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Clinical significance of acquired somatic mutations in aplastic anaemia

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Abstract Aplastic anaemia (AA) is frequently associated with other disorders of clonal haemopoiesis such as paroxysmal nocturnal haemoglobinuria (PNH), myelodysplastic syndrome (MDS) and T-large granular lymphocytosis. Certain clones may escape the immune attack within the bone marrow environment and proliferate and attain a survival advantage over normal haemopoietic stem cells, such as trisomy 8, loss of heterozygosity of short arm of chromosome 6 and del13q clones. Recently acquired somatic mutations (SM), excluding PNH clones, have been reported in around 20–25 % of patients with AA, which predispose to a higher risk of later malignant transformation to MDS/acute myeloid leukaemia. Furthermore, certain SM, such as ASXL1 and DNMT3A are associated with poor survival following immunosuppressive therapy, whereas PIGA, BCOR/BCORL1 predict for good response and survival. Further detailed and serial analysis of the immune signature in AA is needed to understand the pathogenetic basis for the presence of clones with SM in a significant proportion of patients.

Key words Aplastic anaemia · Somatic mutations · Clonal haemopoiesis · MDS · AML

Introduction

Acquired aplastic anaemia (AA) is largely an immune mediated disorder that may present concurrently with clonal haemopoietic stem cell (HSC) disorders, most commonly paroxysmal nocturnal haemoglobinuria (PNH). It may later evolve to myelodysplastic syndrome (MDS) in up to 15–20 % of patients [1–3]. Furthermore, there is overlap between AA and MDS in the form of the entity hypocellular MDS which is often difficult to distinguish from AA on morphological criteria, especially when AA is of the non-severe sub-type [4]. Because AA may be associated with an abnormal cytogenetic clone in up to 12 % of patients, the finding of an abnormal cytogenetic clone does not always help in differentiating AA from hypocellular MDS, although the finding of monosomy 7 usually indicates MDS instead of AA [5]. There are specific acquired somatic mutations (SMs) that characterise these overlapping bone marrow failure (BMF) disorders, in addition to mutations of *PIGA* that occur in PNH. Acquired *STAT3* mutations occur in 40 % of patients with T-large granular lymphocytosis (T-LGL) but can also be detected in 7 % of AA and 3 % of MDS patients with unsuspected T-LGL, that is, with subclinical T-LGL clones. [6] Lastly, SMs that typify MDS and acute myeloid leukaemia (AML) have recently been reported in AA, and which are the main focus of this mini review [7, 8].

Expansion of clones that escape immune attack in aplastic anaemia

Underlying this clonal haemopoiesis is an important interaction with the immune response that occurs in AA resulting in expansion and a proliferative advantage of certain clones that can evade the immune attack, and contribute to haemopoiesis [1–3]. This is best exemplified by *PIGA* mutated HSC. Data supporting an intrinsic survival advantage of PNH HSCs are lacking. Instead, the expansion of PNH clones in AA is more likely due to an extrinsic factor, for example, immune attack, whereby PNH HSCs are

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thought to escape the immune attack that occurs in AA because they lack expression of the glycosyl phosphatidylinositol (GPI) anchor (GPI-AP-), and that the target for the immune attack of normal HSCs is the GPI-anchor itself [9, 10]. Other theories include targeting of non-PNH HSCs by LGL clones [11]. Alternatively, NK cell mediated cytotoxicity may play a role; immunoglobulin-like receptors (KIR) may be differentially expressed in PNH compared to normal, resulting in cytotoxicity of normal HSCs [12]. The presence of GPI-specific, CD1d restricted T cells in PNH supports the hypothesis that PNH HSCs preferentially expands due to immune escape [13].

In both AA and MDS the presence of trisomy 8 (+8) is associated with a high response rate to immunosuppressive therapy (IST). Bone marrow (BM) haemopoietic progenitor cells (HPC) from patients with MDS and +8 show increased expression of WT1 antigen. This induces a specific T cell response to WT1 peptides, leading to suppression of non +8 HPC through a bystander effect by activated CD8 T-cells. In contrast, +8 HPCs survive this immune attack due to increased expression of anti-apoptotic proteins survivin, cyclin D1, and increased proliferation due to increased expression of c-myc, resulting in a proliferative advantage for the +8 HPC [14]. More recent work from Hosokawa and colleagues indicates that the presence of GPI-AP- HPC among patients with +8 may be an important factor associated with the high response to IST. In a series of 53 patients with AA ($n = 22$) and low risk MDS ($n = 31$), 26 % had a PNH clones, as defined by ≥0.005 % for red cells and ≥0.003 % granulocytes. Of 26 patients who received IST, the response rate was 88 % for those with a PNH clones compared to only 41 % without a PNH clone. Furthermore, the response rate to IST in patients with +8 was lower (56 %) than for patients with normal cytogenetics (81 %). Thus, patients with +8 and a PNH clones were more likely to respond to IST compared to those patients lacking a PNH clone [15].

Using SNPA karyotyping in more than 300 AA patients, Katagiri and colleagues showed that the most common genetic lesion was copy number neutral loss of heterozygosity involving the short arm of chromosome 6 (LOH6p) in 13 % of patients [16]. This is an acquired genetic event since it was not detected in CD3+ T-cells, and it involved multiple haemopoietic cell lineages as well as early (CD34+) BM HPCs. LOH commonly affected the HLA locus with loss of expression of HLA-A antigen. In AA, the targets of cytotoxic T-lymphocytes (CTLs) are HPC that present autoantigen expressed by class I HLA molecules, particularly certain HLA-A*02:01, A*02:06, A*03:01, and B*40:02. If there is LOH6p, then the HPC have lost the target for immune attack by CTLs and hence escape the immune attack, resulting in a growth advantage and clonal expansion over unaffected HPCs.

Del13q is closely associated with *PIGA* mutant clones. In a study from Japan, patients with isolated del13q all responded to IST and none progressed to MDS/AML. All had PNH clones, and using cytoFISH, the del13q cells were only present in the non-mutant GPI-AP+ cells. Following IST, expansion of del13q clone occurred more frequently than a decrease in the clone size. So in this situation, both the del13q HSCs and the *PIGA* mutant HSCs underwent preferential expansion and contributed to haematological recovery [17].

So, could a similar process of immune escape explain the existence of some of the other acquired SMs that have been recently described in some patients with AA?

Age-related clonal haemopoiesis: clonal haemopoiesis of indeterminate prognosis (CHIP)

Before considering further the significance of acquired SMs in AA, the recent observation that acquired SMs occur with increasing age in healthy individuals is highly. Several large population based cohort studies have demonstrated that between the ages of 70–79 years, SMs are detected in 10 % of people, and rising further with increasing age thereafter [18–20]. The most frequent genes mutated were *DNMT3A, TET2, ASXL1* and less frequently *TP53, JAK2, SF3B1*, that is, genes that are frequently mutated in MDS/AML. The presence of a SM was associated with an increased risk of haematological malignancy, and increased mortality due to increase in cardiac, cerebral events and diabetes. Most frequently, there was only one SM per patient and the median allele burden (MAB) was low at 9 %, although for those individuals who later developed a haematological malignancy, the MAB was higher at around 20 %. The incidence of SMs, especially DNMT3A R882, rises with increased sensitivity of method used for detection, raising the important question of what clone size is definitely clinically relevant [21]. This entity of age-related clonal haemopoiesis has recently been termed 'clonal haemopoiesis of indeterminate potential' ('CHIP') [22].

Somatic mutations in AA

In recent years, a number of studies have reported the presence of acquired SMs in AA, often associated with low level clones (Table 1). Lane et al. screened for 219 genes in 39 patients, and found SMs in 9 (23 %), comprising *ASXL1* ($n = 2$), *DNMT3A* ($n = 1$) and *BCOR* ($n = 1$). The MAB was <10 % in 7 patients [23]. Heuser et al. found 3 mutations in 2 of 38 patients (*SLIT1*, and *SETBP1* with *ASXL1*) using a smaller panel of 42 genes and excluding SM with a MAB of <15 % [24]. However, the patient with SETBP1 and ASXL1 was tested at time of progression to MDS. In a small cohort of predominantly paediatric patients, SMs

Table 1 Summary of studies reporting SM in aplastic anaemia

	Number of patients screened	Number of genes screened	Number (%) of patients with a SM	Most frequent SM detected	VAF %
Lane 2013	39	219	9 (23 %)	ASXL1, DNMT3A, BCOR	<10 % in 7
Heuser 2014	38	42	2	SLIT1, SETBP1 + ASXL1[a]	25-50 %
Kulasekararaj 2014	150	832 (in 1st cohort of 57; rest targeted sequencing of SM detected in 1st cohort)	29 (19 %)	ASXL1, DNMT3A, BCOR	20 % (<10 % in 41 % patients)
Yoshizato 2015	439	106	24 % (36 % including PIGA)	ASXL1, DNMT3A, BCOR/BCORL1, PIGA	9.5 %
Babushok 2015	22 (mostly children)	WES	16 (72 %); 2 (9 %) with an MDS-associated SM	PIGA, LOH6p, STAT5B, CAMK2b	>20 %

WES whole exome sequencing, *VAF* variant allele frequency

[a] This patient was reported to have most likely progressed to MDS at time of investigation

were detected in 72 %, most frequently involved in immune escape (*PIGA, LOH6p*) and signal transduction (*STAT5B, CAMK2G*), and MDS-associated SM were found in only 9 % of patients [25].

From our King's College Hospital database of 345 patients with idiopathic BMF, we were the first to describe the molecular profile in a large cohort of 150 AA patients with no morphological evidence of MDS and who had stored BM and skin/buccal mucosa samples. We excluded all patients with a known constitutional BMF disorder or a family history of BMF or cancer [26]. The first cohort of 57 patients was screened using a custom panel of 832 gene exons and for the second cohort of 93 patients, more targeted sequencing was performed for genes identified in the first cohort. We identified a subgroup (19 %) with pathogenetically relevant SMs in a relatively small number of genes (*ASXL1* in 12 patients, *DNMT3A* in 8, *BCOR* in 6 and one each for *SRSF2, U2AF1, TET2, MPL, IKZF1* and *ERBB2*. The MAB was 20 %, and for 41 % of SMs the MAB was <19 % clone. SMs, when examined together, predicted for risk of later evolution to MDS; the risk was 38 % compared to 6 % in the absence of a SM ($p < 0.001$), and if the disease duration of the AA was >6 months, the risk of MDS was even more significant at 40 % compared to 4 % without a SM ($p < 0.0002$) (see Table 2). *ASXL1* and *DNMT3A* mutations were associated with evolution to monosomy 7 in 4 AA patients. We also showed that presence of a SM was associated with shorter telomere length compared to patients who lacked a SM. Patients in the first cohort of 57 patients were also screened for *PIGA* mutations. 23 of the 57 had a PNH clone by flow cytometry, and of these, 17 had a PNH clone size >10 %. In 7/17 a single *PIGA* mutation was found, double *PIGA* mutations were present in 6, and a *PIGA* mutation with another SM in 4 patients (*BCOR* in 2, and one each with *ASXL1* and *IKZF2*). A *PIGA* mutation was not detected in any of the 6 patients where the PNH clone size was <10 %, indicating that flow cytometry is far more sensitive than *PIGA* sequencing at detecting small PNH clones [26].

Subsequently, a combined Japanese and USA study reported targeted sequencing of 106 genes in 439 AA patients, with whole exome sequencing in 52, and serial sampling in 82 patients [27]. The most frequently mutated genes were similar to the King's College Hospital study with the exception of *BCORL1* (which was not in the King's panel) and *PIGA* (in the Kings study, PIGA was only screened for in the first cohort of 57 patients). SMs were found in 36 % of AA patients, and in 24 % of patients if *PIGA* SMs were excluded. SMs increased with increasing age (except for *BCOR/BCORL1* and *PIGA*). Most of the SMs were present at a lower MAB at diagnosis compared to 6 months after IST. The NIH cohort identified so-called 'favourable' SMs, *BCOR* and *PIGA,* which were associated with better response to IST and better overall survival, in contrast to 'unfavourable' SMs (*DNMT3A, ASXL1, TP53, RUNX1, JAK2, JAK3, or CSMD1,* although predominantly *DNMT3A* and *ASXL1*) as a group were associated with poorer response to IST and worse survival (see Fig. 1) and progression to MDS/AML. They also showed that monosomy 7 detected at 6 months was associated with poor survival and progression to MDS. The impact of unfavourable SMs was even more significant in patients aged <60 years, raising the possibility that in future, factors such as 'unfavourable' SMs may help identify patients who might be

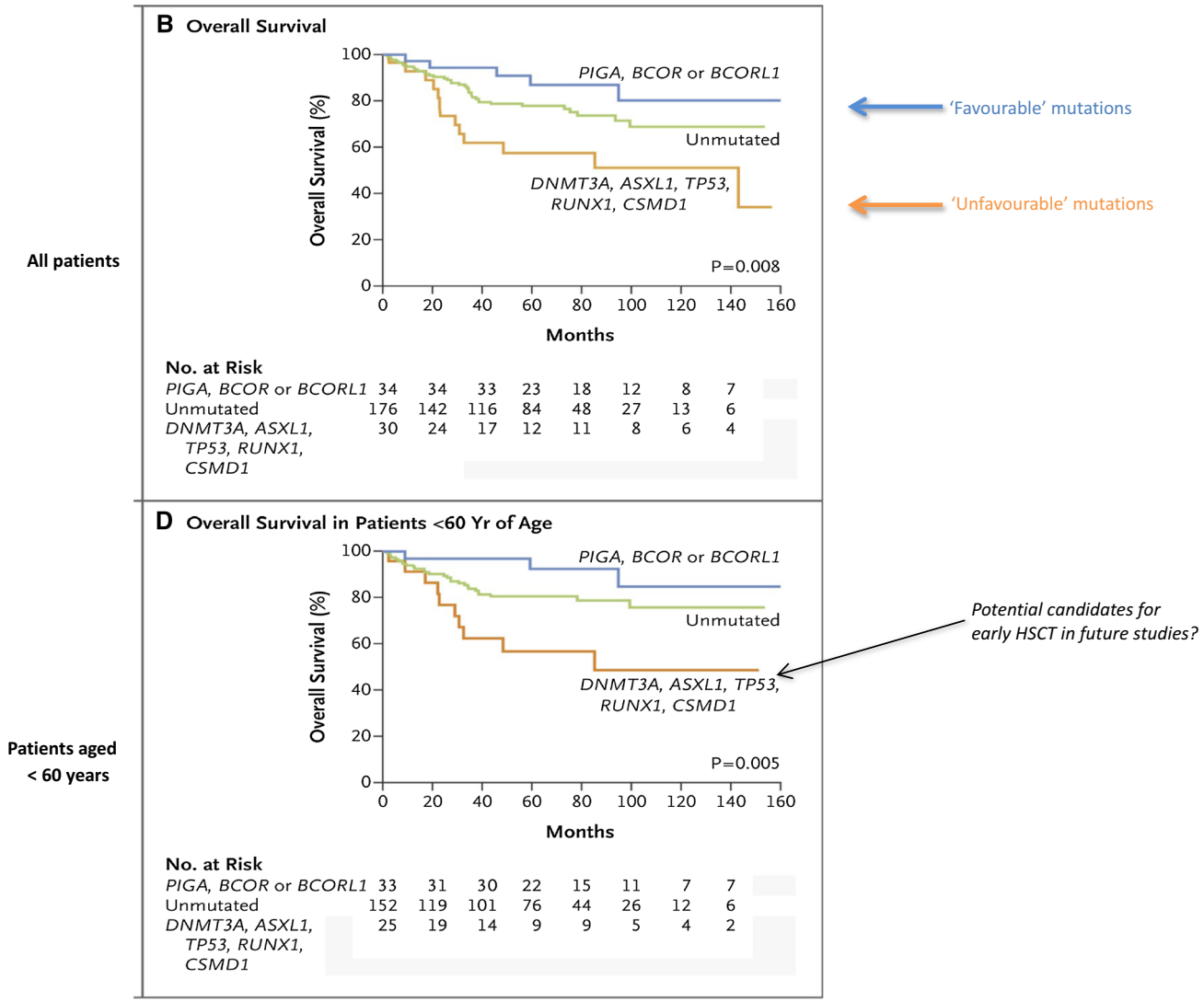


Fig. 1 Impact of somatic mutations on survival after immunosuppressive therapy (IST). Aplastic anaemia patients with so-called 'unfavourable' SMs (*ASXL1, DNMT3A*) show significantly worse response to and overall survival after IST compared to patients with 'favourable' SMs (*PIGA, BCOR/BCORL1*). The impact of 'unfavourable 'SMs was more marked in patients aged <60 years. Modified from Yoshizato et al. [27], reproduced with permission

considered for alternative treatment strategy such as early allogeneic haemopoietic stem cell transplantation (HSCT). Using whole exome sequencing to examine clonal architecture, highly variable patterns were seen. In most patients, clonal haemopoiesis originated from a minor clone present at diagnosis. In some cases, clones were stable over many years. When they examined the pattern of change in clone size for individual SMs, the 'unfavourable' clones (*ASXL1* and *DNMT3A*) more often continued to enlarge, but not in all cases and some even disappeared over time. In contrast the 'favourable' SMs (*BCOR, PIGA*) were more likely to remain stable or decrease in size. The NIH group also highlighted in a separate study the contribution of increased telomere loss to the subsequent acquisition of SMs and emergence of monosomy 7 in a cohort of 13 SAA patients treated with IST and sampled serially post IST [28].

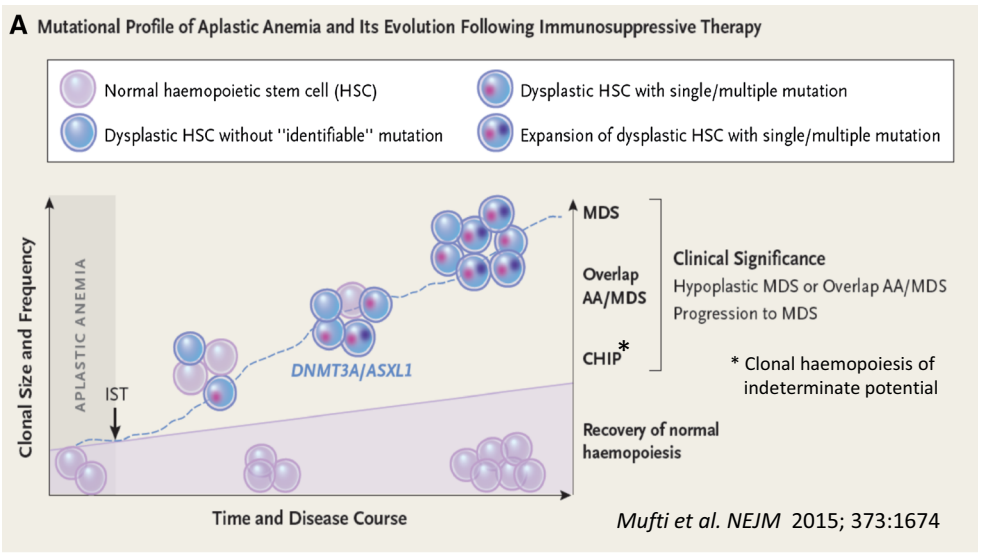
Features of SMs in AA compared to MDS, clonal cytopenia of uncertain significance (CCUS) and normal individuals

Acquired SMs occur more frequently in AA than in older age healthy individuals, but less frequently than in MDS (see Table 3). Many of the SMs seen in AA are similar in type to MDS, clonal cytopenia of uncertain significance (CCUS) which comprises 35 % of patients with idiopathic cytopenia of uncertain significance (ICUS) and who have a SM, and healthy people, except that there are more cases of *BCOR* and

Table 2 Risk of evolution to MDS in aplastic anaemia patients with an acquired somatic mutation

Disease duration	Mutations ($n = 29$)	Wild type ($n = 121$)	P value
<6 months ($n = 63$)	9	54	
Transformation to MDS	3	3	<0.03
Median mutant allele burden <10 %	7	NA	
>6 months ($n = 87$)	20	67	
Transformation to MDS	8	3	<0.0002
Median mutant allele burden <10 %	4	NA	

A somatic mutation was found in 29/150 (19 %) of patients with AA. In the presence of a SM, the risk of MDS is 11/29 (38 %) compared to 6/121 (6 %) $p < 0.001$. If the disease duration is >6 months, the risk of MDS is 40 % compared to 4 % without a SM. Reproduced with permission from Kulasekararaj et al. [26]



B Frequency of 4 Commonly Mutated Genes in Aplastic Anemia From the Two Studies

Top 4 Genes	Kulasekararaj et al	Yoshizato et al
DNMT3A	8.3%	8.4%
ASXL1	8%	6.2%
BCOR/BCORL1	4%*	9.3%
PIGA	NA	7.5%
Median VAF	20%	9.3%

[Kulasekararaj. Blood 2014; 124: 2698
Yoshizato. NEJM 2015; 373:35]

Fig. 2 Mutational profile of aplastic anaemia and its evolution following immunosuppressive therapy, and frequency of 4 commonly mutated genes in aplastic anaemia. Reproduced with permission from Mufti et al. [31]

PIGA and a lower frequency of splicosome mutations, *RUNX1* and *TET2* in AA. In AA, one can identify good and poor prognosis SMs as discussed above. The MAB is in most AA patients low and lower than in MDS or CCUS [29]. Lastly, the number of SMs per patient in AA is less than in MDS and more similar to the number in older aged healthy individuals.

Questions and future perspectives

AA patients who are treated with IST, may recover normal HSC, or they may acquire sequentially SMs and dysplastic changes resulting in clonal expansion to MDS/AML. Complicating this picture is the so-called 'overlap AA/hypocellular MDS' syndrome of patients where it is not possible on morphological grounds to clearly distinguish AA from hypocellular MDS (see Fig. 2) [30]. Current data reviewed above now demonstrate that SMs (excluding *PIGA*) occur in around 20–24 % of patients who clearly have AA and with no morphological evidence of MDS [26, 27]. These patients have with an increased risk (40 % if the duration of AA is >6 months) of later developing MDS/AML [26]. Furthermore,

Table 3 Features of somatic mutations in aplastic anaemia (excluding PIGA mutations)

	Normals[a] (CHIP)	AA[b,c]	CCUS[¥]	MDS[d]
Frequency	~2 % (40–49 years) ~3 % (50–59 years) 5.6 % (60–69 years) 9.5 % (70–79 years) 11.7 % (80–89 years)	19 % (Med age 44 years)[b] 24 %[c]	35 % of ICUS cases	70–80 % (median age 72 years)
Most common	DNMT3A, TET2, ASXL1, JAK2	ASXL1, DNMT3A, BCOR/BCORL1, DNMT3A, ASXL1	TET2, DNMT3A, ASXL1, TP53	SF3B1, TET2, ASXL1, DNMT3A, SRSF2, RUNX1
Prognosis	↑ Risk haematological cancer, coronary heart disease, ischaemic stroke, DM type 2	Good prognosis: BCOR/BCORL1; poor prognosis: ASXL1, DNMT3A[c] For all SM together, 38 % risk of later MDS/AML[b]	↑ Risk of MDS/AML	Good prognosis: SF3B1 Poor/neutral: the rest
Mean VAF %	9 %	20 % (<10 % in 40 % pts)[b] 9.3 %[c]	30 %	30.4 %
Number of mutations per patient	1 (in 93 % individuals)	1 (in 64 % patients[c] 1 (in 90 % patients)[b]	Mean 1.3	Median 3 (0–12)

CHIP clonal haemopoiesis of indeterminate potential, *CCUS*[¥] clonal cytopneia of uncertain significance, *ICUS* idiopathic cytopenia of uncertain significance, *VAF* variant allele frequency

[a] Jaiswal S. N Engl J Med. 2014;371:2488

[b] Kulasekararaj AG. Blood. 2014;124:2698

[c] Yoshizato T. N Engl J Med. 2015;373:35

[d] Haferlach T. Leukemia. 2014;28:241; Papaemmanuil E. Blood. 2013;122:3616

patients with an 'unfavourable' SM (*ASXL1*, *DNMT3A*) have a lower response rate to IST and worse survival; in contrast, 'favourable' SMs (*BCOR, PIGA*) are associated with good response and survival after IST [27]. However, these exciting new data have raised several as yet unanswered and important questions (Table 3).

1. What is the clonal architecture, mutational hierarchy and chronology of genetic events in AA? To help answer this, the European Blood and Marrow transplant (EBMT) Severe Aplastic Anaemia Working Party has established a prospective randomised trial for newly diagnosed SAA patients of standard IST with horse antithymocyte globulin (ATG) and ciclosporin with or without eltrombopag ('RACE' trial) [31]. The main outcome measure is complete response at 6 months. Patients will be closely monitored for morphological and cytogenetic evidence of later transformation to MDS/AML in view of the reported risk of MDS/AML (with high frequency of monosomy 7) in 18 % of patients when eltrombopag is used as a single agent in the treatment of refractory SAA [32–34]. As part of this clinical trial, blood and BM samples will be taken pre and at set time points after ATG for a key research study, the aims of which are to examine the evolving clonal architecture and mutational hierarchy at the genomic level with serial sampling, and to correlate results with the immune signature that predicts response and later risk of evolution to MDS/AML.
2. Are SMs in AA related to ageing? Age-related clones occurring at low level may represent a predilection/founder stage that requires later cooperating mutations for clonal expansion and disease. In AA, the detection of both small and relatively large disease clone populations in AA likely indicates different stages of clonal evolution rather than normal ageing [26].
3. What is the significance of low-level mutant clones? They may represent sub-populations of disease clones present at an early stage, but are they relevant to disease progression? Might they fluctuate over time in a similar manner to PNH clones or abnormal cytogenetic clones? Do they arise by selective protection from immune destruction analogous to PNH [9], LOH6p [16], +8 [14, 15] and del13q [17] clones? Other clones may be kept under control by process of immune surveillance leading to their elimination.
4. Do SMs in AA indicate a diagnosis of hypocellular MDS rather than AA? It is evident that new diagnostic criteria are needed to help differentiate these two disorders, and molecular testing should now be incorporated into new diagnostic criteria.
5. 'Unfavourable' SMs, *DNMT3A* and *ASXL1,* help to identify patients with poor response to IST and worse

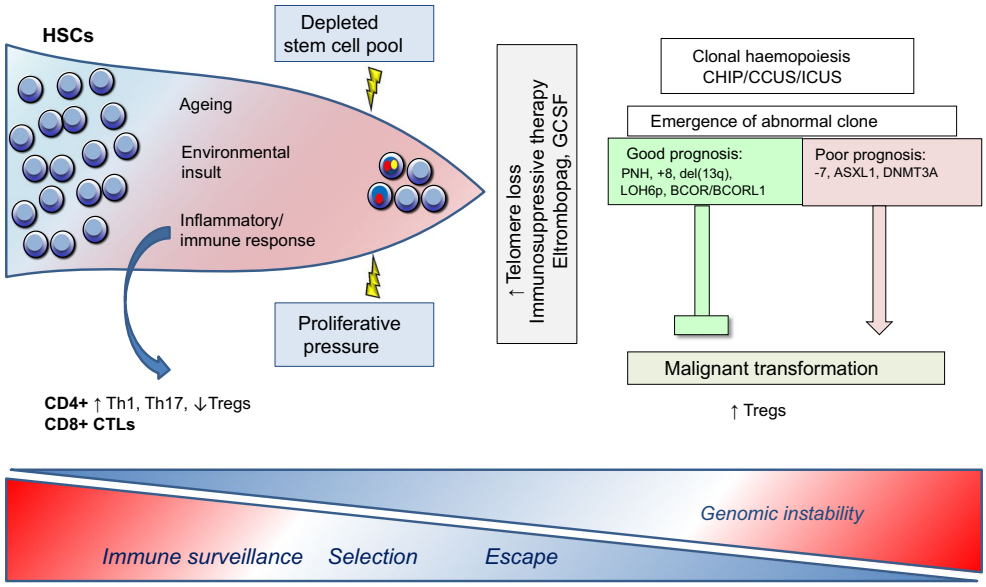


Fig. 3 Clonal haemopoiesis in acquired aplastic anaemia. An initial insult to the BM HSCs triggers an inflammatory immune response with an increase in CD4+ T-helpers and reduction in Tregs, which are dysfunctional in terms of suppressing auto-reactive CD8+ T-cells, resulting in oligoclonal expansion of CD8 + CTLs. The depleted stem cell pool and increased proliferative pressure contribute to increased telomere attrition. A shift in the immune response from immune surveillance through selection to escape with increased Tregs, and concurrent increasing genomic instability, result in emergence of abnormal clones. Repeated courses of IST, eltrombopag and prolonged and high doses of G-CSF are associated with increased risk of MDS/AML. Poor prognosis clones include monosomy 7, ASXL1 and DNMT3A, with increased risk of malignant transformation to MDS/AML, whereas good prognosis clones such as PNH clones, +8, del(13)q and BCOR/BCORL1 are not associated with increased malignant transformation. *Tregs* regulatory T-cells; *CTLs* cytotoxic T-lymphocytes

survival, especially patients aged <60 years [27] and SMs in general predict for a high risk of later MDS/AML [25]. Should we now incorporate these results into other known poor prognostic factors such as short TL [35], low absolute lymphocyte and reticulocyte counts at the time of diagnosis? [36]. Should such patients now be considered for an alternative treatment strategy, specifically allogeneic HSCT? This is now timely in the light of recent improved outcomes of fludarabine-based HSCT, especially those using alemtuzumab, which is associated with a very low risk of GVHD [37–40].

6 Lastly, what is the correlation of the immune response with the emergence and clonal architecture of SMs in AA, and is there a specific immune signature that predicts for malignant transformation? Following an initial insult to the BM HSC, likely viral, an inflammatory immune response is initiated characterised by an increase in CD4+ T-helpers, Th1 (clonal expansion), and Th17 cells, and a reduction in Tregs which are also dysfunctional in terms of suppressing auto-reactive CD8+ T-cells, resulting in oligoclonal expansion of CD8+ CTLs [3]. The depleted stem cell pool and the increased proliferative pressure contribute to increased telomere attrition. A shift in the immune response from a state of immune surveillance through selection to escape with increased Tregs, with concurrent increasing genomic instability, results in emergence of abnormal clones. Repeated courses of IST [41], eltrombopag [33] and prolonged and high doses of G-CSF [42] are associated with increased risk of MDS/AML, especially with emergence of monosomy 7. Poor prognosis clones include monosomy 7, ASXL1 and DNMT3A, with increased risk of malignant transformation to MDS/AML, whereas good prognosis clones such as PNH clones, +8, del(13)q and BCOR/BCORL1 are not associated with increased malignant transformation (see Fig. 3). Finally, further understanding of the immune signature that occurs with the acquisition of SMs will be key to determining the pathogenetic mechanism for clonal transformation in SAA, using, for example, multi-dimensional mass cytometry, as recently reported by our group [43].

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