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Review Article

TP53-Mutated Myelodysplasia and Acute Myeloid Leukemia

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Abstract. TP53-mutated myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) form a distinct and heterogeneous group of myeloid malignancies associated with poor outcomes. Studies carried out in the last years have in part elucidated the complex role played by TP53 mutations in the pathogenesis of these myeloid disorders and in the mechanisms of drug resistance. A consistent number of studies has shown that some molecular parameters, such as the presence of a single or multiple TP53 mutations, the presence of concomitant TP53 deletions, the association with co-occurring mutations, the clonal size of TP53 mutations, the involvement of a single (monoallelic) or of both TP53 alleles (biallelic) and the cytogenetic architecture of concomitant chromosome abnormalities are major determinants of outcomes of patients. The limited response of these patients to standard treatments, including induction chemotherapy, hypomethylating agents and venetoclax-based therapies and the discovery of an immune dysregulation have induced a shift to new emerging therapies, some of which being associated with promising efficacy. The main aim of these novel immune and nonimmune strategies consists in improving survival and in increasing the number of TP53-mutated MDS/AML patients in remission amenable to allogeneic stem cell transplantation.

Keywords: Myelodysplastic syndromes; Acute myeloid leukemias; TP53; Molecular abnormalities; Gene sequencing; Cytogenetic characterization.

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Introduction.

Genetic classification of AML. The myeloid malignancies form a group of related cancers generated by the malignant transformation of hematopoietic stem/progenitor cells, including acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). AMLs form a heterogeneous group of hematological malignancies characterized by a considerable complexity of molecular alterations, clonal development, and consistent defects in cell differentiation/maturation, associated with expansion of immature leukemic elements.

Acute myeloid leukemia (AML) is a heterogeneous and complex disease, characterized by the uncontrolled proliferation of progenitor leukemic cells that progressively accumulate and display variable degrees of differentiation blockade. The incidence of AML is age-dependent, rising markedly at an age of ≥60 years, with a median age at diagnosis of about 68-70 years.[1,2] The incidence of AML in Europe increased from 3.48 in 1976 to 5.06 cases per 100,000 people in 2013, a phenomenon at least in part related to the ageing of the population.[3]

The identification and classification of cellular and molecular abnormalities occurring in AML was of fundamental importance for the understanding of the pathogenesis of these leukemias and for the development

of a more rational approach for their treatment. Thus, the initial classification of AML, the French-American-British (FAB) classification was based on the evaluation of the hematopoietic cell lineage of leukemic cells and of their differentiation stage, based on cytological and cytochemical techniques. The development of techniques in the study of cytogenetic abnormalities introduced new fundamental criteria in the classification of AMLs, reflected in the World Health Classifications of AML proposed in 2001 and 2008.[4,5]

AMLs are a heterogeneous group of hematological malignancies, characterized by a complexity of molecular alterations and clonal development. In the last years, considerable progresses have been made in the characterization of the molecular abnormalities underlying AMLs, with the identification of recurrent chromosomal alterations and of gene mutations, allowing the classification of these leukemias in various subgroups, characterized by different genetic alterations and response to current treatments.[6-9] This molecular classification identified some major molecular subtypes: (i) AMLs characterized by peculiar translocation events (balanced rearrangements) leading to the formation of fusion genes and correspondent fusion proteins, including inv(6), t(15;17), t(8;21), inv(3), *MLL* fusions and t(6;9); (ii) AMLs exhibiting chromatin-spliceosome gene abnormalities, including mutations of genes involved in RNA splicing (*SRSF2, SF3B1, U2AF1, ZRSR2*), chromatin and transcription; (iii) AMLs characterized by *TP53* mutations, complex karyotype alterations and copy-number chromosome alterations; (iv) AMLs displaying mutations of the nucleophosmin 1 (NPM1) gene; (v) AMLs characterized by double CEBPA mutation; (vi) AMLs with *IDH2*^{R172} mutation, defined as a distinct subgroup for the mutual exclusivity with *NPM1* mutation and other class-defining lesions.[8,9] AMLs with mutated *RUNX1* have been included in the WHO classification as a provisional entity in the category of AMLs with recurrent genetic abnormality.[10]

AMLs were characterized in the context of other tumors, solid and hematological tumors, by a relatively low number of mutations in coding genes, but a high number of driver genes, of whom a part is related to leukemia-specific driver genes and driver genes observed also in other tumors.[11]

The genes most frequently mutated in AMLs are represented by: mutations of the tyrosine kinase membrane receptor Flt3, more frequently (about 30% of adult AMLs) with Flt3-Internal Tandem Duplication (*FLT3-ITD*) and less frequently (about 10%) with FLT3-Tyrosine Kinase Domain (*FLT3-TKD*) mutations; mutations of the *NPM1* gene observed in 30-35% of cases; mutations of the methyltransferase *DNMT3A* (DNA methyltransferase 3A) gene (20-30% of AMLs); *NRAS* (15-20% of cases); mutations of the transcription factor *RUNX1* (15% of AMLs); the methylcytosine dioxygenase 2 ten-eleven-translocation (TET) *TET2* gene (15-20% of AMLs); the isocitrate dehydrogenase 2 (*IDH2*) gene (10-15% of AMLs) and *IDH1* gene (5-10%); mutations of the additional sex coombs-like 1 (*ASXL1*), a transcriptional regulator (10-20%); mutations of the transcription factor runt-related transcription factor 1 (*RUNX1*) gene occurring in 5-15% of cases; mutations of the tumor suppressor gene *TP53*, occurring in about 10% of cases; mutations of the transcription factor CCAAT/enhancer-binding protein α (*CEBPA*) (10%); mutations of the zinger finger transcription factor Wilm's tumor 1 (*WT1*) observed in <10% of cases; mutations of the enhancer of zeste homolog 2 (*EZH2*), a histone methyltransferase (5-10%); somatic mutations of the transcription factor *GATA2* (<5%); mutations of the transcription factors BCL6 corepressor (*BCOR*) and BCL6 corepressor like 1 (*BCORL1*) (4%); mutations of the cohesion complex genes (*SMC1A, SMC3, RAD21, STAG1, STAG2*) occurring in 6-12% of cases; mutations of splicing factor genes (*SRSF2, ZRZF2, ZF3B1, U2AF1*) observed in about 18% of cases..[12] The identification of genetic abnormalities in AMLs was of fundamental importance for the understanding of leukemia pathogenesis, for the identification of new therapeutic targets and for the identification of biomarkers suitable to monitor the response to anti-leukemia therapy.[12]

Metzler et al. explored the association of driver gene mutations with clinical characteristics and cytogenetic alterations. The major findings of this analysis showed that: *DNMT3A* and *NPM1* mutations were more common in women than in men; *RUNX1, SRSF2, ASXL1, STAG2* and *BCOR* were less common in women than in men; *FLT3-ITD* mutations were associated with high blast cell counts; mutations in *SRSF2, ASXL1, STAG2, U2AF1, RUNX1* and *PTPN11* are more frequent in secondary AMLs (sAMLs, AMLs developing from a pre-existing myelodysplastic syndrome or a myeloproliferative disorder) than in *de novo*-occurring AMLs; *TP53* mutations were more frequent in therapy-related AMLs (tAMLs); mutations at the level of *DNMT3A, FLT3, NPM1, IDH1, IDH2* and *CEBPA* are present predominantly at the level of patients with normal karyotype.[13]

According to various molecular criteria, the European Leukemia Net stratified AMLs into three risk subgroups, with favorable prognosis (comprising t(15;17), t(8;21), inv(6), biallelic mutated *CEBPA* and *NPM1* mutant (without *FLT3-ITD*), intermediate prognosis (encompassing *NPM1* mutant with *FLT3-ITD*^{low}, t(9;21) and various cytogenetic abnormalities not classified as favorable or adverse) and adverse prognosis (comprising monosomy 7 and 5, deletion of long arm (q) chromosome 7, abnormalities of 3q, 17p and 11q, multiple cytogenetic abnormalities, *NPM1* wt and *FLT3-ITD*^{high}, *TP53* mutations associated with complex karyotype, *ASXL1*

mutations, t(6;9) and t(3;3) groups.[14] Importantly, a recent study by Herold and coworkers on 1116 adult AML patients not selected by genetics validated the ELN-2017 classification and showed that: (i) in 599 patients <60 years, the OS was 64% for ELN-2017 favorable, 42% for intermediate-risk and 20% for adverse-risk AMLs; (i) in 517 patients >60 years, corresponding 5-year OS was 37%, 16% and 6%.[15] Patients with biallelic *CEBPA* mutations or inv(16) displayed a good prognosis; in contrast, patients with *TP53* mutations displayed a particularly poor outcome.[15]

Recently, Fleming and coworkers proposed a machine-learning (ML) approach to develop a hierarchical prognostic risk model that hierarchically categorizes cytogenetic and molecular factors into groupings that accurately predict survival.[16] This approach was used to explore two large cohorts of AML patients: this ML approach allowed to classify the analyzed AMLs into four prognostic groups: good (30%), intermediate (26%), poor (26%) and very poor (18%); the ELN2017 classification evaluated these AML as: good (39%), intermediate (31%) and poor (30%).[16] It is important to note that in this system of AML prognostication a large number of molecular parameters were taken in account: complex karyotype, inv(16), *CEBPA*^{dmut}, inv(3)/t(3;3), *FLT3-ITD*, spliceosome mutations (*U2AF1, SRSF2* or *SF3B1*), *NPM1*^{mut} (in the absence of *FLT3-ITD*), t(8;21), *MLL* translocations, *NRAS*^{mut}, *TP53*^{mut}, *ASXL1*^{mut}.[16] This evaluation system allowed the prognostication of many AML subgroups: (i) in the group characterized by complex karyotype, the presence of high-risk monosomies or chromosomal abnormalities or *TP53* mutations have a very poor prognosis, whereas complex karyotype without these alterations have a better prognosis; (ii) *CEBPA*^{dmut} AMLs have a good prognosis, particularly when associated with *NRAS* mutations; (iii) co-occurrence of *FLT3-ITD* and spliceosome mutations was associated with very negative outcome; (iv) *FLT3-ITD* high allelic ratio (>0.5) have a very poor prognosis when present in the absence of concomitant *NPM1* mutations; (v) triple mutant *NPM1/DNMT3A/FLT3-ITD* display a poor prognosis: (vi) AMLs with spliceosome mutations display a poor prognosis when associated with *ASXL1* mutations or *ASXL1* heterozygous deletion; (vii) among *NPM1*-mutant AMLs, *NRAS* co-mutations identified a subgroup associated with good prognosis, whereas those associated with *IDH1* mutations display an intermediate prognosis; (vii) the presence of *KIT* mutations in t(8;21) AMLs was associated with an intermediate prognosis.[16]

Recently, a functional genomic analysis was performed on a large cohort of 562 AML patients based on whole exome sequencing, RNA-sequencing and *ex vivo* drug sensitivity analyses.[17] This approach showed several relevant findings: (i) a sensitivity of *FLT3-ITD* mutant AMLs to FLT3 inhibitors; (ii) *NRAS*-mutant AMLs resistant to most of drugs, but sensitive to MAPK inhibitors; (iii) *IDH2*-mutant AMLs are sensitive to several drugs, whereas the contrary is true for *IDH1*-mutant AMLs; (iv) *RUNX1*-mutant AMLs are sensitive to PIK3C/MTOR inhibitors; (v) AMLs with mutations of spliceosome genes display a peculiar pattern of drug sensitivity; (vi) triple mutant *NPM1/FLT3/DNMT3A* AMLs are sensitive to ibrutinib.[17] This study was further extended through an integration of functional genomic resources represented by molecular, clinical and drug response data; this approach allowed to identify genetic and cell differentiation state features that predict drug response.[18] Interestingly, modeling of clinical outcome revealed a single gene, *PEAR1*, among the best predictors of patient survival, particularly for young AML patients.[18]

Tazi and coworkers, through the analysis of the genomic profile of 223 AML patients, proposed a classification and risk-stratification. Clustering analysis based on cytogenetic alterations and gene mutations allowed to identify 16 non-overlapping clusters classifying 100% of patients. Some cytogenetic subgroups were identified based on cytogenetic alterations. One cytogenetic subgroup was defined by complex karyotype (≥3 unbalanced cytogenetic abnormalities), corresponding to about 10% of all patients and characterized by frequent *TP53* alterations (about 65%), paucity of other mutations, older age and poor outcomes; another cytogenetic subgroup was characterized by the presence of ≥1 3 trisomies (most frequently involving +8, +11, +13, +21 and +22), corresponding to about 2% of all AMLs and associated with infrequent *TP53* mutations (4%) and with a prognosis more favorable compared to the complex karyotype subgroup, even when ≥3 aneuploidies were present; patients with ≤2 aneuploidies (11% of all AML patients), enriched for MDS-related total or partial monosomies, -7(7q$^-$) or -5(5q$^-$) were clustered with sAML subgroups; other cytogenetic subgroups are those characterized by the presence of translocation events, such as t(15;17), t(8;21), inv(16), t(11;x), t(6;9).[19] The sAML cluster is the second largest cluster (28.4% of all patients) and is characterized by the presence of classifying mutational events, including *SRSF2, U2AF1, SF3B1, ZRSR2, ASXL1, EZH2, BCOR* , *STAG2* as well as *RUNX1, SERTBP1* and *MLL*^{PTD}; the patients comprised in this cluster were characterized by an older age, lower blast counts and higher incidence of antecedent hematological disease (AHD) and displayed a different prognosis according to the number of class-defining gene mutations. The sAML cluster is subdivided into two subgroups: sAML like-1 with single mutations (4.7% of all AMLs); sAML like-2 with ≥2 mutations (23.7% of all AMLs) is enriched in AHD and is associated with worse outcomes; *RUNX1* mutations were observed at similar frequencies in sAML1 and

sAML2 subgroups.[19] *WT1* mutations, when observed in the absence of concomitant *CEBPAbi* and *t(8;21)*, defined a distinct subgroup and represented about 2% of all AMLs and involved patients of younger age and englobed two prognostic subgroups, following the absence (intermediate risk) or the presence (adverse risk) of concomitant *FLT3-ITD* mutations.[19] *DNMT3A/IDH1* or *IDH2* mutant AMLs represent a rare subgroup (1%) of AMLs and are associated with adverse outcomes. 6% of patients, not clustering with any class-defining molecular event, are classified as not otherwise specified (mNOS). *NPM1*-mutant AMLs represent the largest subgroup (31.8% of all AMLs) and display an intermediate or adverse risk following their co-mutational status. About 2% of AMLs displayed apparently not relevant mutational events.[19] *FLT3* and *NRAS* mutations are distributed in the various subgroups and are not class-defining mutations. This genetic classification, together with clinical criteria, allowed to define the probability of response and of disease relapse for the various molecular AML subgroups. Thus, this analysis supported a risk stratification of AML subgroups implying a classification of (i) *NPM1, inv(16), t(8;21), t(15;17), biCAEBPA* and no events subgroups as a favorable-risk AML cluster; (ii) sAML1, *t(6;9),* mNOS, *t(11;x), DNMT3A/IDH1-2* and trisomies is an intermediate-risk AML cluster; (iii) *TP53*-complex karyotype, sAML2 and *inv(3)* is an adverse-risk AML group.[18] The concomitant presence of *FLT3-ITD* in *NPM1* subgroup induced the shift of a part of these AMLs from the favorable to the intermediate risk cluster; the presence of *FLT3-ITD* mutations in AMLs pertaining to the intermediate-risk group induced their shift to an adverse risk condition.[19]

Other recent studies have provided a detailed molecular characterization of AMLs with myelodysplasia-related changes (AML-MRC). Gao et al. reported the results of the genomic profiling of 293 newly diagnosed AML patients and observed that 28.5% of these patients displayed AML-MRC; particularly, several notable differences in rate of mutation of genes recurrently mutated were observed: the mutation rates of *ASXL1* (25% vs 8.7%) *NRAS* (17.9% vs 8.1%), *PTPN11* (11.9% vs 5%), *SETBP1* (6% vs 0.6%), *SRSF2* (11.9% vs 5.5%), *TP53* (16.7% vs 1.2%) and *U2AF1* (17.9% vs 7.5%) were higher in AML-MRC than in those without MRC, while the rates of *FLT3-ITD* (3.6% vs 15.5%), *KIT* (0% vs 6.2%), *WT1* (3.6% vs 9.9%), *NPM1* (1.2% vs 21.7%) and *CEBPA* (4.8% vs 24.2%) were lower in AML-MRC compared to those without MRC.[20] At clinical level, AML-MRC were characterized by older age, low WBC counts and inferior outcomes.[20]

Kang et al. have evaluated 45 AML-MRC patients; genetic aberrations in these patients were analyzed using an RNA-based NGS pane assay; using this approach, 4 gene fusions of *KMT2A-SEPT9, KMT2A-ELL, NUP98-NSD1* and *RUNX1-USP42* were observed.[21] AML-MRC patients have been classified into one of these three subgroups: (i) patients with history of prior MDS or MDS/MPN (AML-MRC-H); (ii) patients with MDS-defining cytogenetic abnormalities (AML-MRC-C); (iii) patients with >50% dysplasia in at least two hematopoietic lineages (AML-MRC-M).[20] 33% of AML-MRC-H, 56% of AML-MRC-M and 96% of AML-MRC-C patients have complex karyotype abnormalities. *TP53* gene was the most frequently mutated gene in these patients and all these patients are included in the AML-MRC-C subgroup; *ASXL1* and *SRSF2* mutations were preferentially associated with the AML-MRC-M subgroup and were frequently co-mutated; *IDH1-2* genes were also frequently mutated and their mutations are distributed in all three AML-MRC subgroups.[21]

The evaluation of genomic profile of AMLs had a clinical value at prognostic level. The presence of some genetic mutations had a clearly negative prognostic impact: (i) a systematic analysis of the literature data showed that in adult AML patients, the presence of *TP53* mutations predicted inferior overall survival compared to patients *TP53*-WT;[22] (ii) a meta-analysis of literature data showed that AML patients with *ASXL1* mutations have a significantly poor prognosis compared to those without mutations;[23] in intermediate risk AML patients, the presence of *WT1* mutations was associated with a significantly increased risk of relapse after transplantation.[24] Secondary AML-like gene mutations other than *ASXL1* (*SRSF2, STAG2, BCOR, U2AF1, EZH2, SF3B1, ZRSR2*) identify a subset of intermediate-risk AML patients (about one-third) with a worse outcome (shorter OS and EFS).[25] The main aim of induction chemotherapy consists in achieving clinical remission and a condition of negativity of measurable residual disease (MRD), a key prognostic factor in AML. The analysis of a cohort of 211 AML patients molecularly characterized by NGS and studies for MRD by immunophenotyping assay after induction chemotherapy and allogeneic stem cell transplantation (allo-SCT).[26] 35% of patients achieved MRD$^-$, 27% MRD$^+$ and 38% persistent disease; after subsequent therapies 34% of patients with MRD$^+$ and 26% of those with persistent disease achieved a condition of MRD$^-$.[26] Mutations in *CEBPA, NRAS, KRAS* and *NPM1* predicted high frequencies of MRD$^-$, while mutations in *TP53, SF3B1, ASXL1* and *RUNX1* and karyotypic abnormalities (inv(3), monosomy 5 or 7) predicted low rates of MRD$^-$.[26] Furthermore, patients with fewer individual clones have a higher probability of achieving MRD$^-$.[26] For patients who underwent allo-SCT, outcomes were favorable for those who achieved a condition of MRD negativity early after induction chemotherapy or after subsequent therapy.[26]

In addition to studies of characterization of genomic

alterations, the gene expression studies have also contributed to capture and to define the heterogeneity of AML disease, showing gene expression changes in large part related to underlying genomic alterations. Particularly, transcriptomic information helped to improve the ENL system of prognostic evaluation of ELN system.[27] The whole transcriptomic RNA sequencing HAMLET (Human AML Expedited Transcriptomics) was established as a single, comprehensive, and flexible platform for AML diagnostics; this platform allows the simultaneous detection of fusion genes, small variants, tandem duplications, and gene expression.[28] HAMLET showed the potential to provide accurate comprehensive diagnostic information relevant for AML classification.[28] Using a base pairing approach, eliminating batch effects across heterogeneous patient cohorts and transcriptomic data, Kong and coworkers developed and immunity and pyroptosis-*related* prognostic signature, consisting of 15 genes, that predicts consistently and accurately AML patients' survival, with a better performance compared to other 10 existing signatures.[29]

Several studies exploring gene expression profile of AMLs identified transcriptomic signatures whose scoring may complement the European Leukemia Net classification. Thus, through the analysis of genes differentially expressed in different types of cytogenetically defined AML subtypes, Nehme et al. identified 22 CODEG (commonly deregulated genes) that provided a robust prognostic signature that was predictive of outcomes of AML patients.[30] An artificial neural network -based machine learning approach to a publicly available data set for a large cohort of AML patients led to the identification of a 3-gene signature comprising *CALCRL, CD109* and *LSP1*, which was predictive of outcomes; this 3-gene signature separated the AML patients classified following ELN 2017 into subgroups with different risk probabilities and allowed the identification of AML patients with high-risk features.[31] Docking et al. used expression data derived from 145 AML patients to develop a novel prognostic score strongly associated with patient outcomes; this risk score combined with standard molecular guidelines, allowed the re-stratification of more than 20% of AML patients into correct risk groups.[32] Furthermore, this transcriptomic analysis allowed to identify a subset of high-risk AML patients characterized by dysregulated integrin signaling and *TP53* or *RUNX1* mutations, potentially treatable with inhibitors of focal adhesion kinase.[32]

Another approach was based on the characterization of genes whose expression was deregulated in leukemic stem cells (LSCs), the cells that initiate and maintain the leukemic process and that, for their quiescent state, are resistant to therapy and are responsible for relapse. Thus, Ng et al. identified 17 genes that are differentially expressed in LSC^+ cells fractions compared to LSC^- cell fractions.[33] The investigation of this LSC17 score in five independent cohorts of AML patients showed its capacity to accurately predict initial therapy resistance; furthermore, patients with high LSC17 scores showed poor outcomes with current treatments, including allo-SCT.[33] Bill and coworkers have evaluated the association between the LSC17 score status and the mutational profile in AML patients and showed that some mutations are significantly less frequent in LSC17-gene^{high} compared to LSC17-gene^{low} (*biallelic CEBPA, GAT2, KIT*), while other mutations were significantly more frequent in LSC17-gene^{high} patients that in *LSC17*-gene^{low} patients (*ASXL1, DNMT3A, FLT3-ITD, KMT2A, RUNX1, SRSF2, STAG2, TET2* and *TP53*).[34] Furthermore, AMLs with complex karyotype or with inv(3) have much more frequently a high LSC17-gene score; however, a part of patients with an adverse risk following ELN2017 display a LSC17-gene score low.[34] Importantly, two large cohorts of AML patients, one of younger (<60 years) and another one of older (>60 years) patients, showed that a high LSC17 gene score was associated with a significantly shorter PFS and OS compared to those with a low LSC17 gene score.[34] Given the results of these studies, Ng and coworkers have developed the LSC17 test in the context of a certified diagnostic laboratory, thus generating a clinical grade test.[35] Values from the LSC17 test to clinical outcome were established in a large cohort of AML patients, thus determining a median assay value that can be used for clinical risk evaluation of individual patients with *de novo* diagnosed AML.[35] A recent study explored the predictivity of the risk by LSC17 signature in a large cohort (1503 primary AMLs) of pediatric AML patients and provided evidence that while LSC17 scores were prognostic for EFS and OS in every age whole AML category (0-10 years, 10-18 years, 18-30 years), they were no longer predictive of survival within established cytomolecular risk groups.[36] Thus, it was identified a distinct molecular signature, LSC4, englobing all the genes initially found to be upregulated in adult LSCs,[33] that was more predictive than LSC17 in pediatric AML cytomolecular subtypes.[36] The LSC47 signature contributed to build a robust relapse prediction model in pediatric AML patients.[36]

A recent study reported the results of a transcriptome-based classification of 655 Chinese AML patients and allowed the identification through enhanced consensus clustering of 8 gene expression subgroups (G1 to G8) with unique features. The first four subgroups corresponded the well-known *t(15;17)* (G1), *CBFB-MYH11* (G2), *RUNX1-RUNXT1* (G3), biallelic *CEBPA* (G4); The G5 subgroup (myelodysplasia-related/-like) included clinical, cytogenetic and genetic features resembling secondary AML; most *NPM1* mutations and *KMT2A* and *NUP98* fusions clustered into G6-G8,

displaying high expression of *HOXA/B* genes and various differentiation stages: *HOX*-committed (G6), *HOX*-primitive (G7) and *HOX*-mixed (G8).[37] Importantly, each subgroup was associated with distinct prognosis and response to therapy, thus supporting the clinical applicability of this gene expression-based AML classification.[37]

Single cell RNA sequencing studies carried out in the last years have consistently contributed to defining the complex and heterogeneous cellular hierarchies of AMLs. A fundamental study by van Galen and coworkers, through a combination of transcriptomics and mutational analyses in single cells from AML patients allowed to define the existence of multiple functional cellular subsets and their associated genetic drivers.[38] The use of a machine learning classifier allowed to distinguish a spectrum of leukemic cells corresponding at various stages of differentiation, whose abundances greatly varied between patients and between subclones in the same tumor. According to their transcriptional profile six types of leukemic cells have been identified, including HSC-like, Progenitor-like, GMP-like, Promonocyte-like, Monocyte-like, DC-like. seven clusters (A to G) of AMLs have been identified: the cluster A contained mainly t(15;17) AMLs and some *FLT3-ITD* mutated AMLs and have a GMP-like transcriptomic profile; the cluster B consisted exclusively of t(8;21) AMLs and shows a GMP-like transcriptomic profile; the cluster F almost exclusively implies *CBFB-MYH11* AMLs and displays high monocyte-like and DC-like scores; the cluster C involves *TP53* and *RUNX1* mutated AMLs and AMLs with complex cytogenetics and other cytogenetic abnormalities and some AMLs with normal karyotype and shows high HSC-like and Progenitor-like scores; the cluster G involves the same AML types described for cluster C and also *CEBPA*-mutated AMLs and displays a wide spectrum of differentiation types; clusters D and E comprise a large number of AMLs and mainly involve AMLs with normal karyotype, largely represented by *NPM1*-mutant AMLs, but largely different in their cell type compositions, the cluster D being enriched in undifferentiated HSC/Progenitor-like cell signatures and englobes multiple *FLT3-ITD* mutant leukemias, while the cluster E was enriched for monocyte-like and DC-like cell signatures and harbored *FLT3-TKD* leukemias.[38] The analysis of primitive AML cells at single-cell level showed that these cells exhibit a dysregulated transcriptional program, involving co-expression of stemness-related genes and of myeloid priming genes and their number is associated with a negative prognosis.[38]

A second study, in part based on single-cell studies, was performed by Zeng and coworkers who provided an analysis of the cellular and molecular heterogeneity of AMLs through the study of the complex cellular hierarchies present in these leukemias.[39] This study was based on a peculiar strategy through which the cellular hierarchies of more than 1,000 AML patients were characterized by gene expression deconvolution on bulk AML transcriptomes using single-cell reference profiles of distinct AML stem, progenitor, and mature cell types.[39] Using this approach, 864 AML patient samples were analyzed, providing evidence that clustering based on the composition of leukemia hierarchies revealed four distinct subtypes; primitive (shallow hierarchy, LSPC-enriched), mature (step hierarchy, enriched for monocyte-like and cDC-like blasts), GMP (enriched by granulo-monocytic progenitor-like blasts) and intermediate (balanced distribution). The hierarchies of different AMLs were separated according to two principal components (PC1 and PC2): PC1, spanning a continuum from primitive to GMP and PC2, spanning from primitive to mature.[39] Hierarchies generated by cytogenetic alterations are dispersed along the primitive versus GMP axis, with adverse cytogenetic alterations generating primitive hierarchies, while favorable cytogenetic alterations generating GMP-enriched hierarchies.[39] Cellular hierarchies generated by driver mutations and their combinations were distributed along the primitive versus mature axis (PC2), reflecting their effect on cell differentiation.[39] The PC1 axis well captures patient prognosis with GMP-like enriched class being predictive of favorable outcomes, while the primitive-like enriched group being associated with poor outcomes.[39] In contrast to PC1, the PC2 axis was not predictive of prognosis. Hierarchy composition of AMLs consistently changes between diagnosis and relapse with a clear increase of total LSPC populations at relapse.[39] The primitive to mature axis (PC2) correlates with *ex vivo* drug sensitivity.[39] At the level of gene expression, the PC1 axis was well captured by the LSC17 gene expression scoring assay; from the LSC17, through regression on PC2, it was derived a LSC7 that captures the primitive>mature axis and predicted drug sensitivity: a high LSC7 score predicted sensitivity to drugs such as venetoclax and azacitidine active on primitive AMLs, while a low LSC7 score predicted sensitivity to drugs such everolimus or selumetinib preferentially active on mature AMLs.[39] The identification of cellular hierarchies present in the different AMLs represents an important tool to better understand leukemia development and to predict and define drug sensitivity.[39]

De novo, secondary and therapy-related AMLs. AMLs can be classified into three different groups following their origin as *de novo*, secondary (sAML) and therapy-related AML (tAML). sAML and tAML are recognized as AML clinical subtypes. Following the WHO classification of myeloid neoplasms, sAMLs are defined as AMLs occurring after an antecedent myeloid neoplasia, such as a myelodysplastic syndrome (MDS)

or a myeloproliferative neoplasm (MPN), independently of the therapy used for the treatment of these disorders. tAMLs are defined as AMLs occurring as a late complication related to the mutagenic potential of cytotoxic chemotherapy and/or radiotherapy for a neoplastic or non-neoplastic disease.[40]

A Danish population-based study carried on 3055 AML patients diagnosed in the lapse of 13 years from 2000 to 2013 showed that 73.6% of cases correspond to *de novo* AMLs. 19.8% to sAMLs and 8.3% to tAMLs.[41] tAMLs were mostly related to solid tumors or to lymphoproliferative disorders.[41] An antecedent myeloid disorder (sAML) or prior cytotoxic exposure (tAML) was associated with a reduced rate of complete remission and decreased overall survival compared to *de novo* AMLs.[41]

Molecular profiling studies of a large set of AML samples have identified four different groups of mutations: secondary type mutations specific to sAML (*SRSF2, SF3B1, U2AF1, ZRSR2, ASXL1, EZH2, BCOR* and *STAG2);* de novo mutations (*NPM1, CBF*); *TP53* mutations; pan-AML mutations (*FLT3, NRAS, KRAS, RUNX1, CEBPA, GATA2*).[42] Some remarkable differences been shown in the frequency of several molecular abnormalities between sAMLs, tAMLs and *de novo* AMLs, as well as between sAMLs and tAML: (i) the presence of mutations in *SRSF2, SF3B1, U2AF1, ZRSR2, ASXL1, EZH2, BCOR* or *STAG2* was specific for sAMLs; tAMLs frequently displayed *TP53* mutations (23% of cases) and in a 33% of cases harbored secondary-type mutations in *SRSF2, SF3B1, U2AF1, ZRS2, ASXL1, AZH2, BCOER* or *STAG2*.[42] Finally, the group of sAML showed a consistent degree of heterogeneity with a first subset characterized by the presence of secondary type mutations, a second subset characterized by the presence of *de novo* or pan-AML mutations and a third set characterized by the presence of *TP53* mutations.

Nazha et al. confirmed through the analysis of a large set of primary and secondary AMLs that mutations of the genes *DHX29, ASXL1, SF3B1, BCOR, PRPF8, CBL, BCORL1, EZH2, STAGF2, JAK2, U2AF1, TET2* are more specific for sAML, whereas *CEBPA, IDH2, DNMT3A, NPM1* and *FLT3* mutations are more specific for primary *de novo* AMLs.[43] The cytogenetic profile showed that sAMLs were more frequently than pAMLs classified as pertaining to an unfavorable risk category.[43] Patients with tAML are older and display more frequently than patients with pAML cytogenetic abnormalities including monosomal (-7, -5 or $5q^-$, $7q^-$) and complex karyotypes, events associated with a poor outcome.[44] More recent studies on a large set of tAML patients confirmed the decrease of the frequency of normal karyotype (30% vs 46%) and the increase of complex karyotype (29% vs 16% in sAML, compared to pAMLs.[45]

tAMLs represent the most aggressive and chemo-resistant malignancies with a 5-year survival of <10%.[46] The 2016 WHO classification of myeloid neoplasms classified the myeloid neoplasms occurring after therapy, including tMDS, tMDS/MPN and tAML as a unique clinical entity, called tMN (therapy-related myeloid neoplasm).[10] Therefore, several studies have considered tMDS and tAML together. As for tAMLs, tMDSs are observed in patients treated for solid tumors (54%) or hematological disease (43%); tMDSs are observed in patients treated with chemotherapy alone or combined chemo-radiotherapy.[47] tMDSs compared to pMDSs display a higher proportion of cases pertaining to high/very high-risk scoring, a higher proportion of cases with multiple cytogenetic aberrations, and shorter overall survival.[47] Al mutational level, tMDSs show some remarkable quantitative differences compared to pMDSs. Thus, Ok et al. reported a frequency of *TP53* mutations higher in tMDS than in pMDS (35.7% vs 17.7%, respectively).[48] Lindsley and coworkers confirmed that tMDSs have a clearly higher frequency of *TP53* mutations compared to pMDSs (38% vs 14%, respectively); they observed also that tMDSs display a lower mutational rate of *SF3B1, ASXL1, U2AF1* and *JAK2* mutations compare to pMDSs; finally, *DNMT3A* mutations were more frequent in tMDS compared to pMDS.[49] Thus, although there are some remarkable quantitative differences between tMDS and pMDS in cytogenetics, gene mutations and epigenetics, there are no specific markers to distinguish between these two MDS forms.[50]

The ELN2022 guidelines for myeloid neoplasms introduced important changes to the AML classification through the removal of the categories of AMLs with myelodysplasia-related changes (AML-MRC) and therapy-related myeloid neoplasms. These changes were based on two different criteria: (i) a prior history of MDS or prior exposure to therapy are now considered as only diagnostic qualifiers; (ii) genetic characteristics, rather than clinical history, are mostly relevant in classifying different AML subgroups.[51] According to the new proposed classification, three different hierarchical groups are defined: (i) mutated *TP53* with VAF >10% (MDS/AML if blasts 10-19% and AML if blasts >20%); (ii) AMLs with myelodysplasia-related gene mutation (*ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1* and *ZRSR2*); (iii) AMLs with myelodysplasia-related cytogenetic abnormality.

The current pathogenetic interpretation of tAML development implies the origin from the expansion of clonal hematopoiesis clones due to the mutagenic activity of cytotoxic chemotherapy or radiotherapy; alternatively, new mutations occur in the normal HSC compartment and progressively drive the leukemic process. The first mechanism seems to play a major role in the development of tAMLs. Clonal hematopoiesis of

undetermined potential (CHIP) is a biological event associated with age observed in healthy individuals and corresponding to the presence in their blood/bone marrow of clonal mutations at the level of *DNMT3A, TET2* and *ASXL1* genes; a fraction of the individuals with CHIP develops an hematological neoplasm later.[52] In addition to the three genes mentioned above, mutations of the epigenetic modifiers *IDH1* and *IDH2* and of the splicing factor genes *SF3B1. SRSF2* and *U2AF1*, of *TP53* and *JAK2* genes are also observed at the level of CHIP. Pre-AML cases of clonal hematopoiesis are characterized by more mutations per sample, higher mutant allele frequencies and enrichment of mutations in specific genes (such as *TP53*, *IDH1, IDH2, DNMT3A, TET2* and spliceosome genes).[53,54] Detection of clonal mutations ≥0.01 VAF identifies subjects at increased risk for developing AML.[55] The cumulative analysis on CHIP mutations and on the risk of developing AML suggests that the considerable variation observed in variant allele frequencies among individuals is mainly driven by chance differences in the timing of mutation acquisition combined with differences in the cell-intrinsic fitness of variants: thus, CHIP development reflects a stochastic process of acquisition of mutations by hematopoietic stem cells and possible clonal expansion driven by some mutations with increased fitness conferring selective advantage to mutant hematopoietic stem cells.[56]

The observation that CHIP-related mutations involve a set of genes frequently altered in leukemia, supports the view that these mutations may confer an increased fitness to hematopoietic stem cells. Evolutionary models of CHIP evolution in the time suggest that each specific mutation carries a fixed fitness advantage, and this may explain the different relative proportions and clonal sizes of CHIP driven by different mutations.[56] The longitudinal analysis of CHIP clones over time in old individuals showed that more than 90% of clones expanded at a stable exponential rate over the analysis period, with different mutations driving clearly different growth rates, ranging from 5% (*DNMT3A* or *TP53*) to more 50% for *SRSF2*.[57] Different patterns of lifelong clonal behavior were observed in different individuals.[56] *DNMT3A* and *TP53* mutant clones preferentially expanded early in life and expanded slowly in old age, while splicing gene mutations drive clonal expansion only later in life and *TET2*-mutant clones emerged across all ages.[57]

A large screening of a Japanese BioBank cohort comprising 11,234 healthy individuals (672 with subsequent hematological malignancy) provided important information about the frequencies of various gene mutations and their tendency to generate an hematological malignancy. This study was based on targeted sequencing of major CHIP-related genes in blood-derived DNA to assess the frequency of driver mutations/indels and copy number alterations (CNAs).[58] The frequency on individuals with CHIP in this population of >60 years of age was 41.5%; in individuals with CHIP, 67% displayed either mutations alone or CNAs alone, 21% two alterations (either mutations or CNAs alone, or concomitantly both mutations and CNAs), 7.5% and 2.3% three or four alterations, respectively (predominantly mutations and CNAs).[58] *DNMT3A, TET2, ASXL1, PPM1D, TP53, SF3B1* and *SRSF2* were the most frequently mutated genes in CHIP; CHIPs bearing *TP53, JAK2, ASXL1, SF3B1, U2AF1* and *DNMT3A* mutations have the greatest proportion of co-occurring alterations; the proportion of subjects with CNAs within CHIPs harboring *TP53, JAK2, ASXL1, SF3B1, U2AF1* and *DNMT3A* was higher compared to other gene mutations.[58] The most relevant association between CNAs and mutations were those represented by *TP53/17p*LOH, *DNMT3A/2p*LOH, *TET2/4q*LOH and *JAK2/9p*UPD: these mutations/CNAs association leads to biallelic alterations and were associated with higher mortality related to hematological malignancies.[58] In this cohort of individuals a hematological malignancy was observed in 8.2% of CHIP-positive individuals compared to 4.45% in CHIP-negative individuals: interestingly, the limitation of the analysis only to myeloid malignancies showed a frequency of 3.48% in CHIP-positive individuals compared to 0.82% in CHIP-negative subjects.[58] A part of the subjects with CHIP display abnormalities of blood cell counts or isolated cytopenia or multiple lineage cytopenias; compared to individuals with SNVs or CNAs alone, CHIP individuals with both mutations and CNAs display a higher clone size and more abnormal blood counts.[58] In this context, individuals with *JAK2* mutations display high platelet counts and those with *U2AF1* mutations show cytopenias of any type; furthermore, there is a clear association between *tp53, U2AF1* and *SF3B1* mutations and lower hemoglobin levels and lower platelet counts.[58] Other studies have shown the co-occurrence of gene mutations and chromosomal abnormalities in a part of CHIPs; some gene mutations, such as *TP53, PPM1D, DNMT3A, SRSF2, JAK2* and *ATM,* have a pronounced tendency to be associated with chromosomal abnormalities.[59] *TP53* mutations are associated with chromosome 3, 5, 7 and 17 abnormalities, *PPM1D* with chromosome 7 and 17 abnormalities.[59] The association of gene mutations with chromosome abnormalities define CHIPS at high risk of leukemic progression.[58] The study of a large cohort of individuals of two BioBanks led to define two types of CHIPs: one type of CHIP with myeloid drivers (M-CHIP, with *DNMT3A, TET2, ASXL1, TP53, PPM1D, SRSF2* and *S3B1* as most recurrently mutated genes) and another type with lymphoid drivers (L-CHIP, with *DUSP22, FAF1, KMT2D, SYNE1, ATM* and *KMT2C* as most recurrently mutated genes); these two different types of CHIPs are also distinguished by different recurrent chromosome abnormalities.[60] In both types of CHIPs, the

association of mutational events with chromosome abnormalities defines a subset of individuals with increased risk of developing myeloid and lymphoid malignancies, respectively.[60] A recent study based on a exome screening of a very large population of 40,208 carriers of CHIP, through the analysis of genome-wide and exome-wide associations, identified 24 loci, whose germline variation affects predisposition to develop CHIP.[61]

CHIP is a risk factor for blood malignancies and particularly for developing AML; however, it is unclear while some individuals who harbor CHIP driver mutations progress, while other ones do not progress to AML is still unclear. A recent study modeled the interaction between positive and negative selection mechanisms observed in deeply sequenced blood samples derived from patients who subsequently progressed to AML, compared to those observed in normal individuals, using deep learning and population genetics methodology.[62] This study evidenced the existence of purifying selection operating in all individuals and preventing disease-predisposing clones from rising to dominance and from inducing a pre-leukemic process.[62] The balance between evolutionary pressures ultimately drives mutation dynamics and health outcomes in aging blood elements.[62]

An initial study by Wong et al. carried out in 4 tAML patients bearing in their leukemic cells *TP53* mutations, showed that the same mutations were present in 0.0003-0.7% of mobilized blood leukocytes or bone marrow 3-6 years before the development of tAML.[63] In mouse bone marrow chimeras containing both WT and *TP53*(+/-) HSCs/HPCs, the *TP53*(+/-) HSCs preferentially expanded after exposure to chemotherapy.[63] According to the results of this study, it was suggested that *TP53*-mutant HSCs resist cytotoxic therapy and expand preferentially after treatment generating tAML.[63]

Two large studies by Gillis et al[64] and Takahashi et al.[65] provided evidence that patients with CHIP in pre-treatment PB samples have a significantly increased probability to develop tAML after treatment. CHIP can be detected in 70% of patients with cancer who subsequently developed tMN.[65] Not only gene mutations, but also chromosome arm-level copy-number alterations are detectable as CHIP and preexist before exposure of patients to chemotherapy or radiotherapy.[66]

Some mutations are recurrently observed in tAMLs and are related to the previous therapy to which these patients were exposed. Thus, Coombs et al. have assessed in 8,810 cancer patients with solid tumors the occurrence of CHIP: CHIP was identified in 25% of these patients, 4.5% with presumptive leukemic driver mutations (CH-PD).[67] *PPM1D* and *TP53* mutations were associated with prior exposure to chemotherapy.[67] CHIP was particularly frequent in some tumors such as thyroid cancer (possibly because of radioactive iodine exposure) and with the lowest frequency in germ cell cancers (probably because of the younger age of the patients with this malignancy).[67] Among the most common solid cancers, the occurrence of CHIP is more frequent in patients with lung cancer, seemingly because of the enrichment for smokers among lung cancer patients.[67]

Another study confirmed that mutations in the DNA damage response regulator *PPM1D* (protein phosphatase Mn^{2+}/Mg^{2+}-dependent 1D) present in CHIP, are observed in about 1/5 of tAML patients and are correlated with cisplatin exposure.[68] Cell lines with hyperactive PPM1D mutations expand to outcompete normal cells when exposed to cytotoxic DNA damaging agents such as cisplatin and this mechanism could be responsible for their elevated frequency in tAML.[68]

A recent study explored a very large set of cancer patients (24,439 individuals) and observed CHIP in 30% of these patients: 68% of these patients had one mutation in CHIP and 32% two or more mutations; the most frequently mutated genes were the epigenetic regulators *DNMT3A* and *TET2* and the genes involved in DNA Damage Response (DDR) pathway, including *PPM1D, TP53* and *CHEK2*; 90% of the mutations observed in CHIP were classified as driver myeloid mutations.[69] The spectrum of gene mutations observed in CHIP was similar in different cancer types, except for DDR gene mutations, particularly of the *PPM1D* gene, which were enriched in ovarian and endometrial cancers.[69] The presence of specific gene mutations was associated with some pathogenic events: (i) mutations of the spliceosome genes *SRSF2* and *SF3B1* were less frequent than other CH mutations and are clearly associated with age; (ii) CHIP mutations in the DDR genes *TP53, PPM1D* and *CHEK2* were strongly associated with prior oncologic therapy; (iii) CHIP mutations in *ASXL1* gene were strongly associated with smoking.[69] Furthermore, the fitness associated with mutations in epigenetic regulators or splicing regulators was not markedly modulated by oncologic therapy.[69] The environmental factors most strongly associated with development of CHIP myeloid driver mutations are represented by radiation therapy, platinum (mostly carboplatin) chemotherapy and exposure to topoisomerase II inhibitors.[69] The characterization of the clonal dynamics of evolution of CHIP mutations in 525 cancer patients in a median lapse time of 23 months provided evidence that 62% remained stable, 28% increased and 10% decreased in clonal size; the growth rate was most pronounced for CHIP mutations in DDR genes.[69] The incidence of CHIP far exceeds that of tAML and the main determinants of the risk of a CHIP to transform into a therapy-related myeloid neoplasia are related to the type of CHIP mutations (mostly *TP53* and spliceosome genes *SRSF2, U2AF1* and *SF3B1* mutations), the number of CHIP mutations and clonal size.[69] As above discussed, *TP53* is one of the mutated genes frequently involved in tAML:

the analysis of 34 tMN seemingly evolving from CHIP displayed *TP53* mutations in 44% of cases; 73% of these *TP53*-mutant tMNs displayed pre-tMN *TP53* mutations; 73% of *TP53*-mutated tMNs showed complex karyotype alterations, an event acquired at the level of neoplastic transformation, but absent in pre-neoplastic CHIPs.[69]

To understand the mechanisms through which *TP53* mutations may promote clonal hematopoiesis and the development of tAMLs it is fundamental to analyze its possible function in the physiology of normal HSCs. P53 was shown to be an important regulator of HSC quiescence through the modulation of the expression of its target gens Gfi-1 and Necdin.[70] Necdin knockout in mice induced less quiescence and more proliferative activity of the HSC compartment; necdin-null HSCs/HPCs displayed enhanced sensitivity to chemotherapy.[71] These observations supported an important role for necdin as a regulator of DNA damage response in HSCs.[70-71] *TP53* regulates the quiescence of HSCs also through induction of p21, an effect inhibited by CDK19.[72] Mutant *TP53* enhances the repopulating activity of HSCs; furthermore, expressing mutant *TP53* expand in response to chemotherapy and radiotherapy, thus indicating a key role for mutant *TP53* in regulating the response of HSCs to genotoxic stresses.[73] A more recent study by Chen et al. elucidated the mechanisms through which mutant *TP53* promotes expansion of HSCs and HPCs. Mutant *TP53* confers a competitive advantage to HSCs and HPCs following bone marrow transplantation and induces HSC/HPC survival and expansion after stress induced by radiation.[74] At transcriptional level, mutant *TP53* promotes in HSCs/HPCs an enrichment of HSC and AML signatures, which are different from gene expression signatures regulated by WT-*TP53*.[74] In HSCs/HPCs expressing mutant *TP53*, *EZH2* target genes are downregulated and this effect is due to the capacity of mutant *TP53* to interact with EZH2 and to enhance its association with the chromatin, thus increasing the levels of methylated histones (H3K27me3) in genes involved in the regulation of OSPC self-renewal and differentiation; as expected, genetic and pharmacologic inhibition of EZH2 led to a decrease of the repopulating capacity of HSCs.[74] These observations supported a major role for epigenetic mechanisms in the mechanism of *TP53*-mediated effects on clonal hematopoiesis.

A recent study showed that in some patients tMNs are preceded by a condition of clonal cytopenia (tCC). tCC develops earlier after primary diagnosis compared to tMN (34 vs 79 months, respectively) and more frequently received radiation therapy (30% vs 8%, respectively) and less frequently chemotherapy (62% vs 82%, respectively) compared to tMN.[75] tCCs displayed a low rate of cytogenetic abnormalities with absent complex karyotype and chromosomic monosomies.[75] At the level of mutational profile, tCCs were enriched in *TET2* and *SRSF2* mutations compared to tMNs and less frequently displayed *TP53* mutations compared to tMN.[75] At tMN progression, 44% of tCC patients showed clonal evolution.[75]

TP53-mutated MDS and AML.

De novo MDS. The molecular abnormalities present in MDS patients have been explored in detail in the last years. These studies have shown that *TP53* is mutated in about 7-10% of MDS patients and is more frequently mutated in patients with high-risk MDSs; these studies showed also that *TP53*-mutated MDSs are characterized by the frequent association with complex karyotype abnormalities, del(5q) and 17qLOH.[76] About 24% of *TP53*-mutated MDSs are low-grade MDSs; in lower risk MDS, *TP53* mutations showed a lower VAF.[77]

MDSs are mainly observed in older adults with a median age at diagnosis of greater than 65 years; however, more rarely, MDSs are also observed in younger adults of age between 20-50 years. The number of mutations increases linearly with age and on average patients >50 years of age have more mutations (particularly, *TET2, SRFSF2* and *DNMT3A* mutations) than those >50 years old.[78] However, *TP53* mutations represent a notable exception, being observed in more than 20% of MDS patients >50 years old.[78,79] These observations suggest that *TP53* mutations represent early onset ancestral events in the genesis of MDSs.

TP53-mutated MDSs and AMLs represent a peculiar subset of hematological tumors. The frequency of *TP53* mutations in *de novo* MDSs or AMLs under the age of 65 years is evaluated in the order of 5-10%. In MDSs, according to the *TP53* mutational status three sets of patients were identified: 82% had one *TP53* mutation, while 3% displayed two *TP53* mutations and 0.1% three mutations; about 54% of patients with one *TP53* mutation had loss of the wild-type allele, while only 13% of those with more than one *TP53* mutation had loss of the wild-type allele; according to the mutational status and to allelic imbalance, one third of *TP53*-mutant patients displayed monoallelic mutations (single hit) and two third displayed multiple allelic targeting (multi hit) (**Figure 1**).[80] In multi hit patients, no residual *TP53* activity was maintained. Multi hit patients displayed several associations with complex karyotype, few co-occurring mutations (co-mutations occur in less than 25% of cases), high-risk presentation and poor outcomes; furthermore, multi hit state predicted risk of leukemic transformation and of death (**Figure 2**).[80] Monoallelic TP53 patients were less cytopenic and displayed a lower frequency of bone marrow blasts compared to multi-hit patients; furthermore, mono-hit *TP53* patients were enriched in lower risk MDS patients according to IPSS-R and WHO criteria of classification; MDS $5q^-$ predominantly showed *TP53* mono-hit, while patients with MDS-EB2 predominantly displayed a

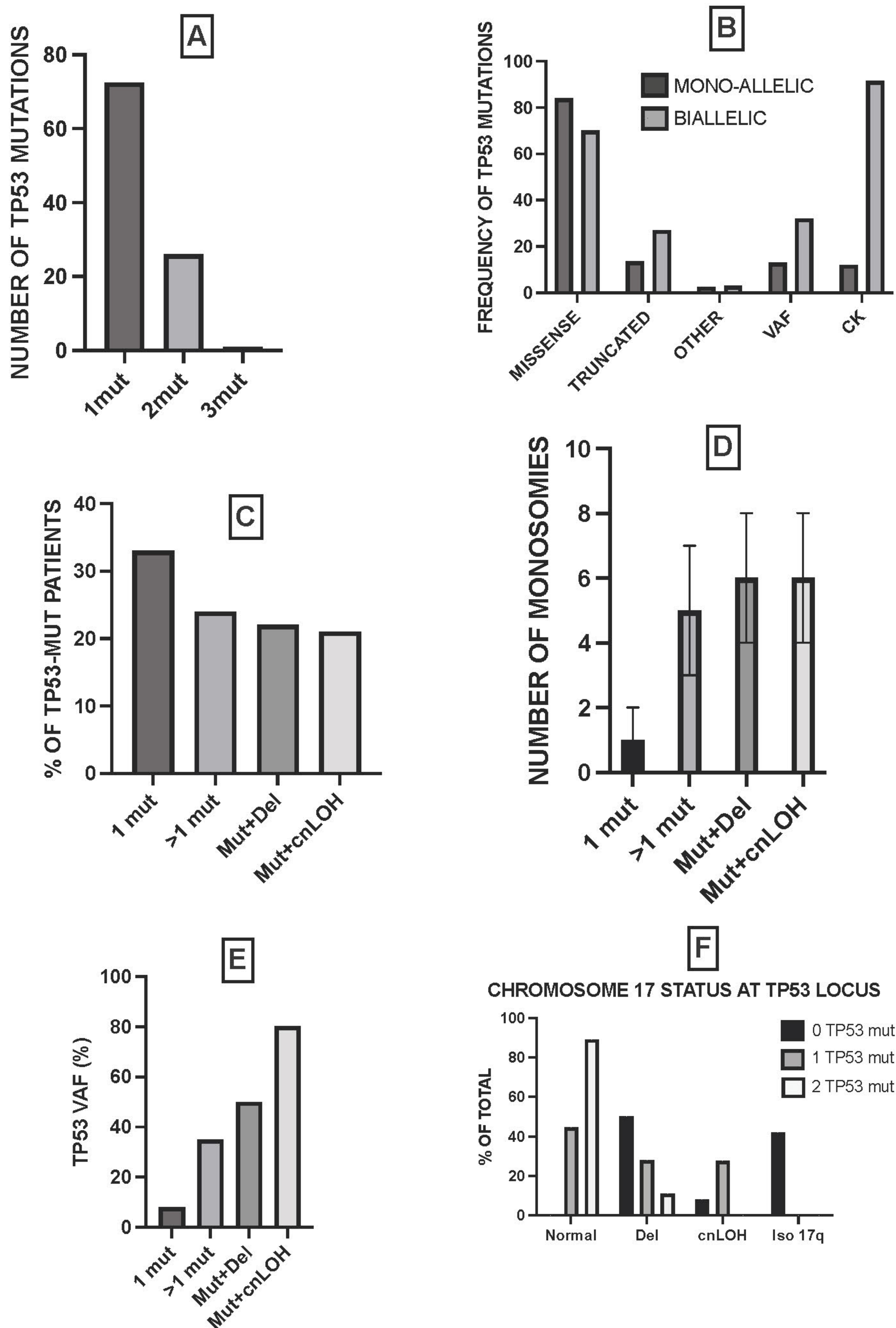

Figure 1. Main molecular properties of TP53-mutated MDS. A: proportion of MDS patients bearing 1, 2 or 3 TP53 mutations. B: MDS patients according to the number of TP53 abnormalities are classified as monoallelic or biallelic, following the involvement of one or both alleles: the types of *TP53* mutations, defined as missense, truncated or other mutations, as well as the VAF of TP53 mutations and the frequency of complex karyotype are shown. C*:* proportion of *TP53*-mutant MDS patients classified into four subgroups following the presence of a single *TP53* mutation (1mut) or of multiple *TP53* mutations (>1 mut) or of *TP53* mutations+chromosome 17 deletions at the level of *TP53* locus (Mut+Del) or of TP53 mutations + cnLOH of TP53 detected only by NGS (Mut + cnLOH). D: Frequency of chromosome monosomies observed in MDS samples classified as above (mean±SEM). E: VAF of *TP53* mutations (median value) observed in four subgroups of *TP53*-mutated MDS, classified as above. F: Frequency of different types of Chromosome 17 abormalities at *TP53* locus into three subgroups of MDS with *TP53* alterations: 0 TP53 mutations, a rare subgroup, with absent TP53 mutations but with structural alterations affecting TP53 expression, 1 and 2 TP53 mutations. The chromosome 17 status at TP53 locus is defined as normal, deleted, cnLOH or isoq17 (isochromosome 17q rearrangement). The data reported in this figure are issued from Bernard et al.[80]

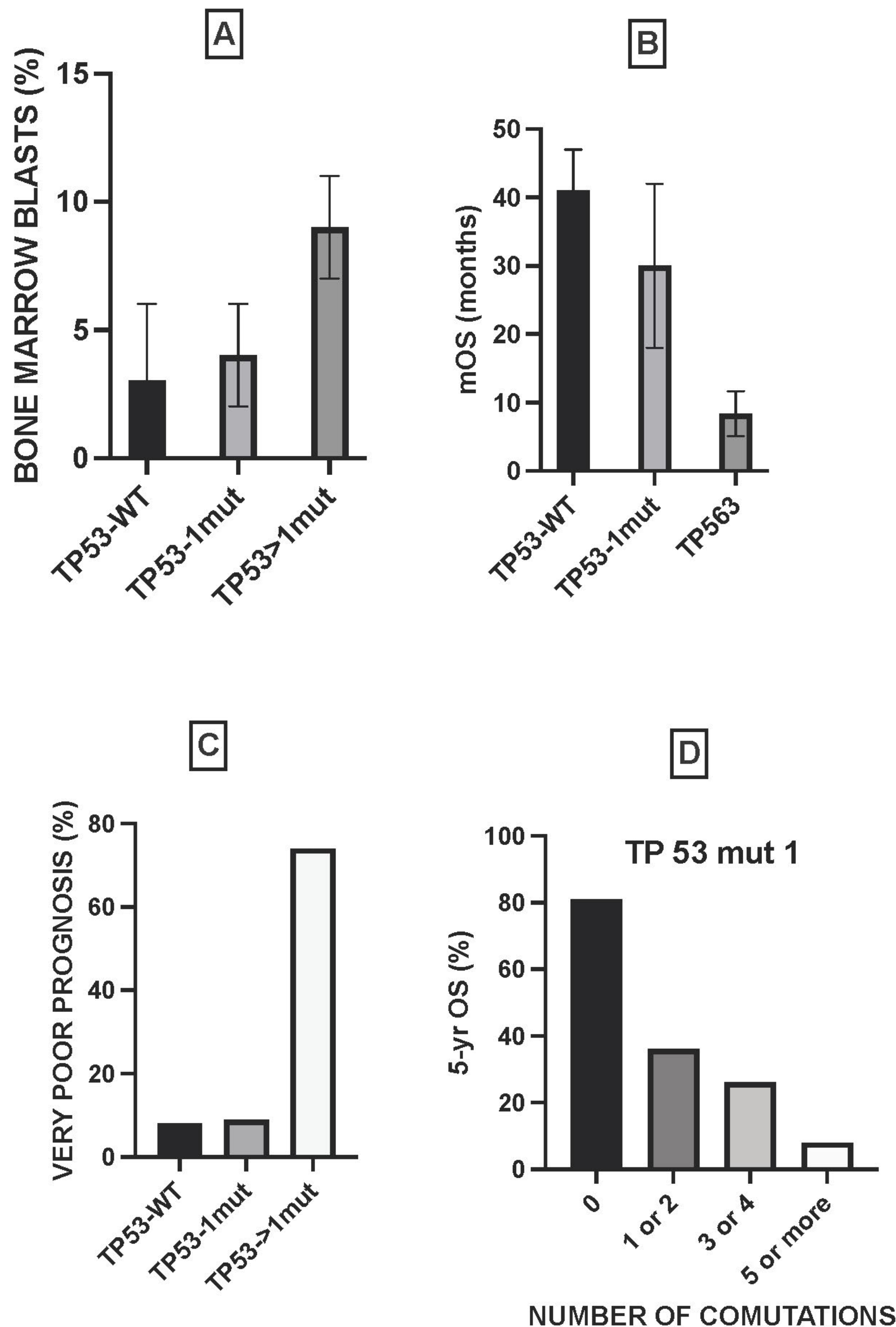

Figure 2. Association between molecular features of TP53-mutated MDSs and clinical parameters. A: Percentage of bone marrow blasts in MDS without TP53 mutations (TP53-WT) and with 1 or >1 TP53 mutations (mean value ± SEM); B: median OS in TP53-WT, TP53 1 mut and TP53 >1 mut (mean value ± SEM); C: frequency of MDS patients with very poor prognosis among TP53-WT, TP53-1 mut and TP53 >1 mut patients; D: 5-yr mean OS in TP53 1 mut patients subdivided into four subgroups according to the number of co-mutations.

TP53 multi-hit. Monoallelic patients displayed outcomes and response to therapy like those observed in WT-*TP53* patients (**Figure 2**).[80] Monoallelic *TP53* mutations more frequently display co-mutations in other genes, particularly *TET2* (29%), *SF3B1* (27%), *ASXL1* (16%) and *DNMT3A* (16%), as subclonal events playing a variable impact on outcomes.[80] Finally, a remarkable difference between the two subtypes of *TP53*-mutant MDSs is that in multi hit state *TP53* mutations are predominantly found in the dominant clone, while in monoallelic *TP53*-mutant MDSs are mainly subclonal.[80] The differential effect of monoallelic and biallelic *TP53* mutations in MDS clinical presentation and outcome are seemingly related to a dose-dependent effect of *TP53* inactivation on genomic instability, as supported by the observation that biallelic *TP53* alterations are associated with an increased number of chromosomal aberrations and an increased frequency of complex karyotypes

compared to monoallelic *TP53* mutations. Finally, the outcome of monoallelic *TP53*-mutated MDSs is strongly influenced by the concomitant presence of comutations; in fact, while monoallelic patients with no other driver mutations have a 5-yr mOS of 81%, it was 36% for patients with one or two mutations, 26% for patients with three or four co-mutations and only 8% for patients with five or more co-mutations; in contrast, the outcomes of patients with multi-hit *TP53* alterations is poor and not influenced by the presence and by the number of additional mutations.[80]

The presence of *TP53* mutations divides MDSs with complex karyotypes (CK-MDSs) into distinct prognostic groups. In a cohort of 359 CK-MDS patients, *TP53* mutations were identified into 55% of these patients. *TP53*-mutated CK-MDSs have fewer co-mutated genes, such as *ASXL1, U2AF1* and *RUNX1* but are enriched for some chromosome abnormalities, such as del(5q) chromosomal abnormality, monosomal karyotype and high karyotype complexity, identified by the concomitant presence of 4 or more chromosomal abnormalities.[81] The presence of *TP53* mutations into CK-MDSs significantly reduced OS (**Figure 3**).[81]

TP53 mutations were detected in 18% of low-risk MDS with del(5q); among these patients, those with *TP53* mutations had a significantly higher risk of AML evolution compared to those without *TP53* mutations (50% vs 15%, respectively).[82] Crisà et al have evaluated *TP53* mutations in MDS patients with isolated partial or total loss of chromosome 7 and observed a higher frequency of *TP53* mutations among patients with 7q loss compared to those with 7 loss (9.8% vs 1.2%, respectively). The presence of *TP53* mutations in these patients had a negative prognostic impact on overall survival.[83] *TP53* mutations, together with *ASXL1, RUNX1* and *CBL* mutations represent the mutations whose presence is associated with an increased risk of evolution to high-risk MDS or AML.[84]

Various studies have supported a prognostic role of *TP53* VAF (variant allele frequency) in MDSs. In low-risk MDSs a *TP53* VAF >6% was associated with shortened OS and inferior progression-free survival; in high-risk MDSs, the level of *TP53* VAF clearly correlates with the occurrence of complex karyotype and a *TP53* VAF >40% was an independent prognostic factor predicting reduced OS.[85,86] The study of a large cohort of 261 MDS patients with *TP53* mutations confirmed the important prognostic role of *TP53* VAF; 67% of these patients had 1 *TP53* mutation, 29% had 2, 4% had 3 and 0.4% had 4; 37% of these patients had mutations in genes other than TP53; 83% of these patients had a complex karyotype and displayed a median *TP53* VAF of 39%; the VAF of *TP53* mutations in patients without a complex karyotype was significantly lower than in those with complex karyotype (5.1% vs 33.9%, respectively).[87] 32% of patients with *TP53* mutations had concomitant

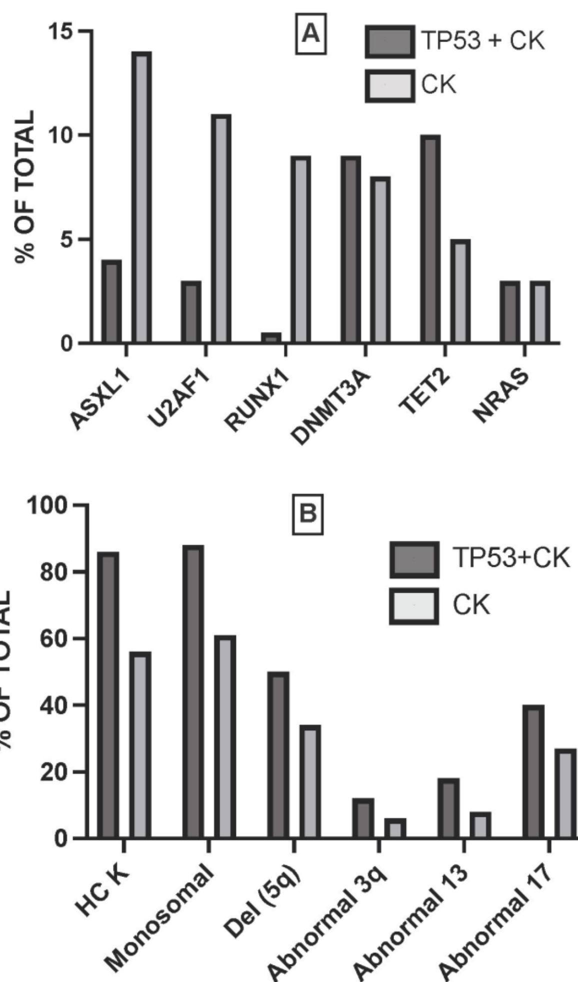

Figure 3. Molecular characterization of MDSs with complex karyotype (CK) in association (CK+TP53) or not (CK) with TP53 mutations. MDSs bearing CK were subdivided into two subgroups according to the presence or not of TP53 mutations. A: frequency of the most recurrent driver mutations observed in CK+TP53 and CK MDSs (mean value ± SEM). ASXL1, U2AF1 and RUNX1 mutations are significantly less frequent in CK+TP53 than in CK MDSs. B: frequency of some relevant chromosomal abnormalities in CK+TP53 and CK MDSs (mean value ± SEM): highly complex karyotype (HCK), monosomal abnormalities, del(5q), abnormal 3q, 13 and 17 chromosome are more frequent in CK+TP53 than in CK MDSs. The data present in this figure are issued from Haase et al.[81]

TP53 deletions; patients with more than 1 *TP53* mutations are less likely to have *TP53* deletions than those with 1 mutation (9.3% vs 42.9%, respectively).[87] *TP53* VAF level was associated with worse prognosis and patients with lower *TP53* VAF respond better to therapy with hypomethylating agents (HMAs): patients responding to treatment with HMAs showed a stable *TP53* VAF just after therapy and a decreased *TP53* VAF at the time of clinical response; patients not responding to HMAs showed an increased *TP53* VAF after therapy.[87] The combination of *TP53* VAF with the presence of complex karyotype defined a subgroup of MDS patients with particularly poor prognosis. Increase in *TP53* VAF was observed in 61% of patients at the time of leukemic transformation.[87]

A recent study reported the analysis of 2355 MDS patients, including 490 (21%) patients with *TP53* mutations: of these, 78% were *biTP53* and 22% *maTP53*. Median OS was worse for *biTP53* subset compared to the *maTP53* subset (1year *vs 1.3* years, respectively*)*; patients with *maTP53* and those with *biTP53* have a doubled and quadrupled risk of death, respectively compared to *TP53-WT* patients; compared to *TP53-WT* the risk of death was higher for *TP53* with CK compared to *TP53* without CK; among patients without CK, allelic *TP53* mutational status had significant impact on outcomes (mOS of 2.8 years for *maTP53* and 1.2 years for *biTP53*); among patients with CK, there was no survival differences between the *maTP53* and the *biTP53* subsets; among patients with low-risk MDS outcomes were worse for patients bearing *TP53* mutations and there were no differences between *maTP53* and *biTP53*.[88] These observations further supported the conclusion that *TP53* mutant MDSs are a heterogeneous group, whose biological and clinical behaviour is influenced by *TP53* allelic mutational status and cytogenetic architecture.[88]

The use of the Evolutionary Action score (EAp53), a computationally derived score to quantify the deleterious impact of different missense *TP53* mutations on the basis of phylogenetic divergence of the mutated sequence position and perturbation due to amino acid substitution, allowed to define a scoring system ranging from 0 to 100, where a higher score indicates a worse impact, and a 0 score indicates wild-type function.[89] This analysis allowed the characterization of a large cohort of *TP53*-mutated MDSs with low-EAp53 score and a favorable prognosis.[89] Low-EAp53-MDSs have a lower frequency of multiple *TP53* mutations and multi-allelic *TP53* alterations, fewer cytogenetic alterations, and a lower frequency of complex karyotype and monosomal karyotype.[89] Furthermore, low-EAp53-MDS more frequently have co-mutations, involving particularly *NRAS* and *RUNX1* mutations.[89]

De novo AMLs. The pivotal study of TCGA on the molecular characterization of 200 *de novo* AML adult patients, with an age of 55±16 years, reported a frequency of 8% of *TP53* mutations, strongly associated with unfavorable risk and with complex cytogenetic abnormalities.[90] Bowen et al. explored 166 AML patients with cytogenetic abnormalities and observed that 31% of these patients had *TP53* mutations; 97% of *TP53*-mutant AMLs had unfavorable cytogenetics and 53% of AML patients with complex cytogenetic abnormalities had *TP53* mutations.[91] Rucker et al. explored 234 AMLs with complex karyotype for *TP53* alterations: 60% of these patients had *TP53* mutations and 40% had *TP53* losses; in total, 70% of these patients displayed *TP53* alterations. Furthermore, *TP53*-altered AMLs more frequently exhibited a monosomal karyotype [-7/7q$^-$ (59%), -5/5q$^-$ (77%), -11/11q$^-$ (13%), -12/12q$^-$ (32%), -18/18q$^-$ (34%) and -3/3p$^-$ (29%)]. This study confirmed also that *TP53* is the most frequently known altered gene in complex karyotype AMLs and that patients with *TP53* alterations were older and had significantly lower remission rates, inferior event-free, relapse-free, and overall survival.[92] Deletions in chromosome 7 (-7) or its long arm (7q$^-$) represent the most frequent adverse cytogenetic events in AML; *T*P53 and -5/5q are the most frequent co-occurring mutations and cytogenetic abnormalities in this AML subset.[93]

TP53 aberrations in AML include gene mutations, mostly involving the DNA binding domain of the gene, and deletions of different sizes implying the *TP53* locus at the level of chromosome 17p13. Functional studies on missense *TP53* mutant variants commonly observed in AML indicate loss-of-function effects and induction of effects comparable to those observed with complete *TP53* inactivation; these findings have suggested a dominant negative effect as the primary force of selection of *TP53* mutations in myeloid malignancies.[94] In addition to somatic *TP53* mutations, *TP53* germline mutations are observed in a minority of AML patients and are more frequent in t-AML.[95]

The prognostic impact of different *TP53* mutations is heterogeneous; in fact, Stengel et al. have explored a large cohort of *TP53*-mutated AMLs: *TP53* mutations were detected in 13% of cases (mutation-only 7%; mutation + deletion 5%; deletion - only 1%); all patients with *TP53* mutations alone or in association with *TP53* deletions, but not cases with *TP53* deletions-only, were associated with a poor prognosis and reduced overall survival.[96]

A recent study reported the most extensive and detailed evaluation and molecular characterization of more than 500 *TP53*-mutant AML patients.[97] About 75% of these patients harbored a *TP53* missense variant, most frequently corresponding to mutations such as R248, R273 and Y220; other genetic variants, including *TP53* deletion, nonsense and frameshift mutations, were less frequent (**Figure 4**).[97] Furthermore, in 70% of cases a *TP53* abnormality was associated with a *TP53* copy-number loss.[97] The concomitant presence of a *TP53* abnormality with a *TP53* copy-number loss or of multiple *TP53* mutations was associated with a worse prognosis.[97] Importantly, this study showed that mutant p53 protein expression patterns by immunohistochemistry evaluated using digital-image-assisted analysis provide an important tool integrating both *TP53* mutation and allelic states in AML patients: some patients (44.5%) displayed a mutant expression pattern characterized by high p53 expression (p53^{high}) and a minority of patients (16.5%) showed a mutant expression pattern with absent p53 expression (p53^{truncated}); other patients (39%), event in the presence of a mutant *TP53* allele, displayed a normal p53 protein

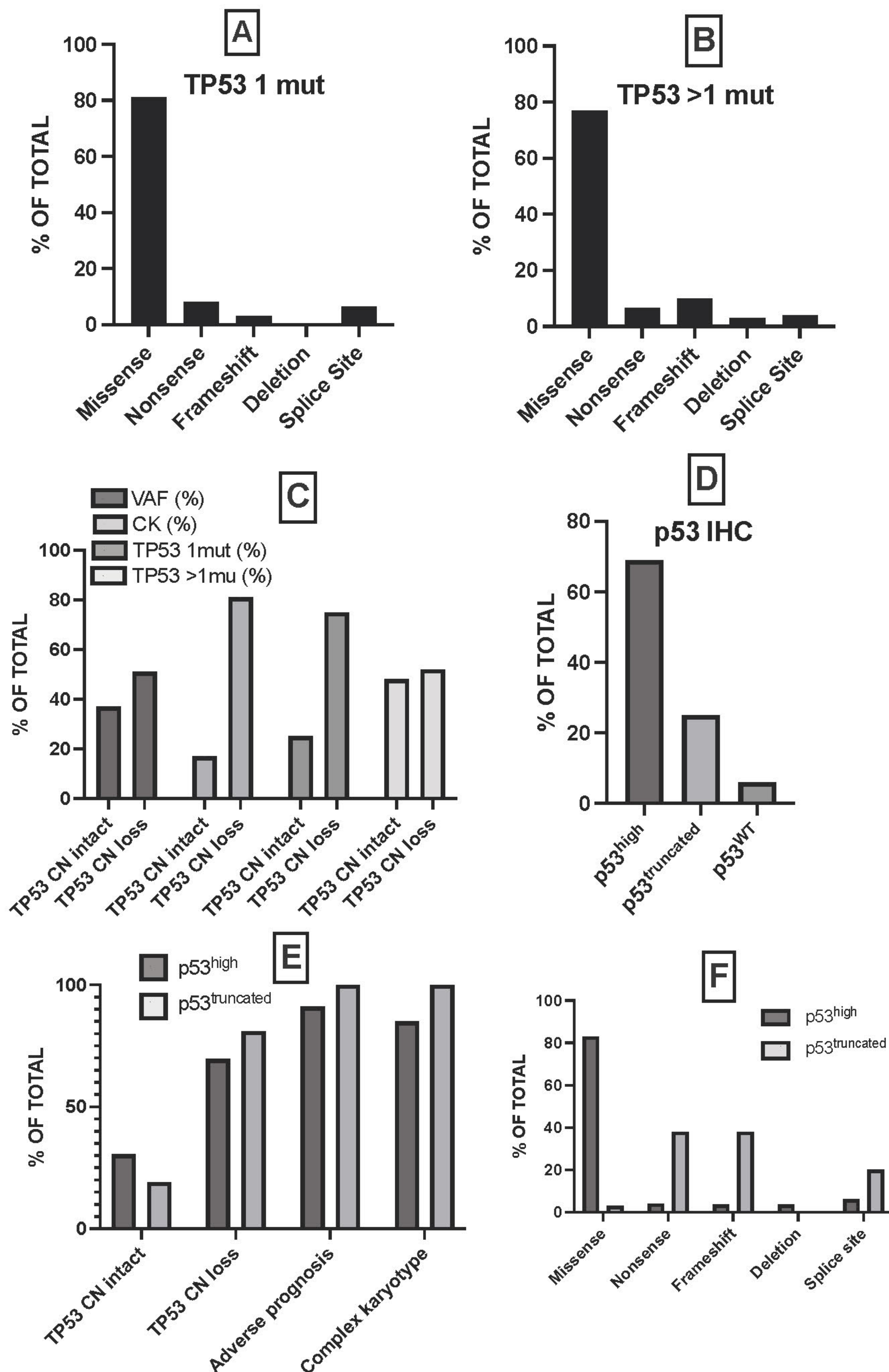

Figure 4. Main molecular properties of TP53-mutated AMLs. A: types of TP53 mutations present in TP53-mutated AMLs bearing 1 TP53 mutation; TP53 mutations were classified as missense, nonsense, frameshift, deletion and splice site. B: types of TP53 mutations present in TP53-mutant AMLs bearing >1 TP53 mutation. C: TP53-mutant AMLs were subclassified into two subgroups according to the presence or not of TP53 copy number alterations (TP53 copy number intact and TP53 CN loss): TP53 VAF was higher in TP53 CN loss than in TP53 CN intact AMLs; CK was markedly more frequent in TP53 CN loss AMLs than in TP53 CN intact; TP53 1 mutations are more frequent in TP53 CN loss than in TP53 CN intact; TP53 with >1 mutation are equally frequent in TP53 CN intact and TP53 CN loss AMLs. D: Immunohistochemical (IHC) classification of TP53-mutant AMLs, with the definition of three subgroups: $p53^{high}$, $p53^{truncated}$ and $p53^{WT}$. E: frequency of TP53 CN intact and of TP53 CN loss in $p53^{high}$ and $p53^{truncated}$ AMLs; frequency of AMLs with adverse prognosis and with complex karyotype in $p53^{high}$ and $p53^{truncated}$ AMLs. F: frequency of different types of TP53 mutants in $p53^{high}$ and $p53^{truncated}$ AMLs. The data present in this figure are issued from Takashori et al.[97]

expression pattern ($p53^{WT}$) (**Figure 4**).[97] These three groups greatly differed for their association with complex karyotype: 79% in $p53^{high}$, 33% in $p53^{truncated}$ and 5% in $p53^{WT}$; similarly, the response to therapy was also different with $p53^{high}$ achieving 18% of CRs, $p53^{truncated}$ 7% and 44% in $p53^{WT}$.[97] Genomic analysis of comutations in *TP53*-mutant AMLs shows a mutated profile involving mainly mutations in genes involved in epigenetic regulation such as *DNMT3A* and *TET2,* RAS-MPK signaling such as *NF1, KRAS/NRAS* and *PTPN11* and RNA splicing such as *SRSF2*; this comutation profile was similar for frontline *TP53*-mutated patients and for those with therapy-secondary *TP53*-mutated AMLs and for those undergoing salvage treatment.[97] In patients with 1 *TP53* mutation the most common co-mutations involved *SRSF2, RUNX1* and *ASXL1*, while those with ≥2 *TP53* mutations most commonly displayed co-mutations involving *KRAS/NRAS, PTPN11* and *RUNX1*.[97]

Prochazka and coworkers have explored the clinical impact of subclonal *TP53* mutations in AML patients.[98] These authors have explored 1537 AML patients (91.6% with *de novo* AML, 4% with sAML and 4.4% with tAML; 98 of these patients (6.4%) were found to harbor *TP53* mutations: 62.2% of these *TP53*-mutant AMLs displayed a VAF (variant allele frequency) of >40%, 19.4% a VAF between 20% and 40% and 18.4% a VAF <20%.[98] The large majority of *TP53* mutations in all three subgroups were missense mutations located in the DNA binding domain of the gene.[98] In either *TP53*-mutated group, patients exhibited a lower rate of complete responses and displayed a lower rate of event-free survival and of overall survival.[98] Another study confirmed the worse prognosis of *TP53*-mutant AML, irrespective of the allele burden, including cases with VAF <20%.[99] At the variance with the two previous studies, a more recent study suggested a prognostic role of mutant *TP53* VAF.[100] Thus, in a retrospective analysis on 202 *de novo* AML patients with a median age of 70 years it was shown that a *TP53* threshold of 40% was predictive of a significant difference in OS, with a median OS of 6.9 months in patients with VAF <40% and an OS of 5.5 months with VAF >40%.[100] Particularly, the *TP53* VAF was predictive of response to cytarabine-based regimens, with a median OS of 7.3 months in patients with VAF <40%, compared to a median OS of 4.7 months in patients with VAF >40%.[100] The *TP53* VAF was also predictive of the response after HSCT.[100]

The prognostic role of *TP53* allelic mutational status is reinforced also by the results of a retrospective study on 983 adult AML patients enrolled in 3 different clinical studies and treated with induction chemotherapy; 83 of these patients displayed *TP53* mutations, 14 *moTP53* and 69 *biTP53*; *biTP53* patients were associated with worse overall survival compared to *moTP53* (2-year OS 4% vs 43%, respectively).[101] Importantly, *moTP53* patients displayed an OS comparable to that observed in AML patients classified as intermediate risk following the ELN 2017 risk classification.[101]

It is important to note that many TP53-mutated AMLs are classified as AML-MRC. Particularly, in the context of AML-MRC, the AML-MRC-C subtype is particularly enriched in *TP53* mutations (40-55%), while the AML-MRC-H and AML-MRC-M subtypes more rarely display *TP53* mutations.[102,103] It is of interest to note that AML-MRC-C subgroup is heterogeneous in that it can be subdivided into *TP53*-mutant and *TP53*-WT cases: the *TP53*-mutant cases have a lower rate of mutations of RNA splicing genes and of *ASXL1, BCOR* and *EZH2* genes compared to those *TP53*-WT.[102,103] *TP53*-mutant AML-MRC-C are associated with cytogenetic abnormalities in 5q, 7q, 17p and complex karyotype and are associated with poor outcome, independently of their multi-hit or single-hit *TP53* mutational status.[102,103]

There are some remarkable differences in the definition of mono-hit and multi-hit *TP53* alterations following either the ICC classification[104] or Grob et al..[105] Multi-hit *TP53* mutations were defined by ICC as ≥2 distinct *TP53* mutations (VAF >10%) or a single *TP53* mutation associated with either: (i) cytogenetic deletion involving chromosome 17p (del(17p) or monosomy 17; (ii) a VAF >50%; any complex karyotype.[104] Grob et al. defined multi-hit *TP53* mutations as: (i) ≥2 *TP53* gene variants irrespective of VAF; (ii) ≥1 *TP53* gene variant co-occurring with a cytogenetic deletion involving chromosome 17; (iii) *TP53* mutations with VAF >55%.[105] A recent study[106] evaluated the potential prognostic impact of *TP53* mutations classified as multi-hit or mono-hit according to both the criteria above reported in a cohort of AML/MDS patients randomized to receive azacitidine + durvalumab (anti-PDL1 antibody) or azacitidine alone; these 205 patients included 61 *TP53*-mutated MDS/AML cases.[107-108] Since there was no difference in the response to these two treatments,[107-108] the patients were pooled in an unique analysis.[106] The results of this analysis showed that outcomes of MDS/AML patients with *TP53* mutations are worse compared to *TP53*-WT, without any significant difference between momo-hit or multi-hit status as defined by either the ICC or Grob et al.[106]

TP53 mutations in AMLs are associated with some copy number alterations, allowing to identify subsets of these patients associated with a very-high risk condition. Ets-regulated gene (ERG) amplification is an event observed in 4-6% of AMLs and is associated with unfavorable prognosis. *ERG* amplification was related to cytarabine resistance.[109] EGR amplification was found to be associated with some chromosome aberrations, including chromotripsis and with *TP53* gene alterations.[110] The association of *ERG* amplification with biallelic loss of *TP53* identified a high-risk subgroup of

AMLs with a median overall survival of only 2.5-3.8 months.[111]

Chromotripsis is a catastrophic event generating multiple genetic alterations reflected by an oscillating pattern of DNA copy number levels in one or few chromosomes. Chromotripsis is an event frequently observed in some tumors. At the level of AMLs, chromotripsis was observed in AML with complex karyotype (34.5% of cases) and was strongly associated with *TP53* mutations, monosomal karyotype, -5/5q- abnormalities; particularly, CK-AML with chromotripsis displayed a frequency of *TP53* mutations of 85%, compared to 53% in CK-AML without chromotripsis.[112] The presence of chromotripsis was associated with a particularly poor outcome.[112] Bochtler et al. observed the occurrence of chromotripsis in about one third of AMLs associated with chromosomal abnormalities; the chromotripsis-positive cases were characterized by a particularly high degree of karyotype complexity, *TP53* mutations, and dismal prognosis.[113] A screening of 395 newly diagnosed AMLs showed the occurrence of chromotripsis in 6.6% of cases, in association with typical features of chromosomal instability, including *TP53* alterations, 5q deletion, higher number of CNAs, complex karyotype, alterations in DNA repair and cell cycle, and focal deletions on chromosomes 4, 7, 12, 16 and 17.[114]

A recent study reported the whole genome sequencing of 42 *TP53*-mutated AMLs and provided evidence that most cases (94%) display *TP53* mutational events.[115] Furthermore, most of cases displayed aneuploidy and chromotripsis.[115] Recurrent structural variants affected chromosome regions that affect *ETV6* on chr12p (45% of cases), *RUNX1* on chr21, and *NF1* on chr17q; interestingly, *ETV6* transcript expression was low in *TP53*-mutated myeloid malignancies with and without structural rearrangements involving chr12p.[115] Finally, telomeric content was found to be increased in *TP53*-mutated MDS/AML compared to other AML subtypes.[115]

Nguyen et al. have analyzed the TCGA dataset on AML patients and through a multi-omic clustering approach have identified three primary clusters; one of these clusters was characterized as a very high-risk molecular subgroup (HRMS), with only about 10% survival at 2 years.[116] At mutational level, this subgroup was characterized by a high *TP53* mutation rate (56%) and a low *NPM1* mutation frequency (4%).[116] Furthermore, this high-risk AML cluster was characterized also by high expression of E2F4, CD34, CD109, MN1, MMLT and CD200 genes.[109] Multi-omic pathway analysis using RNA expression and CNA data identified in the HRMS group over-activated pathways involving immune function, cell proliferation and DNA damage.[116]

The frequency of *TP53* mutations was higher in older AML patients compared to younger AML patients. It is important to note that the mutational pattern of older AML patients was consistently different from that observed in younger AML patients, with: (i) some mutations, such as those of *TET2, SRSF2, AXL1, RUNX1* and *TP53* genes being more frequent in older than in young patients; (ii) other mutations, such as *FLT3-ITD* and *WT1,* being less frequent in older than in younger AML patients; (iii) other mutations, including *NPM1* and *DNMT3A* mutations, have similar frequencies in older and younger AML patients.[117-120] Particularly, Tsai et al. reported a frequency of *TP53* mutations of 4.2% in a group of patients with a mean age of 40 years, compared to 13% in a group of older AML patients with a mean age of 71 years.[117] Prossek et al. reported frequencies of *TP53* mutations of 5%, 14% and 14.5% in a group of patients of less than 60 years, 60-74 and >75 years old, respectively.[120] The increase of the frequency of *TP53* mutations with the age of AML patients is not surprising given their role in clonal hematopoiesis of indeterminate potential (CHIP); somatic mutations of *TP53* are among the five gene most frequently mutated in CHIP and their presence contributes to the progression of CHIP.[74]

TP53 mutations in acute erythroid leukemia. Acute erythroleukemia (AEL) is a rare subtype of AML, defined on the basis of the presence of a high frequency of erythroblasts, including at least 30% of proerythroblasts, associated with recurrent *TP53* mutations. In the International Consensus Classification pure erythroid leukemia is no more recognized as a separate entity and is instead included in a broader category of AMLs with mutated *TP53*;[104] in the 5th edition of the WHO classification of myeloid disorders, the WHO changed the terminology of pure erythroid leukemia with acute erythroid leukemia.[121] A key study by Iacobucci and coworkers reported the first detailed molecular characterization of AEL and defined five age-related subgroups: adult, including *TP53* mutated, *NPM1* mutated, *KMT2A* mutated/rearranged and *DDX1* mutated; pediatric, including *NUP98* rearranged.[122] The molecular characterization of *TP53*-mutated AEL, corresponding to 35.9% of all cases, showed that all but one of the *TP53*-mutated cases displayed alterations of both alleles; *TP53* mutations were predominantly missense mutations occurring in the DNA binding domain and were associated with a poor prognosis.[122] It is important to note that this study involved different types of AMLs implying a significant involvement of the erythroid lineage. A more recent study based on the molecular characterization of a similar range of erythroid-associated AML subtypes confirmed the findings observed in the previous study.[123] Importantly, this study showed also that *TP53*-mutated AELs are characterized by the presence of gain/focal amplifications involving the *EPOR, JAK2* and *ERG/ETS*,

with about 60% of these patients harboring one or more than one of these lesions.[123] It is of interest that most of AELs expressing *TP53* mutations in association with *EPOR, JAK2* and *ERG/ETS* CNAs correspond to cases of pure erythroid leukemia.[123] The association between *TP53* mutations and *EPOR/JAK2* gains and amplifications underlines a sensitivity to JAK2 inhibitors in preclinical models.[123] In line with this last finding, two different reports showed that virtually all cases of pure erythroid leukemia display biallelic *TP53* alterations.[124-125]

TP53 mutations in therapy-related MDS and AML. As above reported, therapy-related myeloid neoplasms (tMN) represent a dramatic consequence of cancer therapy and develop 3 to 7 years after treatment with chemo- and radiation therapies (CRTs) and typically present in a form of tAML or tMDS and are frequently associated with poor prognostic features, such as complex karyotypes and *TP53* mutations. tMDS and tAML are characterized by a peculiar mutational profile: in tAMLs *TP53* resulted to be the gene most frequently mutated, in the range of 25% to 58% of cases.[46,63,126-129] Ok and coworkers have characterized the *TP53* mutational spectrum in 35 tAML/tMDS patients and observed that: *TP53* mutations were mainly clustered at the level of DNA-binding domains, with an allelic frequency of 37%; missense mutations were the most frequent, followed by frameshift and nonsense mutations.[129] This *TP53* mutational pattern was highly comparable to that observed in *de novo* AMLs/MDSs.[130]

Lindsley et al. have evaluated 1514 MDS patients undergoing hematopoietic stem cell transplantation and observed a remarkable difference in the frequency of *TP53* mutations among tMDS compared to pMDS (38% vs 14%, respectively).[49] Interestingly, in tMDS patients it was identified also a higher frequency of mutations of *PPM1D*, a regulator of *TP53*, in tMDS compared to pMDS (15% vs 3%, respectively); thus, 46% of tMDS patients display *TP53* or *PPM1D* mutations.[49] *PPM1D* gene encodes a serine-threonine phosphatase, involved in the cellular response to environmental stress. *PPM1D* mutations alone in MDS did not show any significant association with complex karyotype, while *TP53* mutations are strongly associated with complex karyotype.[49] The proportion of patients with tMDS was about 12% among patients without *TP53* and *PPM1D* mutations, about 40% in patients with *TP53* mutations without *PPM1D* mutations, about 50% among patients without *TP53* mutations and with *PPM1D* mutations and about 55% among patients with both *TP53* and *PPM1D* mutations.[49] *TP53*-mutated MDS patients, independently of their age, have a clearly reduced overall survival after transplantation compared to those without *TP53* mutations.[49]

Hiwase and coworkers have analyzed 245 tMDS and 132 tAML patients; 123 of these patients had *TP53* mutations with VAF >10% or loss-of heterozygosity or copy neutral LOH involving the *TP53* locus: 21 of these patients were classified as single-hit and 102 as multi-hit.[131] Overall survival was not significantly different between single-hit and multi-hit *TP53*-mutant patients; furthermore, there was no difference in the incidence of progression in AML between single- versus multi-hit *TP53*-mutant tMDS.[131] These observations suggest that the *TP53* VAF of 10% is a clinically useful threshold to identify patients with poor outcome.[131]

The analysis of 118 tAML/tMDS patients with complex karyotype confirmed the very strong association between this chromosomal abnormality and *TP53* mutations (90%).[132] Conversely, patients with tAML/tMDS with normal karyotype show distinct genomic and clinical characteristics compared to their counterparts with abnormal karyotype, characterized by a markedly lower frequency of *TP53* mutations.[133,134]

Interestingly, some recent studies have shown some remarkable differences in the cellular and molecular mechanisms of development of tMN in pediatric cancer patients. The mutational profile of pediatric tMN is different from that described in adult TMN and is characterized by frequent Ras/MAPK pathway mutations (*KRAS, NF1* and *NRAS* mutations), *RUNX1* mutations and *KMT2A* rearrangements, while *TP53* mutations are less frequent (6%).[135] At variance with the results observed in adult tMN patients, no evidence of pre-existing minor tMN clones, including also those with *TP53* mutations, was observed; in the majority of cases, tMN development was related to the generation of mutant clones arising as a consequence of cytotoxic therapy.[135] In line with this study, a report on three pediatric neuroblastoma patients developing tMN showed that clonal hematopoiesis, mainly consisting of platinum-induced mutation and no driver myeloid genes, preceded the development of tMN that arose after the acquisition of driver mutations.[136] A recent study provided additional support to the role of chemotherapy in promoting directly or indirectly mutations at the level of the HSPC compartment, responsible for tMN development.[136] Thus, Bertrams and coworkers have explored mutation accumulation in HSPCs before and after cancer therapy in 24 children and observed that post-treatment HSPCs have a considerable increase of mutation burden, comparable to what treatment-naïve cells accumulate during 16 years of life.[136] Particularly, chemotherapy may be mutagenic for hematopoietic cells through three different mechanisms: directly to all exposed cells by DNA cross-linking; directly to dividing cells by base analogue incorporation; indirectly, by mimicking aging processes.[137] Drugs such as platinum-based drugs induce clock-like processes, mimicking in an accelerated way normal aging events.[137] Phylogenetic reconstruction of tumor development in these children

showed that tumor tMN in children originate after the start of treatment and leukemic clones become dominant after chemotherapy exposure.[137] It is important to note that analysis of individual HSPCs purified from human subjects from the birth to 81 years of age showed that these cells spontaneously accumulate a mean of 17 mutations per year after birth and lost 30 base pairs per year of telomere length.[138]

Interestingly, *TP53* germline pathogenic or likely pathogenic variants were identified in 13 of 84 children with a tMN.[135] This observation suggests that individual predisposition to cancer development may play a role in tMN.

In conclusion, mutations of *TP53* are the single most frequent molecular abnormality of tMN and are frequently associated with complex karyotype.[139] *TP53* mutations represent one of the major challenges to ameliorating outcome in t-MN and their presence in tMN is associated with short duration of clinical responses.[139]

The role of TP53 mutations in the development of therapy-related myeloid neoplasias. Two models have been proposed to explain the development and the role of *TP53* mutations in tMN. Following one model, chemo-radiotherapy induces the development of mutations at the level of HSPCs. However, following the other model, chemo-radiotherapy promotes clonal selection of pre-existing mutant HSPCs. Several observations support the second model: the mutational burden of pAML and of tAML is comparable; somatic mutations present in tMN are detectable years before chemo-radiation exposure; somatic mutations in genes involved in the DNA damage response, such as *TP53* and *PPM1D*, are enriched in the blood of patients exposed to chemo-radiotherapy.

Several studies carried out in cancer patients undergoing autologous stem cell transplantation (ASCT) or allogeneic stem cell transplantation (alloSCT) have provided evidence in favour of tMN generated from CHIP bearing mutant genes. Thus, Gibson and coworkers showed that in lymphoma patients undergoing ASCT the presence of CHIP was associated with an increased risk of developing tMN post-transplantation; in 8 of these patients developing tMN, 4 displayed *TP53* mutations and *2 PPM1D* mutations and these mutations were maintained in tMNs.[140] An analysis of 565 Danish lymphoma patients undergoing ASCT showed that 25.5% of these patients displayed CHIP; the global overall survival of patients with CHIP was not significantly inferior to that of those without CHIP in a multivariate analysis; however, patients with mutations in *TP53* and *PPM1D* (corresponding to 35% of all patients with CHIP) had a significantly lower overall survival, in part due to increased rates of therapy-related leukemia.[141] Liu et al. have explored 362 patients with diffuse large B-cell lymphoma (DLBCL) and observed that 29% of these patients displayed CHIP.[142] 7 of these patients developed tMN with a lapse of 6 to 30 months after therapy: all these patients have evidence of CHIP with mutations observed also in tMN; 3/7 of these patients showed CHIP bearing *TP53* mutations expanding at the time of tMN development.[142]

Several studies have explored the mechanism of tMN in multiple myeloma (MM) patients undergoing ASCT. An initial study carried out on 6 MM patients undergoing ASCT and developing tMN showed the presence in the stem cell-enriched bone marrow fractions of leukemia-associated mutations (*TP53* mutations in 5/6 cases) years before the development of tMN.[143] Mouhieddine evaluated a large cohort of 629 MM patients undergoing ASCT: 21.6% of these patients displayed CHIP and 19.8% of these CHIP-positive patients displayed *TP53* or *PPM1D* mutations.[144] The longitudinal analysis on 14 patients who developed tMN showed that 6 displayed CHIP with *TP53* mutations and 9 showed *TP53* mutations at the level of tMNs.[144]

It is important to note that *TP53* and *PPM1D* mutations have a negative impact in lymphoma and MM patients undergoing ASCT not only because they confer an increased risk of tMN development, but also because their presence is associated with a reduced stem cell mobilization at the time of pre-transplantation setting.[145] In line with these observations, Berger and coworkers have studied 18 lymphoma/MM patients developing tMN after ASCT; 70% of these patients displayed CHIP and in 85% of cases tMN mutations were observed in CHIP.[146] Importantly, these patients displayed an impaired stem cell mobilization at the time of transplantation and a delayed regeneration after transplantation.[146] 7 patients were analyzed longitudinally after transplantation and showed initial signs of clonal expansion in the lapse of time comprised from 1 to 14 years after transplantation; in 4 of these 7 patients, the expansion of *TP53*-mutated cells was observed.[146]

The study of Bolton et al. provided evidence about the evolution of CHIP mutations under the effect of anti-cancer therapy through the evaluation of two large cohorts of untreated and treated cancer patients, showing that the frequency of CHIPs bearing DNA damage response genes *TP53, PPM1D* and *CHEK2* is strongly associated with previous exposure to cancer therapy.[68] In contrast, the frequency of CHIPs defined by mutations in epigenetic modifiers, such as *DNMT3A* and *TET2,* and by spliceosome regulators, such as *SRSF2, SF3B1* and *U2AF1,* was not significantly modified by anti-cancer treatments.[68] Mutations in *TP53* and *PPM1D* were most strongly associated previous exposure to platinum or radionuclide or taxane therapy.[68] Serial sampling analysis clearly showed that cancer therapy select for clones with mutations at the level of *TP53, PPM1D* and *CHEK2* and that these clones have lower competitive

fitness in the absence of cytotoxic or radiation therapy.[68] The longitudinal analysis of 35 cancer-treated patients developing tMN showed that in all these cases the CHIP mutation was present at the time of tMN diagnosis; however, leukemic transformation was associated with the acquisition of additional mutations (such as *FLT3, NRAS, KRAS* mutations) and chromosomal abnormalities.[68] 40% of tMN patients displayed *TP53* mutations in 10/14 cases already present at the time of CHIP testing; after therapy, the *TP53* clone consistently attained dominance by the time of tMN development and acquired chromosomal abnormalities.[68] In conclusion, the hematopoietic cells harboring *TP53* mutations are positively selected when exposed to anticancer therapy and may attain clinical dominance with acquisition of additional mutational events and chromosomal aneuploidies.

The study of gynecologic tumor patients undergoing chemo-radiation treatments further suggested a key role of pre-existing *TP53* mutant CHIP clones in the development of tMN. Among gynecological cancer patients, it was estimated that 24% of ovarian cancer, 23% of breast cancer and 25% of endometrial cancer patients have CHIP.[67] Recently, Weber-Lassalle reported the study of 448 ovarian cancer (OC) patients (249 at primary OC diagnosis and 199 at platinum-sensitive recurrence); 17.4% of these patients displayed at least one CHIP gene mutation: *DNMT3A* (7.3%), *PPM1D* (6.6%), *TET2* (2.6%), *ASXL1* (1.8%) and *TP53* (1.5%) were the genes most frequently mutated.[147] *TP53* and *PPM1D* mutations were observed only in patients who received at least one previous line of carboplatin treatment; *TP53* mutations correlated with the number of previous lines of platinum treatment but not with age or BRCA mutational status.[147]

PARP inhibitors have been approved by FDA as frontline maintenance for BRCA-associated advanced stage ovarian cancer and have demonstrated an improvement in relapse-free survival. Kwan et al. explored ovarian cancer patients enrolled in the ARIEL2 and ARIEL3 trials involving treatment with the PARP inhibitor Rucaparib.[148] 20 of these patients developed tMN after Rucaparib treatment: 45% of these patients displayed CHIP in their blood, compared to a frequency of 25% among patients not developing tMN.[147] Interestingly, all tMN developing patients display *TP53* missense mutations; CHIP *TP53* mutations were significantly less frequent in patients not developing tMN (13.6% in those not developing tMN compared to 45% in those developing tMN).[148] Patients with *TP53* variant CHIP have a significantly longer prior exposure to platinum-based therapy. The longitudinal analysis of 5 tMN-developing patients showed a marked increase of *TP53* VAF after rucaparib treatment and before tMN development.[148]

Other studies have shown an increased incidence of tMN in gynecologic cancer patients treated with PARP inhibitors. In a meta-analysis of 28 randomized controlled trials, Morice et al. reported an incidence of myeloid malignancies with PARP inhibitors of 0.73% compared to 0.47% observed in controls.[149] The study of tMNs in breast cancer or ovarian cancer patients treated with PARP inhibitors showed a particularly high frequency of *TP53* mutations estimated in the order of 70-75%.[150-152] Martin et al. have compared the occurrence of CHIP among ovarian cancer patients treated or not with PARP inhibitors as maintenance therapy and observed a higher frequency in those treated with PARP inhibitors compared to those treated without PARP inhibitors (78% vs 18%, respectively); the frequency of *TP53* mutations was higher in CHIP of those treated with PARP inhibitors compared to those treated without PARP inhibitors (64% vs 14%, respectively), while the frequency of *PPM1D* mutations was similar in these two groups of patients (50% vs 43%, respectively).[152] These observations have been confirmed by Bolton and coworkers who explored 10,156 cancer patients for CHIP in their blood and found that patients exposed to PARP inhibitors have an increased frequency of CHIP (33%) compared to that observed in cancer patients undergoing other anticancer treatments (18%) or not yet treated at the moment of blood draw (16%).[153] Studies carried out in experimental models led to the conclusion that the increased frequency of CHIP observed in PARP inhibitor-treated patients could derive from the interaction of previous platinum treatment with PARP inhibitor treatment and *TP53* mutations or mutations of other DNA damage repair pathway genes.[154]

Khalife-Hachem et al. have explored 77 patients with gynecologic and breast cancers developing tMN; 55/77 of these patients showed CHIP, while the remaining 26/77 did not have CHIP; the most frequently mutated genes in these patients at the level of CHIP were those related to aging (*DNMT3A, TET2* and *ASXL1*), while *PPM1D* and *TP53* represent 4.6% and 3.3% of CHIP-related mutations, respectively.[155] The analysis of the mutational profile of tMNs (49 tAML and 28 tMDS) showed three different mutational profiles with 36% of cases showing a *TP53/PPM1D* profile, 25% a MDS-like profile and 39% a *de novo*/pan-AML profile; the *TP53-PPM1D* subgroup displayed a limited number of co-mutation events and the very frequent association with complex karyotype.[155] It is of interest to note that all tMN cases classified as *TP53-PPM1D* are associated with pre-existing CHIP at the time of cancer diagnosis, while tMN cases classified as MDS-like or *de novo*/pan-AML are in part associated with the absence or with the presence of pre-existing CHIP.[155]

2.6% of patients with neuroendocrine tumors develop tMN after peptide receptor radionuclide therapy.[156] Patients with neuroendocrine tumors exposed to peptide

receptor radionuclide therapy display an expansion of pre-existing CHIP containing mutant DNA damage repair genes (*TP53, PPM1D* and *CHEK2*) with development of cytopenia.[157]

Some patients undergoing CAR-T cell therapy for non-Hodgkin lymphomas or for multiple myeloma may develop tMN. Thus, Miller et al have explored 154 patients with NHL (144) or with MM (10) undergoing treatment with CAR-T cell therapy for the occurrence of CHIP. 48% of these patients have a CHIP with a VAF >2%; *PPM1D, DNMT3A, TP53* and *TET2* were the gene more frequently mutated at the level of CHIP.[158] 3 of these patients developed a tMN during the follow-up period, 2 of whom harboured *TP53* mutant CHIP and developed *TP53*-mutant AML [159]. Another study reported the case of a patient with large B-cell lymphoma undergoing CAR-T cell therapy and developing progression of tMDS starting from a *TP53*-mutated CHIP, initially associated with cytopenia.[159]

Somatic mutations in cancer cell genomes are caused by different mutational processes, each of which generates a typical mutation signature. More than 40 mutational signatures have been described in cancer cells related either to endogenous or exogenous factors. Particularly, some mutation signatures are related to exposure to exogenous carcinogens, represented also by some anticancer drugs or radiations. Therefore, some chemotherapies damage DNA and cause mutations in both cancer and healthy cells; therefore, each chemotherapy causes a mutational footprint.[160] The study of chemotherapy mutational footprints in therapy-related AML represents a barcode to determine whether the clonal expansion occurred before or after the beginning of exposure to the drug.[161] In fact, cytotoxic agents introduce hundreds of unique mutations in each surviving cancer cell, detectable by sequencing only in cases of clonal expansion of a single cancer cell bearing the mutational signature; therefore, a unique, single-cell genomic barcode can link chemotherapy to a discrete time window in a patient's life.[161] Using this approach, it was possible to show that multiple myeloma seeding at relapse is caused by the survival and expansion of single multiple myeloma cells following treatment with high-dose melphalan therapy and autologous stem cell transplantation.[161] Using this approach, Pich and coworkers showed that tAMLs originated in patients exposed to platinum-based chemotherapies exhibit a mutational footprint associated with these drugs, related to the capacity of these drugs to induce specific mutagenic events in non-malignant hematopoietic cells. The platinum-based mutational signature was used to determine the clonal expansion originating the secondary AMLs begins after the start of cytotoxic treatment.[162] In cases associated with clonal hematopoiesis the absence of this signature is consistent with the start of the clonal expansion predating the exposure to platinum-based drugs.[162]

Diamond et al. have explored the occurrence of chemotherapy-related signatures by whole genome sequencing in 39 tMNs; 16 of these patients developed tMN after melphalan/ASCT.[163] Five single-base substitution mutational signatures have been observed in these tMNs: SBS1 and SBS-HSC, related to clock-like mutations that accumulate with age; SBS31 and SBS35, related to mutations induced by platinum compounds; SBSM1 induced by the alkylator drug melphalan. In contrast, primary *de novo* and relapsed AMLs display only clock-like mutation processes, in that drugs used in the induction chemotherapy are not linked to distinct mutational signatures.[163] A clear dichotomy was observed, in which tMNs with evidence of chemotherapy-induced mutagenesis from platinum and melphalan are hypermutated and enriched for structural variants deriving from events such as chromotripsis, while tMNs originated in patients treated with non-mutagenic chemotherapies display a mutational profile like that observed in *de novo* AMLs. Pooling together all somatic events occurring in tMN genomes, including SV, CNA and SNV, the cases classified as chemotherapy-positive cases displayed a higher prevalence of *TP53* alterations (62.5% of cases) compared to signature-negative cases (13%); particularly, concerning cases receiving melphalan/ASCT, all six cases with SBS-MM1 signature had an event involving *TP53* compared to 20% in those without the signature.[163] Therefore, in patients with prior MM who were treated with high-dose melphalan and ASCT, tMN can develop from either a reinfused CHIP clone that escapes melphalan exposure and is selected following reinfusion, or from *TP53*-mutant CHIP that survives direct myeloablative conditioning regimen and acquires melphalan-induced DNA damage.[163]

Sperling et al. have analyzed 416 patients with tMN (40% tAML and 60% tMDS) and observed that there is an association between gene mutations and prior cancer treatment exposures.[164] Particularly, significant associations were found between *TP53* mutations and proteasome inhibitors and lenalidomide analogues; multivariate analysis showed the existence of a significant association between *TP53* mutations and prior exposures to thalidomide analogues and vinca alkaloids and negative association with topoisomerase inhibitors; at disease level, *TP53* mutations were particularly associated with multiple myeloma and ovarian cancer.[164] According to these findings, the association between *TP53* mutations and lenalidomide treatment in multiple myeloma patients was explored. Studies in experimental models have shown that *TP53* loss promotes resistance to lenalidomide and confers a selective advantage on *TP53*-mutant HSPCs, determining their outgrowth.[164] In conclusion, these studies have shown the existence of an association

between certain drugs and *TP53* mutations that confer resistance to these drugs and *TP53* mutations that confer resistance to these drugs and promote clonal expansion under the selective effect of these drugs.[164]

TP53 mutations in relapsed/refractory AMLs. The majority of AML patients with newly diagnosed disease achieve complete remission following treatment with intensive induction chemotherapy. However, about two-third patients relapse after frontline therapy and this relapse usually occurs with first 18 months. Late relapses, defined as those occurring after 5 years of remission, are more rarely observed (1-3% of all cases). Basically, early and late relapses are due to resistant clones or subclones of leukemic cells that survive to induction chemotherapy.

Few studies have longitudinally evaluated individual AML patients at primary disease and at relapse. Stratmann et al. reported the longitudinal analysis of 48 adult and 25 pediatric AML patients at diagnosis and at relapse: the genomic mutational landscape at diagnosis and at relapse was highly comparable.[165] Particularly, the frequency of *TP53* mutations was higher in relapsed AMLs compared to primary AMLs; interestingly, *ARID1A* and *CSF1R* mutations are recurrently gained at relapse.[165]

Alwash et al. retrospectively analyzed 200 AML patients who relapsed and were *TP53*-WT at diagnosis; importantly, 29 of these patients developed a newly detectable *TP53* mutation in the context of relapsed/refractory disease.[166] 66% of these patients acquired a detectable *TP53* mutation after the first-line of therapy, 21% after two lines and 14% after three lines of therapy.[166] Some factors increase likelihood of developing a newly detectable *TP53* mutation; particularly, new *TP53* mutations are more common among patients with a baseline chromosome 5 abnormality and with a baseline *IDH2* mutation and among patients treated with intensive therapy compared to those treated with lower intensity.[165] In 45% of these patients, the emergence of *TP53* mutations occurred in the context of complex cytogenetics.[166] In patients who developed *TP53* mutations, the most frequent co-mutations were *DDX41* (30%), *DNMT3A* (22%), *IDH2* (22%) and *NRAS* (18%).[166] The overall survival of these patients acquiring *TP53* mutations was low (4.6 months). Finally, the analysis of 555 AML patients responding to frontline therapy showed that 5 of these patients acquired *TP53* mutations during the remission phase.[166] The results of this study support the monitoring of new emergent *TP53* mutations during AML therapy may have a clinical utility.[166]

Classification of TP53-mutant MDSs and AMLs and their comparison. The International Consensus Classification (ICC) of myeloid neoplasms and acute leukemias recently updated the classification of MDSs and placed these disorders in the context of a broader group of clonal cytopenias, including cytopenias of undetermined significance (CCUS).[167] The presence of multi-hit *TP53* mutations or of *SF3B1* mutation in a cytopenic patient are considered as MDS-defining; furthermore, MDS with biallelic *TP53* gene aberrations are considered a new genetic category of MDSs.[167] Similarly, the ICC created new genetic categories of AML, represented by AML with mutated *TP53* and AMLs with myelodysplasia-related cytogenetic abnormalities and myelodysplasia-related gene mutations.[168]

The WHO defines a single category of MDS with biallelic *TP53* inactivation (MDS-bi*TP53*) irrespective of the blast counts but excludes single-hit *TP53*-mutant MDSs with bone marrow blasts <20%.[121] Similarly, the International Prognostic Scoring System-Molecular considered the poor outcome of multi-hit *TP53*-mutated but excluded single-hit *TP53*-mutated.[169]

According to the latest European Leukemia Network (ELN) 2022 guidelines, the presence of a pathogenic *TP53* mutation, at a VAF of at least 10%, with or without loss of the wild-type *TP53* allele, defines a new entity of *TP53*-mutated AML.[51] In the prognostic hierarchy of AMLs, AMLs with mutated *TP53* constitute the entity with the most adverse prognosis.[51]

Similarly, the ICC guidelines emphasize *TP53*-mutant variant allele frequency >10% regardless of single- or multi-hit status for MDS and AML.[167]

The remarkable differences observed in these different classifications of *TP53*-mutated myeloid neoplasms recently proposed reflect conflicting results observed in different studies. Thus, Bernard and coworkers through the analysis of a large cohort of *de novo* MDS patients reached the conclusion that single-hit MDSs have outcomes similar to *TP53*-WT MDSs, while multi-hit *TP53*-mutated MDSs, associated with complex karyotype, have poor OS.[80] In contrast. Grab et al reported a similarly poor survival for both AMLs and MDSs with excess of blasts, irrespective of single-hit or multi-hit *TP53*-mutant status; however, this study excluded MDS <10% of blasts.[105] Similarly, Weinber et al showed that the survival of MDS and AML with complex karyotype is equally poor independently of single- or multi-hit *TP*53-mutant status.[132]

Recent comparative analysis of the molecular abnormalities of *TP53*-mutated AMLs and of multi-hit *TP53*-mutated MDSs suggests that these two entities probably represent a unique condition.[105,132,170] Thus, Grob et al reported the characterization of *TP53*-mutated cases observed among 2200 *de novo* AMLs and MDS-EB (myelodysplasia with excess of blasts): the molecular characteristics of *TP53*-mutant AML and MDS-EB resulted highly comparable in terms of association with co-mutations and cytogenetic abnormalities; particularly, monosomal karyotype and complex karyotype were

reported at frequencies highly comparable in *TP53*-mutant AML and MDS; similarly, concurrent mutations (*DNMT3A, ASXL1, TET2, RUNX1* and *SRSF2*) were observed at frequencies very similar (**Figure 5**).[105] In both *TP53*-mutant AML and MDS, detection of residual mutant *TP53* was not associated with survival.[105] Furthermore, the clinical outcomes were highly comparable, with a median overall survival around 6 months.[105] In both groups, overall survival was negatively affected by the association with complex karyotype.[105] These similarities between *TP53* aberrant MDS and AML were confirmed by Dunn et al. through the analysis of 84 patients with *TP53*-mutated AML and MDS patients.[170]

A similar conclusion was reached by Weinberg et al. through the analysis of 299 AML and MDS patients with complex karyotype; *TP53* mutations were observed in 83% of these patients (78% in AML patients and 86% in MDS patients; the majority of these mutations were multihit *TP53* mutations). A higher frequency of *TP53* mutations were observed in therapy-related cases.[132] Both in AML and MDS patients, the presence of *TP53* mutations predicted for worse outcome; the clinical features and the response of both *TP53*-mutated or not, AMLs was like that observed in the corresponding MDSs.[132] According to these findings it was concluded that the presence of *TP53* mutations in the context of complex karyotype identifies a homogenously aggressive disease, irrespective of the diagnosis of AML or MDS, of the blast cell count at presentation or therapy-relatedness.[132]

According to all these findings, it was proposed that *TP53*-altered MDS with excess blasts and *TP53*-altered AML should be considered as a unique disease for their treatment in clinical trials.[171]

In line with this unification of *TP53*-mutated MDS and AML in a unique entity recent studies have shown a similar impact of *TP53* mutations on the prognosis of MDS and of AML. Thus, Stengel and coworkers have analyzed a cohort of newly diagnosed MDS (747 cases) and AML (772 cases), including 96 *TP53*-mutated MDS and 84 *TP53*-mutated AML; these patients were classified as single-hit or multi-hit *TP53*-mutated MDS and AML.[172] Overall survival was significantly shorter in patients with *TP53* single-hit compared to patients without *TP53* alterations both in AML and MDS patients (sh vs no hit: AML, 8 months vs 21 months; MDS, 46 vs 70 months); in both MDS and AML, the presence of a multi-hit worsened the prognosis markedly (mh vs no hit: AML, 1 vs 21 months; MDS, 11 vs 70 months).[172]

Shah et al. have explored a large cohort of 327 tMN patients, including 245 tMDS and 132 tAML and showed that patients with *TP53* mutations with VAF >10%, either classified as tMDS or tAML, display a comparably negative prognosis, with similar OS for mono-hit and multi-hit tMDS or tAML.[173] Furthermore, the number of

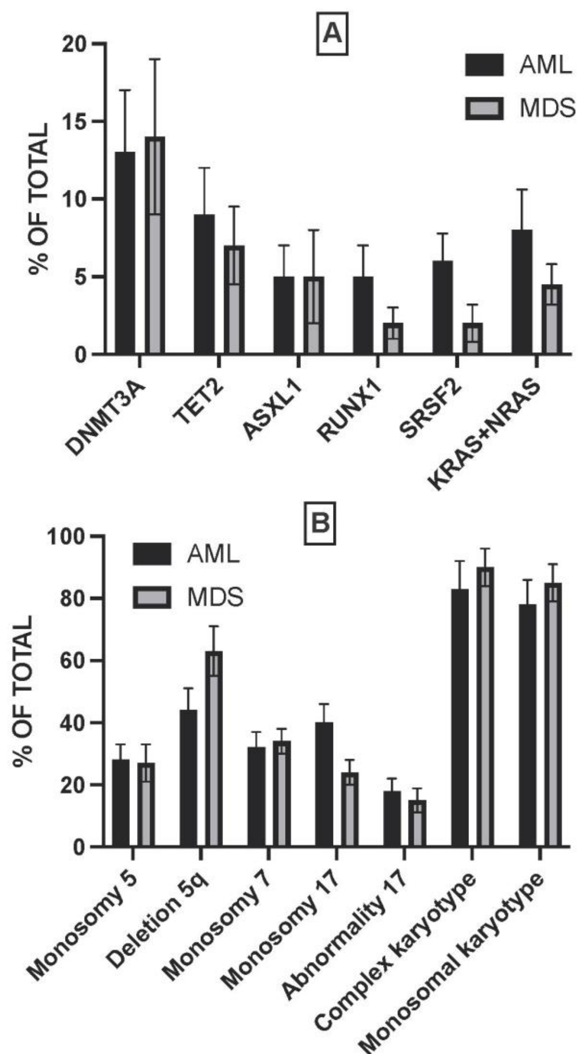

Figure 5. Comparison of the mutational profile (**A**) and chromosomal abnormalities (**B**) in TP53-mutated MDSs and AMLs. Top panel: the frequency of most recurrent driver mutations in reported. Bottom panel: the frequency of most recurrent chromosomal abnormalities is reported.

bone marrow blasts (either <5% 05 >5-9% or 10-19% or >20%) does not affect the OS of the tMN.[173] A similar conclusion was reached by Hiwase et al. showing that in *TP53*-mutated tMN patients the OS in patients with *TP53*-mutant VAF >10%, but not ≤10%, was significantly shorter than in *TP53*-WT patients.[131]

Importantly, the recent classifications of myeloid neoplasms introduced important changes in the classification of tMN. In the ELN[51] and the ICC[104] classification the subcategory of tMN was changed with diagnostic qualifiers instead. In the WHO classification, the tMN and secondary MN were grouped and renamed as myeloid neoplasm after cytotoxic therapy, considering that most of MDS and AML occurring post-cytotoxic therapy have *TP53* mutations and that multi-hit *TP53*-mutant have poor outcome compared with single-hit.[121]

Treatment of TP53-mutated MDS and AML. As above discussed, *TP53* mutations confer resistance

mechanisms to DNA-damaging chemotherapeutic agents, resulting in poor treatment outcomes. Studies carried out in adult AML patients with *TP53* mutations showed a lower complete remission rate, a significantly inferior complete remission duration and overall survival, irrespective of age or the type of treatment received (high-intensity or low-intensity chemotherapy).[174] In the group of *TP53*-mutant AMLs, the *TP53* mutational burden, defined according to the VAF is linked to inferior survival.[99-100]

Induction chemotherapy. *TP53*-mutated AMLs have shown a low response rate to various chemotherapy induction regimens, ranging from 20 to 40% and with a median OS of 4-9 months, using regimens based either on a combination of cytarabine and an anthracycline or cytarabine plus doxorubicin or mitoxantrone-based. The use also of CPX-351, a liposomal form of cytarabine and daunorubicin approved for the treatment of tAML and of AMLs with MRC, did not improve the rate of responses in *TP53*-mutated AMLs with about 30-35% of complete responses and with a very low rate of MRD-negativity (8%).[174-176] Furthermore, mOS was similar for *TP53*-mutant patients treated with CPX-351 compared to 7+3 standard induction chemotherapy.[175]

Since it is unclear what is the optimal induction regimen for AML patients with *TP53* mutations either 7+3 standard induction chemotherapy in patients fit to receive intensive chemotherapy, or, alternatively, venetoclax with hypomethylating agents (VEN/HMA), CPX-351 or various high-dose cytarabine containing regimens; all these treatments were associated with variable results but none of these treatments was more efficacious than the other ones.[176] The same therapeutic regimens are used in *TP53*-mutant AML patients refractory to or relapsing after first-line treatment, with a low rate of complete responses (24%) and with only 13% of patients being able to receive allo-HSCT after achieving response.[177]

Although the response of *TP53*-mutant AML patients to intensive chemotherapy is low, this treatment provides a survival improvement compared to no treatment (8 months vs 1.7 months, respectively).[178]

Hypomethylating agents. The hypomethylating agents include azacitidine (AZA) and decitabine (DEC); these molecules are cytosine analogs that act as inhibitors of methyltransferases, thus inhibiting hypermethylation events occurring in leukemic cells, contributing to the silencing of the expression of some genes, including tumor suppressor genes. These agents have been used with some therapeutic efficacy for the treatment of MDS and of elderly AML patients, not suitable for intensive chemotherapy treatments.

A first prospective uncontrolled study reported encouraging results using 10-day DEC in 113 patients with MDS or AML, including 21 patients with *TP53* mutations; the observed response rate was higher in *TP53*-mutated patients compared to *TP53*-WT patients (100% vs 41% respectively, with no significant difference in the OS of these two different groups of patients).[179] However, a subsequent phase II prospective randomised study in part failed to confirm these results.[180] In this study 5-day and 10-day DEC dosing schedules were compared in elderly AML patients including also 24 *TP53*-mutated patients; a subgroup analysis of these *TP53*-mutated patients showed response rates of 29% and 47%, in the 5-day and 10-day dosing schedules, with a mOS of 4.7 and 4.9 months, not different from those observed in other AML subgroups.[180] Finally, a recent study explored the response to 10-day DEC in a group of refractory/relapsed AML patients; although a part of patients achieved a molecular response with a mOS around 400 days, long-term survival remained poor.[181] In conclusion, although a significant proportion of *TP53*-mutant MDS/AML patients respond to treatment with HMAs in monotherapy, these responses are usually not durable and do not result in a significant improvement of OS; only few patients who achieve either a marked reduction or a clearing of *TP53* mutations, display longer remissions.[182]

In MDS patients with higher risk disease, the HMAs azacitidine and decitabine are the standard of care due to their clinical activity and the capacity to extend overall survival. The study of *TP53*-mutated MDS showed a peculiar sensitivity to DEC. In fact, Chang et al. evaluated the response of 109 MDS patients to DEC and 27.5% of these patients displayed a complete response.[182] *TP53* mutations in these patients predicted response to DEC therapy, with 66% of patients with *TP53* mutations achieving a complete response.[183] 9/10 of these *TP53*-mutated responding patients displayed a complex karyotype. However, in spite the association with a higher response rate, *TP53*-mutated MDSs did not display an improved overall survival.[183] The longitudinal analysis of the mutational profiling of some of these patients showed that 5/7 *TP53*-mutated patients displayed the clearing of *TP53* mutations but the maintenance of other mutations.[183] The HOVON 135/SAKK30/15 trial compared the effect of the association of the Bruton Kinase inhibitor Ibrutinib with DEC to DEC alone in older AML and in high-risk MDS patients.[184] The results of this study showed that Ibrutinib added to 10-day DEC does not improve response or survival in AML and MDS patients compared to DEC alone.[184] Molecular profiling of patients at diagnosis showed that patients with *TP53* mutations had significantly higher response rates to DEC+Ibrutinib treatment.[184]

The *TP53* mutational burden was evaluated in MDS patients undergoing treatment with HMAs. Thus,

Falconi et al. evaluated the VAF of a set of genes recurrently mutated in MDS, including *TP53*, in response to standard treatment with HMAs as a bridge to alloHSCT.[184] This study showed that *TP53* mutations were not predictive of AZA response and, while the allelic frequency of most mutations did not change upon AZA treatment, a significant decrease of *TP53* mutational burden was observed with a decrease of mVAF from 29.5% before treatment to 10.5% after treatment, which was independent of the depth of response.[185] It is important to note that, although the *TP53*-mutant allelic burden significantly decreased upon AZA treatment, *TP53* mutations never became undetectable, also in patients achieving a complete response.[185] Hunter et reported the results of the serial molecular profiling of 108 MDS patients undergoing treatment with HMAs; this study included 35 patients with *TP53* mutations, whose OS was shorter compared to the rest of patients.[186] 46% of patients exhibited clearance of *TP53* mutations and displayed a better mOS (15.6 months) compared to those not achieving claering of *TP53* mutations (7.7 months).[186] The pre-therapy *TP53* mutant VAF of patients achieving mutational clearing was significantly lower than that observed in patients not achieving mutational clearing (12% vs 32%, respectively).[186] 16 *TP53*-mutated patients proceeded to alloHSCT: 7 patients achieving *TP53* clearing before allo-HSCT displayed a trend toward improved OS compared with patients with clonal persistence (25.2 months vs 11.7 months, respectively).[186] These observations suggest that serial sequencing during treatment with HMAs is particularly valuable in *TP53*-mutated patients.

Interestingly, a recent study showed a promising activity of the DEC/cedazuridine (C-DEC) drug association in *TP53*-mutated MDS. This drug association is based on the oral administration of DEC with oral administration of cedazuridine, a cytidine deaminase inhibitor that blocks the rapid metabolism of DEC when orally administered.[187] Recent studies have shown the pharmacological equivalence of oral DEC with oral cedazuridine as compared to intravenous DEC, with overall response rates of 60% and 43% for high-risk MDS in phase II and III trials, respectively. The phase III ASCERTAIN trial involved 133 with intermediate- or high-risk MDS/myelomonocytic leukemia. The study evaluated OS in this patient population, including 44 patients bearing *TP53* mutations.[188] The patients were randomized to receive either: (i) cycle 1 of oral C-DEC followed by cycle 2 of intravenous DEC; or, (ii) cycle 1 of intravenous DEC followed by cycle 2 of oral C-DEC.[188] Patients with *TP53* mutations had worse mOS compared to those with WT-*TP53* (25.5 months vs 33.7 months, respectively); the stratification of *TP53*-mutant into mono-allelic and biallelic showed for those with biallelic alterations a mOS of 13 months which compares favourably with historical results.[188]

BCL2 inhibitors. The BCL2 inhibitor Venetoclax now represents the standard of care for AML patients, newly diagnosed or relapsed/refractory, for elderly AML patients and those who are unfit for intensive chemotherapy treatment, conditions frequently observed among *TP53* mutant AML patients. Therefore, several recent studies have evaluated the response of AML patients with *TP53* mutations to VEN-based regimens.

Initial studies have supported a significant activity of VEN in association with DEC (VEN+DEC) in *TP53*-mutated AMLs. Thus, DiNardo and coworkers reported in *TP53*-mutated treatment-naïve AMLs a CR/CRi rate of response of 47%, with a median duration of response of 5.6 months and a mOS of 7.2 months, findings that seemed favorable as compared to historical controls.[189] Another study retrospectively analyzed 32 *TP53*-mutated AML patients and reported a rate of CR+CRi responses of 67% and 38% in the frontline and in relapsed/refractory condition.[189] Responses were observed either using a 5-day or a 10-day schedule and responder and non-responder patients displayed a similar *TP53* mutational status.[190]

Subsequent studies failed to show a significant benefit of VEN when administered together with DEC compared to DEC alone. Kim et al. performed a post-hoc analysis of a phase II study involving 118 elderly AML patients, including 35 *TP53*-mutated AMLs.[191] Outcomes were worse in patients who had *TP53* mutations compared to those without *TP53* mutations (overall response rate: 66% vs 89%, respectively); overall survival: 5.2 months vs 19.4 months, respectively; relapse-free survival: 3.4 months vs 18.9 months, respectively.[191] Outcomes with DEC+VEN were comparable to historical results with day-10 DEC alone.[191] In a retrospective analysis in 238 AML patients older than 65 years with newly diagnosed *TP53*-mutant AML, patients who received VEN-based regimens had higher response rates than those with non-VEN-based regimens (43% vs 32%, respectively), but exhibited comparable OS with respect to patients treated with non-VEN-based regimens (4.6 vs 5.5 months, respectively).[192] These observations suggested that the addition of VEN to standard treatment regimens did not improve outcomes in younger or older patients who had *TP53*-mutant AML.[192] The analysis of data from a phase III study comparing VEN+AZA or placebo+AZA in poor-risk cytogenetics AML patients subdivided into *TP53*-mutant and *TP53*-WT subgroups: VEN+AZA treatment improved remission rates but not duration of response or overall survival compared to AZA alone in *TP53*-mutant AML patients.[193] In contrast, in *TP53*-WT patients VEN+AZA treatment significantly improved overall survival compared to AZA alone, with outcomes similar to those observed in intermediate-risk AML

patients undergoing a similar treatment.[193] A propensity score cohort of 304 older AML patients treated with DEC+VEN or DEC alone showed that DEC+ZEN significantly improved the response rates and survival outcomes compared to DEC monotherapy; however, some molecular subgroups, such as patients with *TP53* mutations, displayed only a suboptimal response to VEN+DEC treatment (50% of responding patients).[194]

The multiagent therapeutic regimen of fludarabine, cytarabine, granulocyte colony-stimulating factor (G-CSF), and idarubicin is an affective frontline treatment in AML patients suitable for intensive chemotherapy induction. The comparative study of FLAG-IDA and CPX-351 as an induction chemotherapy treatment in high-risk AML and MDS showed that FLAG-IDA was unable to improve the outcomes of *TP53*-mutant AMLs compared to CPX-351.[195] DiNardo and coworkers reported a consistent therapeutic efficacy of VEN in combination with FLAG-IDA induction and consolidation in newly diagnosed and relapsed/refractory AML patients, associated with deep remissions and high rate of transition to successful HSCT.[196] An update analysis of the response to VEN+FLAG-IDA in relapsed/refractory patients showed a high ORR (60%) with 53% CR; 71% of CR patients attained A MRD negative remission status and 68% of responding patients proceeded to HSCT; however, these responses were limited to *TP53*-WT patients: overall survival in *TP53*-WT and *TP53*-mutant AML patients was not reached compared to 5.4 months, with a 12 month overall survival and 17%, respectively.[197]

Daver et al. recently published the results of a meta-analysis englobing 12 clinical trials involving the treatment of *de novo TP53*-mutated AML patients with either intensive chemotherapy or hypomethylating agents or VEN+HMA.[198] The rate of complete remissions and the mean OS were low across the three types of treatments; there was an improved response rate but not in OS in VEN+HMA compared to HMA alone.[198] The median OS was uniformly poor across all three types of treatment: IC 6.5 months, HMA 6.1 months and VEN+HMA 6.2 months.[198] In another study Dover et al. have evaluated the response of 370 AML patients with *TP53* mutations or chromosome 17p deletions to three different treatments: VEN+HMA or HMA alone or intensive chemotherapy; poor OS is observed with all these three treatments and only 8% of the patients can be bridged to allo-HSCT.[199]

Mechanisms of resistance of TP53-mutated MDS and AML to venetoclax. Several studies have explored the cellular and molecular mechanisms mediating the resistance of *TP53*-mutant MDS and AML to VEN. According to the response, DiNardo and coworkers have categorized AML patients treated with VEN into three groups: durable remission (0% with *TP53* mutations); remission, then relapse (24% with *TP53* mutations) and primary refractory (35% with *TP53* mutations).[200] 32% of VEN-treated patients displayed an expansion of *TP53*-mutated cells after VEN treatment, thus suggesting that the presence of *TP53* mutations reduced the sensitivity to VEN or increased relapse-initiating potential.[200]

The study of KO TP53 cells provided a fundamental contribution to define the mechanisms through which *TP53* deficiency may induce a reduced sensitivity to VEN. Several BH3-only proteins, including BAK, BAX, PUMA and NOXA are *TP53* target genes and lower levels of expression of these genes confer resistance to VEN.[201] Furthermore, *TP53* KO resulted in reduced BCL2 and MCL1 levels that contribute to decrease the sensitivity to VEN.[201] *TP53* KO induced some relevant changes in mitochondrial morphology and function: *TP53* KO cells displayed less mitophagy when exposed to stress by a mitochondrial uncoupler and increased cellular respiration, with consequent higher production of cellular reactive oxygen species (ROS).[201] Furthermore, *TP53*KO cells showed also a consistent metabolic deregulation, with increased nucleotide synthesis, associated with decreased glucose, pyruvate, amino acids, and urea cycle intermediate levels, changes that suggest a metabolic shifting on preferential carbon usage to support leukemic cell proliferation.[201] Further exploration of *TP53*KO cells showed that *TP53* deficiency induces a reduced sensitivity not only to BCL2 inhibitors but also to MCL1 inhibitors, when used in monotherapy as single antitumor agent; particularly, BCL2 and MCL1 inhibitors induced only a transient inhibitory killing effect on *TP53*KO cells and some surviving *TP53* deficient cells outgrew *TP53*-WT cells over a longer period of exposure, thus suggesting a competitive survival advantage.[202] Only the concomitant inhibition of both BCL2 and MCL1 increased leukemia cell lethality and durably suppressed leukemia burden, independently of *TP53* mutation status.[202] In line with these findings, Carter et al. showed that co-inhibition of BCL2 with VEN and MCL1 with AMG176 synergistically targets AML cells exhibiting intrinsic or acquired resistance to BH3 mimetics *in vitro* and *in vivo*.[203] Particularly, they showed that primary *TP53*-mutant AML blasts are scarcely sensitive to VEN or AMG176 added in monotherapy but are sensitive to these two drugs added in combination; furthermore, in mouse models inoculated with *TP53*-mutant AML cells only the VEN+AMG176 drug combination was able to significantly prolong animal survival.[203] However, at variance with this study, Mouijalled et al observed that while BCL2 and MCL1 is an efficacious drug combination in many subtypes of poor-risk AMLs, failed to induce an efficient killing of *TP53*-mutant primary AML cells with 7/8 cases resistant to this treatment.[204]

Chen and coworkers showed that the mitochondrial

chaperonin CLBP is upregulated in human AML cells and particularly in those intrinsically resistant to VEN; ablation of CLBP expression sensitizes AML cells, including also *TP53*-mutated AML cells resistant to VEN.[205]

Schimmer et al. through the study of different engineered models of *TP53* deficient cells reached the conclusion that either leukemic cells with *TP53*^{KO} or bearing *TP53* missense mutations equally display a reduced sensitivity to HMAs and VEN, thus suggesting that loss of p53 function, rather than the precise *TP53* allelic configuration determines the inferior efficacy of HMAs and VEN.[206]

It is important to note that a study carried out in different models of resistant AML cells provided clear evidence that the concomitant p53 activation and BCL2 inhibition are synergistically lethal for leukemic cells.[206] At functional level, p53 activation negatively regulates the Ras/Raf/MEK/ERK pathway and activates GSK3 which induces MCL1 phosphorylation and promotes its degradation, thus overcoming AML resistance to BCL2 inhibition; on the other hand, BCL2 inhibition overcomes apoptosis resistance to p53 activation by modifying the cellular response from G1 arrest to apoptosis.[207] These findings imply: (i) the necessity of restoring a p53 activity for an efficient therapeutic response; (ii) the absolute importance of the presence of a mutation in *TP53* when using VEN-based therapy.[207]

Hematopoietic stem cell transplantation (HSCT) in TP53-mutated MDS and AML. Allogeneic HSCT is the only potentially curative treatment for a considerable number of MDS and AML patients. Retrospective studies have shown that transplant efficiency is influenced by the genetic alterations present in the various patients and particularly in *TP53*-mutated MDS and AML patients. In these patients the outcomes of allo-HSCT are considerably affected by the heterogeneous clinical conditions of *TP53*-mutated AML patients and by consistent heterogeneity of these leukemias (related to the *TP53* allelic state, co-occurring somatic mutations, and the position within the clonal hierarchy).

Outcomes of HSCT for *TP53*-mutated AMLs are poor; in fact, a meta-analysis performed in 297 *TP53*-mutated AML patients undergoing allo-HSCT showed a 2-year OS of 29.7% with a relapse rate of 61.4% at 2 years.[208] Similarly, survival after allo-SCT is low for most *TP53*-mutated MDS patients.[209]

The fundamental study of Lindsley and coworkers reported the evaluation of 1514 MDS patients undergoing allo-HSCT.[49] A significantly shorter OS was observed for *TP53*-mutated patients, with an hazard ratio (HR) of 1.71 and shorter time to relapse, with HR of 2.10.[49] The impact of conditioning regimen was also evaluated in these patients, showing that the median survival was similar between myeloablative conditioning regimen (MAC) and reduced-intensity conditioning regimens (RIC) (7.5 months vs 9.2 months, respectively).[49] Yoshizato et al have retrospectively analyzed 797 Japanese MDS patients; 295 of these patients have *TP53* mutations and 98 of them have been transplanted. *TP53*-mutated patients have been subdivided into two subgroups based on the association or not with complex karyotype; the outcomes of these two different subgroups were clearly different: The subgroup with *TP53* mutations and CK (88% of cases) displayed a worse outcome, with a mOS of 4.8 months and with >80% of deaths within 2 years after transplantation; the other subgroup with *TP53* mutations only had a markedly better survival posttransplant with 60% of patients alive at 60 months.[210] Ciurea and coworkers have reported the post-transplant outcomes of 83 MDS/AML *TP53*-mutated patients and median overall survival of less than one year and a 2-year overall survival rate of less than 30%.[211] Three relevant prognostic factors were defined in these patients: the median hematopoietic stem-cell transplantation comorbidity index (HTC-CI) is 4, with a range from 0 to 9; the Karnofsy performance status (KPS); the presence of a complete remission status in first-line (CR1) or in second-line (CR2). A HCT-CI >4, the KPS <80% and the absence of CR1 or CR2 correspond each to 1 point of risk score; *TP53*-mutant patients with a risk 0 have a mean OS significantly better than those with score 1 or score 2.[211]

The analysis of the long-term outcomes of 178 AML patients undergoing allo-HSCT showed that only *TP53* mutations, but not the mutations of other genes, are associated with a significantly shorter OS and RFS and with a higher relapse index.[212] Badar and coworkers have evaluated 370 AML patients with *TP53* mutations: 49 patients received allo-HSCT after first-line therapy and 20 after second-line therapy.[213] In the first-line group, 75% of patients were in complete remission and 70% were MRD-negative at the moment of allo-HSCT; in the second-line group, 50% of patients were in CR and 43% were MRD-negative.[213] The median OS in the first-line group was 30.5 months, compared to 20.2 months in the second-line group; the presence of a condition of CR at day 100 post-transplantation favourably predicted an improved survival post-transplantation.[213] Furthermore, patients in CR with a MRD-negative status at the moment of allo-HSCT had a significantly better OS than those in CR with a MRD-positive condition; however, the presence or not of *TP53*-positivity at transplantation did not affect post-transplantation OS.[213]

Byrne et al. reported the retrospective analysis of 384 *TP53*-mutant MDS/AML patients undergoing allo-HSCT: the post-transplant OS of MDS and AML patients was similar; patients with chronic GVHD displayed a significantly better OS and lower relapse rate than patients without chronic GVHD; patients with

biallelic *TP53* disease or those with CK have a worse outcome compared to those with monoallelic *TP53* disease or without CK, respectively; pre-transplantation *TP53* mutations persistence by NGS predicted post-transplantation relapse, whereas pre-transplantation CR and full donor BM chimerism were associated with a lower rate of relapse.[214]

The European society for Blood and Bone Marrow Transplantation retrospectively analyzed the outcome of 179 AML patients with *TP53* mutations and of 601 AML patients without *TP53* mutations: in patients with *TP53* mutations without CK or chromosome 17p loss the 2-year OS was 65%, while in patients with *TP53* mutations, with either chromosome 17p loss or CK the 2-year OS was 24.6%.[215] Importantly, the 2-year OS of *TP53* mutant patients without 17p loss or CK is like that observed for *TP53*-WT patients (65.2% vs 70.4%). These observations further support the conclusion that *TP53* mutations with concomitant additional cytogenetic feature (CK or 17p$^-$) determine a poor outcome in *TP53*-mutant AML patients.[215]

Three factors limit the efficiency of allo-HSCT in *TP53*-mutated AML patients. (i) Most of *TP53*- mutated AML patients are old and receive reduced intensity conditioning (RIC) to reduce cytotoxicity and RIC is unable in most of cases to induce clearing of *TP53* mutations and to induce a condition of MRD negativity before transplantation, while myeloablative conditioning induces a much higher rate of *TP53* mutations clearing.[216] (ii) In older AML patients molecular associations with MRD positivity and transplant outcomes are driven primarily by baseline genetics, and not by mutations present in remission and baseline *TP53* mutations represent the most unfavourable genetic association.[217] (iii) The negative impact of *TP53* mutations transplant outcomes is related to a very high risk of early relapse after transplantation, thus indicating that *TP53* mutations induce rapid disease progression that outstrips functional engraftment and development of graft-versus-leukemia (GVL) effect.[218]

The proportion of *TP53*-mutated MDS/AML patients which can be transplanted is lower than that observed for patients with other genetic abnormalities. In a recent study, Marvin-Peek et al reported the results of a retrospective analysis of 352 patients with MDS/AML and 91 with *TP53* mutations; the intention to transplant was similar for *TP53*-mutated and *TP53*-WT patients (50 vs 52%), but the real proportion of patients transplanted in the *TP53*-mutant group was significantly lower than in the *TP53*-WT group (19% vs 31%). The *TP53*-mutated MDS/AML patients have an increased number of infections which likely contributes to the lower rates of HSCT in these patients.[219]

The anti-leukemic activity of allo-HSC is related to two main factors: (i) the conditioning regimen; (ii) the immune-related graft-versus-leukemia (GVL) effect. Thus, several studies, above reported, showed that the occurrence of chronic GVHD correlated with improved EFS and OS in *TP53*-mutated MDS/AML patients undergoing allo-HSCT.

Very interestingly, a recent study reported the first results on the combination of a hypomethylating agent and Eprenetapopt sadministered as maintenance therapy post allo-HSCT in *TP53*-mutated AML patients.[220] Eprenetapopt (APR-246) is a small molecule exerting a peculiar effect, restoring WT-*TP53* in *TP53*-mutant AML cells and inducing apoptosis of *TP53*-mutant leukemic cells. Studies that will be discussed below support the use of this drug in combination with a hypomethylating agent in *TP53*-mutant AML cells. A phase II study involved the treatment of 33 MDS/AML patients with AZA+Eprenetapopt in post allo-HSCT: with a median follow-up of 17.0 months, the median OS was 20.6 months and 1-year OS probability was 78%; the observed OS outcomes were encouraging and support prospective randomized studies to define the optimal schedule and duration of this drug association and its therapeutic efficacy.[220]

In conclusion, although there is a low probability of long-term cure and the transplantation is associated with a substantial risk of morbidity and mortality, allo-HSCT can be considered as an appropriate treatment for MDS/AML patients with *TP53* mutations.[218]

CD47 targeting in TP53-mutant AMLs. CD47 is a membrane receptor ubiquitously expressed on the surface of cells and plays a key role in self-recognition. Through interaction with SIRPα, TSP-1 and integrins, CD47 acts as a modulator of cellular phagocytosis by macrophages and of the activation of immune cells.[221] The binding of CD47 to signal-regulated protein α (SIRPα) signals cancer cells to escape from macrophage-mediated phagocytosis, thus promoting tumor progression.[222] CD47 is overexpressed on the surface of many malignant cell types and in some tumors its level of expression is a negative prognostic factor.[222] These observations have supported the rationale of blocking CD47 with inhibitory monoclonal antibodies to promote macrophage anti-tumor mechanisms. CD47 is heterogeneously expressed on AMLs, including the fraction of leukemic stem cells, with 25-30% of these patients displaying high levels of expression; high CD47 expression has been shown to be an independent prognostic factor for poor overall survival in AML patients.[223] CD47 expression was clearly more elevated in AML than in MDS; furthermore, the level of CD47 expression is heterogeneous in the various molecular subtypes: particularly, about 50% of *TP53*-mutant AMLs highly express CD47, while in the remaining cases it was lower.[224]

Preclinical studies using monoclonal antibodies blocking CD47 have shown in *in vitro* and *in vivo* mice

leukemic models a consistent anti-leukemic activity and have supported clinical studies. One of these antibodies, Magrolimab was evaluated in clinical trials involving AML patients. In monotherapy, Magrolimab was unable to induce any CR in patients with refractory/relapsed AML. In subsequent studies, Magrolimab was evaluated in association with other anti-leukemic drugs. A phase Ib study involved the treatment of 52 treatment-naïve AML patients unfit for intensive chemotherapy, with Magrolimab and Azacitidine.[225] In 21 *TP53*-mutant patients, 71% of patients achieved an objective response, 48% a CR; the median overall survival for *TP53*-mutant patients was 12.9 months compared to 18.9 months for *TP53*-WT patients.[225] A phase I/II study involved the enrolment of 18 newly diagnosed AML patients (8 with *TP53* mutations) treated with Magrolimab plus VEN and AZA: in the 7 *TP53*-mutant AML patients evaluable for response, a 100% CR/CRi response was observed, with 57% achieving MRD negativity, as assessed by multicolor flow cytometry assay.[226] A more advanced evaluation of the triplet drug combination showed in 22 frontline *TP53*-mutant AMLs (including 10 tAMLs) a CR+CRi of 63%, compared to 90% in *TP53*-WT AMLs and 1-year OS of 53% compared to 83% for *TP53*-WT AMLs; in 5 sAML patients with *TP53* mutations a CR+CRi of 60% was observed.[226] 30% of *TP53*-mutant AML patients proceeded to allo-HSCT. A phase III placebo-controlled, randomized study to evaluate this drug triplet in newly diagnosed AMLs has been initiated (ENHANCE-3 trial).[227] Finally, Daver and coworkers reported the results of a phase Ib study enrolling 72 frontline *TP53*-mutant AML patients treated with Magrolimab plus azacitidine: a CR+CRi condition was achieved in 41.6% of patients; the longitudinal *TP53* VAF assessment in 8 patients who achieved a CR showed in 5 of these patients A VAF decrease to <5%; the median OS for the 72 treated patients was 10.8 months.[228] A phase III trial in *TP53*-mutant AML (ENHANCE-2) of this drug combination vs standard of care is ongoing.[228]

Johnson and coworkers have analyzed the depth of the molecular response in a group of *TP53*-mutant MDS and AML patients treated with Magrolimab and azacitidine.[229] In patients with *TP53*-mutated MDS, 38% of patients achieved a CR; in these patients, the initial median *TP53* VAF was 0.38 and decreased to 0.07 by cycle 5 of treatment.[229] In patients with *TP53*-mutated AML, 63% of patients achieved a CR; in these patients, *TP53* VAF <0.07 was observed in 54% of patients at cycle 3 and 75% at cycle 5 of treatment.[229]

The ALX Oncology Holdings Inc developed a next generation CD47 blocker, Evorpacept (ALX148): the CD47 binding domain of Evorpacept is an affinity enhanced extracellular domain of SIRPα and its engineered Fc binding domain does not provide the pro-phagocytic signal, but confers to the molecule an antibody-like pharmacokinetic profile. Several ongoing clinical studies are exploring Evorpacept in solid tumors and in hematological malignancies. Recently, the clinical data from the phase Ia (dose-escalation) study ASPEN-05 evaluating Evorpacept in combination with azacitidine and venetoclax fort the treatment of relapsed/refractory or newly diagnosed AML patients were presented.[230] This study showed that: Evorpacept administered with AZA and VEN was generally well tolerated; in 10 relapsed/refractory patients (including 8 patients that progressed after prior VEN treatment and 7 with *TP53* mutations) reduction in marrow blasts was observed in 100% of patients, with 40% objectives responses; in 3 newly diagnosed patients, all with *TP53* mutations, all achieved a response, with 2 complete responses.[230] Another ongoing clinical study (ASPEN-02) is evaluating the safety and the efficacy of Evorpacept in high-risk MDS patients.

Ligufalimab (AK117) is a humanized IgG4 antibody against CD47. AK117 enhanced macrophage-mediated phagocytosis of hematologic cancer and solid tumor cells alone or in combination with other anti-tumor drugs.[231] Ligulifamab is under evaluation in phase I/II ongoing clinical trials.

Lemzopulimab is a peculiar human IgG4 antibody targeting a unique CD47 epitope, enabling CD47 epitope, enabling the sparing of red blood cells but maintaining strong activity against tumor cells. A phase Ib, dose-escalation trial is evaluating the safety and the efficacy of Lemzopilimab in monotherapy in relapsed/refractory AML patients and in high-risk MDS patients; this study showed a good tolerability of Lemzoparlimab with no evident hematological toxicity; one of the five treated patients achieved a morphologic leukemia-free state.[232] Xiao et al. reported the clinical results on 53 newly diagnosed high-risk MDS patients treated with Lemzoparlimab and AZA: the ORR was 82%; an increased pro-phagocytic signal in bone marrow-derived CD33 blasts, as well as an increased percentage of activated macrophages, was observed in 23 responders, but not in 5 non responders; 4/4 patients with *TP53*-mutated MDS achieved a CR or a marrow CR, respectively.[233] These observations preliminary support a promising activity of Lemzoparlimab in high-risk MDS patients exhibiting a higher CALR expression and immune infiltrates in bone marrow.[233]

Pevenodistat. Pevenodistat (PEV) is an inhibitor of NEDD8-activating enzyme (NAE) which is essential for the degradation of some cellular proteins essential for tumor growth and survival. Preclinical studies have supported the evaluation of PEV as a therapeutical agent for the treatment of hematological malignancies. Particularly. These studies supported the evaluation of PEV in combination with hypomethylating agents.

Swords and coworkers have explored the safety and

efficacy of PEV administered together with AZA in elderly AML patients unfit for intensive chemotherapy.[234] 5 of these patients displayed TP*53* mutations and 4/5 of them were responders (CR+CRi+PR) to PEV+AZA treatment.[234] However, subsequent studies failed to confirm this high sensitivity of *TP53*-mutated AMLs to PEV+AZA treatment. Thus Saliba et al. reported the results on the response to PEV+AZA of 9 older AML patients with *TP53* mutations enrolled in the phase II umbrella Beat AML Master trial; these patients were selected according to the presence of *TP53* mutations with a VAF >30%.[235] None of the 9 treated patients attained a CR and 2 patients exhibited a PR.[235] These authors argued that the lower sensitivity of *TP53*-mutated patients observed in this study compared to the previous study of Swords et al could be related to the criteria of selection of these patients (with low *TP53*-mutation VAF in the study of Swords et al. vs with *TP53*-mutation VAF >30% in the study of Saliba et al.).[235] The phase III PANTHER randomized trial explored the safety and efficacy of PEV+AZA vs AZA alone in patients with newly diagnosed high-risk MDS patients.[236] In the whole population of treated patients no significant improvement of OS in the PEV+AZA arm vs AZA alone was observed (21.6 months vs 17.5 months); however, patients receiving >3 cycles or >6 cycles of treatment exhibited a significant improvement in OS compared to AZA arm.[236] This study enrolled a high proportion of *TP53*-mutated MDS patients (28.9% in the PEV+AZA arm and 25.9% in the AZA arm); the ORR in *TP53*-mutated MDS was 25% with PEV+AZA and 28% with AZA alone.[236]

Preclinical studies have supported the rationale of combining PEN+VEN+AZA, showing that this triplet drug association induces a robust activity against primary AML blasts, including also high-risk AML.[237] At mechanistic level, PEV+AZA act as inducers of NOXA expression which enhances VEN-mediated apoptosis.[236] A phase I/II study evaluated the triplet combination of PEN, AZA and VEN in patients with newly diagnosed sAML and MDS with hypomethylating failure.[238] 32 AML patients were enrolled in this study and 34% of them displayed *TP53* mutations: the median OS for patients with *TP53* mutations was 8.1 months and 18 months for *TP53*-WT patients.[238]

Eprenetapopt (APR-246, PRIMA-1). PRIMA-1, a small molecule compound, and its methylated analog known as APR-246 or Eprenetapopt, acts as a suppressor of the growth of an osteosarcoma cell line expressing the *TP53* mutant R272H.[239] This molecule displays the unique property of restoring the DNA binding capacity of p53 mutant protein and, consequently, the growth and tumor-suppressing activities of this protein.[240-241] The restoring capacity was observed for various *TP53* mutants. Preclinical models have supported the anti-tumor activity of APR-246 and its synergistic functional interaction with DNA-damaging anticancer drugs.[242] The pharmacological activity of APR-246 requires its conversion into a methylene quinuclidonone that is able to covalently bind at the level of Cys 124 and 277 of mutant p53 protein, inducing a shift in favour of the WT p53 conformation.[243] A recent study suggested an additional mechanism of anti-tumor activity of APR-246 through induction of oxidative stress mediated by glutathione depletion and induction of ferroptosis.[244]

Preclinical studies have shown synergistic effects of APR-246 and AZA in TP53-mutated MDS and AML cells and have supported the clinical evaluation of this drug association[241] Two phase Ib/II studies have evaluated the association of Eprenetapopt with AZA; the first trial involved the enrollment of *TP53*-mutated MDS (with intermediate or high-risk) and AML (oligoblastic AMLs, with 20-30% of blasts);[245] the second trial involved a similar population of patients, with the exception of the admission of AML patients with any blast percentage and the administration of the two drugs Eprenetapopt and AZA for up to one year in the eventuality of a HSCT.[246] The pooled analysis of 100 patients enrolled in these two studies showed an ORR of 69%, a CR rate of 43%, a NGS *TP53* mutation negativity of 40%, a MRD negativity rate of 6% and a median OS after allo-HSCT of 16.1 months.[247] Responding patients had significant reductions in *TP53* VAF; responding patients had a significantly longer OS compared to non-responding patients.[247] Patients who responded to treatment and proceeded to allo-HSCT had a mOS not reached compared to 9.1 months for patients who did not respond and undergo allo-HSCT.[247]

Other ongoing clinical trials are evaluating Eprenetapopot in other clinical settings and using other drug associations. Thus, Garcia-Manero and coworkers reported the first results on 30 *TP53*-mutant AML patients undergoing treatment with a triplet drug association based on Eprenetapopt in combination with VEN and AZA.[248] A CR rate of 30% and CR+CRi of 53% were observed and the Simon 2-stage efficiency criteria supported future exploration of this drug combination.[248]

A phase III clinical trial comparing Eprenetapopt plus AZA to AZA alone in MDS patients failed to meet its primary endpoint, as announced in a press release of APNEA Company: although the results showed a higher rate of complete responses of 33.3% in the Eprenetapopt+AZA arm compared to 22.4% in the AZA monotherapy arm, the difference between the two arms did not meet the predefined threshold for statistical significance.

A phase II clinical trial evaluated the efficacy and safety of Eprenetapopt in combination with AZA as a post HSCT maintenance therapy in *TP53*-mutated MDS

and AML patients.[249] This treatment was well tolerated with a good safety profile. With a median follow-up of 17 months, the median OS was 20.6 months and 1-year OS probability of 78.8%.[249] It is important to note that 1-year relapse-free survival was of 60% with this treatment that compares favourably with a previous report showing a 1-year relapse-free survival of 30% for *TP53*-mutated MDS patients.[49]

Although a phase I/II clinical trial combining Eprenetapopt with AZA showed an ORR of 71%, 50% CR rate and 47% of molecular remissions, the duration of these remissions was limited due to relapse that occurred with the emergence of the same pre-treatment *TP53* mutations, without secondary mutations, thus suggesting that relapse was not related to the acquisition or selection of subclonal mutations.[247] A recent study provided evidence that resistance to Eprenetapopt could be related to the overexpression of the nuclear exportin XPO1, resulting in shuttling to the cytoplasm of refolded p53, thus leading to therapeutic resistance.[250]

Interestingly, a recent study provided evidence that Eprenetapopt may stimulate anti-immune tumor immunity through a peculiar mechanism, involving increased p53 expression in tumor-associated macrophages.[251] This finding supports the therapeutic association of Epretapopt with immune checkpoint blockers.[251]

Immunotherapy with bispecific antibodies: Flotetuzumab. Studies exploring the therapeutic activity of Flotetuzumab, a CD123xCD3 bispecific dual-affinity retargeting antibody (DART) molecule led to define a significant sensitivity of *TP53*-mutated AMLs to immunotherapy and to discover a peculiar immunological profile of *TP53*-mutated AMLs.

These studies were prompted by recent investigations suggesting that *TP53*, in addition to its well-known function of tumor suppressor, plays also a relevant role in the activation of genes involved in immune responses and inflammation. Particularly, the analysis of transcriptomic data of The Cancer Genome Atlas (TCGA) from 10,000 nonhematologic tumors showed that *TP53* mutations exhibit a correlation with increased leukocyte infiltration and are enriched in wound healing and interferon-γ dominant immune subtypes.[252]

Vadakekolathu et al., through targeted immune gene expression profiling, identified two groups of immune subtypes of AML cells: immune infiltrated and immune depleted.[253] AMLs with immune-infiltrated profiles displayed higher expression of IFN-stimulated genes and T-cell recruiting factors, T-cell markers and cytolytic effectors, counter-regulatory immune checkpoints and molecules involved in antigen presentation and processing; this immunologic profile was associated with suppressed anti-tumor immune reactivity and with response to immunotherapy in solid tumors and in AML.[253] *TP53*-mutant AMLs mostly correspond to immune-infiltrate AMLs. Overall protein expression patterns identified four protein signatures (SIG1, SIG2, SIG3 and SIG4); interestingly, all features of SIG3 group correlated with *TP53* mutational status.[253] SIG3 signatures were enriched in biological processes related to T-cell lineage commitment and T-cell homeostasis; deregulated genes in SIG3 include PD-L1, FoxP3, G2MB, PTEN and BCL2 and were predominantly observed in AMLs with immune-infiltrated mRNA profiles.[253] In parallel, the same authors explored the immune infiltration profiles in AMLs corresponding to various mutational profiles: *TP53* mutated AML cases showed higher immune infiltration, a higher number of mutations and a higher fraction of genome altered, compared to other AML subtypes without *TP53* mutations, including *FLT3-ITD* or *NPM1*-mutant AMLs; concerning immune-related gene, *TP53*-mutated AMLs expressed significantly higher levels of IFN-γ mRNA, CD8A mRNA, PD-L1 mRNA, FoxP3 mRNA, G2MB mRNA and LAG3 mRNA than *TP53*-WT AMLs.[254] This immune gene expression profile suggests that the tumor microenvironment of *TP53*-mutant AMLs is intrinsically proinflammatory and IFN-γ dominant and that these features were associated with poor survival. These observations allowed the discovery of a 34-gene immune classifier prognostic for survival in independent cohorts of AML patients.[254]

The analysis of relapsed/refractory *TP53*-mutated AML patients treated in the context of a clinical immunotherapy trial involving Flotetuzumab provided some interesting information. Flotetuzumab is a bispecific antibody targeting both CD123, a membrane antigen preferentially expressed on leukemic blasts comparted to normal hematopoietic cells and CD3: the use of this bispecific antibody aims to drive an immune response (mediated by CD3) at the level of the sites of leukemic cell development (mediated by CD123).[255] Flotetuzumab was evaluated in 88 adult AML patients with refractory/relapsed disease, showing a CR rate of 26.7%.[256] The analysis of the response of *TP53*-mutant patients enrolled in this study provided evidence of their sensitivity to Flotetuzumab treatment.[254] Particularly, 13 *TP53*-mutant patients were enrolled and 10/13 displayed an increased immune infiltration in tumor microenvironment, while 3/13-clustered in the immune-depleted subgroup; the ORR in these patients was 60%, with 47% of patients achieving a CR; interestingly, the ORR to Flotetuzumab was higher in *TP53*-mutant than in *TP53*-WT patients (60% vs 33.3%); the mOS in *TP53*-mutant patients achieving a CR was 10.3 months.[254] These observations strongly support additional studies based on the treatment of *TP53*-mutated AML patients with Flotetuzumab and with other immunotherapeutic approaches.

Interestingly, a recent study showed that

Flotetuzumab enhances major histocompatibility class II (MHC-II) in AML cells of patients treated with this antibody and this effect is mediated by local production of IFN-γ.[257]

Additional studies further characterized the abnormalities of immune response observed in *TP53*-mutated AMLs. The degree of $CD8^+$ T cell infiltration in AMLs inversely correlates with overall survival, a finding explained by the high dysfunctional state of these cells; in fact, phenotypic and transcriptomic studies have shown that $CD8^+$ T cells present in AML patients display features of exhaustion and senescence.[258] Exhausted T cells express inhibitory receptors (PD-1, CTLA4, TIM3, CD160, CD244) and show a reduced capacity to secrete cytokines and to exert cytotoxic functions. Senescent T cells downmodulate co-stimulatory molecules (CD27 and CD28), express senescence membrane-associated markers, remain metabolically active and secrete cytokines. Following chemotherapy treatment, the phenotypic and transcriptomic profile of $CD8^+$ T-cells diverge from responders and nonresponders, with upregulation of costimulatory pathways and downregulation of apoptotic and inhibitory T-cell signalling pathways in responsders.[258] Senescent-like $CD8^+$ T-cells are unable to kill autologous AML blasts and their proportion negatively correlates with OS.[258] From RNA-sequencing data, an immune effector dysfunction (IED) signature was identified, whose scores correlate with adverse-risk molecular lesions, including *TP53* mutations, stemness and poor outcomes.[259]

Other studies have shown the peculiar immunological features of *TP53*-mutated AML, such as an enrichment of resting memory CD4 T cells and resting NK cells, a high $CD8^+$ T-cell infiltration, a high expression of some immune-related pathways, such as IL2 signal transducer signaling and inflammatory response.[260]

Sallman et al. have explored the immunological phenotype of the malignant clone and alterations of the immune microenvironment of *TP53*-mutant MDS/AML and observed that: (i) PD-L1 expression is significantly increased in stem cells ($CD34^+/CD38^-$ cells) of *TP53*-mutant MDS/AML compared to *TP53*-WT MDS/AML; (ii) patients with *TP53* mutations exhibit reduced numbers of BM-infiltrating $OX40^+$ cytotoxic cells and helper T lymphocytes; (iii) highly immunosuppressive regulatory T cells, such as $ICOS^{high}/PD\text{-}1^-$ and myeloid-derived suppressor cells ($PD\text{-}1^{low}$) are expanded in BM of *TP53*-mutant patients; (iv) a higher proportion of $ICOS^{high}/PD\text{-}1^-$ Treg cells is a highly significant independent predictor of overall survival.[261] According to these observations it was concluded that *TP53*-mutant MDS/AMLs have an immunosuppressive and immuno-evasive environment that favor their development and resistance to therapy and that immunomodulatory therapeutic strategies may provide some benefit.

TIM-3 targeting. T-cell immunoglobulin and mucin domain 3 (TIM-3) is a type I trans-membrane glycoprotein expressed on IFN-γ-producing T-lymphocytes, FoxP3 Tregs and innate immunity cells. It is expressed on leukemic myeloid cells, but normal hematopoietic stem cells lack expression: AML cells overexpress both TIM-3 and its ligand galectin-9, thus generating an autocrine loop that promotes self-renewal of leukemic stem cells.[262]

TIM-3 overexpression on leukemic blasts inhibits their recognition by $CD8^+$ T cell and their destruction by these cells. Sabatolimab is a humanized monoclonal antibody specific for TIM-3; sabatolimab was selected for its binding and inhibitory capacities of TIM-3 and its administration enhances T-cell killing and inflammatory cytokine production by dendritic cells, facilitates the phagocytic uptake and removal of TIM-3-expressing target cells and blocks the interaction between TIM-3 and its ligand galectin-9.[263] Sabotolimab is under evaluation as an agent able to target TIM-3 in both immune and myeloid cells in combination with HMAs in patients with AML and high-risk MDS. A phase Ib study of sabatolimab in combination with HMAs involved the enrollment of 51 high-risk and very-high-risk MDS patients and 40 *de novo* AML patients, showing an ORR of 33% in MDS and of 40% in AML patients.[264] Interestingly, sabatolimab appeared efficacious in *TP53*-mutated patients: 71.4% of ORR in 14 patients with MDS, with a median duration of response of 21.5 months and with 24.5% of these patients proceeding to allo-HSCT; 40% ORR in patients with AML.[264] Based on the promising results observed in this phase I study, the STIMULUS clinical trial program was developed to evaluate the safety and the effectiveness of sabatolimab in various combinations with other drugs in MDS and AML patients. Thus, a phase II clinical trial of sabatolimab in combination with AZA and VEN in newly diagnosed AML patients not suitable for intensive chemotherapy is ongoing (STIMULUS-AML1) and the safety data were recently reported.[265]

Immune checkpoint inhibitors-based regimens. As above discussed, the immune dysregulation observed in the tumor microenvironment implies also an increased PD-L1 expression and a state of immunosuppression, conditions that provide a rationale for evaluating immune checkpoint inhibitors in the therapy of *TP53*-mutated MDS/AML patients.

Nivolumab is an anti-PD1 monoclonal antibody and was evaluated in 70 relapsing/refractory AML patients (16 of whom had *TP53* mutations): only 3 patients with *TP53* mutations responded to this treatment.[266] Another study evaluated the association of nivolumab with induction chemotherapy, based on idarubicin and cytarabine regimen, in 44 patients with AML and high-risk MDS, including 8 cases *TP53*-mutated.[267] At median

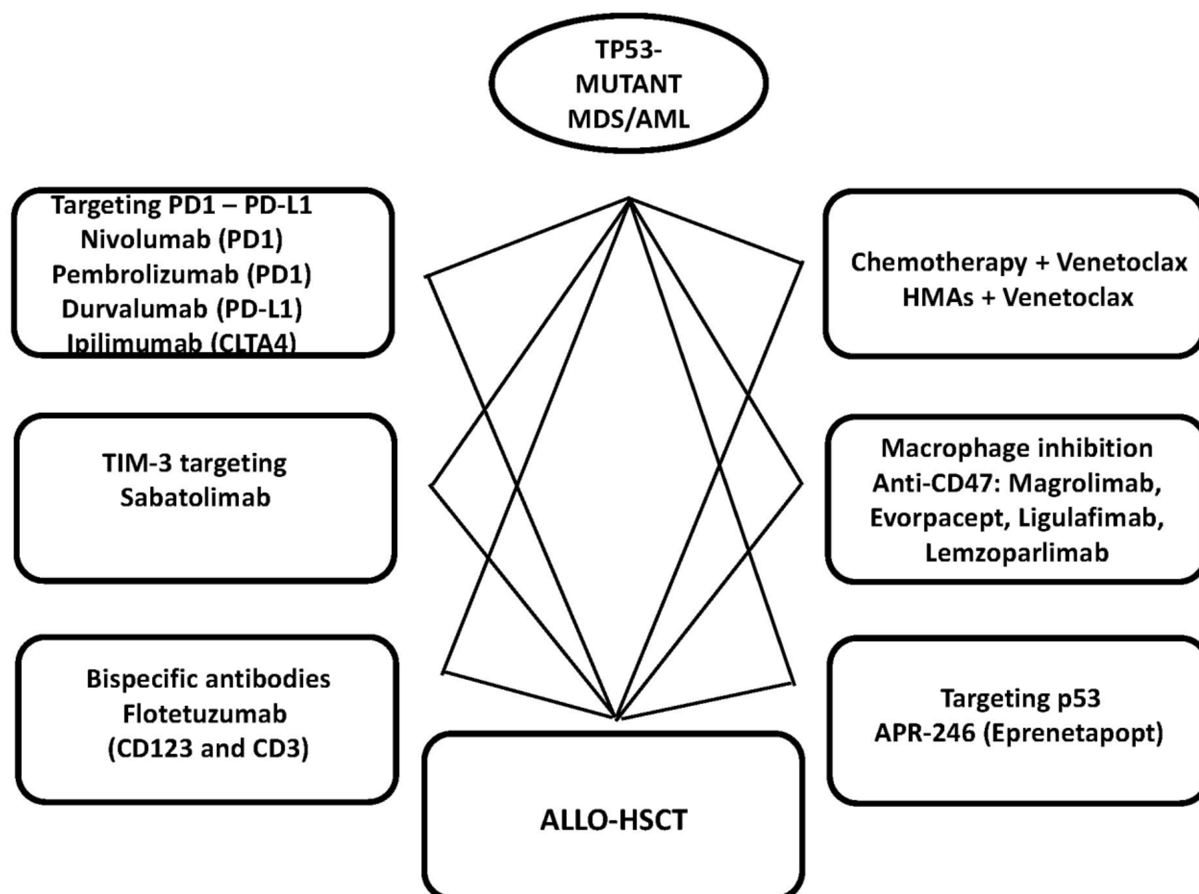

Figure 6. New therapeutic strategies under clinical investigation form the treatment of TP53-mutated MDS/AML.

follow-up of 17 months there was a mOS of 18 months, with 43% of patients achieving a response and proceeding to allo-HSCT.[267] The analysis of mutational profile in responders and non-responders showed that non-responders have more *TP53* mutations than responders (40% vs 12%, respectively).

Other studies have explored the safety and the efficacy of nivolumab as maintenance therapy in high-risk AML patients in remission: however, these studies showed a scarce effect of nivolumab as single agent in eradicating MRD and in extending remission.[268-269] Similarly, the addition of an anti-CTLA4 antibody, Ipilimumab, to AZA and nivolumab failed to significantly improve the response of relapsed/refractory AML patients compared to VEN+AZA or AZA+nivolumab.[270]

An ongoing clinical trial is evaluating the triplet combination of decitabine, VEN and nivolumab. Preclinical studies have shown that PD1 inhibition potentiated the anti-leukemia response in decitabine/VEN-treated AML samples.[271] An initial observation on one patient responding to the triplet drug association showed the clearing of leukemic blasts and of leukemic stem/progenitor cells and the expansion of CD8-positive memory T cells.[271]

Pembrolizumab is another anti-PD1 monoclonal antibody and it was evaluated in combination with high-dose cytarabine in 37 relapsed/refractory AML patients showing a CR+CRi rate of 38% in all patients and in 2/5 (40%) *TP53*-mutated AML patients.[272] Pembrolizumab was evaluated also in combination with azacitidine[273] or decitabine[274] in relapsed/refractory AML patients with a promising efficacy; however, these studies did not provide a specific report on the response of *TP53*-mutated AML patients.

Other studies have evaluated the therapeutic efficacy of durvalumab, an anti-PD-L1 mAb, in high-risk MDS and AML patients. No significant improvement in CR+CRi rates or in OS was observed in 84 first-line high-risk MDS patients[107] or in 129 older/unfit AML patients treated with durvalumab plus azacitidine compared to azacitidine alone.[108] Particularly, in the MDS trial the *TP53* mutant patients experienced poorer outcomes compared to *TP53*-WT patients (41% ORR vs 61% ORR, respectively),[107] in the AML trial, the ORR of both *TP53*-mutant and *TP53*-WT patients was similar (35% vs 34%, respectively).[108] A pooled analysis of the results of these two studies showed that the outcomes of MDS/AML patients with *TP53* mutations are worse compared to *TP53*-WT, without any significant difference between monohit and multihit *TP53* mutational status.[106]

Conclusions. Studies carried out in the last years have considerably improved our understanding of *TP53*-mutated myeloid malignancies. *TP53*-mutated MDS and

AML have been recognized as distinct stem cell disorders; furthermore, recently it was proposed to unify *TP53*-mutated MDS and AML in a unique entity. The identification of *TP53*-mutated MDS/AML as a separate and unique entity is important because it will represent a fundamental condition for the development of dedicated clinical trials.

The molecular characterization of *TP53*-mutated MDS/AML based on the study of large cohorts of cases was of key importance to define the major features of these myeloid malignancies, related either to the characterization of *TP53* alterations (either mutations or gene deletions) or to the associated chromosomal abnormalities (complex karyotype, chromosome monosomy) in the context of a condition of genomic instability and associated co-mutations. These studies have clearly shown that allelic involvement (monoallelic or biallelic), the concomitant presence of chromosome abnormalities, the presence of single or multiple *TP53* mutations and the clonal size of the *TP53* mutant clone and the number of co-mutations at the level of other driver genes are key determinants of the clinical severity of these hematologic malignancies. Therefore, these studies have shown that it is the loss of both copies of *TP53* gene that drives the dismal outcomes of *TP53*-mutated MDS/AML patients rather than the underlying mutation types. These studies underscore the importance of assessing *TP53*-mutant AML/MDS patients through an evaluation of *TP53* mutational status, *TP53* copy number, occurrent of concomitant chromosomal abnormalities and of co-mutations of other driver genes.

TP53-mutated MDS/AMLs are associated with resistance to standard treatments and poor outcomes. Standard treatments, including intensive chemotherapy, HMAs and VEN, induce only a poor survival of newly diagnosed *TP53*-mutated MDS/AML patients. Allo-HSCT is the only treatment capable of achieving a significant improvement of overall survival of these patients. However, the proportion of *TP53*-mutant MDS/AML patients suitable for allo-HSCT is low. The outcomes of *TP53*-mutated MDS/AML patients is related to some parameters TP53-related, such the allelic status of *TP53* abnormalities and the presence of chromosome abnormalities and the achievement of a MRD negativity at transplantation and TP53-not related such as the intensity of the conditioning regimens and the comorbidity index.

Recent studies have identified some peculiar immunological features of *TP53*-mutant MDS/AMLs, predicting their potential sensitivity to immunotherapy. Thus, a promising therapeutic response to immunotherapies using agents that improve macrophage anti-leukemia activity (Magrolimab or other CD47-targeting agents) or T lymphocyte anti-leukemia activity (Flotetuzumab or Sabatolimab) was reported in initial clinical studies. Furthermore, Eprenetapopt, a drug promoting the refolding of mutant p53 protein, showed therapeutic activity in *TP53*-mutant AMLs. Future phase III clinical trials are required to corroborate the clinical efficacy of these new therapeutic strategies, with the specific aim of improving the survival of patients not suitable for allo-HSCT and of increasing the number of patients suitable for allo-HSCT.

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