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TITLE: Revisiting acquired aplastic anaemia: Current concepts in diagnosis and management

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ABSTRACT: Acquired aplastic anaemia is a rare, serious, immunologically-mediated bone marrow failure syndrome, characterised by marrow hypoplasia of varying severity and significant pancytopenia. Careful attention and investigation, including molecular testing, is required to confirm the diagnosis and exclude other mimicking conditions, such as inherited bone marrow failure syndromes. In a proportion of patients, the disease evolves to myelodysplasia or acute myeloid leukaemia and in some there is an association with paroxysmal nocturnal haemoglobinuria. The disease has a major impact on patient quality of life. Haematopoietic stem/progenitor cell transplantation for eligible patients with an available donor is the only current curative therapy. Other patients may receive immunosuppression, most commonly with anti-thymocyte globulin and cyclosporin. An initial response to immunosuppression is often encouraging but relapse is common. Supportive care, including management of transfusion requirements and infections, is central to management. Promising new diagnostic tools and emerging therapies will likely transform approaches to this important, chronic and life-threatening condition.

KEY WORDS: anaemia, aplastic; pancytopenia; bone marrow transplantation; anti-thymocyte globulin; blood transfusion

INTRODUCTION

Acquired aplastic anaemia (aAA) is a rare bone marrow (BM) failure syndrome (BMFS) characterised by BM hypoplasia of varying severity and significant pancytopenia. Patients may present to a range of medical and other health professionals, typically with manifestations of cytopenias, such as infection and bleeding. The condition often poses challenging diagnostic and management issues. At presentation, the differential diagnosis may be broad, as several conditions including inherited BMFS (IBMFS) can mimic aAA. While initial and subsequent treatment strategies are well established, new approaches to relapsed and/or refractory disease have recently emerged. Challenges include the expanding role of molecular lesions in aAA and the complexities of managing infectious risks and long-term transfusion support. This review provides an overview of current aAA diagnosis and management.

EPIDEMIOLOGY

Incidence of aAA varies with age and geographical location. In Europe and North America, the incidence is 2-3 per million per year, and is 2-3 fold higher in East Asia.[1, 2] The observed sex ratio is close to 1:1. Age at onset is bimodal, with peaks in young adulthood and in the elderly.[3] Certain medications and toxins and hepatitis have been implicated in causing or triggering aAA in some patients. Chloramphenicol was considered a common 'cause' of aAA in the 1950s.[4] Other reported agents include anti-epileptics, allopurinol, phenylbutazone, sulfonylureas and sulphonamides, and more recently, some new immunotherapeutics, such as nivolumab.[5]

The Australian Aplastic Anaemia Registry was established to capture and quantify key aspects of aAA such as incidence, causative associations and efficacy of treatment modalities in the Australian population. The Registry consists of a reporting network of essentially all major Australian hospitals that treat the disorder;

the first patient was enrolled in 2013 and more than 130 cases have been registered to date. At the present rate of data accumulation, statistically valid insights into the ethnic, toxin/drug exposures and other influences on disease causation and impact on outcomes of current and emerging treatment programs will be obtained in a manner representative of the overall population. This is in contrast to many previously reported surveys based on selected patient subgroups where genetic, environmental, socio-economic, diagnostic and reporting factors probably contribute to observed differences.

PATHOGENESIS

Progressive BM failure in aAA was formerly attributed to idiosyncratic reactions to drugs or chemicals, such as chloramphenicol and benzene, or certain viral infections. Autoimmune toxicity to the haematopoietic stem/progenitor cells (HSC) in aAA was identified approximately forty years ago as the major causative mechanism; immunosuppressive therapy (IST) with minimal HSC toxicity (typically by infusion of antiserum raised in animals against human T-cell-rich tissue – anti-thymocyte globulin, ATG), was followed by varying degrees of recovery in the majority of aAA patients.[6, 7] Additive benefit from combination with cyclosporin (CsA) further supported this concept.[7, 8]

Considerable *in vitro* evidence demonstrates that toxicity from activated cytotoxic T-cells inhibits haematopoiesis in aAA.[9] In response to a number of putative initiating events, such as environmental exposure to a virus or drug, oligoclonal expansion of T-cells with highly restricted V-beta chain sequences occurs. In most cases however no precipitating viral aetiology is demonstrated.[10] Typically the oligoclonal T-cell population decreases following IST and returns upon relapse, with the degree of V-beta diversity predicting for response to IST.[11] Altered T-cell receptor (TCR) signalling has also been demonstrated, contributing to abnormal T-cell function.[12] Subsequent production of myelosuppressive cytokines, including

interferon gamma and tumour necrosis factor alpha, leads to apoptosis of HSCs. This aberrant response is insufficiently suppressed by normal immunoregulatory mechanisms, with reduced function of regulatory T-cells (Treg), natural killer cells and mesenchymal stem cells.

Certain human leucocyte antigen (HLA) alleles have been associated with susceptibility to aAA and response to IST, possibly through generation of autoreactive T-cell clones or effects on Tregs. Recurrent somatic mutations in HLA Class I genes in aAA suggest autoimmunity is HLA Class I-driven.[13]

Inherited BMFS involve inheritance of genes associated with defective DNA repair and premature tissue ageing, as in Fanconi anaemia (FA), or defective telomere maintenance in dyskeratosis congenita (DC). These IBMFS can present in later childhood or even adulthood, and generally do not respond to IST. Distinction from aAA is important for patient management and family counselling. Detailed discussion of IBMFS diagnosis and management is available elsewhere.[7, 14, 15]

Short telomeres may be seen in the leucocytes of patients with aAA. Normal, age-related telomere loss is largely compensated for by the telomerase repair complex, but mutations in elements of this complex (telomerase RNA component [TERC] and telomerase reverse transcriptase [TERT]) are risk factors for BMFS including aAA, and in aAA are associated with suboptimal haematopoietic recovery after ATG therapy, disease relapse and clonal progression as well as increased incidence of myeloid malignancy.[14, 16, 17]

Clonal evolution to myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) occurs in up to 10-20% of patients.[7, 14, 18] Mutations in myeloid malignancy candidate genes including ASXL1, DNMT3A and BCOR are present in up to half of aAA patients; several of these mutations have prognostic implications for progression to MDS/AML. ASXL1, DNMT3A and RUNX1 mutations are associated with higher rates of

malignant change.[18, 19] There is also some evidence that better outcomes and responses to IST might occur in patients with BCOR and PIGA mutations.[18]

Paroxysmal nocturnal haemoglobinuria (PNH) clones are present in around 50% of patients with aAA.[20] The PNH clone size in aAA is frequently small, with one study describing a median clone size in the peripheral blood (PB) at diagnosis of 0.60% in granulocytes and 0.15% in red blood cells.[21] Flow cytometry is more sensitive than PIGA gene sequencing for detecting the presence of small clones.[22] The clinical significance of PNH clones in aAA is uncertain, but several studies have reported that patients who harbour a PNH clone have a favourable response to IST.[23] In some cases, small PNH clones can expand to cause clinically overt PNH.

PRESENTATION

Patients with aAA usually present with a relatively recent onset of clinical features resulting from cytopenias – commonly fatigue and other anaemia-related symptoms such as exertional dyspnoea, easy bruising, menorrhagia and mucosal and petechial bleeding secondary to significant thrombocytopenia, with clinical manifestations generally proportional to the degree of cytopenias.[7, 24] Infections are less common initially but may be present. In the French Cooperative Group for Epidemiological Study of Aplastic Anemia, 87% of patients had a haemoglobin (Hb) $\leq$100g/L at diagnosis, 94% had a neutrophil count $\leq 1.5 \times 10^9$/L and 99% had a platelet count $\leq 100 \times 10^9$/L.[25] In 5-10% patients, a non-viral marker positive hepatitis may precede aAA.[26]

DIAGNOSIS

The diagnosis of aAA occurs in the setting of cytopenia/pancytopenia and BM hypoplasia/aplasia with exclusion of other known causes of BM failure.[7, 24] Detailed history and physical examination focussing on excluding mimicking disorders is essential. A history of drug, alcohol, occupational and toxin exposures and recent illness or pregnancy should be sought, as should evidence of rheumatologic/autoimmune disease and malignancy. Family history**,** especially in young patients to assess for possible IBMFS, is crucial. Late presentations of IBMFS may occur particularly following stressors such as chemotherapy. Family history should encompass cytopenias, congenital abnormalities, dyskeratosis, malignancy and organ (especially pulmonary and hepatic) dysfunction.

Physical examination should consider signs attributable to cytopenias including pallor, bruising, petechiae and infection. Features in the patient or family members which may suggest an IBMFS include absent digits or radii, microcephaly, micrognathia, short stature, nail abnormalities and abnormal skin and/or hair pigmentation. Cognitive functional deficits and scars from cardiothoracic or other corrective surgery may be present. Classical features in the individual or family are helpful, but importantly their absence does not preclude a diagnosis of IBMFS.

Investigations (see Table 1) aim to document the features and severity of aAA, and exclude alternative causes of cytopenia/pancytopenia.[7, 14, 24]

Diagnosis of aAA requires at least two of the following: Hb <100 g/L, platelets <50 x 10^9/L and PB neutrophils <1.5 x 10^9/L with hypocellular BM and the absence of abnormal infiltrate or fibrosis.[27] The Camitta criteria[24, 28], described in 1975 for selection of aAA patients appropriate for HSC transplant (HCT), are still used today with minor modifications to assess severity; these take into account the degree of PB

cytopenia and BM hypocellularity. Severity is a strong predictor of survival and is determined by the neutrophil count; very severe: 0-0.2 x 10^9/L, severe: 0.21-0.5 x 10^9/L and non-severe: ≥0.5 x 10^9/L. Severe aAA also requires BM cellularity to be ≤25% and in addition to marked neutropenia, requires either a platelet count of ≤20 x 10^9/L or a reticulocyte count of ≤20 x 10^9/L. Very severe cases fulfil severe aAA criteria with a lower neutrophil count.

There may be a history of single lineage cytopenia, particularly thrombocytopenia, assumed to be immune-mediated (ITP) and possibly refractory to corticosteroid. Careful review of previous Full Blood Examination (FBE) abnormalities should be undertaken for clues such as macrocytosis, anaemia and leucopenia. Flow cytometry assesses for both lymphoproliferative disease and PNH clone/s.

Molecular testing is increasingly important, and readily available, with detection of mutations associated with BMFS and myeloid malignancies of major value for both prognosis and therapeutic decision-making. Specific testing for IBMFS is available and should be undertaken to exclude IBMFS even in those without typical extramedullary phenotypic features, especially in younger patients. Molecular testing can be especially useful and informative given the traditionally difficult and hard to reproduce testing methodologies for IBMFS, such as chromosomal fragility testing.

Further investigations such as blood group, red blood cell (RBC) phenotype/genotype, and patient and family HLA typing should be performed as appropriate to inform initial and further management.

TREATMENT

Established algorithms for initial aAA management incorporate symptomatology, severity, age, comorbidities, HLA-matched sibling donor availability and response to IST.[24] IST and HCT can restore haematopoiesis; the latter may be curative. Patients should be referred early to a specialist centre. HLA-typing of siblings should be performed as soon as the diagnosis is made in transplant-eligible patients to confirm whether a matched sibling donor is available and to help guide initial management decisions.
In patients <40 years with an HLA-matched sibling donor, HCT is recommended first-line therapy.[24, 29] Since the first successful allogeneic HCT for aAA was performed in 1972[30] survival has improved due to advances in many aspects of care, especially graft-versus-host disease (GVHD) prophylaxis, with excellent 5- and 10-year survival in young patients with HLA-matched sibling donors.[31] There is a strong age effect, with best survival for patients <20 years, and worst for those >40 years due to higher rates of GVHD and graft failure.[32] Patients who maintain effectively functioning donor BM after HCT have substantially lower rates of development of MDS/AML compared with long term survivors of IST.[33]

Historically, transplant-related mortality (TRM) has been significantly higher with mismatched/unrelated donor transplants, with these typically reserved for patients failing IST, however outcomes have improved and an unrelated donor search is an important part of the work up of transplant-eligible patients without a matched sibling, particularly in paediatrics, where upfront matched unrelated donor and sibling transplants have shown comparable long-term survival rates.[34] Haploidentical transplants are an option for some patients who have failed IST and lack suitable matched donors. Cord blood transplantation is another option if no other donors are available and there is an adequate total donor nucleated cell dose.[24, 32] Recently, haploidentical family member donors have been used with success in HCT for aAA.[35]
Conditioning regimens for HCT vary depending on age, donor type and centre preference. It is important to exclude an IBMFS pre-transplant, as standard regimens can cause fatal toxicity in these patients, and to

avoid using a sibling donor with the same genetic predisposition. BM is the preferred HSC source rather than PB, with lower rates of acute and chronic GVHD and TRM, and comparable risks of rejection.[36]

IST (ATG with CsA) remains standard first-line therapy in patients with very severe/severe aAA >40 years, for all patients with adequate performance status without a suitable donor, and for patients with non-severe aAA who are bleeding, transfusion-dependent or require treatment for other reasons.[24, 37] ATG therapy is frequently associated with fever, rash and worsening thrombocytopenia; prednisolone is used to ameliorate these effects. Horse-derived ATG was associated with increased response rates and 3-year survival when compared with rabbit ATG in a randomised trial in which both were combined with CsA.[38] Data from the Australian Aplastic Anaemia Registry indicate that horse ATG is more commonly used in Australia (personal communication, E Wood, February 2018). Reported response rates with ATG and CsA are 50-70%, with excellent long-term survival in many responders.[8] However, haematological recovery after IST is frequently incomplete, and is poor in approximately 20% of patients, suggesting either extensive loss of HSCs, and/or inadequate immune suppression. One third of responders relapse by two years, but the majority respond to retreatment. Failure to respond can be due to evolution (e.g. to MDS) or incorrect initial diagnosis, such as unrecognised IBMFS.[6-8] Patients who fail to respond or relapse after initial IST, can be considered for allogeneic HCT if a donor is available, or for further IST.[24] Relapse after IST can respond to re-introduction of CsA alone.[6] Rabbit ATG can be considered in this setting, and can be tolerated and effective even after problematic toxicity with previous horse ATG.[24]

Management of disease not suitable for or refractory to IST and/or HCT can be challenging. In addition to supportive care to maintain reasonable quality of life, other therapies may be considered, including a trial of androgen or erythropoietin/G-CSF, or clinical trials, where available. More recently, the thrombopoietin

receptor agonist eltrombopag has shown promise for the treatment of severe aAA. In a phase II study of aAA refractory to IST, eltrombopag was associated with single or multi-lineage cell responses and occasionally sustained responses after tapering of the drug.[39] Eltrombopag was subsequently associated with improved haematological responses compared to historical controls when added to standard IST in treatment-naïve aAA patients.[40] The mechanism of action in aAA is not known but it may directly stimulate proliferation of small numbers of residual HSCs.[40] A large randomised, placebo-controlled trial of eltrombopag in addition to IST, versus IST alone, as front-line therapy in severe aAA is currently underway (ClinicalTrials.gov NCT02099747). Second generation agents are also in clinical trials.

SUPPORTIVE CARE

Supportive care is crucial to all aspects of aAA management in order to maintain best possible quality of life.[24, 41] aAA-specific tools to assess quality of life have been lacking, but are in development.[42]

Psychosocial support: Given the rarity, complexity and impact of this chronic life-threatening disease, age-appropriate psychosocial support for patients and families is extremely important.

Infection management: Infection is the leading cause of death[1, 24] and patients with PB neutrophils <0.5 x 10^9/L are at significantly increased risk of severe infection. Duration of neutropenia is a risk factor for infection. Bacterial infections are the most common cause of serious infections in aAA patients, while invasive fungal infections are the leading cause of death.[43]

Various strategies have been employed for prevention and treatment of infections – mostly extrapolated from studies in acute leukaemia and HCT.[41] There are differences between infection risk and profile in these patient groups, and no randomised controlled trials of prophylactic antimicrobials in aAA, but antibiotic prophylaxis should be considered in patients with severe aAA.[24] Prophylactic fluoroquinolones decrease mortality, febrile episodes and bacterial infections in neutropenic patients with acute leukaemia and

allogeneic HCT.[44] Prophylactic antifungals with a mould-active azole are often recommended, however institutional policies vary. Viral infections are less common, and whilst data are limited, prophylaxis with acyclovir or valaciclovir is often recommended during treatment.[24] *Pneumocystis jiroveci* infection is uncommon.

Tichelli[45] demonstrated that G-CSF in severe aAA patients treated with ATG/CsA resulted in fewer infections and days in hospital but had no effect on overall survival, remission rates, relapse rates or mortality. Concerns exist regarding the potential for increased risk of evolution to MDS/AML with G-CSF use, although this has not been borne out in practice.[32] G-CSF is not presently recommended as a routine adjunct to IST, nor alone in aAA therapy.[24, 32]

Granulocyte transfusions may be considered as adjunctive therapy in individual patients with severe refractory bacterial or invasive fungal infections secondary to severe neutropenia, however benefits remain unproven.[46]

Transfusion support: Transfusion should be used judiciously to relieve symptoms relating to anaemia and thrombocytopenia, bearing in mind the potential for short- and long-term complications, including adverse reactions, alloimmunisation, and iron overload.

Few clinical trial data exist to guide transfusion in patients with BMFS including aAA. Current practice is largely based on expert recommendations and extrapolated from data in other settings.[24, 47] The threshold for RBC transfusion is patient-specific, and dependent on symptoms, comorbidities, Hb level, and impact of anaemia and transfusion on quality of life. Patients may require long-term transfusion support and therefore should have an extended RBC phenotype (or genotype) determined, preferably before their initial transfusion, due to risk of alloimmunisation and subsequent difficulties in provision of compatible RBC.[24] The decision to provide phenotype-compatible RBC from initial transfusion or only after alloimmunisation depends on local policy and availability.

Recommendations for platelet transfusion in aAA come largely from studies in patients with therapy-induced thrombocytopenia from acute leukaemia or HCT.[24] In hospitalised aAA patients on therapy, prophylactic platelet transfusion is generally given to prevent spontaneous haemorrhage when the platelet count is <10 x 10^9/L, or <20 x 10^9/L if there are additional risk factors for bleeding, the presence of fever or sepsis, or if the patient is receiving ATG; higher thresholds may be used in stable outpatients for practical reasons. There is no present evidence to favour apheresis over pooled platelet products. In cases of platelet refractoriness, testing for HLA and HPA antibodies should be performed, and if detected, matched apheresis platelets used if available.

Irradiation of blood components decreases the risk of transfusion-associated GVHD and should be used in aAA patients receiving alemtuzumab- or fludarabine-containing regimens, and those undergoing allogeneic HCT (from time of conditioning, whilst on GVHD prophylaxis and usually for at least 12 months post-transplant or until lymphocytes are >1x10^9/L or if there is evidence of chronic GVHD).[24] Consideration should be given to using irradiated components in other aAA patients receiving immunosuppression; some institutions use these for all aAA patients in an attempt to decrease the risk of allosensitisation.

RBC and platelet products manufactured in Australia and New Zealand are leucodepleted at source. In the most recent guidelines from Australia and New Zealand Society of Blood Transfusion and the UK Advisory Committee on Safety of Blood, Tissues and Organs, these components are considered to be “CMV safe” and suitable for haematology patients and allogeneic HCT recipients.[48, 49] In Australia the use of CMV safe or CMV seronegative blood components for these patients is dictated by institutional policies. Granulocyte transfusions are not leucodepleted so granulocytes from CMV negative donors should be given to CMV negative patients.[48]

Adjunctive agents: Haemostatic agents such as tranexamic acid may be used to minimise mucosal or other bleeding, however no trial data are available to guide management specifically in aAA.[50]

Iron chelation: Patients with aAA receiving regular RBC transfusion are at risk of iron overload.[24, 51] Very high ferritin levels (>3000 microgram/L) and transferrin saturation (>100%) are associated with decreased overall survival following allogeneic HCT and increased HCT-related mortality.[52] Deferasirox is safe and efficacious in aAA[51] and guidelines recommend consideration of chelation therapy when the serum ferritin is >1000 microgram/L.[24] This ferritin threshold is however derived from studies in other conditions. The decision to commence chelation should be patient-dependent, considering factors such as response to therapy and anticipated duration of transfusion support. Phlebotomy is the treatment of choice in iron overload after HCT.

SUMMARY

aAA is a rare, serious immunologically-mediated BMFS. Careful investigation, including molecular testing, is required to confirm the diagnosis and exclude other mimicking conditions, such as IBMFS. In a proportion of patients, the disease evolves to myelodysplasia or acute myeloid leukaemia and in some patients there is an association with PNH. The disease is associated with major morbidity and mortality and has a major impact on quality of life. Haematopoietic stem/progenitor cell transplantation for eligible patients is the only current curative therapy. Other patients may receive immunosuppression, most commonly with ATG and cyclosporin. Initial response to immunosuppressive therapy is often encouraging but relapse is common. Supportive care, including management of transfusion requirements and infections, is central to management. Promising new diagnostic tools and emerging therapies will likely transform approaches to this important, chronic and life-threatening condition.

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Table 1: Investigations in the work up for suspected aAA (including exclusion of mimicking disorders).

Initial tests	
Blood tests	FBE with film, reticulocyte count, renal function, electrolytes, liver function, coagulation, lactate dehydrogenase, haptoglobin, direct anti-globulin test, thyroid function, erythrocyte sedimentation rate, C-reactive protein, flow cytometry/PNH testing [or on BM if appropriate]
BM examination	Aspirate, trephine including reticulin stain, cytogenetic/FISH analysis. Molecular studies may provide evidence for or against the mimicking disorder hypoplastic MDS and may also confer prognostic information in aAA. Testing for genes frequently mutated in myeloid malignancy should be performed and include, but are not limited to DNMT3A, ASXL1, TET2, RUNX1, SRSF2, U2AF1, SF3B1 and TP53. DNA samples should be stored for further analysis as necessary
Exclusion of an alternative diagnosis	
Infection	Serology for Hepatitis A, B and C, HIV, Parvovirus B19, Epstein-Barr virus, Cytomegalovirus and other infections as clinically indicated
IBMFS	Comprehensive genetic studies, including molecular analysis and where relevant chromosomal fragility test, telomere length Organ assessment as directed by history/examination e.g. echocardiogram for cardiac abnormalities and pulmonary hypertension, renal ultrasound, assessment of skeletal abnormalities by plain X-ray
Autoimmune	Antinuclear antibody, extractable nuclear antibody, anti-double-stranded DNA antibody, antineutrophil cytoplasmic antibodies, rheumatoid factor,

	anti-CCP antibody, complement levels.
Nutritional deficiencies and chemical toxins	Iron studies, Vitamin B12, folate, copper and zinc levels, others as appropriate
Lymphoproliferative disorder	Flow cytometry, TCR gene rearrangement studies, selected serum/urine protein studies, imaging e.g. abdominal ultrasound, CXR, CT as appropriate

Acronyms and Abbreviations	
aAA	Acquired aplastic anaemia
AML	Acute myeloid leukaemia
ATG	Anti-thymocyte globulin
BM	Bone marrow
BMFS	Bone marrow failure syndrome
CsA	Cyclosporin
DC	Dyskeratosis congenita
FA	Fanconi anaemia
FBE	Full blood examination
GVHD	Graft-versus-host disease
HCT	Haematopoietic stem/progenitor cell transplant
HLA	Human leucocyte antigen
HSC	Haematopoietic stem/progenitor cells
IBMFS	Inherited bone marrow failure syndromes
IST	Immunosuppressive therapy

ITP	Immune-mediated thrombocytopenia
MDS	Myelodysplastic syndrome
PB	Peripheral blood
PNH	Paroxysmal nocturnal haemoglobinuria
RBC	Red blood cell
TCR	T-cell receptor
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
Treg	Regulatory T-cells
TRM	Transplant-related mortality

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