Review Article

Refractory Thrombocytopenia and Neutropenia: a Diagnostic Challenge

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Abstract. The 2008 WHO classification identified refractory cytopenia with unilineage dysplasia (RCUD) as a composite entity encompassing refractory anemia, refractory thrombocytopenia (RT), and refractory neutropenia (RN), characterized by 10% or more dysplastic cells in the bone marrow respective lineage. The diagnosis of RT and RN is complicated by several factors. Diagnosing RT first requires exclusion of familial thrombocytopenia, chronic auto-immune thrombocytopenia, concomitant medications, viral infections, or hypersplenism. Diagnosis of RN should also be made after ruling out differential diagnoses such as ethnic or familial neutropenia, as well as acquired, drug-induced, infection-related or malignancy-related neutropenia. An accurate quantification of dysplasia should be performed in order to distinguish RT or RN from the provisional entity named idiopathic cytopenia of unknown significance (ICUS). Cytogenetic analysis, and possibly in the future somatic mutation analysis (of genes most frequently mutated in MDS), and flow cytometry analysis aberrant antigen expression on myeloid cells may help in this differential diagnosis. Importantly, we and others found that, while isolated neutropenia and thrombocytopenia are not rare in MDS, those patients can generally be classified (according to WHO 2008 classification) as refractory cytopenia with multilineage dysplasia or refractory anemia with excess blasts, while RT and RN (according to WHO 2008) are quite rare. These results suggest in particular that identification of RT and RN as distinct entities could be reconsidered in future WHO classification updates.

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Background: WHO Classification of MDS. Myelodysplastic syndromes (MDS) are marrow stem cell disorders characterized by ineffective hematopoiesis leading to blood cytopenias, a variable proportion of blasts, and a propensity to evolve to acute myeloblastic leukemia (AML). The first classification of MDS was published by the French-American-British group in 1982, individualizing five entities named refractory anemia (RA), refractory anemia with ringed sideroblasts, RA with excess blasts (RAEB), RA with excess blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMML).[1] This FAB MDS classification, mainly based on the morphologic features of the blood and the bone marrow was refined in 2002[2] and finally in 2008 by the World Health Organization,[3] that shifted the RAEB-T category to AML by lowering the threshold of bone marrow blasts for AML diagnosis from 30% to 20%, also excluded

CMML from MDS, individualized MDS with isolated deletion of the long arm of chromosome 5 (del 5q), and took into account the number of morphologically dysplastic myeloid lineages. This led to separate, in patients without excess of marrow blasts, those with multilineage dysplasia (refractory cytopenia with multilineage dysplasia or RCMD, with or without ringed sideroblasts) from patients with unilineage dysplasia (refractory cytopenia with unilineage dysplasia or RCUD) (**Table 1**).

RCUD as a Distinct Diagnostic Group in the 2008 WHO Classification. RCUD was thus identified as a new MDS group, containing three arbitrarily defined subgroups: refractory anemia (RA), refractory neutropenia (RN) and refractory thrombocytopenia (RT). It is important to consider that these diagnoses are mainly based on the bone marrow finding of a unique dysplastic lineage, contrarily to what their name would intuitively suggest. The characteristics of WHO-defined RCUD are detailed below.

Common characteristics of RCUD. Marrow findings should be unilineage dysplasia defined as the presence of $\geq 10\%$ dysplastic cells in one myeloid lineage. Less than 5% blasts are observed. The blood should contain $< 1\%$ blasts. Cases of unilineage dysplasia with 1% circulating blasts should be classified as MDS-U. If 2-4% circulating blasts are observed, the diagnostic classification is RAEB-1. Even though RARS has unilineage dysplasia, it is recognized as a distinct entity and not included in RCUD. Therefore, RA diagnosis is considered when only erythroid dysplasia is present and if $< 15\%$ ringed sideroblasts.

For the diagnosis of MDS, cytopenias are defined as hemoglobin < 10 g/dL, absolute neutrophil count (ANC) $< 1.8 \times 10^9/L$, and platelet count $< 100 \times 10^9/L$. Importantly, two cytopenias are accepted for the diagnosis of RCUD, provided there is only one dysplastic lineage in the bone marrow. In case of pancytopenia associated with only one dysplasia in the bone marrow, the classification should be MDS-U (**Table 1**). Also, the cytopenia does not always correspond to the bone marrow dysplastic lineage. In a series of 44 patients with a single cytopenia with unilineage dysplasia described by Verburgh et al, 18 (41%) presented with a cytopenia in a lineage not affected by dysplasia.[4] This discrepancy creates an ambiguity in the understanding of the RCUD subgroups, theoretically characterized by one 'refractory cytopenia' (RA, RN, or RT), since a unique cytopenia in a patient with MDS may be associated in some cases with $\geq 10\%$ bone marrow dysplasia in another or several lineages. There is thus an 'unilineage paradox', where the WHO-defined RCUD can be associated with one or two cytopenias not corresponding with the affected lineage in the bone marrow, whereas MDS with only one cytopenia – which could be identified as 'isolated thrombocytopenia' (IT) or 'isolated neutropenia' (IN) – are common. This issue will be discussed below.

In refractory anemia (RA), signs of dyserythropoiesis may be observed on blood smears, such as macrocytosis, anisochromasia or dimorphism, with or without anisocytosis and poikilocytosis, which are markers of clonal heterogeneity in a chimeric bone marrow. Neutrophils and platelets are usually normal in number and morphology. However, the presence of

Table 1. WHO 2008 classification of MDS[3]

	Blood findings	Bone marrow findings
RCUD		dysplasia in $\geq 10\%$ of 1 cell line $< 5\%$ blasts
RA	single or bi-cytopenia	dysplasia in $\geq 10\%$ of the erythroid cell line $< 5\%$ blasts
RN		dysplasia in $\geq 10\%$ of the granulocytic cell line $< 5\%$ blasts
RT		dysplasia in $\geq 10\%$ of the megakaryocytic cell line $< 5\%$ blasts
RARS	anemia, no blasts	$\geq 15\%$ of erythroid precursors with ring sideroblasts, erythroid dysplasia only $< 5\%$ blasts
RCMD	cytopenia(s), $< 1 \times 10^9/L$ monocytes	dysplasia in $\geq 10\%$ of cells in ≥ 2 hematopoietic lineages $\pm 15\%$ ring sideroblasts, $< 5\%$ blasts
RAEB-1	cytopenia(s) ≥ 2-4% blasts $< 1 \times 10^9/L$ monocytes	unilineage or multilineage dysplasia no Auer rods 5-9% blasts
RAEB-2	5-19% blasts $< 1 \times 10^9/L$ monocytes	unilineage or multilineage dysplasia or Auer rods or 10-19% blasts
5q-	anemia, platelet levels normal or increased	unilineage erythroid dysplasia, isolated del(5q) $< 5\%$ blasts
MDS-U	cytopenias	unilineage dysplasia with pancytopenia or no dysplasia but characteristic MDS cytogenetics, $< 5\%$ blasts

moderate neutropenia or thrombocytopenia remains consistent with the diagnosis of RA. Bone marrow cellularity is generally increased, but can be normal or decreased. Dyserythropoiesis is defined as 10% or more dysplastic erythroid precursors. Dysery-thropoiesis is not specific for RCUD compared to other types of MDS. If a dysplasia is present in a second lineage, it should always be < 10%.

In refractory neutropenia (RN), dysgranulopoiesis can be identified in the blood by the presence of nuclear hypolobation and hypogranulation of neutrophils. In the bone marrow, dysplasia in the granulocytic lineage is ≥10%, with no significant dysplasia (<10%) in the erythroid or megakaryocytic lineage.

Refractory Thrombocytopenia (RT) is mainly characterized in the blood by isolated thrombo-cytopenia. A second cytopenia may be associated. In the bone marrow, RT is characterized by ≥10% dysplasia evaluated on at least 30 megakaryocytes. Dysmegakaryopoiesis may include hypolobated megakaryocytes, multinucleated megakaryocytes and micromegakaryocytes. The other cell lineages are not affected, or may display non-significant dysplasia (<10%).

Differential Diagnosis of RT. Following the exclusion of pseudothrombocytopenia, isolated thrombo-cytopenia of RT should mainly be distinguished from chronic immunologic thrombocytopenic purpura (ITP) and familial thrombocytopenia (**Table 2**). RT may be overlooked if bone marrow evaluation is not performed. For this reason, the bone marrow examination should be performed in any patient with an isolated confirmed thrombocytopenia above the age of 60 years.[5] A complete workup for thrombocytopenia should be performed with viral serology, careful medical history with an inquiry about all possible concomitant medications is needed. Cytogenetic studies are of clear interest in this distinction, since 20q deletion has frequently been reported in RT,[6–8] or more rarely other cytogenetic abnormalities such as del(5q).[9] Furthermore, even in MDS, an autoimmune destruction of platelets can contribute to thrombocytopenia. Platelet lifespan studies (and of their sequestration) by radioisotopic methods can be of interest to analyze the various mechanisms of thrombocytopenia,[10] and help in therapeutic decision-making.[11] Anti-platelet autoantibodies have a low sensitivity for the diagnosis of ITP,[12] and, although they are frequently positive in MDS[13] but they do not help very much to identify a mixed pathophysiology of thrombocytopenia.[10] Platelet morphology on blood smears can be helpful for diagnostic orientation. Giant platelets or microthrombocytes can be secondary to hereditary thrombocytopenias of childhood,[14] or associated infections. Associated morphological abnormalities

Table 2. Differential diagnosis of RT

| Pseudothrombocytopenia |
| Congenital |
| Familial thrombocytopenia |
| Wiskott-Aldrich syndrome |
| Gray platelet syndrome |
| Bernard-Soulier syndrome |
| X-linked thrombocytopenia |
| Acquired |
| Autoimmune |
| Immunologic Thrombocytopenic Purpura |
| Aplastic anemia |
| Septicemia |
| Medications |
| Heparin-induced thrombocytopenia |
| Drug-induced immune thrombocytopenia |
| Disseminated intravascular coagulation |
| Splenomegaly |
| Portal hypertension, cirrhosis |
| Gaucher's disease |
| Myelofibrosis with myeloid metaplasia |
| Viral infections |
| HIV |
| HCV |
| Microangiopathy |
| TTP |
| Hemolytic uremic syndrome |
| Malignancy |
| MDS |
| Leukemia |
| Lymphoma |
| CLL |

Abbreviations: CLL, chronic lymphocytic leukemia. HCV, Hepatitis C virus. HIV, human immunodeficiency virus. MDS, myelodysplastic syndrome. TTP: thrombotic thrombocytemic purpura.

such as Pelger-Huët bilobed nuclei, or evidence of dysgranulopoiesis may be suggestive of MDS, whereas abnormal hematopoietic cells may orient the diagnosis towards a hematologic malignancy.

Differential Diagnosis of RN. During workup for neutropenia, sepsis-associated, drug induced, hemodialysis-associated, auto-immune, familial or "ethnic" neutropenia, should be ruled out (**Table 3**).[15] Acute or cyclic neutropenias are not consistent with the diagnosis of RN. Post-infectious neutropenia is mostly seen after viral infections such as varicella, rubella, influenza, measles, hepatitis, Epstein-Barr virus or HIV infections, and may sometimes be prolonged. Chronic moderate isolated neutropenia can be secondary to concomitant medications (such as clozapine, chlorpromazine, ticlopidine, or sulfasalazine), auto-immune disorders, and ethnic/familial neutropenias, characterized by an excessive margination of granulocytes.[16] Autoimmune neutropenia is mainly associated with autoimmune diseases such as lupus erythematosus (LE) or rheumatoid arthritis (Felty's syndrome),[17] or large granular lymphocyte leukemia.[18] Neutropenia associated with other cytopenias may be suggestive of splenomegaly, dietary deficiencies or hematologic malignancies, and should be explored appropriately.

Table 3. Differential diagnosis of RN

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Congenital
   Constitutional neutropenia
   Ethnic neutropenia
   Benign familial neutropenia
   Cyclic neutropenia
Acquired
   Autoimmune
      LE
      Felty syndrome
   Drug-induced
      Agranulocytosis
      Mild neutropenia
      Late neutropenia
   Infection-associated
      Active infection
         Viral infections
         Severe sepsis
      Post-infectious
   Hemodialysis
   Splenomegaly
   Malignancy
      Acute leukemia
      MDS
      LGL leukemia
      Myeloma, lymphoma
      Myelophthisic processes
   Dietary
      B12, folate deficiency
      Copper deficiency
      Malnutrition
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Abbreviations: LE, lupus erythematosus. MDS, myelodysplastic syndrome. LGL, large granular lymphocyte.

Getting Appropriate Material for Morphological Diagnosis. The diagnosis of MDS, and particularly RCUD, relies on the availability of high quality bone marrow samples, and on the exclusion of other diseases. Morphological bone marrow examination, with an iron stain and cytogenetic study still represents the cornerstone of MDS diagnosis. In a study comparing bone marrow smears, bone marrow imprints, and bone marrow biopsies, the best accuracy in 86 MDS was achieved with BM smears. Interestingly, for patients with a diagnosis of RCUD, inter-observer accuracy was 100% with BM smears, compared with only 60% with BM sections.[19]

Distinguishing between RCUD and Borderline Entities. The WHO 2008 classification proposed an entity named idiopathic cytopenia of unknown significance (ICUS), defined as a condition with less than 10% dysplastic cells, fewer than 5% blasts in the bone marrow and no cytogenetic abnormalities.[3,20] These patients most often present with mild cytopenias, and if the morphologist is unaware of the complete medical history, the diagnosis might be reported as "abnormalities not sufficient for the diagnosis of MDS", when the cytogenetic study is normal. Differential diagnosis of ICUS, like for RCUD, includes autoimmune disorders, drug intake, chronic infections, paroxysmal nocturnal hemoglobinuria, and appropriate explorations need to be carried out.[21,22]

ICUS patients should be followed to document or exclude hematological evolution to an authentic MDS, most importantly by repetition of the BM examination with cytogenetic studies if the cytopenia worsens or if a second cytopenia develops. One should also bear in mind that dysplastic changes can be seen in up to 9,5% of the erythroid or granulocytic bone marrow cells in elderly persons and in smokers.[23]

Another borderline entity is idiopathic dysplasia of unknown significance (IDUS). This is a rare condition characterized by no or only mild cytopenias (hemoglobin ≥ 11 g/dL, neutrophils ≥ 1500/mm3, and platelets ≥ 100000/mm3, associated with > 10% dysplasia in one lineage.[24] Most patients are asymptomatic young patients referred to the hematology departments because of macrocytosis or detection of Pseudo-Pelger-Huët abnormalities. As for ICUS, these patients should have regular follow-up and repeated diagnostic investigations in case of hematologic evolution, likely to detect overt MDS. To harmonize the identification of the minimal changes sufficient for MDS diagnosis, a recent collaborative work has set up a list of morphological findings with a high sensitivity/specificity, a high reproducibility and a high prognostic value of a morphology-based score.[25]

The role of cytogenetic analysis is important in the identification of RCUD, since cytogenetic abnormalities will support the diagnosis of MDS as opposed to ICUS.[21] The most common cytogenetic abnormality in RCUD is del(20q). In a cytogenetic and mutational study of 305 MDS with del(20q) whose samples were referred to the MLL Munich Leukemia laboratory, the most represented diagnostic category was RCUD (133 patients, 43.6%), among which 80.5% had del(20q) as sole abnormality.[26] High-throughput sequencing can also help in the diagnosis of MDS in difficult cases by detecting mutations frequently associated with MDS, including TET-2, ASXL1, SF3B1, SRSF2, RUNX1 and DNMT3A.[27,28] On the other hand isolated mutations of TET2, ASXL1 or DNMT3a can be found in elderly apparently healthy persons.[29]

RT and RN are Rare. Apart from RA, the other RCUD (RT and RN) appear to be rare. In a cytomorphologic study of 3156 MDS patients from the Düsseldorf MDS registry, the diagnosis of RCUD was made in 218 (7%). When the Düsseldorf group revaluated, by WHO 2008 diagnostic criteria, 193 RA according to WHO 2001, the following diagnoses were found: 37 RCUD (19%), 6 MDS-U (3%), 111 RCMD (58%), and 39 5q- syndromes (20%), but a higher proportion of RCUD (45%) was found in the Japanese registry.[30] To assess the RCUD and MDS-U categories in 196 patients with less than 5% marrow blasts, Maassen et al. found 28% RA, 6% RT, 13% RN, 20% patients with no cytopenia, and 34% patients with bicytopenia.[31] Another retrospective study on 293

MDS in a single institution identified 5 RN (1.7%) and 6 RT (2.0%) only.[32] Furthermore, in a study combining 228 MDS patients from the Italian, Düsseldorf and GFM registries presenting with isolated neutropenia (IT) (< 1.5 x 10^9/L) or isolated thrombocytopenia (IT) (< 100 x 10^9/L) and no anemia, we found only 3 (1%) RT and no RN (Gyan et al., submitted). The most frequent diagnosis found in patients with IT or IN was RCMD (32 %) and RAEB-1 (18 %), which occurred at similar frequency in both types. Furthermore, during evolution, RT or RN patients often develop additional cytopenias,[33] which is consistent with the hypothesis that RT and RN are early presentations of refractory cytopenias with multilineage dysplasia. This observation further suggests that real WHO-defined RT and RN are very rare – if they even exist – whereas MDS patients with only one cytopenia most often show dysplasia in multiple lineages.

Another important issue adds to the difficulty of identifying RT and RN. Following publication of the WHO 2008 classification, a study evaluating the inter-observer variability in MDS diagnosis found a discrepancy rate of 27%, mostly in the categories with unilineage dysplasia.[34] This was recently confirmed by a study of 50 cases of unilineage dysplasia where an agreement of only 21% was present between observers. Additionally, the threshold of 2% blasts for the revised IPSS calculation was subject to a 30% discordance rate.[35] The diagnosis of RT or RN thus remains difficult and does not to date reflect an international and reliable consensus on diagnostic criteria. The fact that these extremely rare entities are at the frontiers of RCMD and ICUS/IDUS may be a likely explanation.

Prognosis of RT and RN. RCUD is associated with a more favorable outcome than RCMD.[4,36] In a comparative study between the Düsseldorf and the Japanese MDS registries, median overall survival of RCUD and RCMD was 202 months vs. 109 months in the Japanese cohort, respectively, and 142 months vs. 36 months in the German cohort, respectively, with statistical significance.[30] It is important to try to distinguish RCUD patients with a high and low risk of evolution to RAEB or AML. In a series of 126 patients with RCUD, RT diagnosis was associated with shorter OS (median 15.9 months) then RA (median 48.2 months) and RN (median 35.9 months, p<0.001).[33] In another study, the number of RT and RN was too low to identify a statistically different outcome, but median survival was 32.5 months and 72 months for RT and RN, respectively.[32] In a bone marrow flow cytometry analysis of patients with RCUD, Oka et al. described a lower content of CD19+ or CD10+ lymphoid cells in the marrow blast region (CD45^{int}/side scatter^{low}) of patients in whom circulating blasts appeared during follow-up, compared to patients who did not experience disease evolution to higher risk MDS or AML.[37]

In a study evaluating the prognostic value of multilineage dysplasia, Verburgh et al. found a favorable impact of unilineage dysplasia and of a single dysplasia.[4] ANC < 500/mm^3 has been described as an adverse prognostic factor in Low/Int-1 risk MDS by two independent teams, with a shorter leukemia-free survival but surprisingly, no increase in infection-related deaths.[38,39] Beyond the number or cytopenias, the depth of neutropenia and thrombocytopenia have been incorporated as prognostic factors into the revised IPSS prognostic score.[40]

Diagnostic Tools for the Diagnosis of RT and RN. Flow cytometry (FC) is able to identify aberrant expression patterns of lineage antigens in the erythroid, granulo-monocytic and lymphoid lineages, and a collaborative effort has proposed guidelines for the FC recognition of dysplasia.[41] Since RCUD displays a variable level of dysplastic cells in one lineage only, FC may be a valuable tool for the identification of MDS FC signatures. Moreover, a FC score may help to distinguish MDS from other nonmalignant reactive or secondary cytopenias,[42,43] and support the diagnosis of IDUS,[24] which may represent a pre-phase of MDS. The Ogata score, based on a 4-color analysis of 13 antigens, has shown a sensitivity of 70% and a specificity of 92% in the whole MDS group.[43] For RCUD, the sensitivity was 62%, and a specificity reaching 97% in distinguishing MDS from immune cytopenias.[43] Additionally, a FC score is likely to bring prognostic information in MDS even when the blast count is below 5%, with a high correlation with transfusion dependency, cytogenetics, and the IPSS score.[44] In addition, a higher number of aberrantly expressed antigens detected by FC has been associated with worse survival.[45] Altogether, the available data support the use of FC as a diagnostic tool to increase the accuracy of RCUD diagnosis, as well as for the diagnosis of differential conditions, such as PNH.

Identification of recurrent mutations with deep sequencing, such as TET-2, ASXL1, TP53, RAS, SF3B1, SRSF2, RUNX1 and others[46] may help to delineate RN and RT from other non-MDS conditions. However, as said above, mutational analysis as a tool for RT or RN diagnosis may be hampered by the fact that mutations of TET2, DNMT3a and ASXL1 can be seen individually in elderly healthy persons.[29]

Conclusions. The challenge of RT and RN resides in the paucity of diagnostic criteria, the possible overlap with non-MDS disorders, and in the rarity of true cases of these subgroups of RCUD. Furthermore, isolated refractory cytopenias are frequent in other MDS categories. The workup of such patients should include a complete screening for differential diagnosis, cytogenetic analysis, an expert review of the bone marrow smears, and the help of emerging diagnostic tools such as flow cytometry and molecular biology.

The clinical relevance of their distinction from RA or RCMD could be reconsidered in a future revision of the WHO classification of MDS.

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