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Challenges in Diagnosing Myelodysplastic Syndromes in the Era of Genetic Testing: Proceedings of the 13th Workshop of the European Bone Marrow Working Group

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Bone marrow · Workshop · Myelodysplastic syndrome · Next-generation sequencing · Classification · Cytopenia

Abstract

The 13th workshop of the European Bone Marrow Working Group in Utrecht, The Netherlands, was devoted to studying myelodysplastic syndromes (MDS) and their boundaries. The panel received 44 cases submitted to the 3 invited categories, which included: reactive cytopenias with dysplasia, idiopathic cytopenia of undetermined significance, clonal haematopoiesis of indeterminate potential, idiopathic dysplasia of uncertain significance and overt MDS. For this summary, we have selected 17 cases that highlight difficulties in separating true MDS from other causes of cytopenia and the

intricate relationship between clonal haematopoiesis and true MDS. In addition, cases of overt MDS with challenging features were also selected. All cases were stained for p53 expression. Using instructive submitted cases we discuss the following: (1) cytopenia with clonal haematopoiesis not fulfilling MDS criteria, (2) cytopenia and/or dysplasia with germline mutations and/or familial history suggesting an underlying gene defect, (3) MDS based on a recurrent chromosomal abnormality and (4) overt MDS with diagnostic difficulties due to concurrent treatment or disease. The lively discussion in the open forum of the workshop illustrated the need for better integrative understanding of the evolution of acquired genetic abnormalities in haematopoiesis, and the challenge of diagnosing true MDS in cytopenic patients with genetic abnormalities, either germline or acquired.

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Introduction

The diagnostic evaluation of patients with cytopenia requires the combination of clinical, laboratory and/or genetic data. Dysplasia in at least one haematopoietic cell line is mandatory for a diagnosis of myelodysplastic syndrome (MDS) [1]. The current work-up of cytopenic patients includes cytogenetic and often molecular genetic testing to help establish clonality and to provide prognostic information if abnormal. A relatively large number of patients are detected with genetic alterations in the blood or bone marrow, otherwise lacking sufficient criteria for the diagnosis of MDS. The designation “clonal cytopenia of undetermined significance” (CCUS) has been proposed for such instances [2]. Diagnostic criteria of CCUS are discussed in this issue of *Pathobiology* [3]. The true risk of developing overt MDS in CCUS, depending on the involved gene and the type of genetic alteration, is still under investigation [4, 5]. In addition, some cytogenetic aberrations are considered to be diagnostic of MDS, even in the absence of sufficient dysplasia to diagnose MDS on morphologic grounds. These aberrations include monosomy 7 or 13, loss of 5q and balanced translocation of chromosome 3, among others [1].

Genetic analysis of patients with suspicion of MDS can also reveal an underlying germline genetic defect predisposing them to develop a myeloid neoplasm. Although patients with germline mutations can present with clinical symptoms or develop an overt myeloid neoplasm at any age, most patients present in childhood, often in the context of a specific clinical syndrome. Such genes include *TERC* [6] and *GATA2* [7] that are now recognized in the group of genetic abnormalities predisposing to myeloid neoplasms in the updated World Health Organization (WHO) classification [8]. The general diagnostic criteria of MDS also apply to these cases, but establishing a diagnosis of overt MDS can be challenging in cases with congenital dysplasia or in cases characterized by an evidence of bone marrow failure associated with a hypocellular bone marrow. It is also important to note that even in the context of overt dysplasia in a cytopenic patient, the diagnosis of MDS can be difficult because of cytotoxic treatment or concurrent diseases that can induce cytopenia or dysplasia or mask an underlying MDS.

The 13th workshop of the European Bone Marrow Working Group (EBMWG), held in Utrecht, The Netherlands, invited submission of cases on 3 topics (1) reactive cytopenia and dysplasia, (2) clonal haematopoi-

esis of indeterminate potential (CHIP), idiopathic cytopenia of undetermined significance (ICUS) and idiopathic dysplasia of unknown significance (IDUS), and (3) overt MDS. In total, 44 cases were received. Immunohistochemical staining for p53 using the DO-7 antibody was performed on trephine biopsies of all workshop cases (Table 1).

For this summary, we made a selection of cases that did not completely fulfil clinical, morphological and/or genetic features of MDS as well as cases of overt MDS with challenging features (Table 1). They are discussed below, grouped in the following categories: (1) cytopenia with clonal haematopoiesis not fulfilling MDS criteria, (2) cytopenia and/or dysplasia with germline mutations and/or family history suggesting an underlying gene defect, (3) MDS based on a recurrent chromosomal abnormality and (4) overt MDS with diagnostic difficulties due to a concurrent treatment or disease. Each category starts with brief case descriptions followed by a more extensive discussion, including the take-home messages for the practical diagnostic approach to such cases.

Patients and Results

Cytopenia with Clonal Haematopoiesis Not Fulfilling MDS Criteria

Case 1: Panel Diagnosis: CCUS (Differential Diagnosis of MDS with Single Lineage Dysplasia [MDS-SLD])

A 50-year-old man, otherwise healthy, had been followed for neutropenia and thrombocytopenia for 15 years prior to the bone marrow biopsy (BMB). The normocellular bone marrow showed left-shifted erythropoiesis with occasional megaloblastic changes and rare hypolobated micromegakaryocytes (<10%) without a blast increase. The cytogenetic studies revealed 45,X,-Y[6]/47,XY,+8[4]/46,XY[15], and a next-generation sequencing (NGS) myeloid panel identified a mutation of *ZRSR2* (variant allele frequency [VAF] 77%).

Case 2: Panel Diagnosis: CCUS with Later Progression to Overt MDS-SLD; Plasma Cell Neoplasm (Monoclonal Gammopathy of Undetermined Significance that Progressed to Smouldering Plasma Cell Myeloma)

A 68-year-old man was diagnosed with macrocytic anaemia, mild thrombocytopenia and monoclonal gammopathy of undetermined significance (MGUS; IgG lambda 0.4 g/L) after a 2-month period of B-symptoms. BMB displayed subtle dysplasia in maturing granulocytes, megaloblastic erythropoiesis, occasional hypolobated megakaryocytes, and occasional clusters of lambda-restricted atypical plasma cells (<5% of all cells; Fig. 1a). The karyotype was normal and morphologic features were insufficient for MDS, but pathogenic mutations in *ASXL1*, *EZH2*, *SETBP* (VAF 34–37%), and *TET2* (VAF 72%) were identified. The diagnosis of CCUS and plasma cell neoplasm, MGUS, was made. A follow-up BMB after 6 months showed a hypercellular marrow

Table 1. Selected cases from the 13th workshop of the EBMWG

Case No. author	Clinical data	Panel diagnosis	Genetic abnormality	P53 (DO-7) staining*	Special features	Follow-up
<i>1 CCUS</i>						
1 F. Fend, Eberhard-Karls University, Tübingen, Germany	M 50, cytopenia since 15 years	CCUS	45.X-Y[6]/47.XY,+8[4] ZRSR2 (p.Met71le, c.21G>A) VAF 77%	Negative	Prolonged clinical presentation	No MDS
2 M.J. Kluk, Weill Cornell Medical College, New York, USA	M 68, bicytopenia	CCUS	ASXL1 (c.1730C>G; p.S577*) VAF 35%, EZH2 (c.73C>T; p.R25*) VAF 37%, SETBP1 (c.2612T>C; p.1871T) VAF 34%, TET2 (c.4044+1G>C) VAF 72%	Negative	Combined with plasma cell neoplasia	After 6 months progression to MDS-SLD
3 G.C. Capomietti, University of Pennsylvania, Philadelphia, USA	F 69, pancytopenia	CCUS	DNMT3A (c.1784T>C; p.L595P) VAF 22%	Negative	PNH clone, evolving AA?	None
4 E. Ballesteros, University of Connecticut, Farmington, USA	M 67, bicytopenia, neutrophilia	Not diagnostic for MDS	47, XY,+8[14] SRSF2 (c.284C>A; p.Pro95His) VAF 47%, IDH2 (c.419G>A; p.Arg140Gln) VAF 46%	Negative	RA, steroids, MTX, radiotherapy for conjunctival MZL	After 2 years cytopenia suspicious for MDS
<i>3 Germline predisposition</i>						
5 K.K. Reichard, Mayo Clinic, Rochester, USA	M 15, immunodeficiency, infections, lymphedema	Predisposition syndrome, progression to MDS-SLD?	Germine GATA2 365 bp deletion, ASXL1 (c.2638dup; p.Thr880Asnfs*2) VAF 11%	Negative	Clinical symptoms for 10 years	Progression to MDS-SLD?
6 M.A. Klimkowska, Karolinska University, Stockholm, Sweden	M 17, neutropenia, skin lesions	BMF with mutation	Homozygous USH1 (c.623A>G; p.His208Arg)	Negative	Clinical symptoms for 15 years	No MDS yet
7 S.H. Rizk, Cairo University, Cairo, Egypt	M 18 months, pancytopenia, dysmorphic	Hereditary BMF with dysplastic features	Normal karyotype No NGS data	Negative	Early onset, family history	Died 3 months after diagnosis
8 M. Yabe, Memorial Sloan Kettering Cancer Center, New York, USA	F 3, cytopenia	RCC	Monosomy 7 (59%) No mutations (30 gene myeloid panel)	Negative	Since age 3 months bruising, at 3 years dysplasia	Early postnatal presentation: genetic background?
<i>4 MDS with recurrent chromosomal abnormality</i>						
9 A. Chiu, Mayo Clinic, Rochester, USA	M 76, bicytopenia	MDS-U (cyto)	46,XY,inv(3)(q21q26.2)[3], GATA2, MECOM No NGS data	Negative	Poor prognosis, early disease?	None
10 A.M. Bogusz, University of Pennsylvania, Philadelphia, USA	F 66, bicytopenia	MDS-U (cyto)	46,XX,del(13q)(q12q22)[2] ETV6 (c.380G>A; p.R127Q [us*])	Negative	Only ATG/Cs/prednisone treatment	2 years stable
11 I. Kuzu, Ankara University, Ankara, Turkey	F 48, pancytopenia	MDS-U (cyto)	Monosomy 7 No NGS data	Negative	After 6 mo Cs for AA, minimal dysplasia	16 months stable with G-CSF and danazol
<i>5 MDS with concurrent treatment or diseases</i>						
12 P.A. Wright, Cambridge University Hospitals NHS Trust, Cambridge, UK	F 28, pancytopenia	Therapy-related MDS	46,XX,der(6)t(3;6)(q25;p23-24)[10] Extra copy MECOM (70%) No NGS data	Positive, >5-<10%	Melphalan ingestion	After 2 years progression to AML, died 3 months later
13 L. Boudova, Charles University, Pilsen, Czech Republic	M75, bicytopenia	MDS with isolated deletion (5q)	46,XX,del(5)(q13q32-34)[13] EGR1 deletion (80%), ATM (c.1510_1516delAACTTGG, p.Asn504fs) VAF 17%, ASXL1 p.G646fs*12 c.1934dupG VAF 29%, JAK2 (c.1849G>T, p.Val617Phe) VAF 9%	Positive, <5%	Vit. B12 deficiency due to atrophic gastritis	Improvement on lenalidomide treatment

Table 1. (continued)

Case No. author	Clinical data	Panel diagnosis	Genetic abnormality	P53 (DO-7) staining*	Special features	Follow-up
14 A. Chadburn, Weill Cornell Medical College, New York, USA	M 88, leukocytosis, anemia and thrombocytopenia	MDS, not further classifiable	<i>RUNX1</i> , VAF 47%, <i>TET2</i> , VAF 46%	Positive, <5%	Post infection	WBC normalized within days
15 L. Raso-Barnett, Cambridge University Hospitals NHS Trust, Cambridge, UK	M 80, mild anemia and thrombocytopenia	MDS-EB-2	Del 5q (48%), monosomy 7 (43%), extra copy <i>MEGOM</i> (52%) No NGS data	Positive, >20%	Azacitidine related erythroid hyperplasia/dysplasia	Died within a year of clinical onset
16 E.M. Hyjek, University of Chicago, Chicago USA	M 58, microcytic anemia	MDS-MLD (hypoplastic)	46,XY,del(7)(q22q36) (10%), <i>ASXL1</i> (c.2338C>T, p.Q780*), <i>U2AF1</i> (c.470A>C, p.Q157P), <i>ATRX</i> (c.7016del, p.T2339Kfs*4)	Negative	Acquired thalassemia, clonal T-cell expansion	6 months post-SCT relapse of del7q
17 S. Song, University of Pennsylvania, Philadelphia, USA	F 73, pancytopenia for 7 years	MDS-EB-1	<i>SRSF2</i> (c.284C>A, p.Pro95His) VAF56%, <i>ASXL1</i> VAF 51%, 2 <i>TET2</i> mutations VAF 47% and 49%	Negative	Left shifted granulopoiesis post steroids (autoimmune cytopenia)	Progression to MDS-EB-1 7 years after clinical onset

* p53 staining results refers to the presence of bone marrow cells with strong nuclear staining.

AA, aplastic anemia; BMF, bone marrow failure; Cs, ciclosporin; F, female; M, male; MDS-SLD, MDS with single lineage dysplasia; RCC, refractory cytopenia of childhood; us, uncertain significance; VAF, variant allele frequency.

with dysplastic, hypolobated megakaryocytes and an increased plasma cell population (10%; Fig. 1b). The mutation results were unchanged.

Case 3: Panel Diagnosis: Hypocellular Marrow with Paroxysmal Nocturnal Haemoglobinuria-Clone and CCUS, Suggestive of Evolving Aplastic Anaemia

A 69-year-old woman with pancytopenia and low reticulocyte count showed a hypocellular marrow with decreased myeloid and erythroid cells with full maturation, no dysplasia, and only very scarce, occasionally dysplastic megakaryocytes (Fig. 1c). Flow cytometry revealed a paroxysmal nocturnal haemoglobinuria (PNH)-clone (2% of red blood cells, 11% of neutrophils). The karyotype was normal. NGS showed a *DNMT3A* mutation (VAF 22%).

Case 4: Panel Diagnosis: Hypercellular Bone Marrow on Active Therapy, Suspicious but Not Diagnostic of MDS or MDS/MPN (Myeloproliferative Neoplasia), Recommend Repeat Bone Marrow after Stopping Methotrexate

A 67-year-old man with rheumatoid arthritis treated with methotrexate and prednisone for several years and a prior history of low-dose radiotherapy to the orbit because of conjunctival marginal zone lymphoma showed persistent normocytic anaemia, thrombocytopenia and neutrophilia for 2 years, but no monocytosis. The bone marrow was hypercellular with megakaryoblastic erythropoiesis and normal myeloid maturation. Megakaryocytes were adequate in number and highly variable in appearance, although <10% of cells were dysplastic (Fig. 1d). The karyotype was 47,XY,+8[14/20]. NGS identified pathogenic mutations of *SRSF2* (VAF 47%) and *IDH2* (VAF 46%). Testing for *JAK2*, *MPL*, and *CALR* mutations and *BCR-ABL1* rearrangement was negative.

All the above cases presented with cytopenia and one or more pathogenic MDS-type mutations, but they lacked significant dysplasia or a diagnostic clonal cytogenetic abnormality, required for a diagnosis of MDS [1]. Cases 1 and 4 showed trisomy 8, which is not sufficient to diagnose MDS. In addition, all had interfering factors that would discourage rendering an MDS diagnosis. These were a very long history of clinically stable cytopenias (15 years, case 1), drugs known to cause cytopenia/dysplasia or neutrophilia (methotrexate and prednisone, respectively, case 4, see also “Overt Myelodysplastic Syndrome with Diagnostic Difficulties Due to Concurrent Treatment or Diseases”), aplastic anaemia (AA) with a PNH-clone (case 3) or a concurrent plasma cell neoplasm (case 2).

In suspected MDS, an abnormal flow cytometric immunophenotype can help to identify low-grade MDS cases (e.g., reflected by the so-called Ogata score [9]) and to predict progression to overt MDS [10]. In CCUS, several additional clues may indicate an increased risk to develop MDS. These include the presence of 2 or more pathogenic mutations, a large size of the mutant clone as indicated by a high VAF and certain specific mutations or their combinations [5, 11, 12]. The “high-risk” myeloid gene mutation pattern in CCUS includes spliceosome gene mutations (especially *SF3B1*, but also *SRSF2*, *U2AF1*, *ZRSR2*); or a *DNMT3A*, *TET2* or *ASXL1* mutation in combination with any other mutation (all mutations should be at >10% VAF) [5, 11]. In contrast, isolated mutations in *TET2*, *DNMT3A* and *ASXL1*, which can be seen in healthy elderly people without cytopenia (CHIP), carry a lower risk of a subsequent myeloid neoplasm. According to its working definition, CHIP cases do not exhibit morphological evidence of a haec-

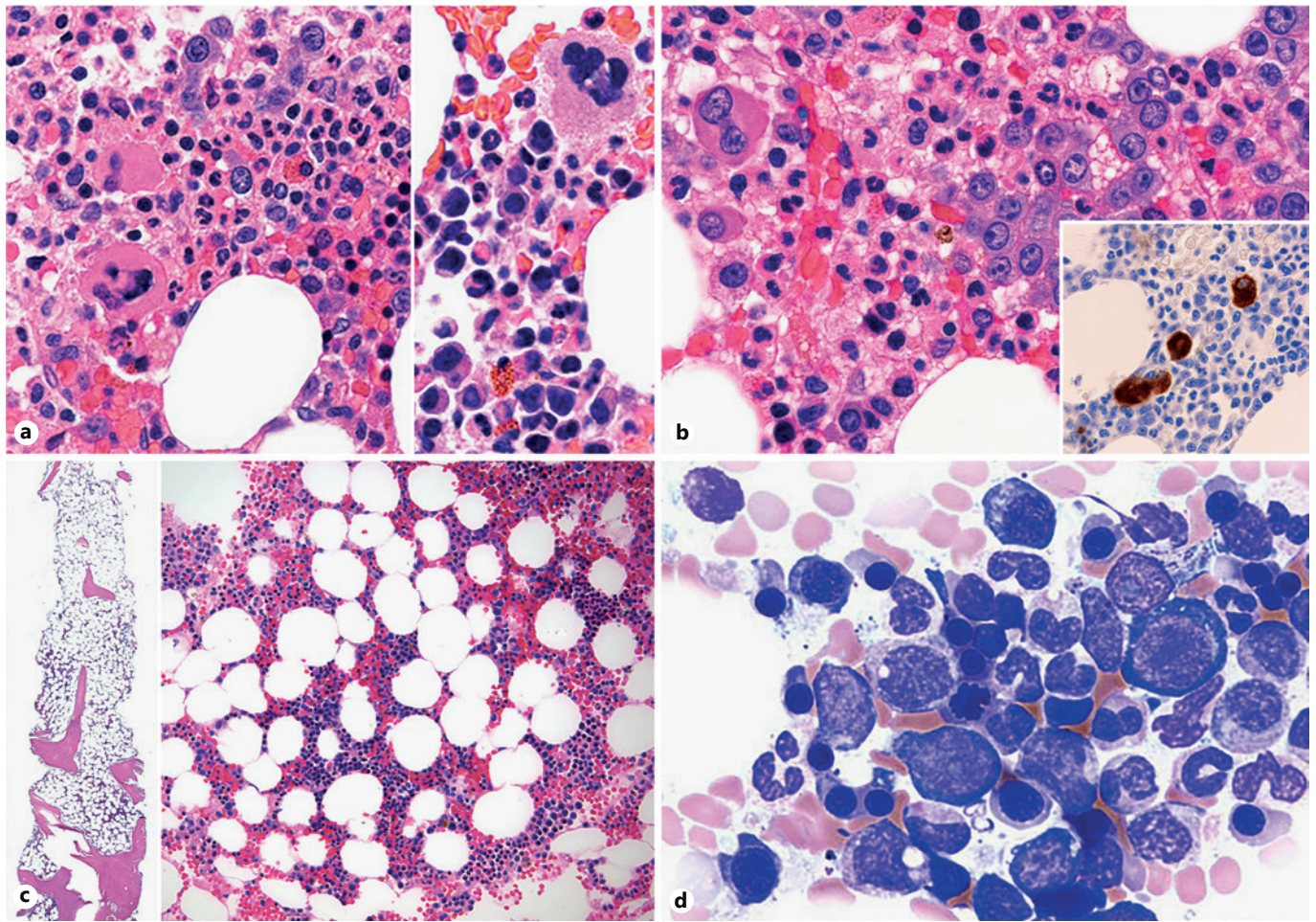


Fig. 1. Case 2: clonal cytopenia of undetermined significance (CCUS) and monoclonal gammopathy of undetermined significance (MGUS). **a** The bone marrow biopsy (BMB) shows mostly normal megakaryocytes (right and left, H&E). **b** The same case, 6 months later with progression to MDS-SLD (and smoldering plasma cell myeloma). Dysplastic, small hypolobated megakaryocytes are present on the BMB (H&E), as confirmed by CD42b IHC (inset). **c** Case 3: Hypocellular marrow with PNH clone and CCUS,

suggestive of evolving aplastic anaemia (AA). The trephine biopsy is markedly hypocellular (left). On higher magnification, the cellularity is mostly maturing erythroid and myeloid elements, with very scarce megakaryocytes (H&E). **d** Case 4: Hypercellular bone marrow on active therapy, suspicious but not diagnostic of MDS or MDS/MPN. The aspirate smear shows megaloblastic erythroid and normal myeloid maturation; rare dysplastic erythroid cells (<10%) are appreciated (wright Giemsa).

matological neoplasm, they do not meet diagnostic criteria for PNH, MGUS or monoclonal B-cell lymphocytosis, and they contain somatic mutations associated with haematological neoplasia at a VAF of at least 2% [2, 5, 13]. Case 2 illustrates that the presence of a pathogenic mutation combination (*TET2* and *ASXL*) at a high VAF permits early identification of evolving MDS and helps to rule out benign cytopenia (“reactive” dysplasia) even in the setting of concomitant plasma cell neoplasm.

The clinical significance of isolated mutations in the most commonly mutated spliceosome genes remains unknown. An isolated *ZRSR2* mutation was found in case 1 that was characterized clinically by prolonged and stable neutropenia and thrombocytopenia. Fleischman et al. recently reported 2 CCUS cases with an isolated *ZRSR2* mutation and refractory macrocytic anaemia with a clinical course similar to case 1 [14].

Case 4 shows a combination of iatrogenic factors (chronic prednisone and methotrexate treatment for rheumatoid arthritis) that can together account for the normocytic anaemia, thrombocytopenia, neutrophilia and dysplasia (as further described below in “Overt myelodysplastic Syndrome with diagnostic Difficulties Due to Concurrent Treatment or Diseases”). The additional trisomy 8 and mutations in the spliceosome gene *SRSF2* and the DNA methylation gene *IDH2*, however, suggest an underlying MDS, although dysplasia criteria were not yet fully met. The possibility of a therapy-related myeloid neoplasm associated with a previous short, low-dose radiotherapy focused on the orbit appeared unlikely, despite the fact that the WHO classification criteria for therapy-related myeloid neoplasms do not take into account the radiation dosage or degree of bone marrow exposure, and thus criteria for therapy-related MDS might be formally met in this case [15].

It has to be stressed that various parts of the findings have to be weighed and interpreted in an appropriate context of other clinical and laboratory findings for a reliable diagnosis. Solitary MDS-type mutations or chromosomal abnormalities in a cytopenic patient may sometimes represent CHIP [2] combined with reactive cytopenia. Significant (>10%) dysplasia may occur in non-MDS conditions too, as discussed below in “Overt Myelodysplastic Syndrome with Diagnostic Difficulties Due to Concurrent Treatment or Diseases”. Even if mutations and dysplasia are present, other possible causes of cytopenia must always be excluded. Repeated bone marrow sampling may be needed to establish the diagnosis, and, in some cases, to identify the progression to overt MDS.

MDS-type mutations indicative of clonal haematopoiesis occur frequently in AA as well; the majority of patients with AA have evidence of clonal haematopoiesis [16, 17]. An overlap of certain immunomorphologic and genetic features of AA, MDS and PNH is documented in case 3 combining CCUS with a *DNMT3A* mutation, a PNH clone and a hypocellular bone marrow, suggestive of evolving AA. *DNMT3A* mutations can be observed in MDS (5–13% of cases), AA (6–12%) [17], and acute myeloid leukaemia (AML; 22%) [18], but also in otherwise healthy people (2% of persons over 70 years) [16, 17, 19]. The pathophysiology of PNH clone expansion in AA or MDS is not well understood; nevertheless, small PNH clones may be found in 20–35% of patients with MDS, and 30–50% of cases of AA may evolve into classic PNH [20, 21], and/or eventually into MDS with or without subsequent AML [22]. These diagnostic challenges underline the role of bone marrow histology with a panel of immunohistochemical markers in the work-up of unclear cytopenias with/without dysplasia. Immunohistochemical staining for p53 may provide additional information to help differentiate hypoplastic MDS from AA [23, 24]. Strong nuclear p53 staining has in previous studies been shown to be useful as both a diagnostic and a prognostic marker [25, 26], and may be associated with an underlying *TP53* mutation [27]. All cases submitted to the bone marrow workshop were stained for p53 using clone DO-7 (DAKO) on unstained bone marrow slides (results in Table 1). Only one of the cases that did not fulfill MDS criteria showed bone marrow cells with strong nuclear p53 staining (case 6 in “Cytopenia and/or Dysplasia with Germline Mutations and/or Familial History Suggesting an Underlying Gene Defect”).

Another interesting feature illustrated by cases 4 and 17 (“Overt Myelodysplastic Syndrome with diagnostic Difficulties Due to Concurrent Treatment or Diseases”) is the possible relationship between pre-existing autoimmune disease (rheumatoid arthritis and autoimmune cytopenia) and the development of MDS. A recent review summarizes the evidence for immune-mediated pathophysiological mechanisms shared by autoimmune cytopenias, ICUS, IDUS and low-risk MDS, including cytokine dysregulation, increased apoptosis and the role of toll-like receptors [28].

Cytopenia and/or Dysplasia with Germline Mutations and/or Familial History Suggesting an Underlying Gene Defect

Case 5: Panel Diagnosis: Germline *GATA2* Mutation
Predisposition Syndrome (with Questionable Progression to MDS-SLD)

A now 15-year-old male had experienced recurrent streptococcal infections and bacteremias, and human papillomavirus-driven warts on the extremities from the age of 5 onwards. Lymphangiectasia in the right thigh region led to recurrent scrotal and penile

lymphedema. Immunodeficiency testing revealed B lymphopenia without hypogammaglobulinemia, an inverted CD4/CD8 ratio, and slight neutropenia and monocytopenia. His bone marrow was hypocellular (10–20%) with decrease of all cell lines, occasional dysplastic megakaryocytes but no blast increase. A *GATA2* mutation was detected in his blood and the bone marrow showed an additional *ASXL1* mutation (VAF 11%).

Case 6: Panel Diagnosis: Bone Marrow Failure in a Patient with *USB1* Gene Mutation

A 17-year-old male patient with neutropenia and lymphopenia was born in a consanguine family. He was affected by frequent infections (that required hospitalization) in his childhood and had remarkably dry skin with increasing, itchy hyper- and hypopigmented macules and patches all over his body (Fig. 2a). The skin on his palms and feet was thickened, his nails were hard and malformed (Fig. 2a insert), one toenail was missing, his fingers were clubbed and he had an epicanthus. The bone marrow was slightly hypocellular for his age with hypolobated megakaryocytes and eosinophilia (Fig. 2b). The aspirate smear showed neutrophils with hyposegmented or clumpy nuclei, pseudo-Pelger forms, large metamyelocytes, intercytoplasmic bridging and occasional megaloblastic changes in the erythroid precursors (Fig. 2c). On immunohistochemistry (IHC), blasts were slightly increased (3–4%), without clustering. Approximately 10% of myelopoietic cells strongly expressed p53 (Fig. 2d), and on flow cytometry some maturing granulocytic cells showed aberrant expression of CD56 (a non-specific finding, which in isolation does not imply a diagnosis of MDS). A genodermatosis was suspected and Fanconi anaemia was excluded by chromosome breakage studies. Panel sequencing revealed a homozygous mutation of the *USB1* gene, confirming the diagnosis of poikiloderma with neutropenia (Clericuzio type), a rare autosomal-recessive genodermatosis with associated bone marrow failure, predisposing to malignancy.

Case 7: Panel Diagnosis: Hereditary Bone Marrow Failure Syndrome with Dysplastic Features, Probably Autosomal Recessive

A 18-month-old boy with physical dysmorphism (long ears, short philtrum) had siblings’ history of transfusions and family members with a history of skin cancer. He was born in a consanguine family and presented with pancytopenia requiring transfusions early after birth. He showed a hypochromic microcytic anaemia, thrombocytopenia and neutropenia with dysplastic hypersegmented neutrophils. His karyotype was normal and t(8;21) and inversion 16 were excluded. The bone marrow was slightly hypocellular for his age with a reduction of the megakaryopoiesis with some pyknotic forms, megaloblastic erythropoiesis and considerable dysmaturation of the myelopoiesis. There was also siderosis. The child died 3 weeks later under supportive treatment without growth factors. No mutation screening was performed.

Case 8: Panel Diagnosis: Refractory Cytopenia of Childhood

A 3-year-old girl was referred with extensive bruising commencing 3 months after birth. She showed pancytopenia, lymphocytosis and monocytosis with increased foetal haemoglobin (5.5%). The bone marrow was severely hypocellular (10–30% cellularity) with dyserythropoiesis and dysplastic megakaryocytes, while granulopoiesis was markedly decreased and left shifted. Blasts were not increased. Fluorescence in situ hybridization (FISH) of the bone mar-

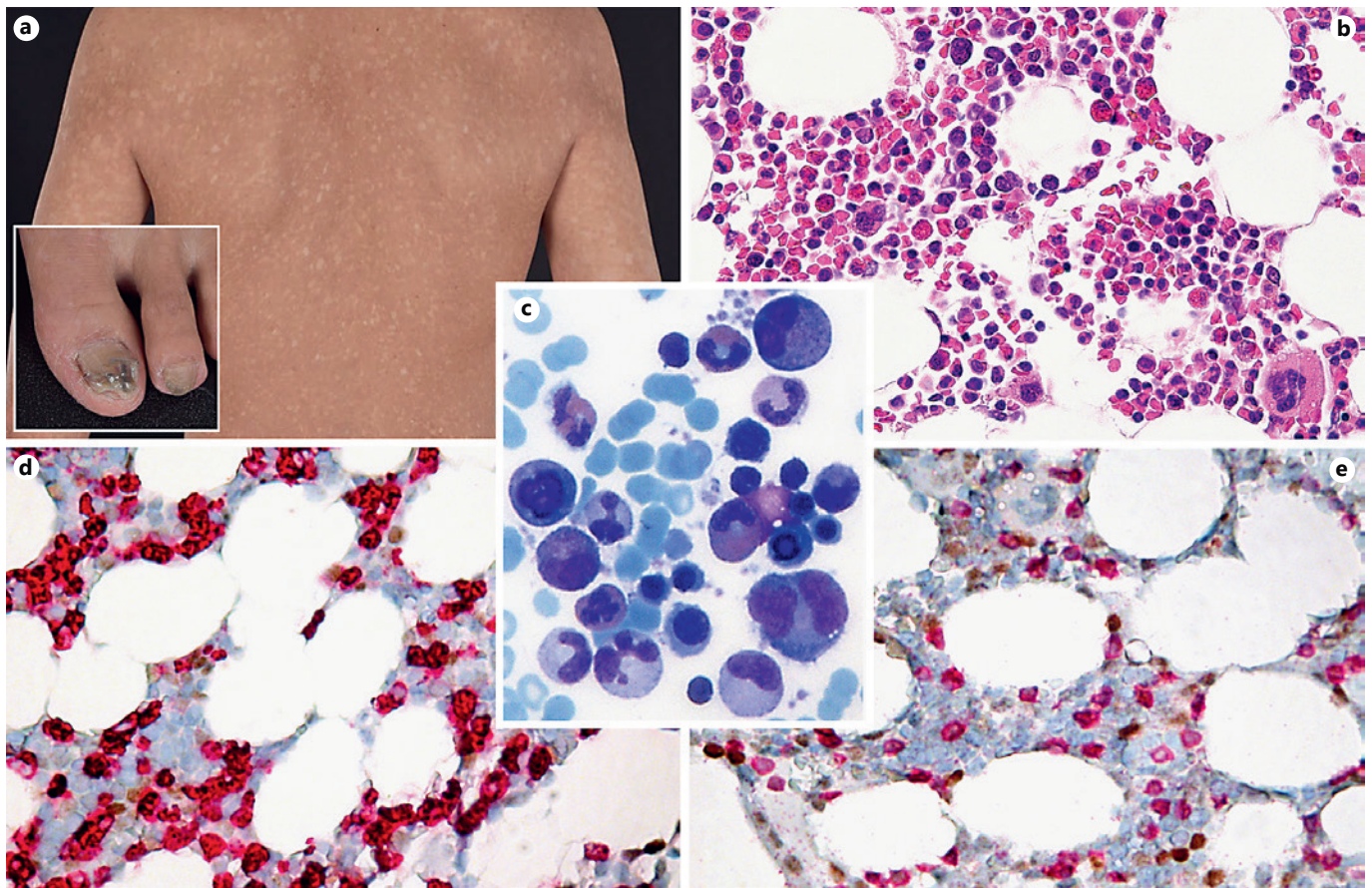


Fig. 2. Case 6: 17-year-old male patient affected by poikiloderma with neutropenia due to bi-allelic *USB1* germline mutation. **a** Clinical image with itchy hyper- and hypopigmented macules and patches all over the body and (insert) onychodystrophy. **b** Bone marrow biopsy (BMB) with slight hypocellularity and eosinophilia (H&E). **c** Bone marrow aspirate smear with dysgranulopoiesis (hypogranulated neutrophils with hyposegmented and clumpy

nuclei, pseudo-Pelger forms, large metamyelocytes, intercytoplasmic bridging) and occasional megaloblastic changes in the erythroid precursors (wright Giemsa). **d** p53 (brown)/myeloperoxidase (red) and **e** p53 (brown)/CD3 (red) double-stainings showing that approximately 10% of the myelopoietic cells abnormally co-express p53, while T-cells remain p53 negative, in line with the severe morphologic dysgranulopoiesis.

row showed monosomy 7 in 59% of the cells. A 30 myeloid gene NGS panel showed no mutations. The family history was not available.

Children and young adolescents that present with bone marrow failure, as in the above cases, require an integrative diagnostic approach, taking into consideration age, detailed clinical and family history, physical examination, and peripheral blood smear and bone marrow examination to narrow down the number of specific differential diagnoses and guide additional genetic studies to establish a diagnosis.

Of the major inherited bone marrow failure syndromes, Fanconi anaemia and dyskeratosis congenita have an especially high risk of developing MDS or AML, while the risk is lower in Diamond-Blackfan anaemia and Shwachman-Diamond syndrome [29]. Long-term granulocyte colony stimulating factor (G-CSF) therapy in severe congenital neutropenia increases MDS and AML risk to a similar level as in Fanconi anaemia [30].

Several other predisposing disorders with germline mutations have been acknowledged in the revised WHO classification [8].

Among these is *GATA2* deficiency due to a germline mutation that has a high risk of progression to MDS at an early age. The clinical presentation is quite diverse, ranging from lymphedema, severe mycobacterial or viral infections to AA [31], as in case 5. Only 20% of the patients initially present with a myeloid neoplasm, but most develop MDS or AML in the years after diagnosis [31].

Monosomy 7 as in case 8 is a frequent acquired aberration in both paediatric and adult MDS, either alone or in combination with other generic alterations [32]. In adults it is commonly associated with therapy-related myeloid neoplasms following chemotherapy with alkylating agents or occupational exposure to chemical toxins. Monosomy 7 has an adverse prognostic impact in both children and adults, depending on the presence of additional genetic abnormalities [32–34]. It is the most frequently acquired abnormality in MDS/AML associated with inherited bone marrow failure syndromes and is associated with a germline *GATA2* mutation in 37% of cases [35]. The incidence of monosomy 7 in refractory cytopenia of childhood is up to 49% of cases [33]. The early

onset of clinical symptoms in case 8 suggests a prodromal phase before overt refractory cytopenia of childhood at age 3. In similar cases, an undiscovered hereditary predisposition should be carefully investigated by dedicated gene screening. Another special condition that should be considered is familial monosomy 7, typically characterized by early-childhood onset and bone marrow insufficiency in at least one affected family member, and association with increased risk for MDS or AML [36]. Most patients present with evidence of bone marrow insufficiency such as petechiae, easy bruising and/or anaemia as in case 8. The monosomy 7 is not present at birth but is acquired within haematopoietic cells due to an inherited genetic predisposition that leads to mosaicism of chromosome 7 in peripheral blood and/or bone marrow. Germline *SAMD9* and *SAMD9L* variants have been recently linked to familial and sporadic cases of paediatric MDS with monosomy 7 [37]. Abnormal haematologic findings and mosaic monosomy 7 can already appear in young children with familial monosomy 7. The prognosis after occurrence of the monosomy is poor, with rapid progression to MDS/AML [36].

Non-cyclic cytopenias in patients with congenital malformations, dysmorphic features, nail dystrophies, café-au-lait spots, hypopigmentation or premature greying are suspicious of genodermatoses and require further genetic testing. Diagnoses that have to be excluded are dyskeratosis congenita and Fanconi anaemia. Poikiloderma with neutropenia caused by bi-allelic *USB1* germline mutations as in case 6 can overlap clinically with dyskeratosis congenita and shares the risk of developing myeloid neoplasia [38, 39]. The derived *USB1* protein has a role in RNA processing. Acquired mutations in *USB1* have been observed in a small subset (2/140 tested cases) of haematopoietic malignancies, supporting the role of the *USB1* gene in the development of myeloid neoplasms [40].

MDS Based on a Recurrent Chromosomal Abnormality

Case 9: Panel Diagnosis: MDS-Unclassifiable (Based on Cytogenetics in the Absence of Sufficient Dysplasia)

Macrocytic anaemia and thrombocytopenia were found in a 76-year-old male in the background of a hypercellular bone marrow with decreased iron stores, mild dyserythropoiesis and left-shifted myeloid maturation without increased blasts or significant dysmegakaryopoiesis (Fig. 3a), except for an occasional non-lobated megakaryocyte (Fig. 3b). The karyotype showed 46,XY,inv(3)(q21q26.2)[3]. *GATA2*, *MECOM* involvement was confirmed by FISH (Fig. 3b). No follow-up data was available.

Case 10: Panel Diagnosis: MDS-Unclassifiable (Based on Cytogenetics)

A 66-year-old female presented with persistent pancytopenia and a normocellular bone marrow with an increase in erythroid precursors but no dysplasia or increase of myeloid blasts. Initially an *ETV6* mutation with unknown significance but normal karyotype was found. A repeated biopsy after 6 months showed additional del(13)(q12q22) in 2/20 metaphases. The patient was stable for 2 years with transfusions and antithymocyte globulin/ciclosporin/prednisone treatment.

Case 11: Panel Diagnosis: MDS-Unclassifiable (Based on Cytogenetics)

A 48-year-old female had AA with pancytopenia and a hypoplastic bone marrow (15% cellularity) without dysplasia and with a normal karyotype. After 6 months of treatment with ciclosporin,

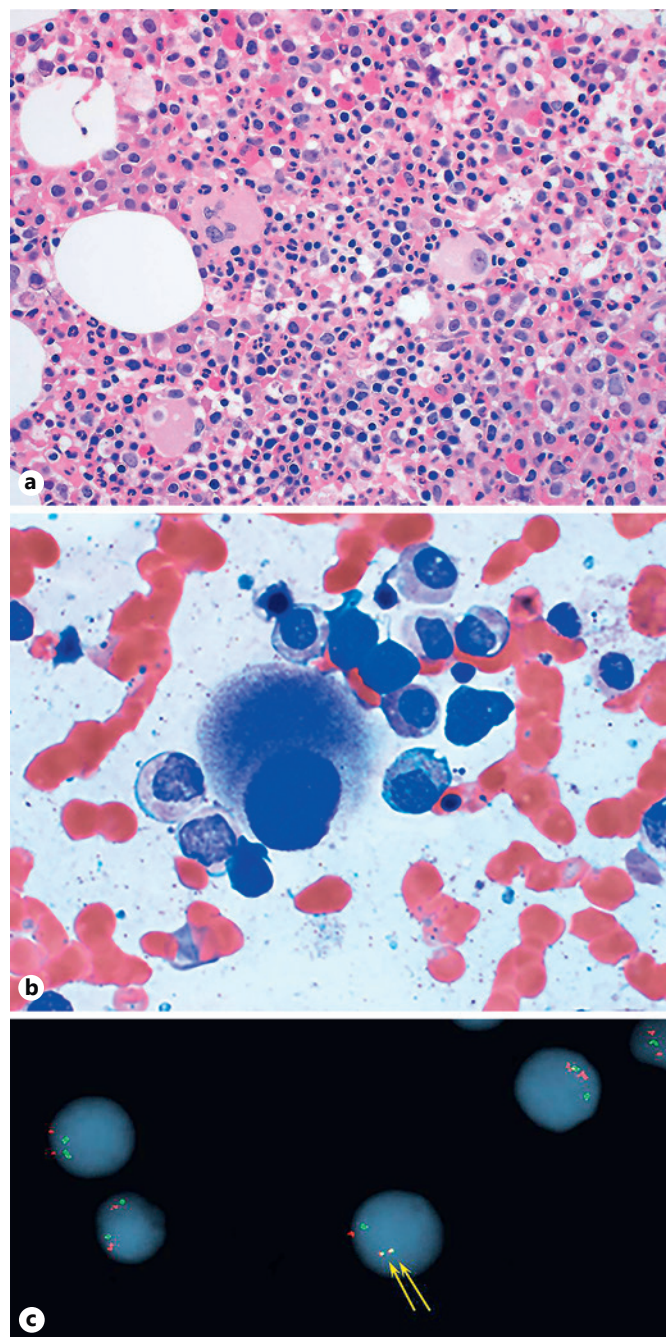


Fig. 3. Case 9: 76-year-old male with no past medical history who presented with macrocytic anaemia and thrombocytopenia. **a** Hypercellular BMB without significant dysplasia (H&E). **b** A single non-lobated megakaryocyte. Usually (but not in this case) non-lobated and bi-lobated megakaryocytes are easily identifiable in cases of myeloid neoplasms associated with inv(3) (q21.3;q26.2) or with t(3;3) (q21.3;q26.2). **c** FISH shows several normal cells (2 red and 2 green spots) and a rearrangement of the RPN1 enhancer (green spot) to the MECOM gene (red spot) on chromosome 3, indicative of inversion 3.

the clinical and laboratory parameters did not change. Under a temporary gluten-free diet and G-CSF treatment, transfusion need decreased and the bone marrow became hypercellular due to an increase of erythroid precursors. Megakaryocytes and myeloid precursors were decreased. At this stage, monosomy of chromosome 7 was found, while significant dysplasia was still lacking. Danazol was added to the G-CSF support and the patient no longer required transfusions for a follow-up of 16 months since presentation.

The presence of specific chromosomal abnormalities is sufficient for a diagnosis of MDS-unclassifiable, in patients with refractory, unexplained cytopenia, even if dysplasia and blast increase are absent [41]. These include (partial) loss of chromosomes 7 (case 11) or 13 (case 10), and inversion of chromosome 3, that is, inv(3)(q21.3;q26.2), or balanced translocation t(3;3)(q21.3;q26.2) (case 9). The latter cytogenetic abnormalities are often associated with the presence of small megakaryocytes with non-lobated or bilobated nuclei (Fig. 3b). In contrast, workshop case 9, that seems to represent an early clinical manifestation of the disease, lacks evidence of significant morphologic dysplasia.

The aggressive clinical course seen in patients with myeloid neoplasm with deregulation of *MECOM* and *GATA2* is unrelated to the number of blasts, that is, of the subclassification of the case as MDS or AML. Prognosis is even worse for cases with a complex or monosomal karyotype [42].

Deletion of 13q is found with a low frequency in AA and MDS, especially in cases lacking clear dysplasia as in case 10 [43, 44]. Associated characteristics are a good response to immunosuppressive therapy, frequent PNH clones, and a relatively low risk of progression to high-grade MDS or AML. This suggests an autoimmune/cytokine role in the disease pathogenesis [41].

Several other chromosomal abnormalities (loss of chromosome Y, trisomy 8 and del(20q) and MDS-related gene mutations (e.g. *DNMT3A*, *TET2*, *ASXL1*) are not sufficient to diagnose MDS, since they can be found as acquired clonal events in the haematopoietic cells of healthy people and patients without myeloid neoplasms [45, 46]. Since these mutations can drive clonal expansion of haematopoiesis, they are considered within the spectrum of CHIP, but they appear to show a relatively low risk of progression towards myeloid neoplasm [2].

Pathogenic germline mutations (including *RUNX1*) require a different approach as they can predispose to myeloid neoplasms, but by themselves are not sufficient to render a diagnosis of MDS, as described in “Cytopenia and/or Dysplasia with Germline Mutations and/or Familial History Suggesting an Underlying Gene Defect”.

Overt Myelodysplastic Syndrome with Diagnostic Difficulties Due to Concurrent Treatment or Diseases

Case 12: Panel Diagnosis: Therapy-Related Myeloid Neoplasm (Therapy-Related MDS). Note: Subsequent Progression to Therapy-Related AML

A 28-year-old female was admitted with vomiting, petechial rash and severe pancytopenia requiring transfusions, and was diagnosed with AA with a normal karyotype. Five months later, she collapsed and two partly empty bottles of melphalan tablets were found besides her. She admitted ingestion of melphalan prior to the first admission. Because of persisting thrombocytopenia after 10 months, a BMB was performed and showed reduced megakaryocytes. Almost 2 years after the first admission, an additional BMB following progressive pancytopenia revealed a cellular marrow

with myeloid and erythroid dysplasia, left-shifted, erythroid expansion and a reduced number of megakaryocytes, which were dysplastic. Interestingly, p53 staining revealed >5% cells with strong nuclear staining, while the initial (hypoplastic) bone marrow sample stained negative, indicating clonal evolution. The karyotype showed many abnormalities, including deletions, duplications and unbalanced translocations. With FISH, an additional copy of *MECOM* was seen. A diagnosis of a therapy-related myeloid neoplasm was made. During the work-up for allogeneic haematopoietic stem cell transplantation (HSCT), she progressed to full blown AML with a monosomal karyotype, including monosomy 7. The AML was refractory to chemotherapy and she died within 3 months from pneumocystis pneumonia.

Case 13: Panel Diagnosis: MDS with Isolated del(5q)

Incidentally, macrocytic anaemia and leucopenia were discovered in a 75-year-old male and were attributed to an underlying atrophic gastritis with very low vitamin B12 levels. Vitamin B12 therapy did not correct the blood values and he developed fatigue, dyspnea and transfusion dependency. A BMB showed a hypercellular marrow with increased, megaloblastic erythropoiesis and increased, frequently monolobated megakaryocytes but no blast increase. P53 staining revealed the presence of strongly positive staining precursor cells at a low frequency (<5%). The karyotype showed loss of chromosome 5(q13q32–34), and FISH confirmed loss of *EGR1* and *PDGFRB* genes on 5q, leading to a diagnosis of MDS with isolated del(5q). NGS did not detect any mutations of TP53, but of several other genes (*ATM* VAF 17%, *ASXL1* VAF 29%, *JAK2* VAF 9%). The patient improved markedly on lenalidomide with normal blood values and did not require transfusion.

Case 14: Panel Diagnosis: MDS Not Further Classifiable (no Excess Blasts). Note: Presence of Erythroblastopenia and Neutrophil-Rich Bone Marrow after Infection

An 88-year-old-male on antibiotics for a foot infection was seen with shortness of breath, fatigue and bilateral lower extremity oedema. Blood values showed leukocytosis ($19 \times 10^9/L$), anaemia and low platelets. Abdominal ultrasound showed a perihepatic lesion suspicious for an abscess, which was attributed to recent trauma. A BMB was 90% cellular with increased left-shifted granulopoiesis and 2% CD34+/CD117+ blasts. P53 staining showed 2% cells with strong nuclear staining. Erythropoiesis and megakaryopoiesis were markedly decreased with a few small, hypolobated megakaryocytes. Immunophenotyping by flow cytometry showed abnormal myeloid maturation, including CD56 expression on granulocytes and monocytes. FISH and karyotyping were normal. No mutations in MPN-related genes were detected, but mutations in *RUNX1* (VAF 47%) and *TET2* (VAF 46%) were present. The white blood cell count normalized on follow-up, while anaemia and thrombocytopenia persisted.

Case 15: Panel Diagnosis: MDS-EB-2. Note: Erythroid Proliferation Post Azacitidine Treatment

A BMB was performed in an 80-year-old previously healthy male because of normocytic anaemia and mild thrombocytopenia. The marrow was hypercellular with dyserythropoiesis, left-shifted hypogranular myelopoiesis, monolobated megakaryocytes, 5–10% CD34 positive blasts and >20% cells with strong p53 staining, including megakaryocytes (Fig. 4a). A repeated aspirate showed 15% blasts. FISH revealed loss of chromosome 5q, monosomy 7 and an

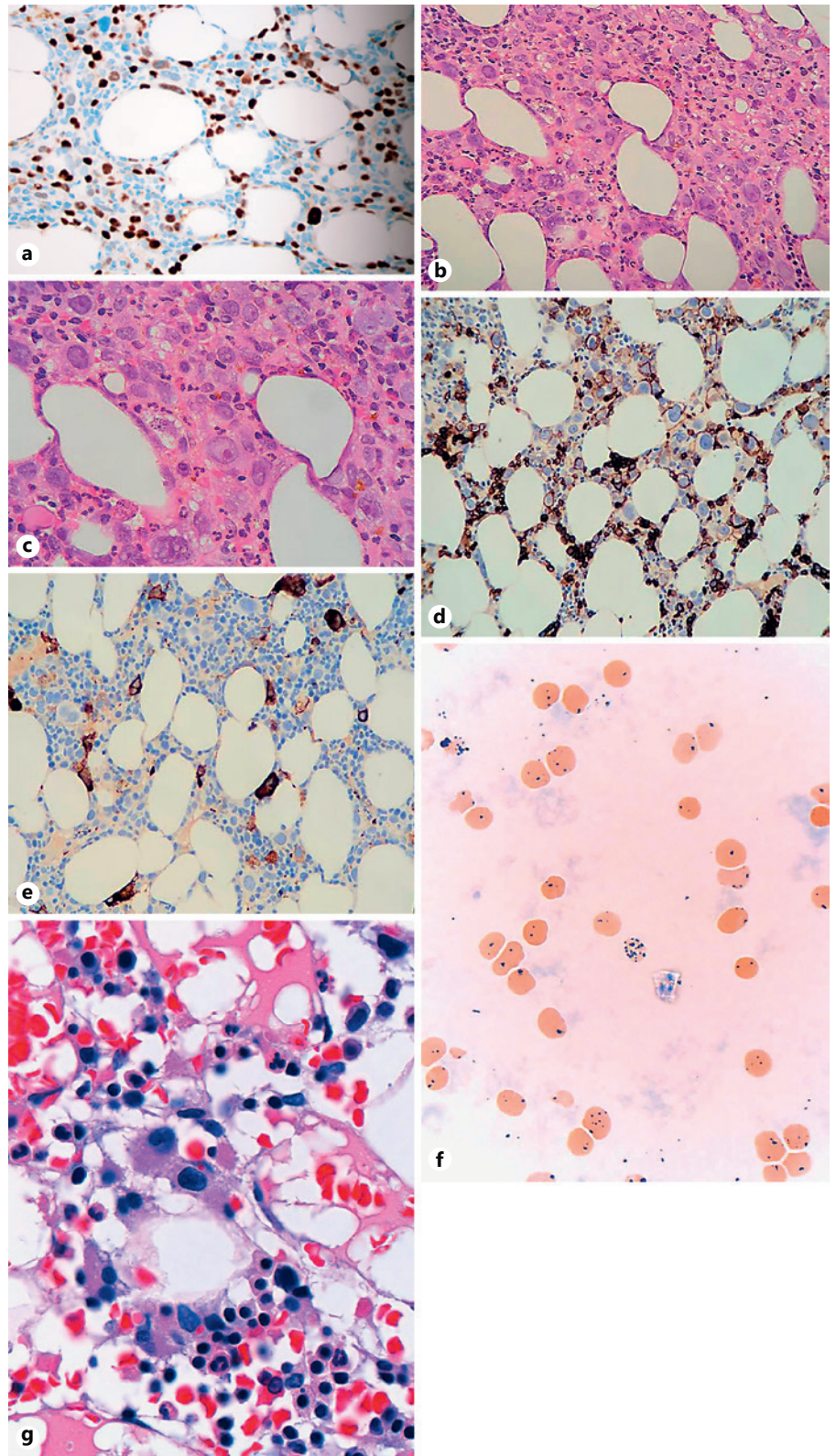


Fig. 4. Case 15: Bone marrow biopsy of an anaemic 80-year-old male. **a** P53 immunohistochemical staining shows an increased number of progenitor cells with strong nuclear staining. **b, c** Erythroid proliferation with dysplastic features and the dysmegakaryopoiesis (H&E). **d** Increased megaloid erythropoiesis is illustrated by glycophorin A. **e** CD61 highlighting the small dysplastic megakaryocytes. **f, g** Case 16. Acquired alpha-thalassemia and MDS in a 58-year-old male with microcytosis and anisopoikilocytosis. **f** Blood smear with haemoglobin H inclusions in the erythrocytes (wright Giemsa). **g** A hypocellular bone marrow biopsy with striking dysmegakaryopoiesis (H&E).

additional copy of MECOM in about 50% of the cells. MDS-EB-2 was diagnosed and azacitidine treatment was started. Although blood values temporarily normalized, after 6 cycles the clinical condition deteriorated to transfusion-dependent pancytopenia without circulating blasts. A new BMB showed a somewhat less cellular marrow with an increased, markedly anaplastic erythropoiesis with giant forms and prominent bright eosinophilic nucleoli and variable CD117 expression (Fig. 4b–d), accounting for up to 40% of all nucleated cells. There was profound dysmegakaryopoiesis, which included both micromegakaryocytes and small dysplastic forms with hypolobated nuclei (Fig. 4e). The aspirate showed trilineage dysplasia and 5% myeloid blasts. FISH remained unchanged with loss of chromosome 5q, monosomy 7 and an additional copy of MECOM. Azacitidine was discontinued. The patient remained severely pancytopenic and passed away 2 months later.

Case 16: Panel Diagnosis: MDS with Multilineage Dysplasia (MDS-MLD; Hypoplastic)

An anaemic 58 year-old-male presented with microcytosis and marked anisopoikilocytosis, including target cells, elliptocytes, acanthocytes and haemoglobin H (HbH) inclusions (haemoglobin electrophoresis revealed 9.9% variant HbH; Fig. 4f). His bone marrow was hypocellular with dysmegakaryopoiesis, dyserythropoiesis and mild dysgranulopoiesis but no blast increase (Fig. 4g); p53 staining was negative. Further genetic analysis showed del(7)(q22q36) in 2 out of 20 metaphases and pathogenic mutations in *ASXL1*, *U2AF1* and *ATRX*. The latter confirmed the diagnosis of acquired alpha-thalassemia in the context of MDS-MLD. In addition, a clonal T-cell population was detected in the bone marrow using IHC and molecular analysis. The patient underwent allogeneic HSCT with initial engraftment. Six months post-HSCT decreased chimerism heralded a cytogenetic relapse in a markedly hypocellular bone marrow.

Case 17: Panel Diagnosis: MDS-EB-1. Note: Left-Shifted Granulopoiesis after Steroids; Prior History of Autoimmune Cytopenia

Three years after the first discovery of mild pancytopenia at the time of diagnosis of breast carcinoma (radiation and hormonal therapy only), a 65-year-old female had a BMB for worsening pancytopenia. The marrow was mildly hypercellular without dysplasia. The karyotype was normal. She received steroids and several transfusions for presumed autoimmune cytopenia. Four years later, her pancytopenia worsened and she showed only a mild response to steroids. The bone marrow was now hypercellular with markedly increased and left-shifted myelopoiesis with 5% blasts and 58% myelocytes and promyelocytes. Megakaryocytes were dysplastic. The karyotype remained normal, NGS showed *SRSF2* (VAF 56%), *ASXL1* (VAF 51%) and 2 *TET2* mutations (VAF 47 and 49%). A subsequent BMB performed approximately 6 months later included the same mutations: *RUNX1* (VAF 34%), *SRSF2* (VAF 60%), *ASXL1* (VAF 41%), *TET2* (VAF 43% for both variants). The decreased percentage in the VAFs in the second biopsy suggests that these were not germline mutations.

Coincidence of MDS with another disease or co-morbid condition can pose a diagnostic challenge. MDS can be erroneously disregarded because of a concurrent disorder or treatment that seems to explain the cytopenia(s). Drugs in general can cause a wide range of haematologic abnormalities [47, 48]. Extreme myeloid hyperplasia, dysplastic features and/or left-shifted myelopoiesis

with increase of blasts can result from severe infection (case 14), cytotoxic therapy as Melphalan (case 12), G-CSF treatment or trauma. Glucocorticoid treatment alone (cases 4 and 17) can cause neutrophilia and induce dysplastic changes in myeloid cells [49].

Marked erythroid hyperplasia with/without dysplasia can occur in severe blood loss, deficiencies of folic acid or vitamin B12 (case 13), PNH, acquired α -thalassemia (case 16), azacitidine (case 15) or erythropoietin treatment. Azacitidine is an anti-metabolite that inhibits DNA methylation and disrupts RNA metabolism and protein synthesis with a direct effect on erythroblast maturation [50].

Germline mutations in the *ATRX* gene induce a neurological syndrome, the so-called Alpha-Thalassemia, mental Retardation X-linked (*ATRX*) syndrome. Acquired inactivating *ATRX* mutations do occasionally occur in the context of MDS, leading to microcytic anaemia, anisopoikilocytosis, and HbH inclusions as in case 16 [51]. Almost all reported patients are elderly males and the *ATRX* mutation does not seem to influence the chance of leukemic transformation.

Two of the cases in this category (cases 13 and 15) had loss of chromosome 5q. Both showed the characteristic non-lobated megakaryocytes of MDS with isolated del(5q), but this diagnosis is not applicable to case 15 due of the accompanying monosomy 7. Because of the adverse prognostic impact of monosomy 7, the presence of this additional abnormality excludes classification as MDS with isolated del(5q) that otherwise has a favourable outcome and responds to lenalidomide [52]. *TP53* mutations occur in MDS with del(5q) and, while they do not exclude this diagnosis, the presence of a mutation may be associated with resistance to lenalidomide. Thus, encountering such *TP53* mutations, even at low VAF, in otherwise typical del(5q) MDS appears to indicate more aggressive disease that may warrant other therapeutic approaches [27]. In the revised WHO 2016 classification, immunohistochemical staining for p53 is recommended particularly for this subgroup of MDS [52]. However, we recommend p53 staining in the diagnostic work-up of any unclear cytopenia with/without dysplasia and in all MDS subtypes, both at diagnosis and during follow-up, since it may be helpful as a diagnostic and prognostic marker and may indicate disease progression (case 12). Strong nuclear p53 staining may indicate an underlying *TP53* mutation; however, this should be confirmed by molecular analysis. The lack of immunohistochemical expression may in rare cases result from a nonsense mutation leading to formation of a truncated, non-immunoreactive protein. However, p53 IHC detects a large fraction of the *TP53* mutations in MDS, which are predominantly missense mutations [53]. Finally, it should be emphasized that the type of fixation, decalcification procedures and antibody selection may impact staining results when studying p53 in tissue sections in MDS, as results may vary with different antibodies as shown in previous studies [24].

Conclusion

MDS is one of the most challenging areas of diagnostic bone marrow pathology. While the disease is defined by the combination of cytopenia(s), dysplasia and clonal haematopoiesis, each of these features are not limited

to MDS. The 13th workshop of the EBMWG was devoted to the spectrum of benign simulators, borderline cases and overt MDS with unusual features. Here we have summarized a selection of the workshop cases illustrating the following themes: CCUS not fulfilling MDS criteria, cytopenia and/or dysplasia with germline mutations and/or familial history suggesting an underlying gene defect, MDS-unclassifiable based on a recurrent chromosomal abnormality and overt MDS with diagnostic difficulties due to concurrent treatment or diseases.

This workshop illustrates that integration of all data, including the knowledge of concurrent diseases and therapy and the patient's family history, as well as flow cytometry and molecular genetic findings is of utmost importance to avoid the pitfalls of over- and underdiagnosing MDS.

Summary

The most important lessons learnt from the workshop are:
In order to avoid overdiagnosis of MDS, be aware that:

- Significant (>10%) dysplasia may occur in non-MDS conditions.
- MDS-type mutations in a cytopenic patient can be seen in cases of co-occurrence of CHIP and non-MDS cytopenia (CCUS).
- Significant megakaryocyte dysplasia may be seen in germline mutation syndromes without MDS and can sometimes be induced by drugs.

In order to avoid underdiagnosing MDS, keep in mind that:

- MDS morphologic features can be obscured by concurrent diseases or treatment effects on the bone marrow, therefore, repeated bone marrow sampling may be necessary to establish the diagnosis.
- In the challenging setting of CCUS, it is most helpful to identify a “high-risk” myeloid gene mutation pattern. This helps to predict the cases that are probably “true” MDS precursors that will show progression, as illustrated by some of the workshop cases.
- Additional clues in CCUS that may indicate a “true” pre-MDS condition are flow cytometry abnormalities and mutations with high VAF.

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