAGE. Als gevolg van overlappende kenmerken tussen CDAII en andere anemieën en de zeldzaamheid van CDAII, resulteerde dit jaren lang in een verkeerde diagnose en verkeerde behandeling van patiënten.

Uiteindelijk in 2009, na een genoomwijde SNP-analyse van Schwarz et al. en Bianchi et al, zijn mutaties gevonden van het SEC23B gen bij patiënten met CDAII. Tot nu toe zijn SEC23B veranderingen voornamelijk vastgesteld door directe genomische sequentie bepaling van het coderende gebied van het gen. Sinds de eerste identificatie van SEC23B mutaties in CDAII patiënten, zijn 59 mutaties geïdentificeerd. Wij beschrijven 4 nieuwe mutaties in SEC23B gen en voor de eerste keer het effect van de mutaties op mRNA-niveau in erytroblast cellen en in lymfocyten van de patiënt. Wij

vergeleken de resultaten en hebben geconcludeerd dat lymfocyten mRNA een valide startmateriaal is om een moleculaire diagnose CDAII te stellen. Ook hebben we de effecten van mutaties aangetoond op het Sec23B eiwitgehalte van de rode bloedcel voorlopers, hieruit blijkt een verlaging van het eiwitgehalte van 30% bij patiënten met een SEC23B mutatie.

In hoofdstuk 3 beschrijven we onze bevindingen met betrekking tot aangeboren erytrocytose, dat gedefinieerd wordt door een toename van het aantal erytrocyten en de hematocrietwaarde (HCT). Het erytropoëtine niveau geeft een indicatie voor de oorzaak van erytrocytose. Patiënten met een aangeboren erytrocytose hebben deze aandoening sinds de geboorte en kunnen onderverdeeld worden in een van de twee types, diegene met erytropoëtine (EPO) niveaus onder het normale bereik waar kan worden aangenomen dat er fouten in het EPO pathway zijn, in tegenstelling tot mensen met normale of verhoogde EPO niveaus die defecten hebben in de zuurstof detectie pathway. We bestudeerden aangeboren erytrocytose (aangeboren polycythemia) als gevolg van mutaties in twee van de genen van de zuurstof detectie pathway: VHL en HIF-2α. Genetisch onderzoek in Tsjoevasjië (Rusland) identificeerde meer dan 100 mensen uit ongeveer 80 families, getroffen met polycythemia. Ze waren allemaal homozygoot voor een C598T mutatie in het Von Hippel Lindau (VHL)-gen. Patiënten hebben een hoog hemoglobinegehalte, een verhoogd plasma erytropoëtine (EPO) niveau, spataderen, pulmonale hypertensie, vertebrale hemangiomen, lage bloeddruk en een verhoogde serumconcentratie van de vasculaire endotheliale groeifactor (VEGF). Chuvash polycythemia patiënten overlijden vroegtijdig, vooral als gevolg van cerebrale vasculaire gebeurtenissen of perifere trombose. We identificeerden een ander groot cluster met dezelfde mutatie op het eiland Ischia (Italië) en door de vergelijking van de haplotypes van de Russische patiënten met de Italianen, hebben we geconcludeerd dat al deze patiënten afstammen van een gemeenschappelijke voorouder. Ook mutaties in HIF-α zijn gevonden als een genetische oorzaak van aangeboren erytrocytose. We beschrijven twee HIF-2α mutaties in 2 patiënten met erytrocytose, maar interessant genoeg vertonen deze geen verhoogde serum EPO niveaus. We identificeerden een nieuwe variant: Ile533Val. De andere mutatie (Gly537Arg) is een de novo-variant, wat suggereert dat exon 12 van het HIF-2α gen een hotspot voor mutaties kan zijn en een cruciale rol heeft voor de normale eiwit functie aangezien alle mutaties die zijn gerapporteerd over dit gen gelocaliseerd zijn in dit exon.

Tenslotte thrombocytopenie, dat we beschrijven in hoofdstuk 4, 5 en 6 ontstaat wanneer het aantal plaatjes in het beenmerg verlaagd is, beneden de normale waardes of wanneer de bloedplaatjes die geproduceerd worden een veranderde functie hebben. De meer voorkomende oorzaak van trombocytopenie is idiopathische trombocytopenische purpura (ITP). Dit is een auto-immuunziekte gekenmerkt door thrombocytopenie als gevolg van auto-antilichamen welke binden aan de antigenen van de bloedplaatjes waardoor ze vroegtijdig vernietigd worden. ITP diagnose is gebaseerd op een laag aantal bloedplaatjes in de afwezigheid van andere hematologische afwijkingen of andere oorzaken van trombocytopenie. De jaarlijkse incidentie van pediatrische ITP is ongeveer 4 tot 6 gevallen per 100.000. Ongeveer 50% van de infantiele ITP gevallen ontstaan acuut na een virale of bacteriële infectie die meestal binnen enkele weken tot maanden zonder behandeling geneest, maar een kwart van deze patiënten ontwikkelt een chronische ziekte. Het endocannabinoïde systeem is betrokken bij immuunregulatie door het onderdrukken van cel activatie, het

moduleren van een aantal signaalmoleculen en het remmen van pro-inflammatoire cytokine productie. We zien hoe de aanwezigheid van de homozygote CNR2 genvariatie rs35761398 (Q63R) een dubbel risico op ITP ten opzichte van heterozygote of wild-type haplotype kinderen. En in het bijzonder het risico voor het ontwikkelen van chronische ITP. Meer zeldzame oorzaken van trombocytopenie zijn degenen die als gevolg van een genmutatie ontstaan, ook wel erfelijke trombocytopenie. Wanneer trombocytopenie niet-syndromaal is, incidenteel en zonder duidelijke afwijking van de bloedplaatjes-morfologie, kan het erg moeilijk te onderscheiden zijn van idiopathische trombocytopenische purpura of zelfs onmogelijk wanneer er geen andere familieleden aangedaan zijn en er geen eerder bloedbeeld heeft aantoont dat trombocytopenie aanwezig was sinds de geboorte . Zo lopen patiënten met een niet specifieke genetische trombocytopenie het risico op een verkeerde diagnose en onnodige therapieën. De klinische en moleculaire karakterisering van nieuwe vormen van erfelijke thrombocytopenie is zeer belangrijk omdat het een differentiële diagnose geeft tussen erfelijke en verworven vormen, wat de behandeling vergemakkelijkt. In de laatste jaren is grote vooruitgang geboekt in de identificatie en karakterisatie van een autosomaal dominante vorm van thrombocytopenie gedefinieerd als Trombocytopenie 2 (THC2, MIM 188000). Deze vorm werd oorspronkelijk beschreven in slechts 2 families, een uit de Verenigde Staten en de andere uit Europa (Italië), waar de THC2 locus (OMIM188000) in kaart werd gebracht. Gandhi et al. wees de microtubuli geassocieerde serine-threonine kinase aan, zoals het (*MASTL*) gen , als een mogelijke genetische oorzaak van thrombocytopenie in de Amerikaanse familie gekoppeld aan de THC2 locus. We hebben dit gen gescreend in de leden van de Italiaanse familie, maar vonden geen *MASTL* gen mutaties. In plaats hiervan vonden we een mutatie in het gen *ACBD5* naast het *MASTL* gen. Deze mutatie was aanwezig in alle aangedane leden van de Italiaanse THC2 familie en niet in 200 Italiaanse controles, maar daarna hebben we in een kort stuk van 22 bp van de 5 'UTR van het gen *ANKRD26*, binnen de THC2 locus, zes verschillende mutaties gevonden in andere Italiaanse stambomen. Om de rol van *ANKRD26* beter te begrijpen in megakaryocyt ontwikkeling hebben we een knockdown van het Ankrd26 gen in zebravissen uitgevoerd. Dit resulteerde in een duidelijk spier fenotype dat aangeeft dat Ankrd26 een cruciale rol zou kunnen hebben in de vroege ontwikkeling. Aangezien het gen opgereguleerd lijkt te zijn in trombocytopenie patiënten is het gebruik van een transgeen dier nodig om het pathogene mechanisme te verduidelijken van de 5 'UTR *ANKRD26* mutaties.

About the Author

Francesca Punzo was born in Naples, Italy.

After finishing the secondary education in 2000, at the Scientific Lyceum in San Giorgio a Cremano (Italy), she started studying Biotechnologies at the Faculty of Medicine, Second University of Naples, Italy. Three years later she obtained her BSc with top grades and *cum laude.* After that, she started working in the laboratory of Molecular Biology of the Pediatric Department of Second University of Naples under supervision of Dr. Silverio Perrotta.

Two years later (2005) she started her master in Medical Biotechnologies at the same University and in 2006 she came to The Netherlands for 6 months internship at the department of Clinical Genetics, Erasmus Medical Center, Rotterdam.

Upon her graduation in 2007, with top grades and *cum laude*, Francesca worked as PhD student in the department of Clinical Genetics of the Erasmus Medical Center in Rotterdam, under supervision of Prof. Dr. B.A. Oostra and Dr. Silverio Perrotta and Dr. Aida Bertoli-Avella. The results obtained from this PhD research have been published in international, peer-reviewed scientific journals. After her PhD she is willing to continue her academic career with a post-doctoral fellow.

List of Publications

1. Mutations in SLC30A10 Cause Parkinsonism and Dystonia with Hypermanganesemia, Polycythemia, and Chronic Liver Disease.
   Quadri M, Federico A, Zhao T, Breedveld GJ, Battisti C, Delnooz C, Severijnen LA, Di Toro Mammarella L, Mignarri A, Monti L, Sanna A, Lu P, **Punzo F**, Cossu G, Willemsen R, Rasi F, Oostra BA, van de Warrenburg BP, Bonifati V. Am J Hum Genet. 2012 Mar 9;90(3):467-77. Epub 2012 Feb 16.
2. Congenital Dyserythropoietic Anemia Type II: molecular analysis and expression of the SEC23B Gene.
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3. CNR2 functional variant (Q63R) influences childhood immune thrombocytopenic purpura.
   Rossi F, Mancusi S, Bellini G, Roberti D, **Punzo F**, Vetrella S, Matarese SM, Nobili B, Maione S, Perrotta S. Haematologica. 2011 Dec;96(12):1883-5. Epub 2011 Aug 9.
4. Mutations in ANKRD26 are responsible for a frequent form of inherited thrombocytopenia: analysis of 78 patients from 21 families.
   Noris P, Perrotta S, Seri M, Pecci A, Gnan C, Loffredo G, Pujol-Moix N, Zecca M, Scognamiglio F, De Rocco D, **Punzo F**, Melazzini F, Scianguetta S, Casale M, Marconi C, Pippucci T, Amendola G, Notarangelo LD, Klersy C, Civaschi E, Balduini CL, Savoia A. Blood. 2011 Jun 16;117(24):6673-80. Epub 2011 Apr 5.
5. Mutations in the 5' UTR of ANKRD26, the ankirin repeat domain 26 gene, cause an autosomal-dominant form of inherited thrombocytopenia, THC2.
   Pippucci T, Savoia A, Perrotta S, Pujol-Moix N, Noris P, Castegnaro G, Pecci A, Gnan C, **Punzo F**, Marconi C, Gherardi S, Loffredo G, De Rocco D, Scianguetta S, Barozzi S, Magini P, Bozzi V, Dezzani L, Di Stazio M, Ferraro M, Perini G, Seri M, Balduini CL. Am J Hum Genet. 2011 Jan 7;88(1):115-20.
6. Novel ATP13A2 (PARK9) homozygous mutation in a family with marked phenotype variability.
   Santoro L, Breedveld GJ, Manganelli F, Iodice R, Pisciotta C, Nolano M, **Punzo F**, Quarantelli M, Pappatà S, Di Fonzo A, Oostra BA, Bonifati V. Neurogenetics. 2011 Feb;12(1):33-9. Epub 2010 Sep 21.
7. A mutation in the acyl-coenzyme A binding domain-containing protein 5 gene (ACBD5) identified in autosomal dominant thrombocytopenia.
   Punzo F, Mientjes EJ, Rohe CF, Scianguetta S, Amendola G, Oostra BA, Bertoli-Avella AM, Perrotta S. J Thromb Haemost. 2010 Sep;8(9):2085-7.
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Savoia A, Noris P, Perrotta S, **Punzo F**, Rocco DD, Oostra BA, Balduini CL. Platelets. 2009 Feb;20(1):72-3.

9. ROBO2 gene variants are associated with familial vesicoureteral reflux.
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11. Von Hippel-Lindau-dependent polycythemia is endemic on the island of Ischia: identification of a novel cluster.
    Perrotta S, Nobili B, Ferraro M, Migliaccio C, Borriello A, Cucciolla V, Martinelli V, Rossi F, **Punzo F**, Cirillo P, Parisi G, Zappia V, Rotoli B, Della Ragione F. Blood. 2006 Jan 15;107(2):514-9. Epub 2005 Oct 6.
12. Inherited disorders of bilirubin metabolism.
    Rossi F, Francese M, Iodice RM, Falcone E, Vetrella S, **Punzo F**, De Vita S, Perrotta S. Minerva Pediatr. 2005 Apr;57(2):53-63. Review. Italian.

PhD Portfolio

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Francesca Punzo Erasmus MC Department: Clinical Genetics Research School: Medical Genetic Centre (MGC)	PhD period: June 2007 – June 2012 Promotor: Ben A. Oostra Supervisor: Aida M. Bertoli-Avella	
PhD training		
	Year	**Workload (Hours/ECTS)**
General academic skills		
Molecular and Cell Biology	2009	6 ECTS
Safely working in the Lab	2009	0,25 ECTS
Biomedical English Writing and Communication	2010	4 ECTS
Research skills		
Biomedical Research Techniques	2009	32 hours
SNPs and Human diseases	2010	32 hours
Basic SPSS	2011	16 hours
Photoshop and Illustrator	2011	16 hours
Writing a successful grant proposal	2011	8 hours
Presentations		
Clinical Genetics Monday Morning Presentation	2007	32 hours
Clinical Genetics Monday Morning Presentation	2008	36 hours
Clinical Genetics Monday Morning Presentation	2009	32 hours
Clinical Genetics Wednesday Morning Presentation	2010	32 hours
Clinical Genetics Wednesday Morning Presentation	2011	36 hours
Clinical Genetics Wednesday Morning Presentation	2012	36 hours
International conferences		
Telethon conference (Poster presentation)	2011	48 hours
European congress of Human Genetics (Poster presentation)	2011	50 hours
Retreats and Workshops		
PhD Day	2009	8 hours
PhD workshop Cologne	2010	4 days
Get Out of the Lab days	2011	3 days
PhD workshop Maastricht	2011	4 days
Didactic skills		
Teaching Master Student: Elodie Jadot	2009	80 hours
Social Activities		
Cluster 15 Party committee	2008-2009	150 hours
Cluster 15 Pantomime committee	2010-2011	100 hours

Acknowledgments

So here we are, at the End of these 7 Chapters but also the End of a big Chapter of my life. How much things, people, parties, and happenings during these 5 years! …Somebody says that life is all about who do you meet. And I always considered myself very lucky because in my life I met really beautiful persons.

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On the Cover:

Shane Wright, Carmen Ferrajolo, Giulia Festinese, Emanuela Punzo, Kristaps Pozarskis, Luna (Ruvalic) Buijs-Offerman, Francesca Punzo, Davide Perrotta, Bianca De Graaf, Simona Polge, Daniel Gonzales-Bertoli, Anna Koopmans, Mariangela Di Paola, Claudia Pivonello, Nadia di Silvia Mancusi, Anna Szczerbinska, Linde Kegel, Beatrice Punzo, Luigi Figliolia, Ludovico di Gaia Callea, Marialuisa Quadri, Elodie Jadot, Lalini Raghoebir, Zeli Fonseca Soares, Chris Vink, Giorgia Festinese, Luigi Maione, Romana Nijman, Cintia Bombardieri, Christan Rohe, Saverio Scianguetta, Gianluca Trifirò, Matteo Barone, Camilla Gabriele, Ralph Stadhouders, Petra Schwertman, Alessandra Di Paola, Christina Merakou, Artemis Mulugeta-Achme, Lorenzo Iacobelli.

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