EWOG-SAA

Current standard diagnostics for severe aplastic anemia in children and adolescents

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1) Definition of aplastic anemia

Acquired aplastic anemia (AA) is a rare hematological disorder characterized by peripheral blood pancytopenia and hypoplastic bone marrow. In the majority of cases, the underlying pathophysiological mechanism is an immune-mediated damage to hematopoietic stem cells.

Historically, acquired AA has been defined as pancytopenia with at least two of the following criteria: absolute neutrophil count (ANC) < 1.5 x 10^9/L, platelet count < 50 x 10^9/L and hemoglobin < 10 g/dl and bone marrow cellularity below 25%. Following the Camitta criteria, the severity is classified as follows:

Severe aplastic anemia (SAA):

- ANC < 0.5 x 10^9/l,
- platelets < 20 x 10^9/l,
- reticulocyte count < 20 x 10^9/l, and
- < 25 % of normal cellularity in bone marrow biopsy.

Very severe aplastic anemia (VSAA) is defined by the same criteria except ANC < 0.2 x 10^9/l.

Patients fulfilling the original criteria of aplastic anemia, but not the ones for SAA or VSAA are frequently referred to as moderate or non-severe AA. In the majority of these patients pancytopenia is associated with other defined disorders and they should not generally be included in SAA protocols.

2) Initial work-up and differential diagnosis

Differential diagnosis of AA: To establish the diagnosis of acquired AA, exclusion of inherited bone marrow failure syndromes (IBMFS) or other causes of pancytopenia by careful past and present history, family history and thorough physical examination. Patients with acquired AA usually have only symptoms caused by cytopenia. Careful differential diagnosis is necessary in case of unusual manifestation with other non-hematological abnormalities.

Patients with Fanconi anemia, telomere biology disease (TBD), Shwachman Diamond syndrome (SDS), congenital amegakaryocytic thrombocytopenia (CAMT), SAMD9/9L syndrome, GATA2 deficiency and MECOM deficiency can present with severe pancytopenia and hypoplastic bone marrow, although the histological picture of these patients is rather compatible with refractory cytopenia of childhood (RCC) than that of AA. The differential diagnosis of RCC and SAA is made by histological evaluation of bone marrow biopsy. Elevated MCV and fetal hemoglobin suggest RCC or IBMFS rather than AA. Screening tests such as chromosome breakage test to exclude Fanconi anemia (FA) and telomere-length analysis to exclude TBD are mandatory in patients with suspected diagnosis of AA. Presence of PNH clone support acquired bone marrow failure rather than IBMFS. Short telomere-length suggests an underlying TBD and indicates extensive physical examination, family history and further molecular analysis. However, patients with AA and RCC can have short telomere-length.

Additional genetics test are initiated based on the clinical phenotype. Next generation sequencing panels to identify the increasing number of germline mutations associated with IBMFS and/or predispositions syndromes and mutations associated with myeloid malignancies are being established. These analyses are not currently standard diagnostic work-up for AA but are performed individually in case of clinical features or family history suggesting a predisposition syndrome.

Patients with varied immunodeficiency or autoinflammatory disorders such as ligase IV deficiency, adenosine deaminase 2 deficiency and SAP deficiency can present with pancytopenia and bone marrow hypoplasia. Rarely patients with acute lymphoblastic anemia present with pancytopenia and severely

aplastic bone marrow. Morphological differentiation between leukemic blasts and hematogones can be difficult. In such case, flow-cytometry and molecular analysis are necessary for the differential diagnosis.

Hepatitis associated AA and AA following viral infection: Prior non-A, -B, -C, -D, -E and -G hepatitis is reported in about 5 to 15 % of patients with AA and called hepatitis associated AA. Typically hepatitis associated AA occurs in young male patients concurrently or within 6 months after hepatitis. Patients with hepatitis AA typically have severe lymphopenia (CD4+T lymphopenia). Rarely, viral infections such as hepatitis viruses, EBV, CMV, HIV-1, parvovirus, COVID and VZV can cause AA.

Bone marrow examination: The diagnostic evaluation of bone marrow morphology and histology is mandatory to confirm the diagnosis of AA. It can be difficult to differentiate AA from hypoplastic RCC and IBMFS. Therefore, they need to be evaluated by experienced reference pathologists. If the result of the first bone marrow biopsy is not conclusive for the diagnosis of aplastic anemia, second bone marrow examination with a biopsy should be done **within 1-2 weeks** to confirm the diagnosis. Bone marrow examination with a trephine biopsy and cytogenetic analysis is necessary. If an insufficient number of metaphases (< 20 metaphases) are obtained, FISH analysis to exclude monosomy 7 and trisomy 8 is necessary.

Importance of early diagnosis and start of therapy: Although the precise diagnosis of SAA and exclusion of other diagnoses such as RCC and IBMFS are important, start of immunosuppressive therapy (IST) should not be delayed due to prolonged diagnostic procedures, which can lead to morbidity and mortality due to infection and inferior response to IST.

Table 1 Checklist of initial work-up in patients with suspected diagnosis of aplastic anemia

Past history	➢ Previous CBC ➢ Unexplained cytopenia, chronic thrombocytopenia or neutropenia prior to pancytopenia ➢ Episode of hepatitis (maximal transaminase and bilirubin, time of onset, serology) (*Hepatitis associated AA*) ➢ Previous infections (bacterial, viral, fungal) ➢ Joint pain, rash and diarrhea (*immunological disease*). ➢ Nutrition status (Vitamin B12, folate, iron, zinc and copper). ➢ Medication: benzenes, chloramphenicol, cytotoxic medications, ionizing radiation
Present history	➢ Time of onset of symptoms ➢ Characterization of symptoms ➢ Current infection
Family history	➢ Parental consanguinity ➢ Familial thrombocytopenia, leukemia, other malignancy and immunodeficiency ➢ Skin abnormalities such as café-au-lait spots, abnormal pigmentation, nail dystrophy, leukoplakia, liver / lung fibrosis ➢ Early childhood death, miscarriages

Physical examination	Signs suggesting other cause of pancytopenia: short stature, failure to thrive, microcephaly, abnormal limbs, lymphadenopathy, hepatosplenomegaly, joint swelling, skin rash, icterus, café-au-lait spots, nail dystrophy, leukoplakia, other congenital anomalies and facial abnormalities, hematuria
Basic laboratory test	➢ Complete blood count (CBC) including a full differential blood count, MCV and reticulocyte count ➢ Microscopic evaluation of smears ➢ Regular laboratory examination including LDH, liver transaminase, blood urea nitrogen, creatinine, total and direct bilirubin, haptoglobin ➢ Hemoglobin F ➢ Direct and indirect Coombs test ➢ ANA screening
Immunological test	Lymphocyte subsets (CD3, CD4, CD8, CD19, CD56) Immunoglobulin G, M and A In cases of suspected immune deficiency/immune dysregulation additional immunological tests might be indicated.
Virus specific serology	Hepatitis A, B, C, E (serology) Herpes Simplex (serology) EBV (serology and PCR) CMV (serology and PCR) Parvovirus B19 (serology and PCR) HIV (combined antigen/antibody test, or serology and PCR)
BM examination	➢ Bone marrow aspirate including a full differential count and smears (evaluated by national reference cytologist/pathologist) ➢ Bone marrow biopsy (evaluated by national reference cytologist/pathologist) ➢ Metaphase cytogenetics (evaluated by national reference cytogenetist) ➢ Fluorescence in situ hybridization (FISH) including at least chromosome 7 and 8
Additional mandatory tests	➢ Exclusion of FA by DEB or MMC testing (growth arrest in flow cytometry or increased chromosomal breakage) (molecular genetic analysis of FA associated genes alone is not recommended) ➢ Telomere-length analysis (qPCR) or Flow FISH is mandatory in patients with suspected AA ➢ PNH clone ➢ HLA typing of the patient and family

	➢ Donor search if no matched sibling donor is available
Genetic test	➢ Analysis of *GATA2* and *SAMD9/9L* genes are currently the standard therapy in the EWOG-SAA study, which should be performed in the reference laboratory. ➢ Additional genetic tests are indicated based on the clinical phenotype. Next generation sequencing panels to identify the increasing number of germline mutations associated with bone marrow failure and/or predispositions syndromes and mutations associated with myeloid malignancies are being established. These analyses are not currently standard diagnostic work-up for AA but are performed individually in case of clinical features or family history suggesting a predisposition syndrome.