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Towards a Conceptualization of Measurable Residual Disease in Myelodysplastic Syndromes

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Abstract:
Approximately 90% of patients with myelodysplastic syndromes (MDS) have somatic mutations in the malignant cells that are known or suspected to be oncogenic. The genetic risk-stratification of MDS has evolved substantially by the introduction of the clinical-molecular International Prognostic Scoring System (IPSS-M) that establishes next-generation sequencing at diagnosis as a standard of care. Furthermore, the International Consensus Classification (ICC) of myeloid neoplasms and acute leukemias has refined MDS diagnostic criteria with the introduction of a new myelodysplastic syndrome/acute myeloid leukemia (MDS/AML) category. Monitoring measurable residual disease (MRD) has historically been used to define remission status, improve relapse prediction, and determine the efficacy of antileukemic drugs in patients with acute and chronic leukemias. However, in contrast to leukemias, assessment of MRD including tracking of patient-specific mutations has not yet been formally defined as a biomarker for MDS. This article summarizes current evidence and challenges, and provides a conceptual framework for incorporating MRD into the treatment of MDS and future clinical trials.

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MRD in MDS

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Author contributions

All authors researched the data for the article and made a substantial contribution to the discussion of the content. ES wrote the first draft, and all authors reviewed and edited the manuscript before submission.

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Key words

Myelodysplastic syndrome; measurable residual disease; acute myeloid leukemia; stem cell transplantation; response criteria.

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Abstract

Approximately 90% of patients with myelodysplastic syndromes (MDS) have somatic mutations in the malignant cells that are known or suspected to be oncogenic. The genetic risk-stratification of MDS has evolved substantially by the introduction of the clinical-molecular International Prognostic Scoring System (IPSS-M) that establishes next-generation sequencing at diagnosis as a standard of care. Furthermore, the International Consensus Classification (ICC) of myeloid neoplasms and acute leukemias has refined MDS diagnostic criteria with the introduction of a new myelodysplastic syndrome/acute myeloid leukemia (MDS/AML) category. Monitoring measurable residual disease (MRD) has historically been used to define remission status, improve relapse prediction, and determine the efficacy of antileukemic drugs in patients with acute and chronic leukemias. However, in contrast to leukemias, assessment of MRD including tracking of patient-specific mutations has not yet been formally defined as a biomarker for MDS. This article summarizes current evidence and challenges, and provides a conceptual framework for incorporating MRD into the treatment of MDS and future clinical trials.

Introduction

Measurable residual disease (MRD), the detection of residual malignant cells during complete hematologic remission, allows for disease monitoring and is the most important predictor of survival for acute leukemias. [1-3] Although myelodysplastic syndromes (MDS) are considered malignant preleukemic myeloid neoplasms and may share many features with subtypes of acute myeloid leukemia (AML), MRD has not yet been effectively applied as a biomarker in MDS. [4-8]

MDS are a heterogeneous group of biologically and clinically distinct sub-entities characterized by ineffective and dysplastic hematopoiesis; therefore, standardizing clinical response criteria has been difficult. [8] The most recently proposed International Working Group (IWG) 2023 response criteria for higher risk MDS is the first to consider MRD status as an exploratory endpoint and recommends its reporting as a response category. [9] However, the IWG 2023 criteria do not provide details about the application of MRD testing or a formal definition of MRD response.

The available evidence shows that MRD assessment in MDS is likely to be context-dependent and influenced by biological and clinical prognostic factors like genetic subtype, disease stage, and treatment strategy. Furthermore, analytical performance and applicability (subgroup versus general testing) of diagnostic tests as well as timepoints and sample sources are important and must be considered in the assessment of MRD in MDS. Widespread implementation of MRD diagnostics in MDS is currently limited by cost and proven clinical utility.

We propose that MRD can be an important biomarker in MDS, which would allow for pharmacodynamic assessment, prediction of survival, disease monitoring and treatment decision making. In this manuscript, we will first review methodologic considerations, such as multiparametric flow cytometry (MFC) versus next generation sequencing (NGS) as well as bone marrow (BM) versus peripheral blood (PB) as source material. We will next consider MRD in the

50 context of clonal hematopoiesis (CH) and for the different clinical settings of non-intensive
51 treatment of older or frail MDS patients, and allogeneic hematopoietic cell transplantation (HCT).
52 Finally, we will consider open questions and prospects for the future, including emerging
53 technologies and efforts toward standardization of MRD evaluation.

54 Methodologic Considerations

55 Multiparametric Flow Cytometry or Next Generation Sequencing

56 MFC-MRD, which is considered technically difficult to standardize but has a short
57 turnaround time, quantifies MRD as progenitor cells that express a leukemia-associated or
58 different from normal aberrant immunophenotype (LAIP/DfN), identified in approximately 90% of
59 AMLs but probably less frequently in MDS. [3,10] MFC-MRD has a limit of detection (LOD) of 0.1%
60 to 0.01% (10^{-3} to 10^{-4}) although higher sensitivities (10^{-5} to 10^{-6}) are reported for leukemic stem
61 cell (LSCs) detection by immunophenotypic aberrant hematopoietic stem cell populations. [11]
62 MFC assessment of different from normal 'dysplastic' maturation[12-14] could supplement MFC-
63 MRD quantitation of aberrant blast or stem cells (**Figure 1**). [15] However interpretation of MFC-
64 MRD in MDS may be limited by residual CH-related changes of hematopoietic cells as well as
65 the challenge of discriminating between lower risk dysplastic clones and leukemic blasts. [16,17]

66 MFC has been used as an MRD test for MDS in a few studies that included high-risk
67 MDS patients in older AML cohorts. [16,18] Evidence from non-intensive treatment trials in AML
68 patients ineligible for HCT shows a significantly higher relapse risk for MFC-MRD positive
69 patients. [19-22] In the peri-transplant setting, tracking of leukemic blasts could be accomplished by
70 MFC-MRD, [23,24] but few studies have examined this approach outside of AML treatment. [25-28]

71 The genetic landscape of MDS has been studied and reviewed in detail by several
72 authors. [7,29-32] About 90% of MDS patients will have at least one oncogenic lesion but no single
73 mutation is pathognomonic for MDS. [29,30,32] As recurrent hotspot mutations and gene fusions that
74 are detectable by real-time quantitative polymerase chain reaction (qPCR) are less frequent in

75 MDS compared to AML, an alternate approach, such as targeted error-corrected NGS is
76 possibly the most useful method for MRD assessment in MDS. [6,32-36] However, no single MRD
77 method has perfect sensitivity and specificity in MDS.

78 NGS-MRD also has several known limitations that have to be addressed prior to broader
79 application in MDS. [37] From a technological perspective, the two most important limitations are
80 the standardization of the bioinformatics analysis platform and the intrinsic error rate due to rare
81 events in a given sample interfering with the clear discrimination of the target from noise. [3,36] Due
82 to its intrinsic error rate, conventional NGS now commonly used at diagnosis of MDS/AML has a
83 LOD of about 2% to 5% variant allele frequency (VAF). [35,36] Although a positive MRD test result
84 above this LOD during complete remission (CR) could be useful prognostically, a deeper LOD is
85 needed to give a meaningful discrimination of relapse risk between positive and negative tests in
86 most instances. Technical advancements like molecular tagging (unique molecular identifiers,
87 UMI) and duplex sequencing allow for error-correction leading to LODs far below 0.1% that are
88 mainly determined by the amount of input DNA/ number of cells and costs. [35,36]

89 From a biological point of view, results of NGS-MRD do not provide the full picture of
90 MDS/AML defined by clonal diversity and evolution with sometimes indetermined potential due
91 to CH (**Figure 2**). [38-40] Consequently, distinguishing between residual hematopoietic stem cells
92 (HSCs) carrying clonal mutations of no pathogenic significance and LSCs by NGS-MRD is
93 challenging. Furthermore, the ability of tracking MRD by NGS is also limited in MDS with
94 germline predisposition when no additional genetic markers are present because germline
95 mutations are noninformative for MRD (**supplementary Table 1**). [3-5] Fundamentally, two
96 approaches of target selection in NGS-MRD can be distinguished – sequencing of predefined
97 target panels versus patient-specific mutation monitoring, but to date it is unknown which
98 approach is superior. This is a moving target, and the decision will depend on a fine balance
99 between costs versus additionally acquired information and evidence related to outcome benefit.

100 **Source Material: Bone Marrow or Peripheral Blood**

101 Studies in AML patients demonstrated a comparable clinical impact of MRD testing
102 between PB and BM aspirate specimens. [41-45] PB as a source for MRD testing may be more
103 suitable than BM in MDS because PB is generally more informative about CH, is not affected by
104 dilution or fibrosis, and is more easily accessible thereby showing greater consistency in addition
105 to lower cost for serial examinations. However, sensitivity is considered to be about 1 log lower
106 in PB and there are concerns about the accurate quantification of the myeloid clonal burden
107 during phases of neutropenia and concurrent relative lymphocytosis. [3,46] MRD testing is most
108 useful in patients who reach complete remission (CR) and show no morphological signs of the
109 underlying disease. However, in cases where patients do not recover hematopoiesis due to drug
110 toxicity or limited stem cell reserve, skewed lymphoid to myeloid cells ratio may be a relevant
111 problem. [46] In a research context, precise calculations of VAF of somatic mutations could be
112 crucial for monitoring the pharmacodynamics of new drugs in refractory patients who fail to
113 achieve CR. Until prospective studies confirm that PB can effectively replace BM in MRD testing
114 for MDS and that both molecular and flow cytometric testing yield comparable results in PB, BM
115 should continue to be regarded as the current gold standard. Circulating cell-free tumor DNA
116 could provide an alternative MRD target in PB during the phase of neutropenia. [47,48]

117 The European LeukemiaNet (ELN) MRD working party actively pursues the goal of
118 standardization and published a detailed consensus document in 2021 updating the
119 recommendations on MRD in AML. [3,49] The currently recommended MRD threshold that has
120 been established by prospective trials for AML in first CR is 1 in 1,000 cells (0.1%; 10^{-3}). We
121 propose that the ELN MRD recommendations on optimized technical requirements, minimal
122 detection limit and standardized reporting should also be implemented in the MRD assessment
123 of MDS (**supplementary Table 1**).

Clonal Hematopoiesis of Indeterminate Potential and Clonal Cytopenia of Undetermined Significance

The prevalence of CH is generally age-related and its detection is assay dependent. [50-52] When sensitivity of sequencing reaches approximately 1% VAF, 85% of persons with an age of 80 years or older will have age-related CH. [53] CH of indeterminate potential (CHIP), defined by somatic mutations with a VAF of 2% or higher, and clonal cytopenia of undetermined significance (CCUS), defined by CHIP with persistent cytopenia, are potentially preneoplastic states and inherent features in the pathogenesis of MDS. [4,5,53-55] However, the occurrence of somatic mutations in CH, CHIP and CCUS are stochastic events and the kinetics of clone growth leading to progression to MDS/AML is unpredictable in most cases (**Figure 2**). [38-40,56-58] Complicating matters, copy number alterations, independently or co-occurring with single nucleotide variants, have also been shown to play an important role in leukemogenesis. [59,60]

The number, combinations and VAFs of somatic mutations show a strong association with progression from CCUS to myeloid neoplasm. [38,50,61-63] CH is a risk factor for therapy-related myeloid neoplasms in patients who received cytotoxic treatment for primary malignancies. [64-67] CH involving somatic mutations in *TP53* and *PPM1D* is common in patients developing therapy-related MDS. [65,67-69] Recent evidence suggests that also thalidomide analogs like lenalidomide provide a growth advantage to *TP53* mutated hematopoietic stem cells (HSCs). [67] Longitudinal measurements of mutant driver genes and clone size may allow for early identification of progression into MDS. However, there is currently insufficient evidence to suggest that monitoring of CH or CCUS could be beneficial for high-risk populations such as individuals with somatic *TP53* mutations that received cytotoxic therapy. Reduction in cost and further improvements in sequencing and data analyses could lead to clone-specific targeted interventions as part of a secondary prevention.

Studies in AML and MDS patients suggest that persistence of CH, especially somatic mutations in one or more of the *DTA* (*DNMT3A*, *TET2*, *ASXL1*) genes, during CR after chemotherapy or before HCT is not associated with increased risk of relapse. [3,70-73] It is important to bear in mind that only AML entities that are characterized by certain driver mutations (*NPM1*, bZIP in-frame mutated *CEBPA*) or gene fusions (*CBFB::MYH11*, *RUNX1::RUNX1T1*) are typically cured without allogeneic HCT, presumably because their LSCs are chemotherapy-sensitive. [49] AML with adverse risk genetic abnormalities including mutated *TP53* and myelodysplasia-related gene mutations or cytogenetic abnormalities, whether primary or secondary, should receive HCT as part of their therapy. [49] Recipient's CH should disappear after HCT which can be tracked by MRD testing but the timepoint at which residual *DTA* mutations should not be detected after transplantation is not established. [42,47,74,75] Additionally, donor-derived CH must be carefully excluded especially if untargeted NGS is used for MRD monitoring. [76,77] A retrospective NGS-MRD study of 131 AML patients who underwent HCT showed that residual *DTA* mutations had no prognostic significance at day 90 and day 180 after HCT. [71] This study indicated that kinetics — an increase in VAF of *DTA* mutations between two timepoints – may be a better prognosticator of relapse. [78] In the future, serial single-cell sequencing analyses will likely provide an answer to which mutations or combinations of mutations of residual CH have an impact on clinically relevant endpoints. [79,80]

Clinical Considerations

Adapting the MRD assessment approach based on treatment goals together with considerations of cost and inconvenience is reasonable. Since effective treatment options are currently lacking for most MDS patients who are not eligible for HCT, MRD testing may not be justified for the majority of "real world" patients receiving palliative treatment outside of clinical trials. The subsequent sections will explore various clinical scenarios that may have different implications for MRD results.

173 **Non-intensive Treatment of MDS**

174 Cytogenetic response, a complete or partial disappearance of chromosomal
175 abnormalities, was introduced as a response criterion for MDS by the IWG in 2000 to enable
176 prospective evaluation and comparability between clinical trials although no data were available
177 at that time to support a relationship between cytogenetic response and clinical outcome. [81]
178 Since then, most clinical trials that have included cytogenetic response criteria as an endpoint
179 have demonstrated this association. [8] We argue that defining MRD criteria for MDS is necessary
180 for the same reasons that cytogenetic response criteria were established, to ensure successful
181 clinical research and clear comparisons between trials.

182 Regular MRD assessment of MDS patients who are not transplant eligible should
183 currently be focused on clinical research. With the exception of hypoplastic MDS or MDS with
184 less than 5% BM blasts and isolated 5q deletion (MDS-del[5q]), treated with immunosuppressive
185 agents or lenalidomide, respectively, most patients with low-risk MDS will initially receive
186 supportive care when they need treatment because of cytopenia. [7]

187 We advocate that reporting MRD responses is important for understanding the efficacy of
188 investigational new drugs. One example is the phase 2 portion of the MDS3001 study, which
189 evaluated the efficacy of imetelstat, a competitive inhibitor of telomerase activity, in 57 red blood
190 cell (RBC) transfusion-dependent patients with lower-risk MDS. [82] Treatment with imetelstat
191 resulted in a clinically meaningful 37% reduction in the 8-week RBC transfusion dependence
192 rate. It should be emphasized here that the reduction of the VAF of somatic *SF3B1* mutations
193 correlated with transfusion independence suggesting that *SF3B1* VAF could be a surrogate
194 molecular marker that predicted response (prolonged transfusion independence).

195 Residual mutations of CH further complicate MRD analysis following non-intensive
196 therapies because they represent the remaining founder clone with residual hematopoietic
197 potential that cannot be eradicated without the use of HCT thus far. [39,83] An improvement in

198 treatment efficacy targeting culprit subclones would make MRD testing more attractive as a
199 surrogate marker for PFS. Since it is biologically implausible that increasing VAF of mutations
200 paralleling progression of subclones would not influence critical outcomes, [84] incorporating MRD
201 analysis in response criteria and in definitions of progressive disease seems to be a reasonable
202 goal.

203 This premise would also apply to future drugs with a mechanism of action that causes
204 differentiation of neoplastic cells into normal blood cells instead of eradication, thereby improving
205 suboptimal hematopoiesis but potentially not leading to a reduction in clonal burden. Only after
206 studying such associations we can learn about the role of MRD and clinical benefit.
207 Consequently, MRD assessment should be incorporated into the design of clinical trials
208 investigating new agents for the treatment of MDS, while implementing recommendations of the
209 US Food and Drug Administration on regulatory considerations for the use of MRD as a
210 surrogate efficacy end point. [85]

211 HSCs with del(5q) are selectively resistant to lenalidomide. Tehranchi et al. showed that,
212 similar to a molecular MRD measurement, the 5q deletion remained detectable in all patients
213 with MDS-del(5q) by fluorescence in situ hybridization of sorted CD34+, CD38-/low, CD90+
214 HSCs at the time of CR during lenalidomide treatment, even in patients with complete
215 cytogenetic response (CCyR). [86] A retrospective analysis of the phase 2 MDS-003 and the
216 phase 3 MDS-004 studies showed that 103/181 (57%) patients achieved a cytogenetic response
217 with lenalidomide of whom 84/103 (81.6%) also achieved RBC transfusion independence at ≥ 26
218 weeks. [87] The case of lenalidomide and MDS-del(5q) is a good example demonstrating that
219 MRD testing on the one hand shows the efficacy of specific treatment at the genetic level and on
220 the other hand provides evidence that a cure in the strict sense is not possible because the
221 malignant stem cell is not eradicated.

222 Patients with low-risk MDS-del(5q) who are treated with lenalidomide have a median
223 AML-free survival of approximately 3.5 years. [87] Transplant eligible patients may benefit from
224 early detection of subclonal *TP53* mutations at diagnosis and regular monitoring during
225 lenalidomide treatment. [67,88-91] In a prospective multicenter study of the German MDS study
226 group involving 67 MDS-del(5q) patients, median overall survival (OS) was significantly different
227 between patients with (N=59) and without (N=8) a *TP53* mutation at diagnosis (3.55 years
228 versus not reached; P=0.002). [89] As the expansion of a *TP53* subclone is associated with
229 treatment failure and progression during treatment with lenalidomide, *TP53* MRD testing would
230 allow better stratification of patients for early HCT or clinical trials. [90]

231 High-risk MDS is treated with hypomethylating agents (HMA) and response is associated
232 with the number and type of somatic mutations. [43,84,92-95] The decrease in VAF of certain high-risk
233 or clearly transforming mutations indicating partial or complete elimination of subclones is
234 associated with better PFS after treatment with HMAs such as azacitidine or decitabine, alone or
235 in combination with other drugs, in several cohort studies (**Table 1; supplementary Table 2**).
236 There seems to be a strong concordance between molecular and clinical responses but the
237 exact threshold of mutation clearance indicating highest outcome difference during treatment
238 with HMAs is not known. VAF thresholds of 1% and 5% have been described to be meaningful
239 in this setting and have to be put in context of baseline risk groups such as *TP53*. [83,84,96]

240 Treatment response is usually short-lived with currently available agents, which may
241 explain why MRD assessment has not been useful in the palliative setting of high-risk MDS in
242 routine care. However, this does not mean that MRD assessment has no merit, but may instead
243 indicate that the current therapeutic options for MDS are limited. What would it mean if HMA
244 therapy did not lead to a temporary suppression of *TP53* mutated clones? [84,92,94,96-99] The answer
245 is that such a therapy would be less effective and bridge fewer patient with MDS/AML to HCT
246 which is the only chance for cure. [84,94,100-102]

247 **Pre-transplant Setting: Prognostication and Treatment Decision Making**

248 Evidence has emerged indicating that MDS with 10% to 19% BM blasts shares important
249 biological and clinical similarities with AML when entities are stratified by genetics. [5,6] Many
250 studies that investigated the role of MRD in AML included a subgroup of MDS/AML, which
251 allowed basic principles of MRD analysis to be applied to results of studies that enrolled AML
252 patients as majority (**Table 1; supplementary Table 2**). [16,18,23,70,103] The creation of the new
253 entity MDS/AML in the recently published International Consensus Classification (ICC) has
254 introduced facts that affect the care of many MDS patients outside of clinical trials. [5] It is a reality
255 that many academically affiliated transplant centers will use available MRD technologies,
256 including less sensitive conventional techniques, in individual cases with the intent to improve
257 the survival of their transplant-eligible MDS patients. Ideally, MRD measurements should be
258 performed in special reference laboratories.

259 When non-intensive or intensive treatments are used as a bridge to HCT, pre-transplant
260 MRD assessment can provide valuable prognostic information to influence the conditioning
261 regimen and the post-transplantation plan. [26] Many retrospective studies have evaluated the
262 prognostic impact of somatic mutations at the time of HCT on the outcome of MDS patients and,
263 without implementing MRD assessment, proposed different genes associated with unfavorable
264 prognosis. [104-110] Factoring in all consistent results and giving most weight to the largest study
265 (Lindsley et al. [108]), which analyzed PB of 1514 MDS patients by NGS (reporting VAF threshold
266 of 2%) before performing allogenic HCT, we can draw the following conclusions. First, mutations
267 in *TP53* are consistently associated with the highest risk of relapse and decreased OS[28,104-
268 107,109,110] that is not influenced by conditioning intensity. [108] Second, mutations in RAS pathway
269 genes are associated with shorter OS due to increased risk of relapse, [107,109] specifically among
270 patients 40 years of age or older who may not have received myeloablative conditioning. [108]

271 Post-hoc analyses of prospective studies in MDS/AML which incorporated MRD
272 assessment after intensive treatment and/or before HCT consistently show a higher risk of
273 relapse for patients with MRD positivity. [16,18,23,70,111] By performing 10-gene NGS-MRD in 48 CR
274 samples from a randomized trial of transplant eligible younger patients up to 65 years of age,
275 Dillon et al. demonstrated that myeloablative conditioning mitigated the relapse risk associated
276 with MRD positivity of non-*DTA* mutations in MDS. [70] Since most MDS patients are older than 70
277 years or have other adverse factors beyond genetics, myeloablative conditioning is frequently
278 not an option and other strategies to reduce relapse risk and improve OS must be explored. In a
279 trial comparing reduced intensity regimens that included MDS patients (33% of 244), Craddock
280 et al. showed that achieving a complete donor T-cell chimerism at 3 months – a potential
281 surrogate marker for graft-versus-leukemia effect – but not the intensification of the conditioning
282 regimen reversed the negative impact of pre-transplant MFC-MRD positivity on relapse
283 incidence and OS. [23] Pretransplant MRD positivity is also not a contraindication to HCT because
284 clinical trials like the VidazaAllo Study have demonstrated a better OS after HCT compared to
285 continuation of HMA treatment. [101] In sum, these data suggest that MDS patients without MRD
286 may avoid myeloablative conditioning and that MRD positivity is useful to steer high-risk patients
287 into clinical trials. [94,101,102]

288 **Post-transplant Setting: Avoiding Relapse**

289 Since relapse of MDS after HCT is associated with a very poor prognosis, there is a
290 great need for early detection and prevention through targeted intervention. [112] MFC, NGS, PCR
291 and CD34+ sorted donor chimerism analyses have been successfully employed to detect MRD
292 in the post-transplant setting (**Table 1; supplementary Table 2**). Duncavage et al. performed
293 NGS-MRD in BM samples from 86 consecutive adult patients with MDS and secondary AML at
294 30 and 100 days after HCT to assess mutation clearance and related risk of relapse. [113] Before
295 HCT, 96% (86/90) of analyzed patients had at least one detectable somatic mutation by whole
296 exome sequencing and 79% (68/86) with the use of a generic myeloid NGS panel of 40

297 recurrently mutated genes. At day 30 posttransplant, 30% (26/86) of patients were MRD-positive
298 – only one patient had a sole DTA variant – defined by a VAF of ≥0.5% in the myeloid NGS
299 panel. After adjustment for conditioning regimen, MRD positivity ≥0.5% was associated with a
300 lower 1-year PFS compared to no detectable mutations at this threshold at 30 days
301 posttransplant (30.8% versus 57.1%; HR for progression or death, 2.09; 95% CI, 1.18 to 3.70; P
302 = 0.02). Importantly, patients with mutations detectable at VAF >0.1% at day 30 had a
303 statistically higher risk of progression (P <0.003 by Gray's test) and a shorter progression-free
304 survival (P = 0.021 by proportional hazards, chi-square test). However, only results of a more
305 elaborate NGS, which also detects patient specific non-myeloid related somatic mutations, were
306 reported for this threshold. Furthermore, MRD positivity at day 100 posttransplant which was
307 detected in 31% (18/58) of patients by incorporating patient-specific non-myeloid related somatic
308 mutations was also associated with a lower 1-year PFS (27.8% versus 77.5%; HR for
309 progression or death, 2.51; 95% CI, 1.26 to 5.01; P = 0.01). In multivariable analysis, age >60
310 years, secondary AML, *TP53* mutation and MRD positivity ≥0.5% at days 30 and 100 were
311 independently associated with disease progression or death.

312 Unfortunately, there are few prospective data on the treatment of MRD of MDS after
313 HCT, almost exclusively from AML studies that included a minority of high-risk MDS patients.
314 [103,114,115] In the RELAZA2 study, Platzbecker et al. used qPCR of leukemia-specific fusion genes
315 or mutant *NPM1* as well as donor chimerism analysis of sorted CD34+ cells from PB (threshold
316 mixed chimerism <80%) to detect MRD and initiate treatment with azacitidine. One-year relapse-
317 free survival was 46% (95% CI, 32% to 59%) in the 53 MRD-positive patients – 5 of whom had
318 MDS, who received the preemptive treatment. [103] Although efficacy of this preemptive approach
319 is also supported by a retrospective study, [116] randomized controlled trials between MRD-
320 positive and MRD-negative patients would be needed to give a definitive answer. Here, an NGS
321 panel-based MRD assay might be more informative than MFC, as posttransplant emerging
322 subclones with therapeutic targets could be better detected. [115]

323 ## Proposition for Future MRD Analysis in MDS

324 ### Tailor MRD to Goals of Therapy

325 MRD assessment, ideally a combination of NGS-MRD and MFC-MRD, should be
326 incorporated in all clinical trials in MDS. Although CR is the ultimate goal of any MDS treatment
327 because of the association with improved OS, we acknowledge that hematological improvement
328 (HI) is also an important and meaningful clinical endpoint associated with improved quality of life
329 that should be explored in clinical trials. [9] Genetic and morphologic responses do not perfectly
330 correlate as shown by complete cytogenetic response (CCyR) which is associated with longer
331 survival in high-risk MDS patients under HMA treatment but does not always lead to CR. [117] For
332 that reason, in contrast to AML, we propose that the complete MRD response (MRD_{CR}) category
333 should always include CCyR and be distinct from morphological responses such as CR or HI.
334 Furthermore, variants in DTA genes should be documented ($DTA^{+/-}$) but generally not
335 considered as MRD positivity (MRD^{+}).

336 Two clinical scenarios – 1) treatment with palliative intent, 2) treatment with curative
337 intent – should be distinguished when applying MRD response criteria. In the former scenario,
338 the application of MRD measurement is currently only reserved for clinical trials; in the latter,
339 MRD assessment may already be offered in individual cases. This would have two advantages.
340 In the palliative setting, where the focus is on PFS and HI, the interaction of morphology and
341 residual subclones would be easier to describe and to investigate (e.g. HI with MRD^{+}-DTA^{+}). In
342 the curative setting, where the main goal is to predict and to prevent relapse, the morphological
343 response might be of lesser importance after induction treatment because of HCT (e.g. marrow
344 CR with MRD_{CR}-DTA^{+}). The proposed provisional MRD criteria (**Table 2**) serve as a basis for
345 discussion and will certainly need to be adjusted by suggestions from the stakeholders'
346 community[9] and results of further studies.

347 An optimal gene panel for NGS-MRD has not yet been defined for MDS. The calculation
348 of the IPSS-M requires analysis of 31 genes for risk stratification at diagnosis. [32] This panel can
349 be used as a starting point for further refinements of NGS-MRD diagnostics in MDS
350 (**supplementary Table 3**). As a minimum, we consider the 10-gene panel, which has been
351 described as prognostic in patients with MDS and AML before conditioning for HCT
352 (**supplementary Table 4**). [70] All detected mutations should be considered potential MRD
353 markers (**supplementary Table 1**).

354 **Timepoints of MRD Assessment**

355 The optimal MRD measurement timepoints are not known and will always reflect the
356 design of published clinical trials that demonstrate outcome differences between MRD-positive
357 and MRD-negative patients. No evidence-based recommendation can be given for the setting of
358 palliative treatment. Outside of clinical trials, a pragmatic suggestion would be to perform MRD
359 testing in patients who have a long-lasting remission with HMAs and wish to reduce therapy, or
360 who have indeterminate cytopenia despite achieving CCyR. For patients treated with the
361 intention of cure, we pragmatically suggest performing MRD testing in BM for remission
362 assessment before HCT as well as on days +30 and +100 after HCT. These timepoints would
363 allow conditioning regimen (myeloablative versus reduced intensity) and immunosuppression
364 (faster vs normal tapering of immunosuppressive agents) to be adjusted as well as optional
365 donor lymphocyte infusion to be planned. If a molecular marker is present, further NGS-MRD
366 assessments could be performed every 4-8 weeks in PB. Any MRD^+ results should be confirmed
367 by further testing to estimate clone kinetics.

368 **Potential Role of New Methodologies**

369 A major drawback of NGS-MRD is that the reported VAF represents the average
370 frequency within a bulk cell population, making it impossible to provide information on the co-
371 occurrence of multiple variants within a single subclone of that cell population. [79] Especially in

372 MDS, where CH is an integral part of its pathogenesis, the inability to distinguish residual CH
373 from LSCs is still an obstacle to clearly establishing the presence of MRD in some cases. Single-
374 cell analysis has great potential to revolutionize MRD assessment in this regard because it is
375 able to resolve clonal architecture. For example, sequencing of single cells from enriched LSCs
376 at diagnosis and during remission could explain which combinations of mutations are found in
377 the same cell and steer more sensitive NGS-MRD detection. Recently, Dillon et al. have shown
378 in a proof-of-principle in three AML patients that a tailored single-cell analysis integrating patient-
379 specific mutations and structural variants from whole-genome sequencing as well as cell surface
380 markers is able to determine which genetic alterations exactly are present in a single cell. [80]
381 Single cell MRD analysis is in the early stages of development. Further studies, ideally in the
382 context of prospective clinical trials, are necessary to demonstrate feasibility on a large scale.

383 Another promising approach to detect MRD is to perform NGS in CD34+ (or alternatively
384 CD117+) selected cells from PB after magnetic cell separation or flow cytometric sorting. [75] In an
385 analysis of 40 MDS/AML patients in CR after HCT, Stasik et al. demonstrated an impressively
386 high sensitivity of 100% and specificity of 91% for detecting molecular relapse. [75] The lower limit
387 of MRD detection was 10^{-6}, about 10-fold more sensitive than the measurement of donor
388 chimerism as performed in the RELAZA2 study, and PB was superior to BM as a source of
389 CD34+ cells.

390 Regarding minimally invasive MRD assessment, serial analysis of circulating cell-free
391 tumor DNA for leukemia-specific mutations in serum may be the optimal approach for cytopenic
392 MDS/AML patients. Previous studies in the post-HCT setting in patients with MDS/AML have
393 demonstrated the principal feasibility of this methodology that must be standardized and
394 prospectively investigated in different clinical scenarios. [47,48]

395 **Standardization Efforts**

396 The standardization of MRD methods is the key to accomplish reproducibility and
397 comparability. The MRD working group of the ELN has published a blueprint on how to
398 successfully carry out such an endeavor in AML. Reproducibility has to be demonstrated in
399 clinical trials using a published standardized methodology. This means that, in addition to
400 technological advancement, considerable standardization efforts will also be necessary in MDS
401 in the future. A first step should be the definition of uniform MRD criteria.

402 Open Questions

403 Because the extent of discordance between MRD measured by MFC and NGS is
404 currently unknown in MDS, we recommend that both methods be prospectively studied in
405 parallel to determine clinically meaningful detection thresholds. Additionally, when NGS-MRD
406 testing is used at specific timepoints in clinical trials, comparison of BM and PB source materials
407 is recommended. The potential role of LSC based detection of MRD is unknown for MDS and
408 should be explored. If patients are randomized between intensive versus non-intensive therapy,
409 MRD assessment should be used to answer the question whether MRD negativity has the same
410 value after both treatment types and what specific mutations are affected by either strategy.
411 Copy number abnormalities and allelic imbalances including copy-neutral loss of heterozygosity
412 are important in the pathogenesis of MDS but have rarely been discussed in the context of MRD.
413 Furthermore, the significance of uncommon mutations from agnostic NGS approaches should be
414 explored in more granularity to answer the question whether all non-DTA mutations or
415 combinations thereof are predictive for relapse or progression. Single-cell sequencing is
416 providing increasing insight into the role of subclones in treatment resistance and relapse. This
417 technology could be used to determine the stage – diagnosis or relapse – at which escape
418 clones emerge and thus possibly predict their occurrence.

Summary

A negative MRD test result indicates that there is no evidence of disease present, above a predefined test threshold. However, although MRD measurements give an important prognostic estimate, this estimate is not absolute because relapse is also observed in MRD-negative patients and MRD assessment is potentially hampered by source material processing, technique used, benign CH and timepoint of investigation. The landscape of MRD in MDS continues to evolve, with the introduction of new methods such as single-cell sequencing, however, a formal working MRD definition is needed now. We propose MRD response criteria built on currently available evidence. Since there remains no curative therapy for most MDS patients, implementation of MRD testing is an important part of clinical trial design and should be a secondary endpoint to achieve inter-trial comparability and efficacy quantification, and to improve our understanding of the relationship between residual CH and relapse. Clinically useful evidence to establish MRD as a biomarker will require both high quality randomized controlled trials and large collaborations.

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Conception of the work: E.S., S.Z.P; Interpretation of Data: E.S., S.F., P.D.A., S.Z.P; Drafted the manuscript: E.S., S.F., P.D.A., S.Z.P. All authors have seen and approved the manuscript being submitted.

Conflict of interest

E.S.: Amgen (Honoraria).

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Disclaimer

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767 **Figure and Table Legends.**

768 **Figure 1.** Measurable Residual Disease Monitoring of MDS by Flow Cytometry: Potential
769 Approaches.

770 **Figure 2.** Schematic depiction of the polyclonal evolution and trackable somatic mutations from
771 clonal hematopoiesis, through CHIP and CCUS to MDS/AML. Each colored dot within the cell
772 represents a distinct mutation, with two different transformed clones (dark circles) developing
773 over time and one outcompeting the other (so called clone sweeping[40]). NGS of the bulk
774 population can only detect genetic alterations with a frequency above the detection limit (LOD)
775 which depends on the error-corrected sequencing methodology used. The variant allele
776 frequency (VAF) represents the variant frequency within the bulk population without information
777 on the co-occurrence of variants within a single subclone of that population that is under
778 constant intrinsic competition and extrinsic pressure (treatment). Depending on the bulk
779 composition, the same VAF can represent different mutational states on a single-cell level
780 (demonstrated by chromosomes in the right lower corner of the figure) such as biallelic versus
781 monoallelic mutation, homozygous versus hemizygous or heterozygous mutations. Importantly,
782 such allelic imbalances, e.g. biallelic *TP53* mutation, are not limited to MDS/AML but can also be
783 found in CH, CHIP and CCUS. CR, complete remission; CTx, chemotherapy; HMA,
784 hypomethylating agent; LOD, limit of detection. The figure was adapted and modified from
785 Stauber et al. [56]

786 **Table 1.** Summary of important studies with MDS patients and reported MRD results including
787 allogeneic HCT. A more extensive version of this table can be found in the supplementary data
788 (**supplementary Table 2**).

789 **Table 2.** Proposition for MRD response criteria in MDS.

790 **Table 1.**

Study	Population	Study design	Intervention	MRD methodology	Results
Mixed populations including palliative therapy					
Welch et al., 2016[97]	MDS (N=26), AML (de novo, N=54; relapsed, N=36)	Prospective, uncontrolled trial (N=84) and extension cohort (N=32)	10-day or 5-day decitabine	WES, NGS gene panel (LOD not specified)	Rate of any mutation clearance associated with morphological response
Hunter et al., 2021[94]	MDS (N=210), MDS/MPN (N=16), AML (N=102), t-MN (N=60)	Retrospective	HMA therapy (7% additional agents)	NGS gene panel (VAF ≥5%)	*TP53* mutation clearance associated with longer median survival (15.6 [negative] versus 7.7 [positive] months; P=0.001)
Sallman et al., 2021[96]	MDS (N=40), AML (N=11), MDS/MPN (N=4)	Phase 1b/2	Eprenetapopt plus azacitidine	NGS (PB; LOD 0.1%)	*TP53* mutation clearance associated with CR
Steensma et al., 2021[82]	ESA relapsed/ refractory lower-risk MDS (N=57)	Phase 2	Imetelstat	NGS (BM, PB)	*SF3B1* VAF reduction correlated with duration of transfusion independence
Yun et al., 2021[83]	MDS (N=95), secondary AML (N=52), MDS/MPN (N=10)	Retrospective	HMA (74%), intensive chemotherapy (45%), HCT (24%)	NGS gene-panel (BM, PB; MRD VAF ≥5%)	MRD negativity (median OS not reached versus 18.5 months; P=0.002) and *TP53* mutation clearance <5% were associated with better OS
Sallman et al., 2023[99]	MDS (N=95)	Phase 1b	Magrolimab plus azacitidine	MFC (LOD 0.02%)	- Small, heterogeneous high-risk cohort with 26% *TP53* mutant MDS - CR rate 33%, MRD negativity rate 23% - Trend for improved OS in patients who became MRD-negative
Nannya et al. 2023[84]	MDS (N=384)	Retrospective	Azacitidine	NGS gene panel (≥1%; LOD not specified)	Except for *DDX41*, post-treatment (≥ 4 cycles) clone size correlated with response

Pretransplant					
Festuccia et al., 2016[26]	MDS (N=285; 23% had advanced to AML before HCT) CMML (N=4)	Retrospective	HCT	MFC-MRD (LOD 0.001%-0.1%) plus cytogenetics/FISH	MRD status associated with CIR
Dillon et al., 2020[70]	MDS (N=48)	Subgroup analysis of a prospective phase 3 trial	RIC (N=23) versus MAC (N=25)	NGS 10-gene panel (PB)	- MRD status associated with OS (55% versus 79%; P=0.045) and CIR (40% versus 11%; P=0.022) at 3 years - Higher relapse rate in MRD-positive patients randomly assigned to RIC versus MAC: 60% versus 8% (P=0.010)
Craddock et al., 2021[23,111]	AML (N=164), MDS (N=80)	Phase 2 randomized trial	Standard RIC (N=108) versus intensified FLAMSA-Bu RIC (N=108)`	MFC (BM; LOD 0.02%-0.05%)	Pretransplant MRD positivity associated with 2-year CIR in MDS: 50.0% versus 21.1% (P=0.020)
Ma et al., 2023[118]	MDS-EB (N=103)	Retrospective	HCT	MFC (BM; LOD <0.01%-0.05%)	MRD status associated with DFS and OS
Posttransplant					
Bernal et al., 2014[25]	AML (N=49), MDS (N=38)	Retrospective	MAC (16%), RIC (84%)	MFC (BM; >0.01%)	- Positive pre-transplant MRD associated with positive MRD at day +100 - Positive MRD at day +100 associated with relapse (OR 6.55)
Duncavage et al., 2018[113]	MDS (N=90)	Retrospective	RIC (42%), MAC (58%)	NGS (BM; VAF ≥0.5%)	- 37% of patients were MRD positive at day +30, 31% at day +100 - MRD-positivity at days +30 and +100 associated with higher risk of disease progression or death

Nakamura et al. 2019[47]	AML (N=37), MDS (N=14)	Retrospective	HCT (MAC 100%; 92% cord blood)	Personalized droplet digital PCR assay (circulating tumor DNA from serum or DNA from matched BM; median LOD 0.04%)	- MRD positivity (either BM or serum) at 1 and 3 months associated with higher 3-year CIR and risk of death - ≥1.5-fold increase in ctDNA between 1 and 3 months post HCT associated with highest risk of relapse (HR=28.5; P=0.0001) and death (HR=17.4; P=0.0009)

AML, acute myeloid leukemia; BM, bone marrow; CIR, cumulative incidence of relapse; CIR, cumulative incidence of relapse; CMML, chronic myelomonocytic leukemia; CR, complete remission; DFS, disease-free survival; *DTA*, DNMT3A, TET2, ASXL1; ESA, erythropoiesis-stimulating agent; FISH, fluorescence in situ hybridization; FLAMSA-Bu, fludarabine, cytarabine, amsacrine, busulfan; HCT, allogeneic hematopoietic cell transplantation; HMA, hypomethylating agent; HR, hazard ratio; LOD, limit of detection; MAC, myeloablative conditioning; MDS, myelodysplastic syndrome; MDS-EB, MDS with excess blasts; MFC, multiparameter flow cytometry; MPN, myeloproliferative neoplasm; NGS, next generation sequencing; OR, odds ratio; OS, overall survival; PB, peripheral blood; RIC, reduced intensity conditioning; t-MN, therapy-related myeloid neoplasms; WES, whole exome sequencing.

791

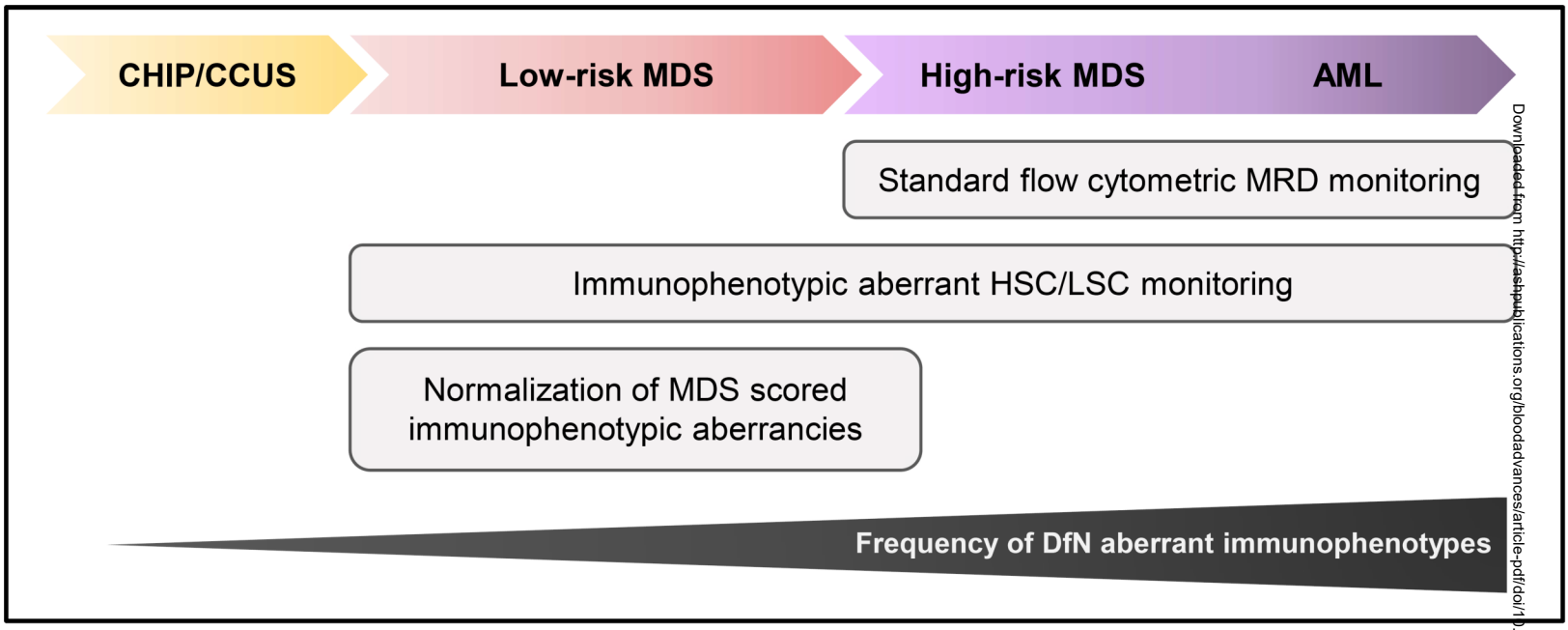
792 **Table 2.**

Category	Defining criteria
MRD_{CR}	1. Complete cytogenetic response* or normal karyotype, **and** 2. Complete MRD response: Negative results (lower limit of detection at least 0.1%) in all MRD tests (NGS, MFC, PCR) that were used
MRD_{LL}	1. Complete cytogenetic response* or normal karyotype, **and** 2. Any MRD above the detection limit of the assay but below the level of 0.1%
MRD^{+}	1. Complete cytogenetic response* or normal karyotype, **and** 2. Any MRD tests positive ≥0.1%
$–DTA^{+/-}$	Used as an additional MRD test qualifier: e.g. MRD_{CR}-DTA^{+}; MRD^{+}-$DTA^{–}$
MFC-$MRD^{–}$	1. MFC is used as a stand-alone test without other genetic or molecular tests 2. MFC-MRD negative: No detection of any leukemic clones by MFC (lower limit of detection 0.1%)
MFC-MRD^{+}	1. MFC is used as a stand-alone test without other genetic or molecular tests 2. MFC-MRD positive: Detection of leukemic clones by MFC with a frequency ≥0.1%
MRD relapse	1. Previous documentation of MRD_{CR}, MRD_{LL} or MFC-$MRD^{–}$ after treatment, **and** 2. MRD relapse confirmed in a second consecutive samples, **and** 3. Newly detected MRD^{+}, **or** 4. Newly detected MFC-MRD^{+}, **or** 5. ≥1 log_{10} increase of VAF of previously detected DTA variants after day +100 of allogeneic HCT†

DTA, *DNMT3A*, *TET2*, *ASXL1*; LL, low level of detection (<0.1%); HCT, hematopoietic cell transplantation; HI, hematologic improvement; MFC, multiparameter flow cytometry; MRD, measurable residual disease; VAF, variant allele frequency.
*International Working Group 2023 response criteria (unchanged from IWG 2006).[9]
†Corroboration by sorted donor chimerism analyses recommended.

793

Figure 1



Approach	Targets	Potential Advantages
Standard flow cytometric MRD monitoring	Aberrant immunophenotype of baseline blasts (LAIP) and/or that is different from normal progenitors (DfN)	– Sensitive – Prognostically validated in combined AML/high-risk MDS cohorts
Immunophenotypic aberrant HSC/ LSC monitoring	Aberrant marker presence on $CD34^{+}CD38^{-}$ progenitors	– Sensitive – Potentially applicable to low- and high-risk MDS (~30% MDS patients have target)
Normalization of MDS scored immunophenotypic aberrancies	Overlap with DfN – Lymphoid marker presence: CD5, CD7, CD56 – Altered maturation pattern: • Neutrophils CD13 vs CD16 • Monocytes HLA-DR vs CD11b • Erythroid CD71 decrease	– Potentially applicable to low-risk MDS – MDS aberrancies/score validated for diagnosis – Assesses myeloid/erythroid differentiated subsets in addition to myeloid progenitors

Figure 1.

Figure 2

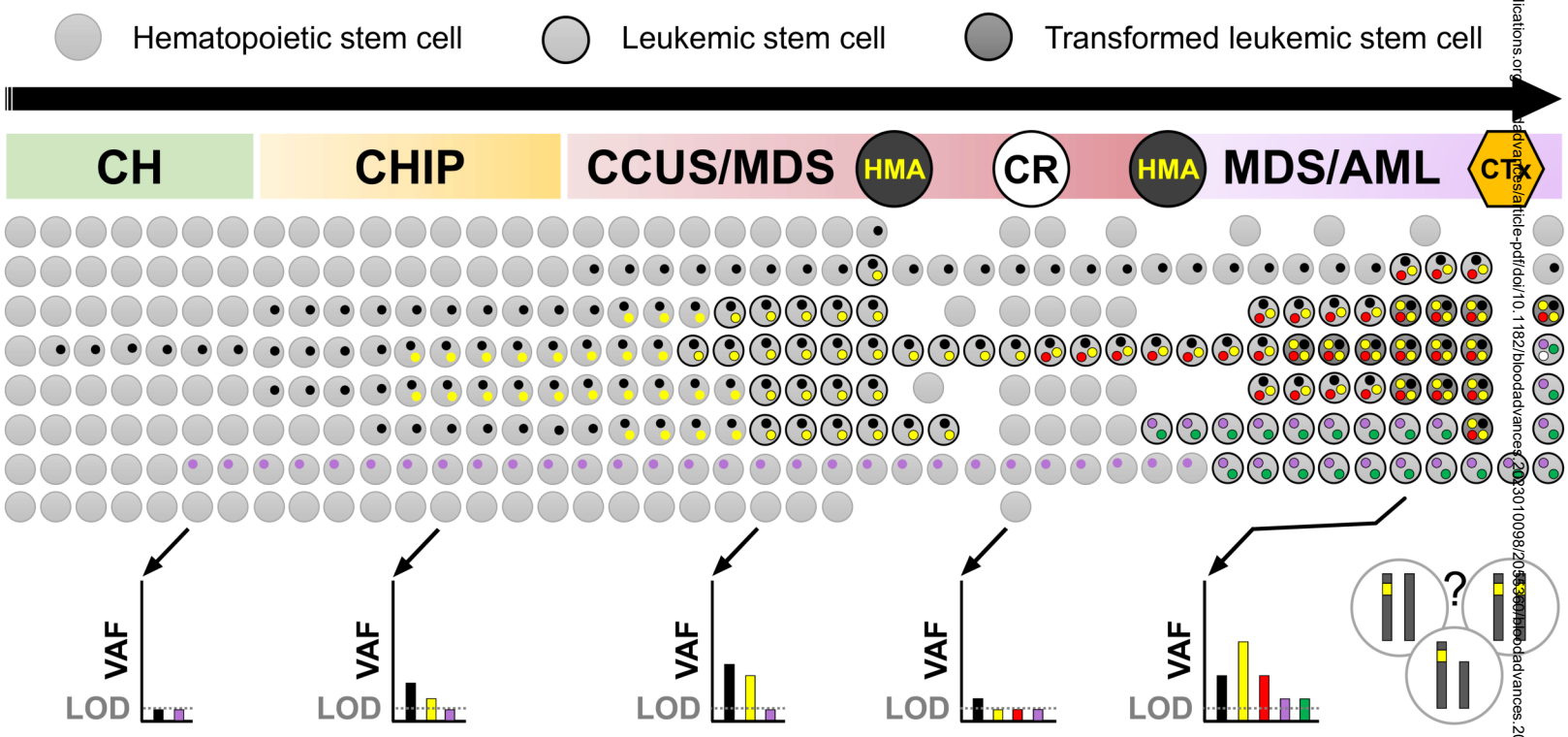


Figure 2.