Bone marrow failure syndromes and refractory cytopenia of childhood

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Bone Marrow Failure Syndromes and Refractory Cytopenia of Childhood

ISBN 978-94-6259-134-9

Design and layout
Promotie In Zicht, Arnhem

Print
Ipskamp Drukkers, Enschede

Cover
Refractory cytopenia bone marrow trephine biopsy; courtesy of dr. King H. Lam, Department of Pathology, Erasmus MC, Rotterdam

The research for this thesis was performed within the framework of the Erasmus Postgraduate School of Molecular Medicine at the Department of Pediatric Oncology/Hematology and at the Department of Immunology, Erasmus MC, Rotterdam, The Netherlands, and at the Hematology Branch, NHLBI, National Institutes of Health, Bethesda, MD, USA, and was funded by Stichting KiKa.

Printing of this thesis was financially supported by: Erasmus Universiteit Rotterdam, Stichting Kinderoncologisch Centrum Rotterdam, BD, Novartis, and Alexion.

Bone marrow failure syndromes and refractory cytopenia of childhood

Beenmergfalen en refractaire cytopenie op de kinderleeftijd

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
13 mei 2014 om 15.30 uur

door

Anna Maartje Aalbers
geboren te Tiel

ERASMUS UNIVERSITEIT ROTTERDAM

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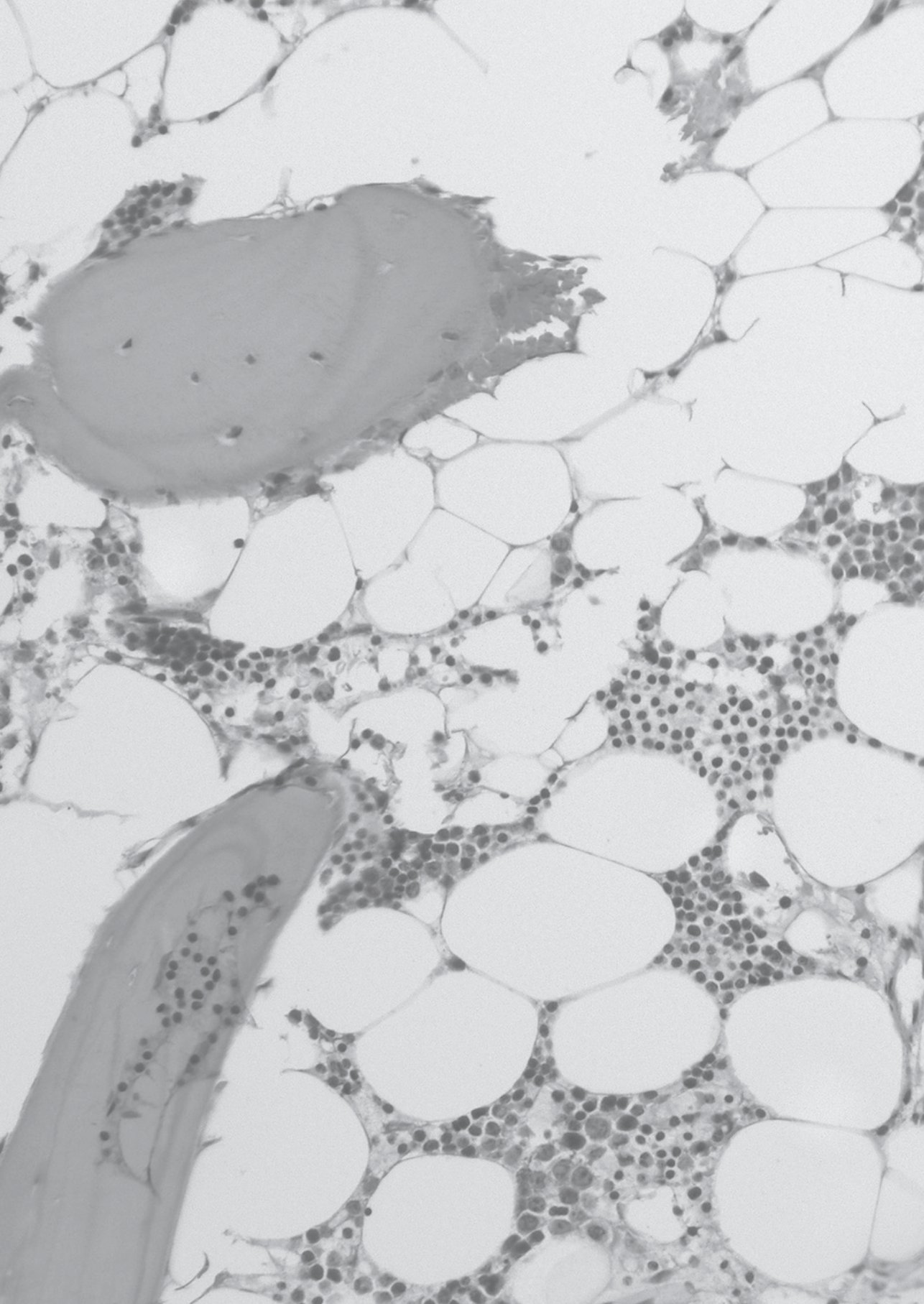

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General Introduction and Aims of the Thesis

GENERAL INTRODUCTION

1. HEMATOPOIESIS

Hematopoiesis, or blood cell production, is sustained through hematopoietic stem cells, which are self-renewing cells that reside in the bone marrow, and that are capable of producing daughter cells that proliferate and mature to provide all adult blood effector cells,[1] including erythrocytes or red blood cells, leukocytes or white blood cells, and thrombocytes or platelets. Erythrocytes are the most numerous cell types in the blood, and have as main task to transport oxygen through the lungs to peripheral tissues; leukocytes are responsible for elimination of bacteria and viruses, and thrombocytes for blood clotting. Hematopoiesis can be subdivided into myelopoiesis and lymphopoiesis. Myelopoiesis comprises the generation of granulocytes, monocytes, mast cells, megakaryocytes (the precursors of thrombocytes), and erythrocytes. Lymphopoiesis encompasses the generation of B cells, T cells, and NK cells; collectively referred to as lymphocytes. Although recent insights suggest that cell fate determination during hematopoiesis is more plastic than previously thought,[2] the classic, hierarchical model of determination of hematopoietic cell lineages is depicted in Figure 1 (adapted from Orkin and Zon[3]).

2. DISORDERS OF MYELOPOIESIS

2.1 Bone marrow failure syndromes

A bone marrow failure syndrome is defined as the condition in which myelopoiesis is insufficient, thereby resulting in unilineage or multilineage peripheral blood cytopenia. Bone marrow failure can be either inherited or acquired; both subtypes will be discussed below.

Inherited bone marrow failure syndromes

The most frequently reported inherited bone marrow failure syndrome are Fanconi anemia, Diamond-Blackfan anemia, Shwachman-Diamond syndrome, and dyskeratosis congenita (reviewed by Shimamura and Alter); less frequently reported are severe congenital neutropenia, congenital amegakaryocytic thrombocytopenia, and thrombocytopenia absent radii syndrome.[4] Clinical presentation of these disorders is heterogeneous. Although the first clinical sign of an inherited bone marrow failure syndrome can be bone marrow failure, patients might also present with myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), pulmonary fibrosis, or a solid tumor.[4] In the following paragraphs, clinical characteristics, underlying genetic defects, and treatment of the four most frequently reported inherited bone marrow failure syndromes are discussed (summarized in Table 1).

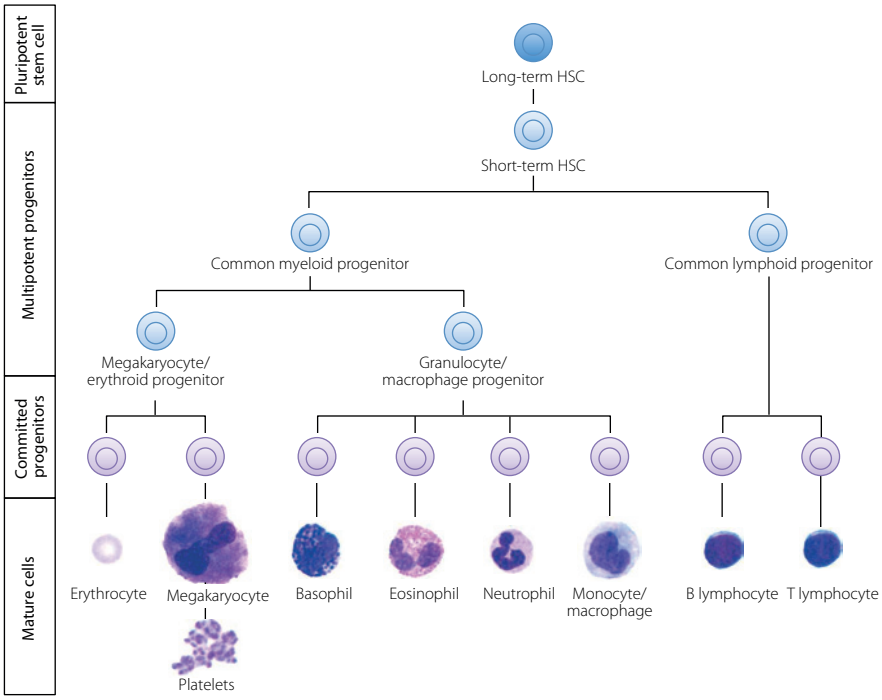

Figure 1 Hierarchical model of hematopoiesis.

Hematopoietic stem- and progenitor cells are depicted schematically, while morphologic pictures of mature cells are shown. HSC, hematopoietic stem cell. Modified from Orkin et al., Cell 2008 and from *Schema hematopoiese,* Laboratorium speciële hematologie, Sophia Children's Hospital, Rotterdam (courtesy of Mrs. Rolinda Stigter).

Fanconi anemia

Fanconi anemia, occurring in about 1 in 100,000 births,[5] is a genetically heterogeneous disorder that is characterized by progressive bone marrow failure, which generally develops in childhood or adolescence, and an increased risk of solid tumors, MDS, and AML.[4] Pancytopenia is the most frequent presenting feature, and the majority of patients display congenital abnormalities, most commonly short stature, hyper- or hypopigmentation of the skin, café-au-lait spots, thumb abnormalities, microcephaly, microphthalmia, renal abnormalities, and hypogonadism.[4] Fanconi anemia is caused by biallelic mutations in Fanconi anemia genes, which are involved in DNA interstrand crosslink repair, preventing tumorigenesis and promoting stem cell function,[6] and which constitute 16 complementation groups.[7] Recently, it was shown that *Fancd2* counteracts the toxic effects of endogenous aldehydes in mice, and it was suggested that these naturally occurring aldehydes might

Table 1 Characteristics of inherited bone marrow failure syndromes.

Syndrome	Phenotype	Affected genes	Mode of inheritance	Treatment	Cancer risk
Fanconi anemia	Pancytopenia, congenital abnormalities including short stature, abnormal skin pigmentation, thumb abnormalities, microcephaly, microphthalmia, renal abnormalities, hypogonadism	*FANC* genes (encoding DNA interstrand crosslink repair proteins)	Autosomal recessive (*FANCB*: X-linked)	HSCT	Increased risk of solid tumors, MDS, and AML
Diamond-Blackfan anemia	Red cell aplasia, congenital abnormalities including short stature, craniofacial abnormalities, abnormal thumbs	*RPS* and *RPL* genes (encoding ribosomal proteins)	Autosomal dominant	Corticosteroids, HSCT	Increased risk, but to lower extent than in other IBMFS, of solid tumors, MDS and AML
Shwachman-Diamond syndrome	Pancytopenia, exocrine pancreatic insuficiency, skeletal abnormalities	*SBDS* (involved in ribosome biogenesis, possibly extraribosomal function)	Automosomal recessive	G-CSF, HSCT	Increased risk of MDS and AML
Dyskeratosis congenita	Pancytopenia, abnormalities including oral leukoplakia, nail dystrophy, abnormal skin pigmentation, pulmonary fibrosis, liver cirrhosis, short telomeres (except *TCAB1* mutants)	*DKC1*, *TERC*, *TERT*, *TINF2, NOP10*, *NHP2*, *TCAB1*, *C16orf57*, *RTEL1* (mainly involved in telomere biology)	X-linked *(DKC1*), autosomal dominant (*TINF2*, *TERC*, *TERT*), autosomal recessive (*TERT*, *NOP10*, *NHP2*, *TCAB1, C16orf57*, *RTEL1*)	Androgens, HSCT	Increased risk of squamous cell carcinoma, MDS and AML

For references, the reader is referred to the main text.

contribute to abnormal development, bone marrow failure, and cancer predisposition in Fanconi anaemia patients.[8,9] The diagnosis of Fanconi anemia is confirmed by chromosomal breakage tests and complementation group analysis; furthermore, gene sequencing can be performed.[4] Hematopoietic stem cell transplantation is the only curative treatment for the hematologic complications of the disease.[4]

Diamond-Blackfan anemia

Diamond-Blackfan anemia is an autosomal dominant bone marrow failure syndrome characterized by red cell aplasia, usually presenting in early infancy, and congenital abnormalities, present in 25-50% of patients, most commonly short stature, craniofacial abnormalities, and abnormal thumbs.[4,10] Diamond-Blackfan anemia has an estimated incidence of 1 in 100.000 to 1 in 200.000 (reviewed by Vlachos et al.[11]). The risk of malignancy, varying from MDS, AML, colon carcinoma, osteogenic sarcoma, to female genital cancers, in Diamond-Blackfan anemia is increased compared to the general population, but to a lower extent than the risk of malignancy in Fanconi anemia and dyskeratosis congenita.[12] After the identification of heterozygous mutations in *RPS19*, encoding a component of the small ribosomal subunit,[13] mutations in genes encoding components of the large 60S and other components of the small 40S ribosomal subunits were described.[10] Spontaneous hematologic remission has been described in some patients.[4] Treatment of Diamond-Blackfan anemia may consist of corticosteroids, to which the majority of patients respond. Some patients lose response or suffer from side effects such as hypertension and diabetes mellitus. The only curative treatment for the hematologic complications of Diamond-Blackfan anemia is hematopoietic stem cell transplantation.[4]

Shwachman-Diamond syndrome

Shwachman-Diamond syndrome is an autosomal recessive condition, characterized by pancreatic exocrine insufficiency, skeletal abnormalities, bone marrow failure (at initial presentation usually neutropenia, followed later by anemia and thrombocytopenia), and an increased risk of MDS and AML, the latter occurring in 19-36% of patients.[14] The estimated incidence of Shwachman-Diamond syndrome varies from 1 in 76.000 to 1 in 168.000.[15,16] Compound heterozygous mutations in *SBDS* are identified in the majority of Shwachman-Diamond syndrome patients. Of the two most frequently found mutations in *SBDS*, 183-184TA>CT and 258+2T>C, at least one is present in approximately 90% of affected individuals. These mutations are located in exon 2, and result from gene conversion with *SBDSP*, the *SBDS* pseudogene.[17] Although its exact function remains unclear, the SBDS protein appears to have a role in ribosome maturation, and might have additional extraribosomal functions.[18,19] Similar to Fanconi anemia and Diamond-Blackfan anemia, hematologic complications in Shwachman-Diamond syndrome can be treated with hematopoietic stem cell transplantation. Neutropenic patients with recurrent or severe infections may benefit from treatment with G-CSF.[4]

Dyskeratosis congenita and telomere disease

Dyskeratosis congenita is a bone marrow failure syndrome that may present with the classic triad of oral leukoplakia, nail dystrophy, and abnormal skin pigmentation. Bone marrow failure usually develops before the age of 20, with 80% of patients showing signs of bone marrow failure before the age of 30.[20] Nonetheless, the clinical presentation of dyskeratosis can be heterogeneous, even among family members carrying the same genetic defect. Patients with dyskeratosis congenita are at increased risk of developing malignancies, the most frequent being head and neck squamous cell carcinoma, AML, and MDS.[21] Inheritance of dyskeratosis can be X-linked, autosomal dominant, or autosomal recessive. In patients with X-linked dyskeratosis, over a decade ago mutations were identified in a gene located on chromosome Xq28 referred to as *DKC1*.[22] *DKC1* is a small nucleolar protein that, among other functions, associates with *TERC*, the RNA template of the telomerase complex.[23] In highly proliferative cells, the telomerase complex prevents critical shortening of telomeres, which are the structures that protect the ends of linear chromosomes. After the identification of *DKC1* mutations in dyskeratosis, short telomeres were described in dyskeratosis patients,[24] and mutations in *TERC* were discovered.[25] Subsequently, mutations in *TERT* (encoding telomerase reverse transcriptase), *NOP10*, *NHP2*, and *TCAB1* (encoding other components of the telomerase complex), *TINF2* (encoding a component of the shelterin telomere protection complex), *C16orf57* (encoding USB1, a phosphodiesterase essential for the U6 snRNA 3' end processing[26]) (reviewed in Dokal[20]) and *RTEL1*[27,28] (encoding a replisome-associated helicase promoting telomere and genome-wide replication[29]) were identified. Although the anabolic steroid oxymetholone can result in improvements in hematopoiesis in about two thirds of patients with dyskeratosis congenita, hematopoietic stem cell transplantation is the only cure for bone marrow failure in dyskeratosis congenita.[20] The preparative regimen used before transplantation, however, may exacerbate the intrinsic propensity in dyskeratosis congenita for pulmonary fibrosis and liver cirrhosis.[4]

Defective telomere maintenance and accelerated telomere attrition as a result of telomerase complex and shelterin gene mutations do clinically not only present as dyskeratosis congenita, but can manifest as a spectrum of disorders, or telomere disease, consisting of aplastic anemia, AML, liver cirrhosis, pulmonary fibrosis, and the severe variants of dyskeratosis congenita, Hoyeraal-Hreidarsson syndrome and Revesz syndrome.[30]

Acquired bone marrow failure syndrome: aplastic anemia

Aplastic anemia is an acquired bone marrow failure syndrome characterized by hypocellular bone marrow and low peripheral blood cell counts. The disease is subdivided into severe aplastic anemia (neutrophil count 0.2-0.5x10^9/L) and very severe aplastic anemia (neutrophil count <0.2x10^9/L).[31] An immune-mediated pathophysiology is inferred from an expansion of oligoclonal cytotoxic T-cells, likely directed against hematopoietic stem- or progenitor cells, and a response to immunosuppressive therapy (IST), consisting

of antithymocyte globulin (ATG) and cyclosporin A (CsA), in a significant proportion of patients with aplastic anemia (reviewed by Young et al.[32,33]). Although immunosuppressive therapy is effective, response to horse ATG (which has a superior response over rabbit ATG in adult as well as in pediatric patients[34-36]) is observed in only about 65% of pediatric aplastic anemia patients,[36] and clonal evolution to MDS or AML after IST occurs in 3-15% of patients.[37] Therefore, in pediatric patients, hematopoietic stem cell transplantation to replete the exhausted stem cell pool is recommended when a matched sibling donor is available.[38] When a matched sibling donor is not available, immunosuppressive therapy including horse ATG is recommended to be given,[36] while nonresponders to immunuppressive therapy at 6 months should be offered an (unrelated) stem cell transplantation.[36,39,40]

2.2 Clonal disorders of myelopoiesis

Clonal myelopoiesis is defined as the condition in which a significant proportion of cells are derived from the same progenitor cell that has acquired a mutation. The non-malignant clonal disorder paroxysmal nocturnal hemoglobinuria (PNH) and the malignant clonal disorders MDS and AML will be discussed below. The relationship between AML, MDS, PNH and inherited and acquired bone marrow failure syndromes is depicted in Figure 2.

Paroxysmal nocturnal hemoglobinuria

PNH is an acquired, non-malignant hematologic disorder caused by clonal expansion of one or more hematopoietic stem cells (HSCs) carrying a loss of function mutation in the X-chromosomal phosphatidylinositol glycan A (*PIG-A*) gene, required for the synthesis of the glycosylphosphatidylinositol (GPI) anchor.[41] As a result of this mutation, daughter cells of the *PIG-A* mutated HSC, generally erythrocytes, granulocytes, monocytes, but also lymphocytes, lack GPI-anchored proteins on their cell membranes. Of interest, recently a PNH case was described without mutations in the *PIG-A* gene, but with a germline and an acquired mutation in *PIG-T*, involved in assembly of the GPI anchor to proteins.[42] PNH is categorized according to the International PNH Interest Group into: 1. classic PNH; 2. symptomatic PNH in the setting of another bone marrow failure syndrome; or 3. subclinical PNH in the setting of another bone marrow failure syndrome.[43]

Classic PNH and symptomatic PNH in the setting of another bone marrow failure syndrome

Classic PNH and symptomatic PNH in the setting of another bone marrow failure syndrome are characterized by the presence of large GPI-deficient clones, intravascular hemolysis, and by an increased risk of thrombosis. A certain degree of bone marrow failure is usually present in classic PNH, while aplastic anemia or MDS are present in symptomatic PNH in the setting of another bone marrow failure.[43] Intravascular hemolysis results from an increased sensitivity of erythrocytes to complement mediated lysis due to absence of the

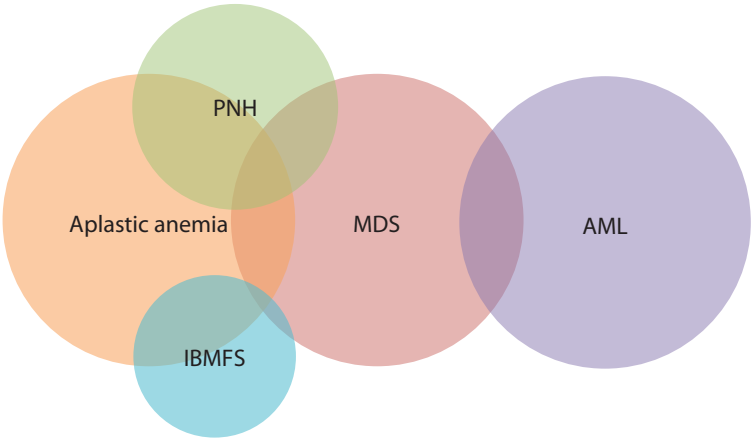

Figure 2 Diagram showing relationship between bone marrow failure syndromes, PNH, MDS, and AML.

Overlapping areas indicate a possible shared pathophysiology or diagnostic difficulties. PNH, paroxysmal nocturnal hemoglobinuria; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; IBMFS, inherited bone marrow failure syndromes. Modified from Young et al., Blood 2006[32] and Shimamura et al., Blood Reviews 2010.[4]

GPI-anchored proteins CD55 (decay-accelerating factor or DAF, inhibiting C3 convertase) and CD59 (membrane inhibitor of reactive lysis, inhibiting formation of the membrane attack complex). Classic PNH can be cured by a hematopoietic stem cell transplantation only,[44] but may be symptomatically treated with eculizumab.[45] Eculizumab is a humanized monoclonal antibody against terminal complement protein C5, and blocks the formation of the cytoloytic membrane attack complex, which consists of complement components C5b, C6, C7, C8 and multiple molecules of C9. Classic PNH in childhood is rare.[46]

Subclinical PNH

Subclinical PNH clones are frequently present in adult patients with aplastic anemia or low-grade MDS. The mechanisms by which PNH clones arise in immune-mediated bone marrow failure syndromes are incompletely understood. It is hypothesized that GPI-deficient cells have a conditional growth advantage by evading an immune attack directed against normal HSCs,[47] or that, next to the *PIG-A* mutation, they gain a second mutation, conferring the GPI-deficient clone a selective advantage over normal HSCs.[48] Another recent hypothesis is that GPI-deficient cells, which can be detected at very low numbers (approximately 0.002%) in bone marrow and peripheral blood of healthy individuals,[49-51] can expand without a conditional or selective advantage, especially in

conditions with reduced stem cell numbers.[52] Small PNH clones are unlikely to contribute to anemia or increase the risk of thrombosis in patients with aplastic anemia and MDS, and hence do not require treatment with complement inhibition.[47]

Myelodysplastic syndrome

MDS is a clonal, stem cell-derived myeloid disorder, that is characterized by an ineffective hematopoiesis, resulting in peripheral blood cytopenias, myeloid dysplasia, and risk of transformation to AML.[53] In adults, MDS is a common hematologic malignancy, with an incidence of 2-13 per 100,000 people per year, rising to 15-50 per 100,000 above the age of 70 years.[54,55] The mean age at presentation is 70 years,[56] and the incidence of MDS is a factor 1.8 higher in men than in women.[55] In contrast, MDS in childhood is rare and has an estimated annual incidence of about 0.8 to 1.8 per million children aged 0 to 14 years.[57-59] The median age at presentation is 6.8 years,[60] and the incidence of MDS is similar in boys and girls.[57-59,61] In the only large series of advanced pediatric MDS, the median age at presentation was 11 years, and the incidence was 2 times higher in boys than in girls.[62] Classification, diagnosis, and treatment of childhood MDS, as described below, have improved greatly in recent years through efforts from the European Working Group of MDS in Childhood (EWOG-MDS).

Classification of pediatric MDS

Pediatric MDS is classified according to the second WHO classification (2008) into refractory cytopenia of childhood (RCC), with < 5% bone marrow blasts and <2% peripheral blood blasts, refractory anemia with excess blasts (RAEB), with 5-19% bone marrow blasts and/or 2-19% peripheral blood blasts, and RAEB in transformation (RAEB-t), with 20-29% bone marrow and/or peripheral blood blasts. Patients with 30% or more bone marrow and/or peripheral blood myeloid blast cells are classified as AML. MDS that develops in acquired or inherited bone marrow failure syndromes, as familial disease (such as in families carrying germline *GATA2* mutations),[63-66] or after radio- or chemotherapy is categorized as secondary MDS; all other cases as primary MDS.[67] Minimal diagnostic criteria for pediatric MDS are described in Table 2. Morphologic evaluation is based on bone marrow aspirate, bone marrow biopsy, and peripheral blood smear.

Refractory cytopenia of childhood

RCC is the most common subtype of childhood MDS, accounting for about half of all cases.[57,67] Patients may present with anemia, infection, and an increased bleeding tendency. At diagnosis, approximately 50% of all patients have an absolute neutrophil count $<1x10^9$/L, and 25% $<0.5x10^9$/L; about 75% have a platelet count of $<150x10^9$/L; and about 50% a hemoglobin level of <10mg/dL, (or <6.2 mmol/L). Hemoglobin F and the mean corpuscular cell volume are elevated in more than 75% of patients.[68] In contrast to adults with low-grade MDS, about 80% of children have a hypocellular bone marrow.[53]

Table 2 Minimal diagnostic criteria for pediatric MDS.

Minimal diagnostic criteria for MDS (at least two of the following):
Sustained unexplained cytopenia (neutropenia, thrombocytopenia, or anemia)
Morphologic myelodysplasia (present in two different myeloid cell lines or exceeding 10% in one single cell line)
Acquired clonal cytogenetic abnormality in hematopoietic cells
Increased blast count (≥5%)

Table adapted from Hasle et al., Leukemia 2003.[107]

Karyotype is normal in the majority of patients with RCC, in contrast to patients with MDS-RAEB(-t), of whom more than 50% have and abnormal karyotype.[60] Monosomy 7 is the most common aberration, found in about 20% of successful cytogenetic analyses in patients with a hypocellular marrow, followed by trisomy 8. RCC patients with monosomy 7 have a high risk of progression to advanced MDS or AML, with a median time to progression of less than 2 years, while disease may be stable for a prolonged time in patients with trisomy 8 and other karyotypes.[68]

Differential diagnosis of refractory cytopenia of childhood

Due to the hypcellular bone marrow and normal cytogenetics in the majority of RCC patients, differentiating RCC from aplastic anemia or from inherited bone marrow failure syndromes can be challenging. Nonetheless, making the distinction between the different entities has therapeutic consequences and is therefore relevant: while RCC is generally considered to be cured by hematopoietic stem cell transplantation, aplastic anemia can successfully be treated in most patients solely with IST.[53] Histopathological criteria for the diagnosis of RCC and aplastic anemia are defined in the 2008 WHO classification, and summarized in Table 3.[67] Based on these criteria, of which the presence of a patchy, left shifted erythropoiesis in RCC, and the absence thereof in aplastic anemia is the main differentiating criterion, the vast majority of aplastic anemia and RCC cases can be reproducibly differentiated.[37] Inherited bone marrow failure syndromes may present with myelodysplasia and have to be excluded by past medical and family history, physical examination for subtle dysmorphic features, organ and skeletal abnormalities, and appropriate laboratory tests. Fanconi anemia has to be excluded in all children with primary MDS.[60]

Pathophysiology of refractory cytopenia of childhood

Driver mutations in adult MDS include mutations in genes involved in RNA splicing, DNA methylation, chromatin modification, transcription regulation, DNA repair, signal transduction, and the cohesin complex.[69-72] In addition, the observation that IST, consisting

Table 3 WHO 2008 histopathological criteria of RCC and severe aplastic anemia.

	RCC	Severe aplastic anaemia
Erythropoiesis	Patchy distribution	Lacking foci or a single small focus with <10 cells with maturation
	Left shift	
	Increased mitoses	
Granulopoiesis	Marked decrease	Lacking or marked decrease, very few small foci with maturation
	Left shift	
Megakaryopoiesis	Marked decrease	Lacking or very few, no dysplastic megakaryocytes
	Dysplastic changes	
	Micromegakaryocytes	
Lymphocytes	May be increased focally or dispersed	May be increased focally or dispersed
CD34+ cells	No increase	No increase

Table adapted from Baumann et al., WHO classification 2008.[67]

of ATG and/or CsA, is effective in some adults with low-grade MDS[73-83] suggests that a T cell-mediated immune response directed against hematopoietic progenitor cells is present in a proportion of patients.[47] In vitro studies in adult low-grade MDS show that autologous peripheral blood lymphocytes inhibit granulocyte colony formation in an MHC class I-dependent manner,[84-87] which can be abrogated by treatment with ATG.[84] Analysis of the T-cell receptor (TCR) β-chain variable (Vβ) domain usage by flow cytometry and PCR-based methods shows oligoclonal expansions of mainly $CD8^+$ T cells in MDS patients.[84,86,88-91] Although recent studies reported that treatment with CsA and ATG is also effective in RCC, and a retrospective study showed that oligoclonality of the TCRVβ is also present in RCC patients, further studies to confirm a possible immune-mediated pathophysiology in RCC had not been performed before the start of the studies described in this thesis.[92-94]

Treatment of refractory cytopenia of childhood

Treatment of RCC, depending on bone marrow cellularity, cytogenetics, and transfusion dependency, may consist of a watch-and-wait strategy, immunosuppressive therapy, or a hematopoietic stem cell transplantation with myeloablative or a reduced intensity conditioning regimen, based on the algorithm according to EWOG-MDS RC06 (the first prospective study by EWOG-MDS in childhood MDS) (Figure 3). RCC patients with a hypocellular bone marrow, normal karyotype or karyotype other than monosomy 7, del(7q), or complex karyotype, and transfusion dependency or absolute neutrophil count

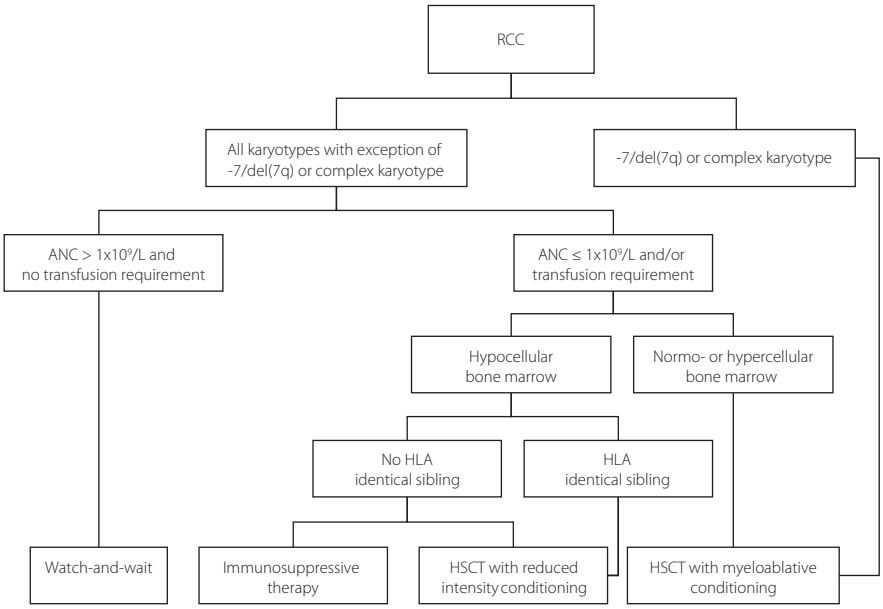

Figure 3 Treatment algorithm of RCC patients according to EWOG-MDS RC06.

ANC, absolute neuthrophil count; HSCT, hematopoietic stem cell transplantation.

(ANC) below 1x10^9/L are eligible for a hematopoietic stem cell transplantation from an HLA-matched donor after a reduced-intensity conditioning regimen or for IST. Based on the results from EWOG-MDS RC06, response to IST with horse and rabbit ATG was recently reported to be 74% and 53% at 4 years, with a failure-free survival of 58% and 48% at 4 years, an overall survival of 91% and 85% at 4 years, and a risk of clonal evolution of 14% and 4% at 4 years, respectively.[94] In a recent pilot study of EWOG-MDS, hematopoietic stem cell transplantation with a reduced-intensity conditioning regimen resulted in an overall survival of 84% at 3 years, and an event-free survival of 74% at 3 years, which is comparable to transplantation following a myeloablative conditioning regimen in RCC.[95]

Acute myeloid leukemia

Pediatric AML is a heterogeneous malignant disease, characterized by abnormal proliferation and impaired differentiation of myeloid cells. Acquired cytogenetic aberrations such as t(8;21)(q22;q22) (AML-ETO), inv(16)(p13q22) (CBFβ-MYH11), t(15;17)(q22;q21) (PML-RARα), rearrangements involving the mixed lineage leukemia gene (*MLL*),[96] and molecular aberrations in genes such as *FLT3*,[97] *NPM1*,[98] *CEBPA*,[99,100] and *WT1*[101,102] are involved in AML pathogenesis and are prognostic factors for treatment outcome. The risk of AML is

increased in patients carrying germline mutations in the genes *CEBPA*,[103] *RUNX1* (or *CBFA2*),[104] or *GATA2*.[63-66] Furthermore, the risk of AML is increased in patients with DNA repair deficiency syndromes, Noonan syndrome and neurofibromatosis type 1, Down syndrome,[105] and, as discussed above, in patients with bone marrow failure syndromes and MDS. Treatment of AML consists of chemotherapy commonly including cytarabine and anthracyclins, aiming at remission induction, and consolidation therapy. In patients with a high risk of relapse, chemotherapy is followed by hematopoietic stem cell transplantation. Current treatment regimens result in 5-year overall survival rates reaching 70%.[106]

AIMS AND OUTLINE OF THE THESIS

Inherited bone marrow failure syndromes, acquired aplastic anemia, PNH, MDS, and AML are part of a spectrum of myeloid disorders. The clinical presentation of inherited bone marrow failure syndromes can be heterogeneous, and a pathophysiologic overlap between parts of the spectrum might be present. In this thesis, we aimed to better characterize this spectrum of myeloid disorders in childhood.

In the first chapters of this thesis we explored whether germline mutations causing inherited bone marrow failure syndromes underlie seemingly sporadic AML and acquired aplastic anemia. **Chapters 2 and 3** describe whether germline mutations in *SBDS*, *TERT* and *TERC* can be found in sporadic AML, and in **Chapter 4**, a novel mutation is characterized in the promoter region of *TERC*, leading to telomere disease, which presented in the affected family with aplastic anemia, leukemia, and liver cirrhosis. Similar to acquired aplastic anemia, clinical and laboratory evidence suggest a T-cell-mediated pathophysiology in a subset of adult patients with low-grade MDS. In the next chapters, we explored whether an immune-mediated pathophysiology is also present in RCC. **Chapter 5** describes the frequency or PNH clones, which might be indicators of an immune-mediated pathophysiology, and their association with response to immunosuppressive therapy in RCC. In **Chapter 6** the frequency of skewing of the T-cell receptor β-chain variable domain in RCC is described, and the association of skewing with T-cell subset distribution. Although an overlap in the pathophysiology of RCC and aplastic anemia seems likely, differentiating between the two disorders remains relevant, because the risk of clonal evolution might be increased in RCC compared to aplastic anemia. This differentiation, however, is challenging, and developing novel diagnostic tools is relevant. **Chapters 7** and **8** investigate whether RCC can be recognized and differentiated from aplastic anemia by different flow cytometry immunophenotyping scoring systems. In **Chapter 9**, our main findings and future perspectives are discussed.

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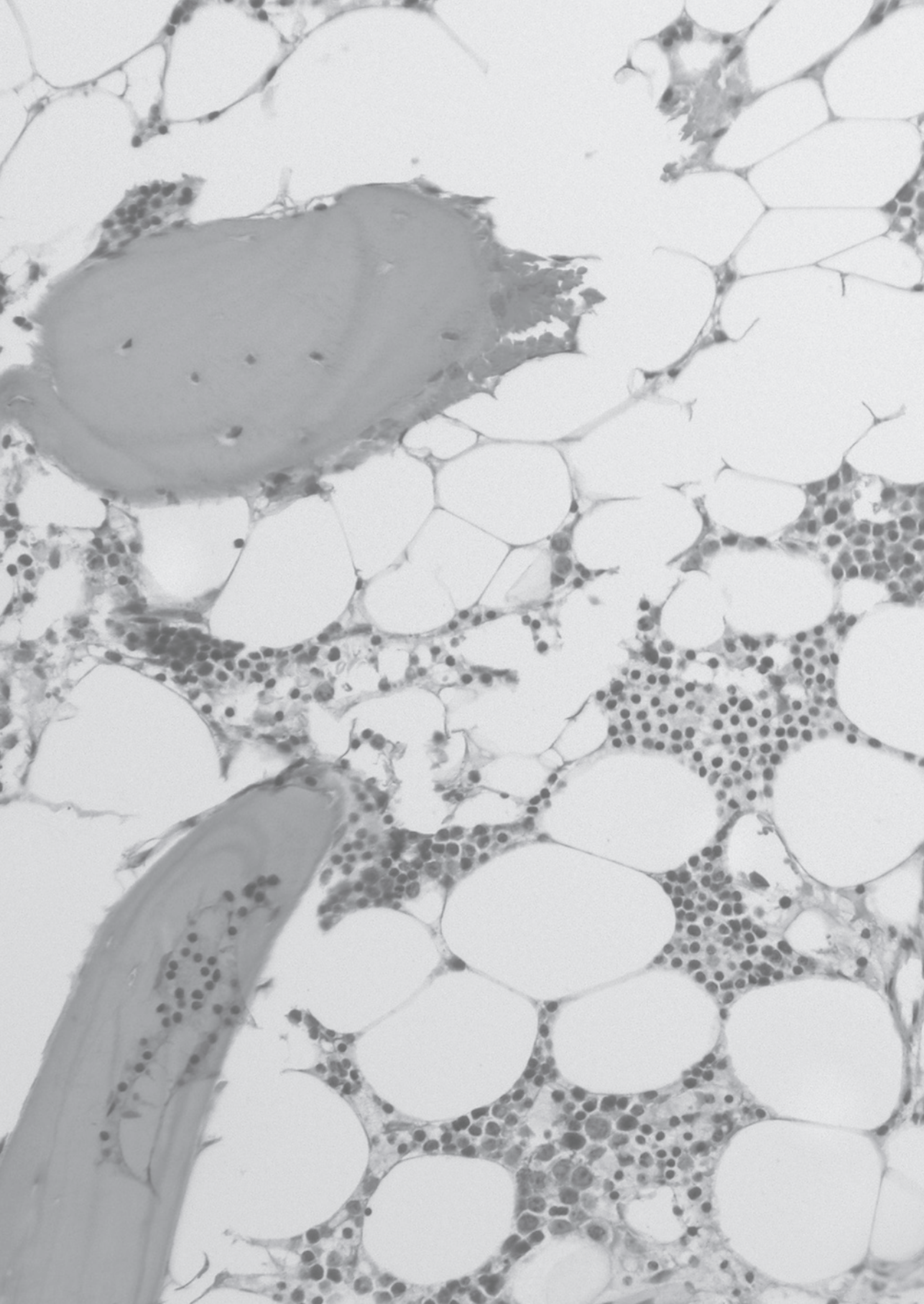

2

Absence of *SBDS* mutations in sporadic pediatric acute myeloid leukemia

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***Br J Haematol* 2012; 158 (6): 800-3**

TO THE EDITOR:

Shwachman-Diamond syndrome (SDS, OMIM #260400) is an autosomal recessive condition, characterized by pancreatic exocrine insufficiency, skeletal abnormalities, bone marrow failure, and an increased risk of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), the latter occurring in 19-36% of patients.[1] Compound heterozygous mutations in *SBDS* are identified in the majority of SDS patients. Of the two most frequently found mutations in *SBDS*, 183-184TA>CT and 258+2T>C, at least one is present in approximately 90% of affected individuals. These mutations are located in exon 2, and result from gene conversion with *SBDSP*, the *SBDS* pseudogene.[2] Although its exact function remains unclear, the SBDS protein appears to have a role in ribosome maturation, and might have additional extraribosomal functions.[3, 4]

Because of the increased risk of AML, but lack of a clear genotype-phenotype relationship in SDS,[5] we hypothesized that compound heterozygous *SBDS* mutations might be present in seemingly sporadic pediatric AML. Furthermore, we hypothesized that heterozygous mutations in *SBDS* might be present at increased frequency in sporadic AML compared to healthy controls, and might thus be a risk factor for AML development. Given the significant toxicity of standard chemotherapy and transplantation conditioning regimens in SDS patients with MDS or AML,[1] but the reduction in morbidity after reduced-intensity conditioning regimens,[6] the identification of AML patients carrying *SBDS* mutations seems clinically relevant.

In leukemic blast cells derived at diagnosis from 160 pediatric AML patients (median age: 9.6 years (range: 0-18.5 years); 90 (56.3%) male, 70 (43.7%) female), who were enrolled in consecutive AML/BFM, DCOG/MRC, and LAME treatment protocols between 1987 and 2008,[7] we specifically amplified *SBDS* and not *SBDSP*, as previously described, and sequenced exon 2 of *SBDS*.[8] Germline material of the AML patients was not available, and we here assume that *SBDS* gene variants found in leukemic blast cells are constitutional and not acquired variants.

Two AML patients carried a heterozygous 258+2T>C mutation (carrier frequency 0.013). This mutation disrupts the donor splice site of intron 2 and results in the use of a cryptic donor splice site in exon 2, leading to a frameshift and premature protein truncation at codon 84.[2] Furthermore, 28 of 160 AML patients carried the silent variant 201A>G (carrier frequency 0.175) (Figure 1). No compound heterozygous mutations in exon 2 of *SBDS* were detected. Of 168 Dutch blood bank donors, 1 carried the heterozygous 258+2T>C (carrier frequency 0.006). Furthermore, 3 of 168 blood bank donors carried a heterozygous 183-184TA>CT (carrier frequency 0.018), introducing a premature stop codon at amino acid 62. The silent variants 141C>T and 201A>G were present in 2 (carrier frequency 0.012) and 32 (carrier frequency 0.190) controls, respectively (Table 1). In previously published controls cohorts, 183-184TA>CT was present in 1 of 70 individuals (carrier frequency 0.014)[9] and 0 of 100 individuals,[2] whereas 258+2T>C was absent in three published controls cohorts of 70, 100 and 276 individuals each.[2, 8, 9]

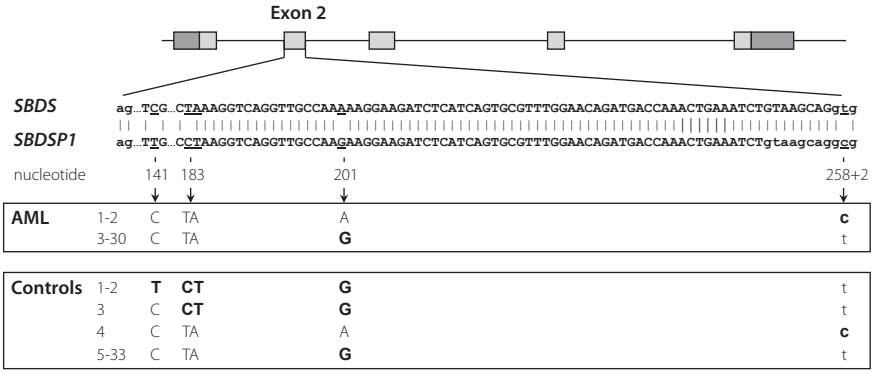

Figure 1

Graphical representation of pediatric AML patients and controls carrying *SBDS* nucleotide changes, depicted in bold, resulting from gene conversion events with *SBDSP* in and around exon 2. The absence of *SBDSP*-like sequences at nucleotide 141, 183-184, and 201 in AML patients, and the absence of *SBDSP*-like sequences at nucleotide 141, 183-184, 201, or 258+2 in controls, indicate the specificity of amplicons for *SBDS*. Figure adapted from Boocock *et al*, Mutations in SBDS are associated with Shwachman-Diamond syndrome. *Nature Genetics*. Jan 2003;33(1):97-101.

Table 1 *SBDS* gene variants resulting from gene conversion in pediatric AML patients and controls.

Nucleotide change	Amino acid change	AML patients (n=160)	Controls (n=168)
Het. 141C>T	-	-	2 (0.012)
Het. 183-184TA>CT	K62X	-	3 (0.018)
Het. 201A>G	-	28 (0.175)	32 (0.190)
Het. 258+2T>C	C84fs3	2 (0.013)	1 (0.006)

Values represent the number of individuals carrying a variant (carrier frequency).

We conclude that in a cohort of 160 pediatric AML patients, homozygous or compound heterozygous mutations in *SBDS* are absent, and heterozygous mutations in *SBDS* are present at frequencies comparable to healthy controls. Our findings confirm a previous report in which no mutations in exon 2 of *SBDS* were found in a smaller cohort of 48 children with de novo AML and 48 children with AML in remission.[10] Taken together, these results suggest that children with seemingly sporadic AML are unlikely to have underlying SDS.

Acknowledgements

AMA was supported by the KiKa Foundation, Amstelveen, The Netherlands, and the René Vogels Foundation, Oirschot, The Netherlands. This research was supported in part by the NIH (NHLBI) Intramural Research Program.

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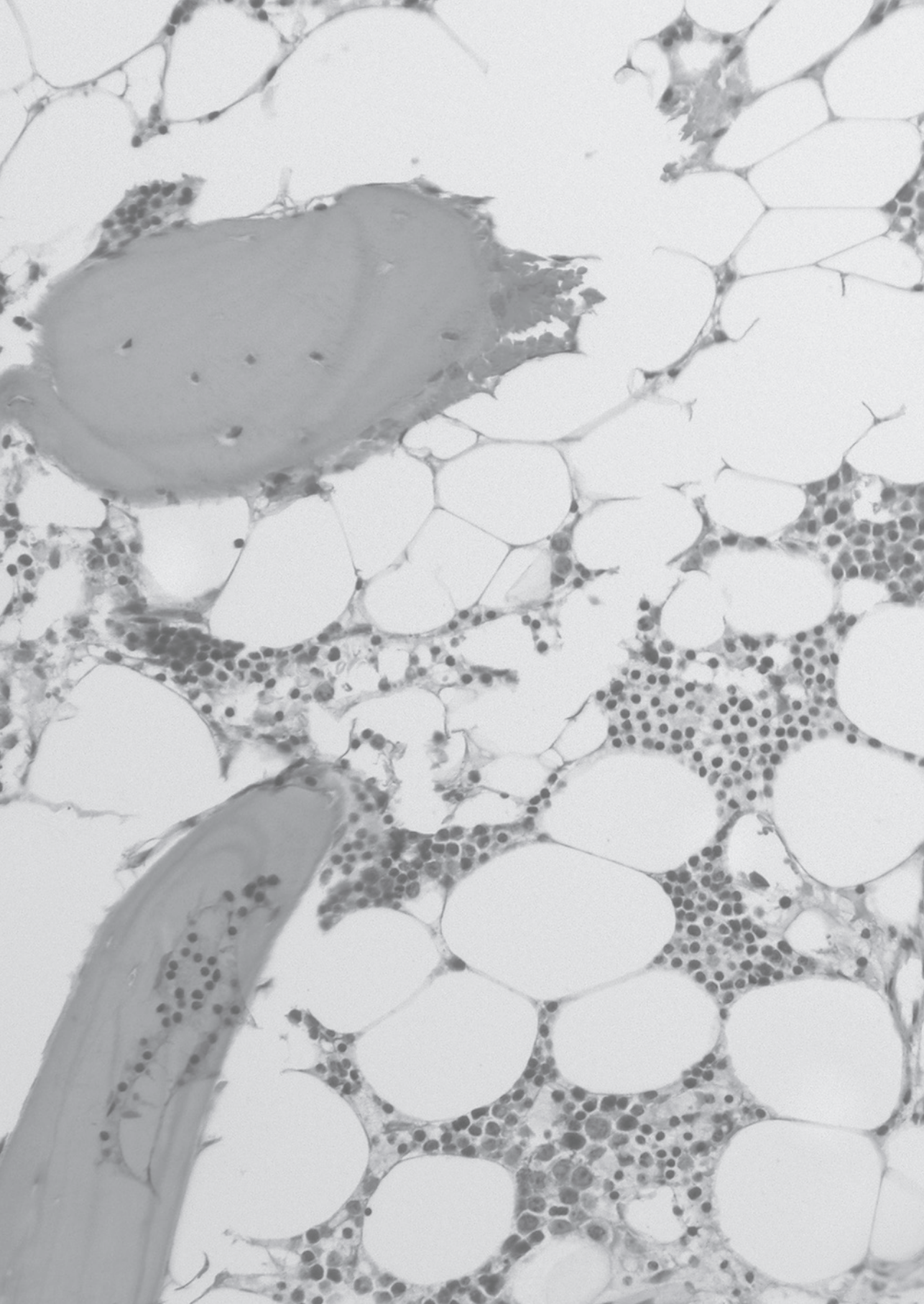

3

Telomere length and telomerase complex mutations in pediatric acute myeloid leukemia

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***Leukemia* 2013; 27 (8): 1786-9** (Published as Letter to the Editor)

ABSTRACT

Inherited loss-of-function mutations in telomerase complex genes, resulting in critically short telomeres and chromosomal instability, have recently been implicated as risk factors for acute myeloid leukemia (AML) in adults. In pediatric AML, the frequency of such mutations, and the association of telomere length with cytogenetic, molecular, and clinical characteristics and outcome, are unknown. In a cohort of 168 pediatric AML patients, compared with 406 geographically matched controls, we did not detect an increased frequency of *TERT* and *TERC* gene variants. Telomere length of leukemic cells was not associated with age, sex, prognostic cytogenetic subgroup, complex karyotype, or expression levels of telomerase and shelterin complex genes. However, patients carrying the high-risk molecular aberration *FLT3*/ITD had significantly shorter telomeres than did patients with favorable *NPM1* mutation or *CEBPA* double mutations. Telomere length was not associated with overall survival, event-free survival, or cumulative incidence of relapse. We conclude that, in pediatric AML, telomerase complex mutations do not confer a risk for leukemia development, and although short telomeres correlate with the high-risk molecular aberration *FLT3*/ITD, telomere length at diagnosis does not correlate with adverse outcome in this pediatric AML cohort.

INTRODUCTION

Pediatric acute myeloid leukemia (AML) is a heterogeneous malignant disease, characterized by abnormal proliferation and impaired differentiation of myeloid cells. Acquired cytogenetic aberrations such as t(8;21)(q22;q22) (AML-ETO), inv(16)(p13q22) (CBFβ-MYH11), t(15;17)(q22;q21) (PML-RARα), rearrangements involving the mixed lineage leukemia gene (*MLL*),[1] and molecular aberrations in genes such as *FLT3*,[2] *NPM1*,[3] *CEBPA*,[4,5] and *WT1*[6,7] are involved in AML pathogenesis and are prognostic factors for treatment outcome.

Recently, constitutional loss-of-function mutations in telomerase complex genes have been implicated as risk factors for AML in adults.[8] The telomerase complex is expressed in highly proliferative cells, and is responsible for maintaining telomeres, which cap the ends of chromosomes and protect genomic DNA from eroding during cell divisions. Impaired telomerase function can result in extremely short telomeres, which limits the proliferative capacity of progenitor cells, and also lead to chromosomal instability, thus predisposing to malignant transformation.[9] This pathogenesis is manifested in dyskeratosis congenita, an inherited bone marrow failure syndrome characterized by mucocutaneous, pulmonary, and liver abnormalities, in which patients are at increased risk for malignancies, including AML,[10] and in which telomerase complex mutations are etiologic.[11-13]

To date, the significance of telomerase complex mutations as risk factor for AML in children is unknown. Furthermore, although extremely short telomeres in leukemic cells of adult AML patients have been reported to be associated with cytogenetic aberrations and complex karyotype, the clinical implications of these short telomeres are unknown.[14,15]

In the current study, we assessed the frequency of mutations in the telomerase complex genes *TERT*, telomerase reverse transcriptase, and *TERC*, the RNA template of the telomerase complex, in a cohort of 168 pediatric AML patients. Furthermore, we measured telomere length in these patients at diagnosis and determined its relationship with clinical and molecular characteristics and outcome.

MATERIAL AND METHODS

Patients

Bone marrow (BM) or peripheral blood (PB) samples, obtained at diagnosis from 168 children with AML (French-American-British (FAB) classification M0-M7), were provided by the Dutch Childhood Oncology Group, the AML-BFM Study Group, the Czech Pediatric Hematology Group, the Saint-Louis Hospital in Paris, France, and the MRC/CCLG, United Kingdom. Institutional review boards of participating institutions approved the study, and patients, parents, or legal guardians of patients provided written informed consent for study participation. Each national study group performed central review of morphologic and cytogenetic classification, as well as clinical follow-up.

Leukemic cells were isolated and enriched by removal of contaminating non-leukemic cells using immunomagnetic beads (Dynabeads CD3, Invitrogen Dynal AS, Oslo, Norway).[16] After enrichment, samples contained 80% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa stained cytospins. DNA was extracted from leukemic cells using TRIzol Reagent (Invitrogen Life Technologies, Breda, Netherlands).

Samples were routinely screened for cytogenetic abnormalities by national study groups with standard chromosome banding. Karyotypic abnormalities were counted as previously described.[17] Samples were further analyzed for recurrent non-random genetic abnormalities, including t(15;17), inv(16), t(8;21), and *MLL* gene rearrangements, using reverse transcription polymerase chain reaction (RT-PCR) and/or fluorescent in situ hybridization, as reported.[18]

Germline DNA, obtained from remission material, was available for 3 patients carrying *TERT* variant A1062T, but not for the 2 patients carrying *TERT* variants T726M and E327D and 3 other patients carrying A1062T.

Treatment protocols

Patients were treated according to consecutive AML-BFM, DCOG/MRC, and Leucémie Aiguë Myéloblastique Enfant (LAME) treatment protocols between 1987 and 2008. Details of treatment protocols and outcome data have been previously published.[19-21] Treatment consisted of four to five blocks of intensive chemotherapy with a standard cytarabine and anthracycline backbone.

Controls

Genomic DNA isolated from peripheral blood leukocytes obtained from 406 healthy Dutch blood bank donors served as control for typing of *TERT* sequence variants that were identified in patient genomic DNA. Genomic DNA isolated from peripheral blood leukocytes obtained from 298 healthy NIH blood bank donors served as control for telomere length measurement. Written informed consent for participation in scientific research was given by both control groups.

Mutational analysis

TERT and *TERC* sequencing of patient genomic DNA, and *TERT* sequencing of control genomic DNA were performed as previously described.[22] Mutation status of *CEBPA*, *FLT3*, *NPM1* exon 12, and *WT1* exons 7-10, determined in our laboratory, was available for the majority of AML patients as described.[18]

Functional analysis of *TERT* mutants

Functional analysis of the *TERT* mutant E327D was performed as previously described.[8] Briefly, mutagenesis of wild-type pcDNA3-Flag-TERT was performed and confirmed by sequencing of the whole insert by Mutagenex (Somerset, NJ) to generate E327D. Dr. Hinh

Ly (Emory University, Atlanta, GA) kindly provided pcDNA3-Flag-TERC. One microgram of pcDNA3-Flag-TERC and one microgram of either wild-type or mutant pcDNA3-Flag-TERT were transfected into telomerase negative WI-38 VA13 cells (ATCC) at 60% confluence in each well of 6-well plates using Superfect Transfection Reagent (Qiagen, Valencia, CA), according to the manufacturer's instructions. Transfections were carried out in two independent experiments.

Telomerase activity was measured using the telomerase fluorescent repeat amplification protocol (Trapeze XL, Chemicon/Millipore, Temecula, CA), as previously described.[8] Briefly, cells were scraped off the dishes 48 h after transfection and washed 3 times in PBS. Subsequently, protein was isolated using CHAPS lysis buffer, and total cellular RNA was extracted with RNeasy (Qiagen). Telomerase activity was measured in triplicate. Telomerase activity was corrected for transfection efficiency of pcDNA3-Flag-TERT by determining TERT expression by RT-PCR.

Telomere length by quantitative PCR

Telomere length was assessed in genomic DNA extracted from leukemic cells by quantitative polymerase chain reaction (PCR) as previously described with some modifications.[23,24] Quantitative PCR reactions were set up by a QIAgility liquid handling robot (Qiagen) and were performed in a Rotor Gene-Q (Qiagen) in triplicate, using the following PCR conditions: 95°C 5 m; 98°C 7 s, 60°C 10 s (25 cycles) (telomere reaction) and 95°C 5 m; 98°C 7 s, 58°C 10 s (35 cycles) (single copy gene reaction). Each reaction contained 1.6 ng genomic DNA, 7.2 pg of each primer (12 pg of the single copy gene reverse primer), and 16 uL Rotor-Gene SYBR Green PCR Master Mix (Qiagen), in a total volume of 20 uL. Telomere length was expressed as a telomere to single copy gene (T/S) ratio and normalized to the T/S ratio of a reference sample ($2^{-(\Delta Ctsample-\Delta Ctreference)}$), which was also used for the standard curve. The coefficient of variation (CV) of this method in 51 pediatric AML samples that were measured in two independent runs, calculated as mean of the standard deviation (SD) per repeated measurement divided by the mean of all measurements, was 12%. Terminal restriction fragment (TRF) length analysis by Southern hybridization was performed to validate the quantitative PCR-based method, and comparison of results showed a high correlation between the methods ($R^2 = 0.86$; TRF (kb) = T/S ratio* 5.5969+ 3.0263). No deletions or amplifications involving chromosome 12q24.2, harbouring the single copy gene *36B4/RPLP0*, were noted in 148 patients for whom complete karyotypes were available; a hyperdiploid karyotype was only present in two patients.[25] Telomere length of healthy controls was determined previously in our laboratory[26] in genomic DNA extracted from peripheral blood leukocytes of NIH blood bank donors using the above described method.

Gene expression profiling

Gene expression profiling (HGU133 Plus 2.0 microarray, Affymetrix) data, acquired in our laboratory, were available for 157 of 168 pediatric AML cases and were analyzed as previously described.[27] Briefly, data were acquired using 'expresso' (Bioconductor Software

Package 'affy'), and probe-set intensities were normalized by variance stabilization normalization (Bioconductor Software Package 'vsn') in R, version 2.11.1. An empirical Bayes linear regression model was used to compare signatures of patients in quartile 1 versus quartiles 2-4. Moderated T-statistics *P*-values were corrected for multiple testing by use of the false discovery rate method defined by Benjamini and Hochberg.

Statistical analyses

Statistical analyses were performed with SPSS 17.0 (IBM) and SAS/STAT (SAS Institute). Categorical variables were compared using the Chi-square test or, in case of low numbers, Fisher's Exact test. Continuous variables were compared using the Mann-Whitney-U test or the Kruskal-Wallis test when more than 2 groups were compared, and median values were reported because of skewed distributions. To assess outcome, the 5-year probabilities of overall survival (pOS) and event free survival (pEFS) were estimated by the Kaplan-Meier method and compared using the log-rank test. Events for OS were defined as death from any cause; events for EFS as non-remittance, relapse, secondary malignancy, or death from any cause. EFS was calculated from the date of diagnosis to the first event; events for patients with refractory disease were set on day 0. Cumulative incidence of relapse (CIR) (calculated from date of CR to first relapse) and non-response curves were constructed by the method of Kalbfleisch and Prentice and were compared using Gray's test.[28] All reported *P*-values are two-sided, and values < 0.05 were considered statistically significant.

RESULTS

Patient characteristics

Patient and disease characteristics are described in Table 1 and Supplemental Table 1. Clinical features were comparable with the AML-BFM98 series[29] with respect to age, sex and FAB type, with the exception that, in the present cohort, more FAB M4 cases were included (31% vs 20%, *P*=0.003), and white blood cell (WBC) count was higher (31 versus 20% of patients > $100x10^9$/L, *P*=0.01) (Supplemental Table 1). Five-year probabilities of overall survival (pOS) and event-free survival (pEFS) were 58±4.2% and 43±4.2%, respectively. Median follow-up time of surviving patients was 5.8 years (range, 0-24 years).

TERT and *TERC* variants in pediatric AML

To determine the frequency of mutations in the telomerase complex genes *TERT* and *TERC*, 168 diagnostic pediatric AML samples, containing at least 80% leukemic cells, were analyzed. No mutations in *TERC* were detected. In *TERT*, three non-synonymous variants were present in a total of 8 patients (carrier frequency 0.048). In one patient, a novel heterozygous variant in a well-conserved amino acid in exon 2 in the N-terminal extension of *TERT*, E327D (mRNA NM_198253.2: 1039G/T) was detected. Furthermore, a heterozygous

Table 1 Patient characteristics by telomere length quartile.

Characteristic	All patients	Telomere length quartile				P-value
		1	2	3	4	
Telomere length range, T/S ratio	0.283-2.27	0.283-0.541	0.541-0.651	0.651-0.838	0.838-2.27	
Patients, No.	167	42	42	42	41	
Sex, No. (%)						
Male	92 (55.1)	26 (61.9)	20 (47.6)	24 (57.1)	22 (53.7)	0.606 [b]
Female	75 (44.9)	16 (38.1)	22 (52.4)	18 (42.9)	19 (46.3)	
Age, median, y [range]	9.7 [0-18.5]	9.1 [1-16.7]	9.7 [0.3-18.5]	10.4 [0.4-18.4]	9.7 [0-17.7]	0.715 [a]
Age < 10 y, No.	86	23	22	20	21	0.930 [b]
Age > 10 y, No.	81	19	20	22	20	
WBC, median (x 10^9/uL) [range]	47.3 [2-378]	58.1 [2-378]	43.1 [2-200]	33 [3-267]	40.2 [2-230]	0.154 [a]

Abbreviations: mt, mutant; wt, wild-type; WBC, white blood cell count

[a] Kruskall-Wallis test

[b] Chi-square test

variant in exon 6 in the reverse transcriptase domain of *TERT*, T726M (mRNA NM_198253.2: 2235C/T), previously described in aplastic anemia,[30] was found in another patient. Both mutations were absent among 406 healthy Dutch blood bank donors. No remission material was available to confirm the germ-line origin of these mutations. Six out of 168 patients carried a heterozygous variant in the C-terminal extension of *TERT*, A1062T (mRNA NM_198253.2: 3242G/A; carrier frequency 0.036). This variant was present in a similar carrier frequency (0.049) in 406 healthy Dutch controls, composed of two independent cohorts (6/192 and 14/214, carrier frequencies 0.031 and 0.065, respectively) (Table 2). In three of the six patients carrying A1062T, remission material was available, in which the germ-line origin of the variant was confirmed. Several synonymous variants and another non-synonymous variant, A279T, all described in healthy controls and not associated with a reduced telomerase function,[31] were present in this pediatric AML cohort (Supplemental Table 2). Minor allele frequencies of two synonymous variants and variant A279T were also determined in healthy controls and were similar to frequencies in AML patients, demonstrating comparability of both populations (Supplemental Table 2).

Telomerase activity of *TERT* variants

To assess whether the novel *TERT* variant E327D resulted in reduced telomerase enzymatic activity, telomerase-deficient VA13 cells were transfected with plasmids containing

Table 2 Non-synonymous *TERT* variants in pediatric AML patients and healthy Dutch controls.

		No. of heterozygotes (carrier frequency)	
Exon	**Codon**	**AML patients (n=168)**	**Controls (n=406)***
2	E327D	1 (0.006)	0 (0)**
6	T726M	1 (0.006)	0 (0)**
15	A1062T	6 (0.036)	20 (0.049)

* Controls were typed for variants identified in AML patients only

** Determined in 401 controls

[b] Chi-square test

wild-type *TERC* and wild-type or mutant (E327D) *TERT*. Telomerase activity of *TERT* codon E327D was similar to wild-type *TERT* (94.7±3.5% (mean ± SD)). Telomerase enzymatic activities of the variants A1062T and T726M have been previously reported. We described an activity of *TERT* codon A1062T of about 60% of wild-type,[8] but a normal activity was reported by others.[32] The activity of T726M, located in the reverse transcriptase domain of telomerase, previously described in a patient with aplastic anemia and very short telomeres,[30] was not associated with a reduced telomerase activity.[31]

Characteristics of patients with *TERT* variants

The patient who carried the *TERT* codon E327D was a female diagnosed at 14.6 years of age with a FAB M2 AML. Karyotype at diagnosis was normal, but leukemic cells carried mutations in the N- and C-terminal domains of *CEBPA* (c.344_348del5bp and c.937_939dupAAG) and a missense mutation in exon 9 of *WT1* (c.1186G/A). *NPM1* and *FLT3* were wild-type. She achieved complete remission and is alive 61 months after diagnosis. Telomere length of leukemic cells (T/S ratio 0.83) was in the third quartile of the cohort of pediatric AML patients. The patient who carried *TERT* codon T726M, a 17-year old female, was diagnosed with a FAB M1 AML, had a normal karyotype, and *CEBPA*, *FLT3*, *NPM1*, and *WT1* were wild-type. She concurrently suffered from xeroderma pigmentosum and melanoma, and died three months after AML diagnosis. Mutational status of nucleotide excision repair genes is unknown. Telomere length of leukemic cells (T/S ratio 0.71) was within the third quartile of the patient cohort. Mucocutaneous features of dyskeratosis congenita were not reported in any patients. Clinical and genetic characteristics of the patients carrying the *TERT* codon A1062T variant, present in a similar frequency in geographically matched healthy controls, are described in Supplemental Table 3.

Telomere length in pediatric AML and association with clinical and biological characteristics

Telomere length was successfully determined in leukemic cells derived from 167 pediatric AML patients at diagnosis. Median telomere length (T/S ratio) was 0.65 (range, 0.28-2.3). When compared to healthy control peripheral blood leukocytes, telomere length in leukemic cells was very short (Figure 1). Because age at diagnosis was not associated with telomere length in leukemic cells (P=0.35) (Figure 1), patients were categorized into quartiles based on telomere length without age-adjustment (Table 1). There was no significant difference in distribution of sex (P=0.61) or WBC count among the quartiles (P=0.15). When evaluated as a continuous variable, there was no significant difference in telomere length among FAB types (P=0.068) (Figure 2A), nor among the AML characteristic cytogenetic subgroups of *MLL*-rearrangements, AML-ETO translocation or inversion of chromosome 16, PML-RARα translocation, normal karyotype, or other cytogenetic aberrations (P=0.15) (Figure 2B). Next, the correlation was assessed between telomere length and the prognostic molecular subgroups of *CEBPA* double mutations and *NPM1* mutations; both favorable aberrations, and *WT1* mutations, internal tandem duplication of *FLT3* (*FLT3*/ITD) or the combination of both, the latter three unfavorable aberrations.

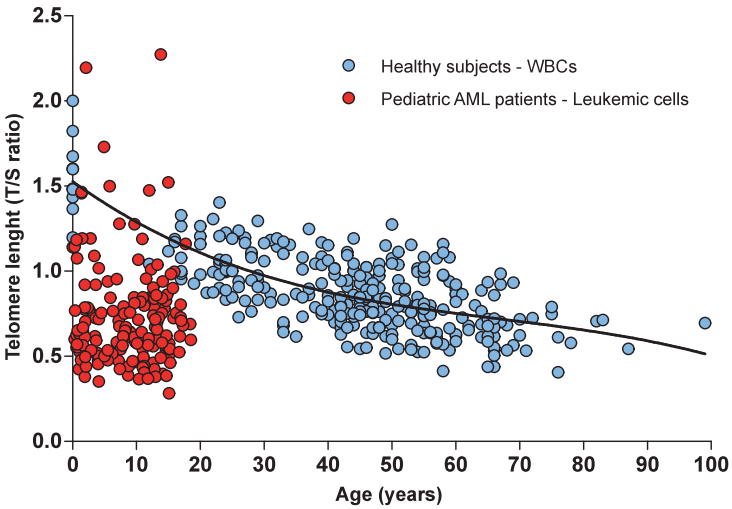

Figure 1 Leukemic cell and leukocyte telomere length as a function of age.

Telomere length, expressed as telomere to single copy gene ratio (T/S), was determined in leukemic cells derived from 167 pediatric AML patients at diagnosis and total leukocytes derived from healthy blood bank donors, and expressed as a function of age (years). Telomere length in leukemic cells is not associated with age (slope -0.0043 ± 0.0045, r^2 0.005, P =0.349), whereas telomere length in total leukocytes decreases as a function of age.

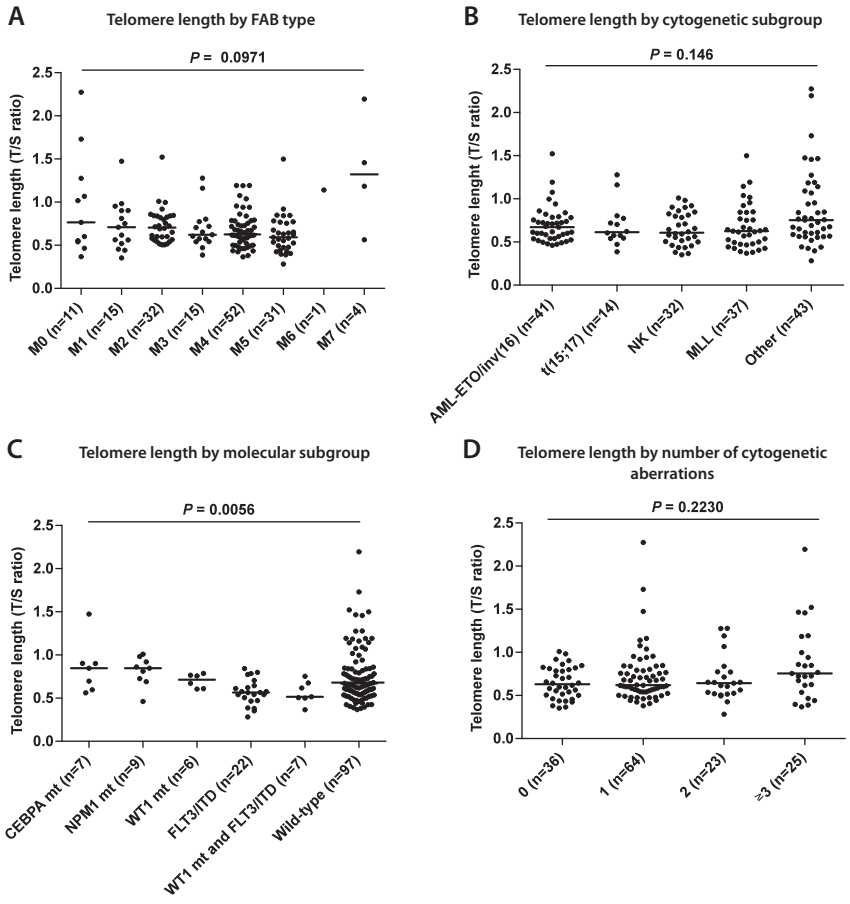

Figure 2 Telomere length in biological subgroups.

(A) Telomere length by FAB type. Telomere length was not associated with FAB type (median T/S ratio M0: 0.77; M1: 0.71; M2 0.71; M3: 0.62: M4: 0.63; M5: 0.59; M6: 1.14; M7: 1.32; median T/S ratio of complete cohort: 0.65). FAB M1-2 (n=1; T/S ratio 0.38) and unknown FAB types (n=5; T/S ratio 1.09) are not shown. (B) Telomere length by cytogenetic subgroup. Telomere length was not associated with cytogenetic subgroup (median T/S ratio *MLL* rearranged cases: 0.63; AML-ETO/ inv(16): 0.67; t(15;17): 0.61; NK: 0.61; other: 0.75; median T/S ratio of complete cohort: 0.65). (C) Telomere length by molecular subgroup. Patients carrying more than one of the screened mutations are excluded; patients carrying both *FLT3/ITD* and a *WT1* mutation are only included in the *WT1* and *FLT3*/ITD group. Telomere length was associated with molecular subgroup (median T/S ratio *CEBPA* double mutants: 0.85; *NPM1* mutants: 0.85; *WT1* mutants: 0.71; *FLT3*/ITD: 0.57; *WT1* and *FLT3*/ITD: 0.52; wild-type (not carrying any of the former aberrations): 0.67). (D) Telomere length by number of cytogenetic aberrations. Nineteen patients, for whom complete karyotypes were not available, are excluded. Telomere length was not associated with the number of cytogenetic aberrations (median T/S ratio 0 aberrations: 0.63; 1 aberration: 0.62; 2 aberrations: 0.64; ≥3 aberrations: 0.76).

When telomere length was evaluated as a continuous variable, telomere lengths between leukemic cells carrying molecular aberrations differed significantly (*P*=0.0056). Specifically, cells carrying *FLT3*/ITD (median T/S ratio 0.57; range, 0.28-0.84) or the combination of a *WT1* mutation and *FLT3*/ITD (median T/S ratio 0.52; range, 0.37-0.75), had shorter telomeres than did *CEBPA* double mutants (median T/S ratio 0.85; range, 0.56-1.5; *P*=0.0087 and *P*=0.026, respectively) and *NPM1* mutants (median T/S ratio 0.85; range, 0.46-1.0; *P*=0.0022 and *P*=0.012, respectively) (Figure 2C). Cells carrying a combination of *FLT3*/ITD and an *NPM1* mutation had telomere lengths similar to cells carrying only *FLT3*/ITD (n=4; median T/S ratio 0.50; range, 0.43-0.66) (not shown). There was no relation between telomere length and number of cytogenetic aberrations (numerical and/or structural), including complex karyotype, defined as three or more cytogenetic aberrations[17] (*P*=0.32) (Figure 2D). In all but two patients with three or more cytogenetic abnormalities, at least one structural abnormality was present.

Prognostic relevance of telomere length in pediatric AML

To assess the influence of telomere length on outcome, telomere length quartiles 2 through 4 were grouped, and outcome was compared with telomere length quartile 1. Five-year pOS in quartile 1 versus quartiles 2-4 (50±8% versus 61±5%, *P*=0.16), pEFS (40±8% versus 44±5%, *P*=0.192), and cumulative incidence of relapse and non-response (50±8% versus 44±5%, P_{Gray}=0.25) were not significantly different (Figure 3).

Gene expression profiling

To gain insight into mechanisms that might contribute to the differences in telomere length in AML blast cells, expression levels, as determined by microarray analysis, of telomere biology-related genes were correlated with telomere length. Expression levels of TERF1, TERF2, TINF2, and POT1, which are components of the shelterin complex,[33] protecting telomeres from recognition as DNA double stranded breaks; WDR79 or TCAB1, facilitating trafficking of telomerase to Cajal bodies;[34] and TERT, DKC1, NHP2, NOP10, and GAR1, components of the telomerase complex,[9] did not correlate with telomere length (Supplemental Table 4). Furthermore, supervised analysis of gene expression levels of 39 pediatric AML cases in telomere length quartile 1 versus 118 cases in quartiles 2-4 did not result in genes that were differentially expressed between the two groups.

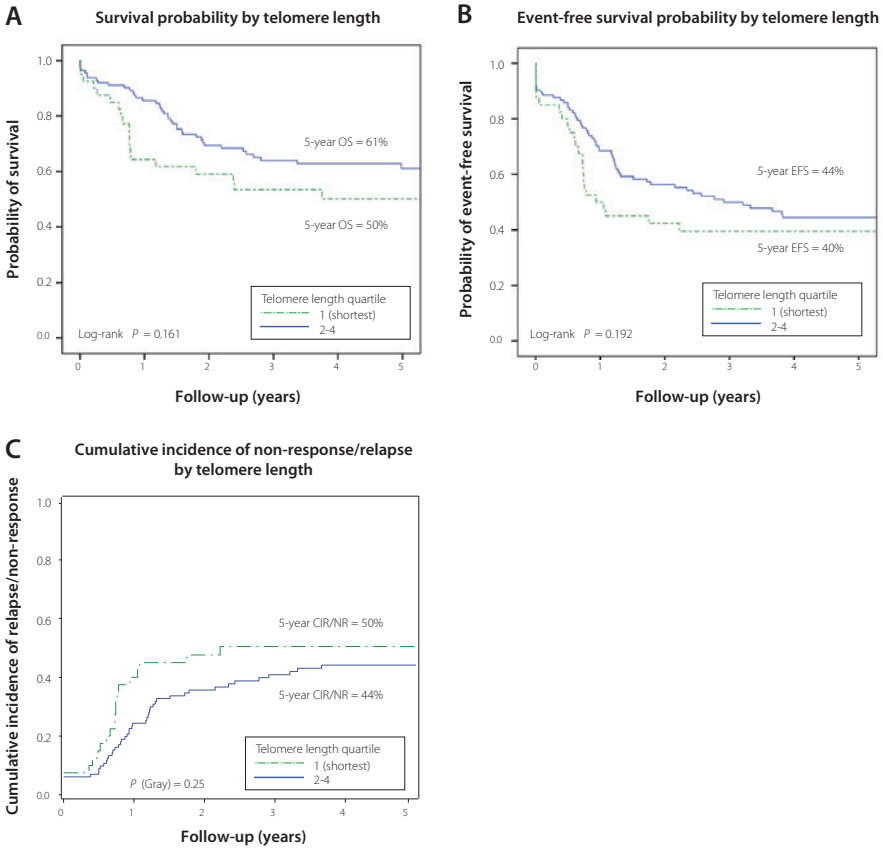

Figure 3 Telomere length and outcome.

(A) Overall survival probability by telomere length quartile 1 versus quartiles 2-4. (B) Event-free survival probability by telomere length quartile 1 versus quartiles 2-4. (C) Cumulative incidence of non-response/relapse by telomere length quartile 1 versus 2-4.

DISCUSSION

In this study, we report the frequency of mutations in the telomerase complex genes *TERT* and *TERC*, and correlate telomere length of leukemic cells with prognostic factors in childhood AML. We describe that mutations in *TERC* are absent, and that variants in *TERT*, the enzymatic component of the telomerase complex, occur at low frequency in pediatric AML. These data are in contrast to adult AML patients, in which constitutional *TERT* mutations appear to be a risk factor for AML.[8] Two unique variants that were found in the

pediatric cohort, E327D and T726M, were absent in healthy controls. However, the relationship of these variants to the etiology of AML is dubious, as the variants showed normal telomerase function when assayed *in vitro*, the karyotype was normal in both patients, and telomere lengths of leukemic cells were within the third quartile of the total cohort.

The most common *TERT* variant in our cohort, similar to the adult AML cohort, was A1062T, located in exon 15, in the E-I motif of the C-terminal extension of *TERT*. The frequency of this variant in adult AML (n=594) (minor allele frequency 0.019) was 3 times higher than in a control cohort (n=1110) (minor allele frequency 0.006) consisting of ethnicity and/or geographically matched healthy controls. Enzymatic activity of telomerase carrying A1062T was reported reduced to 60% of wild-type activity in one,[8] but normal in another report.[32] The variant has been described in aplastic anemia,[22] AML,[8,35] chronic lymphocytic leukemia,[35] diffuse large B-cell lymphoma,[35] liver cirrhosis,[36] but also in healthy individuals.[22,32,37] In our geographically matched healthy controls, A1062T was present at a carrier frequency of 0.049, which was similar to the carrier frequency of 0.048 in the pediatric AML cohort. The frequency of A1062T in Dutch blood bank donors is remarkably higher than the previously published allele frequencies, ranging from 0.006 to 0.007, in large cohorts consisting of controls of different geographic and ethnic backgrounds.[8,36] However, in 600 German controls, similar to the Dutch controls, a carrier frequency of 0.036 was described,[37] which is comparable with a carrier frequency of 0.031 in a 477 white controls from the NCI Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial.[36] This might suggest that in individuals of Caucasian descent, or within specific ethnic subpopulations, the A1062T variant may be less pathogenic than previously thought; and at least in our pediatric AML cohort, it cannot be regarded as a pathologic variant. That *TERT* A1062T seems to have different effects in different ethnic groups might be explained by the complex genomic architecture within ethnicities: gene variants differentially present can influence the effect of *TERT* A1062T. This might be in analogy with the clinical heterogeneity of sickle cell disease in different ethnic subpopulations, which is influenced by genetic modulators, the most powerful of which is fetal hemoglobin.[38]

To summarize, in this pediatric AML cohort, variants in the telomerase complex genes *TERT* and *TERC* do not seem to be a risk factor for developing AML. Clinically, loss-of-function mutations in *TERC* or *TERT* might increase the risk of chemotherapy-related toxicity and are relevant in selecting sibling donors for hematopoietic stem cell transplantation. However, given the results of this study, in the absence of classical features of dyskeratosis congenita, or a family history positive for bone marrow failure, liver cirrhosis, or pulmonary fibrosis, screening for mutations in *TERT* or *TERC* in sporadic AML cases does not seem to be relevant.

Apart from *TERT* and *TERC* seven other genes (*DKC1*, *NOP10*, *NHP2*, *TINF2*, *C16orf57*, *TCAB1* and *CTC1*)[39,40] have been implicated in dyskeratosis congenita pathogenesis, and could therefore hypothetically increase the risk of sporadic AML. We have limited our

analysis to *TERT* and *TERC*, and might therefore miss mutations in other genes leading to increased AML risk. However, together with *TINF2* and *DKC1*, *TERT* and *TERC* are the most frequently mutated genes in dyskeratosis congenita.[39]

Although mutations in the telomerase complex genes *TERT* and *TERC* are infrequent events in pediatric AML, we investigated the possibility whether short telomeres in leukemic cells might be associated with AML characteristics or contribute to an adverse outcome. We here confirm previous reports that telomeres in leukemic cells are very short.[14,15,41-43] Furthermore, we show that leukemic cells carrying the high-risk molecular aberrations *FLT3*/ITD and the combination of *FLT3*/ITD and a *WT1* mutation have significantly shorter telomeres than do *NPM1* or *CEBPA* double mutants. This association might reflect a more extended mitotic history of leukemic cells harboring *FLT3*/ITD as compared to cells carrying favorable prognostic aberrations. We did not observe a relation between leukemic cell telomere length and age, which might suggest that telomere shortening in leukemic cells is so extensive that the effect of age on telomere length of the leukemic cell of origin becomes negligible.

In contrast to studies in adult AML, in which patients with an aberrant karyotype had significantly shorter leukemic cell telomeres than did normal karyotype patients,[14,15] suggesting that short telomeres in AML might induce chromosomal instability, we did not find an association between telomere length and prognostic cytogenetic subgroups or number of cytogenetic aberrations. A limitation of our approach in investigating a possible relation between telomere length and cytogenetic abnormalities is that we studied average leukemic cell telomere length. Rather than the average telomere length, the shortest telomere per cell seems to determine chromosomal stability.[44] Future studies addressing the question whether telomere length is associated with specific chromosomal aberrations in AML might benefit from single telomere length analysis (STELA), in which telomere lengths and fusion events at specific chromosomes can be determined.[45]

In pre-malignant disorders such as Barrett esophagus[46] and ulcerative colitis,[47] short leukocyte or colonocyte telomeres, respectively, are associated with malignant transformation. In severe aplastic anemia, leukocyte telomere length is associated with relapse, malignant transformation, and survival,[24] while in B-cell chronic lymphocytic leukemia and chronic myeloid leukemia, short telomeres of leukemic cells correlate with unfavorable clinical outcome.[48-50] In contrast to these studies, but in accordance with a slightly smaller study in adult AML,[15] leukemic cell telomere length did not predict outcome in the present pediatric AML cohort.

We conclude that in pediatric AML, mutations in components of the telomerase complex are present in low frequency and do not seem to confer a risk for leukemia development. Short telomeres correlate with the high-risk molecular aberration *FLT3*/ITD, but survival is not significantly influenced by telomere length in this pediatric AML cohort.

Acknowledgements

AMA was supported by the KiKa Foundation, Amstelveen, The Netherlands, the René Vogels Foundation, Oirschot, and Erasmus Trustfonds, Rotterdam, The Netherlands. This research was supported in part by the NIH (NHLBI) Intramural Research Program.

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SUPPLEMENTAL INFORMATION

Supplemental Table 1 Comparison present study cohort with AML-BFM 98 cohort.

	Study cohort		AML-BFM 98 cohort[1]		*P*-value
Patients, No.	167		473		
Sex, No. (%)					
Male	92	(55.1)	253	(53.5)	0.72
Female	75	(44.9)	220	(46.5)	
Age, No. (%)					
Age < 10 y	86	(51.4)	259	(54.8)	0.47
Age > 10 y	81	(48.5)	214	(45.2)	
FAB classification, No. (%)					
M0	11	(6.6)	21	(4.4)	0.274
M1	15	(9.0)	68	(14.4)	0.074
M2	32	(19.2)	123	(26.0)	0.076
M3	15	(9.0)	30	(6.3)	0.251
M4	52	(31.1)	94	(19.9)	0.003
M5	31	(18.6)	97	(20.5)	0.589
M6	1	(0.6)	13	(2.7)	0.130
M7	4	(2.4)	25	(5.3)	0.123
Other/ unknown	6	(3.6)	1	(0.2)	0.002
WBC count, (%)					
< 100 x 10^9/L	104	(69.3)	378	(79.9)	0.007
> 100 x 10^9/L	46	(30.7)	95	(20.1)	
Unknown	17				
Karyotype, No. (%)					
Favorable[2]	55	(32.9)	120	(28.4)	0.282
Normal	32	(19.2)	100	(23.7)	0.234
Other[3]	80	(47.9)	202	(47.9)	0.994
Unknown			62		
Molecular aberration, No. (%)					
CEBPA double mt/wt	11/143	(7.1)	n/a		
NPM1 mt/wt	13/149	(8.0)	n/a		
WT1 mt/wt	15/146	(9.3)	n/a		
FLT3/ITD/wt	35/131	(21.1)	n/a		
FLT3/ITD and *WT1* mt/wt	7/154	(4.3)	n/a		

[1] Numbers as reported by Creutzig et al., JCO 2006

[2] Favorable karyotype: inv(16), t(8;21), t(15;17)

[3] Other karyotype includes 37 MLL rearranged cases

Supplemental Table 2 Single nucleotide polymorphisms in *TERT* in pediatric AML patients.

dbSNP rs#	Exon	Codon	mRNA NM_198253.2	Genomic	Proteomic	WT homo-zygote	WT hetero-zygote	Mutant homo-zygote	Minor allele frequency	Carrier frequency
rs61748181	2	279	893	GCC/ACC	A/T	159	9	0	0.027	0.054
rs2736098	2	305	973	GCG/GCA	A/A	89	63	16	0.283	0.470
rs33959226	4	604	1870	GCA/GCG	A/A	164	4	0	0.012	0.024
rs33956095	5	677	2089	GGC/GGT	G/G	167	1	0	0.003	0.006
rs33963617	5	699	2155	GCC/GCT	A/A	163	5	0	0.015	0.030
	5	703	2167	CCG/CCA	P/P	167	1	0	0.003	0.006
	5	709	2185	GTC/GTG	V/V	167	1	0	0.003	0.006
	6	716	2206	GCG/GCA	A/A	167	1	0	0.003	0.006
rs33954691	14	1013	3097	CAC/CAT	H/H	136	32	0	0.095	0.190
rs35033501	16	1108	3382	CCG/CCA	P/P	162	6	0	0.018	0.036

Abbreviation: WT, wild-type. Minor allele frequencies in healthy controls of dbSNP rs61748181, rs2736098, and rs33963617 were 0.032 (401 controls tested: 375 wild-type homozygote, 26 wild-type heterozygote, 0 mutant homozygote), 0.287 (401 controls tested: 206 wild-type homozygote, 160 wild-type heterozygote, 35 mutant homozygote), and 0.013 (399 controls tested: 389 wild-type homozygote, 10 wild-type heterozygote, 0 mutant homozygote), respectively, which is comparable with pediatric AML patients (P=0.75, 0.95, and 0.97, respectively, Chi-square test).

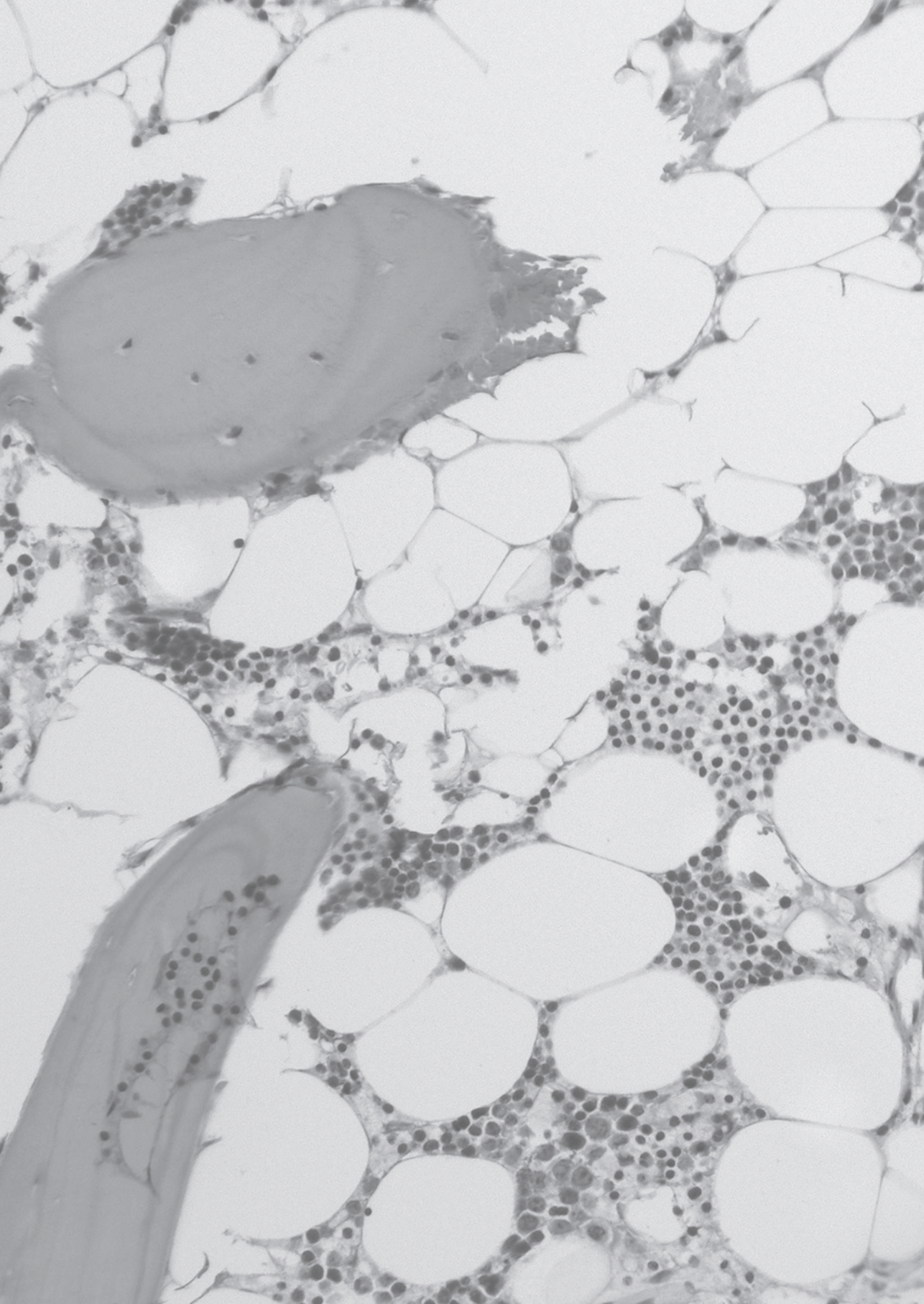
Supplemental Table 3 Clinical characteristics of pediatric AML patients carrying *TERT* variant A1062T.

ID	TERT variant	Sex	Age at Dx (y)	FAB	Karyotype	FISH	Follow up	Telomere length (T/S ratio)	Telomere length quartile
1311	A1062T*	M	0.4	M5	46, XY, der(3)t(3;11)(p21;q23), ins(3;11)(q23,?),der(11)	MLL-NRIP3	CR	0.77	3
1732	A1062T*	M	13.1	M2	45,X,-Y,der(8)t(8;17) (p22;q11) t(8;21)(q22;q22), der(17)t(8;17) (p22;q11), der(21)t(8;21) (q22;q22)[23]/ 46,XY[4]	AML-ETO	CR	0.86	4
3439	A1062T*	M	12	M1	45,X,-Y [PHA 46,XY]		Relapse 33 mo. after Dx; CR	1.47	4
4388	A1062T	M	11.4	M5	46,XY,del(11)(q23)[6]/46,XY[5]	MLL-AF6	Unknown	0.96	4
5056	A1062T	F	5.8	M5b	11q- (?)	MLL-AF6	Relapse 6 mo. after Dx, dead	1.5	4
5157	A1062T	M	10.6	M2	46, XY	AML-ETO	CR	0.82	3

Abbreviations: CR, complete response; Dx, diagnosis; mo., months. Symbols: *, germline origin of variant confirmed.

Supplemental Table 4 VSN-normalized expression levels of telomere length regulating or telomere-associated genes.

Gene	Probe	VSN-normalized expression, median (SD)										*P*-value
		All patients (n=157)		Telomere length quartile 1 (n=39)		Telomere length quartile 2 (n=38)		Telomere length quartile 3 (n=41)		Telomere length quartile 4 (n=39)		
TERT	1555271_a_at	7.260	(0.101)	7.242	(0.106)	7.273	(0.099)	7.250	(0.115)	7.270	(0.084)	0.68
TERT	207199_at	7.600	(0.127)	7.631	(0.124)	7.611	(0.097)	7.570	(0.134)	7.581	(0.147)	0.26
DKC1	201478_s_at	9.005	(0.402)	8.951	(0.447)	8.976	(0.392)	9.046	(0.361)	9.105	(0.412)	0.51
DKC1	201479_at	9.489	(0.442)	9.483	(0.453)	9.404	(0.422)	9.525	(0.426)	9.533	(0.472)	0.60
DKC1	216212_s_at	8.478	(0.234)	8.462	(0.249)	8.484	(0.232)	8.471	(0.183)	8.481	(0.274)	0.99
WDR79	220258_s_at	7.808	(0.138)	7.846	(0.137)	7.790	(0.159)	7.790	(0.114)	7.842	(0.14)	0.49
WDR79	44563_at	8.424	(0.185)	8.452	(0.168)	8.377	(0.21)	8.423	(0.17)	8.451	(0.193)	0.64
NHP2	209104_s_at	11.138	(0.472)	11.177	(0.434)	11.084	(0.416)	11.126	(0.471)	11.076	(0.561)	0.68
NHP2	216583_x_at	7.929	(0.231)	7.929	(0.188)	7.947	(0.2)	7.908	(0.273)	7.928	(0.253)	0.85
NOP10	217962_at	10.946	(0.478)	10.919	(0.557)	10.907	(0.417)	10.908	(0.461)	10.979	(0.468)	0.47
GAR1	219110_at	9.388	(0.426)	9.280	(0.412)	9.274	(0.378)	9.425	(0.336)	9.513	(0.539)	0.08
TERF1	203448_s_at	8.473	(0.353)	8.468	(0.372)	8.518	(0.36)	8.453	(0.306)	8.424	(0.382)	0.97
TERF1	203449_s_at	7.860	(0.319)	7.803	(0.322)	7.854	(0.323)	7.868	(0.262)	7.896	(0.362)	0.32
TERF2	203611_at	8.149	(0.423)	8.113	(0.429)	8.008	(0.4)	8.149	(0.434)	8.211	(0.428)	0.35
TERF2	229790_at	7.627	(0.261)	7.724	(0.243)	7.650	(0.262)	7.613	(0.289)	7.573	(0.249)	0.29
TINF2	220052_s_at	8.289	(0.297)	8.390	(0.295)	8.262	(0.303)	8.305	(0.293)	8.289	(0.295)	0.20
TINF2	223776_x_at	8.897	(0.32)	8.970	(0.314)	8.891	(0.358)	8.848	(0.295)	8.897	(0.301)	0.20
TINF2	224809_x_at	9.173	(0.331)	9.288	(0.312)	9.113	(0.362)	9.161	(0.328)	9.263	(0.309)	0.12
POT1	204353_s_at	7.393	(0.237)	7.354	(0.25)	7.337	(0.259)	7.410	(0.217)	7.429	(0.228)	0.70
POT1	204354_at	7.829	(0.384)	7.881	(0.39)	7.633	(0.384)	7.932	(0.383)	7.862	(0.369)	0.14



4

Human telomere disease due to disruption of the CCAAT box of the *TERC* promoter

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Blood **2012; 119 (13): 3060-3**

ABSTRACT

Mutations in the coding region of telomerase complex genes can result in accelerated telomere attrition and human disease. Manifestations of telomere disease include the bone marrow failure syndromes dyskeratosis congenita and aplastic anemia, acute myeloid leukemia, liver cirrhosis, and pulmonary fibrosis. Here we describe a mutation in the CCAAT box (GCAAT) of the *TERC* gene promoter in a family in which multiple members had typical features of telomeropathy. The genetic alteration in this critical regulatory sequence resulted in reduced reporter gene activity and absent binding of transcription factor NF-Y, likely responsible for reduced TERC levels, decreased telomerase activity, and short telomeres. This is the first description of a pathogenic mutation in the highly conserved CCAAT box, and the first instance of a mutation in the promoter region of *TERC* producing a telomeropathy. We propose that current mutation screening strategies include gene promoter regions for the diagnosis of telomere diseases.

INTRODUCTION

Telomeres, the structures capping the ends of linear chromosomes, consist in vertebrates of hundreds to thousands of TTAGGG repeats. To prevent critical telomere shortening, highly proliferative cells express telomerase (encoded by *TERT*), a reverse transcriptase that adds TTAGGG repeats to telomeres, using TERC as its RNA template. Constitutional loss-of-function mutations in telomerase complex genes result in deficient telomere maintenance and accelerated telomere attrition. Telomere disease in humans manifests clinically as a spectrum of the bone marrow (BM) failure syndromes dyskeratosis congenita (DC) and aplastic anemia (AA), acute myeloid leukemia, cirrhosis, and pulmonary fibrosis.[1] Sequencing strategies for the diagnosis of telomere disease are based on screening exons and their flanking regions of telomerase genes for mutations. Pathogenic mutations in the promoter region of *TERT* or *TERC* have not been firmly established. Here, we report a mutation in the CCAAT box of the *TERC* promoter region, leading to telomere disease.

METHODS

Patients and controls

Diagnosis of AA was performed as previously described.[2] Blood samples were collected after written informed consent, according to protocols approved by the IRB of the National Heart, Lung, and Blood Institute, protocol 04-H-0012 (www.ClinicalTrials.gov identifier: NCT00071045).

Sequence analysis and telomere length measurement

A *TERC* promoter/gene region (-1661 through + 502; +1 is defined as the transcriptional start site of *TERC*) was amplified by polymerase chain reaction (PCR) on leukocyte-genomic DNA (10 ng), followed by bi-directional sequencing. A total of 378 individuals served as controls. Telomere length was measured as described previously.[3,4]

Gel shift assay

The gel shift assay was performed using the LightShift Chemiluminescent EMSA kit (Pierce) with unlabeled (competitors) or 5'biotin end-labeled (probes) oligonucleotide duplexes (wild-type or mutant), covering the hTERC-CCAAT box and adjacent regions (nucleotide -63 through -39). Anti-NF-YA antibody (Rockland Immunochemicals) was used for supershift assay. DNA-protein complexes were analyzed by polyacrylamide gel electrophoresis and the Chemiluminescent Nucleic Acid Detection Module (Pierce).

Plasmid construction, transfection, and luciferase activity assays

Plasmids carrying wild-type or mutant *TERC* promoter regions (starting at positions -798, -436, -272, -107, and -42; ending at a position +62) were constructed based on the pGL4.18[luc2P/Neo] luciferase vector (Promega). After plasmid transfection into HEK293T or HeLa cells ($2.5x10^5$), luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega).

RT-PCR for TERC expression

TERC expression levels were determined on total RNA isolated from peripheral blood mononuclear cells (PBMCs) or skin fibroblasts using the RNeasy Mini Kit (Qiagen). RT-PCR for actin and TERC was performed using the OneStep RT-PCR Kit (Qiagen) and custom made primers and probes.

RESULTS AND DISCUSSION

Telomere disease was suspected in our 39-year old Caucasian index patient (III-3) who was diagnosed with AA and had a family history of AA and leukemia (Figure 1A). Her peripheral blood leukocyte telomere length was 5.4 kb, which is very short in comparison to age-matched healthy controls (Figure 1B). Her sister (III-2), who had normal blood cell counts but a severely hypocellular BM (Figure 1C), and her nephew (IV-1), diagnosed with AA at age 11, also had very short leukocyte telomeres, but an unaffected nephew (IV-2) had normal telomere length (Figure 1B). Neither the proband, her sister, or affected nephew showed classic muco-cutaneous stigmata of DC. No mutations were found in the coding region of *TERC*, *TERT,* or exon 6a of *TINF2*. However, the proband, her sister, and her affected nephew, but not the unaffected nephew, carried a heterozygous mutation in the CCAAT box positioned -58 to -54 (CCAAT>GCAAT) of the *TERC* core promoter region. The mutation was absent in 378 healthy subjects of various ethnic backgrounds. A -714 C insertion, present in 16.7% of healthy controls,[5] was found in the index patient, her sister, and her affected nephew on the same allele as the -58C>G. The remaining upstream region of the *TERC* promoter was wild-type to base -1661.

The CCAAT box is frequently present in promoter regions of RNA polymerase II-transcribed genes, located preferentially -60 to -100 nucleotides from the transcriptional start site.[6] NF-Y, a nuclear protein composed of NF-YA, NF-YB, and NF-YC, binds with high affinity to the CCAAT box.[7] Binding of NF-Y to the CCAAT box of the *TERC* promoter region is crucial for *TERC* promoter activity.[8,9] In humans, a few CCAAT box mutations have been reported but none clearly causing disease. In hereditary persistence of fetal hemoglobin, a benign condition that benefits sickle cell anemia and β-thalassemia patients, mutations in the CCAAT boxes of the *HBG1* and *HBG2* promoters have been reported.[10-13] In β-thalassemia intermedia, a mutation in the CCAAT box of the *HBB* promoter was

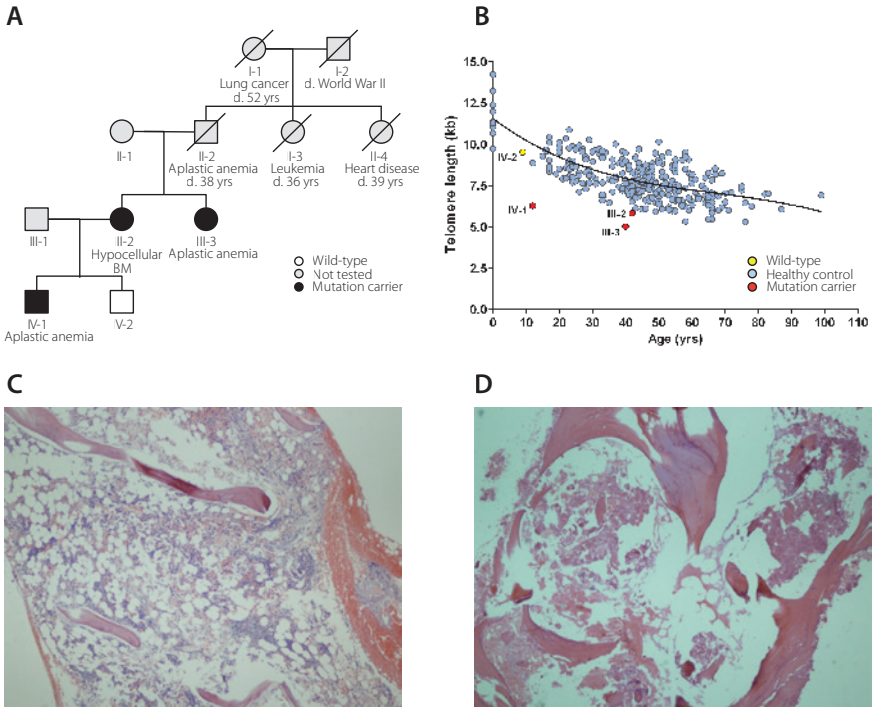

Figure 1 Pedigree, telomere length, and BM histology.

(A) Pedigree of proband (III-3; indicated by an arrow). Individuals II-2 and II-3 are suspected mutation carriers. Slashed symbols indicate deceased (d.) individuals. Neither the proband, her sister, or affected nephew showed abnormal pigmentation of the skin, nail dystrophy, or oral leukoplakia, nor was there evidence of pulmonary or immunological problems, growth retardation, developmental delay, or microcephaly. During cholecystectomy, an enlarged liver was noticed in III-2, however, this finding was not further evaluated after surgery. (B) Blood leukocyte telomere length (kb) as a function of age. Mutation carriers have very short telomeres in peripheral blood leukocytes. The curve marks the 50th percentile of telomere length for control subjects derived from 298 healthy NIH blood bank donors. A sample's telomere length was expressed as a telomere to single copy gene (T/S) ratio which was converted to kilobases (kb). BM biopsy showing profound hypocellularity in the proband (III-3) (C), and hypocellular BM and eosinophilic ground substance in her sister (III-2) (D).

described, but no meaningful functional data supported the hypothesis that this mutation was pathogenic.[14] A *TERC* promoter mutation (-99C>G) was present in a patient with paroxysmal nocturnal hemoglobinuria (PNH)[15] and with myelodysplastic syndrome (MDS).[16] Telomere lengths in these patients were not reported, and functional analyses were inconclusive. The PNH and MDS phenotypes were not explained by this mutation.

To elucidate the effect of the -58C>G mutation in the CCAAT box on *TERC* promoter function, we performed gel shift and reporter gene assays, and determined TERC expression in primary cells. The gel shift assay showed a shifted band of wild-type biotinylated probe, spanning bases -63 through -39 of the *TERC* promoter region; addition of anti-NF-YA antibody resulted in supershift of the band. Mutant probe did not bind to NF-Y (Figure 2A). Whereas wild-type unlabeled oligonucleotide out-competed binding of wild-type probe to NF-Y, mutant unlabeled oligonucleotide did not (Figure 2B). To investigate the effect of loss of NF-Y binding to the mutant box on *TERC* promoter activity, we generated wild-type and mutant *TERC* promoter-luciferase reporter plasmids of different lengths and transiently transfected HEK293T cells with these constructs. The relative luciferase activity of mutant constructs was decreased 3.5- to 9-fold, as compared to the wild-type construct (Figure 2C). Transfection of HeLa cells resulted in comparable patterns (Figure 2D). Co-transfection of wild-type and mutant constructs in a 1:1 ratio in HEK293T cells reduced luciferase activity by about 2-fold as compared to the wild-type construct alone (data not shown). TERC expression was determined by RT-PCR in PBMCs and was lower in affected individuals III-2 and IV-1 as compared to unaffected individual IV-2 (TERC/actin relative expression +/- SEM: 0.00536+/-0.00130, 0.619+/-0.107, 1.00+/-0.232, respectively). TERC expression also was lower in skin fibroblasts from the proband in comparison to an unrelated healthy subject (0.781+/-0.0517, 1.00+/-0.0402, respectively). However, these results should be cautiously interpreted, as TERC expression is highly variable within individuals.[17]

In conclusion, we show absent binding of NF-Y and reduced reporter gene activity with a mutant CCAAT box (GCAAT) in vitro, suggesting that the CCAAT-box disruption may lead to reduced TERC levels, lower telomerase activity, short telomeres, and human telomere disease. We believe that the terms "telomere disease" or "telomeropathy" are more adequate than dyskeratosis congenita to describe the phenotype observed in this family. First, these terms are descriptive of the underlying molecular defect. Second, telomeropathies are a large spectrum of phenotypes, from no clinical manifestations, to macrocytosis, aplastic anemia, pulmonary fibrosis, to the more severe phenotype in infancy Revesz syndrome, all with different prognoses. We prefer to use the term dyskeratosis congenita for patients with the classical clinical presentation and not for all subjects with a telomerase mutation.

We provide the first example of a *TERC* promoter mutation producing a telomeropathy and the first instance of a mutation in a CCAAT box etiologic in human disease. Mutation-screening strategies for the diagnosis of telomere diseases should include promoter regions of major genes related to telomere biology. Similar strategies may also be helpful for marrow failure syndromes such as Diamond-Blackfan anemia or Shwachman-Diamond syndrome, as with current approaches only in around half of inherited marrow failure cases a mutation can be identified.[18]

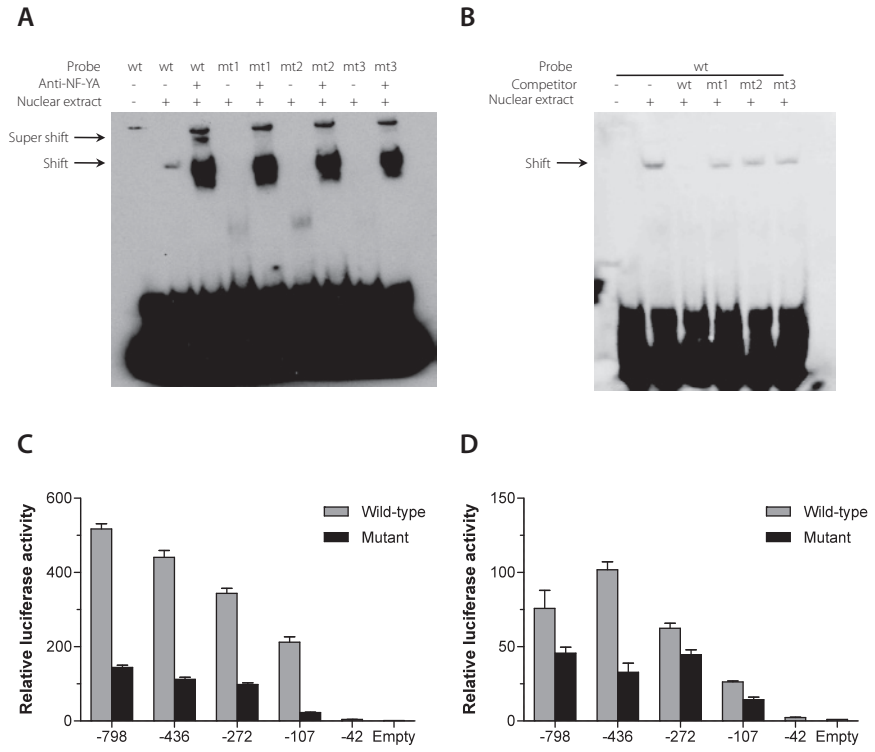

Figure 2 Functional analysis of wild-type and mutant CCAAT boxes of the *TERC* promoter.

(A) Gel shift and supershift assays. Gel shift assay was performed using HeLa nuclear extract with wild-type probe (wt, 5'-Bio/cttggccaatccgtgcggtcgg-3'), a mutant probe (mt1, 5'-Bio/cttgg**g**caatccgtgcggtcgg-3'), and additional mutant control probes (mt2, 5'-Bio/cttgg**agtc**tccgtgcggtcgg-3'; mt3, 5'-Bio/cttggcca**t**tccgtgcggtcgg-3'): bold and underlined letters indicate mutated nucleotides. Anti-NF-YA antibody was used for supershift assay. Arrows show bands which are shifted and supershifted with the wild-type probe, but not with the mt1 probe (-58C>G) and additional mutant control probes (mt2 and mt3). (B) 200-fold molar excess of unlabeled mutant competitor (mt1, mt2, or mt3) does not compete with wild-type binding, whereas wild-type competitor does (indicated with an arrow). Mutant promoter activity in HEK293T (C) or HeLa cells (D) is reduced as compared to wild-type in reporter gene assays. Depicted on the x-axis is the 5'position from the transcriptional start site of *TERC* for each *TERC* promoter-luciferase construct; each construct ends at nucleotide +69. Luciferase activity is reported as relative fold increase, compared with the empty vector pGL4.18[luc2P/Neo] (given an arbitrary value of 1), normalized to protein concentration. Results shown are means of three (HEK293T) or one (HeLa) independent experiment(s) performed in duplicate. Error bars indicate standard error of the mean. Detailed methods including sequences of primers and probes used in this study will be provided upon request.

Acknowledgements

The authors thank Mr. JE Decker and Dr. T Winkler for kindly providing TERC expression data in fibroblasts. This research was supported in part by the NIH (NHLBI) Intramural Research Program. AMA was supported by the KiKa Foundation, Amstelveen, The Netherlands, and the René Vogels Foundation, Oirschot, The Netherlands.

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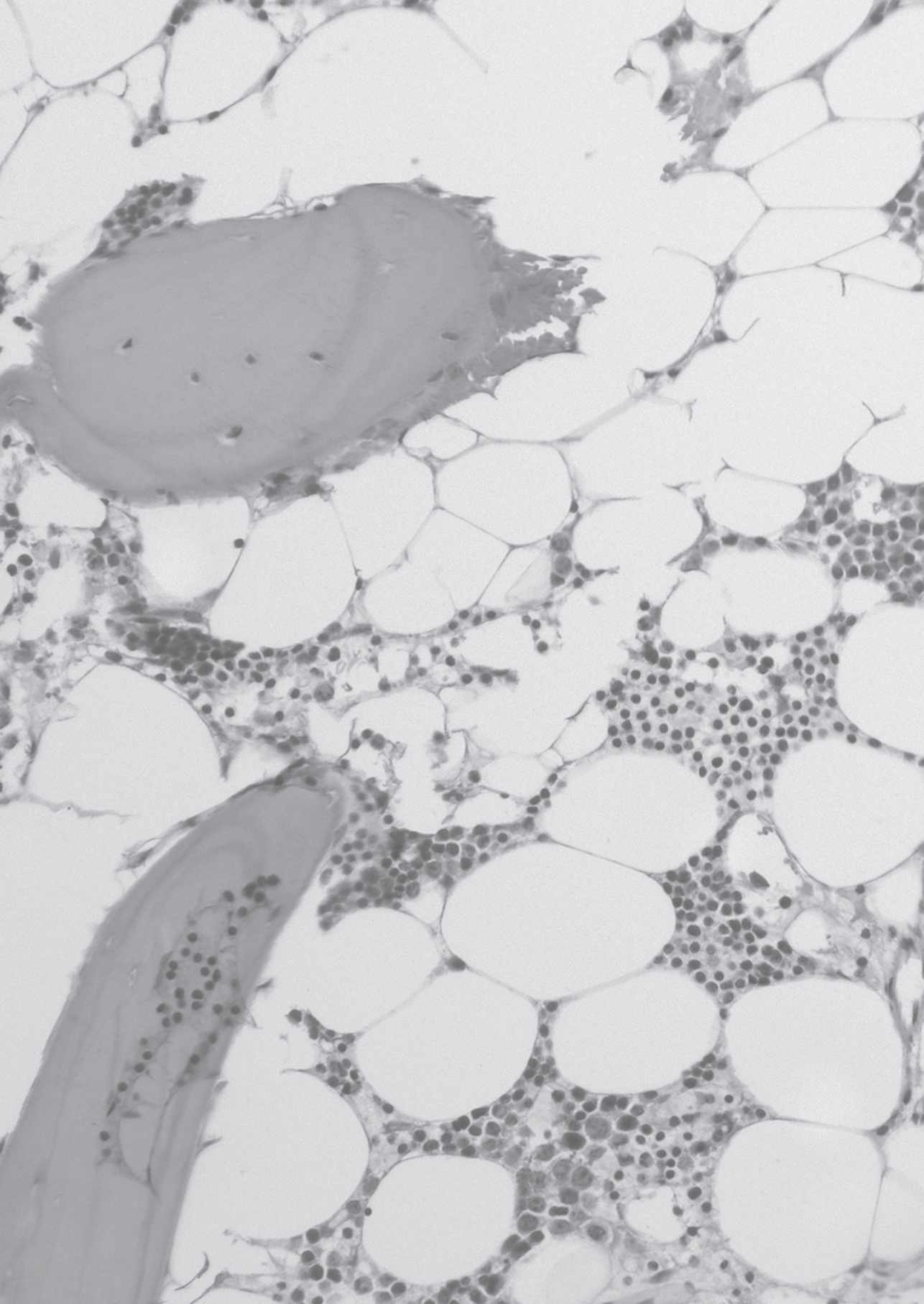

5

The clinical relevance of minor paroxysmal nocturnal hemoglobinuria clones in refractory cytopenia of childhood - a prospective study by EWOG-MDS

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***Leukemia* 2014; 28 (1): 189-92**

ABSTRACT

Minor paroxysmal nocturnal hemoglobinuria (PNH) clones are frequently present in adults with myelodysplastic syndrome (MDS) and are predictive of response to immunosuppressive therapy (IST) in some studies. We recently reported that in refractory cytopenia of childhood (RCC), the most common subtype of childhood MDS, IST might be effective. Data on the frequency and clinical correlates of PNH clones in RCC are lacking, and the value of the presence of a PNH clone as response predictor to IST in RCC is unknown. In this prospective study of the European Working Group of MDS in Childhood (EWOG-MDS), we detected PNH clones (median sizes: 0.06% (range, 0.01-58%) in erythrocytes and 0.9% (range, 0.03-86%) in granulocytes) in 36 of 87 RCC patients (41%) at diagnosis. PNH positive patients were significantly older, had lower blood counts, and were more often HLA-DR15 positive than PNH negative patients. Hypocellular RCC patients with a PNH clone >0.1% were more likely to respond to IST than PNH negative patients (88% versus 40% of patients responded at six months, respectively, P=0.038). We conclude that PNH clones are frequently present in RCC, predict response to IST, and might indicate an immune-mediated pathophysiology in at least a subset of RCC patients.

INTRODUCTION

Myelodysplastic syndrome (MDS) is characterized by ineffective hematopoiesis, resulting in peripheral blood cytopenias, myeloid dysplasia, and risk of leukemic transformation.[1] MDS in childhood is rare and has an estimated annual incidence of about 0.8 to 1.8 per million children aged 0 to 14 years.[2-4] Refractory cytopenia of childhood (RCC), defined as myelodysplasia without an increased blast count,[5] is the most common subtype of childhood MDS, accounting for about half of all cases.[2,6] Karyotype is normal in the majority of patients with RCC, and, in contrast to adults with MDS-refractory anemia (RA), about 80% of children have a hypocellular bone marrow.[1] Although the exact pathophysiology of MDS remains unclear, immunosuppressive therapy (IST), consisting of antithymocytic globulin (ATG) and/or cyclosporine A (CsA), is effective in some adults with MDS,[7-17] suggesting a T-cell mediated immune response directed against hematopoietic progenitor cells in a proportion of the patients.[18] A pilot study conducted by the European Working Group of MDS in Childhood (EWOG-MDS) showed that treatment with CsA and ATG might also be effective in RCC.[19] However, predictors of response to IST in RCC are yet unknown. In some,[20-22] but not all,[16] studies conducted in adult MDS patients, a predictor of good response to IST is the presence of a minor paroxysmal nocturnal hemoglobinuria (PNH) clone.

PNH is an acquired hematologic disorder caused by clonal expansion of hematopoietic stem cells (HSCs) carrying a loss of function mutation in the X-chromosomal phosphatidylinositol glycan A (*PIG-A*) gene, required for the synthesis of the glycosylphosphatidylinositol (GPI) anchor. Classic PNH is characterized by the presence of large GPI-deficient clones, intravascular hemolysis, resulting from an increased sensitivity of erythrocytes to complement mediated lysis due to absence of the GPI-anchored proteins CD55 and CD59, and by an increased risk of thrombosis.[23]

Minor GPI-deficient clones are present in 20 to 68% of adult and pediatric patients with the immune-mediated bone marrow failure syndrome aplastic anemia,[20,24-29] and in 13 to 23% of adults with low-grade MDS.[20,22,28,30,31] The mechanisms by which PNH clones arise in immune-mediated bone marrow failure syndromes are incompletely understood. It is hypothesized that GPI-deficient cells have a conditional growth advantage by evading an immune attack directed against normal HSCs,[18] or that, next to the *PIG-A* mutation, they gain a second mutation, conferring the GPI-deficient clone a selective advantage over normal HSCs.[32] Another recent hypothesis is that GPI-deficient cells, which can be detected at very low numbers in healthy individuals, can expand without a conditional or selective advantage, especially in conditions with reduced stem cell numbers. A mathematical model accurately predicted the known incidence of PNH disease.[33]

Due to the rarity of the disease, no data are available on the frequency and clinical correlates of PNH clones in RCC. In a prospective multicenter EWOG-MDS study, we assessed the frequency of PNH clones with a high-sensitivity flow cytometry assay in a

large cohort of RCC patients, and correlated the presence of PNH clones with clinical characteristics and response to IST.

METHODS

Patients

Peripheral blood samples for PNH analysis by flow cytometry were obtained from 87 primary RCC patients, ≤ 18 years of age, who were not previously treated with IST. Included patients were consecutive, diagnosed between June 2005 and December 2011, and enrolled in the prospective, multi-center studies EWOG-MDS 2006 and EWOG-MDS RC06 (ClinicalTrial.gov identifiers: NCT00662090 and NCT00499070). Institutional review boards of participating institutions approved the studies and patients and/or parents or legal guardians of patients provided written informed consent for study participation in accordance with local law and regulations. RCC was diagnosed according to WHO criteria[6] and confirmed by central review of bone marrow morphology and histology in participating national study centers by reference pathologists of EWOG-MDS. Fanconi anemia was excluded by chromosome fragility testing in all but 9 patients not tested. Data were reported to the coordinating study center of the EWOG-MDS study group through standardized data collection forms.

Detection of GPI-deficient cells

Peripheral blood was collected in heparinized tubes, and analyzed within 48 hours from collection at the immunodiagnostic laboratory of the Department of Immunology, Rotterdam, the Netherlands. The laboratory organizes and participates in quality assurance rounds for PNH diagnostics by the Dutch Foundation for Quality Assessment in Clinical Laboratories (SKML). For detection of GPI-deficient granulocytes, 100 µL whole blood was lysed with ammoniumchloride and stained for 15 minutes at room temperature (RT) with FLAER-AF 488 (Protox Biotech, Victoria, BC, Canada), CD24-PE (ML5, BD Biosciences, Erembodegem, Belgium), CD45-PerCP (2D1, BD Biosciences), CD33-APC (P67.6, BD Biosciences), and CD14-APC-H7 (MO-P9, BD Biosciences). For detection of GPI-deficient erythrocytes, 5 µL whole blood was diluted 1:10 and stained for 15 minutes at RT with CD59-FITC (NAM172-2B5, IQProducts, Groningen, the Netherlands) and CD55-PE (NAM16-4D3, IQProducts). Data were acquired on a FACSCanto II flow cytometer (BD Biosciences). For erythrocytes, 500,000 events were acquired; for granulocytes, a minimum of 15,000 events was acquired. Flow cytometry data were analyzed with BD FACSDiva Software, version 6.1.2 (BD Biosciences). GPI-deficient granulocytes and erythrocytes were defined as $CD24^{-}FLAER^{-}$ and $CD55^{-}CD59^{-}$ cells, respectively. Based on 38 control subjects (median age: 7 years; range: 2-86) without a diagnosis of bone marrow failure or MDS, GPI-deficient populations larger than 0.01% in the erythroid lineage and larger than 0.03%

in the granulocytic lineage were considered abnormal (determined as mean +3 SD). Patients were defined as PNH positive when a GPI-deficient population larger than 0.01% in the erythroid and/or a population larger than 0.03% in the granulocytic lineage were present. Due to severe neutropenia, the minimum number of 15,000 events in the granulocytic lineage could not be acquired in 34 patients, thereby limiting the sensitivity of PNH detection in these patients. Results for monocytes are not reported because too few events could be acquired in the majority of patients for sufficiently sensitive PNH detection.

HLA typing

Patients were HLA-typed by serological or molecular methods for human leukocyte antigen (HLA)-A, -B, -C, -DR and -DQ in participating study centers.

Immunosuppressive therapy

RCC patients included for PNH analysis were either followed with a watch-and-wait strategy, or were treated with IST, or received a hematopoietic stem cell transplantation (HSCT), based on a treatment algorithm according to EWOG-MDS RC06 as summarized in Figure 1. In particular, RCC patients with a hypocellular bone marrow, normal karyotype or karyotype other than monosomy 7, del(7q), or complex karyotype, and transfusion dependency or absolute neutrophil count (ANC) below $1x10^9$/L were eligible for HSCT from an HLA-matched donor after a reduced-toxicity regimen or for IST. IST consisted of horse-ATG (Lymphoglobulin, Sangstat/Genzyme) until 2007 (0.75 ml/kg/day for 8 days); rabbit-ATG (Thymoglobulin, Sangstat/Genzyme or ATG-Fresenius, Fresenius Biotech) from 2007 (3.75 mg/kg/day for 5 days), prednisolone (1-2 mg/kg/day for 14 days, tapered down from day 14 and discontinued on day 28), CsA (5 mg/kg/day with subsequent dose adjustment to maintain blood levels of 100-150 ng/mL by monoclonal assay or 200-400 ng/mL by polyclonal assay, until day 180 with subsequent slow dose tapering), and granulocyte colony-stimulating factor (5µg/kg/day until day 28 in case of ANC below $0.5x10^9$/L).

Response to IST was evaluated on day 180. Complete response was defined as transfusion independency, hemoglobin level within the age-adjusted normal range, platelet count above 150×10^9/L, and ANC above 1.5×10^9/L; partial response was defined as transfusion independency, platelet count above 20×10^9/L, and ANC above 0.5×10^9/L; no response was defined when neither the partial nor the complete response criteria were fulfilled.

Statistical analyses

Statistical analyses were performed with SPSS 17 and 20 (IBM, Chicago, IL, USA). Categorical variables were compared using the Chi-square test or Fisher's exact test. Continuous variables were compared using the Mann-Whitney-U test or the Kruskal-Wallis test when more than two groups were compared. The life-table calculations were performed

according to the Kaplan-Meier method. Event-free survival was calculated with respect to the following events: non-response by day 180, loss of response, clonal evolution, death, second course of IST, and stem cell transplantation at any time point. All results were expressed as the estimated probability of survival with standard error (SE). The two-sided log-rank test was used to compare Kaplan-Meier curves in different subgroups. All reported *P*-values are two-sided and were considered statistically significant when <0.05; *P*-values >0.1 were reported as non-significant (NS), whereas those between 0.05 and 0.1 were reported in detail.

RESULTS

Patient characteristics

A total of 87 treatment-naive pediatric RCC patients (44 male, 43 female), with a median age at diagnosis of 10.2 years (range: 1-18 years), were analyzed by high sensitivity flow cytometry for the presence of PNH clones. Median time from diagnosis to PNH measurement was 1.3 months (range: 0.6 months pre-diagnosis to 49 months post-diagnosis). Hypocellular bone marrow was reported in 73 patients (84%), which is comparable to the frequency of 81% that was previously reported in primary RCC patients in an interim analysis of studies EWOG-MDS 1998 and 2006.[1] Conventional cytogenetics displayed a normal karyotype in 63 cases (85%), which is slightly higher than the frequency of 77% in the interim analysis of EWOG-MDS 1998 and 2006; monosomy 7 in 5 cases (including 1 patient with monosomy 7 and additional aberrations) (7%), and other aberrations in 6 patients (trisomy 8 in 2 patients, rare aberrations in 3 patients, and del(7q) in 1 patient) (8%). In 13 patients, no karyotype was obtained due to insufficient metaphases. In 17 RCC patients a watch-and-wait strategy was applied; 40 RCC patients received a HSCT, and 30 RCC patients were treated with IST. Clinical characteristics are summarized in Table 1.

Minor PNH clones are frequently present in RCC

PNH clones in the erythroid and/or granulocytic lineages were present in 36 of 87 RCC patients (41%). Clone size ranged from 0.011 to 58% (median: 0.057%) in the erythroid lineage, and from 0.03 to 86% (median: 0.93%) in the granulocytic lineage. Clinical symptoms of PNH (hemolysis) were present in one RCC patient, who was 16 years of age at diagnosis. PNH clone sizes in this patient were 58% in the erythroid lineage, and 86% in the granulocytic lineage. The patient received a HSCT three months after diagnosis.

Of the 53 patients in whom the presence of GPI-deficient cells could be assessed with sufficient sensitivity in both erythrocytes and granulocytes, 23 were PNH positive (43%). Of these 23 PNH positive patients, 20 were PNH positive in both erythrocytes and granulocytes. The remaining 3 patients were positive in the erythroid lineage only (Figure 2).

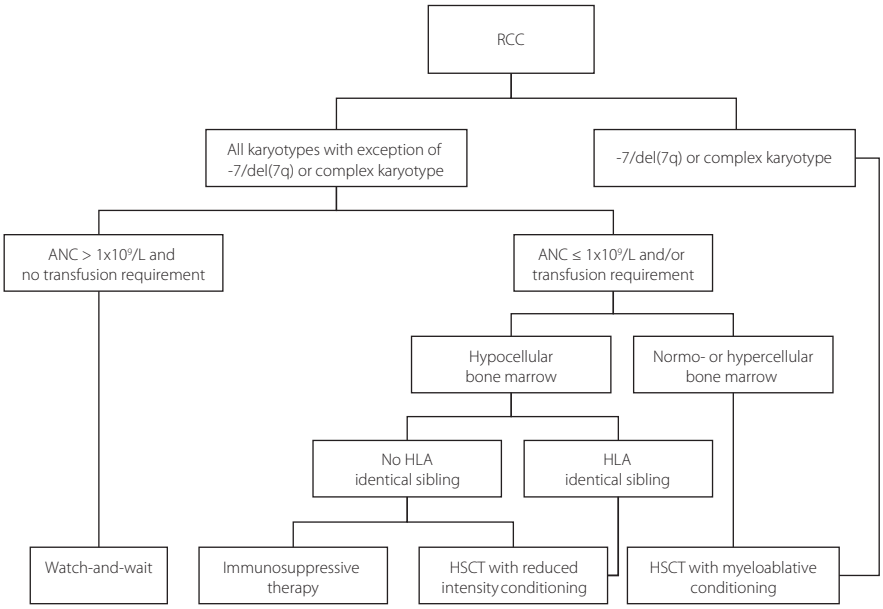

Figure 1 Treatment algorithm of RCC patients included for PNH analysis according to EWOG-MDS RC06.

ANC: absolute neuthrophil count; HSCT: hematopoietic stem cell transplantation.

In patients with a detectable PNH phenotype in both cell types, clones were significantly larger (median difference: 0.76%; range: -0.15 to 84%) in the granulocytic than in the erythroid lineage (Mann-Whitney-U test, *P*=0.0004).

Association of PNH clones with clinical characteristics and HLA-DR15

The 36 RCC patients with a PNH clone were significantly older than the 51 patients without a PNH clone (median: 12.7 versus 7.5 years, respectively, *P*=0.005); male/female distribution was equal. PNH positive patients were more often HLA-DR15 positive than PNH negative patients (42 versus 21%, *P*=0.051). This association was stronger in patients with a PNH clone larger than 0.1% in the erythroid and/or granulocytic lineage compared with patients without PNH clone or a clone smaller than 0.1% (67% HLA-DR15 positive versus 18% HLA-DR15 positive, *P*=0.000). All 5 patients with monosomy 7 who were included in the study were PNH negative (*P*=0.076). Bone marrow cellularity did not differ between PNH positive and negative patients.

At diagnosis, PNH positive patients had a lower leukocyte count (median: 2.7 versus 3.3 x 10^9/L, *P*=0.029), a lower hemoglobin level (median: 7.7 versus 9.5 g/dL, *P*=0.006),

Table 1 Clinical and laboratory characteristics of PNH negative and PNH positive patients.

	All	PNH-	PNH+	*P*
Number of patients	87	51	36	
Median age at diagnosis, years (range)	10.2 (1-18)	7.5 (1.3-17.9)	12.7 (2.3-18)	**0.005**
Male sex, No. (%)	44 of 87 (51)	24 of 51 (47)	19 of 36 (53)	NS
HLA-DR15, No. (%)	22 of 74 (30)	9 of 43 (21)	13 of 31 (42)	**0.051**
Cytogenetics, No. (%)[1]				
Normal	63 of 74 (85)	35 of 44 (80)	28 of 30 (93)	NS
Monosomy 7[2]	5 of 74 (7)	5 of 44 (11)	0 of 30 (0)	0.076
Other[3]	6 of 74 (8)	4 of 44 (9)	2 of 30 (7)	NS
Hypocellular bone marrow, No. (%)	73 of 87 (84)	43 of 51 (84)	30 of 36 (83)	NS
Blood counts at diagnosis, median (range)				
Leukocyte count, $x10^9$/L	2.9 (0.8-10.3)	3.3 (0.9-10.3)	2.7 (0.8-4.9)	**0.029**
ANC, x 10^9/L	0.6 (0.016-5.7)	0.5 (0.016-5.7)	0.6 (0.16-1.7)	NS
Hb, g/dL	9.0 (3-14)	9.5 (4.7-13.8)	7.7 (3.2-11.8)	**0.006**
MCV >97th percentile for age, No. (%)	57 of 81 (70)	28 of 45 (62)	29 of 36 (81)	0.073
Platelet count, $x10^9$/L	31 (1-430)	44.5 (1-430)	20 (1-134)	**0.008**
Transfusion dependency at diagnosis, No. (%)				
Platelets, No. (%)	13 of 77 (17)	9 of 43 (21)	4 of 34 (12)	NS
Erythrocytes, No. (%)	9 of 77 (12)	5 of 43 (12)	4 of 34 (12)	NS

[1] In 7 of 51 PNH negative patients and 6 of 36 PNH positive patients, no karyotype was obtained due to insufficient metaphases (*P*=NS). [2] Includes 1 patient with monosomy 7 with other aberrations. [3] Includes 1 patient with del(7q), 2 patients with trisomy 8 and 3 patients with rare aberrations.
Time from diagnosis to PNH analysis in the PNH negative (median: 1.9 months) versus the PNH positive group (median: 1 month) did not differ significantly (*P*=0.284).

tended to have a higher MCV (above the 97th percentile in 81 versus 62% of patients, *P*=0.073),[3,4] and a lower platelet count (median: 20 versus 44.5 x 10^9/L, *P*=0.008) than PNH negative patients. Transfusion dependency for platelets and/or erythrocytes did not differ significantly between the two groups. Results are summarized in Table 1.

Association of PNH clones with response to immunosuppressive therapy

Twenty-eight hypocellular RCC patients, 14 of whom were PNH positive and 14 PNH negative at diagnosis, were treated with IST and evaluated for response at day 180 after start of therapy. Two additional patients received IST but had a normo- or hypercellular bone marrow.

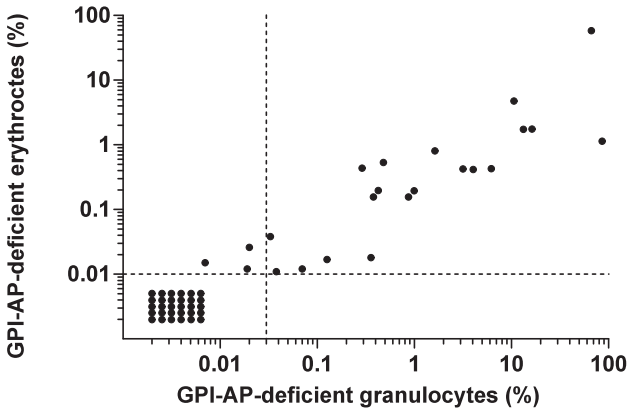

Figure 2 Correlation between proportions of GPI-deficient erythrocytes and granulocytes.

In 53 patients both the erythroid and granulocytic lineage could be assessed with sufficient sensitivity. Cut-off levels for PNH positivity (0.01% for erythrocytes, 0.03% for granulocytes) are indicated by dashed lines. Patients without a PNH clone were given an arbitrary value below 0.03% (x-axis) and 0.01% (y-axis).

Therefore, they were not eligible for IST according to the protocol and were excluded from the analysis. Of the 28 hypocellular RCC patients, one patient, PNH negative, received a HSCT before day 180, and was regarded as a non-responder at day 180. At day 180, a partial response was reached by 10 of 14 (71%) PNH positive, and 5 of 14 (36%) PNH negative patients (P=0.058). None of the patients reached a complete response by day 180.

When only PNH clones larger than 0.1% in the erythroid and/or granulocytic lineage were considered, PNH positive patients responded better to IST than patients with a PNH clone ≤0.1% or no PNH clone: 7 of 8 (88%) PNH positive patients showed a response at day 180, as compared to 8 of 20 (40%) PNH negative patients (P=0.038).

Of note, 2 of 28 patients, both PNH negative, received horse ATG; in 2 patients, information on the type of ATG is unavailable; in the remaining 24 patients, rabbit ATG (Thymoglobulin in 23 patients and ATG-Fresenius in 1 patient) was used.

Association of PNH clones with event-free survival in patients treated with immunosuppressive therapy

Median follow-up time (determined as time from start of IST to last follow-up) of hypocellular RCC patients who received IST was 12 months (range: 3-48 months). Event-free survival rates in PNH positive and negative RCC patients were 63% (SE=14%) and 36% (SE=13%), respectively (log-rank P=0.085) at 2.5 years; event-free survival rates in patients with a PNH clone >0.1% and in patients with a PNH clone ≤0.1% or no PNH clone were 70% (SE=18%) and 40% (SE=11%), respectively (log-rank P=0.089) at 2.5 years.

Evolution of PNH clones in RCC patients followed by watchful waiting

Six RCC patients were followed by a watch-and-wait strategy and studied serially for the presence of GPI-deficient cells. Median follow-up time (determined as the time between first and last PNH measurement) of these patients was 260 days (range: 103-1159 days). Two patients were PNH negative at first measurement; both remained PNH negative during follow-up. The other four patients, who were all PNH positive at first measurement, remained PNH positive during follow-up. Changes in clone size remained within one log-decade in both the granulocytic and erythroid lineage, and thus clone size was relatively stable (Figure 3A-B).

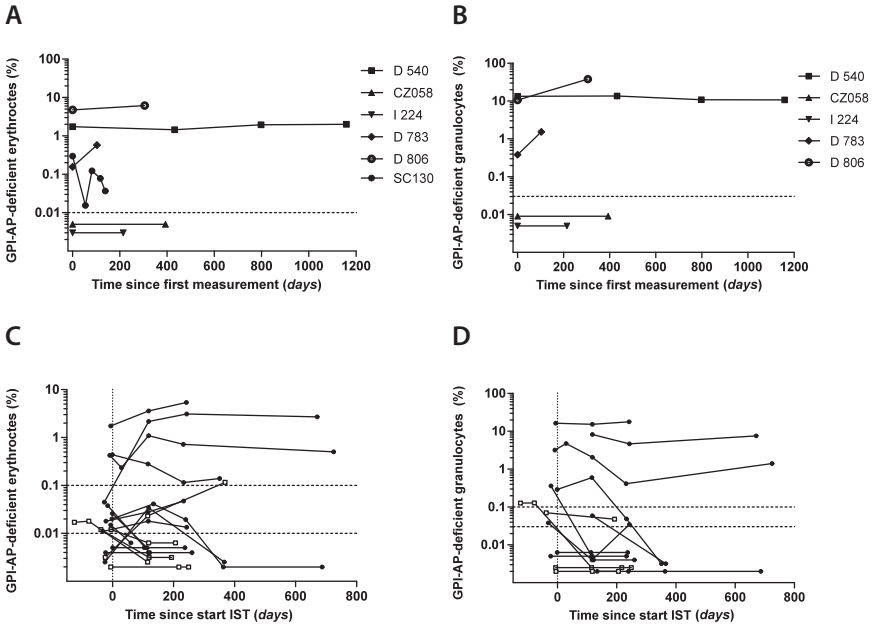

Figure 3 Evolution of GPI-deficient erythrocytes and granulocytes in patients followed sequentially.

(A) Evolution of GPI-deficient erythrocytes in patients followed by watchful waiting (n=6). (B) Evolution of GPI-deficient granulocytes in patients followed by watchful waiting (n=5). Patient SC130 was severely neutropenic, thus limiting the sensitivity of PNH analysis in the granulocytic lineage. (C) Evolution of GPI-deficient erythrocytes in IST treated patients (n=18). (D) Evolution of GPI-deficient granulocytes in IST treated patients (n=12). Dotted lines indicate start of treatment; dashed lines indicate cut-off levels for PNH positivity (0.01% for erythrocytes, 0.03% for granulocytes). Six patients were studied before start of IST in the erythroid lineage only due to a limited number of cells in the granulocytic lineage. IST responders are indicated by circles; IST non-responders by squares.

Evolution of PNH clones in RCC patients treated with immunosuppressive therapy

Of the 30 RCC patients (either with a hypocellular (n=28) or normo- or hypercellular (n=2) bone marrow) who were treated with IST, 18 were studied serially for the presence of PNH clones. Median follow-up time (determined as the time between first and last PNH measurement) of these patients was 255 days (range: 66-734 days); median follow-up time from start of IST to last PNH measurement was 243 days (range: 60-725 days). In PNH positive IST responders, clone size remained relatively stable during follow-up, with changes in size within one log-decade (Figure 3C-D). In one PNH negative patient, who was an IST non-responder, a PNH clone larger than 0.1% (0.12%) emerged in the erythroid lineage during follow-up; clone size in the granulocytic lineage remained between 0.03 and 0.1%. No other significant increases in PNH clone size were noted in this serially studied, IST treated, cohort (Figure 3C-D).

DISCUSSION

In this study among 87 RCC patients conducted by EWOG-MDS, we report that minor PNH clones are present in 41% of patients evaluated before treatment. This frequency is higher than in adults with low-grade MDS (13% to 23%),[20,22,28,30,31] but similar to studies in aplastic anemia in adults and children (20% to 68%).[20,24-28] Because PNH clones might be indicative of immune-mediated bone marrow failure,[18] this increased frequency might point towards a larger proportion of RCC caused by immune-mediated mechanisms than low-grade MDS in adults. Alternatively, PNH clones may more readily emerge by neutral evolution in conditions with reduced stem cell numbers,[33] which might be reflected by bone marrow hypocellularity, occurring much more frequently (80%) in RCC patients than in low-grade MDS in adults.[1]

PNH positive patients were older (median age 12.7 years) than PNH negative patients (median age 7.5 years), a finding for which no clear explanation is available. The median age of PNH positive RCC patients is similar to the age of a cohort of, mainly classic, PNH cases in childhood,[35] while the median age of a cohort of children with PNH positive aplastic anemia was 8.5 years.[27] We furthermore observed significantly lower blood counts and absence of monosomy 7 in PNH positive RCC patients, which is in line with a previous report describing lower platelet counts and a low frequency of karyotypic abnormalities in PNH positive MDS-RA patients.[22] However, PNH positivity does not exclude the possibility of clonal evolution, as is illustrated in one RCC patient in our series, who developed clonal monosomy 7 after partial response to IST.

We describe an increased frequency of HLA-DR15 in PNH positive compared to PNH negative RCC patients, similar to data from previous studies in PNH positive adult MDS and AA patients.[21,22] Additionally, PNH positive hypocellular RCC patients, especially those with

a clone >0.1%, were more likely to respond to IST, and their probability of event-free survival tended to be better than that of PNH negative patients, as described in most,[20-22] but not all,[16] studies in adult low-grade MDS. In contrast to our results in RCC patients, two previous studies in pediatric aplastic anemia patients did not show a superior response to IST in PNH positive patients.[27,36] Of note, although more PNH positive than PNH negative RCC patients showed response, none of the patients reached complete response at day 180. This might be explained in part by the use of rabbit- instead of horse-ATG since 2007, which seems to result in inferior response compared to horse-ATG in both adult and pediatric aplastic anemia patients.[37-40] To adequately change or maintain current treatment recommendations[19] for hypocellular RCC patients, with or without PNH clone, further evaluation of long-term outcome after IST, and evaluation of factors other than PNH that might influence IST response, are currently underway in a larger group of RCC patients.

In patients who were either followed by watchful waiting or treated with IST and serially monitored for evolution of PNH clones, PNH clone size remained relatively stable, even among patients who showed a response to IST. This seems at odds with the immune escape hypothesis, stating that GPI-deficient clones arise in conditions of T-cell mediated marrow destruction: one would expect that with ATG treatment, pathogenic T-cells are deleted or reduced in numbers, after which normal hematopoiesis can reoccur, with a decrease in the proportion of GPI-deficient cells. Although follow-up of RCC patients was relatively short, the lack of change in PNH clone size after response to IST has also been described in adults with MDS and aplastic anemia, but no satisfactory explanation for this observation could be given.[28,31,41]

In summary, in this prospective EWOG-MDS study, we show for the first time that PNH clones are frequently present in RCC, predict response to IST, and might indicate an immune-mediated pathophysiology in at least a subset of RCC patients.

Acknowledgements

AMA and this research were supported by the KiKa Foundation, Amstelveen, the Netherlands. In the Czech Republic RCC diagnosis and treatment were supported by a grant of the Ministry of Health for conceptual development of research organization 00064203 (University Hospital Motol, Prague, Czech Republic).

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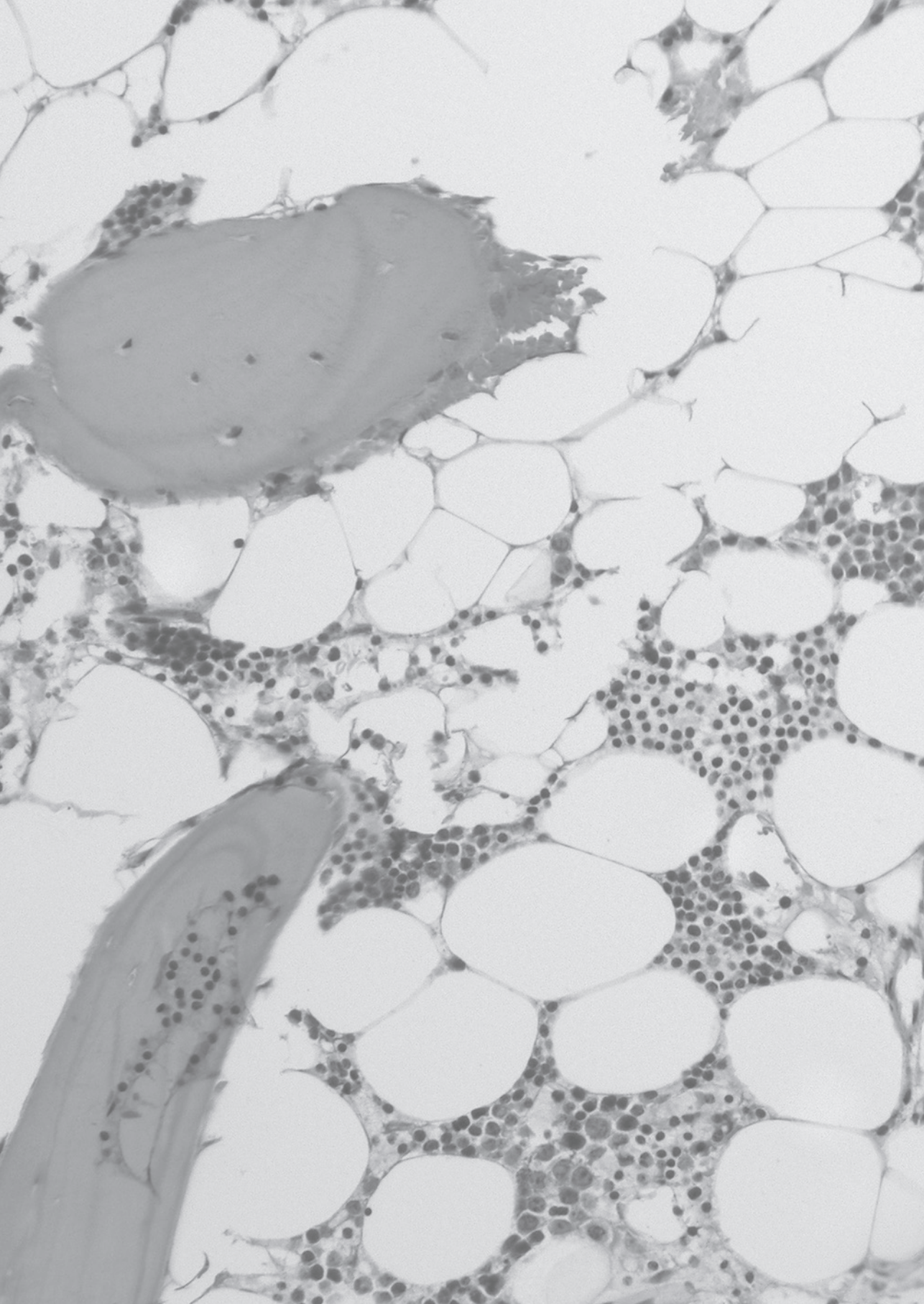

6

T-cell receptor Vβ skewing frequently occurs in refractory cytopenia of childhood and is associated with an expansion of effector cytotoxic T cells - a prospective study by EWOG-MDS

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Blood Cancer J **2014 (accepted)**

ABSTRACT

Immunosuppressive therapy, consisting of antithymocyte globulin and cyclosporine A, is effective in refractory cytopenia of childhood (RCC), suggesting that, similar to low-grade myelodysplastic syndromes (MDS) in adult patients, T lymphocytes are involved in suppressing hematopoiesis in a subset of RCC patients. However, the potential role of a T-cell mediated pathophysiology in RCC remains poorly explored. In a cohort of 92 RCC patients, we prospectively assessed the frequency of T-cell receptor (TCR) β-chain variable domain (Vβ) skewing in bone marrow and peripheral blood by heteroduplex PCR, and analyzed T-cell subsets in peripheral blood by flow cytometry. TCRVβ skewing was present in 40% of RCC patients. TCRVβ skewing did not correlate with bone marrow cellularity, karyotype, transfusion history, HLA-DR15, or the presence of a PNH clone. In 28 patients treated with immunosuppressive therapy, TCRVβ skewing was not clearly related with treatment response. However, TCRVβ skewing did correlate with a disturbed $CD4^+/CD8^+$ T-cell ratio, a reduction in naïve $CD8^+$ T cells, an expansion of effector $CD8^+$ T cells, and an increase in activated $CD8^+$ T cells (defined as HLA-DR^+, $CD57^+$, or $CD56^+$). These data suggest that T lymphocytes contribute to RCC pathogenesis in a substantial proportion of patients.

INTRODUCTION

Myelodysplastic syndromes (MDS), which are characterized by clonal hematopoiesis, impaired differentiation and maturation of myeloid cells, peripheral blood cytopenias, and a risk of progression to acute myeloid leukemia, are rare in childhood, with an estimated annual incidence of 0.8 to 1.8 per million children aged 0 to 14 years.[1-3] The most common variant of pediatric MDS is refractory cytopenia of childhood (RCC), defined as myelodysplasia without an increased blast count. About 80% of children with RCC have a hypocellular bone marrow, and karyotype is normal in the majority of patients.[4,5]

Intrinsic hematopoietic stem cell defects, caused by acquired cytogenetic and molecular aberrations or by epigenetic changes, result in hallmark features of MDS.[6,7] However, evidence obtained in adult MDS patients also suggests that a T-cell mediated immune response directed against hematopoietic progenitor cells contributes to MDS pathophysiology. Clinically, immunosuppressive therapy (IST) consisting of antithymocyte globulin (ATG), which specifically targets T cells, with or without cyclosporine A (CsA), is effective in selected patients.[8-18] Furthermore, in vitro experiments demonstrated that autologous peripheral blood lymphocytes of MDS patients inhibit granulocyte colony formation in an MHC class I-dependent manner;[19-22] this inhibitory effect was abrogated by ATG in the few patients studied.[19] Subsequently, analysis of the T-cell receptor (TCR) β-chain variable (Vβ) domain usage by flow cytometry and PCR-based methods showed oligoclonal expansions of mainly $CD8^+$ T cells in MDS patients.[19,21,23-26] These clonally expanded T cells were revealed to have an activated and effector phenotype.[21,27-29]

We recently reported that CsA and ATG are effective in RCC, and that over half of RCC patients display a skewed TCRVβ complementarity determining region 3 (CDR3) usage,[30,31] which is representative of clonal T-cell expansion. These findings indicate that an immune-mediated pathophysiology might also be present in a proportion of RCC patients. However, apart from the latter small pilot studies, the potential role of a T-cell mediated pathophysiology in RCC remains unexplored. In a prospective study conducted by the European Working Group of MDS in Childhood (EWOG-MDS), we therefore assessed the frequency of TCRVβ skewing in bone marrow and peripheral blood obtained from a cohort of 92 RCC patients, correlated TCRVβ skewing with clinical and laboratory characteristics, and analyzed the T-cell subset composition of peripheral blood. We here describe that T-cell oligoclonality is frequently present in RCC, correlates with a disturbed $CD4^+$/$CD8^+$ T-cell ratio, an expansion of effector $CD4^+$ and $CD8^+$ T cells, and an activated phenotype of $CD8^+$ T cells. Altogether, our data suggest that T cells are actively involved in RCC pathogenesis in a substantial proportion of patients.

METHODS

Patients and controls

Peripheral blood and bone marrow samples for TCRVβ analysis and peripheral blood samples for T-cell subset analysis were obtained from 92 primary RCC patients ≤18 years of age, who had not been previously treated with IST. The patients were consecutive, diagnosed between June 2005 and December 2011, and enrolled in the prospective, multi-center studies EWOG-MDS RC06 and EWOG-MDS 2006 (ClinicalTrial.gov identifiers: NCT00499070 and NCT00662090). Institutional review boards of participating institutions approved the studies and patients and/or parents or legal guardians of patients provided written informed consent for study participation in accordance with local law and regulations. RCC was diagnosed according to WHO criteria[5] and confirmed by central review of bone marrow morphology and histology in participating national study centers by reference pathologists of EWOG-MDS. Data were reported to the coordinating study center of the EWOG-MDS study group through standardized data collection forms. Peripheral blood and bone marrow samples obtained from 29 pediatric patients (median age: 13.2 years; range: 2-18) with (very) severe aplastic anemia ((v)SAA) served as controls for TCRVβ analysis. Peripheral blood samples obtained from 152 healthy subjects (age <2 years, n=53; 2-4 years, n=27; 5-9 years, n=30; 10-15 years, n=20; >15 years, n=22) served as controls for T-cell subset analysis.

HLA typing

Patients were HLA-typed for human leukocyte antigen (HLA)-A, -B, -C, -DR and -DQ by serological or molecular methods in the participating study centers.

Immunosuppressive therapy

RCC patients included for TCRVβ analysis were either followed with a watch-and-wait strategy, or were treated with IST, or received an allogeneic hematopoietic stem cell transplantation (HSCT). In particular, RCC patients with a hypocellular bone marrow, normal karyotype or karyotype other than monosomy 7, del(7q), or complex karyotype, and transfusion dependency or absolute neutrophil count (ANC) below $1x10^9$/L were eligible for either an HSCT from an HLA-matched donor or for IST. IST consisted of horse-ATG (Lymphoglobulin, Sangstat/Genzyme) until 2007 (0.75 ml/kg/day for 8 days); rabbit-ATG (Thymoglobulin, Sangstat/Genzyme or ATG-Fresenius, Fresenius Biotech) from 2007 (3.75 mg/kg/day for 5 days), prednisolone (1-2 mg/kg/day for 14 days, tapered down from day 14 and discontinued on day 28), CsA (5 mg/kg/day with subsequent dose adjustment to maintain blood levels of 100-150 ng/mL by monoclonal assay or 200-400 ng/mL by polyclonal assay, until day 180 with subsequent slow dose tapering), and granulocyte colony-stimulating factor (5μg/kg/day until day 28 in case of ANC below $0.5x10^9$/L). Response to IST was evaluated on day 180. Complete response was defined as transfusion

independency, hemoglobin level within the age-adjusted normal range, platelet count above 150×10^9/L, and ANC above 1.5×10^9/L; partial response was defined as transfusion independency, platelet count above 20×10^9/L, and ANC above 0.5×10^9/L; no response was defined when neither the partial nor the complete response criteria were fulfilled.

TCRVβ analysis by heteroduplex PCR

TCRVβ CDR3 repertoire analysis by heteroduplex PCR was performed as previously described.[31,32] In brief, RNA was isolated from peripheral blood and bone marrow mononuclear cells, separated by density gradient centrifugation. Following cDNA synthesis, TCRB (Vβ-Cβ) PCR, using TCRVβ specific primers for family 1 through 25, was applied. Resulting PCR products were subjected to heteroduplex analysis to determine TCRVβ CDR3 heterogeneity. Skewing was defined when 2 or more TCRVβ families showed an oligoclonal pattern on heteroduplex gel. This cut-off was based on healthy controls, in whom skewing in more than one TCRVβ family was present in only 2 of 18 cases (11%). Skewing was further subdivided into weak skewing, when an oligoclonal pattern was found in 2-5 TCRVβ families, and strong skewing, when an oligoclonal pattern was found in more than 5 TCRVβ families.[31]

T-cell subset analysis by flow cytometry

Peripheral blood samples were collected in heparinized tubes and sent to the Erasmus MC, Rotterdam, The Netherlands. Fresh peripheral blood samples were lysed with NH_4Cl, cells were suspended in PBS/BSA and stained for 15 minutes at room temperature with CD45RO-FITC (UCHL1, DAKO, Glostrup, Denmark), CD45RA-PE (2H4, Beckman Coulter, Woerden, The Netherlands), CD3-PerCP (SK7, BD Biosciences, Erembodegem, Belgium), CD4-PerCP-Cy5.5 (SK3, BD Biosciences), CD27-APC (L128, BD Biosciences), and CD8-APC-Cy7 (SK1, BD Biosciences) (tube 1); or with CD57-FITC (HNK-1, BD Biosciences), CD56-PE (C5.9, Zebra/DAKO, Enschede, The Netherlands), HLA-DR-PerCP (L243, BD Biosciences), CD3-PE-Cy7 (SK7, BD Biosciences), CD38-APC (HB7, BD Biosciences), and CD8-APC-Cy7 (SK1, BD Biosciences) (tube 2). Cells were acquired on a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using FlowJo software version 7.6.5 (Tree Star, Ashland, OR). After exclusion of debris and dead cells based on scatter and CD45 expression, lymphocytes were gated within all nucleated cells in a CD45 and forward scatter (FSC) versus side scatter (SSC) plot. T cells were defined as $CD3^+$ cells within lymphocytes. Naïve, central memory, effector memory, and terminally differentiated effector $CD4^+$ or $CD8^+$ T lymphocytes were defined as $CD45RO^-CD27^+$, $CD45RO^+CD27^+$, $CD45RO^+CD27^-$, and $CD45RO^-CD27^-$ cells, respectively.[33] Activated $CD8^+$ T lymphocytes were defined as the proportion of $CD56^+$, $CD57^+$, HLA-DR^+, or $CD38^+$ $CD8^+$ T cells. Lymphocyte and T-cell subset distributions in RCC patients and healthy controls were compared after categorizing patients and controls into age groups as previously published (<2 years, 2-4 years, 5-9 years, 10-15 years, >15 years),[34] while lymphocyte and T-cell subset

distributions in skewed and non-skewed RCC patients were compared after normalization according to age group using Z-scores as described below.

Statistical analyses

Statistical analyses were performed with SPSS 20 (IBM, Chicago, IL) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Categorical variables were compared using the Chi-square test or Fisher's exact test. Continuous variables were compared using the Mann-Whitney-U test or the Kruskal-Wallis test when more than two groups were compared. To compare relative lymphocyte and T-cell subset distributions between RCC patients with and without a skewed T-cell repertoire, taking age-dependent changes in into account, Z-scores were calculated as $Z = (X–\mu)/\sigma$, with X = raw score, μ = mean of age-matched control group, σ = standard deviation of age-matched control group. All reported *P*-values are two-sided and were considered statistically significant when <0.05; *P*-values >0.1 were reported as non-significant (NS), whereas those between 0.05 and 0.1 were reported in detail.

RESULTS

Patient characteristics

A total of 92 treatment-naïve RCC patients (46 male, 46 female), with a median age at diagnosis of 10 years (range: 1-18 years), were analyzed by heteroduplex PCR for TCRVβ CDR3 skewing in bone marrow and/or peripheral blood. Median time from diagnosis to TCRVβ analysis was 43 days. Clinical characteristics of the included patients are summarized in Table 1. Hypocellular bone marrow was reported in 82% of patients, which is comparable to the previously reported frequency of 81% of primary RCC patients in an interim analysis of studies EWOG-MDS 1998 and 2006.[4] Conventional cytogenetics displayed a normal karyotype in 67 of 78 patients (86%), which is slightly higher than the previously reported frequency of 77%;[4] monosomy 7 in 5 of 78 patients (including 1 patient with monosomy 7 and additional aberrations) (6%), and other cytogenetic aberrations in 6 of 78 patients (trisomy 8 in 2 patients, rare aberrations in 3 patients, and del(7q) in 1 patient) (8%). In 12 patients, karyotype was not obtained due to insufficient metaphases; in 2 patients, no information was available.

TCRVβ skewing frequently occurs in bone marrow and peripheral blood of RCC patients

TCRVβ CDR3 skewing in the bone marrow occurred in 31 of 79 RCC patients (39%) who were successfully analyzed. Of the 31 patients with a skewed TCRVβ CDR3 usage, 20 (25%) displayed weak skewing, and 11 (14%) strong skewing. In the peripheral blood, 33 of 83 analyzed RCC patients (40%) showed a skewed TCRVβ usage, 23 (28%) of whom had a

Table 1 Clinical and laboratory characteristics of included RCC patients.

Characteristic	
Number of patients	92
Median age at diagnosis, years (range)	10.3 (1-18)
Male sex, No. (%)	46 (50)
Hypocellular bone marrow, No. (%)	74 of 90 (82)
Cytogenetics, No. (%) [1]	
Normal	67 of 78 (86)
Monosomy 7 [2]	5 of 78 (6)
Other [3]	6 of 78 (8)

[1] In 12 of 90 patients (13%) no karyotype was obtained due to insufficient metaphases; in 2 patients, no data were available.
[2] Includes 1 patient with monosomy 7 and other aberrations.
[3] Includes 1 patient with del(7q), 2 patients with trisomy 8, and 3 patients with rare aberrations.

weakly skewed and 10 (12%) a strongly skewed repertoire (Table 2). In the 70 RCC patients successfully analyzed in both bone marrow and peripheral blood, skewing was similar in bone marrow and peripheral blood: 29 of 70 patients (41%) displayed weak or strong skewing in the bone marrow, compared to 26 of 70 patients (37%) in the peripheral blood. The frequency of skewing of individual TCRVβ families in peripheral blood and bone marrow within all RCC patients is depicted in Figure 1A: there appeared to be no preferential skewing of specific TCRVβ families, and TCRVβ usage was comparable in bone marrow and peripheral blood.

Table 2 Frequency of TCVβ CDR3 skewing in RCC and (v)SAA patients.

	RCC	**(v)SAA**	***P***
Bone marrow skewing, No. (%)			
No skewing (0-1 families skewed)	48 of 79 (61)	7 of 20 (35)	**0.038***
Weak skewing (2-5 families skewed)	20 of 79 (25)	10 of 20 (50)	
Strong skewing (>5 families skewed)	11 of 79 (14)	3 of 20 (15)	
Peripheral blood, No. (%)			
No skewing (0-1 families skewed)	50 of 83 (60)	17 of 28 (61)	NS*
Weak skewing (2-5 families skewed)	23 of 83 (28)	8 of 28 (29)	
Strong skewing (>5 families skewed)	10 of 83 (12)	3 of 28 (11)	

* No skewing versus weak or strong skewing; Chi-square test.

TCRVβ skewing occurs less frequently in bone marrow of RCC than of (v)SAA patients

In a previous pilot study, RCC and (v)SAA patients displayed a similar frequency of TCRVβ skewing in bone marrow and peripheral blood combined.[31] In the present study, we compared the frequency of TCRVβ skewing in (v)SAA with a larger cohort of RCC patients. TCRVβ skewing in the bone marrow, but not in the peripheral blood, occurred significantly less frequently in RCC patients than in (v)SAA patients: 48 of 79 RCC patients (61%) did not display skewing in the bone marrow, compared to only 7 of 20 (v)SAA patients (35%)

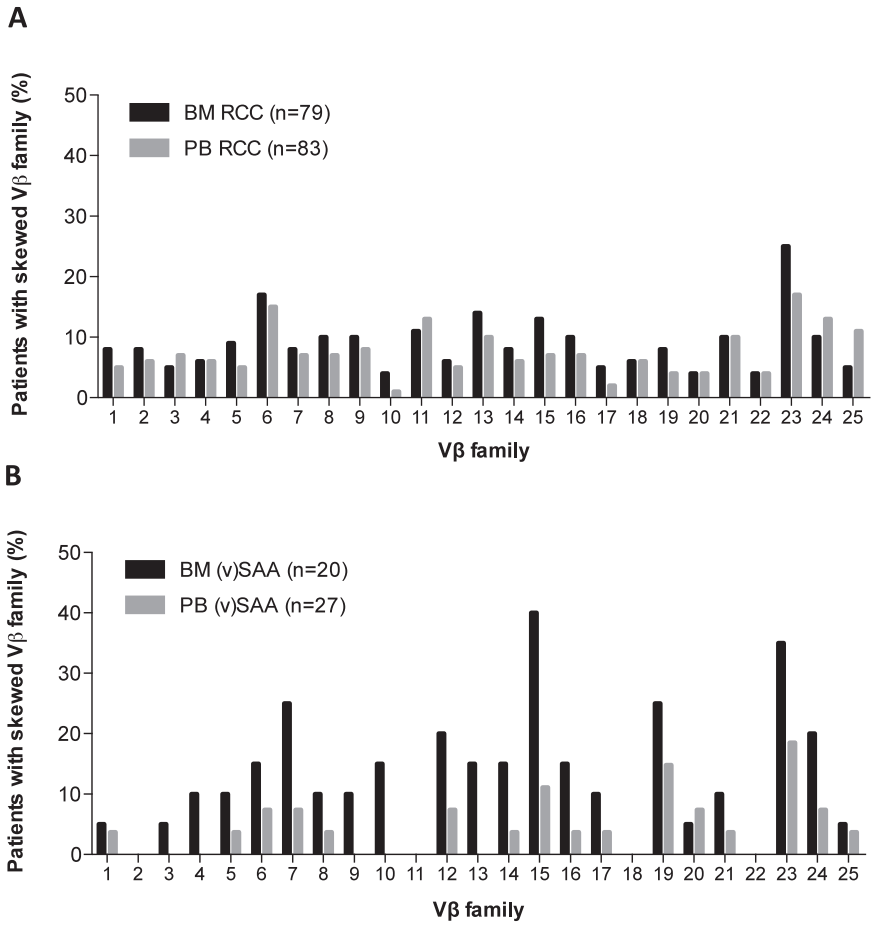

Figure 1 Frequency of skewing of individual TCRVβ families in bone marrow and peripheral blood of RCC and (v)SAA patients.

(A) Frequency of skewing in RCC patients. (B) Frequency of skewing in (v)SAA patients.

(*P*=0.038); 20 of 79 RCC patients (25%) versus 10 of 20 (v)SAA patients (50%) displayed weak skewing in the bone marrow, and 11 of 79 RCC patients (14%) versus 3 of 20 (15%) of (v)SAA patients had a strongly skewed TCRVβ repertoire in the bone marrow (Table 2). The frequency of skewing of individual TCRVβ families in (v)SAA is depicted in Figure 1B.

Clinical characteristics of RCC patients with a skewed TCRVβ repertoire

We next compared clinical characteristics of RCC patients with and without a skewed TCRVβ repertoire. Because characteristics of patients with weak and strong skewing were similar, these patients were grouped for further analyses (Table 3). Patients with a skewed usage of the TCRVβ chain in the peripheral blood were older than patients with a polyclonal T-cell repertoire (median age: 13 versus 10 years, *P*=0.013). A comparable relationship between age and skewing of the T-cell repertoire in the bone marrow was observed, but this difference was not statistically significant. Skewing of the TCRVβ repertoire did not correlate with gender, bone marrow cellularity, or transfusion dependency for platelets or erythrocytes. Because the occurrence of trisomy 8 has been linked with response to IST and the presence of trisomy 8-specific clonal T-cell expansions,[17,35,36] we compared cytogenetic results among patients with and without T-cell skewing. The distribution of patients with a normal karyotype or with monosomy 7 did not differ among the groups with or without a skewed T-cell repertoire, neither in bone marrow nor in peripheral blood. However, all five patients with other abnormalities in the karyotype, including both patients with trisomy 8 that were included in the study, had a skewed T-cell repertoire in the bone marrow (*P*=0.014). In the peripheral blood the same trend was observed, but differences were not statistically significant. Finally, although HLA-DR15 and the presence of minor PNH clones are predictors of IST response in some studies, and thought to be indicators of a T-cell mediated pathophysiology of bone marrow failure,[17,37-40] we observed no differences in the frequency of HLA-DR15 and PNH clone positivity between the groups with and without skewing.

Laboratory characteristics of RCC patients with a skewed TCRVβ repertoire

RCC patients with or without T-cell skewing in the peripheral blood had similar peripheral blood leukocyte, neutrophil, lymphocyte, T-cell, and platelet counts, and comparable hemoglobin and MCV levels (determined at the time of peripheral blood and bone marrow collection for TCRVβ repertoire analysis) (data not shown, all *P*-values >0.01). However, patients with strong, but not those with weak skewing, in the bone marrow had significantly lower leukocyte, absolute lymphocyte, and T-cell counts in the peripheral blood than patients without skewing (median leukocyte count, 3.3 x 10^9/L (range, 0.8-7.5) versus 2.1 x 10^9/L (range, 0.8-2.9); median lymphocyte count, 1.9 x 10^9/L (range, 0.6-6.1) versus 1.1 x 10^9/L (0.1-2.2); median T-cell count, 1.2 x 10^9/L (range, 0.5-3.6) versus 0.6 x 10^9/L (range, 0.4-0.7); *P*=0.020, *P*=0.002, and *P*=0.020, respectively).

Table 3 Clinical characteristics of RCC patients without or with a skewed TCVβ repertoire in bone marrow or peripheral blood.

	TCRVβ skewing BM			TCRVβ skewing PB		
	No skewing	**Skewing**	***P***	**No skewing**	**Skewing**	***P***
Number of patients	48	31		50	33	
Median age at diagnosis, years (range)	10 (1-18)	13 (1-18)	NS	10 (1-18)	13 (4-18)	**0.013**
Male sex, No. (%)	22 of 48 (46)	16 of 31 (52)	NS	27 of 50 (46)	15 of 33 (46)	NS
Hypocellular bone marrow, No. (%)	35 of 47 (75)	26 of 30 (87)	NS	40 of 49 (82)	29 of 33 (88)	NS
Transfusion dependency at TCRVβ analysis, No. (%)						
Platelets	15 of 32 (47)	9 of 18 (50)	NS	16 of 32 (50)	9 of 18 (50)	NS
Erythrocytes	12 of 32 (38)	6 of 18 (33)	NS	12 of 32 (38)	7 of 18 (39)	NS
Cytogenetics, No. (%)						
Normal	35 of 38 (92)	23 of 30 (77)	0.094	34 of 38 (90)	26 of 32 (81)	NS
Monosomy 7	3 of 38 (8)	2 of 30 (7)	NS	3 of 38 (8)	1 of 32 (3)	NS
Other	0 of 38 (0)	5 of 30 (17)	**0.014**	1 of 38 (3)	5 of 32 (16)	0.086
HLA-DR15, No. (%)	13 of 37 (35)	6 of 23 (26)	NS	10 of 39 (26)	10 of 28 (36)	NS
PNH clone at diagnosis, No. (%)						
>0.01%	17 of 43 (40)	14 of 30 (47)	NS	20 of 48 (42)	13 of 33 (40)	NS
>0.1%	10 of 43 (23)	7 of 30 (23)	NS	13 of 48 (27)	8 of 33 (24)	NS
Response to IST, No. (%)	4 of 13 (31)	5 of 8 (63)	NS	8 of 16 (50)	6 of 10 (60)	NS

Association of TCRVβ repertoire skewing with response to IST

Twenty-eight RCC patients were treated with IST and evaluated for response at day 180 after start of therapy. Response to IST was not significantly different between patients with or without T-cell skewing (Table 3). In particular, 5 of 8 patients (63%) with skewing in the bone marrow responded to IST, whereas 4 of 13 patients (31%) without skewing in the bone marrow responded to IST; 6 of 10 patients (60%) with skewing in the peripheral blood responded to IST, whereas 8 of 16 patients (50%) without skewing in the peripheral blood responded to IST. Furthermore, there were no significant differences in the frequency of skewing of specific TCRVβ families (data not shown), and in the median total number of skewed TCRVβ families among IST responding and non-responding RCC patients (medians of 2 and 0.5 expanded families in the bone marrow, respectively, and 1 and 0.5 in the peripheral blood, respectively). In responding and non-responding patients in whom follow-up bone marrow or peripheral blood samples were available (11 responders, median follow-up time after start of IST: 367 days (range: 234-1233 days); and 8 non-responders, median follow-up time after start of IST: 160 days (range: 62-1018 days)), no consistent in- or decreases in the number of skewed TCRVβ families were observed (data not shown).

TCRVβ skewing is associated with a reduction of naïve CD8$^+$ T cells and an expansion of effector CD4$^+$ and CD8$^+$ T cells in peripheral blood

Previous studies have shown that adult MDS patients, in comparison with healthy controls, display a disturbed CD4$^+$/CD8$^+$ T-cell ratio,[29] and a reduction in naïve CD8$^+$ T cells with an increase in CD8$^+$ effector cells.[21,25,29] In the present RCC cohort, we therefore compared the relative distribution of these parameters with healthy children. RCC patients showed a strong relative expansion of lymphocytes (Supplemental Figure 1), mainly due to an absolute reduction in granulocytes and monocytes (data not shown). Within the lymphocytes, T cells were relatively increased (Supplemental Figure 1), mainly due to an absolute reduction in B cells and NK cells (Supplemental Figure 2). Within the T-cell compartment, no consistent differences were observed in the frequency of CD4$^+$ and CD8$^+$ T cells and in the distribution of naïve and effector CD4$^+$ and CD8$^+$ T cells (Supplemental Figure 1) between RCC patients and healthy children. However, RCC patients with a skewed TCRVβ repertoire in the bone marrow had significantly less naïve CD8$^+$CD45RO$^-$CD27$^+$ T cells, and more terminally differentiated effector CD8$^+$CD45RO$^-$CD27$^-$ T cells in the peripheral blood than RCC patients without a skewed T-cell repertoire (Figure 2 and Supplemental Table 1). Moreover, when compared with RCC patients without skewing, patients with a skewed T-cell repertoire in the peripheral blood showed significantly different relative peripheral blood T-cell subset numbers, with less CD4$^+$ T cells, more CD8$^+$ T cells, and consequently a decreased CD4$^+$/CD8$^+$ T-cell ratio, more terminally differentiated effector CD4$^+$CD45RO$^-$CD27$^-$ T cells, less naïve CD8$^+$CD45RO$^-$CD27$^+$ T cells, more effector memory CD8$^+$CD45RO$^+$CD27$^-$ T cells, and more terminally differentiated effector CD8$^+$CD45RO$^-$CD27$^-$ T cells (Figure 2 and Supplemental Table 1).

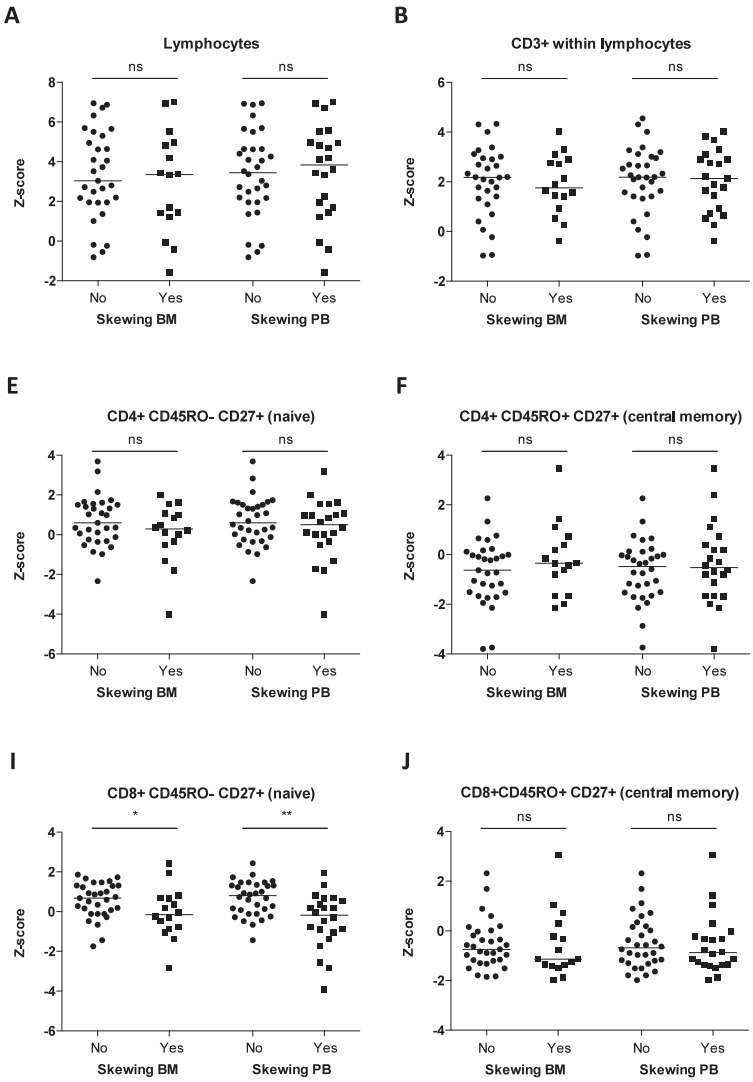

Figure 2 Naïve and effector CD4⁺ and CD8⁺ T cells in RCC patients with or without a skewed bone marrow or peripheral blood TCRVβ repertoire (Z-scores of relative distribution).

A) Lymphocytes within leukocytes. B) CD3⁺/T cells within lymphocytes. C) CD4⁺ within CD3⁺/ T cells. D) CD8⁺ within CD3⁺/ T cells. E) Naïve CD4⁺ T cells. F) Central memory CD4⁺ T cells. G) Effector memory CD4⁺ T cells. H) Terminally differentiated effector CD4⁺ T cells. I) Naïve CD8⁺ T cells. J) Central memory CD8⁺ T cells. K) Effector memory CD8⁺ T cells. L) Terminally differentiated effector CD8⁺ T cells. Lines indicate medians. Z-scores below 0 indicate a decrease, and Z-scores above 0 indicate an increase compared to age-matched controls. Ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

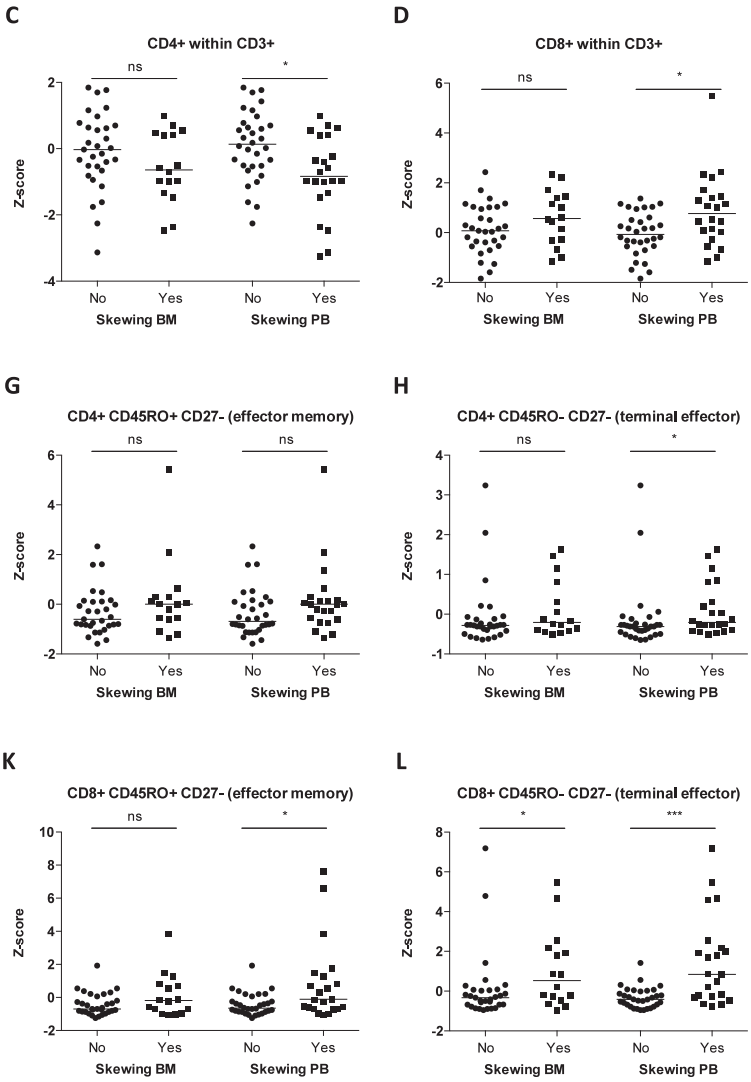

Figure 2 Continued.

TCRVβ skewing is associated with an expansion of activated CD8$^+$ T-cells in peripheral blood

When compared with healthy pediatric controls, no consistent differences were observed in the frequency of activated CD8$^+$ T cells in RCC patients (Supplemental Figure 3). However, the frequency of CD8$^+$ T cells positive for activation markers CD56 and CD57 in patients with a skewed TCRVβ repertoire in the bone marrow and peripheral blood, and HLA-DR in patients with a skewed TCRVβ repertoire in the bone marrow, was significantly increased when compared with patients with a polyclonal T-cell repertoire (Figure 3A-C, Supplemental Table 2). No differences were observed in the frequency of CD8$^+$ T cells positive for the activation marker CD38 (Figure 3D, Supplemental Table 2).

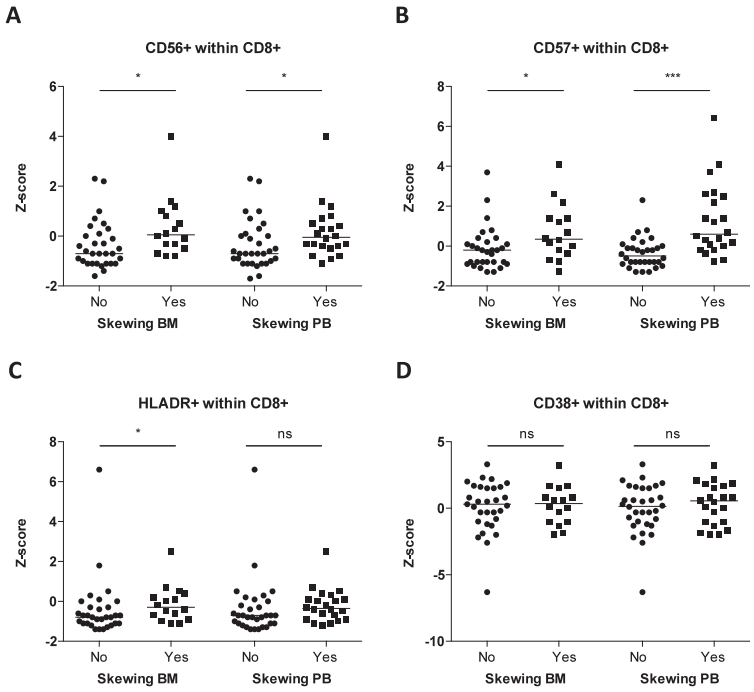

Figure 3 Activation markers on CD8$^+$ T cells in RCC patients with or without a skewed TCRVβ repertoire in bone marrow or peripheral blood (Z-scores of relative distribution).

A) CD56$^+$CD8$^+$ T cells. B) CD57$^+$CD8$^+$ T cells. C) HLA-DR$^+$CD8$^+$ T cells. D) CD38$^+$CD8$^+$ T cells. Lines indicate medians. Ns, not significant; *, P<0.05; ***, P<0.001.

DISCUSSION

In this study we show that TCRVβ skewing occurs in about 40% of RCC patients, suggesting an immune-mediated suppression of hematopoiesis in a considerable proportion of these patients. The frequency of TCRVβ skewing in the bone marrow, but not in the peripheral blood, of RCC patients was lower than the frequency of 65% in pediatric (v)SAA patients, indicating that an immune-mediated pathophysiology of bone marrow failure might be more common in (v)SAA than in RCC. Distinguishing RCC from (v)SAA, however, can be challenging, mainly due to the high frequency of bone marrow hypocellularity and the low frequency of karyotypic abnormalities in RCC, and a pathophysiological overlap between both diseases seems likely.

TCRVβ skewing was most prevalent in older children, but no correlation was found between skewing and bone marrow cellularity, the presence or absence of a normal karyotype or transfusion history. Previous reports in adult MDS patients also failed to show any significant correlation between clinical characteristics and TCRVβ skewing.[25,26] With respect to laboratory characteristics, we observed no difference in hemoglobin or MCV levels, platelet, leukocyte, absolute neutrophil, lymphocyte, and T-cell counts when patients with TCRVβ skewing were compared with those without skewing, which is in line with reports in adult MDS patients.[25,26] However, patients with skewing in more than 5 TCRVβ families had significantly lower absolute lymphocyte and T-cell counts than patients without skewing. This indicates that in patients with strong TCRVβ skewing, but not in patients with skewing in 2-5 TCRVβ families, skewing might reflect a contracted T-cell repertoire. This phenomenon was previously described in aplastic anemia patients after IST.[41] Furthermore, although HLA-DR15 positivity and the presence of a PNH clone are thought to be indicators of an immune-mediated pathophysiology of MDS, we did not find an increased frequency of TCRVβ skewing in HLA-DR15 or PNH positive patients.

TCRVβ skewing was not predictive of IST response in the present pediatric cohort. Although the number of patients treated with IST was small, and results should be interpreted cautiously, this is consistent with studies in adult MDS patients, in whom a predictive role of TCRVβ skewing for IST response has not been found,[23,25,26] although alterations in TCRVβ profiles or loss of clonal dominance of specific TCRVβ families after response to IST were described.[19,24] Likely, the specific T-cell subsets (e.g., $FOXP3^+$ regulatory T cells, Th17 T cells)[42-45] involved in the clonal expansion, the type of IST the patient received, and patient characteristics such as age, HLA-type, bone marrow cellularity, and underlying molecular aberrations all modulate response to IST, and TCRVβ skewing alone is insufficient to predict response to therapy.

TCRVβ skewing was associated with a reduced $CD4^+/CD8^+$ T-cell ratio in peripheral blood, a reduction in naïve $CD8^+$ T cells, an expansion of effector $CD8^+$ T cells, and an increase in activated $CD8^+$ T cells. These data confirm that RCC patients with a skewed TCRVβ repertoire indeed have an expanded population of activated T cells that might mediate the suppression of hematopoiesis that is observed in RCC.

The exact meaning of TCRVβ skewing and expansion of activated effector $CD8^+$ T cells, and presumed T-cell mediated suppression of hematopoiesis in MDS, remains unclear. It might either be a reflection of immune surveillance against hematopoietic cells expressing a neo-antigen, or might be a result of breaking of self-tolerance, leading to an autoimmune-like response against normal hematopoietic progenitor cells, or a combination of both. Evidence for the latter hypothesis was provided in the specific subgroup of MDS patients with trisomy 8, who are likely to respond to IST, but in whom the proportion of trisomy 8 cells increases after IST response.[17,35] T cells of clonally expanded TCRVβ families obtained from these patients selectively inhibited trisomy 8 cell growth.[35] WT1, overexpressed in trisomy 8 positive bone marrow mononuclear cells, but also expressed at a low level in normal hematopoietic stem cells, was subsequently implicated to be one of the antigens inducing T-cell mediated myelosuppression in trisomy 8 positive MDS.[36] Future efforts to gain more insight into the precise nature and antigenic target(s) of the skewed and activated T cells in trisomy 8 positive and other subtypes of MDS could benefit from TCR repertoire deep sequencing approaches, which have now become readily available. These approaches should preferably be performed in sorted T-cell subsets, taking into account differences between HLA types.

In summary, we show that TCRVβ skewing is frequently detected in RCC. TCRVβ skewing is associated with a disturbed $CD4^+/CD8^+$ T-cell ratio, an expansion of effector $CD4^+$ and $CD8^+$ T cells, and an activated phenotype of $CD8^+$ T cells, which suggests that T cells are actively involved in the pathogenesis of RCC in a substantial proportion of patients.

Acknowledgements

AMA and this research were supported by the KiKa Foundation, Amstelveen, the Netherlands. In the Czech Republic RCC diagnosis and treatment were supported by a grant of the Ministry of Health for conceptual development of research organization 00064203 (University Hospital Motol, Prague, Czech Republic). The authors acknowledge excellent technical support by research technicians from the Departments of Immunology and Pediatric Oncology/Hematology, Erasmus MC.

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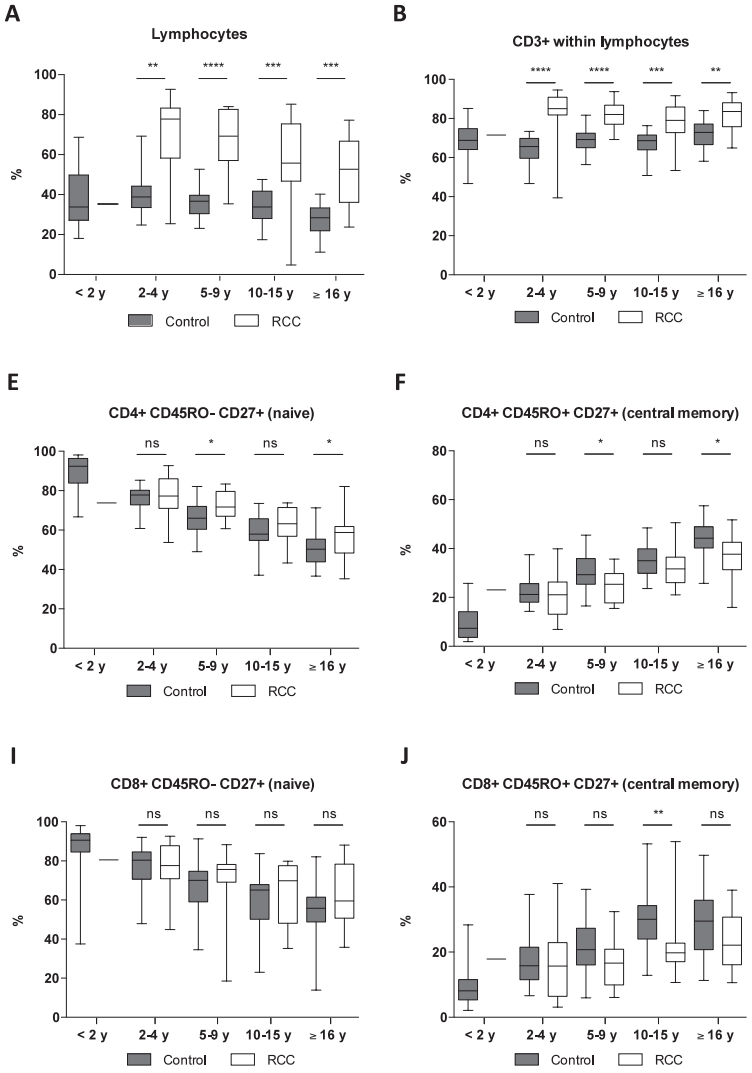
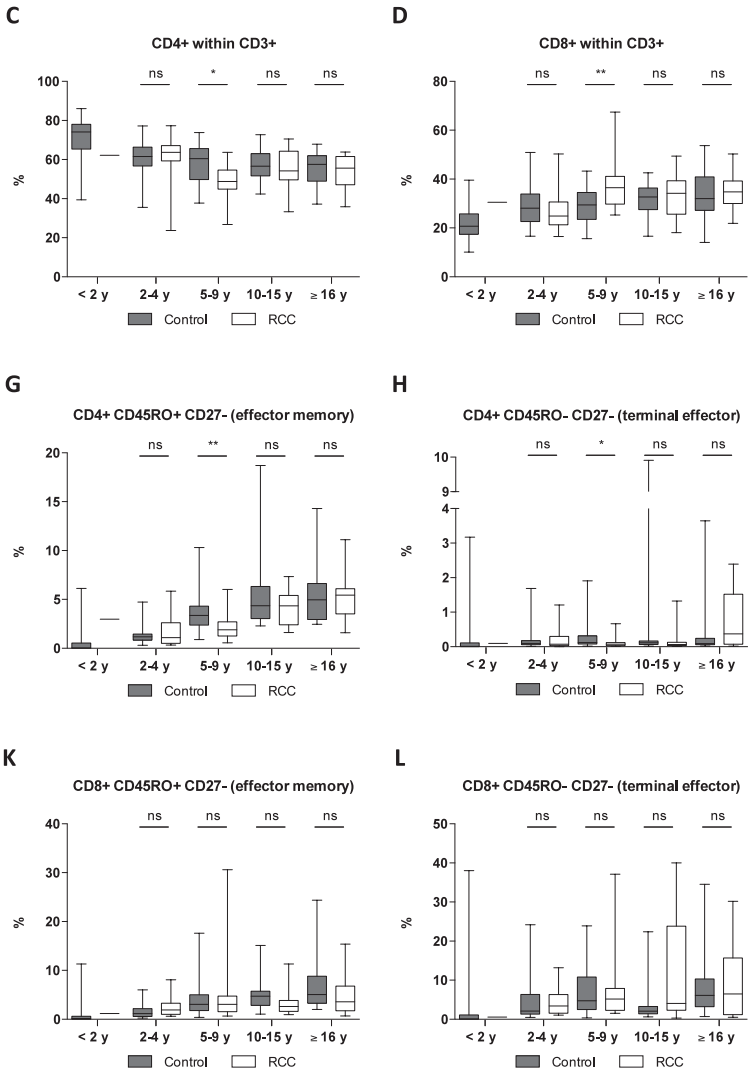
SUPPLEMENTAL INFORMATION

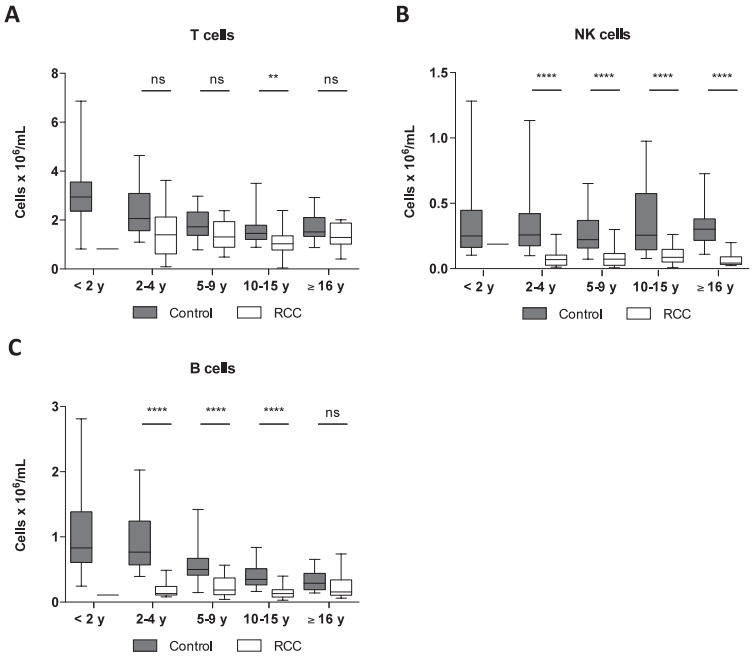


Supplemental Figure 1 Relative T-cell subset distribution in RCC patients and healthy controls.

A) Lymphocytes within leukocytes. B) $CD3^+$/T cells within lymphocytes. C) $CD4^+$ within $CD3^+$/ T cells. D) $CD8^+$ within $CD3^+$/ T cells. E) Naïve $CD4^+$ T cells. F) Central memory $CD4^+$ T cells. G) Effector memory $CD4^+$ T cells. H) Terminally differentiated effector $CD4^+$ T cells. I) Naïve $CD8^+$ T cells. J) Central memory $CD8^+$ T cells. K) Effector memory $CD8^+$ T cells. L) Terminally differentiated effector $CD8^+$ T cells. Lines indicate medians. Boxes extend from the 25th to 75th percentile; lines in the boxes indicate medians, whiskers indicate minimum and maximum values. Ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, ****, $P<0.0001$.

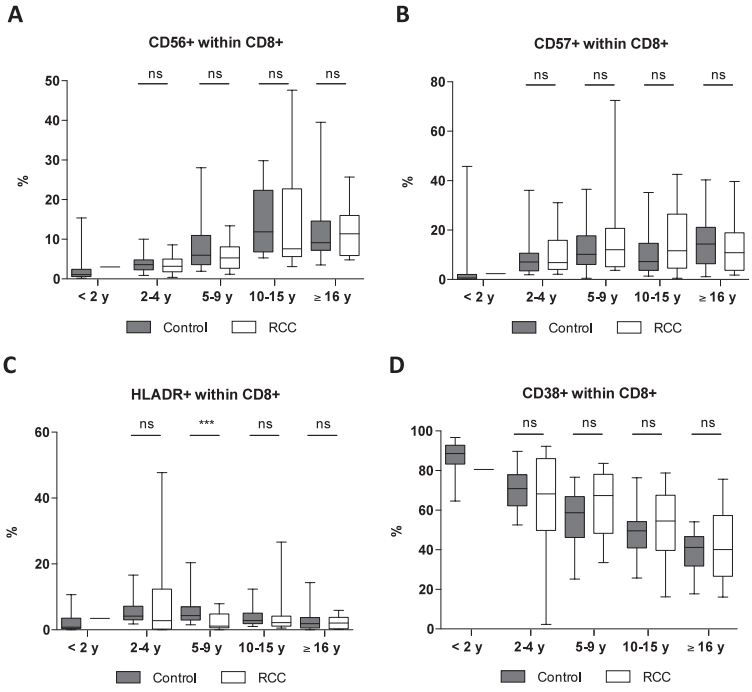


Supplemental Figure 1 Continued.



Supplemental Figure 2 Absolute lymphocyte subset distribution in RCC patients and healthy controls.

A) T cells. B) NK cells. C) B cells. After exclusion of debris and dead cells based on scatter and CD45 expression, lymphocytes were gated within all nucleated cells in a CD45 and forward scatter (FSC) versus side scatter (SSC) plot. Within lymphocytes, T cells were defined as $CD3^+$ cells, NK cells as $CD3^-CD56^+$, and B cells as all other cells. Absolute cell counts were calculated from total white blood cell counts, which were measured with a Coulter Counter Z2 (Beckman Coulter, Woerden, The Netherlands). Ns, not significant; **, $P<0.01$, ****, $P<0.0001$.



Supplemental Figure 3 Activation markers on CD8+ T cells in RCC patients.

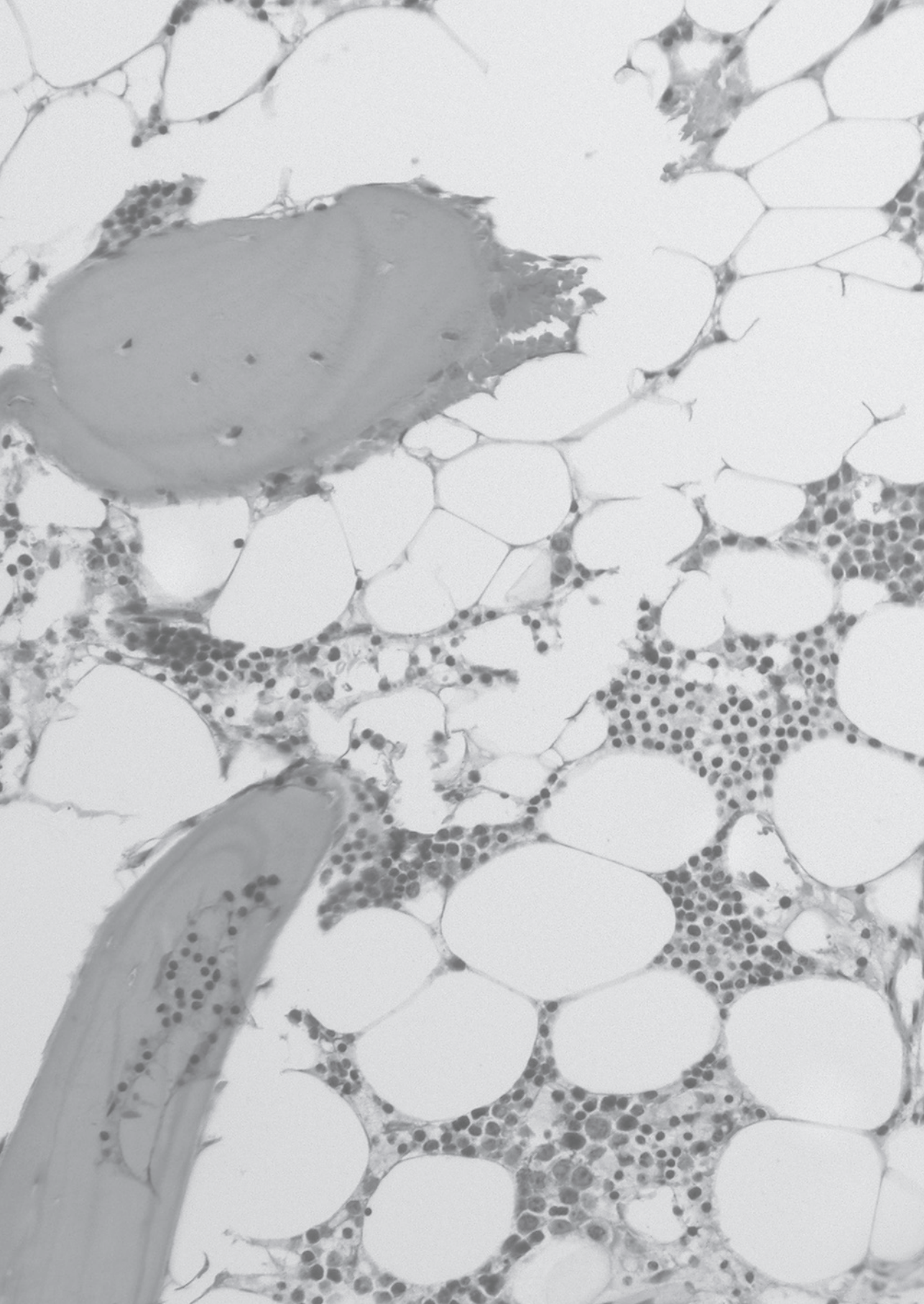
A) $CD56^+CD8^+$ T cells in RCC patients and age-matched healthy controls. B) $CD57^+CD8^+$ T cells in RCC patients and age-matched healthy controls. C) $HLADR^+CD8^+$ T cells in RCC patients and age-matched healthy controls. D) $CD38^+CD8^+$ T cells in RCC patients and age-matched healthy controls. Boxes extend from the 25^{th} to 75^{th} percentile; lines in the boxes indicate medians, whiskers indicate minimum and maximum values. Ns, not significant; ***, $P<0.001$.

Supplemental Table 1 Association of peripheral blood lymphocyte subsets (Z-scores of relative distribution) with bone marrow or peripheral blood TCRVβ CDR3 skewing.

	Vβ skewing BM			Vβ skewing PB		
	No skewing (n=31)	**Skewing (n=16)**	***P***	**No skewing (n=32)**	**Skewing (n=22)**	***P***
Lymphocytes	3.04 (-0.81-6.95)	3.36 (-1.59-6.99)	NS	3.45 (-0.81-6.95)	3.84 (-1.59-6.99)	NS
CD3+	2.18 (-0.97-4.33)	1.76 (-0.37-4.02)	NS	2.19 (-0.97-4.55)	2.13 (-0.37-4.02)	NS
CD4+ within CD3+	-0.03 (-3.13-1.84)	-0.65 (-2.47-0.98)	NS	0.13 (-2.26-1.84)	-0.84 (-3.26-0.98)	**0.010**
CD4+CD45RO-CD27+ within CD4+	0.6 (-2.34-3.69)	0.29 (-4.02-1.99)	NS	0.6 (-2.34-3.69)	0.5 (-4.02-3.19)	NS
CD4+CD45RO+CD27+ within CD4+	-0.63 (-3.8-2.26)	-0.35 (-2.17-3.45)	NS	-0.48 (-3.74-2.26)	-0.53 (-3.8-3.45)	NS
CD4+CD45RO+CD27- within CD4+	-0.6 (-1.59-2.33)	0.01 (-1.36-5.44)	NS	-0.69 (-1.59-2.33)	0 (-1.36-5.44)	0.077
CD4+CD45RO-CD27- within CD4+	-0.28 (-0.64-3.24)	-0.21 (-0.52-1.62)	NS	-0.31 (-0.65-3.24)	-0.21 (-0.52-1.62)	**0.032**
CD8+ within CD3+	0.08 (-1.84-2.43)	0.57 (-1.14-2.34)	NS	-0.07 (-1.84-1.37)	0.77 (-1.14-5.47)	**0.011**
CD8+CD45RO-CD27+ within CD8+	0.68 (-1.75-1.86)	-0.15 (-2.85-2.44)	**0.047**	0.8 (-1.44-2.44)	-0.18 (-3.94-1.95)	**0.002**
CD8+CD45RO+CD27+ within CD8+	-0.74 (-1.85-2.32)	-1.14 (-1.98-3.04)	NS	-0.69 (-1.98-2.32)	-0.88 (-1.96-3.04)	NS
CD8+CD45RO+CD27- within CD8+	-0.69 (-1.25-1.93)	-0.19 (-1.05-3.83)	NS	-0.65 (-1.25-1.93)	-0.11 (-1.05-7.63)	**0.021**
CD8+CD45RO-CD27- within CD8+	-0.33 (-0.95-7.19)	0.53 (-0.95-5.48)	**0.035**	-0.42 (-0.95-1.42)	0.85 (-0.76-7.19)	**0.000**
CD4/CD8 ratio	-0.35 (-1.59-2.6)	-0.73 (-1.57-1.17)	NS	-0.26 (-1.26-2.6)	-0.76 (-1.99-1.17)	**0.015**

Supplemental Table 2 Association of peripheral blood $CD8^+$ T-cell activation markers (Z-scores of relative distribution) with bone marrow or peripheral blood TCRVβ CDR3 skewing.

	Vβ skewing BM			Vβ skewing PB		
	No skewing (n=31)	**Skewing (n=16)**	***P***	**No skewing (n=32)**	**Skewing (n=22)**	***P***
Within CD8+						
CD56+CD8+	-0.7 (-1.6-2.3)	0.05 (-0.8-4)	**0.012**	-0.7 (-1.7-2.3)	-0.05 (-1.1-4)	**0.023**
CD57+CD8+	-0.2 (-1.3-3.7)	0.35 (-1.3-4.1)	**0.024**	-0.5 (-1.3-2.3)	0.6 (-0.8-6.4)	**0.000**
HLADR+CD8+	-0.8 (-1.4-6.6)	-0.3 (-1.1-2.5)	**0.044**	-0.7 (-1.4-6.6)	-0.35 (-1.2-2.5)	0.082
CD38+CD8+	0.3 (-6.3-3.3)	0.35 (-2-3.2)	NS	0.15 (-6.3-3.3)	0.55 (-2-3.2)	NS



7

Applicability of a reproducible flow cytometry scoring system in the diagnosis of refractory cytopenia of childhood

Anna M. Aalbers, Marry M. van den Heuvel-Eibrink, Valerie de Haas, Jeroen G. te Marvelde, Anja X. de Jong, Mirjam van der Burg, Michael Dworzak, Henrik Hasle, Franco Locatelli, Barbara De Moerloose, Markus Schmugge, Jan Stary, Marco Zecca, C. Michel Zwaan, Arjan A. van de Loosdrecht, Jacques J.M. van Dongen, Charlotte M. Niemeyer, and Vincent H.J. van der Velden

***Leukemia* 2013; 27 (9): 1923-5**

TO THE EDITOR:

Myelodysplastic syndrome (MDS) in childhood is rare and has an estimated incidence per year of about 0.8 to 1.8 per million children aged 0 to 14 years.[1-3] The most common subtype of childhood MDS, accounting for about half of all cases, is refractory cytopenia of childhood (RCC), defined as myelodysplasia without an increased blast count.[4,5] Karyotype is normal in the majority of patients with RCC, and, in contrast to adults with MDS-refractory anemia (RA), about 80% of children have a hypocellular bone marrow (BM).[6] Although morphologic criteria for diagnosing RCC are strictly defined,[5] differentiating RCC from the immune mediated BM failure syndrome aplastic anemia can be challenging. Similarly, difficulties can be encountered in differentiating, based on morphologic criteria, non-clonal cytopenias from low-grade MDS in adults, especially in cases without cytogenetic aberrations or specific morphologic abnormalities such as ring sideroblasts. Flow cytometric immunophenotyping has been suggested as valuable addition to morphology in the diagnosis of MDS.[7] Recently, Ogata and others described a simple, reproducible flow cytometric scoring system as diagnostic tool in adult low-grade MDS with a sensitivity of 70% and a specificity of 93%.[8,9] The diagnostic utility of this scoring system in RCC is unknown. Here we describe its applicability in childhood MDS, with emphasis on RCC, using pediatric advanced MDS patients, low-grade adult MDS patients, healthy individuals, and cytopenic non-MDS patients as controls.

BM samples obtained from 82 primary RCC patients (median age: 10.5 years, range: 1.3-17.9), not previously treated with immunosuppressive therapy, were available for analysis by flow cytometry. Patients were diagnosed from June 2005-December 2011 and enrolled in the prospective, multi-center studies EWOG-MDS 2006 and EWOG-MDS RC06 (ClinicalTrial.gov: NCT00662090 and NCT00499070). BM was hypocellular in 66 patients (81%), and normo- or hypercellular in 16 of 82 patients (20%). Cytogenetic analysis was normal in 62 patients (76%), monosomy 7 was present in 5 patients (6%), and other aberrations in 5 patients (6%); in 10 of 82 patients (12%) no result was obtained due to insufficient metaphases. Institutional review boards of participating institutions approved the studies and parents or legal guardians of patients provided written informed consent for study participation. RCC was diagnosed according to WHO criteria[5] and confirmed by central review of BM morphology and histology.[10]

BM samples were collected in heparinized tubes, sent to the Erasmus MC, and generally analyzed within 24 hours from collection. A minimum of 500 µL BM was lysed with NH_4Cl, resuspended in 350 µL PBS/BSA or to a maximum concentration of $20x10^6$ cells/mL, and 50 µL aliquots were stained for 15 minutes at room temperature with CD7-FITC (3A1/1, 7F3, Sanquin, Amsterdam, the Netherlands), CD56-PE (C5.9, DAKO, Glostrup, Denmark), CD45-PerCP (2D1, BD Biosciences, Erembodegem, Belgium), CD19-PE-Cy7 (SJ25C1, BD), CD5-APC (L17F12, BD), and CD34-APC-Cy7 (8G12, BD) in tube 1, and CD3-FITC (SK7, BD), CD16-PE (B73.1, BD) and CD56-PE (C5.9, Zebra/DAKO), CD45-PerCP

(2D1, BD), CD10-PE-Cy7 (HI10a, BD), CD19-APC (SJ25C1, BD), and CD20-APC-Cy7 (L27, BD) or APC-H7 (L27, BD) in tube 2. Cells were acquired on a FACSCanto II flow cytometer (BD Biosciences). After exclusion of debris and dead cells based on scatter and CD45 expression, at least 100,000 cells were analyzed in 61 of 82 RCC patients (75%). In the remaining 21 patients, a median of 54,323 cells could be analyzed (range, 4,359-98,482). Flow cytometric data were analyzed with Infinicyt software (Cytognos, Salamanca, Spain) according to the published strategy.[9] Briefly, in tube 1, lymphocytes were gated within all nucleated cells in a CD45 and forward scatter (FSC) versus side scatter (SSC) plot, and blast cells were gated within CD45 versus CD34 and FSC versus SSC plots. Within this population, myeloid blast cells were identified as SSC^{high} cells in a CD45 versus SSC plot (parameter 1), and B-cell progenitors were identified as SSC^{low} cells (parameter 2). Next, the lymphocyte versus myeloid blast cell CD45 mean fluorescence intensity (MFI) ratio was calculated (parameter 3). In tube 2, lymphocytes and granulocytes were gated in CD45 and FSC versus SSC plots, and SSC peak channel values of granulocytes versus lymphocytes were calculated (parameter 4). Samples were evaluated based on the published cut-offs (determined in 281 adults with low-grade MDS and 257 non-clonal cytopenic adult controls): $CD34^+$ myeloid blast cells (parameter 1) ≥2%, $CD34^+$ B-cell progenitors within $CD34^+$ blast cells (parameter 2) ≤5%, lymphocyte/myeloid blast cell CD45 MFI ratio (parameter 3) ≤4 or ≥7.5, granulocyte/lymphocyte SSC peak channel ratio (parameter 4) ≤6.[9] Patients scored 1 point for each fulfilled criteria; patients scoring ≥2 points were considered likely to have MDS.[9]

To validate the scoring system in our laboratory, 8 low-grade adult MDS patients, 28 cytopenic non-clonal adult controls, and 9 healthy adult controls were evaluated (Figure 1). Seven of 8 MDS patients (87.5%), 2 of 28 cytopenic controls (7.1%), and 0 of 9 healthy controls scored positive (≥2 points) (Table 1), which is comparable to the previously published sensitivity of 70% and specificity of 93%.

Subsequently, 82 RCC patients were analyzed (Figure 1). In these patients, the percentage of myeloid blast cells was generally not greater than 2%, the CD45 MFI of myeloid blast cells was in general not aberrant, the SSC of granulocytes was not decreased, and the percentage of $CD34^+$ B-cell progenitors was very heterogeneous, but in general not decreased. Importantly, in 35 patients, <500 CD34+ blast cells and <25 $CD34^+$ B-cell progenitors (for evaluation of parameter 2), <50 $CD34^+$ myeloid blast cells (for evaluation of parameter 3), and/or <1000 granulocytes (for evaluation of parameter 4) were present for reliable analysis of all four parameters. Thus, of 82 patients, only 47 patients could be evaluated for all four parameters according to the previously published scoring system,[9] limiting its applicability to 57% of patients. Of those patients who could be evaluated completely, 4 of 47 patients (8.5%) scored ≥2 points (Table 1), and were thus likely to have MDS according to the previously published criteria.[9] The patients who scored positive were slightly older than the total RCC cohort (median age at diagnosis: 13 versus 10.5 years); 2 patients carried a monosomy 7, and in 2 patients no cytogenetic result was obtained due to insufficient metaphases. Owing to the low sensitivity in the already

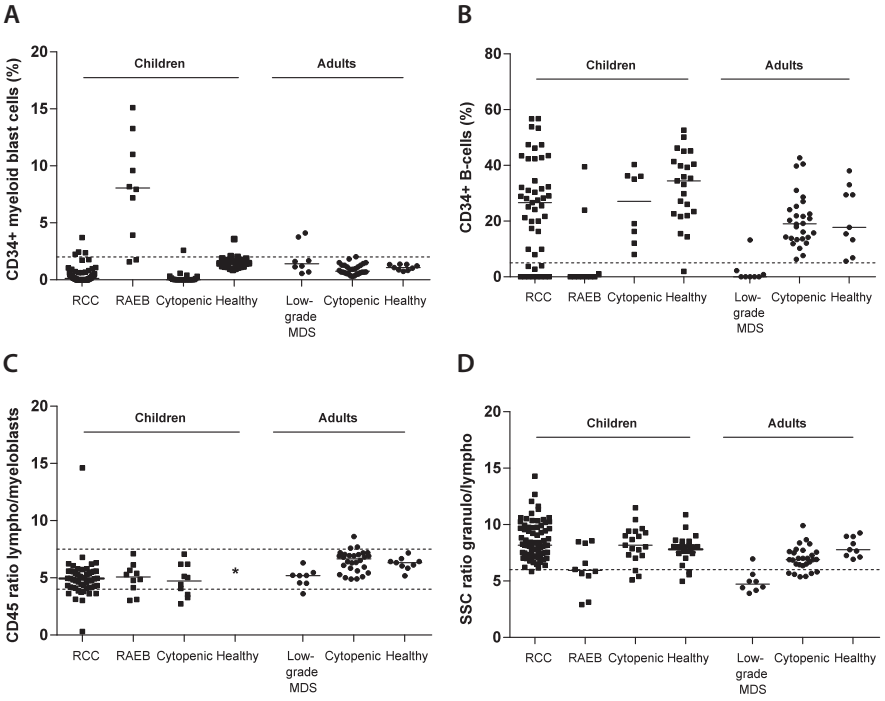


Figure 1 Comparison of four parameters in BM of evaluable healthy and cytopenic controls, RCC and pediatric advanced MDS patients, and adult low-grade MDS patients.

Dashed lines indicate cut-off levels.[9] (A) Percentage of CD34+ myeloid blast cells (parameter 1; cut-off ≥2%). (B) Percentage of CD34+ B-cell progenitors within CD34+ blast cells (parameter 2; cut-off ≤5%). (C) Lymphocyte/myeloid blast cell CD45 MFI ratio (parameter 3; cut-off ≤4 or ≥7.5). Parameter 3 in healthy pediatric controls was not comparable to data obtained in other patients and controls, and is not shown (indicated by asterisk). (D) Granulocyte/lymphocyte SSC peak channel ratio (parameter 4; cut-off ≤6). Sufficient cell numbers for evaluation were present in all 82 RCC patients in A, in 47 RCC patients in B, in 52 patients in C, and in 77 RCC patients in D; consequently, also patients in whom not all four parameters could be evaluated are shown.

limited number of patients that could be evaluated for all 4 parameters, the scoring system published by Ogata and others does not seem applicable in RCC.

To assess whether the scoring system, which is largely based on immunophenotyping of blast cells, might be applicable in pediatric advanced MDS, 10 cases with MDS-RAEB or RAEB-t were evaluated. Of these, 8 (80%) scored positive.

To determine the specificity of the scoring system in a pediatric control population, 22 cytopenic non-clonal pediatric controls (3 Fanconi anemia, 1 transient erythroblas-

Table 1 Flow cytometric scores of evaluable RCC and pediatric advanced MDS patients, adult low-grade MDS patients, and healthy and cytopenic controls. Only individuals in whom all 4 parameters could be evaluated are shown, with the exception of healthy pediatric controls, in whom only 3 of 4 parameters were evaluated.

	0	1	2	3	4	Cases positive (%)
Children						
RCC	29	14	3	1	0	4 of 47 (8.5)
RAEB	2	0	3	3	2	8 of 10 (80)
Cytopenic controls						
FA/ITP/TEC	2	3	0	0	0	0 of 5 (0)
(v)SAA	3	0	0	0	0	0 of 3 (0)
Healthy controls*	17	5	1	0	0	N/A
Adults						
Low-grade MDS	1	0	5	1	1	7 of 8 (88)
Cytopenic controls	20	6	2	0	0	2 of 28 (7.1)
Healthy controls	9	0	0	0	0	0 of 9 (0)

* Only 3 parameters evaluated

topenia of childhood, 1 idiopathic thrombocytopenia of childhood, and 17 (very) severe aplastic anemia ((v)SAA) patients) were analyzed (Figure 1). In 14 (all (v)SAA) of 22 cytopenic non-clonal pediatric controls, insufficient granulocytes and/or blast cells were present for evaluation of all parameters. Of the 8 patients that could be evaluated completely, no patient scored ≥2 points (Table 1). Parameters 1, 2, and 4 of the scoring system were also assessed in a historic control cohort of 23 healthy children (Table 1). It appeared that the CD45 MFI ratio of lymphocytes and $CD34^+$ myeloid blast cells in the healthy pediatric controls was not comparable to the data obtained in other patients and controls. The former data were obtained using 4-color stainings, a different method (stain-lyse-wash) than the one employed in the other samples, and acquired on a FACSCalibur flow cytometer (BD Biosciences), which might explain the difference in CD45 ratio. Consequently, parameter 3 could not be used for the flow cytometric scoring system in our healthy pediatric controls. Out of 23 healthy children, 17 scored 0 points, 5 scored 1 point, and 1 scored 2 points when three of four parameters were analyzed. Theoretically, 6 of 23 healthy children (26%) could have scored 2 or more points had all four parameters been analyzed. It should be noted that the median percentages of $CD34^+$ myeloid blast cells and, particularly, $CD34^+$ B-cell progenitors in healthy children are higher than in healthy adults. Results are summarized in Figure 1 and Table 1. Altogether, based on the analysis of 31 pediatric (23 healthy and 8 cytopenic) control samples, the theoretical

specificity of the flow cytometric scoring system is at least 81% (25 of 31), which is comparable with the specificity obtained in adults.[9]

In conclusion, the scoring system proposed by Ogata and others is not usefully applicable in RCC due to a limited number of patients that can be evaluated for all four parameters, and due to a low sensitivity in those patients that can be evaluated. Furthermore, given the differences in percentages of $CD34^+$ myeloid blast cells and $CD34^+$ B-cell progenitors between adults and children, different cut-off values for parameter 1 and 2 may be needed for evaluation of pediatric patients with advanced MDS. To evaluate whether other immunophenotypic abnormalities are present and might aid in diagnosing RCC, more detailed analyses of myeloid maturation patterns using novel software tools are currently underway.[11,12]

Acknowledgements

AMA was supported by the KiKa Foundation, Amstelveen, The Netherlands.

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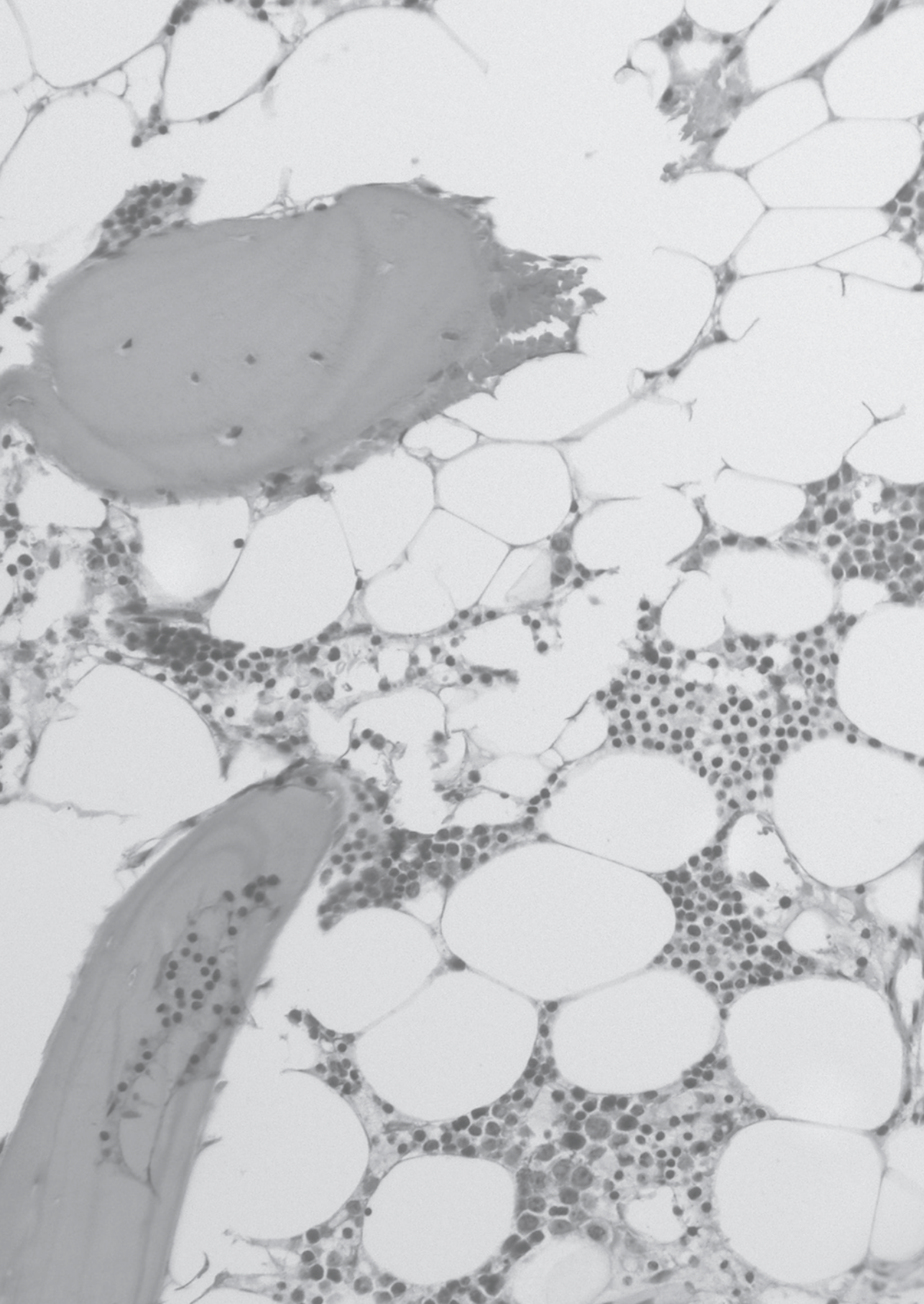

8

Bone marrow immunophenotyping by flow cytometry in refractory cytopenia of childhood – a prospective study by EWOG-MDS

Anna M. Aalbers, Marry M. van den Heuvel-Eibrink, Irith Baumann, Michael Dworzak, Henrik Hasle, Franco Locatelli, Barbara De Moerloose, Markus Schmugge, Ester Mejstrikova, Michaela Reiterova, Marco Zecca, C. Michel Zwaan, Jeroen G. te Marvelde, Anton W. Langerak, Jacques J.M. van Dongen, Rob Pieters, Charlotte M. Niemeyer, Vincent H.J. van der Velden

Submitted

ABSTRACT

Refractory cytopenia of childhood (RCC) is the most common type of childhood myelodysplastic syndrome (MDS). Because the majority of patients with RCC have a normal karyotype and a hypocellular bone marrow, differentiating RCC from the immune-mediated bone marrow failure syndrome (very) severe aplastic anemia ((v)SAA) can be challenging. Flow cytometric immunophenotyping of bone marrow has been shown to be a valuable diagnostic tool in differentiating MDS from non-clonal cytopenias in adults. Here, we performed the first comprehensive flow cytometric analysis of immature myeloid and lymphoid cells, nucleated erythroid cells, granulocytes, monocytes, and lymphoid cells in bone marrow obtained from a large prospective cohort of 81 RCC patients. RCC patients had a strongly reduced myeloid compartment, but not as severe as (v)SAA patients. Furthermore, the number of flow cytometric abnormalities differed significantly between RCC patients and healthy controls, and between RCC and pediatric patients with (v)SAA or advanced MDS. We conclude that, although dysplastic changes in RCC are present in a lower frequency than previously reported in adults with low-grade MDS, flow cytometric immunophenotyping could be a relevant addition to histopathology in the diagnosis of RCC, and might aid in differentiating RCC from (v)SAA.

INTRODUCTION

Myelodysplastic syndrome (MDS) in childhood is rare and has an estimated annual incidence of 0.8 to 1.8 per million children aged 0 to 14 years.[1-3] Refractory cytopenia of childhood (RCC), defined as myelodysplasia without an increased blast count, is the most common variant of pediatric MDS. Diagnosis of RCC is established according to the 2008 WHO criteria.[4] Based on these criteria, a distinction can be made between RCC and the immune mediated bone marrow failure syndrome (very) severe aplastic anemia ((v)SAA), with presence or absence of patchy erythropoiesis, respectively, as main differentiating parameter.[5] Nonetheless, because the majority of children with RCC have a normal karyotype and 80% of patients have a hypocellular bone marrow, differentiating RCC from (v)SAA can be challenging.[4,6] Similar challenges are encountered in distinguishing (v)SAA and other non-clonal cytopenias from low-grade MDS in adults, especially in cases without specific morphologic or cytogenetic aberrations. Flow cytometric immunophenotyping is a valuable addition to morphology in the diagnosis of MDS in adults.[7] Several studies showed that abnormalities detected by flow cytometry in myelomonocytic, erythroid and/or myeloid blast cells[8-17] can be of diagnostic and prognostic relevance in adult MDS.[18-20]

In pediatric MDS, only a limited number of flow cytometric immunophenotyping studies have been reported. In advanced pediatric MDS, CD7 expression on myeloid blast cells was described to correlate with dismal survival.[21] We recently showed that in RCC, a simple and reproducible flow cytometric scoring system, described by Ogata and others as diagnostic tool in adult low-grade MDS,[15,17] cannot be applied due to a low sensitivity in the limited number of RCC patients that can be evaluated completely.[22]

In the present study, we performed for the first time a comprehensive flow cytometric analysis of the maturing granulocytic, monocytic, and erythroid lineages in bone marrow aspirates of 81 RCC patients, collected prospectively by the European Working Group of MDS in Childhood (EWOG-MDS). We evaluated whether flow cytometry can aid in distinguishing RCC from (v)SAA and advanced MDS, and correlated flow cytometric findings with clinical characteristics of RCC patients.

METHODS

Patients and controls

Bone marrow samples for flow cytometric immunophenotyping were obtained from 81 primary RCC patients ≤18 years of age, who were not previously treated with immunosuppressive therapy (IST). Included patients were consecutive, diagnosed between June 2005 and December 2011, and enrolled in the prospective, multi-center studies EWOG-MDS RC06 and EWOG-MDS 2006 (ClinicalTrial.gov identifiers: NCT00499070 and NCT00662090). Institutional review boards of participating institutions approved the studies and patients

and/or parents or legal guardians of patients provided written informed consent for study participation in accordance with local law and regulations. RCC was diagnosed according to WHO criteria[4] and confirmed by central review of bone marrow morphology and histology in participating national study centers by reference pathologists of EWOG-MDS. Data were reported to the coordinating study center of the EWOG-MDS study group through standardized data collection forms. Bone marrow samples obtained from 17 pediatric (v)SAA patients (median age: 9.6 years; range: 1.6-18.1 years), 7 pediatric patient with advanced MDS (refractory anemia with excess blasts (RAEB) or refractory anemia with excess blasts in transformation (RAEB-t) (median age: 14.3, range: 3.9-17.7 years), and 9 healthy adult stem cell donors served as controls. In the RCC cohort, PNH clones and T-cell receptor Vβ skewing were detected as described previously.[23,24]

HLA typing

Patients were HLA-typed for human leukocyte antigen (HLA)-A, -B, -C, -DR and -DQ by serological or molecular methods in the participating study centers.

Bone marrow immunophenotyping by flow cytometry

Bone marrow samples were collected in heparinized tubes, sent to the Erasmus MC, and generally analyzed within 24 hours from collection. A minimum of 500 µL bone marrow was lysed with NH_4Cl, washed, resuspended in 350 µL PBS/BSA or to a maximum concentration of $20x10^6$ cells/mL, and 50 µL aliquots were stained for 15 minutes at room temperature with a monoclonal antibody panel as described in Supplemental Table 1. Cells were acquired on a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using Infinicyt software (Cytognos, Salamanca, Spain) or BD FACSDiva Software, version 6.1.2 (BD Biosciences).

After exclusion of debris and dead cells based on scatter and CD45 expression, cell populations were identified using CD45 expression and forward or sideward scatter (FSC/SSC) properties, and additional markers when indicated. Granulocytes were defined as $CD45^{dim/bright}/SSC^{int/high}$ cells, and $CD33^{dim}$ when indicated; monocytes as $CD45^{dim/bright}/SSC^{int}$, and $CD64^{+}$ when indicated; nucleated erythroid cells as $CD45^{dim/neg}$, SSC^{low} cells, excluding $CD71^{neg}$ and $CD235^{neg}$ cells (other cells) and excluding $CD71^{neg}$ and $CD235^{dim}$ cells (unlyzed erythrocytes). Myeloid blast cells were defined as $CD45^{dim}/SSC^{low/int}/CD34^{+}$ cells, and $CD117^{+}$ when indicated; promyelocytes as $CD45^{dim}/SSC^{low/int/high}/CD34^{-}/CD117^{+}/CD13.33^{+}$ cells. $CD34^{+}$ B-cell precursors were defined as $CD45^{dim}/SSC^{low}/CD34^{+}/CD19^{+}$ cells. Other cell types that were identified were lymphocytes, defined as $CD45^{bright}/SSC^{low}$ cells; B cells as $SSC^{low}/CD19^{+}$ cells; mature B cells as $SSC^{low}/CD19^{+}/CD10^{-}$; B-cell precursors as $SSC^{low}/CD19^{+}/CD10^{+}$ (irrespective of CD34 positivity); T cells as $SSC^{low}/CD3^{+}$; NK cells as $CD3^{-}/CD16.56^{+}$; basophils as $CD123^{+}/HLA\text{-}DR^{-}$, and plasmacytoid dendritic cells (DCs) as $CD123^{+}/HLA\text{-}DR^{+}$. Unless otherwise indicated, percentages of cell populations indicate proportions within all nucleated cells.

Parameters that were evaluated in myeloid and lymphoid progenitor cells, maturing granulocytes, monocytes and erythrocytes are described in Table 1 and are largely in line with European LeukemiaNet (ELN) recommendations for flow cytometry in MDS.[7] Maturation patterns of the myelomonocytic lineage, and expression of CD markers on myeloid blast cells, granulocytes and monocytes were compared with reference images (Infinicyt settings: resolution 128, maximum density increment, linear) that were generated, depending on the combination of antibodies used, by merging flow cytometric data from 6-8 normal bone marrow samples (100.000 events per sample, merged into 100.000 events). Maturation patterns and expression of CD markers were scored as aberrant when the expression of the analyzed population deviated more than 0.5 log decade from the expression of the reference population. Granularity of myeloid blasts ($CD117^{+}$), granulocytes ($CD33^{dim}$), and monocytes ($CD64^{+}$) was expressed as the relative SSC compared to lymphocytes. The relative granularity of myeloid blasts ($CD117^{+}$), granulocytes ($CD33^{dim}$), and monocytes ($CD64^{+}$) of individual RCC patients or controls was scored as abnormal when the relative granularity was less than 2 SD of the mean of the relative granularity of healthy controls. When <50 myeloid blasts, <1000 granulocytes, or <50 nucleated erythroid cells were present, percentages were reported, but cell populations were not further characterized.

The total number of flow cytometric abnormalities was calculated by adding up all flow cytometric abnormalities described in Table 1.

Statistical analyses

Statistical analyses were performed with SPSS 20 (IBM, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Categorical variables were compared using the Chi-square test or Fisher's exact test. Continuous variables were compared using the Mann-Whitney-U test or the Kruskal-Wallis test when more than two groups were compared. All reported *P*-values are two-sided and were considered statistically significant when <0.05; *P*-values >0.1 were reported as non-significant (NS), whereas those between 0.05 and 0.1 were reported in detail.

RESULTS

Patient characteristics

A total of 81 treatment-naïve RCC patients (40 male, 41 female), with a median age at diagnosis of 10.5 years (range: 1-18 years), were analyzed. Median time from diagnosis to analysis was 1.5 months. Clinical characteristics of the included patients are summarized in Table 2. Hypocellular bone marrow was reported in 80% of patients, similar to the previously reported frequency of 81% of primary RCC patients in an interim analysis of studies EWOG-MDS 1998 and 2006.[6] Conventional cytogenetics displayed a normal

Table 1 Analyzed parameters per cell population.

Myeloid and lymphoid progenitors	Erythrocytes	Granulocytes	Monocytes	Other cells
Percentage of $CD34^+$ B-cell precursors	Percentage of erythroid cells ($CD235^+$ and/or $CD71^+$)	Percentage of granulocytes ($CD33^{dim}$)	Percentage of monocytes ($CD64^+$)	Percentage of lymphocytes
Percentage of myeloid blasts ($CD34^+/CD117^+$)	Heterogeneous expression of CD71	Percentage of $CD10^+$ and $CD10^-$ granulocytes	SSC monocyte/lymphocyte	Percentage of T cells
Percentage of promyelocytes ($CD34^-/CD117^+/CD13.33^+$)	Heterogeneous expression of CD36	SSC granulocyte/ lymphocyte	Abnormal expression of CD13	Percentage of NK cells
SSC myeloid blast/ lymphocyte		Abnormal CD11b-CD13 pattern	Abnormal expression of CD14	Percentage of B cells
Abnormal expression of CD11b		Abnormal CD16-CD13 pattern	Abnormal expression of CD36	Percentage of mature B-cells ($CD19^+/CD10^-$)
Abnormal expression of CD117		Abnormal expression of CD33	Abnormal expression of CD33	Percentage of B-cell precursors ($CD19^+/CD10^+$)
Abnormal expression of CD34		Expression of CD34	Abnormal HLA-DR-CD11b pattern	Percentage of basophils
Expression of CD15		Abnormal expression of CD15	Expression of CD34	Percentage of plasmacytoid DCs
Expression of CD64		Abnormal expression of CD64	Abnormal expression of CD15	
Abnormal expression of HLA-DR		Expression of HLA-DR	Abnormal expression of CD64	
Expression of CD7			Abnormal expression of HLA-DR	
Expression of CD56			Expression of CD56	

Table 2 Clinical and laboratory characteristics of included RCC patients.

Characteristic	
Number of patients	81
Median age at diagnosis, years (range)	10.5 (1-18)
Male sex, No. (%)	40 (49)
Hypocellular bone marrow, No. (%)	64 of 80 (80)
Cytogenetics, No. (%) [1]	
Normal	61 of 71 (86)
Monosomy 7 [2]	5 of 71 (7)
Other [3]	5 of 71 (7)

[1] In 9 of 80 patients (11%) no karyotype was obtained due to insufficient metaphases; in 1 patient, no data were available.
[2] Includes 1 patient with monosomy 7 and other aberrations.
[3] Includes 1 patient with del(7q), 2 patients with trisomy 8, and 2 patients with rare aberrations.

karyotype in 61 of 71 evaluable patients (86%, which is slightly higher than the previously reported frequency of 77%),[6] monosomy 7 in 5 of 71 patients (including 1 patient with monosomy 7 and additional aberrations) (7%), and other aberrations in 5 of 71 patients (trisomy 8 in 2 patients, rare aberrations in 2 patients, and del(7q) in 1 patient) (7%). In 9 patients, no karyotype was obtained due to insufficient metaphases; in 1 patient, no information was available.

Cellular composition of RCC versus healthy control bone marrow

Patients with RCC showed a strong decrease in the myeloid and lymphoid progenitor compartment compared to healthy controls. The median percentage of myeloid blast cells ($CD117^+$) was only 0.1% in RCC, compared to 0.9% in controls; similarly, the percentages of promyelocytes and nucleated erythroid cells were decreased. As previously observed in adult MDS patients,[19] the percentage of $CD34^+$ B-cell precursors was significantly decreased in RCC, with a median percentage of 0.0% in RCC versus 0.3% in controls (Table 3).

In parallel with a reduction in immature myeloid cells, the percentage of granulocytes ($CD33^{dim}$) was strongly decreased in RCC (median, 27.5% in RCC versus median, 60.9% in controls), with a relative increase in mature $CD10^+$ granulocytes in RCC (median, 47.9% in RCC versus median, 28.7% in controls). The percentage of monocytes ($CD64^+$) was similar in RCC patients and controls, although monocytes were low or absent in some RCC patients (Table 3). Remarkably, in one RCC patient, 23% of all nucleated cells were monocytes.

Likely partly due to the reduction in myeloid cells, the proportion of lymphocytes in RCC was strongly increased, with a median of 47.7% in RCC versus 11.4% in controls. The median percentage of T cells was 39% in RCC, and 7.7% in controls. The percentages of NK

Table 3 Bone marrow cellular composition in RCC patients and controls.

	NBM	RCC	(v)SAA	RAEB(-t)	RCC versus		
	(N=9)	(N=81)	(N=17)	(N=7)	NBM	(v)SAA	RAEB(-t)
Cell population (%)	*median (range)*	*median (range)*	*median (range)*	*median (range)*	*P-value*	*P-value*	*P-value*
Myeloid and lymphoid progenitors							
Myeloid blasts ($CD117^+$)	0.9 (0.8-1.2)	0.1 (0-2.1)	0.0 (0.0-0.3)	8.9 (1-31.9)	**0.000**	**0.000**	**0.000**
$CD34^+$ B-cell precursors	0.3 (0.1-0.8)	0.0 (0.0-4.6)	0.0 (0.0-0.1)	0.0 (0.0-1.4)	**0.000**	**0.008**	NS
Promyelocytes	2.1 (0.7-2.7)	0.1 (0.0-2.8)	0.0 (0.0-0.3)	1.6 (0.0-5.4)	**0.000**	**0.001**	**0.002**
Erythroid cells							
Erythroid cells	9.8 (4.2-15.6)	3.4 (0.0-43.1)	0.8 (0.0-10.1)	7.9 (2.3-23.8)	**0.001**	**0.004**	**0.035**
Myeloid cells							
Granulocytes ($CD33^{dim}$)[1]	60.9 (57.4-68.5)	27.5 (0.0-73.5)	9.1 (0.0-62.4)	39.7 (7.6-68.4)	**0.000**	**0.035**	NS
$CD10^-$ granulocytes[2]	66.8 (40.8-78)	52.1 (3.1-88)	50.7 (20.3-87.4)	62.5 (44.2-96.3)	**0.047**	NS	0.097
$CD10^+$ granulocytes[2]	28.7 (18.9-49.6)	47.9 (12-96.9)	48.2 (12.2-79.8)	36.2 (3.7-53)	**0.002**	NS	0.069
Monocytes ($CD64^+$)	3.2 (2.8-5.1)	3.4 (0.0-22.8)	1.5 (0.0-6.1)	4.3 (0.0-24.8)	NS	**0.025**	NS
Lymphoid[1] and other cells							
Lymphocytes	11.4 (5.8-13.1)	47.7 (4.5-97.9)	71.9 (21.2-96.2)	18.7 (8.9-64.3)	**0.000**	**0.021**	**0.017**
B-cells	3.1 (1.3-7.3)	6.3 (0.2-24.4)	13.1 (5.3-42.7)	3.6 (1.6-11.8)	**0.017**	**0.000**	**0.031**
Mature B cells, $CD19^+/CD10^-$	2.0 (0.9-3)	5.1 (0.2-21.7)	13.1 (3.1-42.7)	3.3 (1.0-5.5)	**0.001**	**0.000**	**0.037**
B-cell precursors, $CD19^+/CD10^+$	1.3 (0.4-4.3)	0.3 (0-21.5)	0.1 (0.0-4.5)	0.2 (0.0-6.3)	**0.012**	NS	NS
T-cells	7.7 (4.2-9.5)	39 (2.3-86.2)	49.5 (14.1-85.3)	11.3 (6.9-63.5)	**0.000**	NS	**0.030**
NK-cells	1.1 (0.2-2.4)	2.7 (0.2-11.7)	3.9 (1.6-10.2)	0.9 (0.2-2.2)	**0.009**	**0.016**	**0.005**
Basophils	0.2 (0.0-0.3)	0.1 (0.0-1.0)	0.0 (0.0-0.8)	0.0 (0.0-1.3)	0.093	**0.007**	NS
Plasmacytoid DCs	0.3 (0.3-0.4)	0.2 (0.0-1.1)	0.0 (0.0-0.4)	0.3 (0.0-1.5)	**0.001**	**0.005**	NS

[1] Evaluated in 80 RCC patients; no data in 1 patient.

[2] Evaluated in 75 RCC patients, 13 (v)SAA patients, and 6 RAEB/RAEB-t patients due to absence of sufficient granulocytes in remaining patients.

NBM, normal bone marrow.

and total B cells were also increased in RCC, but the proportion of B-cell precursors was reduced. The percentage of basophils in RCC was not different from healthy controls, while the percentage of plasmacytoid DCs was reduced. Results are summarized in Table 3 and Figure 1.

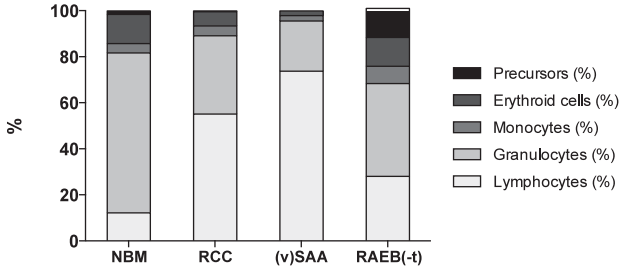

Figure 1 Cellular composition of bone marrow in RCC patients and controls.

For graphical representation, means of the main cell populations were calculated per patient and control group, and scaled to 100%. Precursors consist of myeloid blast cells and $CD34^+$ B-cell precursors.

Cellular composition of bone marrow of RCC versus other controls

Although the proportions of progenitor and mature myeloid cells were strongly reduced in RCC patients compared to healthy controls, in (v)SAA this reduction was even more severe. The median percentage of myeloid blasts, promyelocytes, and $CD34^+$ B-cell precursors was 0.0% in (v)SAA; the proportion of nucleated erythroid cells was 0.8%. The percentage of granulocytes ($CD33^{dim}$), already reduced in RCC compared to healthy controls, was further decreased in (v)SAA (median, 9.1%) compared to RCC, as was the percentage of monocytes ($CD64^+$). The proportion of lymphocytes was strongly increased in (v)SAA compared to RCC, with a median of 71.9%, while the percentage of T cells was similar in (v)SAA and RCC. Percentages of B and NK cells were higher in (v)SAA, while basophils and plasmacytoid DCs were reduced in (v)SAA compared to RCC (Table 3).

Not unexpectedly, compared to patients with RCC, pediatric patients with RAEB(-t) had increased percentages of myeloid blasts ($CD117^+$), promyelocytes, and erythroid cells, while the percentage of $CD34^+$ B-cell precursors, granulocytes and monocytes were equally low. The proportion of lymphocytes, T, NK, and B cells were lower in RAEB(-t) than in RCC patients; the percentages of basophils and plasmacytoid DCs were similar. Results are summarized in Table 3 and Figure 1.

Immunophenotype of myeloid blast cells in RCC and controls

In adult MDS patients, the granularity of myeloid blast cells is frequently decreased, and lineage infidelity markers are often detected.[19] In our RCC series, the granularity of myeloid blast cells ($CD117^{+}$), expressed as ratio to the SSC of lymphocytes, was increased compared to healthy controls, but not different from (v)SAA patients (median, 2.15, 1.76, and 2, respectively, *P*=0.000 and *P*=NS). When observed individually, 1 of 45 RCC patients with sufficient evaluable myeloid blast cells ($CD117^{+}$) had a relative granularity of less than 2 SD of the mean of healthy controls, whereas none of the 2 evaluable (v)SAA patients and none of the healthy controls had. Expression of the lineage infidelity marker CD7 on myeloid blast cells was detected in 6 of 45 evaluable RCC patients (13%), and in none of the healthy controls or (v)SAA patients; expression of CD56 was also detected in 6 of 45 RCC patients, and in none of the healthy controls or (v)SAA patients. In RAEB(-t) patients, multiple abnormalities were detected in myeloid blast cells (Table 4). The median total number of immunophenotypic abnormalities in myeloid blast cells was 0 in RCC patients, healthy controls, and (v)SAA patients, and 1 in RAEB(-t) patients.

Immunophenotype of erythroid cells in RCC and controls

The most frequently observed abnormality in nucleated erythroid cells in RCC was heterogeneous expression of CD71 (transferrin receptor) and CD36 (thrombospondin receptor), which was detected in 42 of 72 RCC patients (58%) with evaluable erythroid cells (Figure 2A-C). In healthy controls, one patient showed heterogeneous expression of CD36; 2 of 11 evaluable (v)SAA patients (18%) and 3 of 7 RAEB(-t) patients (43%) showed heterogeneous expression of CD36 and CD71 (Table 4). In the RCC, (v)SAA, and RAEB(-t) patients with heterogeneous expression of CD71 and CD36, this always co-occurred. The median total number of immunophenotypic abnormalities in erythroid cells was 2 in RCC patients, and 0 in healthy controls, (v)SAA patients, and RAEB(-t) patients.

Immunophenotype of granulocytes in RCC and controls

No differences in the relative SSC of granulocytes ($CD33^{dim}$) were observed between RCC and healthy controls, and between RCC and (v)SAA patients (not shown). Individually, of the 75 RCC patients with sufficient granulocytes ($CD33^{dim}$) to evaluate, 6 (8%) had a relative granularity that was less than 2 SD of the relative granularity in healthy controls, 3 of 13 evaluable (v)SAA patients (23%), and none of the healthy controls had. An abnormal pattern of CD16 and CD13 expression on maturing granulocytes was detected in 14% of evaluable RCC patients, comparable to 15% detected in (v)SAA; none of the healthy controls showed and abnormal CD16-CD13 pattern (Figure 2D-F) (of note, the mere absence of a granulocyte subset was not considered an abnormal pattern). Less frequently occurring abnormalities in RCC and (v)SAA, and abnormalities detected in RAEB(-t) patients are described in Table 4. The median total number of immunophenotypic abnormalities in granulocytes was 0 in RCC patients, healthy controls, and (v)SAA patients, and 1 in RAEB(-t) patients.

Table 4 Flow cytometric abnormalities in RCC patients and controls.

Flow cytometric abnormality	NBM	RCC	(v)SAA	RAEB(-t)
Myeloid blast cells, No. (%)				
Abnormal granularity	0 of 7 (0)	1 of 45 (2)	0 of 2 (0)	3 of 7 (43)
Abnormal expression of CD34	0 of 7 (0)	0 of 45 (0)	0 of 2 (0)	2 of 7 (29)
Abnormal expression of CD117	0 of 7 (0)	1 of 45 (2)	0 of 2 (0)	2 of 7 (29)
Abnormal expression of CD11b	0 of 7 (0)	0 of 45 (0)	0 of 2 (0)	0 of 7 (0)
Abnormal expression of HLA-DR	0 of 7 (0)	0 of 45 (0)	0 of 2 (0)	1 of 7 (14)
Expression CD64	0 of 7 (0)	0 of 45 (0)	0 of 2 (0)	0 of 7 (0)
Expression of CD15	0 of 7 (0)	0 of 45 (0)	0 of 2 (0)	0 of 7 (0)
Expression of CD7 >20%	0 of 9 (0)	6 of 45 (13)	0 of 2 (0)	2 of 7 (29)
Expression of CD56 >20%	0 of 9 (0)	6 of 45 (13)	0 of 2 (0)	0 of 7 (0)
Erythroid cells, No. (%)				
Heterogeneous expression of CD71	1 of 9 (11)	42 of 72 (58)	2 of 11 (18)	3 of 7 (43)
Heterogeneous expression of CD36	0 of 9 (0)	42 of 72 (58)	2 of 11 (18)	3 of 7 (43)
Granulocytes, No. (%)				
Abnormal granularity	0 of 9 (0)	6 of 75 (8)	3 of 13 (23)	1 of 7 (14)
Decreased myeloid/lymphoid ratio[1]	0 of 9 (0)	52 of 81 (64)	13 of 17 (76)	1 of 7 (14)
Expression of HLA-DR	0 of 7 (0)	2 of 75 (3)	0 of 13 (0)	1 of 7 (14)
Abnormal expression of CD64	0 of 7 (0)	0 of 75 (0)	0 of 13 (0)	0 of 7 (0)
Abnormal expression of CD15	0 of 7 (0)	0 of 75 (0)	0 of 13 (0)	0 of 7 (0)
Expression of CD34	0 of 7 (0)	0 of 75 (0)	0 of 13 (0)	0 of 7 (0)
Abnormal expression of CD33[2]	0 of 9 (0)	0 of 75 (0)	0 of 13 (0)	2 of 7 (29)
Abnormal CD11b-CD13 pattern[3]	0 of 9 (0)	4 of 74 (5)	0 of 13 (0)	3 of 7 (43)
Abnormal CD16-CD13 pattern[4]	0 of 9 (0)	10 of 73 (14)	2 of 13 (15)	5 of 7 (71)
Monocytes, No. (%)				
Abnormal granularity	1 of 7 (14)	3 of 74 (4)	0 of 13 (0)	3 of 6 (50)
Abnormal expression of HLA-DR	0 of 7 (0)	0 of 74 (0)	0 of 13 (0)	0 of 6 (0)
Abnormal expression of CD64	0 of 7 (0)	0 of 74 (0)	0 of 13 (0)	0 of 6 (0)
Abnormal expression of CD15	0 of 7 (0)	0 of 74 (0)	0 of 13 (0)	1 of 6 (17)
Expression of CD34	0 of 7 (0)	0 of 74 (0)	0 of 13 (0)	1 of 6 (17)
Abnormal HLA-DR-CD11b pattern	0 of 7 (0)	0 of 74 (0)	0 of 13 (0)	0 of 6 (0)
Abnormal expression of CD33[2]	0 of 9 (0)	3 of 74 (4)	0 of 13 (0)	0 of 6 (0)
Abnormal expression of CD14	0 of 9 (0)	0 of 74 (0)	0 of 13 (0)	0 of 6 (0)
Abnormal expression of CD36	0 of 9 (0)	0 of 74 (0)	0 of 13 (0)	2 of 6 (33)
Abnormal expression of CD13[5]	0 of 9 (0)	3 of 72 (4)	0 of 13 (0)	0 of 4 (0)
Expression of CD56 >20%	0 of 9 (0)	15 of 74 (20)	0 of 13 (0)	0 of 6 (0)

[1] A decreased myeloid/lymphoid ratio was not counted as flow cytometric abnormality in the final sum of abnormalities. [2] When both granulocytes and monocytes had a decreased expression of CD33, this was not considered abnormal. [3] No data available in one RCC patient. [4] In one RCC patient, a PNH clone in the granulocytic lineage of 86% was present; no data available in another RCC patient. [5] In one RCC patient, insufficient monocytes for reliable analysis were present in this labeling; no data available in one RCC patient. In 2 RAEB patients, monocytes could not be reliably gated based on SSC and CD45 expression. NBM, normal bone marrow.

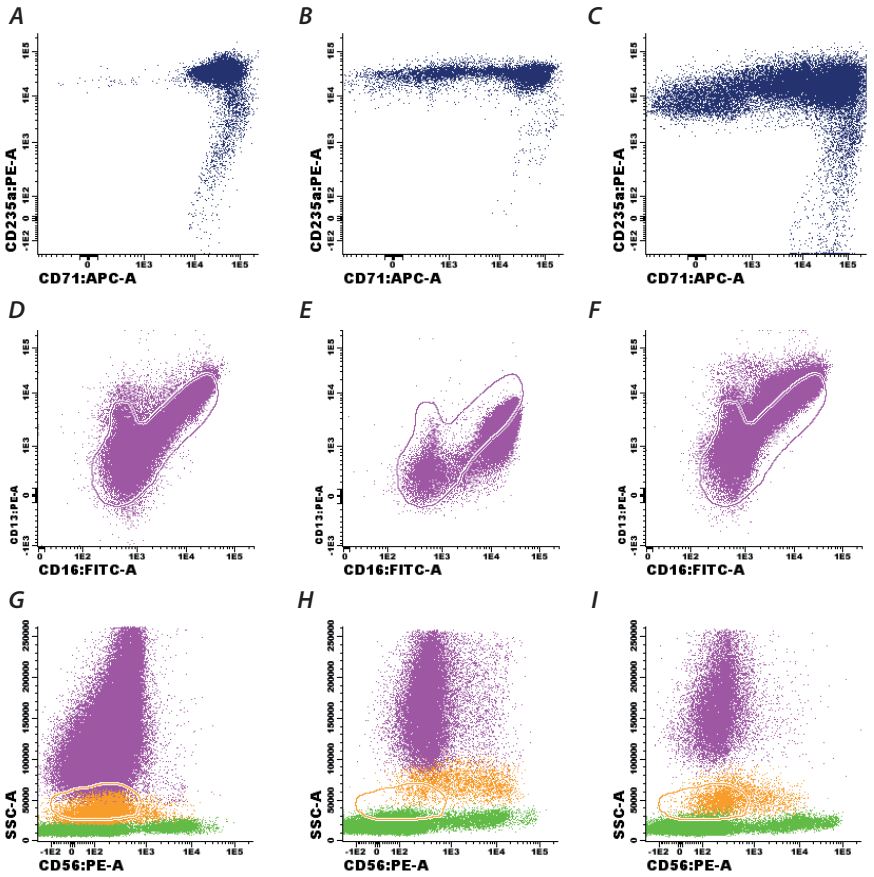

Figure 2 Immunophenotype of nucleated erythroid cells and granulocytes in RCC patients and controls.

(A) Normal expression of CD71 in healthy control bone marrow, ID NBM 023. (B) Heterogeneous CD71 expression in RCC patient, ID CH028. (C) Heterogeneous CD71 expression in RCC patient, ID I 220. (D) Normal pattern of CD16-CD13 expression in healthy control bone marrow, ID NBM 023. (E) Abnormal pattern of CD16-CD13 expression in RCC patient, ID D 663. (F) Abnormal pattern of CD16-CD13 expression in RCC patient, ID D 555. (G) Absence or low level of CD56 expression on monocytes, indicated in orange, in healthy control bone marrow, ID NBM 023. Granulocytes and lymphocytes are indicated in pink and green, respectively. (H) Aberrant expression (>20%) of CD56 on monocytes in RCC patient, ID CZ078. (I) Aberrant expression (>20%) of CD56 on monocytes in RCC patient, ID SC126. Pink and orange lines indicate reference image of normal granulocytes and monocytes, respectively.

Immunophenotype of monocytes in RCC and controls

The relative SSC of monocytes ($CD64^+$) in RCC was increased compared to healthy controls, but was not different from (v)SAA patients (median, 3.1, 2.8, and 3, respectively, *P*=0.006 and *P*=NS). Three of 74 RCC patients (4%) with sufficient cells to evaluate, none of the 13 evaluable (v)SAA patients, and one healthy control had a decreased granularity. Expression of CD56 on more than 20% of monocytes occurred in 15 of 74 RCC patients (20%), and in none of the healthy controls and (v)SAA patients (Figure 2G-I). Abnormalities detected in RCC patients and controls, including RAEB(-t), are described in Table 4. The median total number of immunophenotypic abnormalities in monocytes was 0 in RCC patients, healthy controls, and (v)SAA patients, and 1 in RAEB(-t) patients.

Total number of flow cytometric abnormalities in RCC compared to controls

Of the 81 RCC patients, 57 (70%) displayed one or more flow cytometric abnormalities, and 49 (60%) two or more. In 1 healthy control bone marrow sample, flow cytometric abnormalities were present. This control sample displayed a decreased granularity of the monocytes, and heterogeneous expression of CD71 on nucleated erythroid cells. Of the (v)SAA patients, 6 of 17 (35%) had one or more, and 2 of 17 (12%) had two or more abnormalities. In the RAEB(-t) group, all patients displayed two or more abnormalities. Using a cutoff of 2 abnormalities, flow cytometry has a sensitivity for the recognition of RCC of 60%, and a specificity, using (v)SAA patients as control group, of 88%.

The median number of abnormalities was 2 (range, 0-6) in the RCC patients, which is higher than the number of abnormalities in healthy control bone marrow (median, 0; range 0-2, *P*=0.0018), and higher than in the (v)SAA patients (median, 0; range, 0-3; *P*=0.0012), but lower than in RAEB(-t) patients (median, 4; range, 2-9; *P*=0.0017) (Figure 3).

Of interest, one patient with MDS-RAEB had no detectable $CD34^+$ B-cell precursors, very few $CD19^+$/$CD10^+$ B-cell precursors and NK cells, a large proportion of large granular lymphocyte-type cells ($CD3^+CD16.56^+$), no monocytes, and an increased percentage of myeloid blast cells, that were phenotypically normal. Granulocytes showed an abnormal CD11b-CD13 and CD16-CD13 expression (Supplemental Figure 1). This immunophenotype was previously described in patients with the autosomal dominant and sporadic monocytopenia and mycobacterial infection (MonoMAC) syndrome who developed MDS,[25,26] in whom germline mutations in *GATA2* were later identified.[27-31] Indeed, in the present MDS-RAEB patient, a *GATA2* mutation was detected.

Flow cytometric abnormalities and clinical characteristics in RCC

No significant associations were detected between the number of flow cytometric abnormalities in RCC patients and age or sex, HLA-DR15, bone marrow cellularity, transfusion dependency at diagnosis, the presence of a PNH clone, or skewing of the T-cell receptor Vβ chain (data not shown). Of interest, however, the 5 RCC patients carrying the

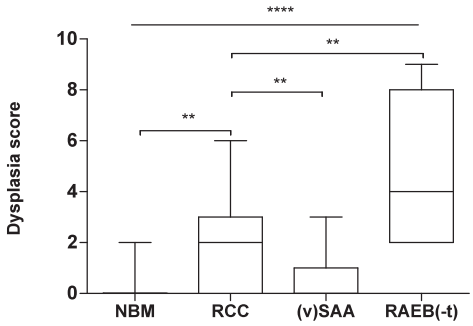

Figure 3 Number of flow cytometric abnormalities in RCC patients and controls.

Lines indicate medians. Boxes extend from the 25th to 75th percentile; lines in the boxes indicate medians, whiskers indicate minimum and maximum values. **, $P<0.01$; ****, $P<0.0001$.

cytogenetic abnormality monosomy 7, associated with a high risk of progression to AML, all had at least two immunophenotypic abnormalities. No relation was detected between the number of flow cytometric abnormalities and white blood cell count, absolute neutrophil count, hemoglobin level, MCV, or thrombocyte count (data not shown). Only 22 RCC patients with a hypocellular bone marrow included in this study were treated with IST. In this limited number of patients, no significant association was observed between the number of flow cytometric abnormalities and IST response (data not shown).

Combination of cytopenia and flow cytometric abnormalities to identify RCC and (v)SAA patients

In the majority of RCC patients, immature myeloid and/or lymphoid cells were reduced in numbers, but still detectable, while in the vast majority of (v)SAA patients, myeloid blast cells and $CD34^+$ B-cell precursors were absent: both cell types were absent in 27 of 81 RCC patients (33%) and in 15 of 17 (v)SAA patients (88%). Two or more immunophenotypic abnormalities were detected in 49 of 81 RCC patients (60%), and in 2 of 17 (v)SAA patients (12%). If a diagnosis of RCC was considered if myeloid blast cells and/or CD34+ B-cell precursors were present, or if two or more immunophenotypic abnormalities were detected, 61 of 81 RCC patients (84%) could be correctly classified, whereas the specificity of this combination, using (v)SAA as a control group, was 76%. In 55 RCC patients with a hypocellular bone marrow, and a normal karyotype or insufficient metaphases to obtain a karyotype, 45 patients were correctly identified as RCC, resulting in a sensitivity of the combined score in this group of patients of 82%.

DISCUSSION

Flow cytometric immunophenotyping has been shown to be of value in distinguishing MDS from non-clonal cytopenias in adult patients.[7] In the present study, we evaluated whether flow cytometry can aid in distinguishing RCC from healthy controls, and from (v) SAA and advanced MDS patients, and correlated flow cytometric findings with clinical characteristics of RCC patients. We analyzed percentages and/or dysplastic features of the immature myeloid and lymphoid, nucleated erythroid, maturing granulocytic, monocytic and lymphocyte compartments.

We describe that in RCC the myeloid compartment, both mature and immature, is severely compromised in comparison to healthy controls. This finding is not unexpected, because bone marrow is hypocellular in the majority of RCC cases. In comparison with (v) SAA, however, the reduction in myeloid cells was less severe in RCC, similar to what has been shown previously by Reiterova and others, although in a smaller cohort of pediatric RCC and (v)SAA patients.[32] An important difference between RCC and (v)SAA patients was the complete absence of myeloid blast cells and $CD34^+$ B-cell precursors in the majority of (v)SAA patients, while in RCC, although reduced, in most patients some progenitor cells were still detectable. Of note, in 5 pediatric patients with other cytopenias than RCC or (v) SAA (3 with Fanconi anemia, 1 with transient erythroblastopenia of childhood, 1 with immune thrombocytopenic purpura), the percentages of $CD34^+$ B-cell precursors, myeloid blast cells, and promyelocytes were significantly higher than in RCC (data not shown). We compared the bone marrow composition of pediatric patients with healthy adult controls, because healthy pediatric controls are hardly available. In comparison with healthy pediatric controls, the reduction in myeloid blast cells and $CD34^+$ B-cell precursors in RCC patients would probably be even more severe, because the precursor compartment in children is relatively larger than in adults.[22,33]

In comparison with studies in adult low-grade MDS, we detected relatively few flow cytometric abnormalities in RCC, apart from the severe reduction of myeloid cells. Furthermore, the type of immunophenotypic abnormalities between RCC and adult low-grade MDS differed. One of the commonly occurring abnormalities in adult MDS is an abnormally decreased SSC of granulocytes, which is reflective of hypogranularity. Although in a few RCC patients the relative SSC of granulocytes was <2SD of the mean of healthy controls, in the total RCC cohort the relative SSC of granulocytes was not different from healthy controls or (v)SAA patients. Expression of lineage infidelity markers on myeloid blast cells, another common finding in adult MDS, was also not observed frequently in RCC. In adult MDS, lineage infidelity marker expression correlates with an increased blast percentage, and a more advanced disease stage.[19,34] That RCC is a low grade MDS subtype may partly explain the low frequency of lineage infidelity marker expression on myeloid blast cells. In RCC, the most frequently occurring immunophenotypic abnormality was heterogeneous expression of CD71 and CD36 on erythrocytes in

58% of cases (discussed below), followed by aberrant expression of CD56 on monocytes in 20% of cases, which might reflect stressed hematopoiesis rather than true dysplasia. All other abnormalities occurred in a lower frequency in RCC.

Of interest, although only 5 patients with monosomy 7 were included in the present study, all of them displayed at least 2 flow cytometric abnormalities. RCC with monosomy 7 confers a high risk of progression to AML, but histopathologically, no differences can be detected between RCC cases with or without monosomy 7.[6] Likely, flow cytometry is able to detect subtle dysplastic changes with a greater sensitivity than histopathology, partly because more cells can be examined by flow cytometry.

The histopathological differentiation between RCC and (v)SAA is mainly based on the presence of patchy erythropoiesis with defective maturation and/or the presence of micromegakaryocytes in RCC, and the absence of erythropoiesis and megakaryopoiesis in (v)SAA.[5] These histopathological differences are reflected by the differences we detected by flow cytometry between RCC and (v)SAA. In our flow cytometric analyses, the percentage of nucleated erythroid cells in RCC was higher than in (v)SAA patients. We furthermore detected heterogeneous expression of CD71 and CD36 in a large proportion of RCC patients. The exact meaning of this heterogeneous expression is unclear, but flow cytometric abnormalities in CD71 and/or CD36 expression on nucleated erythroid cells are specific and recurrent findings in adult MDS patients.[8,10,11,35-37] Flow cytometric evaluation of megakaryopoieis is still only limitedly possible, but it was recently shown that abnormalities detected in peripheral blood platelets by flow cytometry are of diagnostic significance in adult MDS.[38] Future studies will have to reveal whether immunophenotyping of platelets and/or megakaryocytes can be of additional value in distinguishing RCC from (v)SAA as well.

Distinguishing RCC from (v)SAA remains of clinical relevance, even though the pathophysiology of RCC seems, similar to (v)SAA, at least in part immune-mediated, also given the good response in selected RCC patients to IST.[23,39] The rate of clonal evolution after IST in RCC patients, ranging from 4% to 14% (in rabbit and horse ATG, respectively),[39] might be higher than in (v)SAA patients, in whom the probability of clonal evolution had dropped to 3% in a recent interim analysis (type of ATG not reported).[5] Furthermore, while the conditioning regimen before hematopoietic stem cell transplantation is myeloablative (reduced intensity in selected patients) in RCC, this is not the case in (v)SAA patients.[40,41]

One of the limitations of our study and of most other studies evaluating the value of flow cytometry as diagnostic tool in MDS, is that, although determining cell numbers by flow cytometry is fairly objective, scoring of dysplasia remains subjective. Therefore, future studies assessing the value of flow cytometry as a means to differentiate RCC from non-clonal cytopenias should employ more standardized protocols and antibody panels, and novel software tools that might be able to more objectively evaluate myeloid dysplasia.[42,43] When our results can subsequently be reproduced in an independent cohort of RCC and (v)SAA patients, flow cytometry immunophenotyping could be included in

the diagnostic work-up of childhood cytopenias also in laboratories with a more limited expertise in flow cytometry.

In summary, we describe that RCC patients have a strongly reduced myeloid compartment in comparison to healthy controls, but the reduction is not as severe as in (v)SAA patients. We furthermore report that the number of abnormalities detected by flow cytometry differs significantly between RCC patients and healthy controls, and between RCC and pediatric patients with (v)SAA or advanced MDS. Our results indicate that, although flow cytometric abnormalities in RCC patients are present at a relatively low frequency, flow cytometric immunophenotyping might be a relevant addition to histopathology and cytogenetic analysis in the diagnosis of RCC.

Acknowledgements

AMA and this research were supported by the KiKa Foundation, Amstelveen, the Netherlands. In the Czech Republic RCC diagnosis and treatment were supported by a grant of the Ministry of Health for conceptual development of research organization 00064203 and grant NT/14534 (University Hospital Motol, Prague, Czech Republic). The authors acknowledge excellent technical support by research technicians from the Leukemia and Lymphoma Diagnostics group from the Department of Immunology and logistic support from the Department of Pediatric Oncology/Hematology, Erasmus MC.

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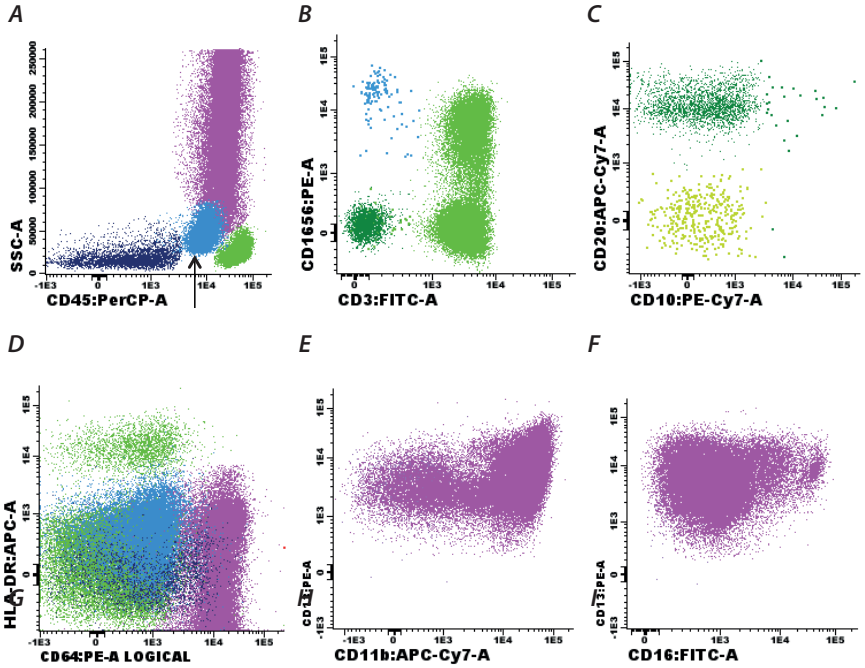
SUPPLEMENTAL INFORMATION



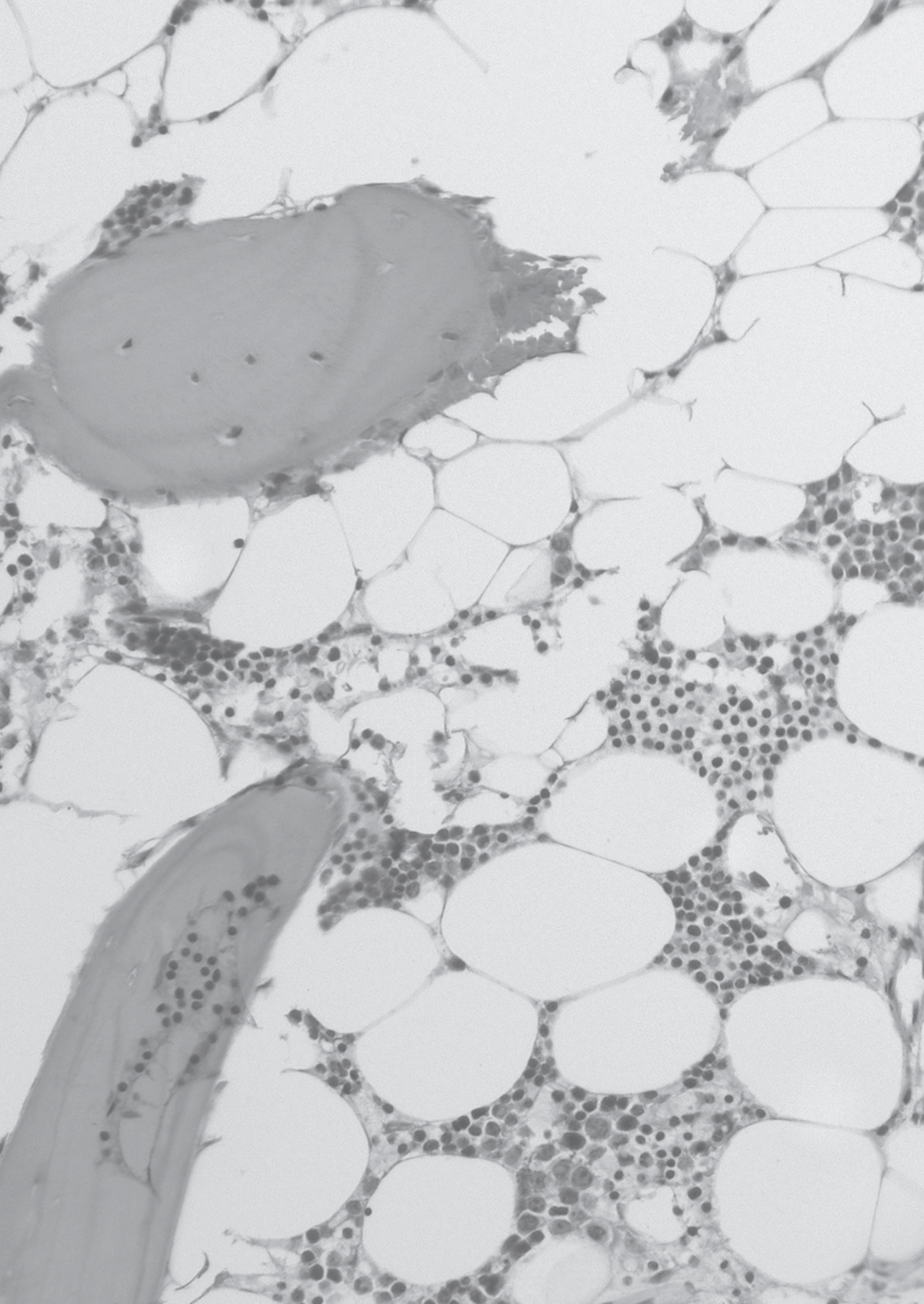
Supplemental Figure 1 Flow cytometric abnormalities in a *GATA2* mutant RAEB patient.

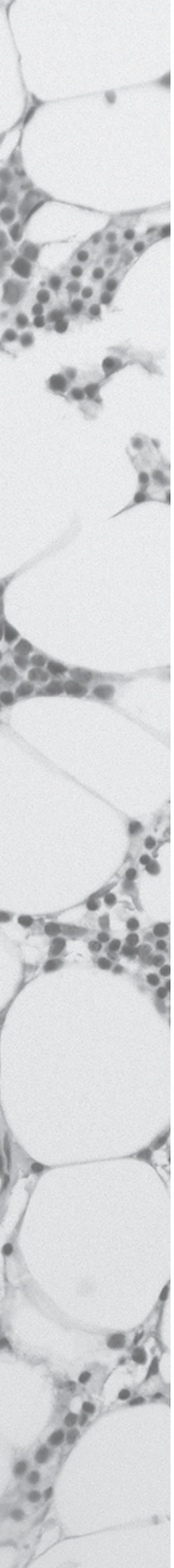
(A) Absence of $CD34^+$ B-cell precursors, indicated by an arrow. Granulocytes, in pink, appear to be hypogranular, but the relative granularity is not smaller than 2SD of healthy controls. (B) Within lymphocytes, low percentage of NK cells ($CD3^-CD1656^+$, 0.13% within white blood cells, 0.4% within lymphocytes), high percentage of T cells ($CD3^+$, 93% within lymphocytes, of which large part is $CD3^+/CD1656^+$). (C) Within $CD19^+$ cells, very low number of B-cell precursors ($CD10^+$). (D) Absence of monocytes ($HLA\text{-}DR^+/CD64^+$), also appreciable from (A). (E) Abnormal CD11b-13 pattern of granulocytes. (F) Abnormal CD16-13 pattern of granulocytes.

Supplemental Table 1 Monoclonal antibody panel.

	FITC			PE			PerCP		
Tube	**Antigen**	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**
1	CD3	SK7	BD	CD16.56	B73.1 and C5.9	BD and Zebra/Dako	CD45	2D1	BD
2	CD16	gran/1,5D2	IBL	CD13	L138	BD	CD45	2D1	BD
3	CD36	CLB-IVC7	IBL	CD33	P67.6	BD	CD45	2D1	BD
4	CD36	CLB-IVC7	IBL	CD235a	JC159	Zebra/Dako	CD45	2D1	BD
5	CD15	MMA	BD	CD64	10.1	Serotech	CD45	2D1	BD
6	CD7	3A1/1, 7F3	Sanquin	CD56	C5.9	Zebra/Dako	CD45	2D1	BD
7	CD34	8G12	BD	CD13.33	L138 and P67.6	BD	CD45	2D1	BD

PE-Cy7/ PC7			APC			APC-Cy7/ APC-H7		
Antigen	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**
CD10	HI10a	BD	CD19	SJ25C1	BD	CD20	L27	BD
CD34	8G12	BD	CD117	104D2	Zebra/Dako	CD11b	ICRF44	BD
CD34	8G12	BD	CD11b	D12	BD	CD14	MO-P9	BD
CD117	104D2D1	Beckman Coulter	CD71	LO 1.1	BD	CD34	8G12	BD
CD34	8G12	BD	HLA-DR	L243	BD	CD11b	ICRF44	BD
CD19	SJ25C1	BD	CD5	L17F12	BD	CD34	8G12	BD
CD123	6H6	e-Bioscience	CD117	104D2	Zebra/Dako	HLA-DR	L243	BD





9

General Discussion and Perspectives

GENERAL DISCUSSION AND PERSPECTIVES

Inherited bone marrow failure syndromes, acquired aplastic anemia, PNH, MDS, and AML are part of a spectrum of myeloid disorders. The clinical presentation of inherited bone marrow failure syndromes can be heterogeneous, and a pathophysiologic overlap between parts of the spectrum is present. In this thesis, we aimed to better characterize different aspects of this spectrum of myeloid disorders in childhood.

Inherited bone marrow failure syndromes

The presentation of inherited bone marrow failure syndromes varies from manifestation in infancy with severe bone marrow failure and congenital abnormalities, to presentation in adulthood with apparently acquired aplastic anemia, myelodysplastic syndrome (MDS), squamous cell carcinoma, pulmonary fibrosis, liver cirrhosis, or acute myeloid leukemia (AML).[1] In the first part of this thesis we explored whether germline mutations causing inherited bone marrow failure syndromes underlie seemingly sporadic AML and acquired aplastic anemia.

Mutations in *SBDS*, *TERT*, and *TERC* are absent in sporadic pediatric AML

Although patients with an established diagnosis of Shwachman-Diamond syndrome (SDS) have an increased risk of acute myeloid leukemia (AML),[2] we did not identify an increased frequency of (compound) hetererozygous or homozygous mutations in *SBDS* in a large pediatric AML cohort, confirming the results from a smaller study in de novo AML.[3,4] We also did not detect an increased frequency of mutations in the telomerase complex genes *TERT* and *TERC*, which underlie dyskeratosis congenita.[5] This is in contrast with a recent study in adult AML patients, in whom germline loss-of-function mutations in *TERT* were detected, presumably resulting in extremely short telomeres, chromosomal instability, and malignant transformation.[6] Loss-of-function mutations in *SBDS*, *TERC*, or *TERT* in AML patients might increase the risk of chemotherapy-related toxicity and are relevant in selecting sibling donors for hematopoietic stem cell transplantation.[1,2] However, given the results of our studies, we conclude that screening for mutations in *SBDS*, *TERT*, or *TERC* in sporadic AML is not indicated in the absence of classical features of Shwachman-Diamond syndrome or dyskeratosis congenita, or a family history positive for bone marrow failure, liver cirrhosis, or pulmonary fibrosis.

Apart from mutations in genes implicated in inherited bone marrow failure syndromes, so far unidentified germline variants might be present in sporadic pediatric AML. More than 100 cancer susceptibility genes have been described,[7] while causal associations between AML and germline variants have only been firmly established for a few genes, including *CEBPA*,[8] *RUNX1* (or *CBFA2*),[9] and *GATA2*[10-13] (besides aberrations underlying the inherited bone marrow failure syndromes and other syndromes as discussed in **Chapter 1**). Whole genome sequencing strategies might therefore identify additional AML susceptibility genes in the near future.[7]

Telomere disease caused by the first pathogenic mutation in the *TERC* promoter

The clinical presentation of the family in whom we identified a mutation in the promoter region of the RNA template of the telomerase complex, *TERC*, illustrates the heterogeneous presentation of inherited bone marrow failure syndromes.[14] The index case in our study presented at 39 years of age with apparently acquired aplastic anemia. Her family history however was positive for aplastic anemia, leukemia, and liver abnormalities. Telomere disease was suspected, and very short telomeres were identified in affected family members. No mutations were identified in the coding regions *TERT*, *TERC*, and *TINF2*, but we identified the first pathogenic mutation in the CCAAT box of the promoter region of *TERC*. We propose that mutation-screening strategies for the diagnosis of telomereopathies should include promoter regions of major genes related to telomere biology. Similar strategies may also be helpful for marrow failure syndromes such as Diamond-Blackfan anemia or Shwachman-Diamond syndrome, as with current approaches only in around half of inherited marrow failure cases a mutation can be identified.[15]

Refractory cytopenia of childhood

Differentiating hypocellular, low-grade subtypes of MDS from the acquired bone marrow failure syndrome aplastic anemia can be challenging, and a pathophysiologic overlap between the two disorders has been suggested.[16] In analogy with the immune-mediated pathophysiology of acquired aplastic anemia, clinical and laboratory evidence suggest a T cell-mediated pathophysiology in part of patients with low-grade MDS. Immunosuppressive therapy (IST), consisting of antithymocytic globulin (ATG), which specifically targets T cells, and/or cyclosporine A (CsA), is effective in some children with refractory cytopenia of childhood (RCC) and in adults with low-grade MDS,[17-28] and in vitro studies in adults suggest a T cell-mediated destruction of hematopoietic progenitor cells. In the second part of this thesis, we explored the occurrence of possible indicators of an immune-mediated pathophysiology in RCC, PNH clones and T-cell receptor (TCR) β-chain variable (Vβ) domain skewing. We also investigated whether flow cytometric immunophenotyping can be employed as an additional tool in the diagnostic work-up of RCC, and in the differentiation of RCC from aplastic anemia. Our studies were conducted in a large, unique, international prospective cohort of RCC patients, in collaboration with the European Working Group of MDS in Childhood (EWOG-MDS).

PNH clones in refractory cytopenia of childhood and implications for clinical practice

PNH clones arise from hematopoietic stem cells (HSCs) carrying an acquired mutation in the *PIG-A* gene, resulting in absence of the GPI anchor and GPI-anchored proteins on daughter cells of the mutated HSC.[29] Minor GPI-deficient clones are present in 20 to 68% of adult and pediatric patients with aplastic anemia,[30-36] and in 13 to 23% of adults with low-grade MDS.[31,35,37-39] How PNH clones arise exactly is not understood, but their presence

in presumed immune-mediated bone marrow failure syndromes led to the hypothesis that GPI-deficient cells have a conditional growth advantage by evading an immune attack directed against normal HSCs.[40] Minor PNH clones, detected by flow cytometry with thresholds ranging from 0.003% to 1%, predict response to IST in some,[31,39,41] but not all,[27] studies in adult MDS patients, and are considered surrogate markers of an immune-mediated pathophysiology in bone marrow failure.[42] In our study in 87 RCC patients, minor PNH clones were present in approximately 40% of patients,[43] a percentage higher than in adults with low-grade MDS,[31,35,37-39] but similar to children and adults with aplastic anemia.[30-36] In 28 patients treated with IST, PNH clones larger than 0.1% predicted response to IST (mainly consisting of rabbit ATG): 88% of patients with a PNH clone responded to IST, compared to 40% of patients without a PNH clone. Due to the limited samples size, the possible independent prognostic value of PNH clones in predicting response to IST could not be tested in a multivariate analysis. The presence or absence of PNH clones at diagnosis should therefore not influence the decision whether or not to start treatment with IST. A recent study in a larger cohort of RCC patients treated with IST failed to identify response predictors to IST, but did not assess the presence of PNH clones.[17] To assess the independent prognostic value of PNH clones for IST response, we therefore propose to extend our study in a larger cohort of RCC patients who will be treated with IST.

PNH clones in RCC patients in general are very small. One RCC patient (16 years at diagnosis) with PNH clone sizes of 58% in the erythroid lineage and 86% in the granulocytic lineage showed clinical signs of PNH, i.e. hemolysis. Because treatment with eculizumab, a C5 complement inhibitor, is available to prevent hemolysis and to reduce the risk of thrombosis in patients with clinically significant PNH clones,[44,45] we propose to screen RCC patients at diagnosis for the presence of PNH clones in both the erythroid and granulocytic lineages with flow cytometry. Finally, our sequential data on PNH clone size changes suggest that clone size remains relatively stable over time, and at subclinical levels, which is in line with studies in adults, showing that patients with subclinical PNH are unlikely to develop clinical PNH.[46,47] Nevertheless, to identify the limited number of patients that might be at risk of symptomatic PNH, we recommend to perform follow-up for the presence of PNH clones in RCC patients followed by watchful waiting or treated with IST yearly, according to recommendations in adults.[48]

T cells in refractory cytopenia of childhood and implications for future research

In vitro studies in adult low-grade MDS show that autologous peripheral blood lymphocytes inhibit granulocyte colony formation in an MHC class I-dependent manner,[49-52] which can be abrogated by treatment with ATG.[49] Furthermore, analyses of the TCRVβ domain usage show oligoclonal expansions of mainly $CD8^+$ T cells in MDS patients.[49,51,53-56] In conjunction with a response to IST in selected patients with low-grade MDS,[31,35,37-39] these studies suggest a T cell-mediated immune response directed against hematopoietic progenitor cells in a proportion of patients.[40] We showed that TCRVβ

skewing is present in approximately 40% of RCC patients (**Chapter 7**), but TCRVβ skewing was not clearly related with treatment response. TCRVβ skewing was furthermore not associated with the presence of a PNH clone, but did correlate with a disturbed $CD4^+$/$CD8^+$ T-cell ratio and an expansion of effector and activated $CD8^+$ T cells. These data might indicate that T lymphocytes contribute to RCC pathogenesis in a proportion of patients. A limitation of our study is that we did not provide direct evidence proving this hypothesis. Future studies should employ co-culture experiments with clonally expanded T cells and autologous bone marrow, using adequate controls, to support the hypothesis that hematopoiesis in RCC is inhibited in a T cell dependent manner.

T cell-mediated suppression of hematopoiesis in MDS could be a result of breaking of self-tolerance, leading to an autoimmune-like response against normal hematopoietic progenitor cells, as likely occurs in aplastic anemia.[57] It could also be a reflection of immune surveillance against hematopoietic cells expressing a neo-antigen. Evidence for a combination of both hypotheses was provided in MDS patients with trisomy 8, who are likely to respond to IST.[27,58] T cells of clonally expanded TCRVβ families obtained from these patients selectively inhibited trisomy 8 cell growth.[58] WT1, overexpressed in trisomy 8 positive bone marrow mononuclear cells, but also expressed at a low level in normal hematopoietic stem cells, was subsequently implicated to be one of the antigens inducing T cell-mediated myelosuppression in trisomy 8 positive MDS.[59] Finally, the T cell-mediated suppression of hematopoiesis might be cytokine-mediated, and not necessarily antigen specific. Patients with aplastic anemia have an increased number of IFNγ positive T cells,[60] and it was recently suggested that, whereas transient production of the pro-inflammatory cytokine IFNγ is likely required to modulate hematopoiesis during infection, chronic IFNγ signaling impairs self-renewal of hematopoietic stem cells.[61]

Our finding that TCRVβ skewing was not associated with IST response is consistent with studies in adult MDS patients,[53,55,56] although alterations in TCRVβ profiles or loss of clonal dominance of specific TCRVβ families after response to IST were described.[49,54] The specific T-cell subsets (e.g., $FOXP3^+$ regulatory T cells, Th17 T cells)[62-65] involved in the T-cell clonal expansion, the type of administered IST, and patient characteristics such as age, HLA-type, bone marrow cellularity, and underlying molecular aberrations might influence response to IST, and TCRVβ skewing alone is insufficient to predict response to therapy. Similar reasons might explain why the presence of a PNH clone, considered as surrogate marker of immune-mediated marrow failure,[42] did not in all cases coincide with TCRVβ skewing.

Insight into the precise nature and possible antigenic target(s) of the skewed and activated T cells in MDS might be derived from TCR repertoire deep sequencing approaches, which should preferably be performed in sorted T-cell subsets, taking into account differences between HLA types.

Advances in the understanding of the pathophysiology in refractory cytopenia of childhood

The response to IST in selected patients with RCC,[17] the frequent presence of PNH clones and their association with response to IST,[43] and the presence of TCRVβ skewing and their association with an expansion of effector cytotoxic T cells (**Chapter 7**) suggest that the pathophysiology of RCC is at least partly immune-mediated. Of interest, acquired mutations in *STAT3* or *STAT5b* were identified in T cell large granular lymphocytic leukemia (LGL),[66,67] a disorder that sometimes shows overlap with aplastic anemia and MDS. Thereafter, acquired mutations in the dimerization and activation domain of *STAT3* were also found in clonally expanded T cells in a small proportion of MDS patients.[68] The authors hypothesized that the *STAT3* mutations were responsible for the persistence of the T cell clones, and therefore for the continuation of the destruction of hematopoietic stem cells.

As a result of large-scale whole-genome or whole-exome sequencing strategies, acquired mutations have now been identified in approximately 90% of adult MDS patients, including mutations in genes involved in RNA splicing, DNA methylation, chromatin modification, transcription regulation, DNA repair, signal transduction, and the cohesin complex,[69-72] and by massive parallel sequencing the clonal architecture in adult MDS has been elucidated. In pediatric MDS, both in RCC and in advanced MDS, such studies are lacking, and should be performed. Although in RCC $CD34^+$ cells are low or absent, studies in adult MDS show that most cells of the myeloid lineage belong to the MDS clone,[69] and consequently, if genetic abnormalities are present in RCC, they will likely be detectable in RCC granulocytes as well.

Flow cytometric immunophenotyping as potential diagnostic tool in refractory cytopenia of childhood

Differentiating RCC and aplastic anemia is challenging with the current diagnostic strategies, consisting of morphologic and histopathologic evaluation of bone marrow. However, the introduction of central histopathological review by EWOG-MDS reference pathologists has resulted in a reproducible distinction in RCC and aplastic anemia based on WHO criteria.[73] Developing novel diagnostic tools nonetheless seems relevant. Flow cytometric immunophenotyping has been suggested as valuable addition to morphology in the diagnosis of MDS.[74] In a prospective study, we showed[75] that RCC could not be recognized by a simple immunophenotyping scoring system that has a high sensitivity and specificity in adult MDS.[76,77] Three of four parameters of this scoring system comprises the evaluation of (myeloid) blast cells, which might partly explain why the sensitivity of the scoring system is limited in RCC: in adult low-grade MDS patients the percentage of myeloid blast cells is usually larger than 2%, which is not the case in children. However, with an extensive flow cytometric characterization of bone marrow cellular composition and analysis of flow cytometric aberrancies on myeloid cells, differences could be found between RCC and healthy controls, and between RCC and aplastic anemia patients

(**Chapter 8**). Of note, there was no significant association in RCC between the presence of flow cytometric abnormalities, PNH clones, or TCRVβ skewing (Figure 1). Furthermore, in 22 RCC patients with a hypocellular bone marrow analyzed, there was no significant association between the presence of flow cytometric abnormalities and IST response. Apparently, a relatively modest degree of flow cytometric abnormalities can coincide with a T cell-mediated suppression of hematopoiesis, reflected by a response to IST, and the dysplasia might even be induced by this T cell-mediated suppression.

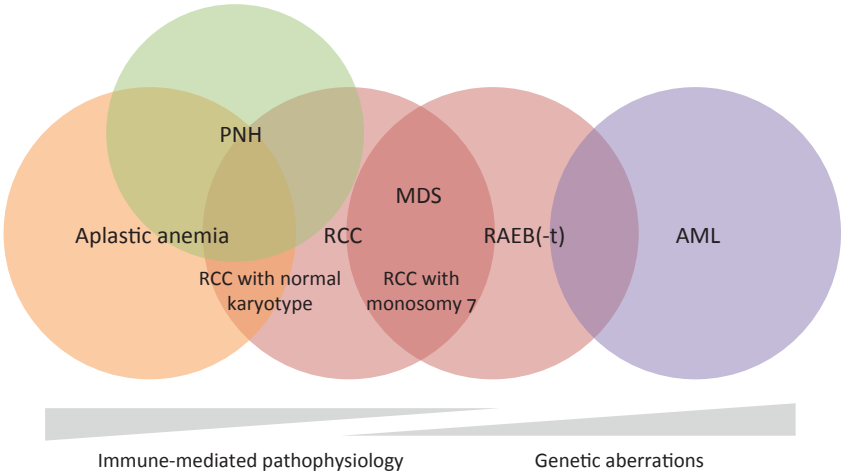


Figure 1 Model depicting the presumed pathophysiologic overlap between aplastic anemia and RCC, and between RAEB(t) and AML.

In a proportion of RCC patients, indicators of an immune-mediated pathophysiology are present, and selected patients respond to IST. Myelodysplasia detected by flow cytometry in RCC is relatively modest. RCC patient with monosomy 7 are less likely to have PNH clones, display more flow cytometric abnormalities, and have a high risk of clonal evolution. In RAEB(-t) patients, genetic aberrations are more frequently detected than in RCC, myelodysplasia is prominent, and patients have a high risk of evolution to AML. PNH, paroxysmal nocturnal hemoglobinuria; MDS, myelodysplastic syndrome; RCC, refractory cytopenia of childhood; RAEB(-t), refractory anemia with excess blasts (in transformation); AML, acute myeloid leukemia.

A limitation of our study, and of studies performed by others, is that scoring of dysplasia is subjective. Therefore, future studies, as are now initiated by the EuroFlow consortium, evaluating the value of flow cytometry as a means to differentiate RCC from aplastic anemia should employ more standardized protocols and antibody panels, and novel software tools that might more objectively evaluate myeloid dysplasia.[79,80] One of the promising possibilities is the EuroFlow-based maturation tool of the flow cytometry

analysis software Infinicyt (developed by Cytognos, Salamanca, Spain). This maturation tool, of which an example is provided in Figure 2, can objectively compare multiple markers of different developmental stages of, for example, granulocytes, between patients and controls (unpublished results). Despite the current limitations, our findings suggest that flow cytometric immunophenotyping might be used as an additional tool for the diagnosis of RCC.

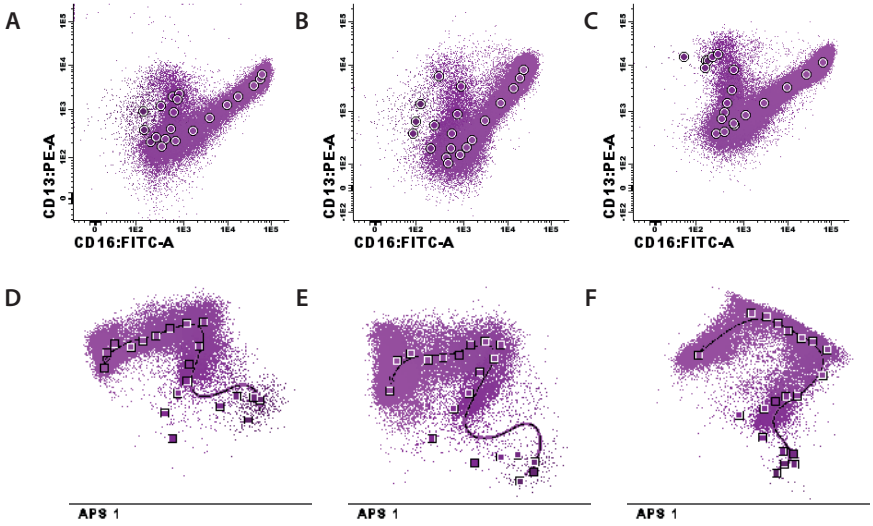


Figure 2 Example of EuroFlow-based maturation tool applied to normal and MDS granulocytes (unpublished results).

(A) 'Classic' two-dimensional representation of granulocytic maturation in normal bone marrow. (B) Two-dimensional representation of granulocytic maturation in bone marrow of MDS patient 1. (C) Two-dimensional representation of granulocytic maturation in bone marrow of MDS patient 2. (D) 'APS' representation of granulocytic maturation, indicated by a line, in normal bone marrow. Dots indicate 20 different maturation stages, which are automatically assigned. The APS representation of a population is based on principal component analysis of all CD markers that are assayed. (E) APS representation of the granulocytic maturation in MDS patient 1. (F) APS representation of granulocytic maturation in MDS patient 2. (G) Expression patterns of multiple CD markers in different maturation stages of granulocytes, compared with normal granulocytes, in MDS patient 1. Except for a CD16 expression below -2SD of normal expression in the most mature stages, CD marker expression is normal, indicating a normal maturation of granulocytes in this patient. (H) Expression patterns of multiple CD markers in different maturation stages of granulocytes, compared with normal granulocytes, in MDS patient 2. CD13 expression on immature granulocytes, and CD10 expression on more mature granulocytes is increased, objectively indicating an abnormal maturation of granulocytes in this MDS patient.

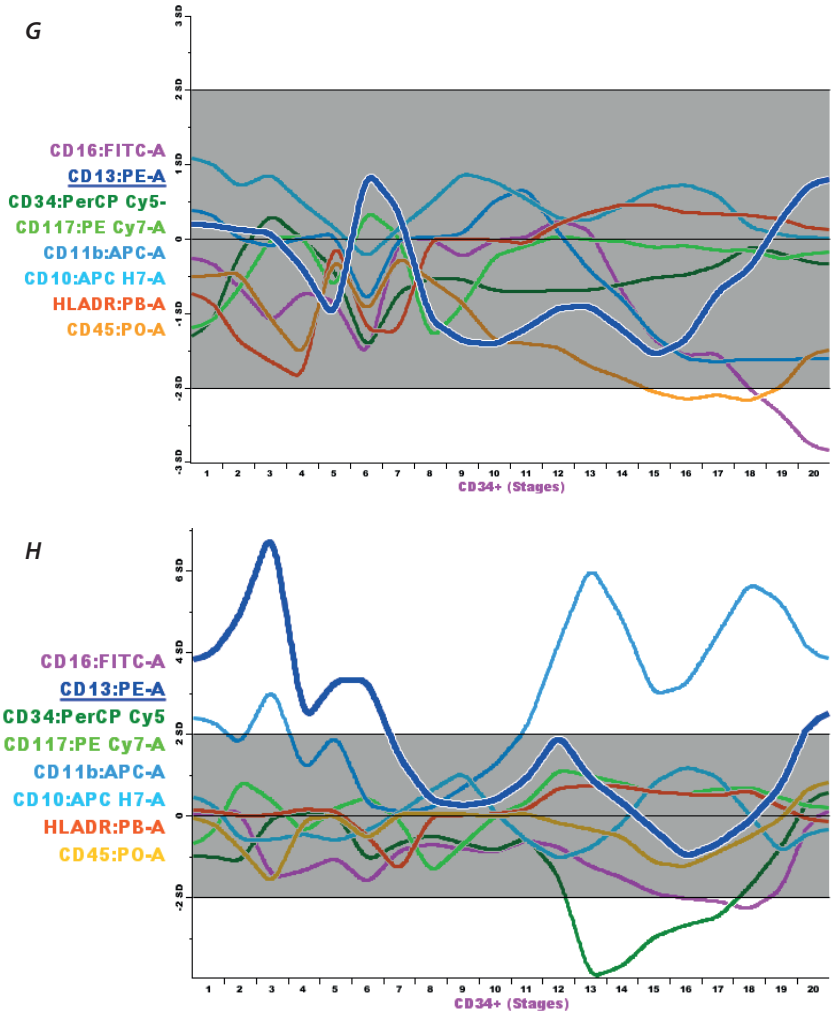


Figure 2 Continued.

Are hypocellular, normal karyotype refractory cytopenia of childhood and aplastic anemia overlapping disorders?

By definition, RCC and aplastic anemia are different disorders, with an apparently different pathophysiology: RCC is a subtype of MDS, and is thus regarded as a malignant clonal disease, while aplastic anemia is a non-malignant immune-mediated bone marrow failure syndrome. The distinction between hypocellular RCC without cytogenetic abnormalities and aplastic anemia is based mainly on histopathologic criteria, which are highly reproducible.[73] The validity of the distinction was supported by a pilot study, showing that

patients with RCC had a lower response rate to IST (consisting of horse or rabbit ATG)[81] than patients with aplastic anemia.[82] Support was furthermore provided by different rates of clonal evolution after IST: in RCC, the probability of clonal evolution ranged from 4% to 14% (in rabbit and horse ATG, respectively),[17] while in a recent interim analysis in aplastic anemia, the probability of clonal evolution had decreased from 15% to 3% (type of ATG not reported) after the introduction of central histopathological review.[73] However, several arguments also support the hypothesis that hypocellular, normal karyotype RCC and aplastic anemia are disorders that share a common pathophysiology. First, our studies show that indicators of an immune-mediated pathophysiology are frequently present in RCC, similar to aplastic anemia ([43] and **Chapter 7**). Second, the assumption that RCC without cytogenetic abnormalities is a clonal disorder, and pediatric aplastic anemia exclusively a non-clonal disorder without molecular aberrations, still has to be proven. Third, the argument that IST response rates in RCC and aplastic anemia patients are different might now be less valid. It was recently shown that horse ATG results in superior response rates over rabbit ATG.[83,84] When only horse ATG was evaluated, response rates were similar in RCC (74%) and aplastic anemia (65%).[17,85] Although we showed flow cytometric differences between RCC and aplastic anemia, the number of dysplastic

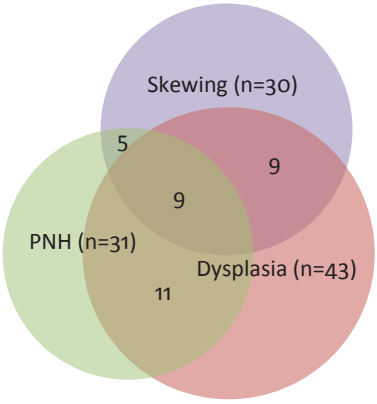


Figure 3 Venn diagram depicting the overlap between flow cytometric dysplasia, PNH clones and TCRVβ skewing in RCC.

A total of 72 RCC patients were assessed for all three parameters. Dysplasia was defined as the presence of two or more immunophenotypic abnormalities in the bone marrow. PNH clones were defined as more than 0.01% of GPI-deficient erythrocytes and/or more than 0.03% of GPI-deficient cells in the peripheral blood. TCRVβ skewing was defined as skewing in two or more Vβ families. Dysplasia was present in 43 patients, a PNH clone in 31 patients, and skewing in 30 patients (http://chart.apis.google.com/chart?chs=1000x300&cht=v&chd=t:43,31,30,11,9,5,9). No dysplasia, PNH clones, or skewing was present in 11 patients.

changes in RCC is relatively modest in comparison with advanced MDS. In Figure 3, the presumed pathophysiologic overlap between normal karyotype, hypocellular RCC and aplastic anemia is depicted. Of note, RCC patients with monosomy 7 (or complex cytogenetic aberrations) have a high risk of progression to AML and are not included in this overlap with aplastic anemia.

CONCLUSION

In this thesis we show that the clinical presentation of myeloid disorders in childhood is heterogeneous, and that differently classified disorders sometimes share a common pathophysiology. Seemingly acquired aplastic anemia can be caused by a mutation in a telomerase complex gene, and hence be a manifestation of telomere disease. RCC, classified as a clonal disorder, might at least in part be caused by an immune-mediated pathophysiology, which provides a rationale for treatment with IST. To further elucidate the pathophysiology of both RCC and aplastic anemia, future research should be directed towards identifying the antigenic targets of the T cell-mediated immune response in RCC and aplastic anemia. In addition, whole genome sequencing strategies should be employed to identify molecular aberrations, which might be present in addition to the aberrant immune response in both patient groups.

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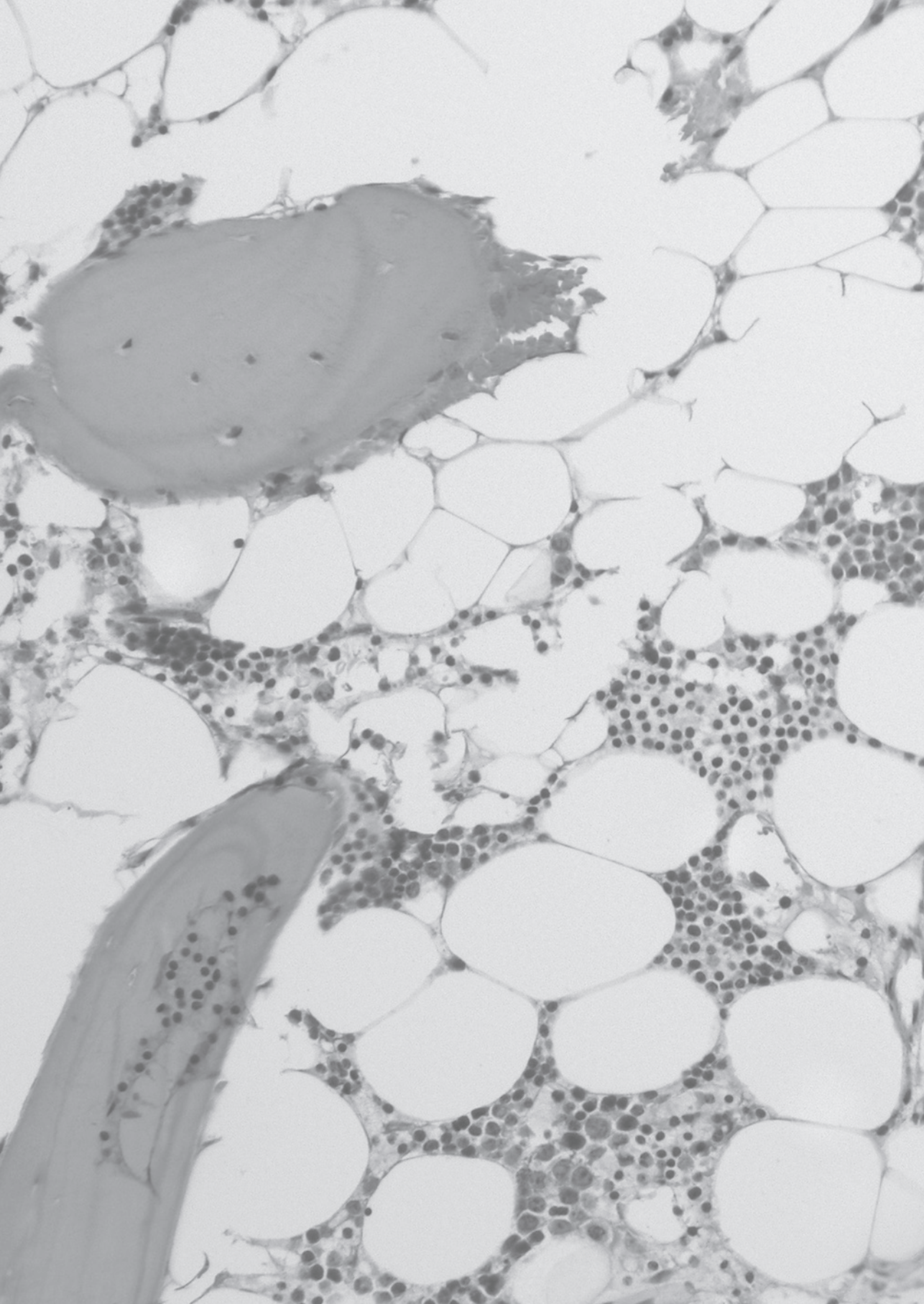
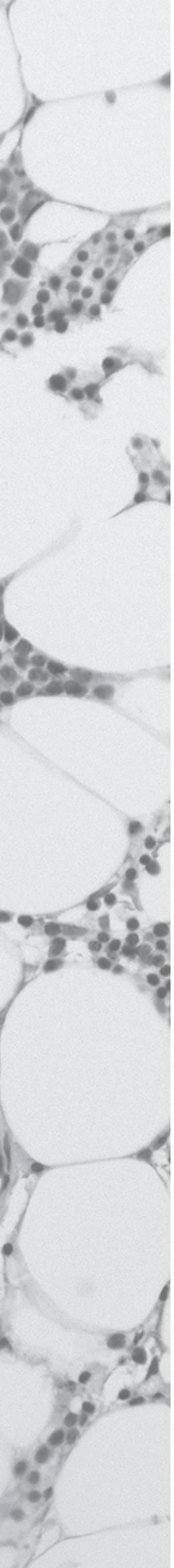




10

Summary

SUMMARY

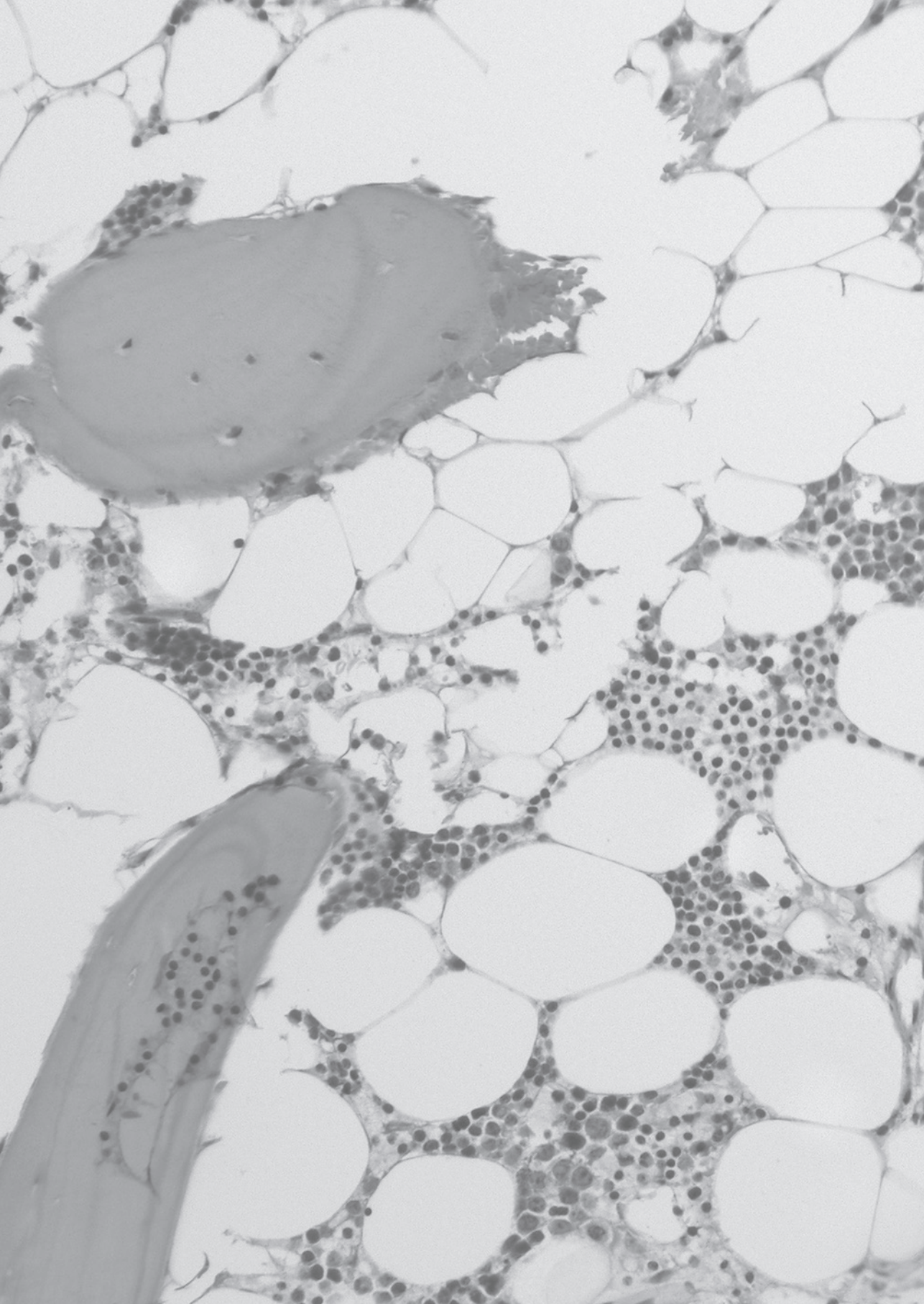
Inherited and acquired bone marrow failure syndromes, paroxysmal nocturnal hemoglobinuria (PNH), myelodysplastic syndrome (MDS), and acute myeloid leukemia (AML) are part of a spectrum of myeloid disorders. The clinical presentation of inherited bone marrow failure syndromes can be heterogeneous, and a pathophysiologic overlap between parts of the spectrum is present. Patients with inherited and acquired bone marrow failure syndromes are at increased risk of developing MDS or AML, and adult patients with low-grade MDS, which is classified as a malignant clonal disease, might have an immune-mediated pathophysiology, similar to the acquired bone marrow failure syndrome aplastic anemia. In this thesis, we aimed to better characterize different aspects of this spectrum of myeloid disorders in childhood.

In the first chapters of this thesis we explored whether germline mutations causing inherited bone marrow failure syndromes underlie seemingly sporadic AML and acquired aplastic anemia. Because patients with Shwachman-Diamond syndrome (SDS) have an increased risk of AML, we hypothesized in **Chapter 2** that *SBDS* mutations, underlying SDS, might be present in seemingly sporadic pediatric AML. However, in a large cohort of sporadic pediatric AML patients, we failed to identify *SBDS* mutations, suggesting that these children are unlikely to have underlying SDS. Similarly, patients with dyskeratosis congenita are at increased risk of developing AML, and germline loss-of-function mutations in telomerase complex genes were recently implicated as risk factors for AML in adults. In **Chapter 3** we report, however, that in pediatric AML, *TERT* and *TERC* gene variants are not present at an increased frequency compared to healthy controls. In **Chapter 4**, we describe a family in which multiple members presented with aplastic anemia, leukemia, or liver cirrhosis, suggestive of telomere disease. We identified a novel pathogenic mutation in the *TERC* promoter, resulting in reduced reporter gene activity and absent binding of transcription factor NF-Y, likely resulting in short telomeres, and we proposed that current mutation screening strategies include gene promoter regions for the diagnosis of telomere diseases.

In analogy with the immune-mediated pathophysiology of acquired aplastic anemia, clinical and laboratory evidence suggest a T-cell-mediated pathophysiology in part of adult patients with low-grade MDS. In the next chapters of this thesis, we aimed to elucidate whether an immune-mediated pathophysiology might also be present in refractory cytopenia of childhood (RCC), the most common subtype of childhood MDS. In **Chapter 5** we show that PNH clones, considered surrogate markers of immune-mediated bone marrow failure, are present in 40% of RCC patients, which is a frequency similar to studies in aplastic anemia, but higher than in adult MDS. We also show that the presence of a PNH clone is associated with response to immunosuppressive therapy (IST). In

Chapter 6 we describe that T-cell receptor β-chain variable domain skewing, a measure of clonal T-cell expansion, is present in almost half of RCC patients, and is accompanied by an expansion of effector cytotoxic T cells, although there was no clear association with IST response. These results suggest that RCC has at least in part an immune-mediated pathophysiology, similar to aplastic anemia, and provide a rationale for treatment of RCC patients with IST. Nonetheless, differentiating between RCC and aplastic anemia remains relevant, because the risk of clonal evolution in RCC might be increased compared to aplastic anemia. In **Chapter 7** we report that RCC cannot be recognized by a simple flow cytometric immunophenotyping scoring system that has a high sensitivity and specificity in adult MDS. However, an extensive flow cytometric characterization of bone marrow composition and myeloid maturation, described in **Chapter 8**, differentiates between RCC and aplastic anemia with a sensitivity of 84% and a specificity of 76%.

In conclusion, this thesis illustrates the heterogeneous clinical presentation of myeloid disorders in childhood. Seemingly acquired aplastic anemia can be caused by a mutation in a telomerase complex gene, and hence be a manifestation of telomere disease. We provide a rationale for IST in RCC, classified as a clonal disorder, by showing that the disease might at least in part be caused by an immune-mediated pathophysiology. Future research in RCC and aplastic anemia might be directed towards identifying antigenic targets of the T cell mediated immune response, and whole genome sequencing strategies could be employed to identify molecular aberrations which might be present in addition to the aberrant immune response in both patient groups.



11

Nederlandse Samenvatting

NEDERLANDSE SAMENVATTING

Hematopoietische, of bloedcelvormende, stamcellen zijn zelfvernieuwende cellen die in het beenmerg dochtercellen produceren. Deze dochtercellen of voorlopercellen kunnen uitrijpen tot alle cellen die voorkomen in het bloed, zoals erythrocyten of rode bloedcellen, die zorgen voor zuurstoftransport, leukocyten of witte bloedcellen, die verantwoordelijk zijn voor de afweer, en trombocyten of bloedplaatjes, die betrokken zijn bij de bloedstolling. De hematopoiese wordt onderverdeeld in myelopoiese en lymfopoiese. De myelopoiese omvat de productie van onder meer granulocyten en monocyten (witte bloedcellen betrokken bij de aangeboren of niet-specifieke afweer), bloedplaatjes, en rode bloedcellen. De lymfopoiese betreft onder meer de vorming van B en T cellen (witte bloedcellen betrokken bij de verworven of specifieke afweer). Een verstoorde myelopoiese kan resulteren in bijvoorbeeld anemie of bloedarmoede, een verhoogde gevoeligheid voor infecties, en/of een verhoogde bloedingsneiging. Er zijn verschillende vormen van en oorzaken voor een verstoorde myelopoiese.

Bij beenmergfalen maakt het beenmerg onvoldoende myeloïde cellen aan. De oorzaak hiervoor kan aangeboren zijn of verworven. Voorbeelden van aangeboren beenmergfalen, waarbij er in het DNA van alle lichaamscellen een specifieke mutatie, of verandering, aanwezig is die resulteert in onvoldoende aanmaak van myeloïde cellen, zijn het Shwachman-Diamond syndroom (SDS) en dyskeratosis congenita. SDS wordt veroorzaakt door een kiembaanmutatie in het *SBDS* gen, en dyskeratosis congenita wordt veroorzaakt door een kiembaanmutatie in een van de telomerase complex genen (bijvoorbeeld de genen *TERT* en *TERC*). Het telomerase complex onderhoudt de lengte van telomeren, de structuren die de uiteindes van chromosomen waarin het DNA is opgeslagen beschermen. De leeftijd waarop aangeboren beenmergfalen zich openbaart en de wijze waarop kan per patiënt variëren. Een hematopoietische stamcel- of beenmergtransplantatie, waarbij de eigen afwijkende stamcellen vervangen worden, is de enige definitieve behandeling voor deze vorm van beenmergfalen. Een voorbeeld van verworven beenmergfalen is aplastische anemie. Aplastische anemie wordt veroorzaakt door een afwijkende afweerreactie van T cellen tegen de myeloïde voorlopercellen, waardoor die geen myeloïde bloedcellen meer produceren. De behandeling van aplastische anemie kan bestaan uit behandeling met afweeronderdrukkende, of immunosuppressieve, therapie (IST), of uit een hematopoietische stamceltransplantatie. Patiënten met beenmergfalen hebben een verhoogde kans op het krijgen van de klonale myeloïde aandoeningen myelodysplastisch syndroom (MDS) en acute myeloïde leukemie (AML).

Bij klonale myeloïde aandoeningen wordt een aanzienlijk deel van de myeloïde bloedcellen geproduceerd door een voorlopercel waarin een mutatie is ontstaan. MDS wordt gekenmerkt door een ineffectieve hematopoiese, dysplasie of een afwijkende vorm en

uitrijping van myeloïde cellen, en een verhoogd risico op AML. De ineffectieve hematopoiese resulteert in bijvoorbeeld bloedarmoede, een verhoogde gevoeligheid voor infecties, en/of een verhoogde bloedingsneiging. Refractaire cytopenie op de kinderleeftijd (RCC) is de meest voorkomende vorm van MDS bij kinderen. Tot voor kort was een hematopoietische stamceltransplantatie de enige behandeling voor RCC, maar sinds kort wordt bij een deel van de kinderen ook IST gegeven. Bij volwassenen met MDS kan in de meeste gevallen een of meer verworven, dus niet aangeboren, mutaties gevonden worden. Echter, bij een deel van de volwassen patiënten met MDS, de laaggradige MDS, lijkt net zoals bij het aplastische anemie een afwijkende immuunrespons tegen de myeloïde voorlopercellen aanwezig te zijn. Of bij kinderen met RCC, net zoals bij patiënten met aplastische anemie, een afwijkende immuunrespons ook een oorzaak voor de ziekte is, is onbekend. AML is een vorm van leukemie, waarbij een gemuteerde voorlopercel niet goed kan uitrijpen en zich extreem vermenigvuldigt, waardoor de normale hematopoiese wordt verdrongen. De behandeling van AML bestaat uit chemotherapie, waarmee de sneldelende leukemiecellen worden gedood, soms gevolgd door een hematopoietische stamceltransplantatie.

Dit proefschrift had als doel om verschillende aspecten van enkele van bovengenoemde myeloïde aandoeningen beter te karakteriseren.

In de eerste hoofdstukken van het proefschrift onderzochten we of de kiembaanmutaties die aangeboren beenmergfalen veroorzaken ook voorkomen bij schijnbaar toevallig voorkomende AML en verworven aplastische anemie. Omdat patiënten met SDS een verhoogd risico hebben op AML, onderzochten we in **hoofdstuk 2** de hypothese dat compound heterozygote mutaties in het *SBDS* gen, de oorzaak voor SDS, ook gevonden kunnen worden bij sporadische AML. Echter, in een cohort van 160 kinderen met AML vonden we geen homozygote of compound heterozygote mutaties in *SBDS*. Verder hadden patiënten en controles een vergelijkbare frequentie van heterozygote mutaties in *SBDS*. Dit suggereert dat kinderen met schijnbaar sporadische AML nooit of zeer zelden onderliggend SDS hebben. Net als patiënten met SDS hebben patiënten met dyskeratosis congenita een verhoogd risico op AML. Kiembaan mutaties in genen die coderen voor het telomerasecomplex, de oorzaak voor dyskeratosis congenita, kunnen leiden tot extreem korte telomeren en chromosomale instabiliteit, en zijn recent gerapporteerd bij volwassenen met AML. Bij kinderen met AML vonden we dit echter niet: in **hoofdstuk 3** beschreven we dat mutaties in de genen *TERT* en *TERC*, die coderen voor onderdelen van het telomerasecomplex, voorkomen in een vergelijkbare frequentie bij kinderen met AML en bij gezonde controles. In **hoofdstuk 4** beschreven we een familie waarvan meerdere familieleden aplastische anemie, leukemie, of levercirrose hadden, en korte telomeren, wat suggestief is voor telomeerziekte, een verzamelnaam voor aandoeningen die net als dyskeratosis congenita door mutaties in telomerasecomplex genen veroorzaakt worden.

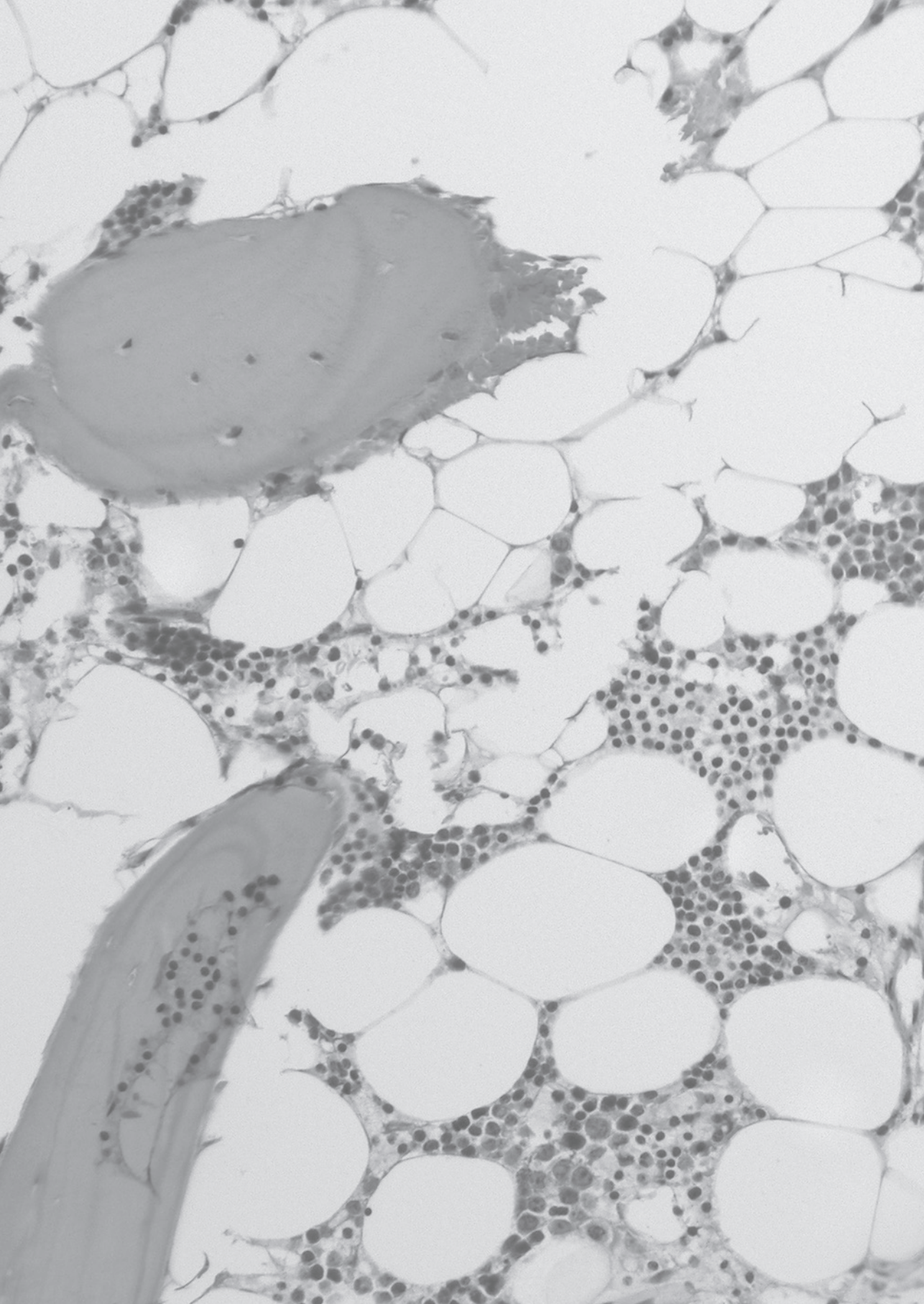
We vonden een niet eerder beschreven pathogene mutatie in de CCAAT box van de *TERC* promoter. De promoterregio van een gen codeert niet voor RNA of eiwit, maar zorgt ervoor dat het overschrijven van het coderende deel van het gen in gang wordt gezet. De mutatie in de *TERC* promoter resulteerde in een verlaagde activiteit van een reportergen, verstoorde binding van de transcriptiefactor NF-Y, met waarschijnlijk korte telomeren als gevolg. Op basis van deze resultaten deden we de aanbeveling dat in de toekomst voor de diagnose van telomeerziekte ook promoterregio's van genen die coderen voor het telomerasecomplex gescreend moeten worden.

In de volgende hoofdstukken van dit proefschrift onderzochten we of er, net zoals bij aplastische anemie en laaggradige MDS bij volwassenen, aanwijzingen zijn voor een immuungemedieerde pathofysiologie bij RCC. In **hoofdstuk 5** tonen we aan dat PNH klonen, die gezien worden als surrogaatmarkers voor immuungemedieerd beenmergfalen, aanwezig zijn bij 40% van de patiënten met RCC. Deze frequentie is vergelijkbaar met de frequentie bij patiënten met aplastische anemie, maar hoger dan de frequentie bij volwassenen met MDS. We lieten verder zien dat PNH klonen geassocieerd zijn met een respons op IST bij patiënten met RCC. In **hoofdstuk 6** beschreven we dat skewing van de T-cel receptor Vβ (TCRVβ) keten, een maat voor klonale expansie van T cellen die gezien kan worden bij auto-immuunziektes, voorkomt bij bijna de helft van de RCC patiënten. De skewing ging gepaard met een expansie van effector cytotoxische T cellen. Er was echter geen relatie met respons op IST. Deze resultaten suggereren dat de pathofysiologie van RCC ten minste bij een deel van de patiënten immuungemedieerd is, vergelijkbaar met aplastische anemie, wat een onderbouwing geeft voor de behandeling van RCC patiënten met IST. Desondanks blijft het maken van een onderscheid tussen RCC en aplastische anemie van belang: het risico op klonale evolutie, wat kan resulteren in AML, is waarschijnlijk hoger in RCC dan in aplastische anemie, en de behandeling van aplastische anemie en RCC is mede om deze reden nog verschillend.

Met de huidige diagnostische methodes is het maken van een onderscheid tussen RCC en aplastische anemie moeilijk. Daarom onderzochten we of RCC herkend kan worden met behulp van immunofenotypering van beenmergcellen met flow cytometrie, waarbij de eigenschappen van grote aantallen cellen gevisualiseerd kan worden. Bij deze methode worden cellen gekleurd met antistoffen, die binden aan specifieke eiwitten in of op de cel, en die gelabeld zijn met fluorochromen. De aan de cel gebonden fluorochromen worden door lasers in een flowcytometer aangestraald, waarna de lichtverstrooiing gedetecteerd wordt. In **hoofdstuk 7** beschreven we dat RCC niet herkend kan worden met een eenvoudige flowcytometrische methode die eerder werd beschreven als methode met een hoge sensitiviteit en specificiteit bij volwassenen met MDS. Echter, in **hoofdstuk 8** beschreven we dat met een uitgebreidere methode, waarbij we de samenstelling van het beenmerg en maturatiepatronen van myeloïde cellen bestudeerden, verschillen kunnen

worden gevonden tussen RCC en aplastische anemie. Dit impliceert dat flowcytometrie gebruikt zou kunnen worden als nieuw diagnostisch instrument om onderscheid te maken tussen de twee aandoeningen.

Concluderend laat dit proefschrift zien dat de klinische presentatie van myeloïde aandoeningen op de kinderleeftijd heterogeen kan zijn, en dat verschillend geclassificeerde aandoeningen dezelfde pathofysiologie kunnen hebben. Schijnbaar verworven aplastische anemie kan veroorzaakt worden door een mutatie in een gen dat codeert voor het telomerasecomplex. RCC, dat geclassificeerd wordt als een klonale aandoening, lijkt in ieder geval deels een immuungemedieerde pathofysiologie te hebben, wat een onderbouwing geeft voor de behandeling met IST. Om de pathofysiologie van RCC en aplastische anemie verder te verhelderen zou in de toekomst onderzoek gedaan kunnen worden naar het identificeren van antigenen die het doel zijn van de T-cel gemedieerde immuunrespons in RCC en aplastische anemie. Verder zou genoomwijd naar aangeboren of verworven mutaties gezocht kunnen worden bij patiënten met RCC en aplastische anemie, omdat naast de afwijkende immuunrespons ook mutaties zouden kunnen bijdragen aan het ontstaan van beide aandoeningen.



Appendices

ABOUT THE AUTHOR

Curriculum Vitae

Anna Maartje Aalbers was born on February 2nd, 1984 in Tiel, The Netherlands. She attended secondary school at the Johan de Witt-gymnasium in Dordrecht, The Netherlands, from which she graduated in 2002 (cum laude). From 2002 onwards she went to medical school at the Leiden University Medical Center (LUMC), Leiden, The Netherlands. From April through December 2006 she performed a scientific internship entitled 'Unraveling the regulation of longitudinal growth: Lessons from experiments of nature' at the Department of Pediatric Endocrinology at the Oregon Health and Sciences University in Portland, Oregon, USA, under supervision of professor Ron Rosenfeld and dr. Vivian Hwa, and professor Jan Maarten Wit (Department of Pediatric Endocrinology, LUMC). She performed her senior clinical rotation at the Department of Pediatric Oncology, LUMC, from September through December 2008, and graduated from medical school in January 2009. Before starting her PhD training, she was a resident (not in training) at the Department of Pediatrics, Reinier de Graaf Gasthuis, Delft. She then did her PhD training from September 2009 through September 2013 at the Department of Pediatric Oncology/ Hematology and the Department of Immunology, Erasmus MC, Rotterdam, under supervision of professor Rob Pieters, dr. Marry van den Heuvel-Eibrink, and dr. Vincent van der Velden. During her PhD training she was a Visiting Fellow at the Hematology Branch, NHLBI/NIH, in Bethesda, Maryland, USA, under supervision of dr. Neal Young and dr. Rodrigo Calado (March 2010 through July 2011). In January 2014 Anna started her residency internal medicine at the Reinier de Graaf Gasthuis, Delft, The Netherlands. The research performed during her PhD studies is presented in this thesis.

List of Publications

Aalbers AM, van den Heuvel-Eibrink MM, Baumann I, et al. T-cell receptor Vβ skewing frequently occurs in refractory cytopenia of childhood and is associated with an expansion of effector cytotoxic T cells - a prospective study by EWOG-MDS. *Blood Cancer J* 2014 (accepted).

Aalbers AM, van der Velden VHJ, Yoshimi A, et al. The clinical relevance of minor paroxysmal nocturnal hemoglobinuria clones in refractory cytopenia of childhood - a prospective study by EWOG-MDS. *Leukemia* 2014; 28 (1): 189-92.

Aalbers AM, van den Heuvel-Eibrink MM, de Haas V, et al, Applicability of a reproducible flow cytometry scoring system in the diagnosis of refractory cytopenia of childhood. *Leukemia* 2013; 27 (9): 1923-5.

Aalbers AM, Calado RT, Young NS, et al. Telomere length and telomerase complex mutations in pediatric acute myeloid leukemia. *Leukemia* 2013; 27 (8): 1786-9.

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Blink M, van den Heuvel-Eibrink MM, Aalbers AM, et al. High frequency of copy number alterations in myeloid leukaemia of Down syndrome. *Br J Haematol* 2012; 158 (6): 800-3.

Aalbers AM, Kajigaya S, van den Heuvel-Eibrink MM, van der Velden VH, Calado RT, Young NS. Human telomere disease due to disruption of the CCAAT box of the TERC promoter. *Blood* 2012; 119 (13): 3060-3.

Aalbers AM, Chin D, Pratt KL, Little BM, Frank SJ, Hwa V, Rosenfeld RG. Extreme elevation of serum growth hormone-binding protein concentrations resulting from a novel heterozygous splice site mutation of the growth hormone receptor gene. *Horm Res* 2009; 71 (5): 276-84.

van Bon BW, Koolen DA, Borgatti R, Magee A, Garcia-Minaur S, Rooms L, Reardon W, Zollino M, Bonaglia MC, De Gregori M, Novara F, Grasso R, Ciccone R, van Duyvenvoorde HA, Aalbers AM, et al. Clinical and molecular characteristics of 1qter microdeletion syndrome: delineating a critical region for corpus callosum agenesis/hypogenesis. *J Med Genet* 2008; 45 (6): 346-54.

PhD Portfolio

Name PhD student: Anna M. Aalbers
Erasmus MC Department: Pediatric Oncology and Immunology
Research School: Molecular Medicine
PhD period: 1 September 2009 - 1 September 2013
Promotor: Prof.dr. R. Pieters
Supervisors: Dr. M.M. van den Heuvel-Eibrink, Dr.ir. V.H.J. van der Velden

PhD training	Year
General courses	
Presenting Skills for Scientists III, MolMed postgraduate school, Erasmus MC	2011
Biomedical English Writing Course for MSc and PhD-students, MolMed postgraduate school, Erasmus MC	2011
Specific courses	
Advanced Course on Molecular Immunology, MolMed postgraduate school, Erasmus MC	2013
Introductory Course on Statistics & Survival Analysis for Research master/ PhD students & MDs, MolMed postgraduate school, Erasmus MC	2012
Cursus Medische Immunologie, Department of Immunology, Erasmus MC, and Avans Hogeschool, Utrecht, The Netherlands	2011
Biomedical Research Techniques VIII, MolMed postgraduate school, Erasmus MC	2009
Seminars and workshops	
Research meetings of Laboratory of Pediatrics, Pediatric Oncology and Immunology, Erasmus MC, *6 informal oral presentations*	2009-2013
17th Molecular Medicine Day, Postgraduate School Molecular Medicine, Erasmus MC, *poster presentation*	2013
16th Molecular Medicine Day, Postgraduate School Molecular Medicine, Erasmus MC, *poster presentation*	2012
9th Annual NHLBI DIR (NIH) Scientific Retreat, Cambridge, MD, USA	2011
8th Annual NHLBI DIR (NIH) Scientific Retreat, Baltimore, MD, USA	2010
14th Molecular Medicine Day, Postgraduate School Molecular Medicine, Erasmus MC	2010
European School of Hematology (ESH) - Conference on Myelodysplastic Syndromes, Mandelieu, France	2009
International conferences and presentations	
45th Congress of the International Society of Pediatric Oncology (SIOP), Hong Kong, China, *2 poster presentations* (meeting not attended)	2013
18th Congress of the European Hematology Association (EHA), Stockholm, Sweden, *2 poster presentations*	2013

PhD training	**Year**
54th American Society of Hematology (ASH) Annual Meeting, Atlanta, Georgia, *poster presentation*	2012
6th International Symposium on MDS and Bone Marrow Failure Syndromes in childhood, Prague, Czech Republic, *poster presentation*	2012
8th Biennial Childhood Leukemia Symposium, Santiago, Chile	2012
Cold Spring Harbor Laboratory - Meeting on Telomeres & Telomerase, Cold Spring Harbor, New York, USA, *poster presentation*	2011
53rd American Society of Hematology (ASH) Annual Meeting, San Diego, California, *poster presentation*	2011
52nd American Society of Hematology (ASH) Annual Meeting, Orlando, Florida, USA	2010
Other international meetings and presentations	
26th Annual Business Meeting of the European Working Group of MDS in Childhood (EWOG-MDS), Madrid, Spain, *oral presentation*	2013
23rd Annual Meeting of the International BFM Study Group, Santiago, Chile, *oral presentation*	2012
24th Annual Business Meeting of the European Working Group of MDS in Childhood (EWOG-MDS), Ghent, Belgium, *oral presentation*	2011
23rd Annual Business Meeting of the European Working Group of MDS in Childhood (EWOG-MDS), Rome, Italy, *oral presentation*	2011
Grants	
Best poster award at the 45th Congress of SIOP	2013
Travel grant for the 18th EHA Congress, awarded by EHA	2013
Travel grant for the 54th ASH Annual Meeting, awarded by the Trustfonds, Erasmus Universiteit, Rotterdam, Netherlands	2012
Travel grant for the 53rd ASH Annual Meeting, awarded by the Trustfonds, Erasmus Universiteit, Rotterdam, Netherlands	2011
Grant for Visiting Fellowship at NIH, awarded by the Renée Vogelstichting, Oirschot, Netherlands	2010
Other	
Visiting Fellowship at the Hematology Branch, National Heart, Lung, and Blood Instititute, National Institutes of Health, Bethesda, MD, USA (supervisors: Dr. Neal S. Young and Dr. Rodrigo T. Calado)	2010-2011

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