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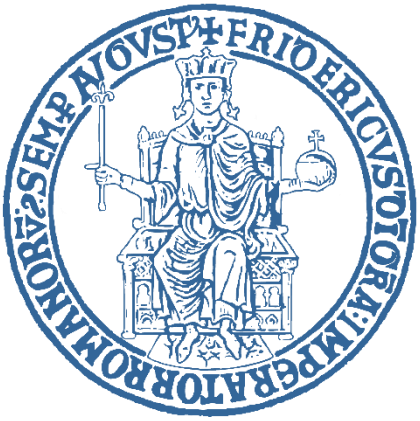

SCUOLA DI MEDICINA E CHIRURGIA

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XXXV Ciclo

Composition and clonal evolution of TP53 mutated acute myeloid leukemia

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INDEX

Main abbreviations:

- ASXL1 : additional sex combs like
- BFU: burst forming unit
- BSA: bovine serum albumin
- CALR: calreticuline
- CFU: colony forming unit
- Chr: chromosome
- HSCs: Hematopoietic stem cells
- DMSO: dimethyl sulfoxide
- DNMT3A: DNA methyltransferase 3 alpha
- FS: frameshift
- G/ GM-CSF: granulocyte/ granulocyte-macrophage colony-stimulating factor
- IDH1/IDH2: Isocitrate dehydrogenase
- JAK2:Janus kinase 2
- AML: acute myeloid leukemia
- PMF: Primary myelofibrosis
- NGS: next generation sequencing
- WHO: World Health Organization
- PBS: Phosphate-buffered saline
- PCR: polymerase chain reaction
- PV : Polycythemia vera

- RPM: revolutions per minute
- s-AML: acute myeloid leukemia that arises from a previous chronic myeloproliferative neoplasm
- MDS: Myelodysplastic Syndrome
- MPN: myeloproliferative neoplasm
- FBS: fetal bovine serum.
- t-AML: therapy-related AML, induced acute myeloid leukemia
- ET: Essential Thrombocythemia
- TET2: Tet methyl cytosine dioxygenase 2
- HU: Hydroxiurea
- PB: Pipobroman

Abstract

Acute myeloid leukemias (AMLs) result from the consecutive acquisition of different genetic lesions. AMLs mutated for *TP53* tumor suppressor gene have been largely demonstrated to be associated with both a complex karyotype and a poor prognosis.

Our study has focused on the investigation of the clonal architecture of TP53-mutated AMLs, by differentiating between *de novo* AMLs, induced AMLs (t-AMLs) and secondary AMLs (s-AMLs) consecutive to a myeloproliferative neoplasm (MPN), by both a standard sequencing and genotypic analysis of progenitor-derived colonies.

We observed several patterns of clonal architecture, which suggest that the amplification of the TP53-mutated clone as enabled by chemotherapy for t-AML, as well as by the acquisition of mutations in epigenetic regulators, such as *DNMT3A* or *TET2* for *de novo* AML.

The s-AMLs following MPN may present a profile similar to that of t-AMLs, with mostly isolated *TP53* mutations, though also to that of *de novo* AMLs. Assuming that the high frequency of *TP53* mutations in s-AMLs may be due to selection of mutated cells due to treatment, we tried to identify small TP53-mutated clones in a series of MPN by high-sensitivity error corrected sequencing (NGS-HS), distinguishing between patients either treated or not with Hydroxyurea (HU).

We observed several mutated clones in both treated (5/12) and untreated (2/5) patients. However, such data need further confirmation on larger sample sizes, as well as by the confirmation of the variants by digital PCR.

We further applied NGS HS method to characterize the molecular profile of sickle cell disease (SCD) patients, to detect the presence of *TP53* subclones, potentially susceptible to clonal expansion following treatment with HU, with consequent onset of myeloid neoplasms.

In conclusion, TP53-mutated AMLs follow several patterns of clonal architecture, where the expansion of TP53-mutated clones seems to require a second event, i.e. either treatment or mutation of an epigenetic regulator involved in age-related clonal hematopoiesis (ARCH). For s-AML patients we observed that the duration of exposure to HU is positively associated with the presence of mutated *TP53* clones ($p<0.001$). Therefore, a complete molecular characterization by NGS, could be useful to detect and to monitoring the presence of *TP53* subclones in both MPN and SCD patients.

Our hypothesis need to be confirmed by specific *in vivo* study.

1. Introduction

1.1 AML: definition, epidemiology and classifications

Acute myeloid leukemia (AML) is an aggressive malignancy with an annual, age adjusted incidence of 4.1 cases per 100,000 adults, rising to 15-20 cases per 100,000 over 60 years old.[1] From a clinical point of view, we can differentiate AML in three different types:

(i) *de novo* AML, the occurrence of which cannot be linked to a previously-known hematologic disorder;

(ii) secondary AML (s-AML), which occurs in a context of either myelodysplastic syndrome (MDS) or myeloproliferative neoplasms (MPN);

(iii) therapy-related AML (t-AML), which develops secondary to cytotoxic and/or radiation therapies.

The 2016 WHO classification[2] of myeloid neoplasms and acute leukemia had defined six subtypes of AML and related neoplasms:

(a) AML with recurrent genetic abnormalities,

(b) AML with myelodysplasia-related changes,

(c) Therapy-related myeloid neoplasms, i.e., t-AML and therapy-related MDS (t-MDS),

(d) AML not otherwise specified (AML NOS),

(e) Myeloid sarcoma, and

(f) Myeloid proliferations of Down syndrome.

Recently,[3] this classification has been revised in order to highlight the huge progresses in understanding and managing these diseases. The latter version withdraws the previously confusing use of the term AML NOS, under which types based on differentiation have been listed. Another fundamental change is the eradication of the 20% blast requirement for AML types with the definition of genetic abnormalities (except for AML with BCR:ABL1 fusion and AML with *CEBPA* mutation).

The removal of the blast cut-off represents a key turning point in correlating morphological findings with molecular genetic studies to confirm that distinct abnormalities drive the disease pathology, .i.e. *TP53* mutated or *NPM1* mutated AML/MDS. Another important new component of the new classification is the introduction of a section on AML with other defined genetic alterations, including uncommon AML subtypes. As such, the new AML classification structure continues

to emphasize integration of clinical, molecular/genetic, and pathologic parameters and their clinicopathologic implications.

However, the WHO describes the diagnostic features, as well as the prognostic factors of myeloid malignancies, though also underlying complex and variegated genetic interactions and ontogenies not properly described and explained by the current classifications.

An AML risk classification scheme based on cytogenetic abnormalities had already been proposed in 1998.[4] In the updated 2017,[5] and 2022,[6] risk stratification of the European Leukemia Net (ELN), mutational aberrations play an increasingly important role.

Risk category†	Genetic abnormality
Favorable	• t(8;21)(q22;q22.1)/*RUNX1*::*RUNX1T1*†,‡ • inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB*::*MYH11*†,‡ • Mutated *NPM1*†,§ without *FLT3*-ITD • bZIP in-frame mutated *CEBPA*‖
Intermediate	• Mutated *NPM1*†,§ with *FLT3*-ITD • Wild-type *NPM1* with *FLT3*-ITD (without adverse-risk genetic lesions) • t(9;11)(p21.3;q23.3)/*MLLT3*::*KMT2A*†,¶ • Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	• t(6;9)(p23.3;q34.1)/*DEK*::*NUP214* • t(v;11q23.3)/*KMT2A*-rearranged# • t(9;22)(q34.1;q11.2)/*BCR*::*ABL1* • t(8;16)(p11.2;p13.3)/*KAT6A*::*CREBBP* • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2, MECOM(EVI1)* • t(3q26.2;v)/*MECOM(EVI1)*-rearranged • −5 or del(5q); −7; −17/abn(17p) • Complex karyotype,** monosomal karyotype†† • Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, and/or ZRSR2*‡‡ • Mutated *TP53*[a]

Figure 1. Acute Myeloid Leukemia (AML) risk stratification. Adapted from Dohner et al. [6]

1.2 Leukemogenesis

The pathogenesis of AML represents a multistep process involving mutagenesis, epigenetic dysregulation and formation of copy number aberrations. During the process of leukemogenesis, multiple genomic or chromosomal aberrations affect hematopoietic stem/progenitor cells (HSPCs), thus resulting into preleukemic/leukemic stem cells and, ultimately, overt leukemia.

These heterogeneous aberrations are classifiable into distinct functional groups according to their known effects on signaling, chromatin modification, DNA methylation, the cohesin complex, transcription factors, NPM1, the splicing machinery and tumor suppressors.[7]

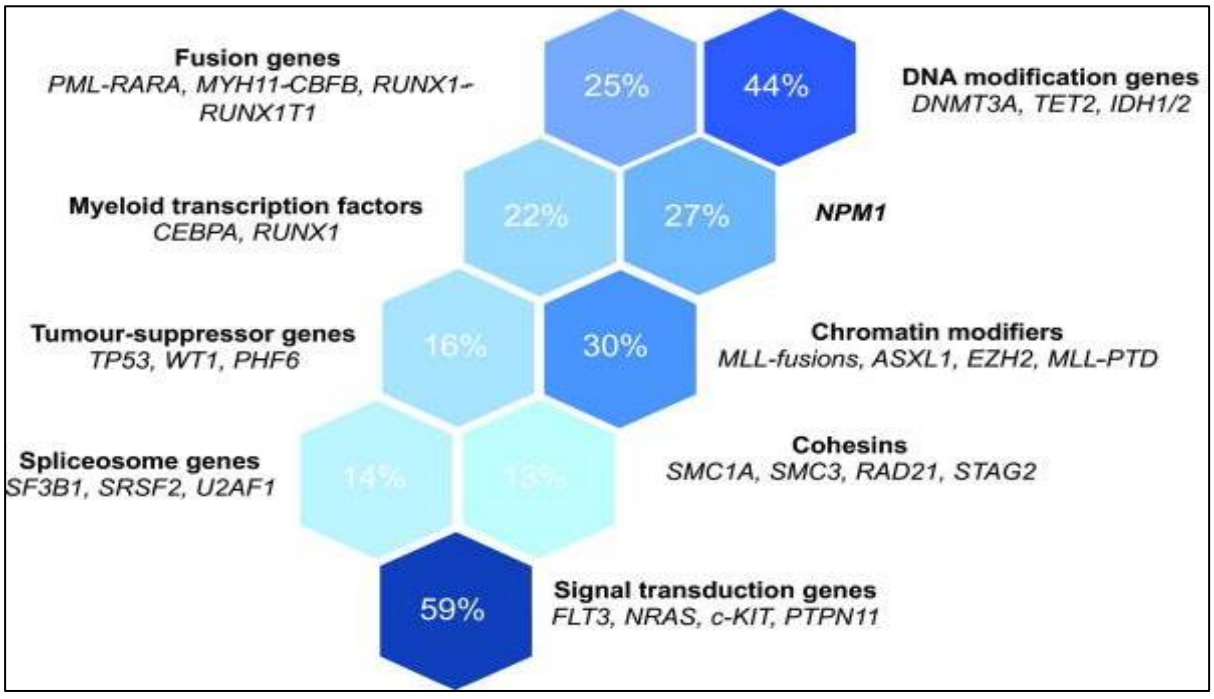


Figure 2: Recurrent mutation groups in de novo AML. Genes recurrently mutated in AML belong to distinct functional groups or pathways. Here we present the most prominent functional groups and genes associated with these. The proportion of AMLs with mutations affecting each of these groups is displayed (data obtained from The Cancer Genome Atlas Research Network, 2013).[7]

Some of these genetic lesions, including *DNMT3A*, *TET2* and *ASXL1* mutations, are considered as initiating events, and it has been hypothesized that they result in clonal expansion of mutant Hematopoietic Stem and Progenitor Cells (HSPCs), thus leading to clonal hematopoiesis of indeterminate potential (CHIP),[8–11] a condition predisposing to the subsequent acquisition of leukemic mutations. These early alterations have been shown to persist at both relapse and complete remission (CR).[12,13]

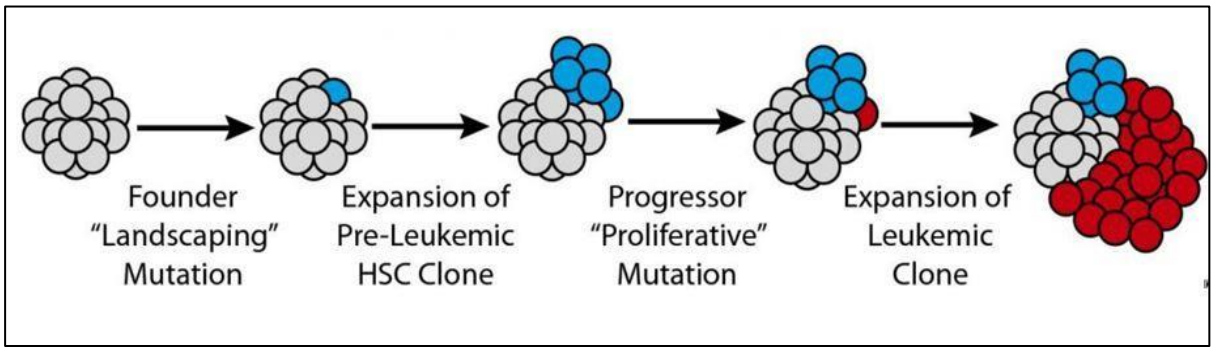


Figure 3: A model for the acquisition of mutations in AML.

The earliest founding mutations occur in "landscaping" genes involved in global regulation of gene expression through epigenetic mechanisms, whereas late progressor mutations occur in genes usually leading to an increase in activated signaling and cellular proliferation, such as *RAS* and *FLT3*. Therefore, CHIP, also defined as age related clonal hematopoiesis (ARCH), consists in the acquisition of somatic mutations which drive clonal expansion in the absence of cytopenia and

dysplastic hematopoiesis, analogously to monoclonal gammopathy of undetermined significance (MGUS) and monoclonal B-cell lymphocytosis, which are precursor states for hematologic neoplasms but are usually benign.[14]

This model of leukemogenesis fits with the results from previous studies which investigated the clonal evolution of AML with normal karyotype, showing for instance that *DNMT3A* mutations foresee *NPM1* or *FLT3* mutations.[12,15,16]

DNMT3A mutations genetically define pre-leukemic stem cells in mouse xenotransplantation models of AML, as they are sufficient to provide to mutant HSPCs a multilineage repopulation potential.[12] However, as *DNMT3A* mutations account for less than 30% of individuals with CHIP or adult AMLs, the genetic variety of these diseases raises the question whether other chromosomal or genetic lesions may represent the initiating pre-leukemic events.

In a particular subtype of AML, acute promyelocytic leukemia, the PML/RARA fusion has been reported as the initiating lesion.[17] In other types of AMLs, some lesions like *TET2* or *IDH2* mutations, as well as core-binding factor (CBF) or *MLL* translocations, have been also hypothesized as initiating events, as they occur either early in the clonal history, or they can lead to pre-leukemic hematopoiesis in xenotransplantation models.[13,15,18,19]

The top three largely known driver mutations are those in *FLT3*, *NPM1*, and *DNMT3A*, especially observed in *de novo* AML with normal karyotype. Some other genes, such as *SRSF2*, *SF3B1*, *U2AF1* or *BCOR*, have been often found as mutated in

s-AML. Mutations in *TP53*, indeed, almost always associated with a complex karyotype, are mainly found in t-AML [9].

In most cases of AML, mutations have been detected as associated with either other recurrent mutations or cytogenetic abnormalities.[20] As a result, fundamental questions have arisen on the exact role of cooperation between different types of mutations, their order of occurrence, and the mechanisms at the origin of the dysregulation of normal proliferation and differentiation of mutated clone, with the possible consequent development of an AML.

The kinetics of development strongly depends on the functional type of the first event initiating the disease, its synergistic effect on the second, the other successive events, and the time of appearance of the mutations during the hematopoietic development. The consequences of the mutation cannot be the same if this occurs in a quiescent HSC or if it appears in a highly proliferative progenitor.

A number of subclones of variable size characterizes AML genome. These subclones may have different pathobiological properties, as well as responses to anti-leukemic treatments.[20,21]

With the advent of next-generation sequencing (NGS) technologies, mutational subclones can now be detected with high sensitivity. The significance of variants detected at lower levels (than 2%) instead still remains unclear.

1.3 TP53 mutation and AML

TP53 is a tumor suppressor gene that encodes for transcription factor p53, containing transcriptional activation, DNA binding, and oligomerization domains (see Figure 4).

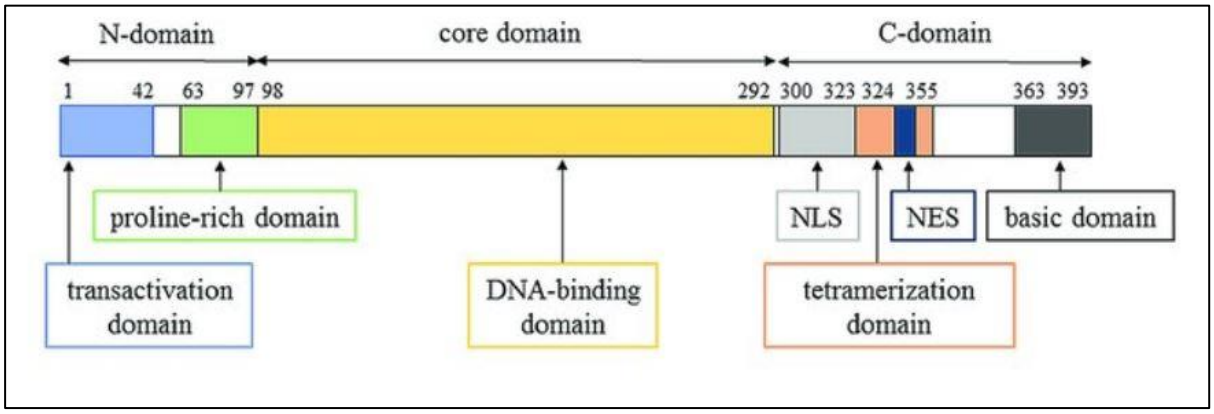


Figure 4: Domain structure of p53. Human p53 is composed of 393 amino acid residues and has a transactivation domain, proline-rich domain, DNA-binding domain, nuclear localization signal area, tetramerization domain, nuclear export signal area, and basic domain. *Oncotarget 2018- adapted from Soussi T Oncogene 2007*

The encoded protein is commonly referred to as "*the guardian of the genome*" and responds to diverse cellular stresses to regulate expression of target genes, thus inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism.

Differently from several other human cancers, a great majority of AMLs displays no genomic TP53 alterations. When observed in AMLs, these aberrations include gene mutations, most of which are located within the DNA binding domain of the gene, and/or deletions of different sizes affecting the TP53 locus on chromosome 17p13.

Somatic *TP53* mutations were identified in CHIP [8–10], and commonly found in therapy-related CHIP.[22,23] Interestingly, some patients with Li-Fraumeni syndrome (LFS), who carry germline TP53 mutations, develop MDS and AML as they age. Indeed, somatic TP53 mutations are present in 10% of MDS and AML cases,[7,24] and in 25-30% of secondary MDS and AML patients or t-AML. Of note, no difference between chemotherapy and radiation regimen concerning the development of t-AML has ever been shown.[23,25]

Recently, evaluation of co-occurring genetic mutations identified the *TP53*-altered AML cluster as a unique disease subgroup. Nevertheless, AML with *TP53* mutations does not correlate with unique transcriptional signatures by standard RNA sequencing. Mutations may co-occur within either the founder clone or subclones, or may be found in separate clones that do not correlate with neoplastic hematopoietic cells.

Two patterns of *TP53* mutation has frequently emerged. As first, mutations in *TP53*, epigenetic genes (*IDH1, IDH2, DNMT3A, TET2*) and transcription factors (*CEBPA, RUNX1, NPM1*) generally in founder clones and, conversely, mutations involving signaling pathways (i.e. *FLT3, RAS, PTPN11, BCOR, JAK2, NF1*) and polycomb and splicing mechanisms (i.e. *SF3B1, KDM6A, SRSF2*), frequently occurred in subclones. Mutations involving the *ASXL1, CBL*, and *U2AF1* genes occur almost in subclones of *TP53*, and *TP53* occurs as a new subclone variant.[26]

How mutant p53 drives the pathogenesis of hematologic malignancies remains still unclear and controversial. Despite being one of the most studied genes since its initial discovery, it has so far been considered "undruggable". This mutation predicts a poor prognosis, specifically with a dismal median survival of 5-10 months, in less than 10% of patients, regardless of the administered therapy.[23,27,28]

Up to now, it is well known that *TP53*-mutated cancers respond poorly to cytotoxic chemotherapy, whose effectiveness is highly dependent on the presence of intact p53 to enable the induction of apoptosis.[29] The largest study, including 3.324 patients, of *TP53*-mutant MDS reports that the presence of multiple *TP53* hits (regardless of the particular mutations) is associated with poor survival and outcomes for patients with monoallelic *TP53* hit did not significantly differ from patients with wild type *TP53*.[30]

Prochaska et al. have further analyzed a large cohort of intensively treated AML patients, focusing on the biological and clinical features associated with subclonal *TP53* mutations. The authors found that these abnormalities accounted for a significant proportion of *TP53*-mutated AML, and were predominantly missense mutations. This study shows that even *TP53*-mutated subclones, defined by VAF <20%, have a significantly negative prognostic impact in terms of complete remission rate, overall survival, and event-free survival. These findings may affect *TP53* screening methods and risk stratification of AML, due to the need to replace traditional Sanger sequencing with a detection limit of 20% VAF for mutant clones with highly sensitive next-generation sequencing approaches in some cases.[31]

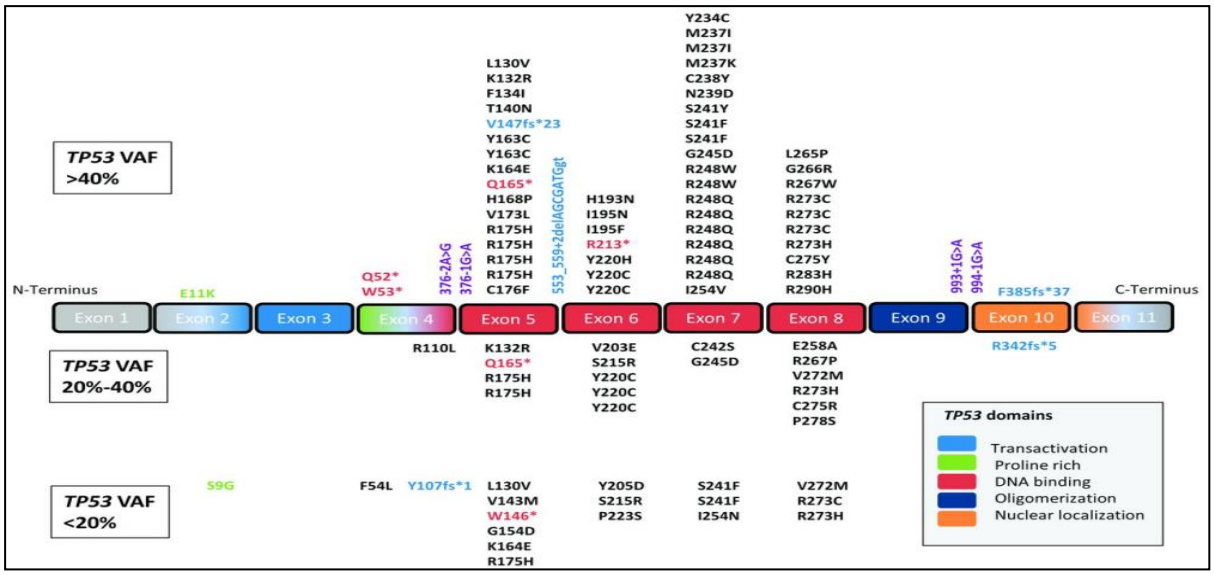


Figure 5. Distribution of 108 TP53 mutations found in diagnostic specimens of 98/1537 patients with acute myeloid leukemia. [31] Top panel: TP53 mutations with a variant allele frequency (VAF) of >40%; middle panel: mutations with a VAF of 20%-40%; lower panel: mutations with a VAF <20%.

Unconventional strategies and *TP53*-targeted therapeutics have currently being tested as either monotherapy or in combination with conventional drugs in order to improve the response rate, especially in relapsed or refractory patients, and to increase the number of patients potentially eligible for hematopoietic stem cells transplantation (H-SCT), which remains the only cure option in *TP53* mutated AML, though not always effective.

Either combined with standard AML chemotherapy or emerging targeted therapies, pharmacological targeting of the *TP53* pathway may provide therapeutic benefit.

An in-depth study of the processes of leukemogenesis is a necessary starting point. Several studies have identified the genetic hierarchies specific for *de novo* AMLs, AMLs with myelodysplastic features, or secondary AMLs, and also genetic hierarchies typical of *TP53*-mutated neoplasms.[20,21,23,25,32]

The team of Wong in 2015, by genome sequencing of 22 t-AMLs, 49 *de novo* AMLs and 8 s-AMLs, have discovered an equivalent number of genetic abnormalities in t-AML and *de novo* AML. Such finding suggests that prior chemotherapy does not increase the amount of mutations detectable by this technology. In four cases of t-AML, *TP53* mutation at diagnosis was detected at a low frequency (<1%) in previous specimens from 3 to 6 years before, including two cases before any chemotherapy. Moreover, somatic mutations in *TP53* were found at a low frequency in an elderly population never treated with chemotherapy.

Wong has proposed a model in which rare HSPCs carrying age-related TP53 mutations are resistant to chemotherapy and preferentially expand after treatment. According to this model, HSPCs harboring *TP53* mutations will expand under the selective pressure of chemotherapy.[23]

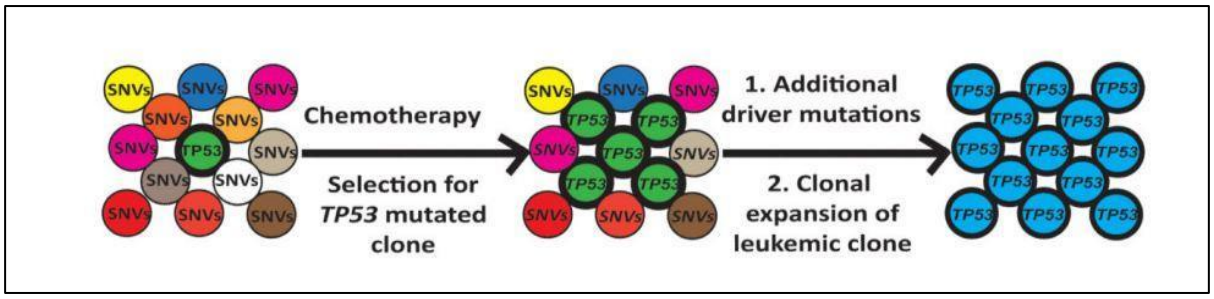


Figure 6: Model of how cytotoxic therapy shapes clonal evolution in t-AML/tMDS. Adapted by Wong et al.[23]

Due to competitive transplantation experiments on a murine model with wild-type and TP53+/- HSCs, this study demonstrated that TP53+/ HSCs did not provide any advantage in the absence of chemotherapy, though gaining a selective advantage after exposure to N -ethyl- N - nitroso urea (ENU).[23]

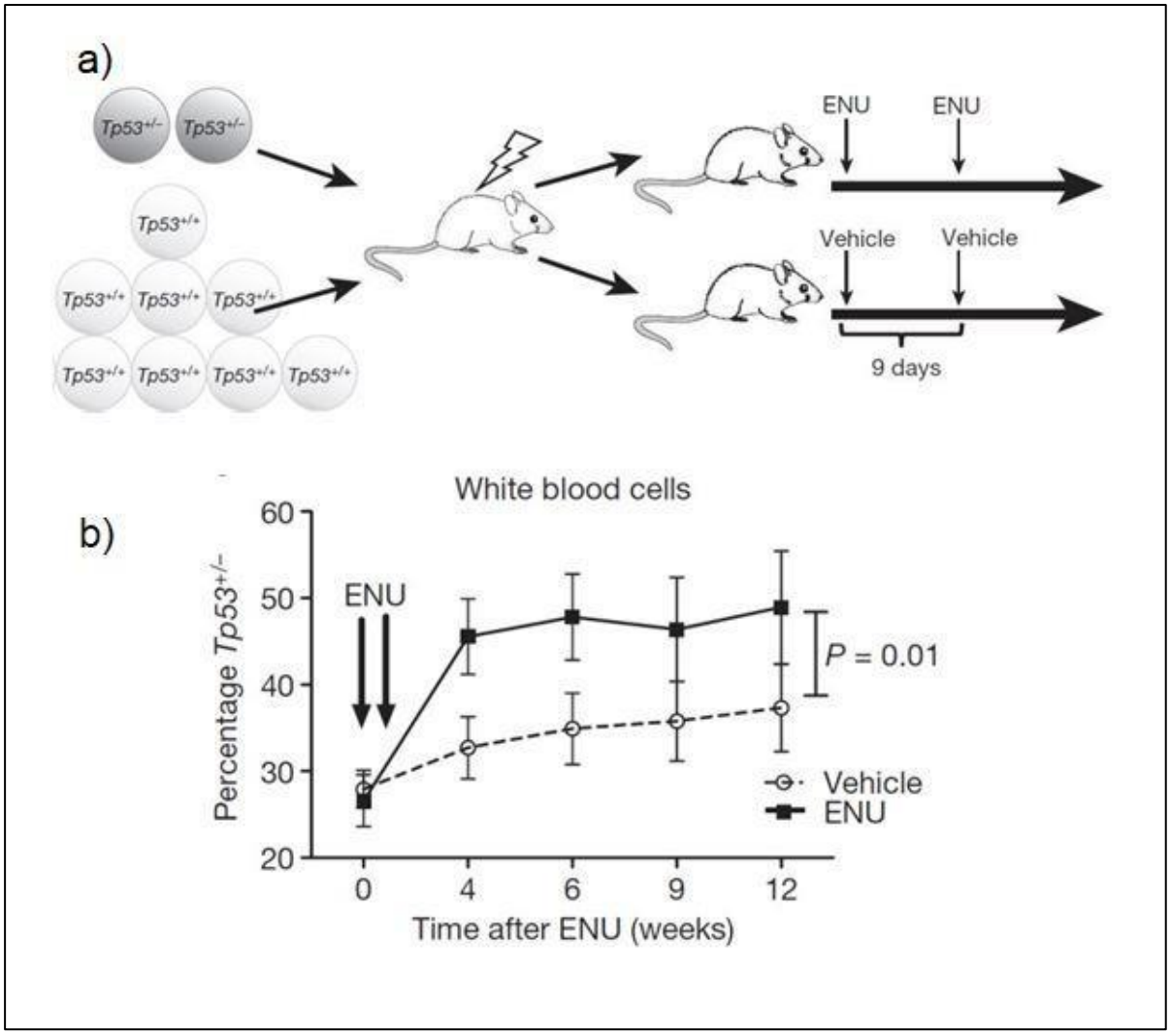


Figure 7: Adapted from Wong et al. (a) Experimental design: competitive xenografting of HSC TP53+/+ and TP53+/- followed by exposure to ENU. (b) Percentage of TP53+/+ HSCs as a function of time, according to exposure or not to ENU.[23]

Papaemmanuil et al. established a new AML sub-entity called *TP53*-aneuploidy, which encompasses *TP53* mutations, complex karyotype, and chromosomal copy-number alterations, such as −5/5q, −7/7q, −17/17p, −12/12p, +8/8q for the most frequent and the most repeatedly associated with *TP53* mutations. Of note, this group presents less *RAS*-pathway mutations than all other sub-groups. Mutation in *NPM1*, transcription factors, such as *CEBPA*, *GATA2*, and *RUNX1* were either never or seldom associated with *TP53* mutations. Among the frequent CHIP mutations, *DNMT3A* mutations were the most frequently-associated, even though this remained a rare association.[20]

By studying the genotype of AML, our group found *DNMT3A* or *TET2* mutations in half of the *de novo* TP53mut AMLs, and never in the induced TP53mut AMLs (therapy related-AML or t-AML)[21]. This result suggests that the expansion of the malignant clone follows a different path in these two types of AML. Clonal expansion in the case of t-AML is induced by chemotherapy, whilst by HCLA mutations in the case of *de novo* AML.

By comparing the clonal architecture of *de novo* AML with and without *TP53* mutation, it seems that for AML without *TP53* mutation, there is an initial clonal expansion phase (after the first mutation), whose signature is the presence of colonies with only one mutation (the initial mutation of *DNMT3A* or *TET2*). On the other hand, for AML with *TP53* and HCLA mutation, there is no evidence of this clonal expansion phase with a single mutation.

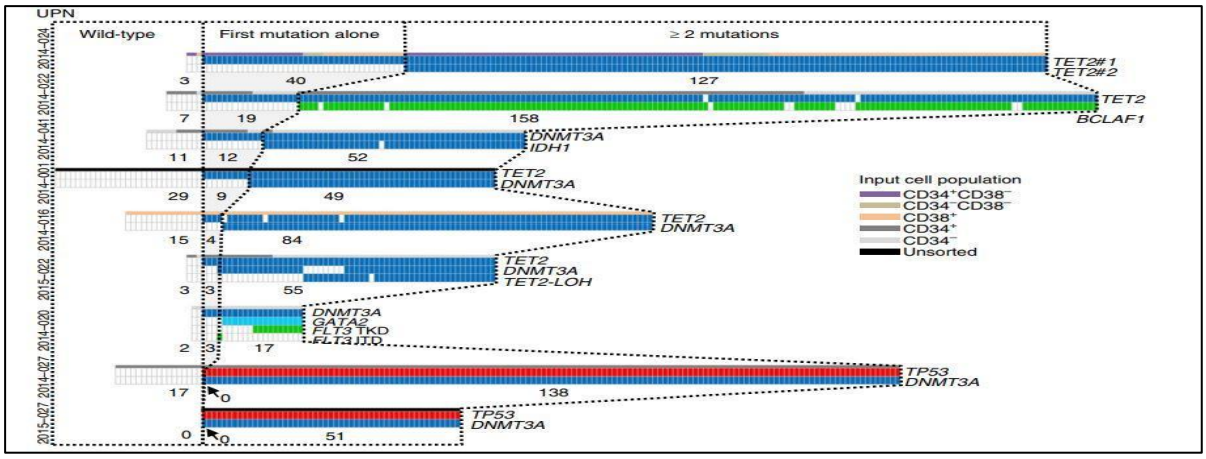


Figure 8: Capture of early clonal expansion steps by single-cell-derived colony analysis[21]. Mutational patterns of individual colonies from patients with early initiating lesions. Cell sorting strategies before culture are indicated above each table. Dotted lines separate wild-type colonies, colonies with one mutation, and colonies with ≥ 2 mutations/lesions. The number of colonies of each category is indicated below the tables.

This suggests that TP53 mutation alone does not provide any proliferative advantage and that clonal expansion occurs only when *TP53* and HCLA mutation are both present in the same HSC. A study published in Blood in 2017, studied the clonal architecture of 8 *TP53*mut AMLs of all types (*de novo*, s-AML and tAML) and was able to demonstrate that *TP53* mutation appears first during leukemogenesis. This team studied a case of s-AML presenting a *TP53* and a *DNMT3A* mutation and found only double mutated clones, consistent with the data previously exposed.[33]

1.4 AML secondary to MPN

MPN are clonal disorders of HSCs including *polycythemia vera* (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). The major mutations in MPN are mutations in *JAK2* (primarily *JAK2* V617F), *CALR*, and *MPL*. A subset of patients affected by MPN transform to secondary AML, generally defined as blast phase (MPN-BP). The 10-year risk of transformation is 1% for ET, 4% for PV, and 20% for PMF. A *JAK2* MPN can be transformed into a mutated *JAK2* AML or into an unmutated *JAK2* AML.[34,35] In addition to driver known mutations, which affect MPN clinical phenotype, several further mutations have been described throughout the course of the disease. Grinfeld et al. proposed a molecular classification which contemporarily takes into account the diversity and interaction of molecular abnormalities, to provide a personalized prediction of the probability of transformation.[36]

The molecular pathway leading to MPN-BP can proceed through at least two distinct routes. First, *JAK2*/*CALR*/*MPL*-positive MPN progresses to *JAK2*/*CALR*/*MPL*-positive AML with the acquisition of additional associated genetic alterations. On the other side, a MPN can experiment loss of pre-existing driver mutation at AML onset, more likely due to the transformation of an antecedent or *de novo* mutant clone leading to MPN-BP, which overcomes the MPN *JAK2*/*CALR*/*MPL*-mutated clone.[37,38]

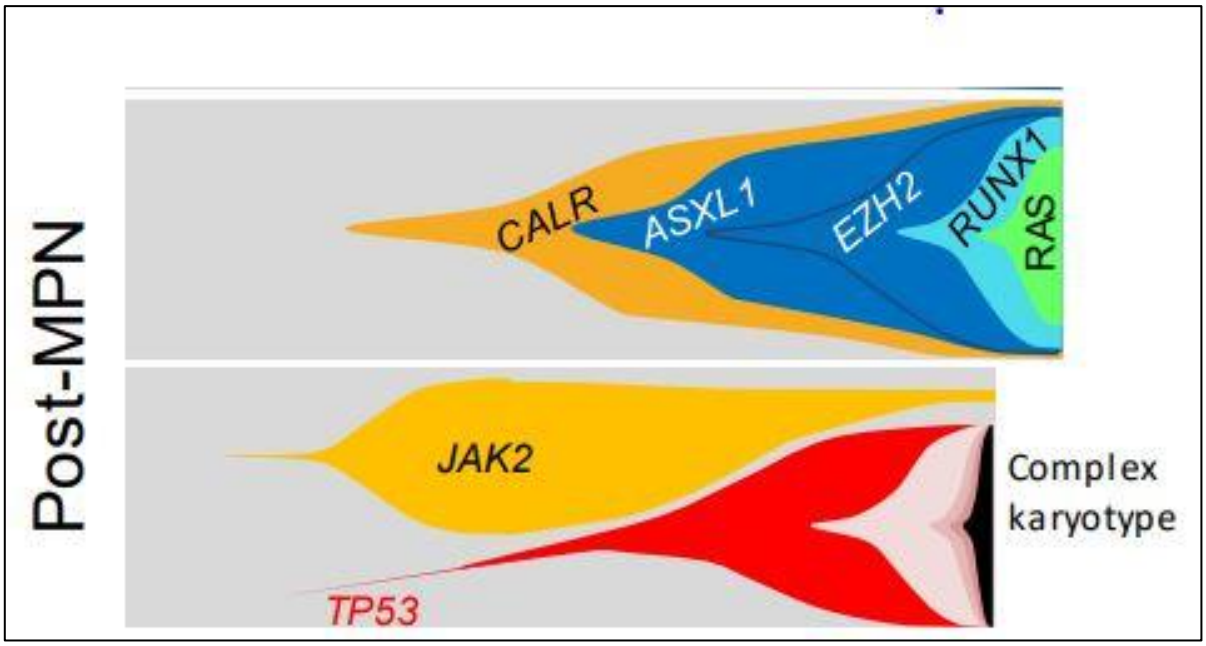


Figure 9: Fish diagrams showing representative (but not complete and exhaustive) clonal AML hierarchies in AML secondary to MPN.

Mutations affecting *TP53* are often consistent with MPN-BP, synergistic with *JAK2* mutations in leukemogenesis, and associated with slower long-lasting transformation.[39]

Mutations involving *TET2* typically predate MPN drivers,[40] but they can occur in subsequent disease phases as well, including at transformation.[41] Mutations occurring in *RUNX1*, *IDH1*/2, and *U2AF1* have been described in cases with a faster leukemic progression[39] . In contrast, *ASXL1* mutations have been reported at all phases of disease, suggesting a specific contribution in clonal evolution.[41]

Abnormalities involving long arm of chromosome 1 have been associated with clonal progression and evolution to MPN-BP, likely due to overexpression of MDM4, a negative regulator of TP53, further underlying the role of this latter in such context.

Mutations in RNA splicing factors (*SF3B1*, *U2AF1*, *SRSF2*, and *ZRSR2*) are relatively frequent in MPN-BP, with a similar incidence to that observed in the chronic phase of MPN. Their occurrence is generally associated with the later phases of MPN course.

TP53 mutations acquisition is considered a key event in the process of transformation of chronic MPN to leukemia, but mutations in this protein alone do not confer a growth advantage to cells, and expansion of mutant clones requires a selection process. MPN patients have been treated for years with various antiproliferative drugs, and some treatments have been clearly shown to favor the transformation process, such as alkylating agents.[42] However, the role of long-term treatment in MPN patients in the clonal selection of *TP53* mutations is still unclear. Particularly, hydroxyurea (HU) has been suspected, though no definite correlation has been established.[43]

Maslah al. recently observed, at a single-cell level, that the presence of additional mutations does not influence the selection of *TP53* mutant cells by MDM2 inhibitor treatment, and described an *in vitro* test allowing to predict the emergence of *TP53* mutated clones favorized by a drug treatment.[44]

1.5 AML in patients with SCD

Sickle cell disease (SCD) is a debilitating disease associated with widespread morbidity and early mortality. HU has been the first drug approved for SCD by the US Food and Drug Administration over 20 years ago. Although studies have not shown an increased incidence of solid tumors in adults with SCD, two large population studies have found an increased incidence of leukemia.[45,46] Case reports describe patients who developed MDS/AML after short- and long-term HU use, but larger studies did not found a significantly increased risk of hematologic malignancies in patients exposed to HU.[47] Ghannam et al reported for the first time in patients with SCD after unsuccessful allo-HCT the progression of baseline high-risk *TP53* clonal abnormalities into myeloid malignancy.[48] Moreover, the association of HU with genotoxicity is inconsistent.[49] In conclusion, the association between HU exposure and leukemia in SCD is occasional and unproven.[50,51]

However, the predictive value and clinical utility of screening for myeloid malignancy-associated mutations, such as *TP53*, in SCD patients is currently unknown.

2. Hypotheses and objectives

The aim of our study is first to determine the succession of genetic events in *TP53* mutated AML, and to define their clonal architecture. We will try to distinguish the different clonal amplification profiles of TP53-mutated AMLs according to whether they are *de novo* or t-AML, in order to determine which event provides a proliferation advantage to the *TP53*mut clone. We hypothesize that HSCs carrying an isolated *TP53* mutation do not have a sufficient selective advantage over their normal counterpart to initiate AML. A *TP53* mutated clone requires a second event to amplify. We assume that this second event is chemotherapy (including HU) for t-AML, and associated mutations for *de novo*-AML, especially those involved in ARCH, such as *TET2* or *DNMT3A*, to gain an advantage over non-mutated HSCs.

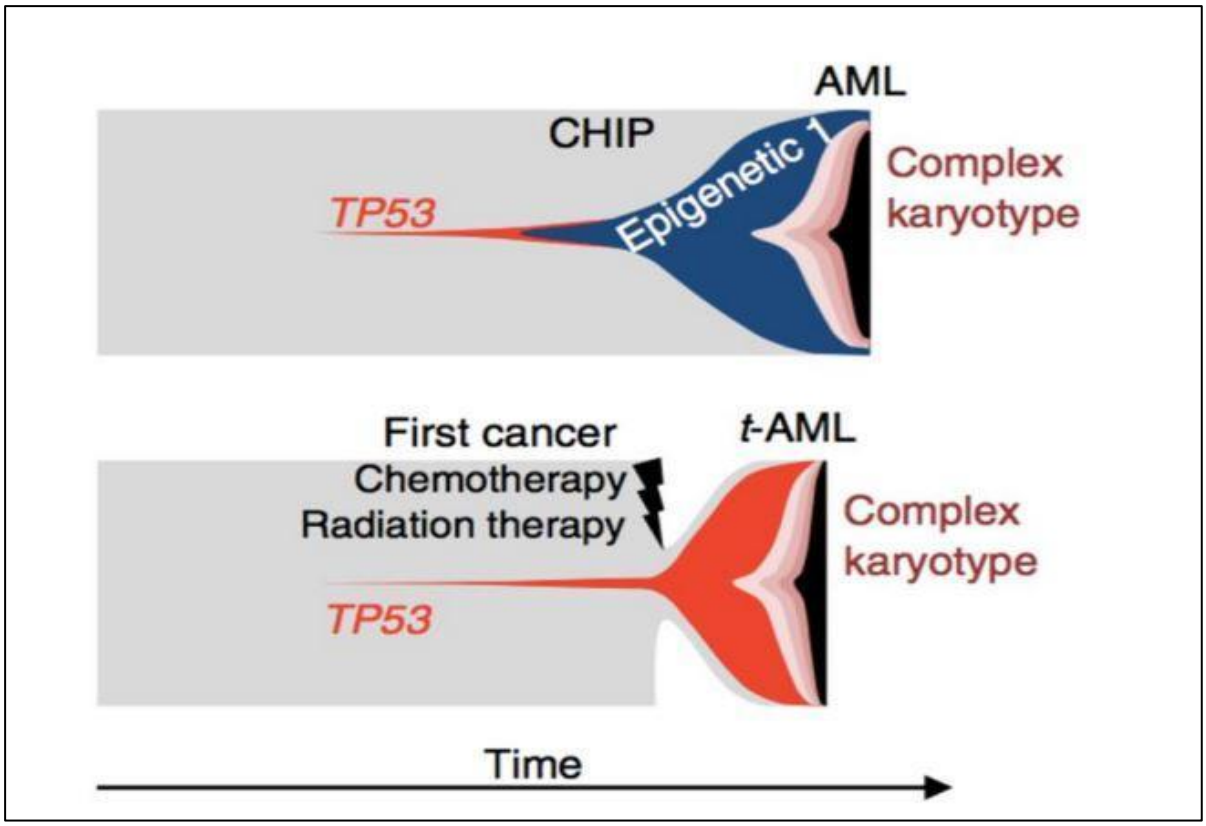


Figure 10: Model of clonal evolution*: de novo* AML TP53-mutated vs t-AML TP53-mutated.

As well, we also aim to study also the cooperation between *TP53* mutations and the main ARCH mutations. A preliminary work allowed us to develop only the tools and to define adjustments for the subsequent experiments necessary to study our working hypothesis.

We will further focus on treated and untreated myeloproliferative neoplasms (MPN). We assume that cyto-reductive therapy (such as HU) selects for pre-malignant pre-existing TP53mut subclones, and gives them a selective proliferation advantage over non-mutated HSCs.

We will characterize the cases of transformation to AML and, secondly, plan to detect, in patients followed for non-transformed MPN, larger and more numerous TP53mut clones in case of cytoreductive therapy rather than in the absence of therapy.

We also aim to study the molecular characterization and clonal evolution of AMLs TP53mutated in patients with a history of SCD treated with HU for a period longer than 7 years, and to screen for myeloid malignancy-associated mutations, such as *TP53*, in newly diagnosed SCD patients. This latter is the objective of a currently ongoing study.

3 Materials and methods

3.1 Patients.

This is a single-center ambispective observational study. Initially, 121 patients diagnosed with AML at Saint Antoine hospital in Paris, between 2013 and 2022. were included in the study, stratified into 3 groups: (a) 20 AML-induced (t-AML) (Table 2a), (b) 43 *de novo* AML selected on the basis of a karyotype potentially associated with TP53 mutations (complex karyotype, del(17p) or del(5q)) (Table 2b), and (c) 58 AML secondary to MPN (Table 3a).

In a second phase, 17 patients who had been followed up for more than 7 years for a non-transformed MPN were included (Table 3b), distinguishing between patients who had received cytoreductive therapy (hydroxyurea or pipobroman), and patients who had never received cytoreductive therapy. In a third phase 5 patients, followed for SCD and, who developed a subsequent AML and 31 SCD patients were further included.

All patients provided written informed consent for the use of their data for clinical purposes. The study was approved by the local Ethics Committee of Saint-Antoine Hospital and is in accordance with the 1976 Declaration of Helsinki and its later amendments.

Table 2a. t-AMLs

Code	Age	Diagnosis (antecedent)	Karyotype
tAML-01	56	AML (breast cancer)	Complex
tAML-02	70	AML/MDS (familial breast cancer)	Complex
tAML-03	20	AML (Hodgkin lymphoma)	46,XX,t(6;9)(p23;q34)[23]/46,XX[3]
tAML-04	66	AML (ovarian cancer)	Complex
tAML-05	75	AML (Burkitt lymphoma)	Complex
tAML-06	58	AML (Burkitt lymphoma)	Complex
tAML-07	54	AML/MDS (breast cancer)	46,XX,t(3;5)(q25;q34)ort(3;5)(q26;q34)[24]/ 46,XX[2]
tAML-08	84	AML (breast cancer)	46,XX,ins(1;8)(q32-41;q21q23)[12]/47, idem, +mar[8]/46,XX[3]
tAML-09	62	AML (multiple myeloma)	Complex
tAML-10	77	AML (bladder cancer)	Complex
tAML-11	63	AML (Hodgkin lymphoma)	Complex
tAML-12	81	AML (LLC)	Complex
tAML-13	63	AML (prostate cancer)	Complex
tAML-14	56	AML (breast cancer)	Complex
tAML-15	77	AML (breast cancer)	Complex
tAML-16	33	AML (breast cancer)	Complex
tAML-17	73	AML (mantle cell lymphoma)	Complex
tAML-18	96	AML (urothelial carcinoma)	Complex
tAML-19	78	AML (breast cancer)	Complex
tAML-20	59	AML (tongue cancer)	Complex

Table 2b. *De novo* AMLs

dnAML-01	78	AML	Complex
dnAML-02	74	AML	Complex
dnAML-03	72	AML/MDS	Complex
dnAML-04	67	AML/MDS	Complex
dnAML-05	85	AML/MDS	Complex
dnAML-06	69	AML/MDS	Complex
dnAML-07	78	AML/MDS	46,XX,t(2;3)(q?21;q26),del(5)(q23q34)[24]
dnAML-08	75	AML/MDS	Complex
dnAML-09	72	AML	Complex
dnAML-10	69	AML	Complex
dnAML-11	60	AML/MDS	46,XX,del(5)(q12-13q34)[24]/46,XX[1]
dnAML-12	74	AML	Complex
dnAML-13	81	AML	46,XY,add(5)(q11) or del(5)(q11q34)[19]/46,XY[5]
dnAML-14	70	AML/MDS	Complex
dnAML-15	74	AML	Complex
dnAML-16	80	AML	Complex
dnAML-17	74	AML/MDS	Complex
dnAML-18	68	AML	Complex
dnAML-19	54	AML	Complex

dnAML-20	51	AML	Complex
dnAML-21	78	AML	Complex
dnAML-22	24	AML	Complex
dnAML-23	85	AML	Complex
dnAML-24	70	AML	Complex
dnAML-25	58	AML	Complex
dnAML-26	82	AML	Complex
dnAML-27	70	AML	Complex
dnAML-28	52	AML	Complex
dnAML-29	20	AML	Complex
dnAML-30	32	AML	Complex
dnAML-31	62	AML	Complex
dnAML-32	47	AML	Complex
dnAML-33	64	AML	Complex
dnAML-34	67	AML	Complex
dnAML-35	57	AML	Complex
dnAML-36	72	AML	Complex
dnAML-37	91	AML	Complex
dnAML-38	65	AML	Complex
dnAML-39	66	AML	Complex
dnAML-40	60	AML	Complex
dnAML-41	52	AML	Complex
dnAML-42	56	AML	Complex
dnAML-43	63	AML	Complex

Table 3a. AMLs secondary to MPN

Code	Age	Diagnosis (antecedent)	Time to progression/ MPN follow-up	Cytoreductive treatment	Duration HU exposure (years)	Karyotype
sAML-01	83	AML (ET)	11	HU	11	46,XX
sAML-02	62	AML (PV)	17	HU+PB	17	complex
sAML-03	71	AML (PV)	10	HU	10	46,XX,del(20)(q11q13)[19]
sAML-04	63	AML (PV)	12	HU+PB	12	complex
sAML-05	62	AML (PV)	16	PB	13	complex
sAML-06	45	AML (MF)	1	HU	1	46,XX
sAML-07	62	AML (MF)	3	none	0	complex
sAML-08	55	AML (ET)	10	HU+PB	10	46,XY,der(7)t(1;7)(q10;p10)[16]/46,XY[10]
sAML-09	71	AML (PV)	6	HU	4	complex
sAML-10	61	AML (TE)	NA	HU	0,1	complex
sAML-11	66	AML (PV)	15	HU+PB	7	46,XX,del(5)(q33-34)[16]/46,XX[7]
sAML-12	75	AML (MF)	1	HU	1	complex
sAML-13	69	AML (PV)	4	HU+PB	3	complex
sAML-14	61	AML (MF)	12	HU	6	complex
sAML-15	68	AML (MF)	2	HU	2	XX, del(17)(p13)[17]/46,?idem[4]/46,XX[2]
sAML-16	56	AML (PV)		NA	-	NA
sAML-17	81	AML (PV)	9	HU	9	complex
sAML-18	63	AML (PV)		NA	-	complex
sAML-19	80	AML (ET)		HU	7	NA
sAML-20	75	AML (PV)	18	HU+PB	18	complex
sAML-21	64	AML (PV)	13	HU	5	complex
sAML-22	81	AML (PV)	6	HU	5	NA
sAML-23	90	AML (ET)	8	HU	8	NA
sAML-24	66	AML (PV>MF)	12	HU	11	complex
sAML-25	71	AML (ET)	13	HU	13	NA
sAML-26	44	AML (ET)	6	HU	-	complex
sAML-27	72	AML (ET >MF)	7	HU	3	complex
sAML-28	60	AML (PV)	12	HU	11	20des, an. cr.3q
sAML-29	69	AML (MPN)	1	HU	1	46,XY,t(5;12)(q

						33;p13)[21]/46, sl,del(2)(q23q37)[2]
sAML-30	64	AML (TE)	20	HU	0,08	45~46,X,-X,del(7)(q13q32),+mar[cp13]/46,XX[2]
sAML-31	69	AML (TE>MF)	26	HU	26	complex
sAML-32	60	PV	4	HU	2	46,XY[28]
sAML-33	91	AML (TE)	7	HU	7	del 20q,7q,17q
sAML-34	77	AML (PV)	5	HU	3	NA
sAML-35	81	AML (TE)	13	HU	13	complex
sAML-36	81	AML (TE)	1	HU	0,5	complex
sAML-37	85	AML (TE)	2	HU	2	46,XY[27]
sAML-38	79	AML (PV)	17	PB	10	complex
sAML-39	71	AML (TE)	11	HU	11	46,XY,der(19)hsr(19)(q1?3)t(6;19)(q22;q1?3)[6]/46,hsr(19)(q1?3)[6]/46,XY[9]
sAML-40	80	AML (ET>MF)	21	HU	16	46,XY[18]
sAML-41	74	AML (ET)	2	HU	2	complex
sAML-42	71	AML (PV>MF)	2	HU	0,6	46,XX[20]
sAML-43	56	AML (ET>MF)	7	HU	2	46,XX, +8, +21[22]/46,XX[3]
sAML-44	65	AML (ET)	11	HU	4	46,XY
sAML-45	83	AML (PV)	7	HU	7	complex
sAML-46	74	AML (ET>MF)	NA	NA	NA	NA
sAML-47	61	AML (PV)	12	HU	11	complex
sAML-48	69	AML (ET)	15	HU	15	complex
sAML-49	87	AML (ET)	NA	NA	NA	NA
sAML-50	59	AML (MF)	10	HU	5	46,Y,t(X;12)(q2?1;q1?3),add(16)(q12)[2]/46,sl,der(6)add(6)(p2?3)del(6)(q11)[13]/47,sdl1,+21[3]/48,sdl2,+mar[3]/45,sdl2,-der(6)add(6)(p2?3)del(6)(q11)[2]/

						46,XY,ins(14;8)(q2?1;q1?2q2?3)[2]/ 46,XY[3]
sAML-51	68	AML (MF)	1	HU	0,8	NA
sAML-52	75	AML (ET)	20	HU	12	complex
sAML-53	83	AML (ET)	20	HU	8	complex
sAML-54	66	AML (PV)	16	HU	3	complex
sAML-55	62	AML (ET>MF)	24	HU	23	complex
sAML-56	80	AML (LMMC)	5	none	-	complex
sAML-57	70	AML (ET)	9	HU	1	46,XX,?add or del(7)(q?),del(9)(q12q33)[3]/ 47,idem,+11[3]/46,XX[4]
sAML-58	25	AML (CMML)	4	none	-	Complex

Table 3b. MPN

MPN-01	79	ET	16	HU	16
MPN-02	70	PV	7	HU	7
MPN-03	67	ET	16	HU	16
MPN-04	52	PV	9	HU	9
MPN-05	41	PV	20	HU+PB	2
MPN-06	65	ET	9	HU	9
MPN-07	53	PV	10	none	-
MPN-08	73	ET	18	none	-
MPN-09	37	ET	10	none	-
MPN-10	71	ET	33	none	-
MPN-11	63	PV	24	none	-
MPN-12	73	PV	15	HU	15
MPN-13	81	PV	8	HU	8
MPN-14	61	ET	10	HU	10
MPN-15	81	ET	4	HU	4
MPN-16	51	PV	NA	HU	NA
MPN-17	72	ET	19	PB	15

Abbreviations: NA= not available.

3.2 Samples.

Bone marrow or blood samples at different time points (AML diagnosis, MPN diagnosis, last molecular follow-up points of MPN and SCD) were collected as DNA, dry pellets/ or cryo-preserved fetal serum (FSS)/DMSO-10% mononuclear cell ampoules. DNA was extracted on Qiagen columns, according to the manufacturer recommendations.

3.3 Cytogenetic.

Conventional cytogenetic analysis was performed in diagnosis samples on R-banding metaphases obtained from 24 h unstimulated culture using standard procedures. Karyotypes were interpreted according to the recommendations of the International System for Human Cytogenetic Nomenclature (complex karyotype was defined by at least three structural or numeral aberrations). Chromosomal rearrangements were confirmed by FISH.

3.4 Targeted next generation sequencing.

Standard panel. libraries were obtained from 112.5 ng of DNA, using HaloPlex Target Enrichment System® (Agilent technologies, Santa Clara, CA, USA), and sequenced on a MiSeq® sequencer (Illumina, San Diego, CA, USA). Two panels were used: a "122-gene" kit and a "diagnostic" kit of 40 genes (Tables 3 and 4). Alignments, variant calling, and allele frequency quantification were realized using SureCall software. All variants were checked using IGV software V2 and a second analysis using Sophia DDM® software version 5.0.12 (Sophia genetics, Lausanne, Switzerland) with a sensitivity of 1% was also performed.

Table 4: Gene list of "122 genes" for standard NGS

ABCB11	*BOC*	*CTCF*	*DNMT3A*	*FZD1*	*IKZF1*	*MAP2K2*	*NOTCH2*	*RB1*	*SMC3*	*TET2*
ACSS3	*BRAF*	*CUL3*	*DOK2*	*GATA1*	*ITGAX*	*MLL*	*NPM1*	*RBMX*	*SPI1*	*TET3*
AKAP13	*BRPF1*	*CUX1*	*DSCAM*	*GATA2*	*JAK1*	*MLL2*	*NRAS*	*RET*	*SRSF2*	*TLE4*
APH1A	*CBL*	*CUX2*	*EED*	*GATAD2B*	*JAK2*	*MPL*	*NSD1*	*RUNX1*	*SSRP1*	*TP53*
ARHGEF2	*CBLB*	*CXXC4*	*EGR1*	*GBP4*	*JAK3*	*MSR1*	*NUMA1*	*SETBP1*	*STAG1*	*TP73*
ASXL1	*CCND3*	*DAAM2*	*ERCC2*	*GDF5*	*JARID2*	*MYBL2*	*PBRM1*	*SF3A1*	*STAG2*	*TYK2*
ASXL2	*CDKN2A*	*DAXX*	*ETV6*	*GLI1*	*KDM6A*	*NCOA7*	*PDS5B*	*SF3B1*	*STAT3*	*U2AF1*
ATM	*CDKN2B*	*DDX1*	*EZH2*	*HJURP*	*KIT*	*NCSTN*	*PHF6*	*SH2B2*	*SUZ12*	*WAC*
BCLAF1	*CEBPA*	*DDX41*	*FBXW7*	*HRAS*	*KRAS*	*NF1*	*PTEN*	*SH2B3*	*TEK*	*WT1*
BCOR	*CHEK2*	*DIS3*	*FLT3*	*IDH1*	*MAML1*	*NMNAT2*	*PTPN11*	*SHKBP1*	*TERC*	*XRCC1*
BCORL1	*CSF3R*	*DNM2*	*FOXP1*	*IDH2*	*MAP2K1*	*NOTCH1*	*RAD21*	*SMC1A*	*TERT*	*XRCC3*
ZRSR2										

Table 5: Gene list of the "diagnostic" kit for standard NGS

ASXL1	*DNMT3A*	*PHF6*	*SRSF2*	*CALR*
ASXL2	*EZH2*	*PTPN11*	*SMC3*	*HRAS*
ATM	*FLT3*	*RAD21*	*STAG2*	*IDH1*
BCOR	*GATA2*	*RUNX1*	*TET2*	*IDH2*
CBL	*JAK2*	*SETBP1*	*TP53*	*KMT2A*
CEBPA	*KIT*	*SF3B1*	*U2AF1*	*KRAS*
CSF3R	*MPL*	*SH2B3*	*WT1*	*NPM1*
DDX41	*NF1*	*SMC1A*	*ZRSR2*	*NRAS*

High Sensitivity Panel. A High Sensitivity (HS) kit was designed to target *TP53*, *CALR*, *MPL* and *JAK2* (Table 4). This method, derived from the Haloplex method, hybridizes the DNA fragments obtained after enzyme digestion to 2 indexes. The first one is sample specific at the standard technique. The second one is a random sequence of 10 nucleotides (unique molecular barcode) which specifically binds to each DNA fragment. This index allows to identify either groups or families of amplicons after amplification according to the original fragment, and thus to limit the errors associated with amplification and sequencing, providing a better sensitivity than the standard technique (Fig. 11). Amplicon libraries were prepared from 57.6ng of DNA per sample using the HaloPlex HS Target Enrichment System® protocol (Agilent technologies) and NGS sequencing was performed on a MiSeq® instrument (Illumina). The results were aligned to a reference genome using the SureCall software, then we looked up for abnormalities and analyzed them manually one by one on the IGV software, setting a sensitivity threshold at 0.002 (VAF

determined by the ratio of the number of variant amplicon families to the total number of amplicon families). In a second step, variants at the most frequently mutated positions of TP53 (Fig. 12) were searched with a theoretical sensitivity of 0.001.

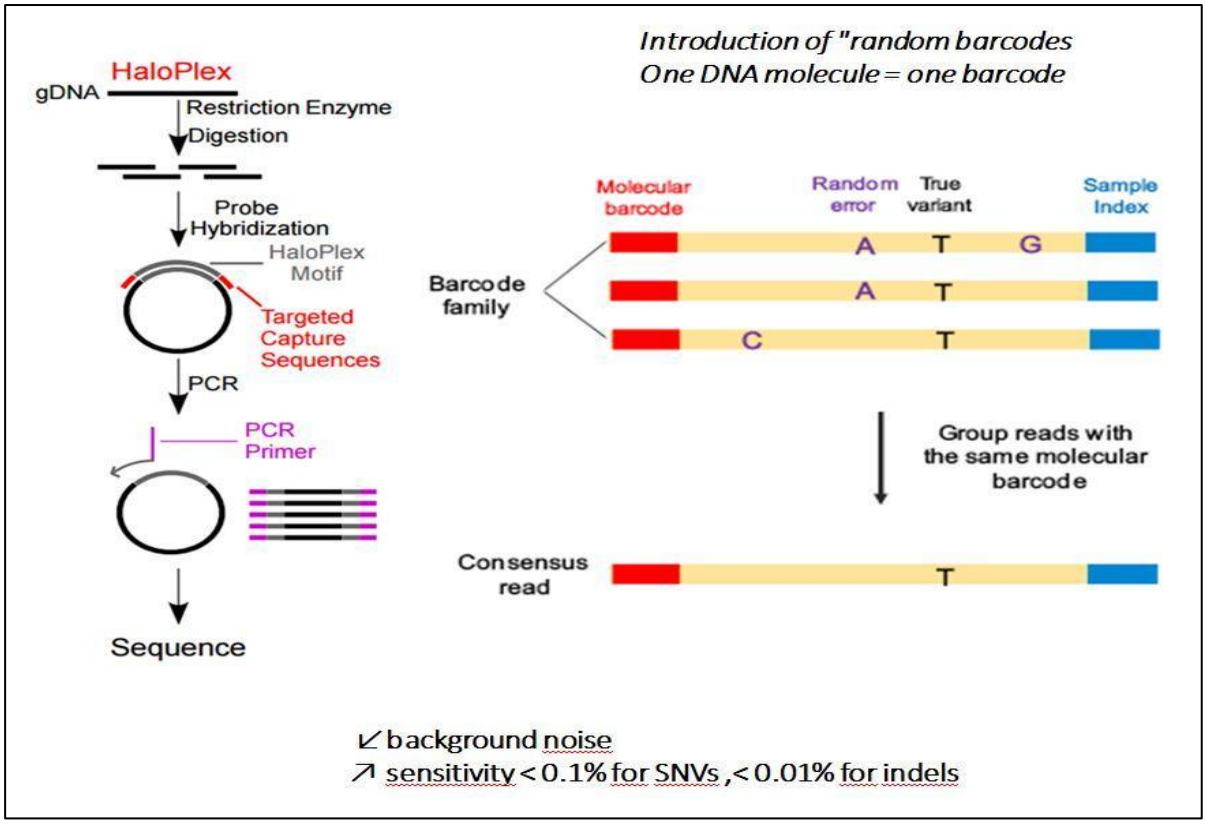


Figure 11 Characteristics of High Sensitivity NGS. Insertion of a random index before amplification allowing the identification of amplicon families

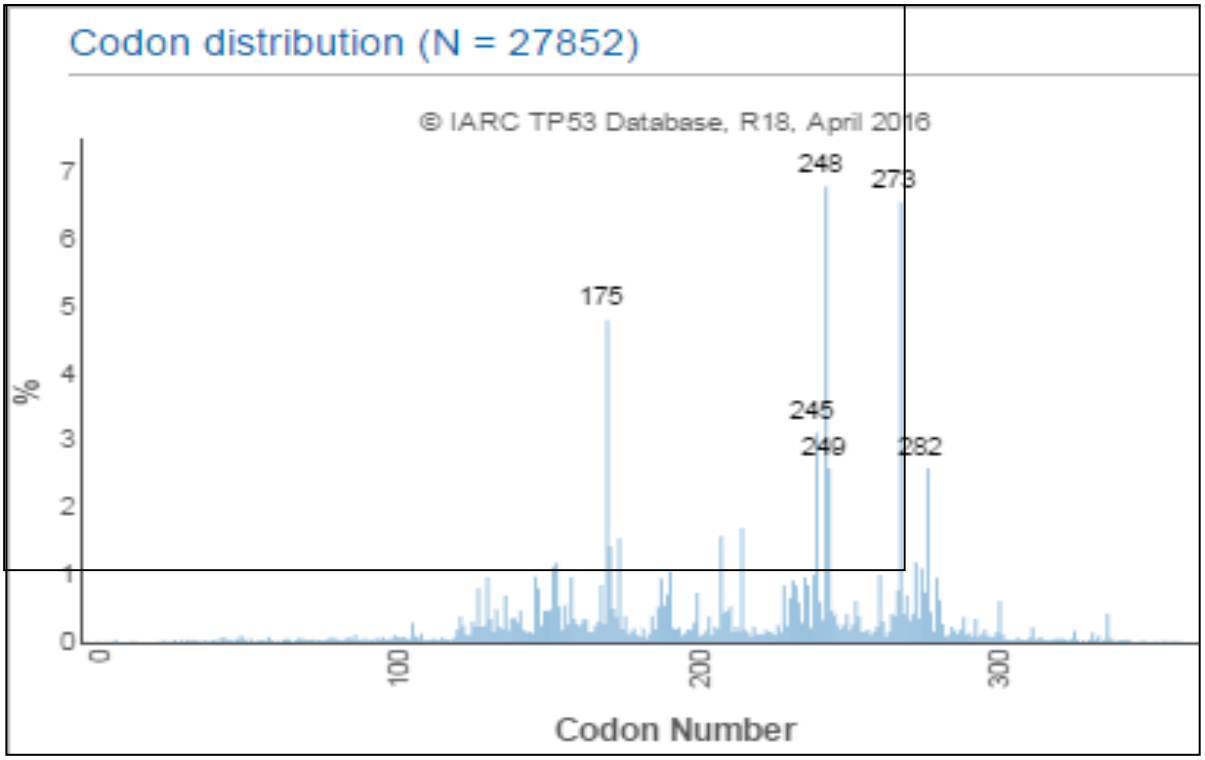


Figure 12: Distribution of the most frequently mutated codons of TP53(from IARC TP53 Database)

Table 6 : List of TP53 targets for high sensitivity sequencing

Chr	beginning	end	gene	exon(s)
chr17	-	-	*TP53*	all encoding exons
chr9	5069925	5070052	*JAK2*	exon12
chr9	5073760	5073780	*JAK2*	exon14
chr19	13054527	13054728	CALR	exon9
chr1	43814998	43815018	MPL	exon10

3.5 Cultures of medullary progenitors/Methyl cellulose assays.

Sorted cells from diagnostic samples were seeded in triplicate at 100 to 300,000 cells/mL of culture dish in 2% standard methylcellulose medium (Stem Cells Technologies) supplemented with: 37% fetal calf serum, 12% bovine serum albumin, 1% L-glutamine, 10 6 M b-mercaptoethanol, 1 IU ml 1 of erythropoietin, 50 ng ml 1 of stem cell factor, 25 ng ml 1 of *FLT3* ligand, 10 ng ml 1 of interleukin 3, 10 ng ml 1 of interleukin 6, 10 ng ml 1 of granulocyte colony stimulating factor, 5 ng ml 1 of granulocyte-macrophage colony-stimulating factor, 10 ng ml 1 of thrombopoietin, 100 IU ml 1 penicillin and 100 mg ml 1 streptomycin.

Colonies were counted after 14 days and picked. When appropriate, after a wash in PBS, individual colonies were split into two cell suspensions to perform both genotyping and FISH analyses. The first cell suspension was gently deposited into 18-well immunofluorescence slides. Slides were dried and fixed for further FISH analyses. The second cell suspension, or whole colonies when no translocation was detected in bulk AML, was stored at 80 C for further genotyping assays.

3.6 DNA Extraction from colonies.

The DNA of the colonies was extracted using a method based on the use of proteinase K (Qiagen).The following preparation was added to each well: 47.3 µl of sterile water, 0.5µl of Tween 20 and 0.972 µL of proteinase K (10 mg/l). The following thermal program was applied by using a thermal cycler to activate and then inactivate the enzyme: 1 hour at 56°C followed by 15 minutes at 95°C. The DNAs were then stored at 20°C.

3.7 Allele-specific PCR.

For SNV mutations, an allele-specific PCR technique was applied. Fluorochrome-labeled primers and probes (FAM and VIC) were designed and engineered by Applied Biotechnologies™ (SNP genotyping assay Kit) (Some examples on Table 7). The PCR reaction was carried out according to the instructions provided by the manufacturer in 96-well plates (15µl/well) on an Applied ABI 7500 time-lapse PCR machine over 50 cycles. Results were interpreted with Applied software, using differential Ct (theoretical cycles) analysis for the wild-type and mutated allele within each colony.

Table 7: Details of allele-specific primers

Variant*	Primer sens	Primer antisens	Probe WT	Probe mut
ATM_p.A2325A	TCTCAAGCAAATGATCAAGAAGTTGGA	AATAACAGTAAAACACTAATCCAGCCAATAAAAAAA	TGTGCAGCGGTTTGT	TGTGCAGCAGTTTGT
NSD1_p.C225C	TCACTGGCTTTTGATTCTGAACATTCT	GTCCTTTTTGGATTATCAGAGCTCTTTCT	AAGCCTTGCGCTAAAT	AAAGCCTTGTGCTAAAT
ETV6_p.T86T	CTCAAGTGGGCTGAAAATGAGTTTT	GCAGGAGAGCTTTGCCATTC	CAGCAACACGTTTGAA	CAGCAACACATTTGAA
SETBP1_p.V1101I	CACCACCTCAGTTCCACACA	CATGCTTGGCTTTATGCTTAGCT	ACTCCCACGTAAAGAT	AACTCCCACATAAAGAT
DNMT3A_p.N649D	GTTCAGCAAAGTGAGGACCATTACT	GACAGGAAAATGCTGGTCTTTGC	CTTTATGGAGTTTGACCTCG	ATGGAGTCTGACCTCG
TP53_p.L111R	GAAAACCTACCAGGGCAGCTA	AGACTTGGCTGTCCCAGAATG	CAAGAAGCCCAGACGGA	CAAGAAGCCCCGACGGA
TP53_p.L198R	AGCCAAAGAAGAAACCACTGGAT	CACTTGATAAGAGGTCCCAAGACTT	AGAATATTTCACCCTTCAGGTAC	TTCACCCGTCAGGTAC

ASXL1_chr11:31022837	CTGTCCTCCCAAACCTCAGTAG	GTTCTAACATCCGGATGCAACTGA	TGCTCCACTAATCTCCA	TGCTCCACTATCTCTCA
JAK2_p.V617F	AAGCTTTCTCACAAGCATTTGGTTT	AGGCATTAGAAAGCCTGTAGTTTTACTT	CTCCACAGACACATACT	CTCCACAGAAACATACT
TP53_chr17:7578115	CTGAGGTCTGGTTTGCAACTG	GTCAAATAAGCAGCAGGAGAAAGC	AGTGGCCCTCCAGGTGA	TGGCCCTCCGGGTGA
TET2_p.P459L	GGGAAGTGAAAATAGAGGGTAAACCT	GGCTGCATACATGTGTAGATGGATT	TGGGAAGGTGGTGCCT	CTGGGAAGTGGTGCCT
TP53_p.R116Q	AACTACATGTGTAACAGTTCCTGCAT	GAGTCTTCCAGTGTGATGATGGT	AACCGGAGGCCCATC	AACCGGAGACCCATC
CTCF_p.S388?	GTCCGTTTCAGTGCAGTTTGTG	TCTCATGTGCCTTTTCAGCTTGT	ATGTGTCCCTGCTGGCAT	ATGTGTCCCTACTGGCAT
TET2_p.T965N	GGTGGCATCTCTTACAGAAGCA	TGACTATGGCAAGACTCAGTTTGG	CAGCAGCAAACACAGC	AGCAGCAAAACACAGC
TP53_p.D281A	GCTTTGAGGTGCGTGTTTGTG	TGCGGAGATTCTCTTCCTCTGT	CGCCGGTCTCTCCCA	CCGGGCTCTCCCA
DDX41_chr5:176940274	TCTGACAAGTCTCCCTGAAGCT	CCCGGTTCCCACAAGGT	ACCCCCGGCTCCAA	ACCCCTGGCTCCAA
ATG2B_p.S8?	CACTATGCCTTGGCCGTTTTC	CCAGGTACCTCTGCAGGAG	CTCTTCTTGATGGACTCC	CTCTTCTTGATAGACTCC
FLT3_chr13:28577554	CAGCCTAAGATTTCTGCAACAACAG	TCTAGGTGATGTATTACTCTTTATGGTAGAACA	CTTCCCCCAATACAAC	CTTCCCCCAGTACAAC
IKZF1_chr7:50465654	TGAAAACGAATGGAAACCAGGCTAT	GCCCCATTATACCATAAAGTACCTTTGA	CATATCCATTACAAAATAAG	ATATCCATTACAGAATAAG

*** protein sequence or Hg19 genomics position**

3.8 Statistical analysis

The sample was described in its clinical and demographic characteristics by descriptive statistics techniques. In depth, qualitative data were expressed as absolute and relative percentage frequency, whilst quantitative variables either by mean and standard deviation (SD) or median and interquartile range (IQR), as appropriate. To verify the Gaussian distribution of quantitative variables, the Shapiro-Wilk test was applied. Between groups differences (p53mut vs. not) in duration of HU exposure, age at AML diagnosis and delay of SMP flare-up were assessed by either Student's t test or non-parametric Mann-Withney U test, in this latter case replaced by Wilcoxon-Pratt signed rank test due to the presence of ties. Statistical significance was set at a $p<0.05$. Analyses were performed with R software version 4.2.0 (CRAN ®, R Core 2022, Vienna, Austria) [52].

4. Results

4.1 Comparison of the clonal architecture of t-AML and de novo AML

4.1.1. Sequencing of t-AML and de novo AML

A summary of the main results of the targeted sequencing of 20 t-AMLs and 43 de novo AMLs are shown in Tables 8 and 9 and Figures 13A-D and 14.

Table 8

ID	Karyotype	Gene	VAF
dnAML-01	Complex	*TP53*	0,72
dnAML-02	Complex	*TP53*	0,90
		TET2	0,45
dnAML-03	Complex	*TP53*	0,64
		NF1	0,13
dnAML-04	Complex	*TP53*	0,77
dnAML-05	Complex	*TP53*	0,90
		TET2	0,50
		TET2	0,11
		CSF3R	0,05
dnAML-06	Complex	*TP53*	0,11
dnAML-07	46,XX,T(2;3)(Q?21;Q26),Del(5)(Q23q34)[24]	*U2AF1*	0,47
		DNMT3A	0,44
dnAML-08	Complex	*EZH2*	0,95
		RUNX1	0,86
		IDH2	0,46
		DNMT3A	0,39
dnAML-09	Complex	*TP53*	0,48
		DNMT3A	0,44
		IDH1	0,43
		TP53	0,41
dnAML-10	Complex	*TP53*	0,47
		DNMT3A	0,44
		TP53	0,42
dnAML-11	46,XX,Del(5)(Q12-13q34)[24]/46,XX[1]	*SF3B1*	0,38

		IKZF1	0,33
		RUNX1	0,28
dnAML-12	Complex	*TP53*	0,70
		MLL	0,45
dnAML-13	46,XY,Add(5)(Q11) Ordel(5)(Q11q34)[19]/46,XY[5]	*ASXL1*	0,33
		RUNX1	0,33
		DNMT3A	0,31
dnAML-14	Complex	*TP53*	0,83
		DNMT3A	0,42
dnAML-15	Complex	*TP53*	0,88
		TP53	0,33
dnAML-16	Complex	*TP53*	0,32
dnAML-17		*DNMT3A*	0,46
	Complex	*TP53*	0,42
		TP53	0,39
dnAML-18	Complex	*TP53*	0,89
		NSD1	0,63
		DNMT3A	0,61
dnAML-19	Complex	*SF3B1*	0,31
		DDX41	0,32
dnAML-20	Complex	*DNMT3A*	0,43
		IDH1	0,43
		RUNX1	0,43
dnAML-21	Complex	*TET2*	0,48
		TET2	0,46
		NRAS	0,08
		KRAS	0,01
		SRSF2	0,50
		ASXL1	0,42
		ETNK1	0,43
		BRAF	0,33
		JAK2	0,02
dnAML-22	Complex	*NRAS*	0,11
dnAML-23	Complex	*TP53*	0,11
		TP53	0,11
dnAML-24	Complex	*TP53*	0,21
dnAML-25	Complex	*TP53*	0,44
		TP53	0,40
		NRAS	0,11
dnAML-26	Complex	*TP53*	0,96
dnAML-27	Complex	*TP53*	0,72
		DNMT3A	0,42
dnAML-28	Complex	*IDH2*	0,38
		ASXL1	0,01

		PHF6	0,33
dnAML-29	Complex	*DNMT3A*	0,03
		NF1	0,19
		NF1	0,10
		PHF6	0,22
		RUNX1	0,25
dnAML-30	Complex	*TP53*	0,35
dnAML-31	Complex	*TP53*	0,22
		TP53	0,30
dnAML-32	Complex	*DNMT3A*	0,25
		DNMT3A	0,01
dnAML-33	Complex	*TP53*	0,49
		TET2	0,50
		TET2	0,23
		TET2	0,09
		TET2	0,07
		KRAS	0,03
		SRSF2	0,22
dnAML-34	Complex	*TP53*	0,31
		DNMT3A	0,26
dnAML-35	Complex	*TP53*	0,18
dnAML-36	Complex	*TP53*	0,81
dnAML-37	Complex	*TET2*	0,60
dnAML-38	Complex	*RUNX1*	0,40
		BCOR	0,44
dnAML-39	Complex	*0*	
dnAML-40	Complex	*BCOR*	0,11
dnAML-41	Complex	*ASXL1*	0,39
		TP53	0,71
dnAML-42	Complex	*TP53*	0,94
		DNMT3A	0,46
		BCOR	0,93
		NRAS	0,02
		PTPN11	0,02
		PTPN11	0,01
dnAML-43	Complex	*TET2*	0,45
		TP53	0,73
		ASXL1	0,44

Table 9.

ID	Karyotype	Gene	VAF
tAML-01	Complex	*TP53*	0,87
		SMC1A	0,46
		EZH2	0,42
		EZH2	0,10
tAML-02	Complex	*TP53*	0,41
		TP53	0,34
tAML-03	46,XX,t(6;9)(p23;q34)[23]/46,XX[3]	*NRAS*	0,38
tAML-04	Complex	*TP53*	0,55
		NRAS	0,21
		NRAS	0,06
		NRAS	0,05
tAML-05	Complex	*TP53*	0,44
		TP53	0,31
tAML-06	Complex	*TP53*	0,52
tAML-07	46,XX,t(3;5)(q25;q34)ort(3;5)(q26;q34)[24]/46,XX[2]	*NOTCH2*	0,52
tAML-08	46,XX,ins(1;8)(q32-	*TET2*	0,80
tAML-09	Complex	*TP53*	0,47
tAML-10	Complex	*BRAF*	0,86
		TET2	0,46
		TET2	0,43
		ATM	0,43
		NOTCH1	0,07
tAML-11	Complex	*TP53*	0,14
		TP53	0,09
tAML-12	Complex	*TP53*	0,29
		TP53	0,11
		TP53	0,07
tAML-13	Complex	*TP53*	0,38
tAML-14	Complex	*TP53*	0,34
		TP53	0,33
tAML-15	Complex	*TP53*	0,41
tAML-16	Complex	*FLT3*	0,06
tAML-17	Complex	*TP53*	0,46
tAML-18	Complex	*TP53*	0,45
tAML19	Complex	*TP53*	0,44
tAML-20	Complex	*TP53*	0,42
		TP53	0,05
		TET2	0,55
		FLT3	0,12
		NRAS	0,31
		NPM	0,48

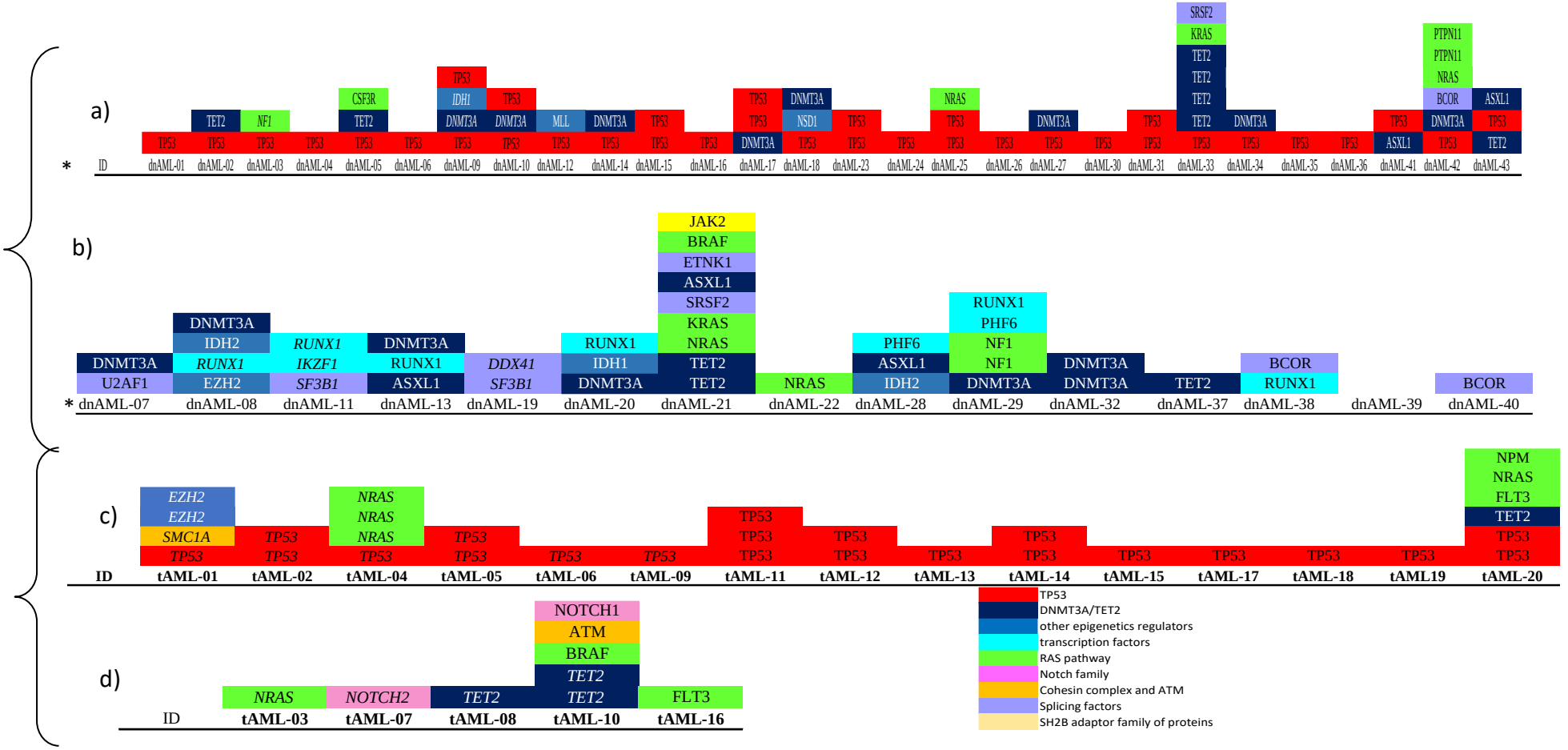


Figure 13. Mutational profile of t-AMLs and *de novo* AMLs. Concomitant mutations in a) *de novo* AMLs TP53 mutated, b) *de novo* AMLs TP53 wild type, c) t-AMLs TP53 mutated, d) t-AMLs TP53 wild type.

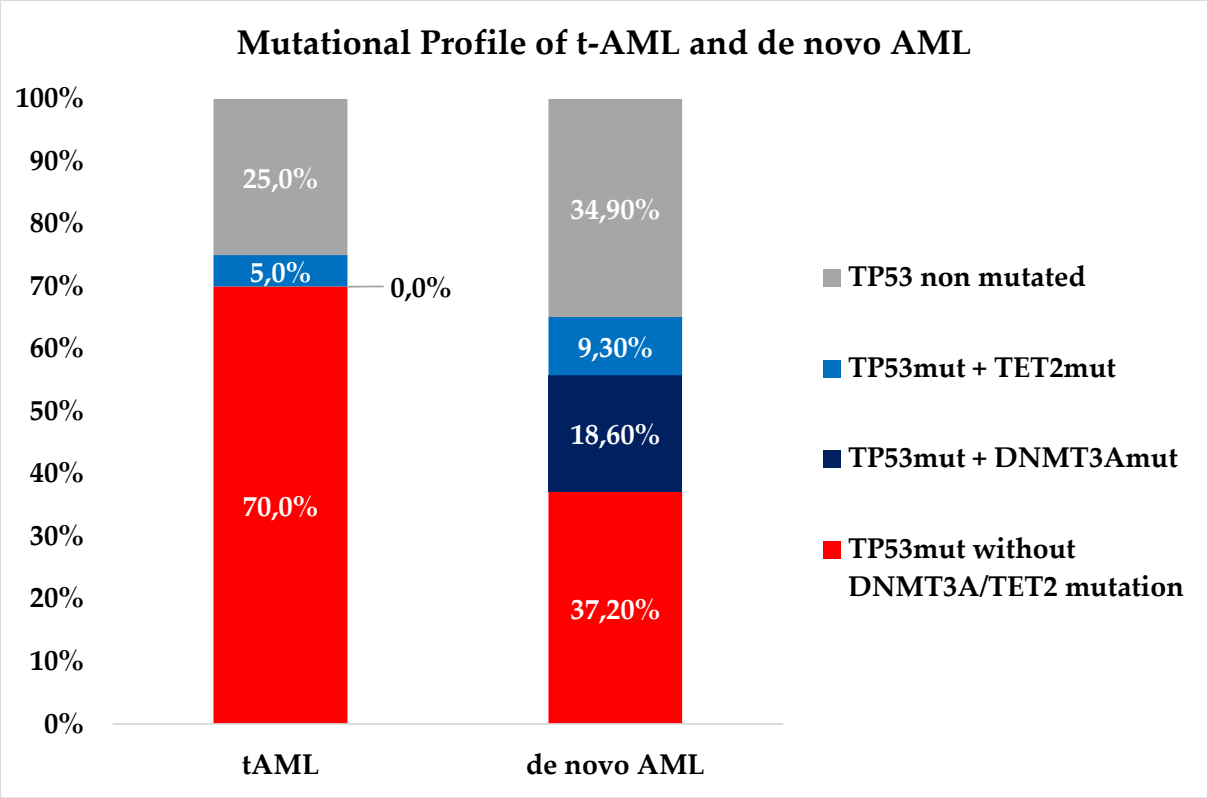


Figure 14. Association of mutations in TP53, DNMT3A and TET2 for t-AMLs and *de novo* AMLs.

As expected, 75% (15/20) t-AMLs and 63% (27/43) *de novo* AMLs in the series had *TP53* mutations. Among the 21 cases of AML not mutated for *TP53* (5 t-AML and 16 *de novo* AMLs), 6 correspond to patients with a non-complex karyotype.

Concerning the major epigenetic regulator mutations belonging to ARCH, there were 8 cases of association of *TP53* and *DNMT3A* mutations and 4 cases of association of *TP53* and *TET2* mutations among *de novo* AMLs, whilst only 1 case of association of *TET2* and *TP53* among t-AMLs. We did not find any case of association of *DMT3A* and *TP53* among t-AMLs. This result suggests an association of *TP53* mutations and altered epigenetic regulators in ARCH, especially *DNMT3A,* in *de novo* AMLs. Thus,

de novo AMLs may follow a partially ARCH-derived clonal evolutionary pattern, unlike t-AMLs.

The analysis of the clonal composition derived from our sequencing results and cytogenetic data indicates that in the 12 cases of *de novo* AML with associations of *TP53* and *TET2* or *DNMT3A* mutations, the associated mutations are present in a dominant clone, representing more than 50% of cells (Table 8). In order to clarify the clonal architecture of these *de novo* AMLs and the order of occurrence of the mutations, we analyzed the colonies of different patients with *TP53* and *DNMT3A* mutations for which we had cryopreserved cells.

4.1.2 Analysis of colonies of some dnAMLs

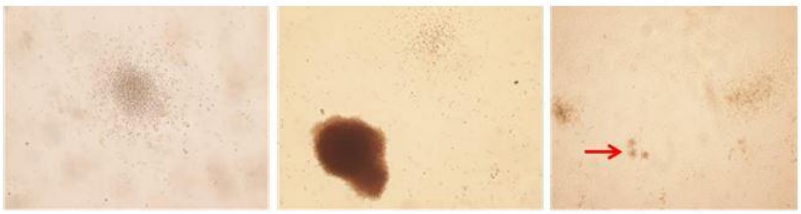
We cultured the CD34+ and CD34- mononuclear cells of patient dnAML-09 at diagnosis and relapse, which occurred 287 days after diagnosis. After 14 days of incubation, we observed the presence of colonies from BFU-E, CFU-G, CFU-GM, as well as small blastic colonies.

a)

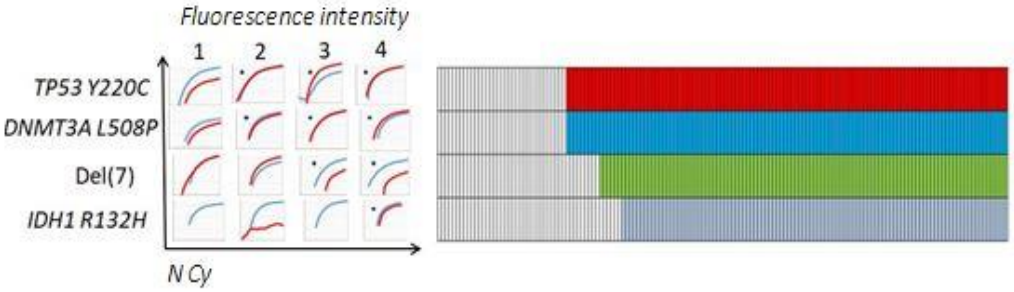
Karyotype: 45, XX, add(3)(q21), del(5)(q21q34), der(7)t(3;7)(q13~21;q21 or q31), -16 [18]

NGS: *TP53 (VAF 0,48) DNMT3A (VAF 0,44) IDH1 (VAF 0,43) TP53 (VAF 0,41)*

b)



c)



d)

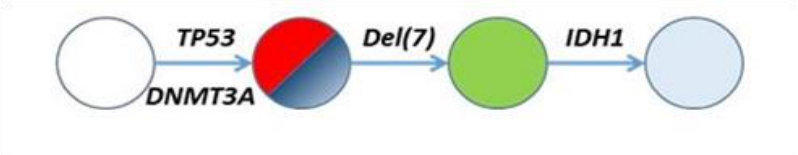


Figure 10: Progenitor-derived colony analysis for dnAML-09. (a) Karyotype and NGS of *dnAML* 09 (b) Photos of dnAML-09 colonies: (from left to right) CFU-GM colony, BFU-E colony, small blast colonies. (c) Identification of the order of acquisition of mutations and chromosomal abnormalities of dnAML-09 by analysis of 155 colonies, from allele-specific PCR amplification curves. (d) Clonal architecture of dnAML-09.

155 colonies were collected and genotyped for mutations in *TP53* Y220C, *DNMT3A* L508P, *IDH1* R132H, and loss of heterozygosity in the long arm of chromosome 7 using an informative polymorphism (Figure 10c). The analysis of the isolated or simultaneous presence of the different lesions indicates that mutations in *TP53*Y220C and *DNMT3A* are the earliest in the clonal architecture. No colony mutated for *TP53* without a *DNMT3A* mutation, nor mutated for *DNMT3A* without a *TP53* mutation, was observed. Therefore, it is not possible to specify the order of occurrence between the two mutations.

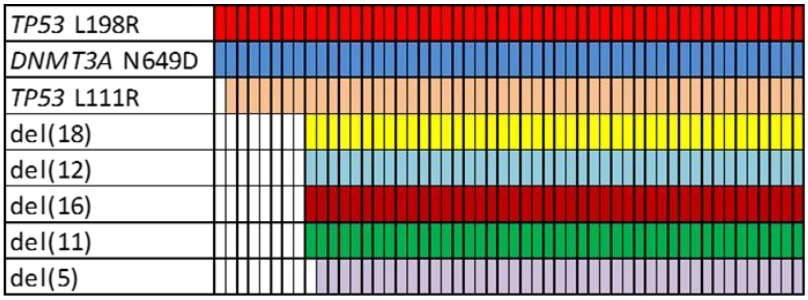
We cultured CD3- / CD34+ and CD34- mononuclear cells from dnAML-10 patient at diagnosis. The colonies observed were all blastic in appearance (Figure. 11b). They were collected and genotyped by allele-specific PCR for the detection of *TP53* L198R, *TP53* L111R, *DNMT3A* N649D mutations, and loss of heterozygosity related to del(18), del(5), del(16), del(12) and del(11) (Figure 11c).

a) **Karyotype:**44,XY,der(5)t(5 ;12),amp(11)(q2 ?2),der(12)t(12 ;17),+13,-15,-16,-18,der(20)t(15 ;20)(q11 ;q11),+mar

NGS: *TP53 (VAF 0,47) DNMT3A (VAF 0,44) TP53 (VAF 0,42)*

b)

c)



d)

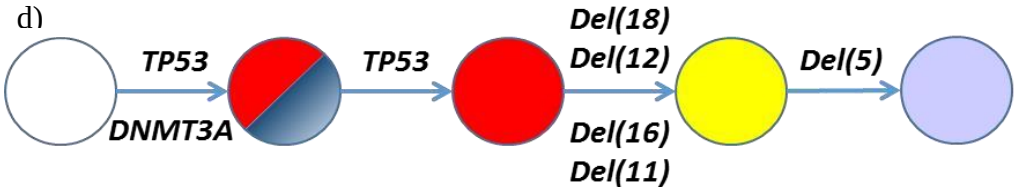


Figure 11: Analysis of dnAML-10 progenitor colonies. (a) Caryotype and NGS of dnAML-10 b)Pictures of dnAML-10 colonies: small blastic colonies.(c) Identification of the order of acquisition of mutations and chromosomal abnormalities in dnAML-10 by analysis of 52 colonies. (d) Clonal architecture of dnAML-10.

Of the 52 analyzable colonies, *TP53* L198R and *DNMT3A* mutations were identified as the earliest mutations in the clonal architecture (Figure 11 d).

Chromosomal abnormalities could not be dissociated, and occurred after all mutations. As for AML-09, the order of occurrence of *TP53* mutations relative to those of *DNMT3* could not be clarified.

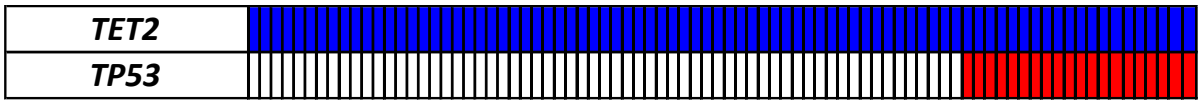
We further cultured mononuclear cells from the dnAML-33, AML-34 et dnAML-43 patients at diagnosis. The colonies observed were all blastic in appearance. For patient AML-33 they were also collected and genotyped by allele-specific PCR for the detection of *TP53* p.Arg248Gln (VAF 0,49) and *TET2* p.Gln1501Thrfs*3 (VAF 0,50) mutations.

a)

Karyotype:48,XY,del(5)(q31q35),+8,+8,der(11)t(11;17)(q11;q?11),der(17)(17pter>17q11::?::11q13->11qter), 20,+21,+mar[?der(7)][17]/46,XY[7]

NGS: TP53 (VAF 0,49), TET2 (VAF 0,50), SRSF2 (VAF 0,22), TET2 (VAF 0,23), TET2 (VAF 0,09), TET2 (VAF 0,07), KRAS (VAF 0,03)

b)



c)

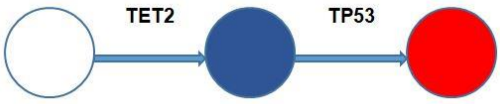


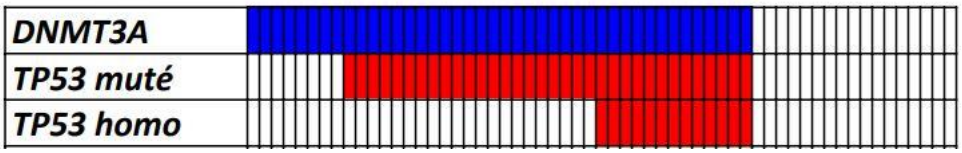
Figure 12: Analysis of dnAML-33 progenitor colonies. (a) Karyotype and NGS of *de novo AML*-33 (b)Identification of the order of acquisition of mutations and chromosomal abnormalities in dnAML-33 by analysis of 82 colonies. (c) Clonal architecture of dnAML-33.

For patient AML-34 the colonies were collected and genotyped by allele-specific PCR for the detection of TP53 p.Arg175Gly and DNMT3A mutations as well.

a) **Karyotype:** 46,XX,del(1)(p?21p3?3),3,del(5)(q11q34),add(7)(q21),ins(11;7)(q14;q21q3?2),add(17)(q2?5),+1 ,-21,+mar[der(3)],+mar,+dmin,inc[cp11]/46,XX[7]

NGS: TP53 (VAF 0,31), DNMT3A (VAF 0,26)

b)



c)

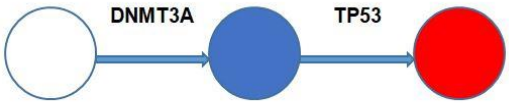


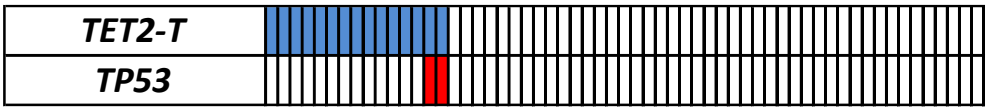
Figure 14: Analysis of dnAML-34 progenitor colonies. (a) Karyotype and NGS of *de novo* AML 34 (b)Identification of the order of acquisition of mutations and chromosomal abnormalities in dnAML-34 by analysis of 59 colonies. (c) Clonal architecture of dnAML-34.

Finally, for patient AML-43 the colonies were collected and genotyped by allele-specific PCR for the detection of TP53 p.Val143Met, and TET2 p.Pro929Leufs*24 mutations.

a) Karyotype: complex

NGS: TET2 (VAF 0,45), TP53 (0,73), ASXL1 (0,44)

b)



c)

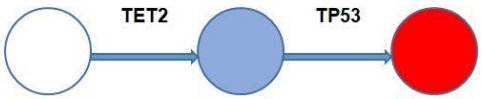


Figure 15: Analysis of dnAML-43 progenitor colonies. (a) Karyotype and NGS of *de novo* AM- 43. (b) Identification of the order of acquisition of mutations and chromosomal abnormalities in dnAML-43 by analysis of 59 colonies. (c) Clonal architecture of dnAML-43.

For patients AML-33, AML-34 and AML-43, among the analyzable colonies, ARCH mutations were identified as the earliest mutations in the clonal architecture. However, we have not identified any colony with TP53 mutation alone.

For patient AML-41 the colonies were collected and genotyped by allele-specific PCR for the detection of *TP53* p.Asp228*fs*1, *BCOR* p.(Asp 284 Glu;Ser 286 Lys fs* 15) and *DNMT3A* p.Arg882Hys mutations.

a) Karyotype:44,XY,der(3)t(3;?19)(p11;?q11),-5,-7,

19,+mar[4]/44,idem,der(4)del(4)(p15p12)del(4)(q11q25)[16]

NGS: TP53 (VAF 0,94), BCOR (VAF 0,93), DNMT3A (VAF 0,46)

b)

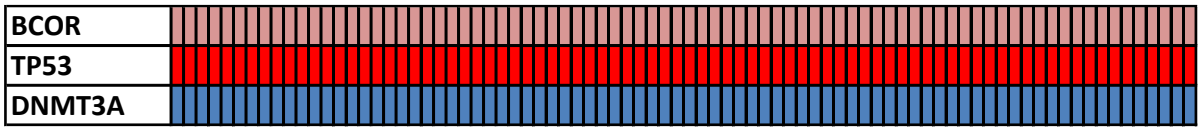


Figure 16: Analysis of dnAML-41 progenitor colonies. (a) Karyotype and NGS of *de novo* AML-41. (b) Identification of the order of acquisition of mutations and chromosomal abnormalities in dnAML-41 by analysis of 82 colonies.

No colony mutated for TP53 without a DNMT3A mutation, nor mutated for DNMT3A without a TP53 mutation, was observed. Therefore, it is not possible to specify the order of occurrence between the three mutations.

For patient AML-42 the colonies were as well collected and genotyped by allele-specific PCR for the detection of *TP53* p.Arg273 Cys and *ASXL1* p.Gli 710* mutations.

a) Karyotype: complex

NGS: ASXL1 (VAF 0,39), TP53 (VAF 0,72)

b)

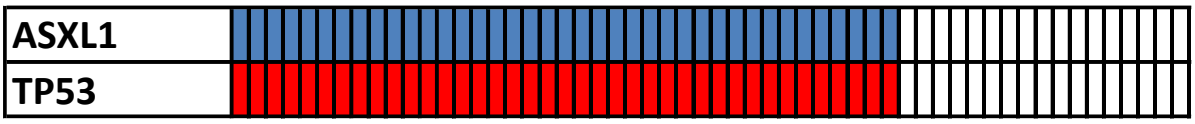


Figure 17: Analysis of dnAML-42 progenitor colonies. (a) Karyotype and NGS of *de novo* AML-42(b) Identification of the order of acquisition of mutations and chromosomal abnormalities in dnAML-42 by analysis of 55 colonies.

No colony mutated for TP53 without an ASXL1 mutation, nor mutated for ASXL1 without a TP53 mutation, was observed. Once again, it is not possible to specify the order of occurrence between the two mutations.

The NGS and colony results allow us to develop two models of clonal expansion in TP53-mutated AMLs, by distinguishing between t-AMLs, in which chemotherapy confers a proliferative advantage on the TP53 clone, and a portion of *de novo* AMLs, in which expansion of TP53 clone is enabled by the concomitant mutations in ARCH-related epigenetic regulators. We can observe that in no case is the TP53 mutation present alone: we can find the presence of the TP53 mutation or on a clone already mutated for an epigenetic factor or it is impossible to establish which mutation occurred first, which suggests that TP53 alone does not carry the clone.

4.2 Clonal architecture of s-AML and identification of TP53 mutated clones in myeloproliferative neoplasms

In this context, we studied the clonal architecture of s-AML, in order to clarify whether they follow a clonal expansion pattern corresponding to that of t-AML or *de novo* AML.

4.2.1 Clonal architecture analysis of AMLs secondary to MPN: sequencing and focus on some s-AMLs colonies

The results of targeted sequencing performed at the time of diagnosis on 58 s-AML are presented in Table 10, Figures 18 and 19.

Table 10.

ID	Karyotype	Gene	VAF
sAML-01	46,XX,del(20)(q11q13)[19]	MLL	0,414
		NOTCH1	0,407
		DNMT3A	0,371
		IDH2	0,354
		GATA2	0,244
		FLT3	0,0605
sAML-02	Complex	JAK2	0,999
		TP53	0,401
sAML-03	46,XX,del(20)(q11q13)[19]	JAK2	0,957
		CTCF	0,361
		SMC1A	0,356
		BCLAF1	0,070
sAML-04	Complex	CUX1	0,402
		TP53	0,359
		JAK2	0,18
		TP53	0,167
		TP53	0,119
sAML-05	Complex	TP53	0,702
		IDH2	0,085

sAML-06	46, XX	GATA2	0,469
		ASXL1	0,468
		PHF6	0,459
		GATA2	0,449
		PTPN11	0,447
		NRAS	0,396
		EZH2	0,386
		FLT3	0,060
sAML-07	Complex	JAK2	0,642
		RUNX1	0,290
		PTPN11	0,062
sAML-08	46,XY,der(7)t(1;7)(q10;p10)[16]/46,XY[10]	RUNX1	0,232
		IDH1	0,219
		CALR	0,138
sAML-09	Complex	JAK2	0,723
		TP53	0,485
sAML-10	Complex	NF1	0,651
		IKZF1	0,405
		ASXL1	0,348
		CALR	0,213
sAML-11	46,XX,del(5)(q33-34)[16]/46,XX[7]	TP53	0,764
		DNMT3A	0,395
		JAK2	0,177
		RUNX1	0,176
		GATA2	0,068
sAML-12	Complex	TP53	0,452
		TP53	0,433
		U2AF1	0,419
sAML-13	Complex	TP53	0.684
		JAK2	0.164
sAML-14	Complex	JAK2	0,343
		NF1	0,134
sAML-15	XX, del(17)(p13)[17]/46,?idem[4]/46,XX[2]	JAK2	0,950
		TP53	0,725
		TET2	0,515
		ASXL1	0,470
		TET2	0,331
		SRSF2	0,306
sAML-16	NA	JAK2	0,915
		ASXL1	0,815
		DNMT3A	0,249
sAML-17	Complex	TP53	0,823
		TP53	0,055
		JAK2	0,010

sAML-18	Complex	TP53	0,855
sAML-19	NA	JAK2	0,980
		U2AF1	0,482
		TP53	0,476
		TET2	0,433
		ASXL1	0,346
		NF1	0,056
sAML-20	Complex	JAK2	0,658
		SF3B1	0,430
sAML-21	Complex	BCLAF1	0,333
		TP53	0,217
		JAK2	0,213
		TP53	0,185
sAML-22	NA	JAK2	0,77
		IDH1	0,410
		TP53	0,370
		TP53	0,210
sAML-23	NA	JAK2	0,840
		TP53	0,800
		TET2	0,78
sAML-24	Complex	TP53	0,38
		PTPN11	0,04
		PTPN11	0,09
		TP53	0,35
		NRAS	0,01
		JAK2	0,07
sAML-25	NA	TET2	0,18
		CBL	0,35
		CBL	0,37
		JAK2	0,48
		TP53	0,96
sAML-26	Complex	TP53	0,64
		JAK2	0,49
sAML-27	46,XX,del(5q)(q13;q13),-7,+11	CALR	0,54
		TP53	0,23
		TET2	0,25
sAML-28	Del 3q13	IDH2	0,50
		ASXL2	0,08
		DDX41	0,52
		BCORL1	0,26
		BCOR	0,36
		MPL	0,36
		JAK2	0,43
		CEBPA	0,30

sAML-29	46,XY,t(5;12)(q33;p13)[21]/46,sl,del(2)(q23q37)[2]	GATA2	0,25
		BCOR	0,44
sAML-30	45~46,X,-X,del(7)(q13q32),+mar[cp13]/46,XX[2]	CALR	0,44
		NF1	0,21
sAML-31	Complex	RUNX1	0,15
		TP53	0,42
		U2AF1	0,34
		RUNX1	0,32
		RUNX1	0,17
		JAK2	0,43
sAML-32	46,XY[28]	RUNX1	0,32
		SRSF2	0,54
		CBL	0,42
		JAK2	0,43
		IDH2	0,45
sAML-33	Del 20q,7q,17q	JAK2	0,44
sAML-34	NA	ZRSR2	0,72
		SF3B1	0,80
		TP53	0,88
		JAK2	0,33
sAML-35	Complex	TP53	0,91
		JAK2	0,43
		EZH2	0,04
sAML-36	Monosomy 7 et del 17p	NF1	0,73
		SRSF2	0,60
		SETBP1	0,44
		ASXL1	0,40
		PTPN11	0,44
sAML-37	46,XY[27]	TET2	0,97
		STAG2	0,94
		SRSF2	0,50
		ASXL1	0,47
		CEBPA	0,45
		BCOR	0,07
		RUNX1	0,01
		CEBPA	0,38
sAML-38	Complex	TP53	0,52
		TP53	0,18
		TP53	0,18
		JAK1	0,21
		CBL	0,20
sAML-39	Complex	TP53	0,31
		ASXL2	0,39
		TET2	0,50

		TET2	0,37
		BCOR	0,22
		ZRSR2	0,79
		CBL	0,22
		CBL	0,09
		EZH2	0,03
		ASXL1	0,02
		JAK2	0,11
sAML-40	46,XY[18]	MPL	0,88
		ZRSR2	0,71
		DNMT3A	0,02
sAML-41	Complex	TP53	0,44
		TP53	0,32
		NF1	0,24
sAML-42	46,XX[20]	IDH2	0,41
		SRSF2	0,43
		ASXL1	0,37
		JAK2	0,36
		RUNX1	0,06
sAML-43	46,XX, +8, +21 [22]/46,XX[3]	IDH2	0,24
		EZH2	0,52
		EZH2	0,43
		CALR	0,90
		ASXL1	0,45
		RUNX1	0,10
		ATM	0,09
sAML-44	46,XY	DNMT3A	0,01
		TET2	0,34
		STAG2	0,42
		JAK2	0,33
sAML-45	Complex	JAK2	0,55
		TP53	0,62
		TP53	0,35
		TET2	0,47
sAML-46	NA	TP53	0,44
		TP53	0,03
		TET2	0,32
		JAK2	0,61
sAML-47	Complex	TP53	0,45
		TP53	0,33
		JAK2	0,14
sAML-48	Complex	JAK2	0,88
		TP53	0,35
		TP53	0,32

		TP53	0,02
sAML-49	NA	SF3B1	0,19
		ASXL1	0,16
		CBL	0,01
		ASXL1	0,04
		ASXL1	0,01
		CALR	0,65
sAML-50	Complex	DNMT3A	0,42
		KRAS	0,02
		EZH2	0,44
		ZRSR2	0,85
		RUNX1	0,46
		ASXL1	0,47
		JAK2	0,92
sAML-51	NA	EZH2	0,76
		ASXL1	0,23
		SH2B3	0,13
		STAG2	0,22
		U2AF1	0,44
		CSF3R	0,13
sAML-52	Complex	TP53	0,24
		TP53	0,22
		DNMT3A	0,17
		TET2	0,18
		TET2	0,14
		JAK2	0,36
		ZNF687	0,19
sAML-53	Complex	TP53	0,24
		TET2	0,22
		TET2	0,22
		EZH2	0,27
		EZH2	0,02
		JAK2	0,20
		SH2B3	0,05
		RBBP6	0,22
		PPM1D	0,01
		NF2	0,02
sAML-54	Complex	SRSF2	0,12
		JAK2	0,47
sAML-55	Complex	TP53	0,53
		ASXL1	0,31
		JAK2	0,88
		EP300	0,03
sAML-56	Complex	TP53	0,80

		TET2	0,52
		TET2	0,45
		DNMT3A	0,43
		DNMT3A	0,07
		KRAS	0,05
		ASXL1	0,02
		CBL	0,02
		CBL	0,02
sAML-57	Complex	IDH1	0,18
		U2AF1	0.24
		JAK2	0,16
sAML58	Complex	ASXL1	0,44

Abbreviations. NA = not available

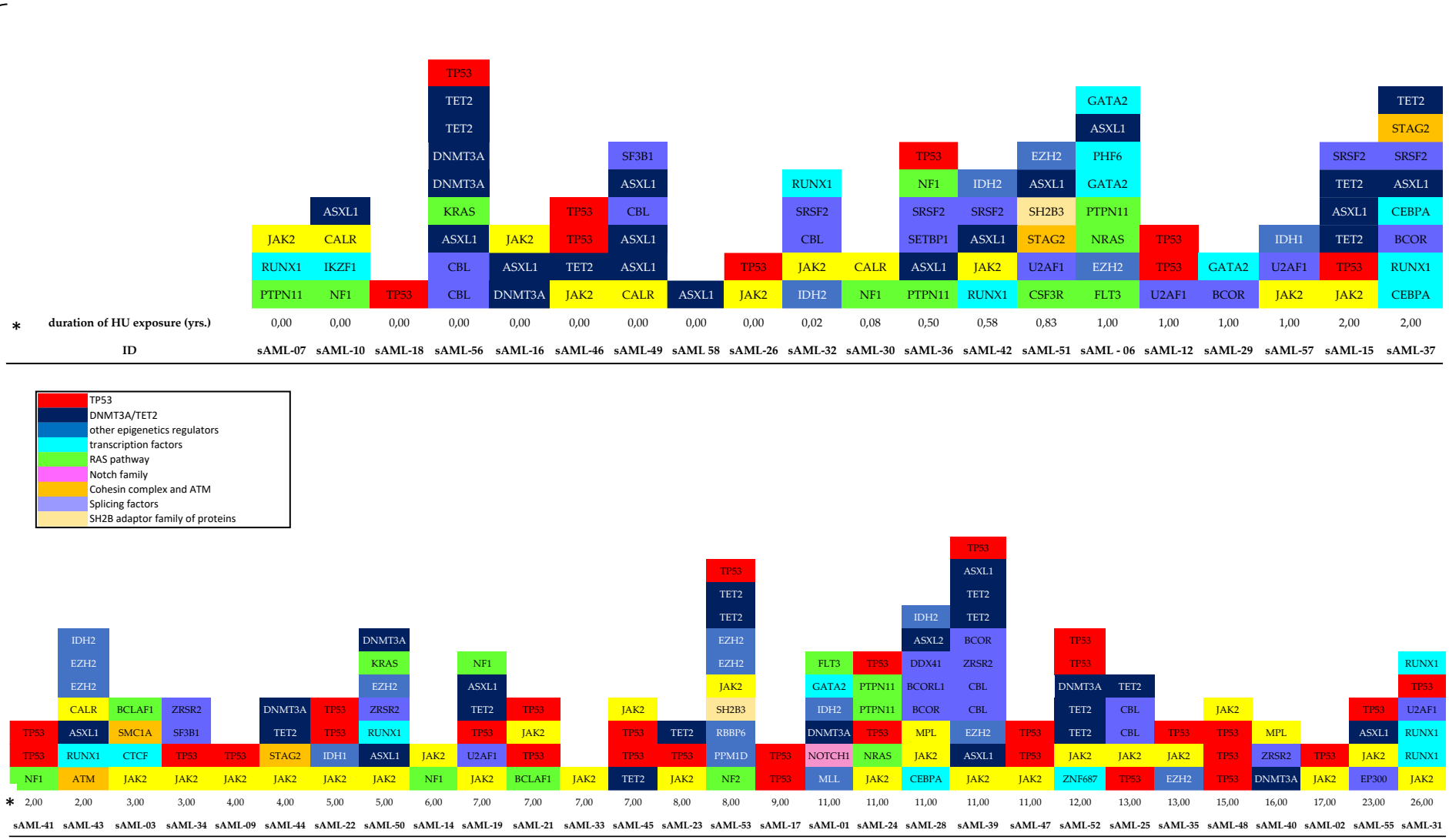


Figure 18. Mutational profile of s-AMLs according duration of Hydroxyurea exposure.

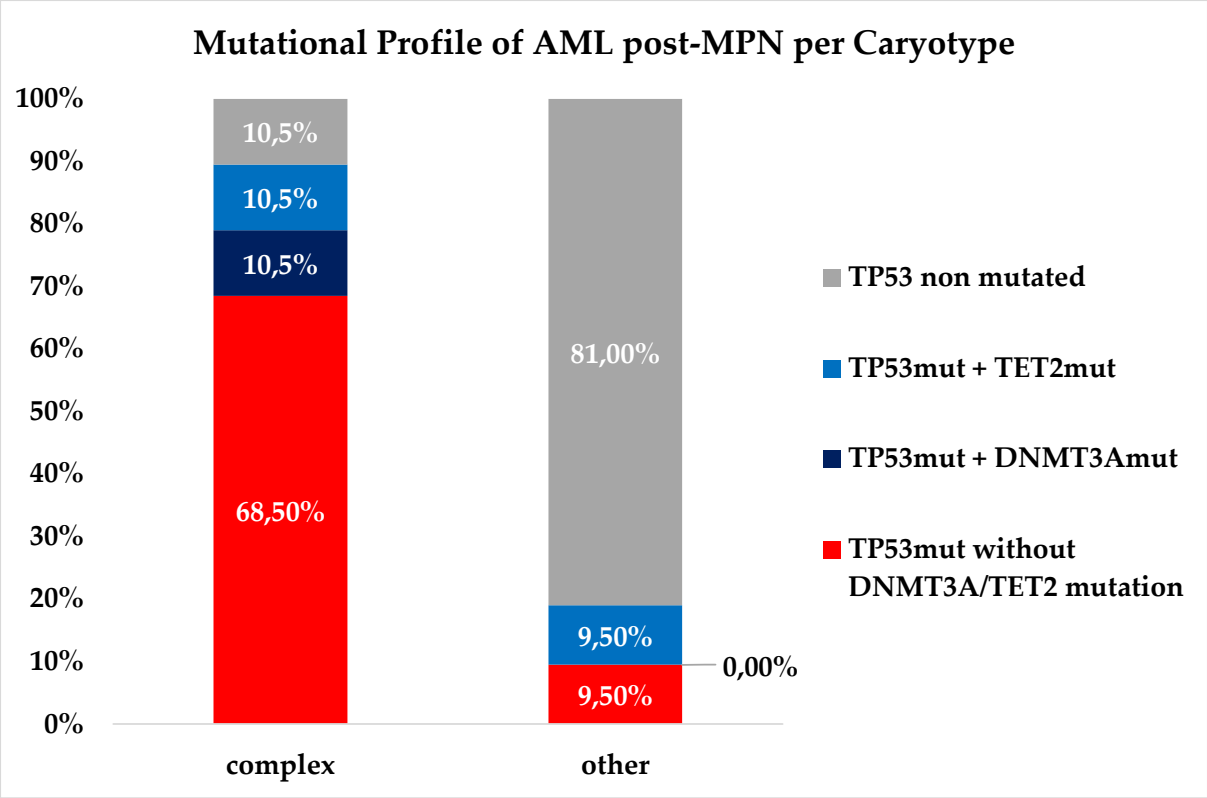


Figure 19: Mutational profile of secondary AMLs. Association of *TP53 DNMT3A* and *TET2* mutations for s-AMLs per karyotype.

Thirty-three of the 58 s-AMLs in our series (57%) are TP53 mutated (or had a numerical alteration of 17p) and among these 21 have a complex karyotype. Among *TP53* mutated patients, twelve also carry mutations of epigenetic factors involved in ARCH (*DNMT3A*, *TET2* or *ASXL1*), and 25 are *JAK2* mutated.

In order to better clarify the clonal evolution of *TP53*- and *JAK2*-mutated AML, we performed high-sensitivity sequencing on samples taken at the diagnosis of MPN for 3 patients (s-AML-15, s-AML-17, s-AML-22), and on an intermediate follow-up sample for patient s-AML-23. In this way, we have sought for TP53 sample at low level at these time points.

For patient s-AML-15, the *JAK2* V617F and *TP53* R248Q mutations identified with VAF of 0.95 and 0.73, respectively, at the time of AML onset were found manually, at the diagnosis of MPN, with VAF of 0.55 and 0.0009, respectively (Fig. 20).

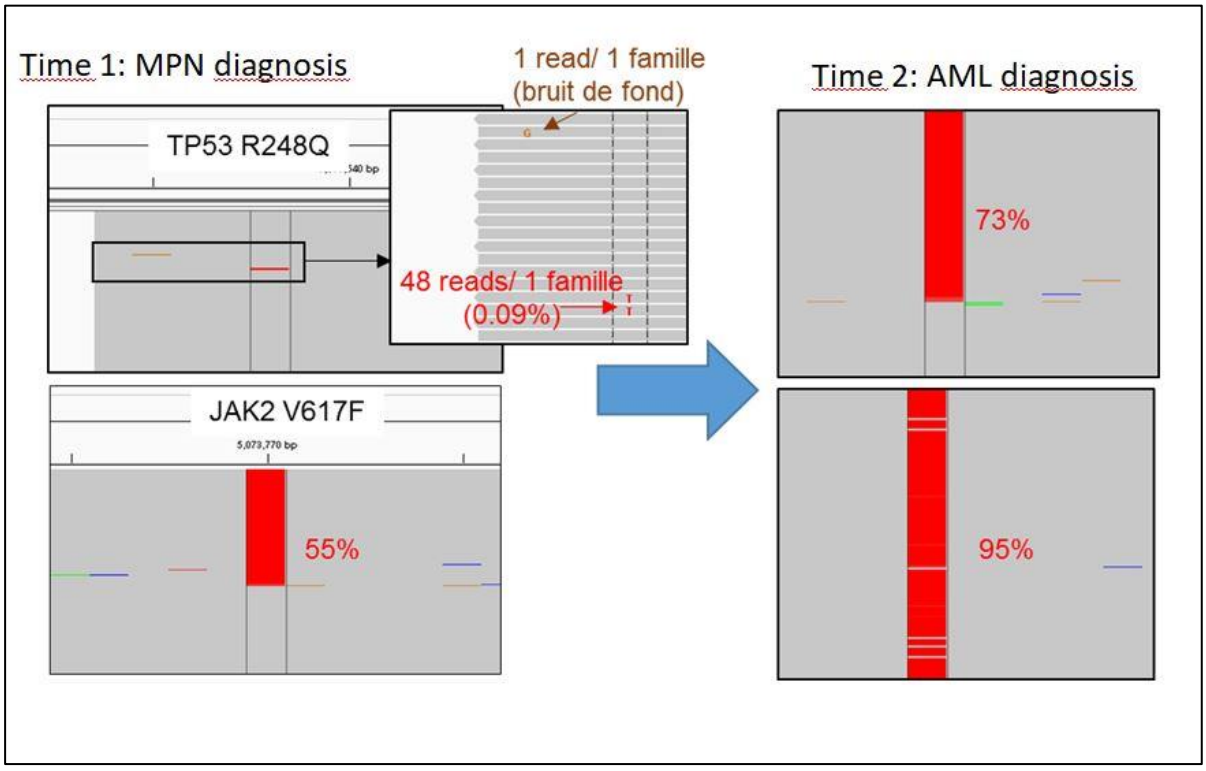


Figure 20: Evolution of allele frequencies for mutations TP53R248Q and JAK2V617F between diagnostics of MPN and acuteness at s-AML-15 (Visualization on IGV).

For patient s-AML-17, the *JAK2* V617F mutation was found with a VAF of 0.01 in acute phase and 0.58 at diagnosis of MPN. The *TP53* 7578176C>T (splice site) mutation identified with a VAF of 0.82 at AML onset was not detected at diagnosis of MPN (Fig. 21).

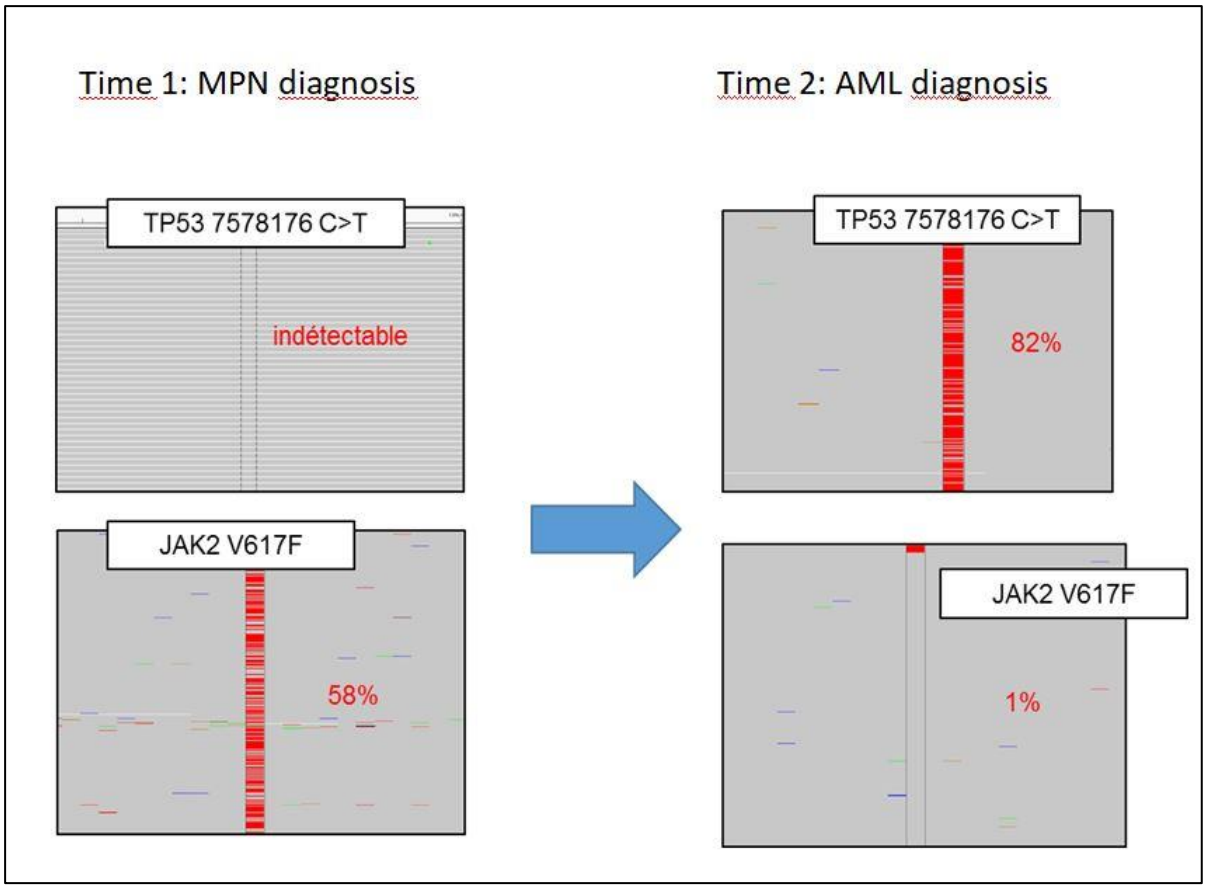


Figure 21: Evolution of frequencies of mutated alleles for TP537578176C>Tet and JAK2V617F between diagnosis of MPN and acutization in s-AML-17. (Visualization on IGV).

For patient s-AML-22, *JAK2* is mutated with a VAF at 0.77, *TP53* R282W at 0.37, and *TP53* R213L at 0.21 at AML onset. At MPN diagnosis, *JAK2* was found at 0.32, and mutations in *TP53* R282W and R213L were identified at VAFs of 0.0003 and 0.0007, respectively.

For patient s-AML-23, mutations in *JAK2* and *TP53* H179Q are found at VAFs of 0.84 and 0.8 at AML onset. Three years earlier, at an intermediate follow-up point, the VAFs of these mutations were 0.41 and 0.22, respectively.

These results show the pre-existence of *TP53* clone at the time of MPN in 3 out of 4 patients. The case of s-AML-17 suggests that *TP53*-mutated clone is derived from a different clone than the *JAK2*-mutated clone, with a dissociated course between MPN diagnosis and AML onset.

To support this hypothesis, we cultured CD34+ and CD34- mononuclear cells from patient s-AML-13, whose transformation was characterized by a decrease in *JAK2* V617F allelic burden as compared to that measured at diagnosis (0,95) by allele-specific PCR, and for which we had cryopreserved bone marrow cells. We observed numerous small leukemic-looking colonies, and only one colony derived from BFU-E. These were collected and genotyped in the laboratory by allele-specific PCR for *TP53* D149A, JAK2V617F mutations and loss of heterozygosity related to del(5), del(7), del(13) and 14q uniparental disomy (DUP) (Figure 22).

Karyotype: complex

NGS: TP53 (VAF 0.68) JAK2 (0.16)

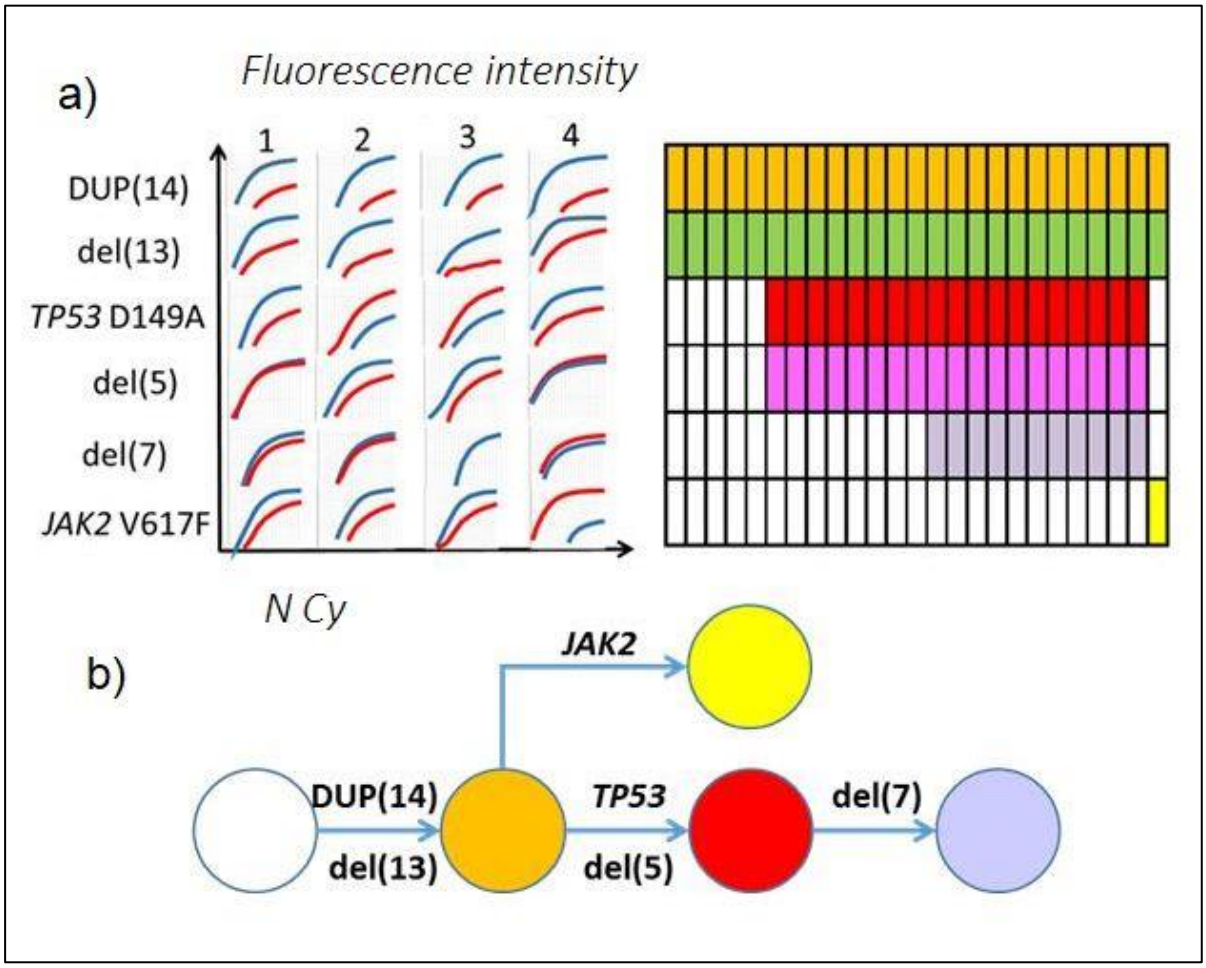


Figure 22: Analysis of colonies from progenitors for s-AML-13. (a) Identification of the order of acquisition of mutations and chromosomal abnormalities in s-AML-13 by analysis of 25 colonies based on allele-specific PCR amplification curves. (b) Clonal architecture of s-AML-13.

From the results of 25 analyzable colonies, we observed that the earliest abnormalities of clonal architecture are del(13) and DUP(14q), followed by TP53 D149A and del(5), then del(7). The only colony derived from BFU-E contains del(13), DUP(14q) and JAK2 V617F. Analysis of these colonies shows that *TP53* mutated clone is independent of the *JAK2* mutated clone. The del(13) and DUP(14) foresee *TP53* and *JAK2* mutations.

For s-AML-15, which carries a deletion of the short arm of chromosome 17 at the karyotype, we observe that del(17p) and TP53 R248Q have respective VAFs of 0.89 and 0.78, which implies that del(17p) is earlier than TP53 R248Q. To further clarify the clonal architecture, we cultured s-AML-15 mononuclear cells collected at the diagnosis of SMP. We observed numerous and large colonies that were rather suggestive of non-leukemic hematopoiesis. The results of specific-randomized PCR on the 33 analyzable colonies for JAK2V617F, the ASXL1 frameshift mutation, the two TET2 frameshift mutations (P459Lfs and T965Nfs), TP53 R248Q, and del(17) are shown in Figure 23.

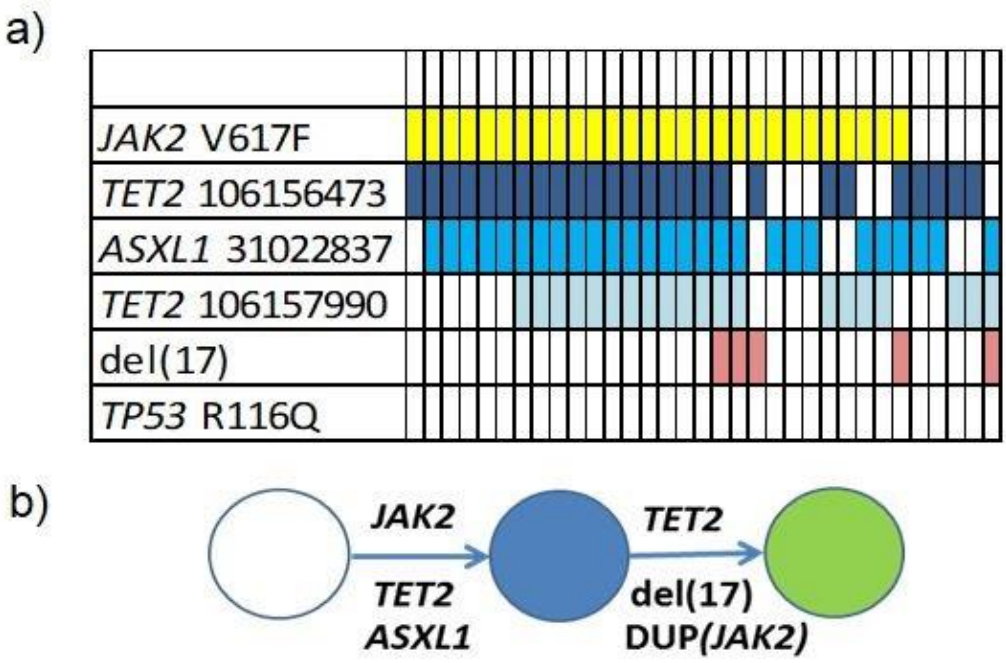


Figure 23: Analysis of progenitor-derived colonies for s-AML-15.(a)Identification of the order of acquisition of mutations and chromosomal abnormalities in s-AML-13 by analysis of 33colonies, according to JAK2 V617F status.(b) Clonal architecture of s-AML-15.

Mutations in *JAK2*, *TET2* P459Lfs and *ASXL1* seem to be the earliest, followed by *TET2* T965Nfs and then del(17p). There is no detectable *TP53* R248Q mutation at this stage, whereas a del(17p) is present in 5 colonies, which confirms that a del(17p) appears prior to TP53 mutation.

There is also a cluster of 5 colonies in which JAK2 is not mutated, though it otherwise seems to follow the same clonal architecture. Mitotic recombination with loss of JAK2 mutation, likely occurring after TET2 P459Lfs, might be at the origin of these non JAK2 mutated colonies.

4.2.2 **Research of TP53 mutated clones in MPN by high sensitivity sequencing**

The results of NGS HS sequencing performed on 17 patients followed for MPN for more than 7 years, on s-AML-15, s-AML-17, s-AML-23 at the diagnosis of SMP, and on s-AML-22 at the intermediate follow-up point are presented in Table 11. We first searched manually for TP53 variants using IGV software with a sensitivity threshold of 0.002 and then for variants at the most frequently mutated positions with a sensitivity threshold of 0.001.

Of the 17 patients followed for more than 7 years, 5 out of 12 who had received cytoreductive therapy and 2 out of 5 who had never received it carried mutated TP53 clones.

Considering the total of 58 patients diagnosed with s-AML (post-MPN), for 55 patients we had information about the cytoreductive treatment received for MPN: 41 patients received HU, 3 patients Pipobroman (PB), 6 patients PB and then HU, whilst 5 patients did not receive any cytoreducing treatments.

Table 11. Details of TP53 variants detected in high-sensitivity NGS for a sensitivity threshold of 0.002 and then for a threshold greater than 0.001 for codons 175, 245, 248, 249, 273 and 282; with classification of patients according to the duration of cytoreductive treatment received

ID	Duration of cytoreductive treatment	Detection threshold	Position	Ref. Base	Mutated Base	VAF	AA
MPN-01	16	>0,002	none				
MPN-03	16	>0,002	none				
		Targeted search	7577094	G	A	0,001	R282W
MPN-12	15	>0,002	none				
			7576541	G	A	0,004	Non-ref
		>0,002	7577120	C	T	0,003	R273H
MPN-17	15		7577547	C	T	0,003	G245D
		Targeted search	7577094	G	A	0,001	R282W
MPN-14	10	>0,002	7577547	C	T	0,002	G245D
			7578177	C	A	0,006	E224D
			7577030	C	A	0,004	S303I
MPN-17	9	>0,002	7576900	G	T	0,004	P316T
			7577121	G	A	0,002	R273C
			7576900	G	A	0,002	P316S
MPN-04	9	>0,002	none				
MPN-06	9	>0,002	none				
MPN-23	8	>0,002	7578393	A	T	0,22	H179Q
		Targeted search	7577547	C	T	0,001	G245D
MPN-02	7	>0,002	none				
		>0,002	7578406	C	A	0,002	R175L
			7577547	G	T	0,001	
MPN-22	5	Targeted search	7578211	C	A	0,0007	R213L
			7577094	G	A	0,0003	R282W

We then focused on patients treated exclusively with HU, excluding patients who had received PB, to avoid the likely impact of this latter on the leukemogenesis process. We characterized the molecular profile of these patients at s-AML diagnosis and tried to evaluate the existence of an association between the duration of exposure to HU and TP53/aN 17p status.

Table 12 Characteristics of patients treated with HU stratified for the presence of TP53/aN17p status (n=41).*

	TP53/aN17p status		
	Positive (N=24)	**Negative (N=17)**	**p**
Duration of HU exposure, years	8.0 (4.5-12.5)	2.0 (1.0-6.0)	**<0.001**
Age at AML diagnosis, years	72.1 (10.0)	68.1 (11.6)	0.253
Delay of MPN flare-up, years	10.1 (6.6)	8.2 (6.2)	0.368

* Data are expressed as either mean and standard deviation (SD) or median and interquartile range (IQR). Gaussian distribution was verified by the Shapiro-Wilk's test. P-values were computed by either Student's t test or non-parametric Wilcoxon-Pratt signed rank test (due to the presence of ties).
HU: Hydroxyurea, AML: Acute myeloid leukemia, MPN: myeloproliferative neoplasm, TP53/aN 17p positive status: presence of TP53 mutation and/or numerical anomaly cr.17p, TP53/aN 17p negative status: absence of TP53 mutation and/or numerical anomaly cr.17p

4.2.3 Sequencing analysis of AMLs in patients with SCD

Of 5 patients with AML and SCD, 4 were TP53 mutated AMLs and the remaining patient was diagnosed with CBF (t 8;21) leukemia. All 5 patients experienced early relapse and/or post-transplant complications/death, including the patient with CBF leukemia.

Table 13. Cases of AML in patients affected by SCD at Saint Antoine Hospital.

Code	Duration of HU exposure (years)	Myeloid Neoplasm	AML NGS			
			Gene	Nucleotide	Protein	VAF
SCD/AML 01	>20	AEL	TP53	c.455C>T	p.Pro152Leu	0,24
			TP53	c.524G>A	p.Arg175His	0,22
			PTPN11	c.213T>A	p.Phe71Leu	0,16
SCD/AML 02	>20	AEL	TP53		p.T377P	0,04
SCD/AML 03	7	AML/MDS	TP53	c.658T>G	p.Tyr220Asp	0,67
* SCD/AML 04	17	AML	t (8;21)			
**SCD/AML 05	15	AEL	TP53	c.413 C>T	p.Ala138Val	0,35
			TP53	c.993+1G>A	p?	0,31
			TP53	c.537G>T	p.(His179Gln)	0,02
			JAK2	c.1849G>T	p.Val617Phe	0,88

Abbreviations: AEL: Acute Erythroid Leukemia; AML: Acute myeloid leukemia, SCD: sickle cell disease, HU Hydroxyurea, NGS: next generation sequencing, VAF: variant allele frequency

* For patient SCD/AML 04 we detected a TP53 subclone by NGS HS (P.Arg174Glu, 0.19%, 8 families of reads)

** Patient with concomitant MPN

4.2.4 **Research of TP53 mutated clones in SCD by high sensitivity sequencing**

Among 31 patients analyzed, by NGS, followed for SCD: 8 are the patients for whom anomalies have been detected by standard and high sensitivity technique and among these 4 were *TP53* mutated, as confirmed by the number of families of reads (>7).

Twenty-two patients did not have any mutation, and 4 patients had some particularities detected by NGS HS, but not frank mutations.

Table 14. Research of TP53 mutated clones in patients with SCD (n=31).

ID	Mutated Gene	NGS		
		Protein	VAF	FR
SCD 01	none			
SCD 02	none			
SCD 03	none			
SCD 04	none			
SCD 05	none			
SCD 06	none			
SCD 07	none			
SCD 08	none			
SCD 09	none			
SCD 10	none			
SCD 11	none			
SCD 12	none			
SCD 13	none			
SCD 14	none			
SCD 15	none			
SCD 16	none			
SCD 17	none			
SCD 18	none			
SCD 19	none			
SCD 20	none			
SCD 21	none			
SCD 22	none			
*SCD 23	TP53	p.Asp281Gly	0.6	
SCD 24	MPL	p.Gln387*	48	
SCD 25	DNMT3A	p.Gly707Ser	1	
	JAK2 V617F	traces		
*SCD 26	TP53	p.Thr230Ala	0.7	
*SCD 27	TP53	p.Pro152Ser	0.7	
SCD 28	TP53	p.Arg251His	0.2	10
	JAK2 V617F	traces		
SCD 29	TP53	p.Glu11lys	0.28	8
SCD 30	TP53	p.Tyr181Cys	0.79	43
SCD 31	TP53	p.Arg174*	0.27	13

Abbreviations: SCD: sickle cell disease, NGS: next generation sequencing, VAF: variant allele frequency, FR: families of reads. Red: mutated patients, black: non-mutated patients, blue: peculiar cases.

*The result for patient SCD23 was obtained by standard NGS. The results for patients SCD26 and SCD27 were not confirmed by NGS HS

5. Discussion

The study of the clonal architecture carried out on 121 AML by sequencing and colony analysis allows us to describe three profiles of AML carrying TP53 mutations according to their *de novo*, therapy-related/induced or secondary character. Among the 27 *de novo* AMLs with *TP53* mutations, 12 also carry mutations in *DNMT3A* or *TET2*, which is controversial with some published data which do not show this association of mutations.[7,53] Colony analysis confirmed that these are the earliest mutations in clonal architecture, but they did not distinguish the order of occurrence between *TP53* mutations and epigenetic regulator mutations in all cases. There is no evidence of a clonal expansion phase with *TP53* as single mutation. Such finding supports the hypothesis that the expansion of a *TP53* mutated clone requires the co-occurrence of a mutation in an epigenetic regulator gene involved in ARCH. Associations of mutations in *TP53* and *DNMT3A* or *TET2* have been described in ARCH, but the number of observations is much higher than expected, given the individual frequencies of mutations in the three genes in ARCH (6 cases of mutations for an expected value of 0.89).[9,10,25] We plan to confirm such hypothesis in future studies focused on the *in vivo* effect of these mutations, through murine xenografts of HSCs depleted in *TP53*, *DNMT3A* and/or *TET2* by RNA interference.

For s-AML, our series included only 4 patient who did not receive cytoreduction therapy (pipobroman or hydroxyurea), regardless of our selection criteria, since most patients with MPN at Saint-Antoine Hospital receive prior treatment. The mutational

profile of MPN transformations revealed a high percentage of mutations in *TP53* (33/58; 57%), frequently associated with a decrease in the allelic load of *JAK*2V617F, thus resulting in the development of AML from a separate clone of the *JAK*2V617F clone. Apart from MPN-specific mutations, MPN transformations carrying *TP53* mutations seem to have mutational profiles summarizing those of t-AML and *de novo* AML, with 12 cases of associations between *TP53* mutations and HCLA mutations. Moreover, it is interesting to note that the analysis of the colonies of patient s-AML-13 allows us to identify the deletions on chromosomes 13 and 14 as the earliest in the clonal architecture, occurring before the mutations of *JAK2* and *TP53*, which belong to two distinct subclones.

It is well known that the long arm of chromosome 14, including the *ATG2B* and *GSKIP* genes, may be involved in rare predispositions to MPNs [54], and that rare cases of uniparental disomia, 14q could acquire mimicits this situation [55]. These data suggest that the acquired duplication of the region including *ATG2B* and *GSKIP* could represent an early initiating event of MPN and its evolution into AML. We can question about the relationship between cyto-reducing treatments and clonal evolution.

Likewise the role of chemotherapy in t-AML leukemogenesis, we hypothesize that cyto-reducing therapies provide a proliferation advantage to mutated clones for *TP53*. This hypothesis is supported by the discovery of multiple clones mutated for *TP53* at low frequencies at the time of MPN diagnosis by the amplification of these

mutations observed at the time of AML onset. Our first findings on untransformed MPN, with the detection of mutated *TP53* clones in 5 out of the 12 patients who did receive cyto-reducing therapy, and 2 out of the 5 patients who did never receive cyto-reducing therapy, do not allow us to draw any conclusion at this stage. We plan to study additional patients followed for MPN.

We will also use digital PCR to confirm identified variants, as our HS sequencing results should be viewed with caution. Indeed, the sensitivity threshold of the technique for nucleotide substitutions was established at 1/1000, a value around which most of VAFs of the clones we detected were located.

We focused on patients treated with HU alone, excluding those who received PB, as it is known to provide leukemias. We sought to characterize the molecular profile of these patients at the time of s-AML diagnosis and assess the existence of an association between duration of exposure to HU and *TP53*/aN17p status. Duration of exposure to HU positively correlates with the presence of mutated *TP53* clones ($p<0.001$).

Finally, we will further test the effect of HU *in vivo* in the xenograft experiments above mentioned, aiming to observe whether this triggers the amplification of the clones mutated for *TP53*.

We can question the place of cyto-reducing treatments in MPNs, and the relevance of personalized monitoring, which would detect and monitor small clones mutated for *TP53* in treated patients.

We believe that this reflection may be even more relevant for patients affected by diseases other than MPNs, in which HU has always been considered a life-saving drug. SCD patients. In this manuscript are reported some data concerning the molecular characterization by NGS standard and/or NGS HS for about 30 patients followed for SCD. Therefore, a complete molecular characterization, by NGS, could be useful to detect and to monitor the presence of *TP53* subclones in patients with SCD.

6. Acknowledgements

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