## Title

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# Clinical and Molecular Determinants of Clonal Evolution in Aplastic Anemia and Paroxysmal Nocturnal Hemoglobinuria

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abstract

**PURPOSE** Secondary myeloid neoplasms (sMNs) remain the most serious long-term complications in patients with aplastic anemia (AA) and paroxysmal nocturnal hemoglobinuria (PNH). However, sMNs lack specific predictors, dedicated surveillance measures, and early therapeutic interventions.

**PATIENTS AND METHODS** We studied a multicenter, retrospective cohort of 1,008 patients (median follow-up 8.6 years) with AA and PNH to assess clinical and molecular determinants of clonal evolution.

**RESULTS** Although none of the patients transplanted upfront (n = 117) developed clonal complications (either sMN or secondary PNH), the 10-year cumulative incidence of sMN in nontransplanted cases was 11.6%. In severe AA, older age at presentation and lack of response to immunosuppressive therapy were independently associated with increased risk of sMN, whereas untreated patients had the highest risk among nonsevere cases. The elapsed time from AA to sMN was 4.5 years. sMN developed in 94 patients. The 5-year overall survival reached 40% and was independently associated with bone marrow blasts at sMN onset. Myelodysplastic syndrome with high-risk phenotypes, del7/7q, and *ASXL1*, *SETBP1*, *RUNX1*, and *RAS* pathway gene mutations were the most frequent characteristics. Cross-sectional studies of clonal dynamics from baseline to evolution revealed that *PIGA*/human leukocyte antigen lesions decreased over time, being replaced by clones with myeloid hits. *PIGA* and *BCOR/L1* mutation carriers had a lower risk of sMN progression, whereas myeloid driver lesions marked the group with a higher risk.

**CONCLUSION** The risk of sMN in AA is associated with disease severity, lack of response to treatment, and patients' age. sMNs display high-risk morphological, karyotypic, and molecular features. The landscape of acquired somatic mutations is complex and incompletely understood and should be considered with caution in medical management.

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## INTRODUCTION

Aplastic anemia (AA) is a prototypic bone marrow (BM) failure syndrome manifesting with pancytopenia of autoimmune origin.[1] Although hematopoietic stem-cell transplant (HSCT) represents a curative option for younger cases, older patients and those lacking a suitable donor are treated with immunosuppressive therapy (IST).[2] Particularly in such patients, the refinement of IST during the past few decades has dramatically improved survival outcomes.[3]

Nevertheless, with increased numbers of survivors, a higher risk for clonal evolution to both myeloid neoplasia (MN) and paroxysmal nocturnal hemoglobinuria (PNH) emerged, corroborating the hypothesis described by Dr Dameshek's riddle 50 years ago as to the intertwining relationship of such entities: what do aplastic anemia, paroxysmal nocturnal hemoglobinuria, and hypoplastic leukemia have in common?[4,5] Indeed, retrospective studies with long follow-up revealed that up to 20% of patients with AA treated with IST may develop either myelodysplastic syndromes (MDSs) or acute myeloid leukemia.[6,7] These events are distinguished by dismal outcomes and absence of obvious clinical predictors. Furthermore, as it has happened for granulocyte colony-stimulating factor in the past,[8,9] the use of thrombopoietin-receptor agonists has recently raised concerns as to the increased incidence of clonal evolution, further stressing the importance of studies aiming at the identification of predictors of such deadly complications.[10]

Single nucleotide polymorphism array karyotyping and, more recently, next-generation sequencing (NGS)

ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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## CONTEXT

**Key Objective**

Secondary myeloid neoplasms (sMNs) are late events in nontransplanted acquired aplastic anemia (AA) or paroxysmal nocturnal hemoglobinuria (PNH) patients. This retrospective, multinational study (N = 1,008) reports how clinical, cytogenetic, and molecular characteristics influence the risk of sMN, suggesting rational diagnostic approaches.

**Knowledge Generated**

sMN presented high-risk morphological, cytogenetic, and molecular features. Cross-sectional analyses revealed an inversion in clonal dynamics: *PIGA*/human leukocyte antigen mutations, predominant at baseline, were replaced by myeloid drivers at transformation. Lack of response to nontransplant treatments, older age, and myeloid driver mutations at AA/PNH onset conferred higher risk of progression.

**Relevance**

Patients with AA or PNH need close, prolonged monitoring with marrow morphology and cytogenetics. Morphological progression to sMN or occurrence of monosomy 7 should prompt clinicians to consider transplantation in eligible patients. The landscape of somatic mutations is complex and incompletely understood. Clinicians should carefully interpret the molecular profile with morphological and cytogenetic data for therapeutic decisions.

revealed somatic mutations with a wide range of frequencies (5% up to 72%) in patients with AA and PNH according to the testing timing (diagnosis *v* follow-up), the number of interrogated genes, and technique (targeted *v* whole exome/genome sequencing).[6,11-17] In addition to *PIGA* mutations, responsible for the emergence of PNH clone(s), the most frequently mutated genes are *DNMT3A*, *ASXL1*, and *BCOR/L1*, whose effects on AA and PNH clonal dynamics are far from being completely understood.[5,11] In the paucity of HSCs surviving after AA immune attack, mutations generating a survival advantage such as *PIGA* or myeloid driver hits (eg, *RUNX1* and *SETBP1*) may arise or acquire clonal dominance if already present.[18]

These observations suggest that the pathogenesis of secondary myeloid neoplasms (sMNs) may be linked to clonal hematopoiesis (CH), characterized by somatic mutations typical of MN with a peculiar MDS-related signature.[6,11,12,17] However, low burden mutations may be transient events and not contributory to later evolution, whereas others may persist, thereby heralding progression. This is reminiscent of CH of indeterminate potential (CHIP) found in healthy individuals or clonal cytopenia of undetermined significance.[19-21]

Unlike leukemic driver mutations, *PIGA* hits produce an escape phenotype (blessing in disguise) in the context of AA immune-mediated attack.[22] The theory of clonality as escape[23] posits that *PIGA*-mutant HSCs do not significantly sustain BM hematopoiesis unless the T-cell–mediated immune attack selectively targets the *PIGA* wild-type HSCs.[24] Similarly, somatic hits in the human leukocyte antigen (HLA) genomic region have also been identified in AA and tentatively assigned to an immune escape of the stressed BM, paralleling findings observed in cancer.[16,17,25-27]

The rarity of AA and the timing of clonal evolution have made prospective controlled studies aiming to characterize factors associated with evolution difficult. Perhaps the long-term follow-up of the recently completed RACE trial[3] may be able to do so; but it will take years to get the full picture. Thus, retrospective analyses including large numbers of patients followed for extended periods of time are the sole able to decipher this complex field. Herein, we performed a comprehensive study of late clonal complications in 1,008 consecutive patients with AA and PNH from a multicenter international cohort to define predictors of malignant progression. Furthermore, we examined clinical and molecular features of progression, being able to track the dynamics of clonal evolution in a subgroup of patients in a cross-sectional and longitudinal fashion.

## PATIENTS AND METHODS

### Study Design and Patients

We devised a retrospective, multicenter study to investigate the influence of baseline characteristics of patients with AA and PNH on the risk of late clonal complications (eg, sMN and secondary PNH) and to characterize the clinical and genomic features of evolution. All consecutive patients diagnosed with acquired AA and/or PNH between 1972 and 2020 at the three participating centers were enrolled in this study after obtaining written informed consent (Table 1 and Fig 1). The review of medical records was approved by the internal Institutional Review Boards of each center in agreement with the Declaration of Helsinki. Full details on study design, patients, and definitions are available in the Data Supplement (online only).

### Genomic Studies

Samples were collected and subjected to multiple NGS platforms as previously described[28-30] and according to the manufacturer's protocols. Full details are provided in the Data Supplement.

**TABLE 1.** Main Clinical Characteristics of the Study Cohort

| Variable | Modalities | n = 999 | AA (n = 817) | AA/PNH (n = 75) | PNH (n = 107) |
|---|---|---|---|---|---|
| Sex | Female | 502 (50.3) | 399 (48.8) | 43 (57.3) | 60 (56.1) |
| | Male | 497 (49.7) | 418 (51.2) | 32 (42.7) | 47 (43.9) |
| Age at diagnosis, years | Median [IQR] | 34.0 [20.1-54.3] | 34 [19.1-56.7] | 30.5 [20.5-43.9] | 36.1 [27.3-46.9] |
| PNH clone at diagnosis (n = 832)[a] | No | 433 (52) | 433 (66.5) | 0 (0) | 0 (0) |
| | Yes | 399 (48) | 218 (33.5) | 74 (100) | 107 (100) |
| PNH clone (percentage) | Median [IQR] | 11 [2.4-61.4] | 3.3 [1.2-8] | 48 [30.8-82.7] | 83.5 [61.4-94.5] |
| Camitta criteria | Nonsevere | | 176 (21.5) | 48 (64) | |
| | Severe | | 641 (78.5) | 27 (36) | |
| Karyotype at diagnosis (n = 780)[a] | Normal | 725 (93) | 598 (91.6) | 64 (100) | 63 (100) |
| | Failure | 43 (5.5) | 43 (6.6) | 0 (0) | 0 (0) |
| | Others[b] | 12 (1.5) | 12 (1.8) | 0 (0) | 0 (0) |
| Cohort | RPH | 116 (11.6) | 98 (12) | 14 (18.7) | 4 (3.7) |
| | CCF | 396 (39.7) | 336 (41.1) | 17 (22.7) | 43 (40.2) |
| | SLS | 487 (48.7) | 383 (46.9) | 44 (58.6) | 60 (56.1) |

NOTE. For categorical variables, numbers in parentheses indicate percentages.

Abbreviations: AA, aplastic anemia; CCF, Cleveland Clinic Foundation; IQR, interquartile range; PNH, paroxysmal nocturnal hemoglobinuria; RPH, Riberão Preto Hospital; SLS, Hôpital Saint-Louis.

[a]The available sample size because of missing data.

[b]Including sex chromosome abnormalities and age-related findings not configuring an alternative diagnosis (also see the Data Supplement).

### Statistical Analysis

Cumulative incidences of clonal complications were calculated in a competing risk setting, where death and HSCT were considered competing events. The nonadjusted effect was evaluated using log-rank and Gray's tests for overall survival (OS) and cumulative incidence outcomes, respectively. The multivariate impact of baseline variables was evaluated using the multivariate Cox cause–specific model.

All statistical tests were two-sided, and a $P$ value $< .05$ was considered statistically significant. All analyses and data visualization were generated using the statistical computing environment R (4.0.0 R Core Team, R Foundation for Statistical Computing, Vienna, Austria), Excel Microsoft Office 365 (Redmond, WA), and GraphPad Prism (8.4.0, San Diego, CA). Further details on statistics are provided in the Data Supplement.

## RESULTS

### Determinants of Late Clonal Complications and Survival

A total of 1,008 patients were assessed for inclusion. Because of the absence of follow-up data (n = 3) and overt diagnosis of MN at PNH presentation (n = 6), nine patients were excluded from further analysis (Table 1 and Fig 1). In agreement with our previous report,[7] none of the patients undergoing HSCT upfront (n = 117, Data Supplement) experienced late clonal complications (either sMN or secondary PNH) at a median follow-up time of 8.2 years (interquartile range [IQR], 5.7-10.6), registering a 10-year OS of 86.7% (77.8-92.1).

We thus studied nontransplanted patients with AA, AA/PNH overlap, and classical hemolytic PNH (n = 882, Data Supplement). Their 10-year OS reached 76.4% (median follow-up time 8.6 years, Data Supplement), and the 10-year cumulative incidence of sMN was 11.6% (Data Supplement). A trend toward a higher risk of malignant progression was observed in AA (12.8%) and AA/PNH (13.1%) as compared with classical hemolytic PNH (3.4%, both $P = .06$, Fig 2A). Consequently, classic PNH cases had the best OS with 95.2% alive at 10 years from diagnosis versus 72.1% ($P < .001$) and 88.8% ($P = .99$) in patients with AA and AA/PNH, respectively ($P < .001$ globally by the log-rank test, Data Supplement).

Besides the presence of PNH hemolytic clones, outcomes of AA were also affected by disease severity. While showing similar rates of sMN progression (13.6% *v* 12.4%, $P = .86$; Fig 2B), nonsevere cases had better OS (81.3% *v* 71.0%, $P < .001$) but a slightly higher risk of secondary PNH (15% *v* 9%, $P = .12$) when compared with patients with severe disease (Data Supplement).

In severe AA (n = 504, Data Supplement), the risk for malignant evolution was higher in older (> 35 years) cases (20% *v* 6.6% in younger patients; $P < .001$) and in those with partial or no response to IST (15.7% *v* 8.5% in complete responders; $P = .02$). Older age at presentation (hazard ratio [HR], 1.37 [95% CI, 1.20 to 1.56]; $P < .001$) and poor response to IST (HR, 2.60 [95% CI, 1.39 to 4.84]; $P = .003$) were independent predictors of sMN progression and survival (Figs 2C and 2D and Table 2). When studying patients with available molecular information (n = 302),

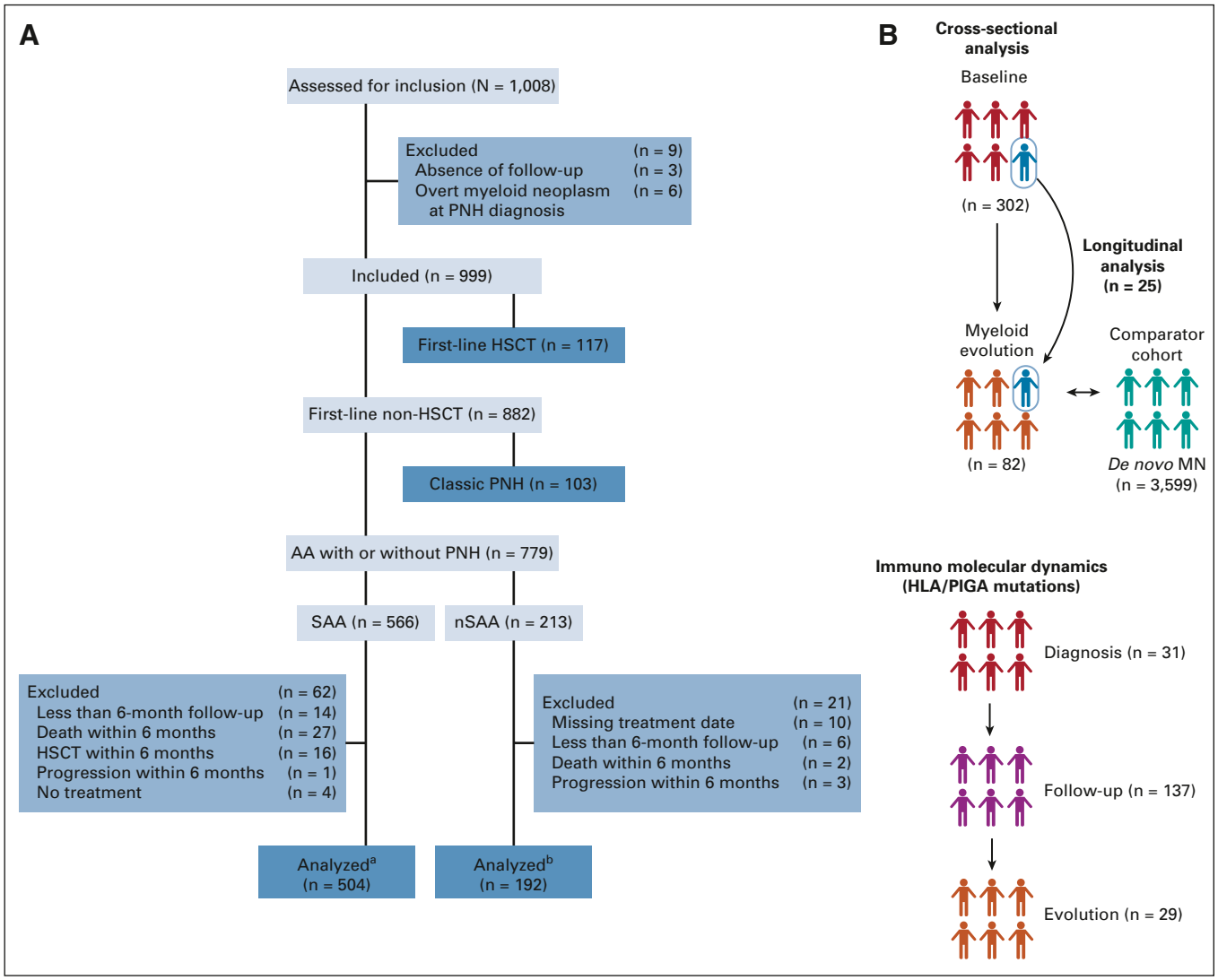


**FIG 1.** Study design. (A) CONSORT diagram of patient inclusion with details of sequenced patients for (B) cross-sectional and longitudinal analyses. [a]Landmark: 6 months after diagnosis. [b]Landmark: 6 months after first-line (or diagnosis if no treatment). AA, aplastic anemia; HSCT, hematopoietic stem-cell transplant; MN, myeloid neoplasia; nSAA, nonsevere aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria; SAA, severe aplastic anemia.

overall, the presence of myeloid mutations at disease onset did not seem to affect evolution (Data Supplement). The presence of a small PNH clone characterized patients with better survival outcomes (83.9% *v* 70.7% at 10 years; $P = .02$) and was strongly associated with evolution to secondary hemolytic PNH (19.8% *v* 4.7%; $P < .001$; Data Supplement). A lower incidence of secondary PNH evolution was instead observed in poor IST responders (5% *v* 15.8%, $P < .001$) and in male patients (6.0% *v* 12.1% in females; HR, 0.49 [95% CI, 0.27 to 0.89]; $P = .02$ in a multivariate setting).

A similar analysis on nonsevere AA (n = 192, Data Supplement) identified nontreated patients (accounting for 34% of cases) as the group with the highest risk of sMN evolution (21.9% *v* 8.9% and 8.3% at 10 years in nontreated, poor, and good IST responders, respectively; $P = .04$; Data Supplement). A slightly increased risk was also observed in AA patients with hemolytic PNH (16.4% *v* 11.5%; $P = .03$). It is noteworthy that in nonsevere cases the risk of sMN progression was not affected by age, which instead still influenced survival outcomes as well as did the presence of PNH clones (Data Supplement).

## Clinical and Molecular Features of Secondary Myeloid Neoplasms

After a median time of 4.5 (IQR 1.8-7.7) years, 94 patients (M:F ratio 1.61; median age 61 years, IQR 34-69) evolved to sMN. The majority of progressed cases were patients with severe AA or those not registering complete responses to either first or salvage therapies. Of note is that nearly all patients undergoing second-line treatment (excluding cases rescued with HSCT) showed poor responses, with only one patient achieving a complete

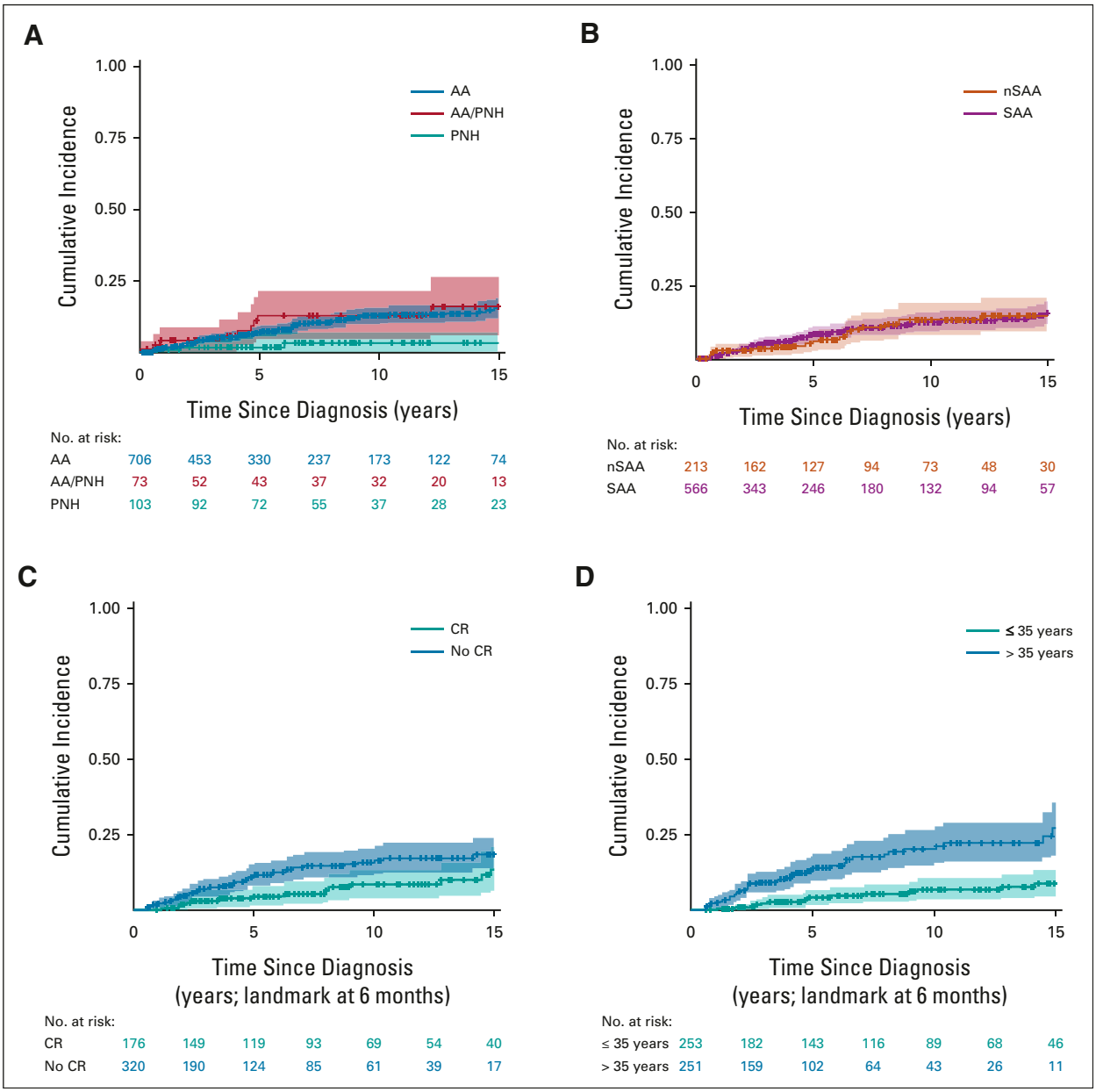


**FIG 2.** Incidence and study of factors influencing malignant progression. (A) The cumulative incidence of malignant progression according to disease phenotypes. At 10 years from diagnosis, the risk of progression to secondary myeloid neoplasms was 12.8% (10.0-15.9) for AA, 13.1% (6.0-22.9) for AA/PNH overlap syndrome, and 3.4% (0.9-9.0) for PNH ($P = .13$ globally, $P = .47$ in AA *v* AA/PNH, $P = .06$ in AA *v* PNH, and AA/PNH *v* PNH by Gray's test). According to disease severity, (B) the 10-year cumulative incidence of malignant progression was 12.4% (9.4-15.8) for severe and 13.6% (8.6-19.6) for nonsevere cases ($P = .86$ by Gray's test). Cumulative incidence curves for myeloid progression according to (C) treatment response and (D) age at diagnosis. At 10 years, the cumulative incidence of progression was 15.7% (11.5-20.6) in patients with partial or no response to treatment versus 8.5% (4.4-14.2) in cases achieving complete response ($P = .02$). At 10 years, the cumulative incidence of progression was 6.6% (3.7-10.8) in younger cases (age ≤ 35 years) versus 20.0% (14.3-26.2) in older patients ($P < .001$). Numbers at risk are color-coded and indicated below each curve. AA, aplastic anemia; CR, complete response; nSAA, nonsevere aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria; SAA, severe aplastic anemia.

response (Data Supplement). MDS (75%) was the most frequent diagnosis at evolution, followed by acute myeloid leukemia (18%) and MDS/myeloproliferative neoplasm (7%; Data Supplement). With a median follow-up of 4.7 years, the 5-year OS after sMN diagnosis reached 40% (Data Supplement) and was independently influenced by blast ≥ 5% (HR, 3.64 [95% CI, 1.82 to 7.30]; $P < .001$; Figs 3A and 3B and Data Supplement).

In contrast to the usual characteristics of the MDS population, the majority of MDS cases had higher R-IPSS

  

**TABLE 2.** Multivariate Analysis for Severe AA Cases (n = 504)

| | Overall Survival | | sMN Incidence | | sPNH Incidence[a] | |
|---|---|---|---|---|---|---|
| **Variable** | **HR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** |
| Sex | | | | | | |
| Female | 1 | | 1 | | 1 | |
| Male | 1.00 (0.69 to 1.45) | .99 | 1.39 (0.82 to 2.33) | .22 | 0.49 (0.27 to 0.89) | **.02** |
| Response to treatment | | | | | | |
| CR | 1 | | 1 | | 1 | |
| No CR | 4.45 (2.54 to 7.77) | **< .001** | 2.60 (1.39 to 4.84) | **.003** | 0.76 (0.41 to 1.41) | .39 |
| Age (10-year effect) | 1.29 (1.16 to 1.43) | **< .001** | 1.37 (1.20 to 1.56) | **< .001** | 0.88 (0.75 to 1.02) | .1 |
| Year at transplant (10-year effect) | 1.35 (0.96 to 1.88) | .08 | 1.14 (0.76 to 1.72) | .52 | 1.47 (0.95 to 2.28) | .09 |
| Center effect as frailty | | **.015** | | .97 | | **.02** |

NOTE. Bold indicates significant *P* values.

Abbreviations: AA, aplastic anemia; CR, complete response; HR, hazard ratio; SAA, severe aplastic anemia; sMN, secondary myeloid neoplasia; sPNH, secondary paroxysmal nocturnal hemoglobinuria.

[a]Only SAA.

scores because of the over-representation of poor cytogenetic risk groups. Overall, myeloid mutations (n = 82 of 94) were found in at least 80% of sMN, with 61% of mutants harboring ≥ 2 lesions (Fig 3C). Apart from cases ineligible/unfit for active treatment (44.4%), patients received chemotherapy (12.2%), hypomethylating agents (18.9%), or HSCT (36.2%), either upfront (21.1%) or as a consolidation strategy (15.1%).

Analysis of molecular characteristics resulted in the distinction of four different sMN groups (Data Supplement). In particular, cases harboring del7/7q and complex karyotype had a similar mutational burden and survival outcomes to those with normal karyotype, differing instead from patients carrying other cytogenetic alterations. *ASXL1*, *SETBP1*, *RUNX1*, and *RAS* pathway gene mutations constituted the unique signature of del7/7q carriers, and classical leukemogenic drivers such as *DNMT3A*, *FLT3*, and *NPM1* were typical of normal karyotype, whereas cases classified as others and complex karyotype had diverse molecular configurations (Figs 3D and 3E).

To further establish the discrete peculiarities of myeloid disorders arising from AA, we then applied a propensity score matching to reduce any possible confounding effects between our sMN cases (n = 94) and an internal control cohort (n = 94 matched patients with a 1:1 ratio from 3,599 de novo cases) by using age, sex, BM blast percentage, and disease type as relevant baseline covariates (confounders). As expected, we found that higher-risk R-IPSS scores (54% *v* 37%) and chromosome 7 aberrations (53% *v* 11%) and *ASXL1* (24% *v* 12%) and *RUNX1* (21% *v* 8%) mutations were more frequent in sMN as compared with de novo cases, emphasizing that MN arising post-AA constitutes a distinct nosologic entity (Data Supplement).

### Immunomolecular Dynamics of Clonal Evolution

In a cross-sectional study of clonal dynamics from baseline (n = 302, Data Supplement) to evolution, overall, 18% of patients harbored myeloid mutations at primary disease onset with a higher frequency of mutant cases found among patients with AA as opposed to PNH (Fig 4A). After exclusion of *PIGA*, mutated in up to one third of cases, the most recurrent alterations at baseline were found in *BCOR/L1*, *ASXL1*, and *TET2* genes (Fig 4B).

In cases sequenced at baseline, follow-up, and evolution, the frequency of *PIGA* mutations dramatically decreased at sMN progression, consistent with a reciprocal expansion of PNH clones and evolution to secondary hemolytic PNH in nonprogressors. The same trend was observed for somatic HLA aberrations that decreased during follow-up and at progression, whereas an opposite effect was noticed for myeloid lesions. These quantitative changes were paralleled by dynamics of mutational burden (Fig 4C and Data Supplement).

We then classified our patients on the basis of features (myeloid driver mutations only = group 1; *PIGA*/PNH clone presence and *BCOR/L1* = group 2, and no mutation = group 3) previously hypothesized to be associated with sMN evolution, with the exclusion of pure hemolytic PNH, which we have shown to behave as a distinct clinical entity with regard to sMN progression. Accordingly, a Cox proportional hazard model showed that group 1 had a higher risk of sMN ($P < .001$). Of note, the latter category of patients was characterized by older age at AA presentation when compared with the most favorable group carrying *PIGA* and *BCOR/L1*, whereas no relationship with IST response was observed (median age 37 [IQR 27-73] *v* 26 [19-48] years in groups 1 and 2, respectively; $P = .04$; Fig 4D and Data Supplement).

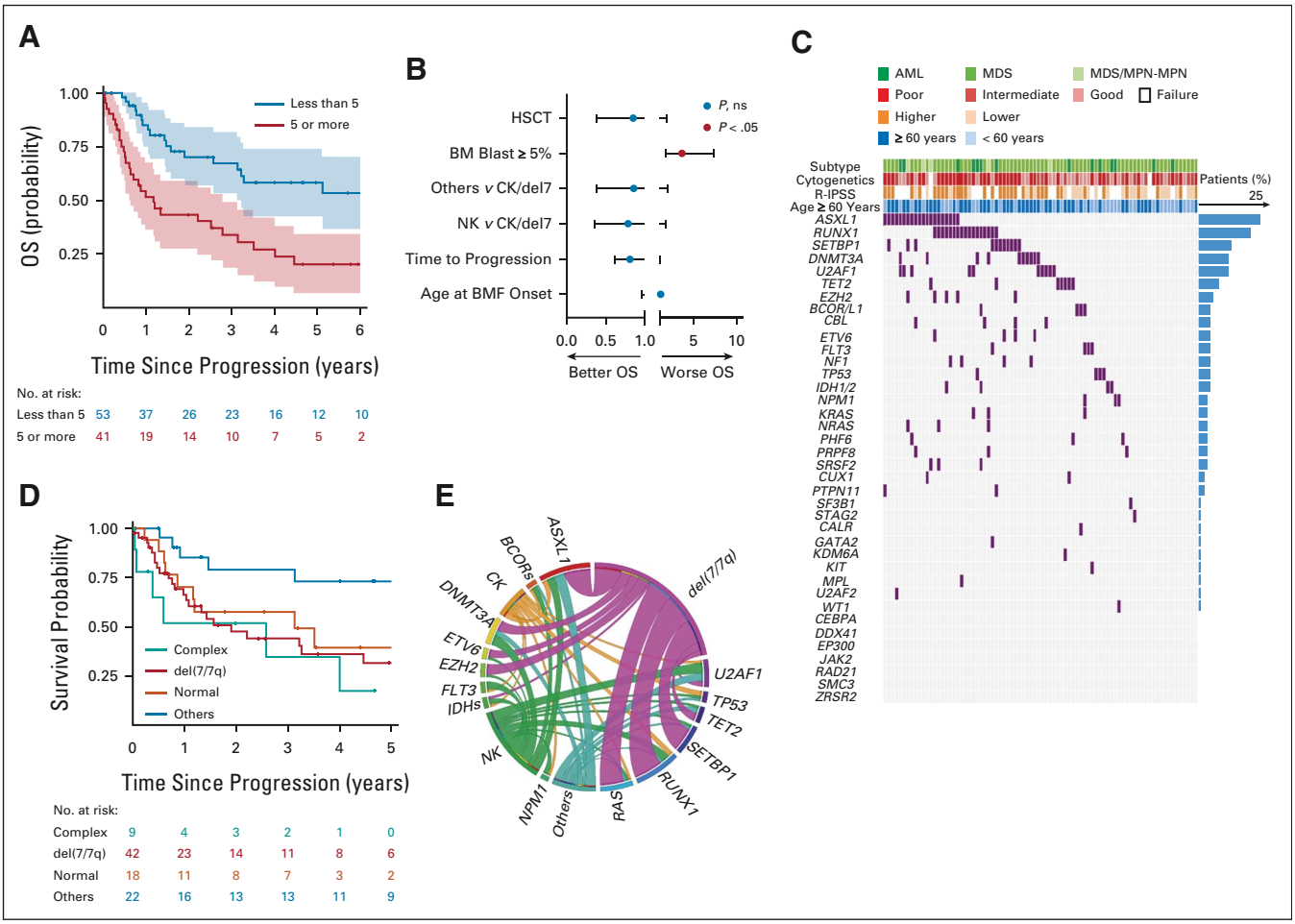


**FIG 3.** Features of malignant progression. (A) The 5-year OS according to bone marrow blast percentage at secondary myeloid neoplasia onset (58.2% [41.1-71.9] in patients with < 5% *v* 20.2% [8.6-35.3] in those with ≥ 5%). The Forest plot in (B) illustrates multivariate analysis for OS using a multivariate Cox cause–specific model. In (C), a waterfall plot depicts the genomic landscape of progressors (n = 82 patients with available molecular information). Disease subtype, cytogenetic risk groups according to R-IPSS and R-IPSS risk group (higher > 3.5; lower ≤ 3.5), and age categories (age < 60/≥ 60 years) are color-coded on top, whereas on the right side, frequencies of mutations for each gene are shown. (D) The survival curves of main cytogenetic risk groups (numbers at risk for each subgroup are color-coded and provided below the curves). In (E), a circle plot illustrates the relationship between mutations and cytogenetics. Three cases failed the cytogenetic testing and thereby were removed from the panels including cytogenetics features. AML, acute myeloid leukemia; BMF, bone marrow failure; CK, complex karyotype; HSCT, hematopoietic stem-cell transplant; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; NK, normal karyotype; ns, not significant; OS, overall survival.

The results of previous cross-sectional analysis have been confirmed longitudinally in 25 serial cases (AA onset and sMN evolution; Data Supplement). As expected, when compared with baseline, we found higher mutational (median number of mutations 0 [IQR 0-1] *v* 2 [1-2], $P < .001$) and clonal burdens (median variant allele frequency 20% [IQR 5-27] *v* 30% [19-44]; $P = .01$) at myeloid progression (Data Supplement). Interestingly, the dissection of the genomic architecture of such cases showed that 10% of mutations, which constituted the bulk of disease at evolution, were ancestral events already present at the time of BM failure onset or early in its course. Conversely, we noticed that *CUX1* mutations disappeared at progression with concomitant acquisition of deletions affecting chromosome 7 (Data Supplement). Nevertheless, no difference in time to progression was observed in patients with and without mutations shared at both time points.

## DISCUSSION

Over the course of 10 years from diagnosis, one fifth of patients with AA treated with IST will experience late clonal complications (eg, sMN and secondary PNH). Early studies on X-chromosomal inactivation and karyotyping already indicated that CH may be present in a substantial number of AA cases, possibly as a prodromal sign of the increased risk for clonal evolution.[17,31] Among these clonal cytogenetic aberrations, partial or total loss of chromosome 7 represents the

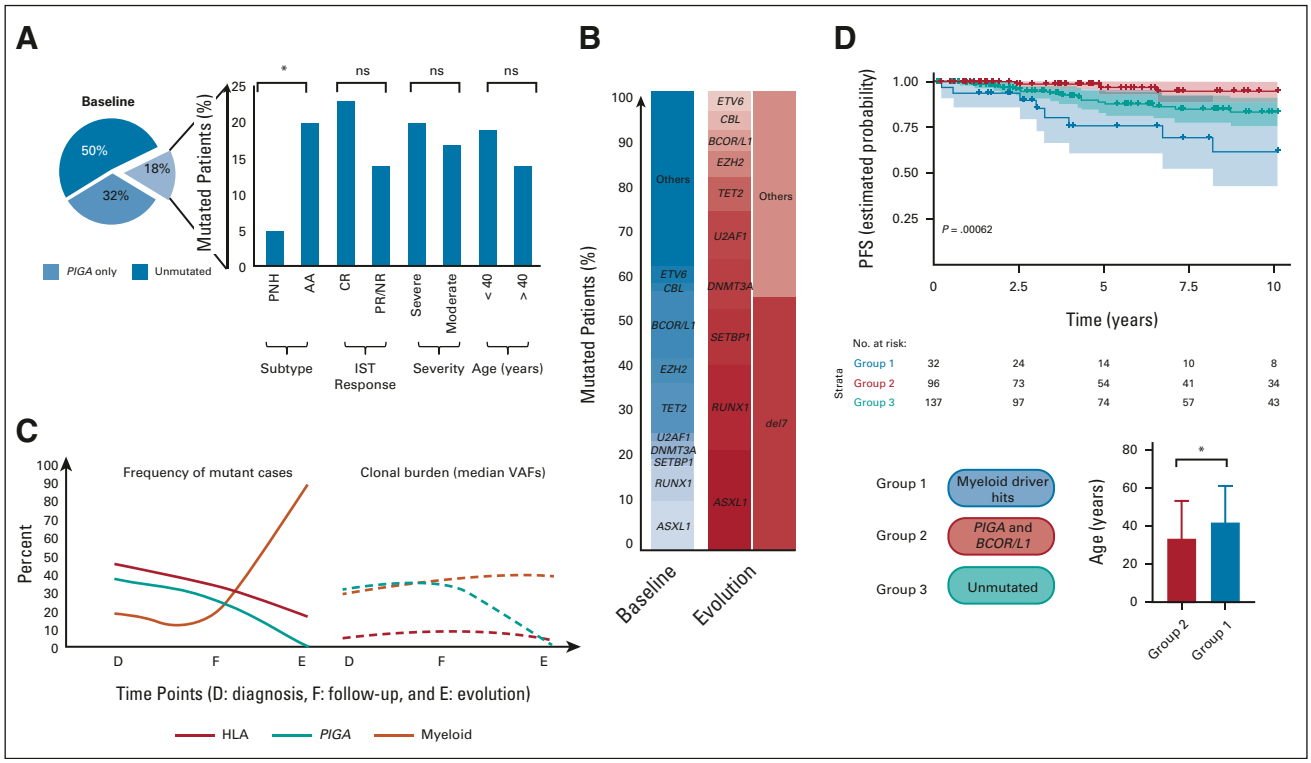


**FIG 4.** Clonal dynamics in AA. In (A), the pie chart shows the frequency of mutants for *PIGA* and myeloid mutations with a breakdown of cases according to characteristics at onset and treatment response (n = 302). Bar charts in (B) illustrate the difference in molecular spectra between baseline and myeloid progression with frequencies estimated on the total of mutants (n = 302 at baseline; n = 82 at evolution). (C) The dynamics of changes in molecular lesions (HLA, *PIGA*, and myeloid) in patients sequenced at baseline (time point D, diagnosis), follow-up, (time point F), and evolution (time point E, see the Data Supplement for details). Frequencies are shown as solid lines on the left, and clonal burdens (median VAFs at each time point) are shown as dashed lines on the right with each color representing a different set of mutations. In (D), a stratified Cox proportional hazard function adjusting for age was fit to estimate baseline progression hazard rates and generate PFS curves of patients with AA and AA/PNH (n = 265). Patients were classified on the basis of features (myeloid driver mutations only = group 1; *PIGA*/PNH clone presence and *BCOR/L1* = group 2, and no mutation = group 3) previously hypothesized to be associated with myeloid evolution (also see the Data Supplement; numbers at risk are indicated below the curves). Particularly, n = 7 patients (one evolved to secondary myeloid neoplasia) with concomitant *PIGA*/PNH clone and myeloid mutations were considered as belonging to group 2. Pairwise comparisons: group 1 versus group 2, $P < .001$; group 1 versus group 3, $P = .026$; and group 2 versus group 3, $P = .025$, log-rank test. The bar chart on the lower right illustrates the difference in age at onset in an analysis of patients with pure AA phenotype (n = 58). AA, aplastic anemia; CR, complete response; HLA, human leukocyte antigen; IST, immunosuppressive therapy; NR, no response; ns, not significant; PFS, progression-free survival; PNH, paroxysmal nocturnal hemoglobinuria; PR, partial response; VAF, variant allele frequency.

most frequent and worrisome alteration.[31] Subsequently, a systematic application of NGS further underscored how widely spread CH occurs in AA.[3,11] To that end, diverging theories on AA oligoclonality have been proposed, ranging from contraction of normal HSC pool and bystander CHIP-like phenomena to immunologic tumor surveillance reactions triggered by abnormal clones.[5] Despite progress, these issues remained unresolved because of the rarity of AA and the long latency of sMN evolution, whose today study is amenable to only retrospective analyses of large cohorts of patients, waiting for the long-term follow-up of the randomized RACE trial in which prospective sampling has been performed.[3]

In the present study, we have now precisely estimated the incidence, timing, and clinical associations of late clonal complications including response to IST. Furthermore, the availability of a large cohort of patients followed for a median of 8.6 years enabled us to characterize the clinical and genetic features of sMN evolution, by compiling the largest cohort of cases so far described.

Our results confirm that older age at diagnosis is an independent risk factor for malignant progression.[7,32] An interesting implication on this association stems from the correlation between age and CHIP in healthy individuals and the lower incidence of CH in pediatric AA cases, paralleling findings on age-related CH.[17,40] For instance, AA cases presenting at an older age may already carry somatic age-related mutations, which could be positively selected after multiple rounds of IST because of the inherent increased fitness advantage of oligoclonal over normal HSCs. On the other hand, the differential diagnosis with hypoplastic MDS (without excess blasts) occurring in older age is not trivial, even in

expert centers. Whatever the scenario is, this shares mechanistic analogies with therapy-related MN, where preexisting clones are preferentially selected, and is also reminiscent of anecdotal PNH clones expansion/contraction under specific environmental conditions.[30,33] In line with previous studies, we found that myeloid hits were present in older patients and heralded sMN progression.[11] Indeed, the acquisition of mutations in *ASXL1* and *RUNX1* genes may provide increased HSC self-renewal capabilities offering a fertile soil for clonal sweeping in the context of an immunologically stressed hematopoiesis.[11,34]

Besides age, poor response to standard IST was also found to be independently associated with an increased risk for myeloid evolution, typically accompanied by a history of failing multiple lines of treatment.[7,35] In either case, progression of CHIP, age-related CH acceleration, or Darwinian selection phenomena in the context of AA immune responses may be invoked to explain the relationship of age and IST responses with regard to myeloid evolution. One could even speculate that the same mutation may hold a diverse prognostic significance when occurring in two patients with different ages at disease onset (eg, 20 *v* 60 years). Besides biological implications, in such a scenario, therapeutic choice would be strongly influenced by age, with patients undergoing HSCT possibly protected from the development of sMN because of the complete subversion of BM hematopoiesis (eg, from oligoclonal in AA to normal, polyclonal after HSCT). This is the reason why sMN is a problem typical of the nontransplant setting, where clonal trajectories are sculpted by the re-expansion of the individual patient's hematopoiesis after IST (so-called bottleneck effect), which not only increases the number of HSCs replications with a non-negligible BM stress/telomere attrition but also generates processes of clonal dominance if mutations are already present (selection of the fittest clone).

Our results show that AA progression to sMN has unique molecular characteristics with the distinction of different subgroups characterized by discrete cytogenetic and molecular features. The high frequency of chromosome 7 abnormalities, *ASXL1*, *RUNX1*, and *SETBP1* mutations, and their dynamics with other lesions putatively derived from AA immune-pressure (*PIGA* and HLA mutations) indicate a peculiar signature of clonal evolution, different from that of other MN subtypes.[5] It is noteworthy that sMN cases acquiring monosomy 7 or del(7q) have been reported to have a shorter time to progression and a lower tendency toward association with del(5q) or *TP53* disruption as opposed to primary or treatment-related MN.[6] Indeed, we found that the majority of our cases who progressed to sMN within 5 years from the initial diagnosis carried chromosome 7 alterations. Remarkably, the aforementioned deletion 7 gene signature (*ASXL1*, *RUNX1*, and *SETBP1*) has been recently found to be associated with sMN arising in children with *SAMD9/SAMD9L* syndromes[36] and in other congenital BM failure syndromes, MN developing postautologous HSC transplantation or chemotherapy, and in the context of expanding hematopoiesis after gene therapy.[37,38]

Besides somatic myeloid mutations, other players have been identified as possible determinants of AA and PNH clonal dynamics. We have previously shown that germline HLA configurations imprint different risk of clonal progression (to both secondary PNH and sMN) and survival outcomes.[39] In this scenario, immune-privileged lesions or dysregulation of immune pathways may favor the evasion from T-cell attack and provide a sustainable balance in the context of CH, cooperating with myeloid genes in intricate ways reported as a war of clones struggling for dominance.[18,27,40]

In conclusion, we propose that AA malignant evolution may be the consequence of a relentless autoimmune attack producing a maladaptive response to immunosuppression (Data Supplement). Our results call for a close monitoring of patients with AA with classical BM smear examination and cytogenetics. Furthermore, information derived from targeted deep NGS at regular intervals throughout the follow-up time must be integrated in the survivorship plan of such patients to allow early interventions. That said, although morphological progression to MDS and occurrence of monosomy 7 are obvious triggers to discuss transplantation, the occurrence (or expansion) of isolated molecular alterations as an indication for transplant is, nowadays, highly debated (except for *TP53* mutations) and must be judiciously interpreted within the context of other variables such as age, donor's availability, and response to IST.


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## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

**Clinical and Molecular Determinants of Clonal Evolution in Aplastic Anemia and Paroxysmal Nocturnal Hemoglobinuria**

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