Syndromes predisposing to leukemia are a major cause of inherited cytopenias in children

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Abstract

Prolonged cytopenias are a non-specific sign with a wide differential diagnosis. Among inherited disorders, cytopenias predisposing to leukemia require a timely and accurate diagnosis to ensure appropriate medical management, including adequate monitoring and stem cell transplantation prior to the development of leukemia. We aimed to define the types and prevalences of the genetic causes leading to persistent cytopenias in children. The study comprises children with persistent cytopenias, myelodysplastic syndrome, aplastic anemia, or suspected inherited bone marrow failure syndromes, who were referred for genetic evaluation from all pediatric hematology centers in Israel during 2016-2019. For variant detection, we used Sanger sequencing of commonly mutated genes and a custom-made targeted next-generation sequencing panel covering 226 genes known to be mutated in inherited cytopenias; the minority subsequently underwent whole exome sequencing. In total, 189 children with persistent cytopenias underwent a genetic evaluation. Pathogenic and likely pathogenic variants were identified in 59 patients (31.2%), including 47 with leukemia predisposing syndromes. Most of the latter (32, 68.1%) had inherited bone marrow failure syndromes, nine (19.1%) had inherited thrombocytopenia predisposing to leukemia, and three each (6.4%) had predisposition to myelodysplastic syndrome or congenital neutropenia. Twelve patients had cytopenias with no known leukemia predisposition, including nine children with inherited thrombocytopenia and three with congenital neutropenia. In summary, almost one third of 189 children referred with persistent cytopenias had an underlying inherited disorder; 79.7% of whom had a germline predisposition to leukemia. Precise diagnosis of children with cytopenias should direct follow-up and management programs and may positively impact disease outcome.

Introduction

Prolonged cytopenias are a non-specific sign with a wide differential diagnosis, including acquired and inherited disorders.[1-2] Among inherited disorders, predisposition to leukemia has recently emerged as an important clinical

entity with immediate implications for follow-up and management programs of patients and their family members.[3] The clinical presentation of syndromes predisposing to leukemia is variable, and may include isolated thrombocytopenia, neutropenia, anemia or pancytopenia, with or without bone marrow failure. Diagnosing such syndromes before the emergence of leukemia is essential for proper treatment and cure.

Classical inherited bone marrow failure syndromes (IBMFS) are well recognized as predisposing to myeloid malignancies.[4,5] Non-classical IBMFS with pathogenic variants in an increasing number of genes has been profusely described.[6] IBMFS differ in their extra-hematopoietic congenital anomalies, the age of onset of BMF, and the risk and age of developing myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). Refractory cytopenia of childhood (RCC) is the most common form of MDS in children.[7] In contrast to MDS in adults, pediatric MDS is more commonly associated with genetic predisposition.[8] Genetic alterations in *GATA2* and *SAMD9L* have emerged as the most common cause of inherited pediatric MDS.[9] Familial platelet disorder, associated with MDS and leukemia, was first described in 1999, with the identification of deleterious heterozygous *RUNX1* variants in families with inherited thrombocytopenia (IT) and AML.[10] Later, additional genes causing thrombocytopenia and a predisposition to MDS/AML were described, for example, *ETV6* and *ANKRD26*.[11] *RUNX1* and *ETV6* variants also predispose to lymphocytic malignancies.[12] The presentation of IT in individuals with a predisposition to MDS may include isolated mild to moderate thrombocytopenia, and normal platelet size and morphology; and thus overlaps with classical IT.[9,13]

An accurate diagnosis of inherited syndromes with a predisposition to leukemia has major therapeutic implications, including intensive follow-up of patients; this may entail an annual bone marrow examination.[14] The diagnosis is also crucial for determining the management program, including decisions regarding hematopoietic stem cell transplantation (HSCT) prior to the development of acute leukemia, and for selecting the appropriate conditioning for transplant. In addition, accurate molecular diagnosis is essential for identifying asymptomatic family members who may be at risk for myeloid transformation, for providing genetic counseling to affected patients and family members, and for selecting unaffected related donors for transplantation.

Genetic workup may be initiated with Sanger sequencing, when a single gene is the major cause of a particular disorder. However, due to the non-specific clinical and laboratory presentation of syndromes causing cytopenias, an unbiased diagnosis, as offered by next-generation sequencing (NGS) technologies, is usually essential.[11,15,16] Indeed, several studies have demonstrated advantages of NGS technologies for improving diagnosis,[11,13,15-18] and reported genetic diagnostic rates of 13-54%. However, differences were evident in the patient populations (IBMFS only, MDS only, IT only or all these conditions), age (children, adults or both), sequencing method (NGS panel or whole exome sequencing [WES]), the type of variants detected (germline variants or both somatic and germline variants) and the types of sequence variants reported (pathogenic and likely pathogenic [P/LP]) only or also variants of unknown significance [VOUS]). Therefore, comparing the results of those studies is challenging.

We hypothesized that a substantial fraction of children with persistent cytopenias may have inherited syndromes predisposing to MDS/AML and should be identified prior to the development of leukemia. We hereby present the results of a comprehensive Israeli nationwide study of germline variants detected in children with prolonged cytopenias, referred to our laboratory during a 4-year period. Only P/LP sequence changes were reported. Our study revealed that almost one third of the children had identifiable inherited disorders, of whom 79.7% predisposed to leukemia. Based on our findings, we suggest that children and young adults presenting with prolonged cytopenias should undergo genetic evaluation.

Methods

Patients

The study included children and adolescents (aged 0-20 years at presentation) with prolonged cytopenias, evaluated between January 2016 and December 2019. The patients were classified into five subgroups according to referral diagnoses: (i) suspected IBMFS, based on the presence of extra-hematopoietic manifestations and early onset cytopenias; (ii) MDS with BM dysplasia and less than 20% blasts or BM cytogenetic abnormality;[7,8,19] (iii) severe aplastic anemia (SAA), based on an abrupt onset of severe pancytopenia and marrow aplasia;[20] (iv) a refractory single cytopenia including thrombocytopenia (platelet count <150X10^9/L) or neutropenia (absolute neutrophil count <1X10^9/L up to 1 year of age or <1.5X10^9/L in older children and adults).[21] Exclusion criteria were hemolytic and microcytic anemias, overt leukemia, and a known pathogenic variant in the family.

Data extracted from medical charts included age at presentation, current age, ethnic origin, family history, extra-hematopoietic manifestations, and therapy. The laboratory data recorded comprised complete blood counts, hemoglobin electrophoresis, fluorescent *in situ* hybridization (FISH) telomere length, chromosomal breakage tests and BM morphology, cellularity (based on trephine biopsies in the majority of patients), cytogenetics and FISH-MDS panels for detecting common chromosomal changes.[22]

The study was approved by the Rabin Medical Center Institutional Review Board.

Sanger sequencing

We initiated the genetic workup by Sanger sequencing in patients with a clear clinical presentation and when variants in a single gene were a common cause of the specific suspected diagnosis. This approach included the genes *FANCA* for Fanconi anemia (FA), *ELANE* for severe congenital neutropenia (SCN), *RPS19* for Diamond Blackfan anemia (DBA), *SBDS* for Shwachman Bodian Diamond syndrome (SBDS) and *DKC1* for dyskeratosis congenita (DC). Sanger sequencing was also used to confirm NGS findings and family segregation.

Next-generation sequencing

Our custom-made NGS panel is continuously updated. The latest version included 226 known genes that cause inherited cytopenias (*Online Supplementary Table S1*). Importantly, the panel includes the *TERC* gene encoding for the RNA component of the telomerase, the non-coding 5'UTR regions of *DKC1* and *ANKRD26*, and intron 5 of *GATA2*, in which disease-causing variants have been described.[23] Panel design, library preparation and sequencing were performed as previously described.[24] The DNA used for genetic analysis was extracted from peripheral blood. Germline variants were identified by the variant allele frequency. In patients for whom it was uncertain whether the variant was of a germline origin, we performed Sanger sequencing on DNA extracted from fibroblasts.

Whole exome sequencing

WES was performed as described in Goldberg *et al.*[25]

Sequencing results and bioinformatics pipeline

Sequencing reads were aligned against a reference genome (GRCh37/UCSC hg19) and variants were called and annotated using the SureCall software (v.3.5.1.46; Agilent Technologies). SNP filtering was established as previously described,[24] using both an in-house platform and the Emedgene AI-based genomic analysis platform (Emedgene Technologies, Tel-Aviv, Israel). Genetic variants were reported according to the American College of Medical Genetics guidelines.[26,27] Only P/PL results were reported.

Copy number variant detection

Copy number variant (CNV) analysis was performed using "Rainbow"-Genoox CNV Caller. The Rainbow caller employs a machine-learning based anomaly detection algorithm, in which variants are determined based on exon-level coverage, using a cohort of samples (n>30). The model considers several factors including GC content, coverage variance over multiple samples and neighboring gene coverage (Genoox,Tel-Aviv, Israel). CNV findings were confirmed by multiplex ligation-dependent probe amplification (MLPA, MRC, Amsterdam, Holland) available for: *FANCA*, *RPS19*, *RPL5*, *RPS26*, *RPL11*, *RPS17*, *RPL35A*, *TERT* and *DKC1* and by cytoscan-high density SNP-array.[28]

Statistical analysis

Fisher exact test was used to compare the clinical and demographic characteristics between patients who were and were not diagnosed with inherited disorders. A *P*-value of <0.05 was considered statistically significant.

Table 1. Referrals and genetic diagnoses in our cohort.

Referral diagnosis	N of pts	N of genetically diagnosed pts (%)	Diagnosis according to molecular findings (N of pts)
IBMFS	48	29 (60.4)	IBMFS (29)
MDS	26	6 (23.1)	MDS predisposition (3)
			IBMFS (2)
			IT with MDS predisposition (1)
SAA	31	1 (3.2)	IBMFS (1)
Isolated thrombocytopenia	33	17 (51.5)	Classical IT (9)
			IT with MDS predisposition (8)
Isolated neutropenia	51	6 (11.8)	Congenital neutropenia (6)
Total	189	59 (31.2)	

IBMFS: inherited bone marrow failure syndromes; IT: inherited thrombocytopenia; MDS: myelodysplastic syndrome; SAA: severe aplastic anemia; No: number; pts: patients.
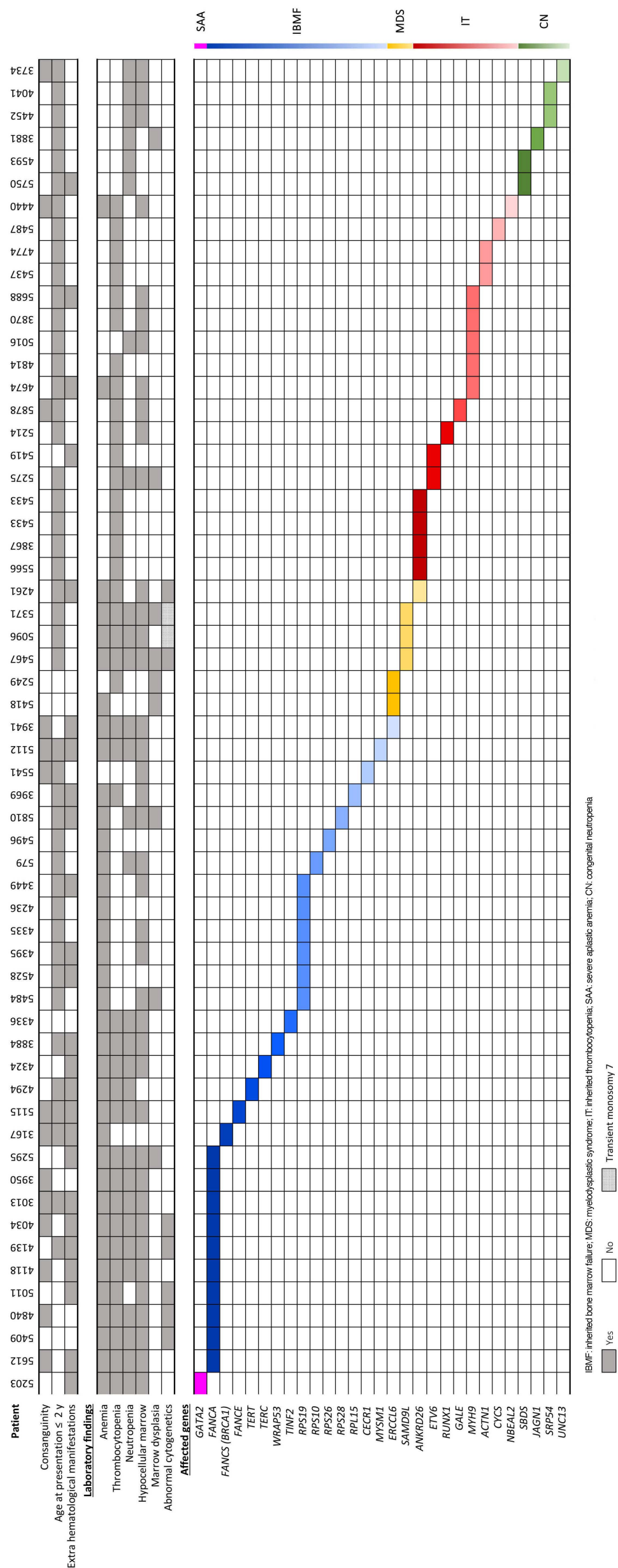
Figure 1. Integrated matrix of the 59 patients diagnosed with inherited cytopenias. The upper part of the panel shows demographic, clinical and laboratory details of our patients; the lower part shows the affected genes. Genes are ordered according to the diagnoses. The column on the right and the box colors relate to the referral diagnoses, and demonstrate the overlapping phenotypes caused by some affected genes. IBMFS: inherited bone marrow failure syndromes; MDS: myelodysplastic syndrome; SAA: severe aplastic anemia; CN: congenital neutropenia.

Results

Clinical diagnosis

During the study period, 189 DNA samples of children presenting with persistent cytopenias from twelve pediatric hematology centers in Israel were sent to the Molecular Hematology Laboratory in Schneider Children's Medical Center of Israel. The clinical referral diagnosis, defined by the treating hematologists, was IBMFS (48 patients), MDS (26 patients), SAA (31 patients), isolated neutropenia (51 patients) and isolated thrombocytopenia (33 patients) (Table 1).

Most patients were Israelis (96%). Eighty-seven (46%) of our patients were of non-Jewish origin, mainly Arabic, which is considerably higher than their proportion in the Israeli population (26.1%).[29] Almost one-third of the patients originated from consanguineous families (*Online Supplementary Table S2*). The median age at clinical presentation was 1 year (range, 0-20) and the median age at referral was 8 years (range, 0.5-41). Patients were referred at a median of 5.8 years following their first clinical presentation.

Genetic diagnosis

Of the 189 children referred for genetic evaluation, 49 had a clinical presentation suggestive of a specific diagnosis and, therefore, Sanger sequencing of commonly mutated genes was initially performed (*Online Supplementary Figure S1*). Diagnosis was reached for 13 (6 were homozygous for *FANCA* variants, 5 had heterozygous variants in *RPS19* and 2 were compound heterozygous for variants in the *SBDS* gene). Fourteen patients with a negative Sanger sequencing result were further evaluated using our NGS panel and 22 did not undergo further genetic workup (11 recovered, 6 are clinically stable and are being followed, 4 underwent successful HSCT and one was lost to followup). In total, 140 patients were initially directly referred for NGS panel diagnosis (*Online Supplementary Figure S1*).

Overall, P/LP variants were identified in 59 of 189 patients (31.2%). Of the diagnosed patients, 47 (24.9% of the whole cohort and 79.7% of the diagnosed patients) had an inherited predisposition to leukemia, while nine had IT affecting platelet production and function, and three had congenital neutropenia not currently known to cause leukemia predisposition (Figure 1; Tables 2 to 7; *Online Supplementary Table S3*). Eleven sequence variants, in ten patients, were defined as VOUS; these patients are not included in the analyses (*Online Supplementary Table S4*). In addition, 12 patients with a clinical and genetic diagnosis compatible with benign ethnic neutropenia were not included in the analyses.

Overall, 28.6% of the patients had congenital anomalies, with a statistically significant higher proportion among patients that were genetically diagnosed (39% and 23.8% in diagnosed and undiagnosed patients, respectively; *P*-value =0.038, *Online Supplementary Table S2*).

Patients presenting with inherited bone marrow failure syndromes

Of 48 patients who were referred with IBMFS, five had increased chromosomal breakage and three had short telomeres (Table 2; Table 3). In 29 of 48 (60.4%) patients, genetic variants explaining the clinical phenotype were found; of those, 27 had classical and two had non-classical IBMFS. Twelve patients were diagnosed with FA: ten were homozygous for *FANCA* variants, one had a homozygous *FANCS* (BRCA1) variant and one had a *FANCE* variant. Ten patients were diagnosed with DBA: six had heterozygous variant in *RPS19*, and one each had a heterozygous variant in the genes *RPS10*, *RPS15*, *RPS26* and *RPS28*. One additional patient carried a homozygous variant in the *CECR1* gene, which caused a DBA-like phenotype. Four patients with DC had genetic alterations in the *TERT*, *TERC*, *WRAP53* and *TINF2* genes. Two patients had non-classical IBMFS, with homozygous variants in the *ERCC6L2* and *MYSM1* genes (Figure 1; Table 3; *Online Supplementary Table S3*). Both were of consanguineous Arab families. The patient with the biallelic *ERCC6L2* variant also had congenital anomalies and developmental delay. The patient with a *MYSM1* homozygous variant had early onset pancytopenia, with a hypocellular marrow and a paucity of red cell precursors and mild developmental delay and short stature. This phenotype was similar to that previously described for a few individuals.[30,31]

Patients presenting with myelodysplastic syndrome

Twenty-six patients in this study had MDS, including 19 with RCC and seven with MDS and excess blasts (MDS-EB). Germline disease-causing variants were found in six of the 19 patients with RCC (31.6%). Three patients had variants in the *SAMD9L* gene, two in *ERCC6L2* and one in *ANKRD26* (Figure 1; Table 4; *Online Supplementary Table S3*). No germline pathogenic variants were detected in the seven patients presenting with MDS-EB. All three patients diagnosed with *SAMD9L* variants had monosomy 7, which was subsequently resolved in two (7 months and 4 years later) (Figure 1). The two patients with biallelic variants in *ERCC6L2* originated in consanguineous Arab families with no known relatives with MDS. Both were referred with pancytopenia and had BM dysplastic changes.

One child presented with a variant in the initiation codon of *ANKRD26*. He was born at term following a normal pregnancy, with a low birth weight (1.9 kg), a single umbilical artery and dysmorphic features. He had severe recurrent infections including *Pneumocystis Jiroveci* and *Cytomegalovirus* lung infections. Rechavi *et al.*[32] extensively studied this patient, who is now 8 years old, and found a mosaic monosomy 21, as well as defects in immunoglobulin

Table 2. Clinical characteristics of patients referred with the diagnosis of inherited bone marrow failure syndrome (diagnosed by Sanger sequencing).

Patient	Ethnic origin/ Consanguinity (+/-)	Gene	Disease/ Inheritance	MHGVS Coding	Age at presentation/ diagnosis	Hematological presentation	BM, cytogenetics and functional tests	Extra hematological manifestations	Outcome
4118	Arab Muslim (+)	FANCA	FA/AR	NM_000135: c.3490C>T-Hm	6y/6y	Pancytopenia	Hypocellular marrow, reduced megakaryocytes	Short stature, café au lait spots, pelvic kidneys	Post HSCT
4139	Arab Christian (-)	FANCA	FA/AR	NM_000135: c.4261-2A>C- Hm	1-7d/25y	Pancytopenia	Hypocellular marrow	Short stature, dysmorphic features	Post HSCT
4034	Arab Muslim (+)	FANCA	FA/AR	NM_000135.4): c.3382C>T-Hm	5y/5y	Pancytopenia	Hypocellular marrow, trisomy 1q, increased chromosomal breakage	Short stature, dysmorphic features	Followup
3013	Jewish (+)	FANCA	FA/AR	NM_000135.4: c.3788_3790delTCT- Hm	2y/4.5y	Pancytopenia	Aplastic marrow	Short stature, dysmorphic features, café au lait spots, skeletal anomalies	Died of SCC, post HSCT
3950	Arab Muslim (+)	FANCA	FA/AR	NM_000135.4: c.3788_3790delTCT- Hm	10y/10y	Pancytopenia	Hypocellular marrow, increased chromosomal breakage	None	Post HSCT
5295	Jewish (-)	FANCA	FA/AR	NM_000135.4: c.2172dupG- Hm	11y/15y	Pancytopenia	Hypocellular marrow, dyserythropoiesis, increased chromosomal breakage	Short stature, schizophrenia	Post HSCT
4528	Jewish (-)	RPS19	DBA/AD	NM_001022.3: c.98G>A-Ht	1-7d/0.9y	Anemia	Not done	Short stature	Followup
4337	Jewish (-)	RPS19	DBA/AD	NM_001022.4): c.184C>T-Ht	8-28d/0.5y	Anemia	Early erythroid maturation arrest	Short stature	Followup
4335	Other (-)	RPS19	DBA/AD	NM_001022.4): c.184C>T-Ht	1-7d/3y	Anemia	Hypocellular marrow, pure red cell aplasia	None	Followup
4236	Jewish (-)	RPS19	DBA/AD	NM_001022.3: c.98G>A-Ht	0.2y/16y	Anemia	Not done	None	Followup
3449	Jewish (-)	RPS19	DBA/AD	NM_001022.3: c.134T>C-Ht	8-28d/0.9y	Anemia	Hypocellular marrow, early erythroid maturation arrest	Short stature, hearing defect	Followup

BM: bone marrow; BMF: bone marrow failure; AR: autosomal recessive; AD: autosomal dominant; y: years; d: days; FA: Fanconi anemia; DBA: Diamond–Blackfan anemia; SCC: squamous cell carcinoma; Hm homozygous; Ht: heterozygous; HSCT: hematopoietic stem cell transplantation.

switching. Thrombocytopenia was noted at birth. When referred to a pediatric hematology center (at age 6 years) he had macrocytosis (MCV 98fL), thrombocytopenia (48X10^9/L), substantially high fetal hemoglobin (30%) and a hypoplastic BM with dysplastic changes.

Patients presenting with thrombocytopenia

Thirty-three patients presented with thrombocytopenia and an inherited cause was found in 17 (51.5%). Of them, nine (52.9%) had IT that was known to affect platelet production and function and eight (47.1%) had variants in genes that were known to cause thrombocytopenia with a predisposition to leukemia (Figure 1; Table 5; *Online Supplementary Table S3*). Of the nine patients with IT due to platelet production or function defects, five had variants in the *MYH9* gene, two in *ACTN1* and one each in *NBEAL2* and *CYCS*. Their thrombocytopenia ranged from mild to severe (7-125x10^9/L), with a normal to high MPV, 8-17.7 fL (normal values 5.6-12.1 fL[33]). Of the eight patients with thrombocytopenia and an inherited predisposition to MDS/AML, four had variants in the 5'UTR of *ANKRD26*, 2 in *ETV6*, 1 in *RUNX1* and one in the *GALE* gene. Seven patients had mild to moderate thrombocytopenia (45-117x10^9/L), with a normal MPV, 8.5-12 fL (normal values 5.6-12.1 fL[33]). The patient diagnosed with a variant in *GALE* had giant and pale platelets (MPV 17 fL), as we previously described.[34] He was of Bedouin origin and not related to the original family we described.

Patients presenting with neutropenia

Of the 51 patients presenting with neutropenia 6 (11.8%) were molecularly diagnosed (Figure 1; Table 6; *Online Supplementary Table S3*). Two patients had compound heterozygous variants in the *SBDS* gene. One had a homozygous variant in a known neutropenia causing gene, *JAGN1*[35]. One additional patient, presenting with familial neutropenia and recurrent infections, was subsequently diagnosed by WES with a novel SRP54 variant causing SCN and SBDS.[25,36] This gene was not known to cause neutropenia at the time the NGS study was performed. We later incorporated this gene into the subsequent versions of our NGS panel and detected a variant in this gene in one more patient that had a re-do of the NGS panel. An additional patient who presented with isolated neutropenia was later diagnosed by WES with a homozygous variant in *UNC13D*.

Twelve patients in our neutropenia group (8 patients of Muslim Arab origin and 4 of Yemenite Jewish origin), were homozygous to the known polymorphism in the *DARK* promoter (rs2814778, -30 T>C)[37]. They all had absolute neutrophil counts (ANC) >0.5x10^9/L (0.6-0.76x10^9/L) and no recurrent infections, compatible with the diagnosis of benign ethnic neutropenia. This same polymorphism was also found in one additional patient of Arabic origin, who presented with severe neutropenia (ANC levels <0.2x10^9/L) and recurrent infections, and therefore underwent HSCT. His phenotype suggests the presence of a yet undiscovered gene causing congenital neutropenia; the patient underwent successful HSCT.

Patients referred with suspected severe acquired aplastic anemia

Thirty-one patients with suspected SAA were referred to rule out IBMFS or other MDS predisposing syndromes. A pathogenic variant in the *GATA2* gene was found in one patient (3.2%) and his diagnosis was amended to IBMFS (Figure 1; Table 7; *Online Supplementary Table S3*). This patient had mild to moderate neutropenia (0.6-1.2x10^9/L) 2 years prior to the diagnosis of SAA. He had normal baseline monocyte counts, which were reduced to 0.02-0.2 K/micL, together with the development of neutropenia.

Discussion

In this paper, we presented the results of the genetic diagnosis of 189 children with prolonged cytopenias. P/LP germline variants were identified in 59 children (31.2%). Most of the diagnosed children (47/59, 79.7%) with persistent cytopenias had leukemia predisposition, while 12 children (20.3%) had either congenital thrombocytopenia with impairment of platelet production (9 children) or congenital neutropenia not currently known to predispose to malignant transformation (3 children).

In most children referred for genetic evaluation of cytopenias, NGS diagnosis was performed upfront. In the minority, when the clinical picture suggested a known disorder commonly caused by variants in a single gene, we initiated the genetic workup with Sanger sequencing. Of the various NGS methods, we used NGS panels as they offer a uniformly high depth of sequencing of the genes of interest. An advantage of our panel is that it was designed also to include non-protein coding regions such as the 5'UTR of *ANKRD26* and *DKC1*; the RNA component of the telomerase, encoded by the *TERC* gene; and intronic regions in *GATA2*. We report only P/LP sequence changes. The drawback of the NGS panel method is that the time lag between the identification of a new gene and its insertion into the panel requires frequent panel updating. We modified our panel seven times during the 4-year study period. All children referred for a molecular workup had a provisional diagnosis determined by their treating hematologist. For most patients, the genetic diagnosis supported the referral diagnosis. One patient with suspected SAA, who was evaluated to rule out an inherited disorder, was indeed diagnosed with a germline *GATA2* variant; this changed the diagnosis to IBMFS. Three patients had pathogenic variants in the *ERCC6L* gene; two of them were

Table 3. Clinical characteristics of patients referred with the diagnosis of Inherited bone marrow failure syndrome (diagnosed with next generation sequencing panel).

Patient	Ethnic origin/ Consanguinity (+/-)	Gene	Disease/ Inheritance	MHGVS Coding	Age at presentation/ diagnosis	Hematological presentation	BM, cytogenetics and functional tests	Extra hematological manifestations	Outcome
5612	Arab Muslim (+)	FANCA	FA/AR	NM_000135.4: c.3749_3750insT-Hm	7y/8y	Pancytopenia	Hypoplastic marrow, increased chromosomal breakage	Short stature, café au lait spots	Followup
5409	Jewish (-)	FANCA	FA/AR	1. NM 000135.2: c.2172-2173 insG-Ht 2.NM_000135.4: c.891_893+1delICTGG-Ht	6y/6y	Pancytopenia	Hypoplastic marrow, monosomy 7, increased chromosomal breakage	None	Post HSCT
4840	Arab Muslim (+)	FANCA	FA/AR	NM_000135.2: c.4069 4070insT-Hm	6y/7y	Pancytopenia	Hypoplastic marrow, del7q	Short stature	Lost to followup
5011	Yazidi (+)	FANCA	FA/AR	NM 000135.2: c.1361_1374delinsGAG -Hm	4y/14y	Neutropenia, thrombocytopenia	Severe aplastic marrow, complex karyotype	Café au lait spots	Lost to followup
3167	Arab Muslim (+)	FANCS (BRCA1)	FA/AR	NM_007300.4: c.1115G>A – Hm	1-7d/4y	Anemia	Normal	Café au lait spots, short stature, dysmorphism, developmental delay	Followup
5115	Arab Muslim (+)	FANCE	FA/AR	NM 021922.2: c.1114-8G>A-Hm	1y/10y	Pancytopenia	Aplastic marrow	Café au lait spots, short stature	Post 2nd HSCT
4294	Jewish (-)	TERT	DC/AD	NM_198253.3: c.2834_2842delACTACTCCA insCACCT-Ht	1-7d/30y	Pancytopenia	Red cell hyperplasia	Short stature, splenomegaly	Followup
4324	Arab Muslim (-)	TERC	DC/AD	NR_001566: r.172A>G -Ht	4y/8y	Pancytopenia	Hypoplastic marrow, short telomeres	Café au lait spots, short stature	Post HSCT
3884	Jewish (-)	WRAP53	DC/AR	NM_0011143990.1: c.936c>G-Hm	1-7d/4y	Pancytopenia	Hypocellular marrow, short telomeres	Short stature	Followup

Patient	Ethnic origin/ Consanguinity (+/-)	Gene	Disease/ Inheritance	MHGVS Coding	Age at presentation/ diagnosis	Hematological presentation	BM, cytogenetics and functional tests	Extra hematological manifestations	Outcome
4336	Arab Muslim (-)	*TINF2*	DC/AD	NM_001099274.3: c.813dupA-Ht	4y/5y	Pancytopenia	Hypoplastic marrow, short telomeres	None	Lost to followup
5484	Jewish (-)	*RPS19*	DBA/AD	NM_ 001022.4: c.384_385delAA -Ht	1-7d/2.5y	Anemia	Hypoplastic and mild red cell dysplasia	None	Followup
579	Jewish (-)	*RPS10*	DBA/AD	NM_001202470.2: c.71A>G-Ht	8-28d/30y	Anemia	Aplastic marrow	None	Post HSCT
5496	Arab Muslim (-)	*RPS26*	DBA/AD	NM_001029.5: c.23delA-Ht	8-28d/41y	Anemia	Not done	None	Followup
5810	Arab Muslim (-)	*RPS28*	DBA/AD	NM_001031.4: c.2T>C -Ht	1-7d/2y	Anemia, neutropenia	Mild dysplastic red cell precursors	None	Followup
3969	Jewish (-)	*RPL15*	DBA/AD	NM_001253379.2: c. 29T>C – Ht	1-7d/0.4y	Anemia	Pure red aplasia	Preterm 27 weeks	Followup
5541	Arab Christian (+)	*CECR1*	DBA-like/ AR	NM_001282225.1: c.1397_1403del-Hm	8-28d/20y	Anemia	Pure red aplasia	None	Followup
5112	Arab Muslim (+)	*MYSM1*	BMF/AR	NM_0010085487.2: c.2329-2A>G-Hm	1-7d/1.2y	Pancytopenia	Hypocellular marrow	Dysmorphic features	Followup
3941	Druze (+)	*ERCC6L 2*	BMF/AR	NM_020207.4: c.3525+2T>G – Hm	7y/9y	Pancytopenia	Hypocellular marrow	Short stature, dysmorphic facies, café au lait spots, hearing loss, developmental delay, schizo-affective disorder	Followup

NGS: next generation sequencing; BM: bone marrow; BMF: bone marrow failure; AR: autosomal recessive; AD: autosomal dominant; y: years; d: days; FA: Fanconi anemia; DC: Dyskeratosis Congenita; DBA: Diamond-Blackfan anemia; Ht: heterozygous; Hm: homozygous; HSC: hematopoietic stem cell transplantation.

referred with MDS and one with a suspected IBMF. Similarly, of the five patients with variants in *ANKRD26*, four had isolated thrombocytopenia and one had MDS (Table 1; Figure 1). This emphasizes the variable phenotypic presentation of children with inherited cytopenias and supports an unbiased diagnostic approach.

Inherited bone marrow failure syndromes
Of the 48 patients referred for an NGS workup with a diagnosis of IBMFS, 60.4% were genetically diagnosed. All but two had classical IBMFS, including FA, DBA and DC (Figure 1; Table 2; Table 3; *Online Supplementary Table S3*). These results are similar to those obtained in recent studies, in which the diagnosis rate of IBMFS was 48-59%.[38,39] The relatively high accurate referral diagnosis of patients with IBMFS is probably related to the classical clinical presentation, including typical congenital anomalies (in 70.8% of the patients in this group; *Online Supplementary Table S2*), as well as to the availability of functional screening tests for FA (chromosomal breakage

test) and DC (telomere length) that support the diagnosis.

Myelodysplastic syndromes
Of 26 children referred with MDS, based on cytopenias and BM morphology, with or without cytogenetic abnormalities, six (23.1%) were found to have germline variants: three in *SAMD9L*, two in *ERCC6L2* and one in *ANKRD26* (Figure 1; Table 4; *Online Supplementary Table S3*). Interestingly, although germline variants in *GATA2* are commonly found in pediatric patients with MDS,[9] none of our patients referred with MDS had genetic alterations in this gene. Feurstein *et al.* recently found that 19% (13/68) of young adults with MDS/AML had sequence variants in leukemia predisposition genes,[17] including in *GATA2*, *ERCC6L2*, *RUNX1*, *ANKRD26*, *CSF3R*, DC genes, *FANCA* and *PARN*. None of them had *SAMD9/L* variants; the latter have been commonly described in children with MDS.[9] In our study, all pathogenic variants were found in patients with RCC, while none of the seven patients with MDS-EB car-

Table 4. Clinical characteristics of patients referred with a diagnosis of myelodysplastic syndrome.

Patient	Ethnic origin/ Consanguinity (+/-)	Gene	Disease/ Inheritance	MHGVS Coding	Age at presentation/ diagnosis	Hematological presentation	BM, cytogenetics and functional tests	Extra hematological manifestations	Outcome
5467	Jewish (-)	*SAMD9L*	MDS/AD	NM_152703: c.4736A>G-Ht	0.4y/0.8y	Pancytopenia	Hypocellular with myelodysplasia, monosomy 7	None	Followup
5096	Jewish (-)	*SAMD9L*	MDS/AD	NM_152703.4: c.4045C>G-Ht	0.9y/5.5y	Pancytopenia	Hypocellular marrow, monosomy 7 -resolved	None	Followup
5371	Eritrean (-)	*SAMD9L*	MDS/AD	NM_152703.2: c.2957G>A -Ht	1.2y/2y	Pancytopenia	Hypocellular, dysplastic megakaryocytes, monosomy 7 -resolved	None	Followup
5418	Arab Muslim (+)	*ERCC6L2*	BMF/AR	NM 001010895.2: c.535A>G- Hm	20y/27y	Anemia, followed by pancytopenia	Dysplastic red cell precursors	None	Died of sepsis
5249	Druze (+)	*ERCC6L2*	BMF/AR	NM_020207.7: c.3492+2T>G Hm	16y/16y	Anemia, thrombocytopenia	Hypercellular dysplasia	None	Died of sepsis
4261	Jewish (-)	*ANKRD 26*	IT-MDS/AD	NM_001256053.1: c.3G>A -Ht	1-7d/6y	Thrombocytopenia	Hypocellular marrow, monosomy 7, monosomy 21 (germline)	Short stature, mildly dysmorphic features, combined immune deficiency, bicuspid aortic valve	Followup

BM: bone marrow; BMF: bone marrow failure; IT: inherited thrombocytopenia; MDS: myelodysplastic syndrome; AR: autosomal recessive; AD: autosomal dominant; y: years; d: days; Ht: heterozygous; Hm: homozygous.

ried variants in known relevant genes. Similarly, of 43 patients with germline variants in *SAMD9/L* enrolled in the European Working Group of MDS in childhood (EWOG-MDS), 39 (91%) had RCC and only four (9%) had MDS-EB.[40] The reason for this observation is currently unclear.
We observed phenotypic variability among the three patients with biallelic variants in *ERCC6L2*: one is a 7-year-old boy who presented with IBMFS and two patients, aged 16 and 20 years, presented with MDS. The *ERCC6L2* gene encodes for a transcription–coupled nucleotide excision repair protein and when mutated, causes genomic instability and affects mitochondrial function, leading to increased levels of reactive oxygen species.[31] Most patients described so far were children presenting with IBMFS.[41] Bluteau *et al.* found *ERCC6L2* variants in seven (8%) of 86 patients diagnosed with IBMFS. However, two of these seven patients, both aged 22 years, had marrow dysplastic changes; and one patient developed AML at the age of 43 years.[6] Poor prognosis AML-M6 was recently identified in adult members (aged 39-59 years) of four Finnish families,

Table 5. Clinical characteristics of patients referred with isolated thrombocytopenia.

Patient	Ethnic origin/ Consangui-nity (+/-)	Gene	Disease/ Inheritance	MHGVS Coding	Age at diagnosis/ presentation	Hematological presentation	BM, cytogenetics and functional tests	Extra hematological manifestations	Outcome
4674	Jewish (-)	*MYH9*	IT/AD	NM_002473.6: c.287C>T -Ht	8-28d/18y	Thrombocytopenia	Mild myeloid hypoplasia	Mental retardation, renal failure	Followup
4814	Jewish (-)	*MYH9*	IT/AD	NM_002473.5: c.4270G>A -Ht	1-7d/0.3y	Thrombocytopenia	Not done	None	Followup
5016	Arab Muslim (-)	*MYH9*	IT/AD	NM_002473.4: c.5797C>T -Ht	1-7d/5y	Thrombocytopenia	Reduced megakaryocytes	None	Followup
3870	Jewish (-)	*MYH9*	IT/AD	NM_002473.6: c.287C>T-Ht	1y/5y	Thrombocytopenia ("ITP")	Reduced megakaryocytes	None	Followup
5688	Jewish (-)	*MYH9*	IT/AD	NM_002473.5: c.4641G>T-Ht	0.9y/15y	Thrombocytopenia	Normal	None	Followup
5437	Jewish (-)	*ACTN1*	IT/AD	NM_001130004.1: c.1019C>T -Ht	1-7d/10.5y	Thrombocytopenia	Not done	None	Followup
4774	Jewish (-)	*ACTN1*	IT/AD	NM_001130004.1: c.1019C>T-Ht	8-28d/17y	Thrombocytopenia	Not done	None	Followup
5487	Arab Muslim (-)	*CYCS*	IT/AD	NM_018947.5: c.274A>G-Ht	0.5y/5y	Thrombocytopenia ("ITP")	Not done	None	Followup
4440	Arab Muslim (+)	*NBEAL 2*	IT/AR	NM_015175.3: c.7225-1G>C-Hm	8-28d/4y	Thrombocytopenia	Reduced megakaryocytes	None	Followup
5566	Jewish (-)	*ANKRD 26*	IT-MDS/AD	NM_001256053: c.-134G>A -Ht	8-28d/4y	Thrombocytopenia ("ITP")	Not done	None	Followup
3867	Jewish (-)	*ANKRD 26*	IT-MDS/AD	NM_014915.2: c.-127A>G –Ht	8-28d/20y	Thrombocytopenia	Not done	None	Followup
3610	Jewish (-)	*ANKRD 26*	IT-MDS/AD	NM_014915.3: c.-128G>T-Ht	8-28d/1.5y	Thrombocytopenia ("ITP")	Normal	None	Followup
5433	Jewish (-)	*ANKRD 26*	IT-MDS/AD	NM_001256053: c.-134G>A -Ht	1-7d/1y	Thrombocytopenia	Not done	None	Followup
5275	Jewish (-)	*ETV6*	IT-MDS/AD	NM_001987.4: c.1103T>G -Ht	0.7d/18y	Thrombocytopenia ("ITP")	Hypocellular marrow	None	Followup
5419	Arab Muslim (-)	*ETV6*	IT-MDS/AD	NM_001987.4 c.1104C>G-Ht	5.5d/6y	Thrombocytopenia	Not done	Developmental delay, short stature	Followup
5214	Jewish (-)	*RUNX1*	IT-MDS/AD	NM_001001890.2: c.532+1_532+10del GTAAGTGCAT-Ht	8-28d/2.5y	Thrombocytopenia	Reduced megakaryocytes	None	Followup
5878	Arab Muslim (+)	*GALE*	IT-MDS/AR	NM_000403.3: c.151C>T –Hm	8-28d/1.5y	Thrombocytopenia	Not done	None	Followup

BM: bone marrow; IT: inherited thrombocytopenia; MDS: myelodysplastic syndrome; AR: autosomal recessive; AD: autosomal dominant; y: years; d: days; Ht: heterozygous; Hm: homozygous; ITP: immune thrombocytopenia.

with variants in *ERCC6L2*.[42] Future studies will help elucidate the full natural history of this disorder.

Monoallelic nucleotide substitutions at the *5'UTR* of the *ANKRD26* gene were found in 18% of the patients with IT and are the most common cause of IT with a predisposition to MDS/AML.[11,43] These variants cause gain of function and disrupt the downregulation of the expression of *ANKRD26* by *RUNX1* and *FLI1*. This causes suppression of megakaryopoiesis, which leads to thrombocytopenia and to MDS/AML later in life.[44] We describe here an initiation codon variant in *ANKRD26* (c.3G>A), in an 8-year-old boy with mosaic monosomy 21 who presented with MDS. This variant was previously described in two adult patients with AML and was shown to cause *ANKRD26* overexpression as well.[45]

Inherited thrombocytopenia

Of the 33 patients referred with thrombocytopenia, 17 (51.5%) had IT (Figure 1; Table 5; *Online Supplementary Table S3*). Of them, nine (27.3% of 33) had variants affecting late megakaryopoiesis and platelet production, without a predisposition to leukemia (in *MYH9*, *ACTN1*, *NBEAL2* and *CYCS*); while eight patients (24.2% of 33) had variants in genes encoding for growth factors involved in early megakaryopoiesis and a predisposition to leukemia (4 had variants in the 5'UTR of *ANKRD26*, 2 in *ETV6* and 1 in *RUNX1*; and 1 had a biallelic genetic alteration in *GALE*). Molecular

diagnosis of IT is essential, not only for identifying patients with a predisposition to leukemia but also for offering accurate diagnosis and avoiding the false diagnosis of immune thrombocytopenia (ITP), with possible subsequent futile treatment including splenectomy.[46,47] Indeed, five of our IT patients (29.4%), diagnosed with *ANKRD26*, *ACTN1*, *ETV6*, *CYCS* and *MYH9* variants, were considered to have chronic ITP and were treated as such (Table 5). These results emphasize that persistence of mild to moderate thrombocytopenia from birth, and a lack of response to ITP therapy should alert physicians to suspect IT.

Congenital neutropenia

In six of 51 (11.8%) pediatric patients with a clinical and laboratory diagnosis of congenital neutropenia we detected variants explaining the clinical phenotype (Figure 1; Table 6; *Online Supplementary Table S3*). Two patients were diagnosed with SBDS; one patient was diagnosed with a *JAGN1* homozygous variant. Two other patients who had negative panel results were later diagnosed by WES, with variants in *SRP54* and in *UNC13D*, a rare cause of neutropenia.[48] Another patient was diagnosed with *SRP54* following its incorporation into the NGS panel.

Since benign ethnic neutropenia is not rare in our population, we subsequently looked for the known homozygous polymorphism in the *DARK* promoter (rs2814778, -30 T>C), which was previously described in people of African and

Table 6. Clinical characteristics of patients referred with isolated neutropenia.

Patient	Ethnic origin/ Consanguinity (+/-)	Gene	Disease/ Inheritance	MHGVS Coding	Age at presentation /diagnosis	Hematological presentation	BM, cytogenetics and functional tests	Extra hematological manifestations	Outcome
3881	Jewish (-)	*JAGN1 (CN)*	CN/AR	NM_032492.3: c.3G>A -Hm	2y/20y	Neutropenia	Early myeloid maturation arrest	Recurrent infections	Followup
4452**	Jewish (-)	*SRP54*	CN/AD	NM_003136.3: c.349_351de-lACA	2y/30y	Neutropenia	Hypoplastic marrow, early myeloid arrest	Recurrent Aphthae	Followup
4041	Arab Christian (-)	*SRP54*	CN/AD	NM_003136.3: c.349_351de-lACA	8-28d/11y	Neutropenia	Late myeloid arrest	Recurrent infections	Followup
5750*	Jewish (-)	*SBDS*	SBDS/AR	1. NM_016038: c. 183_184 delTAinsCT - Ht 2. NM_016038: c.258+2 t>c: splice - Ht	0.3y/1y	Neutropenia	Normal marrow, abnormal exocrine pancreatic functions	Short stature, shortened long bones, small kidneys	Followup
4593*	Jewish (-)	*SBDS*	SBDS/AR	1. NM_016038: c. 183_184 delTAinsCT - Ht 2. NM_016038: c.258+2 t>c: splice - Ht	0.2y/1.5y	Neutropenia	Normal	Short stature	Followup
3734**	Arab Muslim (+)	*UNC13*	CN/AD	NM_199242.2: c.679C>T- Hm	8-28d/2.4y	Neutropenia, later pancytopenia	Hypocellular marrow	None	Post HSCT

BM. bone marrow; CN: congenital neutropenia; AR: autosomal recessive; AD: autosomal dominant; y: years; d: days; SBDS: Shwachman-Bodian-Diamond syndrome; Ht: heterozygous; Hm: homozygous; HSCT: hematopoietic stem cell transplant. * Diagnosed by Sanger sequencing, **diagnosed by whole exome sequencing.

Table 7. Clinical characteristics of patients referred with a presumptive diagnosis of severe aplastic anemia.

Patient	Ethnic origin/ Consanguinity (+/-)	Gene	Disease/ Inheritance	MHGVS Coding	Age (years) at presentation/ diagnosis	Hematological presentation	BM, cytogenetics and functional tests	Extra hematological manifestations	Outcome
5203	Jewish (-)	*GATA2*	BMF/AD	NM_001145661.1: c.1186C>T -Ht	11/11	Pancytopenia	Severe hypoplastic marrow	Mild dysmorphic features	Post HSCT

BM: bone marrow; BMF: bone marrow failure; AD: autosomal dominant; Ht: heterozygous; HSCT: hematopoietic stem cell transplant.

Arab ancestry, and in Yemenite Jews.[37] Thirteen of our patients were indeed found to be homozygous for the described polymorphism. In twelve, the clinical phenotype was compatible with ethnic neutropenia, while one patient had a severe phenotype and underwent successful HSCT, suggesting the presence of a yet undiscovered gene causing congenital neutropenia.

Notably, most patients referred with prolonged neutropenia had no genetic diagnosis. Using both NGS panels and WES, Blombery *et al*. did not identify a molecular diagnosis in ten of 11 patients with IBMFS who were referred with suspected SCN.[38] Using an NGS panel, Galvez *et al*. successfully diagnosed only three of 25 patients with prolonged neutropenia.[18] Overlap with primary immunodeficiencies,[49] new unrecognized genes (estimated to be the cause in about 25% of patients with SCN)[50] and acquired neutropenia probably contribute to the low yield of pathogenic variant detection among these patients. The overall poor results that we and others achieved calls for better selection of candidates for genetic diagnosis due to isolated neutropenia.

Severe acquired aplastic anemia

Only one patient of 31 (3. 2%) referred with SAA was found to have IBMFS with a variant in GATA2 (Figure 1; Table 7; *Online Supplementary Table S3*). Most patients in this group underwent an NGS workup. However, three patients underwent only a partial workup by Sanger sequencing. Keel *et al*. found that among 98 pediatric and young adult patients who underwent HSCT due to what was considered SAA, five (5.1%) had germline genetic alterations in *DKC1*, *MPL* and *TP53*.[15] In their study of germline variants in young adults with aplastic anemia, Feurstein *et al*. found that six of 39 (15,4%) patients had MDS germline predisposition.[17] Our patient was already on immunosuppressive therapy when the genetic test results were received. Immunosuppressive therapy was discontinued, and he successfully underwent HSCT. Interestingly, asymptomatic neutropenia was documented 2 years prior his development of pancytopenia. The low number of patients with SAA diagnosed with an underlying inherited syndrome in our cohort may be related to our routine use of an extensive workup to exclude inherited disorders, including chromosomal breakage test and evaluation of telomere length.

A main limitation of this nationwide study is that it was conducted retrospectively and that patients were referred at the discretion of the treating physicians. All the patients referred during the study period were included in our cohort, yielding a wide variety of clinical presentations. Therefore, we divided the patients to subgroups according to referral diagnoses and analyzed the results of each subgroup independently.

In summary, using NGS panels, we performed a nationwide study of 189 children who presented with prolonged cytopenias. To the best of our knowledge, this is the first comprehensive genetic study of children presenting with a wide range of clinical manifestations including IBMFS, MDS, SAA, thrombocytopenia, and neutropenia. We were able to identify P/LP variants in 31.2% of the children, the majority in genes predisposing to leukemia. Positive diagnostic results were most often achieved in children with suspected IBMFS and with isolated thrombocytopenia. We conclude that applying Sanger sequencing and NGS panels to children with persistent cytopenias is important for identifying inherited syndromes, especially those predisposing to leukemia. This may direct close monitoring and intervention prior to the development of overt leukemia.

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