Title: Pathogenesis, risk factors and therapeutic options for autoimmune haemolytic anaemia in the post-transplant setting

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Summary

Autoimmune haemolytic anaemia (AIHA) is a rare complication of allogeneic haematopoietic stem cell transplantation (HSCT), observed with an incidence of 1-5%. Paediatric age, diagnosis of non-malignant disease, lympho-depleting agents in the conditioning regimen, use of unrelated donor, graft *versus* host disease and infections have been associated with a higher risk of AIHA post HSCT. Post-HSCT AIHA is associated with high mortality and morbidity, and it is often very difficult to treat. Steroids and Rituximab are used with a response rate around 30-50%. These and other therapeutic strategies are mainly derived from data on primary AIHA, although response rates in post-HSCT AIHA have been generally lower. Here we review the currently available data on risk factors and therapeutic options. There is a need

for prospective studies in post-HSCT AIHA to guide clinicians in managing these complex patients.

Short running title: AIHA post HSCT

Keywords

- Autoimmune haemolytic anaemia
- Haematopoietic stem cell transplantation
- Autoimmunity
- Primary immunodeficiency
- Metabolic disease
- Rituximab

Review

Introduction

Autoimmune haemolytic anaemia (AIHA), together with other less common autoimmune cytopenias (AIC), has been increasingly reported as a complication of allogeneic hematopoietic stem cell transplantation (HSCT). A growing amount of literature has been published on this subject, mostly retrospective studies analysing incidence and risk factors among recipients of HSCT. The incidence of post-HSCT AIHA is around 1-5% but it has been described in up to 20% of specific patient cohorts, in particular children with non-malignant disorders.[1-3] Factors such as use of unrelated donor, lympho-depleting agents in the conditioning regimen, presence of graft *versus* host disease (GVHD) and infections are thought to contribute to the development of this complication. Post-HSCT AIHA is difficult to treat and it is associated with high morbidity and increased risk of mortality. Therapeutic strategies are mainly derived from primary AIHA, although there is lack of robust scientific evidence and post-HSCT AIHA is associated with other comorbidities (immunocompromised host, infections, presence of GVHD) that complicate further the treatment.[4] Patients with AIHA have double the risk of mortality than other patients post HSCT.[5] Recent reports have shown a reduced mortality for patients treated in the last 10 years, suggesting that the availability of

new drugs (Rituximab, Sirolimus, Bortezomib) may have been beneficial in controlling the disease and reducing the toxicity associated with prolonged steroid use and splenectomy.

This review presents a comprehensive update on the current literature regarding pathogenesis, incidence, risk factors and available treatment options.

Autoimmune haemolytic anaemia

AIHA is a disorder characterized by an autoimmune destruction of red blood cells (RBC). It is an acquired condition, which presents as a primary disease or secondary to other conditions (most commonly infections, malignancies and autoimmune disorders). AIHA classification is based on the type of autoantibodies: warm AIHA (wAIHA), cold (cold agglutinin disease [CAD]), mixed or atypical forms. Two third of patients with primary AIHA have wAIHA, around one third CAD, and approximately 5% the mixed type.[6]

AIHA is classically characterized by: normocytic anaemia, increased mean corpuscular haemoglobin concentration (MCHC), unconjugated hyperbilirubinemia, decreased haptoglobin (which binds free haemoglobin [Hb] chains), increased lactate dehydrogenase and increased reticulocytes. The cornerstone of AIHA diagnosis is the direct anti-globulin test (DAT), where autoantibodies bound to the RBC surface are detected through human anti-globulins. In the context of haemolysis, a positive DAT indicates an autoimmune origin. DAT is positive for IgG alone or IgG and complement (C3d) deposition in typical wAIHA. IgM autoantibodies usually detach from RBC during processing, so that DAT is positive for C3d only, and negative (or weak positive) for IgG in CAD. A cold agglutinin titre (above 1:64 at 4°C) confirms the diagnosis of CAD. In some cases, DAT may be negative because of the low sensitivity of the assay, or in case of warm IgM or IgA autoantibodies. Other tests, particularly the polybrene test, could be useful in diagnosis cases of DAT-negative AIHA.[7] Ultimately, DAT-negative AIHA can be diagnosed on the basis of the clinical picture, exclusion of other causes of haemolysis and response to steroids. Importantly, DAT can be positive in the absence of haemolysis in various situations: in healthy donors (<0.1%), in patients with paraproteinemia and autoimmune conditions, as well as a result of therapy such as intravenous immunoglobulin (IVIG), anti-thymocyte globulin (ATG) and Daratumumab.[8,9]

In wAIHA, RBC destruction is mediated by IgG, autoantibodies that bind antigens on the RBC surface. Macrophages in the spleen and liver are able to phagocyte the coated RBC through the Fc receptor for Fc fragment of IgG. Haemolysis occurs mainly in the extravascular compartment. Occasionally, a small amount of complement fixation can occur causing intravascular haemolysis. Autoantibody targets include peptides from the rhesus system, band 3 protein and glycophorin A. Sometimes, no specificity can be determined.[10]

In CAD, IgM autoantibodies bind RBC at low temperature in the extremities and directly activate the classical complement pathway. C3b remains bound on RBC surface and triggers RBC phagocytosis by Kuppfer cells in the liver, causing chronic extravascular haemolysis. Intravascular haemolysis occurs during exacerbations due to the activation of the terminal complement and RBC lysis via the membrane attack complex. The antigens targeted in CAD are from the Ii blood group system.[11]

As AIHA autoantibodies target shared RBC antigen, haemolysis can also occur against transfused RBC, an important consideration when transfusion support is needed (see specific section).

A number of mechanisms play a role in why autoimmune antibodies arise. In patients with infections, it has been postulated that molecular mimicry is the mechanism at the basis of autoantibodies production: pathogen antigens which share similarity to RBC surface molecules may induce cross-reactive antibodies. Drugs can directly bind to the RBC surface and induce specific antibodies, or they can mediate the formation of immunocomplexes with IgM.[12] Moreover, in patients with infections or lymphoproliferative disease, polyclonal B and T-cell activation may contribute to the origin of an autoimmune process. The T-cell compartment is also thought to play a role in the autoimmune process. A skewed T-cell repertoire in favour of a T-helper (Th) type 2 phenotype due to increased interleukin (IL)-10, IL-4 and IL-2 and decreased interferon-γ,[13] and Th17 phenotype[14] have been demonstrated in patients; defects in regulatory T cells (T reg) were shown in animal models.[15]

AIHA post haematopoietic stem cell transplantation

Published data on the incidence and risk factors for AIHA and AIC post HSCT are summarized in Table I. The incidence of AIC varies from 2 to 7% in most reports, but can reach 22-56% in particular settings.[2,3] AIHA accounts for most cases of post-HSCT AIC with a variable incidence from 0.7% to 5.6%. A particularly high incidence (19-21%) has been reported in infants with severe combined immune deficiency (SCID) undergoing haploidentical HSCT (haplo-HSCT)[1] and in children with metabolic diseases treated with umbilical cord blood transplantation (UCBT).[2] In the majority of cases, AIHA occurs at 5-10 months post HSCT.

Most patients who develop autoimmune haemolysis in the post-transplant setting experience wAIHA.[16] The diagnostic approach is similar to primary AIHA, although mild forms can easily be unrecognized due to the common occurrence of anaemia by various causes post-HSCT.

The mechanisms that underlie autoimmunity post-HSCT have not been fully elucidated but poor immune-reconstitution, resulting in loss of self-tolerance, appears to be critical. The thymus-derived self-tolerant T cells are lacking in the early phase post-HSCT, as thymus is damaged by numerous insults (the conditioning regimen, steroids, infections, and GVHD).[17] Peripheral tolerance, mediated by T cells, is thus the predominant mechanism. This is highly affected by lympho-depleting agents (particularly Alemtuzumab) and in certain settings (haplo-HSCT), with an imbalance in reconstitution of T reg, compared to effector and helper T. Horn *et al.* found that >70% of children with AIHA had abnormal T cell reconstitution with reduced CD4 and CD8 numbers as well as abnormal proliferative responses. They hypothesized that the delayed T cell reconstitution with lack of T reg, characteristic of the T-deplete haploidentical setting, was responsible for the emergence and persistence of self-directed B cells.[1] Koo *et al*. demonstrated a reduced number of CD4 and CD8 in children with post-HSCT AIHA compared to controls.[18] Moreover, an imbalance in T cell reconstitution compared to B cells, with relative increase in B cell number, has been described.[19,20] Infections and presence of GVHD could possibly trigger an expansion of B and T cells, with the development of autoimmune clones. Indeed, cytomegalovirus (CMV) infection has been reported as a risk factor for post-HSCT AIHA. CMV could elicit both B cell expansion with production of autoantibodies, and CD8 expansion with further imbalance of the T-cell repertoire.[21] The cytokine profile in children with post-HSCT AIHA has also been described as defective, with a Th2 prevalence that is known to favour autoimmunity.[21]

The majority of reported cases of AIHA occurs in the context of full donor chimerism, suggesting that autoantibodies are derived from donor plasma-cells against donor RBC.[5,18,20,22] However, a recipient *versus* donor response have been postulated in a small number of cases with mixed donor chimerism.[3,5,23] Cwynarski *et al* reported 6 cases of AIHA happening in the context of mixed chimerism and molecular relapse of chronic myeloid leukaemia. As AIHA resolved after administration of donor lymphocyte infusion (DLI) and reversal to full donor chimerism in 3 out of 5 patients, a recipient-anti-donor process was hypothesized by the authors.[23]

Incidence of AIHA post HSCT

Table I reports the results of studies evaluating incidence and risk factors of AIHA post HSCT.

Adults

One of the first adult series reported 12/272 (4.4%) cases of AIHA post HSCT, identifying the use of unrelated donor and presence of chronic GVHD (cGVHD) as risk factors in multivariate analysis. AIHA was not the primary cause of death but added morbidity in these patients.[24] A study from Eurocord analysed the incidence of autoimmune diseases (AID) in 778 recipients of UCBT (both adults and children). AIHA occurred in 2.5% of patients. In multivariate analysis, a diagnosis of non-malignant disease and a short interval between primary diagnosis and UCBT were identified as risk factors for AID.[22] The group from King's College, London, reported a cumulative incidence (CI) of AIHA of 3.6% in adults post HSCT. The presence of AIHA increased both the overall mortality and the transplant-related mortality (TRM) in this cohort. Indeed, 4/19 patients died as a direct consequence of AIHA. The only risk factor associated with AIHA was the use of unrelated donor.[5] In the largest report, a Spanish multicentre study, AIHA incidence was 1.5% among 4099 adults and children who received HSCT. Disease free survival (DFS) was 52% at 40 months for the whole cohort. Factors associated with a better DFS at 40 months in multivariable analysis were paediatric age (DFS 89% if ≤15 years *vs* 19% if >15 years) and response to treatment (DFS for those achieving complete remission [CR] was 74% *vs* 22% if partial remission or no remission).[16] Lv *et al* analysed a series of 1377 adults transplanted for malignant diseases: 3-year incidence of AIHA was 2.2%. After multivariate analysis, they identified the presence of cGVHD and haplo-HSCT as risk factors for AIHA.[25]

Children

The first case series of AIHA post-HSCT in children appeared 20 years ago and reported high incidence of AIHA (19.5%) among 41 children with SCID who underwent T-depleted haplo-HSCT. Use of peripheral blood stem cells (PBSC) was the risk factor identified in this cohort. AIHA significantly contributed to morbidity and mortality in that setting.[1] In another cohort of 439 children, 5-year incidence of post-HSCT AIHA was 5% and a diagnosis of metabolic disease increased significantly the risk. Ten out of 19 patients died: 3 directly of AIHA, 5 of infection during AIHA treatment.[26] One of the largest analysis was performed among 1574 paediatric HSCT: 3-year incidence of AIC was 2.1%, almost half of the cases were AIHA. Risk factors for AIHA occurrence in multivariate analysis were non-malignant disorder and alternative donor source. In this report, a high rate of response to Rituximab (RTX), 100% in AIHA, was highlighted.[27] In a single centre report from Leiden, incidence of post-HSCT AIC among children was 5% at 3 years, with AIHA accounting for 46% of cases. In multivariate analysis, the following factors resulted significantly associated with an increased risk of AIC: CMV reactivation (hazard ratio [HR] 3.4), non-malignant diagnosis pre HSCT (HR 3.5) and

Alemtuzumab use (HR 2.5). In this cohort, patients with AIHA did not achieved remission with steroids and all needed another line of therapy (mostly RTX) with good outcome.[21] Three other recent reports analysed paediatric HSCT cohorts[18,20,28] with incidence of AIHA of 3.6%, 0.3% (6.3% incidence of Evans syndrome) and 3.7%, respectively.

Risk factors for AIHA post HSCT

Risk factors associated with post-HSCT AIHA are identified among recipient characteristics (paediatric age, non-malignant disorders), transplant variables (use of unrelated or haploidentical donor, use of lympho-depletion) and post-transplant complications (presence of GVHD and infections).

Paediatric age

It appears that children are more susceptible to AIHA occurrence post HSCT.[2,16,22,26,27] Some authors found that infants have a higher risk of developing post-HSCT AIHA than children[2] and younger children than older.[26] This could be due to the immaturity of infantile immune system with uncompleted thymic maturation, hampered by the use T-cell depleting agents (ATG, Alemtuzumab) and calcineurin inhibitors.[2] Secondly, children who undergo HSCT have a high prevalence on non-malignant diseases (immunodeficieny, metabolic disorders, haemoglobinopathy) that have been identified *per se* as risk factors by a number of reports.[3,20–22,26,27]

Non-malignant diseases

Diagnosis of non-malignant disease appears to be a risk factor for AIHA and AIC post HSCT. A high incidence of AIHA (19%) has been reported in children transplanted for SCID[1] as well in those receiving UCBT for metabolic diseases.[2,3] O'Brien demonstrated that a diagnosis of metabolic disease was in fact the only risk factor for AIHA among children who underwent HSCT[26]. In 2 other large reports, including both children and adults,[22,27] and in 1 report on children[21] a diagnosis of non-malignant disease was a significant risk factor after multivariate analysis. Szanto *et al* used a different definition (no chemotherapy before HSCT) demonstrating a similar impact on risk of AIHA.[20] It is likely that the intact immune system of

these patients (as compared to patients with malignancies who received chemotherapy) and the allo-immunization consequent to multiple transfusion (especially in thalassemia) may play a role in the pathogenesis of post-HSCT immune dysregulation.

Conditioning regimen and lympho-depleting agents

Both ATG and Alemtuzumab, used as lymphocyte depleting agents, increase the risk of AIC post HSCT. In a cohort of 380 children, use of ATG or Alemtuzumab increased the risk of AIC with a HR of 8, the highest among different variables.[20] Alemtuzumab, in particular, has been associated with a higher risk of post-HSCT AIC when compared to ATG. Alemtuzumab causes a deeper and more prolonged lympho-depletion compared to ATG, with subsequent skewed immune reconstitution, that can be characterized by an uncontrolled expansion of self-directed lymphocytes.[21,28]

The intensity of the conditioning regimen did not correlate with AIHA occurrence in most reports.[5,18,21,22,28] However, patients with aplastic anaemia who were transplanted with a reduced intensity conditioning (RIC) appeared predisposed to AIC.[29] It is not clear weather this reflects an underlying predisposition to autoimmunity or the intensive lympho-depletion used in conditioning.

Use of unrelated donors

Another risk factor reported by several studies is the use of unrelated donor[5,16,24,26,27,30] or haploidentical donor.[25] Transplants from mismatched donors are characterized by a slow immune-reconstitution due to lympho-depleting strategies (*in-vivo* or *ex-vivo*) and by a high incidence of GVHD. These factors may interplay in the post-transplant setting and induce autoimmune phenomena.

Source of stem cells

Studies involving patients who received UCBT showed a high incidence of AIHA.[2,31,32] The use of cord blood as cell source was associated with AIC in univariate analysis in three large studies[16,20,27] but was not confirmed in multivariate analysis.

The use of PBSC was demonstrated to be a risk factor for AIC occurrence in two settings: SCID[1] and aplastic anaemia patients.[29]

This could be ascribed to the different T and B-cell reconstitution observed after HSCT from different cell sources. Patients who underwent UCBT, in particular, demonstrated a unique pattern of immune reconstitution with quicker recovery of B cells and slower reconstitution of

CD3+ T lymphocytes and CD8+ compared to patients who received bone marrow transplant, which could allow the emergence of uncontrolled autoantibody secreting plasma cells.[33]

GVHD

A few studies reported GVHD as a risk factor for AHIA: Sanz *et al* found in multivariate analysis that cGVHD was a risk factor for development of AID in adults who underwent UCBT.[31] Similar results were observed in another cohort of adults after haplo-HSCT.[25] Chang *et al* described a cohort of 15 children with AIC post HSCT, 12/15 presented cGVHD although a statistical association was not reported.[34] Szanto and colleagues showed that presence of aGVHD was associated with a higher risk of AIC and hypothesized that autoreactive T cell could originate when donor lymphocytes interact with recipient antigen presenting cells.[20] Moreover, thymus tissue can be damaged by GVHD with consequent impaired development of self-tolerance.[17]

Infections

Infections, particularly viral, are considered to be a predominant trigger of autoimmunity. The pathway leading to autoimmunity after a viral infection is not clear but is thought to be related to molecular mimicry, infection of primary cells, and imbalance of the immune system in response to the infection. In an already compromised immune system post transplantation, the imbalance of effector and regulatory immune cells becomes more marked. An association between post-HSCT AIHA and CMV reactivation has been showed by Kruizinga *et al* in children.[21]

Treatment of AIHA post HSCT

Treatment of post-HSCT AIHA is not standardized and there is a lack of evidence for all the therapeutic options. Most indications are derived from treatment of primary AIHA, with very few prospective trials in support.[35-37] Data on post-HSCT AIHA come only from case series or even case reports with several limitations: the retrospective nature of data, small number of cases, different criteria to report outcome and no discrimination between warm and cold forms of AIHA. Moreover, results on post-HSCT AIHA are often reported together with other AIC, so that it is difficult to understand how is the response to treatment for patients with AIHA only.

Transfusions

Acute haemolysis can cause very rapid drop in Hb, and needs to be treated as an emergency. Due to the frequent presence of pan-reactive autoantibodies that react against common antigens, finding a compatible unit might be challenging and could take several hours. In life-threatening situations, transfusion should not be delayed and the least mismatched ABO, Rh and K compatible unit should be given. In non-urgent situations, indication for transfusion should be based on the clinical assessment and presence of symptoms rather than on the Hb level. This is because even the best matched unit could be targeted by autoantibodies and may contribute to activate further the haemolytic process. Leukodepleted and irradiated blood products should be administered slowly, and first line treatment should ideally start before transfusion.[38,39]

Importantly, a recent report showed that the prevalence of iron overload was significantly higher in patients with post-HSCT AIHA compared to controls, highlighting the importance of judicious use of transfusion and awareness of the possible need for chelation in these patients.[18]

Folic acid supplementation is generally recommended as chronic haemolysis can lead to folate deficiency.[8]

<u>First line treatment</u>

Steroids

Mirroring the treatment for primary forms of AIHA, post-HSCT AIHA is treated with steroids as first line. In primary forms, guidelines suggest methylprednisolone or prednisone/prednisolone at 1-2 mg/kg/day for 2-4 weeks with slow taper over 3-6 months. A response is generally obtained within 1-2 weeks. Patients who do not respond after 3 weeks should be considered for alternative treatment options, and steroids should be tapered and discontinued.[9,39] Response to steroids is around 75-80% in primary adult wAIHA and 15-30% in CAD.[38] A large French observational study has reported a CR post steroids in 58% of children with primary and secondary AIHA.[40]

In post-HSCT AIHA, the response rate to steroids are generally lower (Table II). Overall response rate (ORR) are highly variable among reports from 10% to 90%, but CR with steroids only is generally achieved in 30% or less of cases, with the majority of patients requiring second line treatment. Steroids remain the first line treatment for post-HSCT AIHA, although their use as single agent is limited to non-severe forms with rapid response.

Steroids increase the susceptibility to infections, particularly viral and fungal, and can cause several other well-known side effects including diabetes, hypertension, steroid myopathy and osteopenia. Moreover, children with post-HSCT AIHA showed higher incidence of complications related to steroid treatment like avascular necrosis and cataracts.[18] Therefore, strategies aiming at reducing the overall duration of steroid treatment and at tackling AIHA recurrence after steroid cessation are needed.

It is important to remember that post-HSCT patients on prolonged steroid treatment should receive adequate anti-microbial prophylaxis, including anti-fungals and Pneumocystis prophylaxis. Moreover, current guidelines recommend lifestyle measures, vitamin D and calcium supplements to all patient on long-term glucocorticoids and bisphosphonate in those at high risk of osteoporosis.[41]

Rituximab

RTX is an anti-CD20 chimeric monoclonal antibody that depletes CD20-expressing B cells. Selective B-cell depletion may result in reduction of autoantibodies production, allowing time for normal Th and T reg recovery. A prospective study on adults with primary AIHA demonstrated that RTX (at a low fixed dose of 100 mg/week for 4 weeks) and prednisone (1 mg/kg/day for 1 month, then slow wean) had an ORR of 91.3% at 6 months, relapse free survival 86% at 12 months, 68% at 2 years.[35] A phase III trial comparing steroids alone *vs* steroids and RTX in primary wAIHA demonstrated a higher CR rate (75% *vs* 36%) and more durable remission (70% *vs* 45% at 36 months) in patients treated with combination therapy.[36] These results were confirmed by a double blind randomized controlled trial in adults with primary wAIHA who were treated with steroids and RTX (2 doses) or steroids and placebo.[37]

In 2004, O'Brien and colleagues reported 3 children who received RTX as part of the treatment of post-HSCT AIHA, with response in one child.[26] Other reports in post-HSCT AIHA were published since (Table III). RTX has been used as second line treatment after steroids at the standard dose of 375 mg/m^2/week for 4 weeks with a time to response of 3-6 weeks from start of the treatment and an ORR ranging from 38% to 100%. Similar rates have been observed when used as first line therapy alone,[22,31,42] or in combination with steroids.[18,19,32] It is important to underline once again that the majority of reports are based on very small numbers. In the largest published series, 40 patients (adult and children) received RTX as first or second line therapy. Factors associated with response to RTX were ABO-incompatibility between donor and recipient and higher B-cell number at AIHA onset.[16]

The advantages of RTX are its well-known safety profile and tolerability as it has been used to treat numerous patients with lymphoma and autoimmune conditions.[43] RTX depletes B cells

and antibody production and can increase the risk of infections, particularly in the already fragile population of post-HSCT patients. Progressive multifocal leukoencephalopathy has been reported as a rare but often fatal complication caused by reactivation of latent JC virus in the brain. Another disadvantage is the risk of hypogammaglobulinemia, that seems a frequent event in post-HSCT patients. Indeed, in the recent report by Koo *et al,* 88% of patients with post-HSCT AIC developed persistent hypogammaglobulinemia at a median time of 1.7 years post RTX.[18] Lum *et al* reported that 42% of patients required immunoglobulin replacement at a median time of 10.5 years (range 2.6-15.2 years) after RTX.[28] This risk has to be taken into account as it contributes to the susceptibility to infections and to the reduced response to vaccination. Level of IgG, IgM and IgA should be monitored pre and post RTX regularly and the need for IVIG supplement should be assessed against local policies.

RTX should be considered as a first line option in combination with steroids in severe cases or when a shorter duration of steroid is advisable, or as an early second line treatment in all AIHA post HSCT.[4,31,32]

Second line treatment

As far as further lines of treatment are concerned, there is no consensus around the best treatment, but some considerations can be made based on the current available literature. Results from published studies are depicted in table IV.

Splenectomy

In wAIHA, RBC are predominantly destructed in the extravascular compartment by spleen macrophages. Indeed, splenectomy has been considered for many years the standard second line therapy and the only curative option in idiopathic wAIHA with a response rate around 60-80%.[38] However, it has been moved to ≥ third line therapy in current guidelines for adult primary wAIHA, due to the introduction of new effective treatments and the increased awareness of complications, namely infections and thrombosis.[9,38] A recent large study on 4766 adults, affected mainly by primary AIHA, reported an increased risk of venous thrombosis, abdominal thrombosis and sepsis for those who received a splenectomy.[44] In post-HSCT AIHA, response rate to splenectomy are lower: in the largest series, only 1 out of 7 cases responded.[16] Due to the low rate of success in this setting and the increased risk of infections post HSCT, splenectomy should now be considered only in severe forms after failure of other medical treatments.

Sirolimus

Sirolimus inhibits the mammalian target of rapamycin (mTOR), which is part of the T cell receptor pathway, and induces cell death and apoptosis in lymphocytes. It is used in solid organ transplantation and autoimmune diseases, and its effect has been associated with suppression of Th and effector cells and sparing of T reg. Efficacy has been demonstrated in 5/5 children with AIHA[39] and in patients with AIHA post solid organ transplantation.[45] Recently, 14 patients with primary AIHA received Sirolimus with an ORR of 85%; a significant increase in T reg levels was observed after 6 months in tested patients.[46] The recommended dose is 2-3 mg/m^2 to achieve a serum level of 4–12 ng/ml, with an optimal target of 9 ng/ml, for at least 3 months before evaluating its efficacy.[39,46]

Several case reports and case series has documented the efficacy of Sirolimus in post-HSCT AIHA refractory to other treatment,[18,19,21,28] making this treatment strategy attractive as second/third line. Sirolimus side effects includes immunosuppressive properties with increased risk of infection, mucositis,[46] hypertriglyceridemia, hyperglycaemia. Post-HSCT patients who received Sirolimus for GVHD, compared to those receiving steroids, had a lower incidence of hyperglycaemia, similar incidence of infections and higher occurrence of transplant associated thrombotic microngiopathy (TMA).[47] The risk of TMA increases when Sirolimus is used in combination with calcineurin inhibitors such as Cyclosporine, and this combination should be avoided.[48]

Mycophenolate mofetil

Mycophenolate mofetil (MMF) causes depletion of guanosine nucleotides preferentially in T and B lymphocytes and inhibits their proliferation, thereby suppressing cell-mediated immune responses and antibody formation. Encouraging results have been published in a case series of children treated with MMF for AIHA or Evans syndrome.[39] In post-HSCT AIHA, it has been used in a minority of cases with no reported benefit.[16] As MMF is widely used in the post-HSCT setting for GVHD prophylaxis, the advantage resides in the well-known safety and toxicity profile in this group of patients. MMF may be considered as a steroid sparing agent in the setting of post-HSCT AIHA, with the aim at shorten the duration of concurrent steroid treatment, especially in children.

Bortezomib

Bortezomib is a proteosome inhibitor that causes plasma cells apoptosis, used in the treatment of multiple myeloma. Bortezomib has been investigated in a phase II multicentre study in

adults with CAD: a single course achieved an ORR of 31% in this difficult-to-treat condition.[49] There are case reports of its efficacy in post-HSCT AIHA, although a reporting bias could account for the good outcome reported in refractory cases. Beyond case reports, 2 studies reported efficacy in a total of 3 out of 8 patients.[16,21] Concerns about Bortezomib toxicity are mainly related to cytopenia and peripheral neuropathy (described in patients with myeloma).

Cyclosporin

Cyclosporin (CSA) is a calcineurin inhibitor that impairs cytokine production and proliferation of T lymphocytes. It is a potent immune-suppressive agent and has been used both in primary and post-HSCT AIHA in small number of cases.[16,22] CSA is not currently recommended as a treatment for AIHA; its use could be justified in the post-HSCT setting when AIHA occurs at stopping the immunosuppression.

<u>New agents</u>

Daratumumab and Abatacept have been used in post-HSCT AIHA and results have been published in small case series.

Daratumumab

Daratumumab (anti CD38 monoclonal antibody) targets plasma cells and has been used in treatment of AID. Few case reports have been published on use of Daratumumab in post-HSCT AIHA (Table IV). Again, as only cases with positive outcome tend to be reported, the actual efficacy is not known. Daratumumab was well tolerated in this setting, although it is known to cause cytopaenia and peripheral neuropathy in multiple myeloma and interfere with blood bank test (false positive DAT).[50]

Abatacept

Abatacept, a monoclonal antibody that blocks costimulatory signalling on T cells, has been used in 3 patients with post-HSCT AIHA refractory to other treatment[51] with promising outcome.

Other new agents are under investigation in primary or secondary wAIHA, although their use in post-HSCT AIHA has not been so far reported. Ongoing trials will elucidate the safety and efficacy of these medications.

Fostamatinib

Fostamatinib is an oral spleen tyrosine kinase inhibitor which has been recently licensed for use in immune thrombocytopenia. It blocks Fc receptor (FcR) and B-cell receptor activation, preventing macrophage mediated destruction of antibody coated platelets, and potentially reducing antibody production.[52] An open-label multicentre phase 2 study of Fostamatinib has demonstrated an improvement in Hb levels in 11 of 25 patients with wAIHA.[53] A phase 3 study is ongoing (NCT03764618).

Ibrutinib

Ibrutinib, an inhibitor of Bruton tyrosine kinase, has been reported to be effective in cases of AIHA associated with chronic lymphocytic leukaemia[54,55] and it is currently tested in a clinical trial in wAIHA (NCT04398459). As Ibrutinib has been recently approved for treatment of steroid refractory cGVHD, its safety has been already tested in transplanted patients.[56]

Orilanolimab

Orilanolimab (SYNT001) is a monoclonal antibody that blocks the interaction between the neonatal crystallizable fragment receptor (FcRn) and the Fc portion of IgG, inducing an increased clearance of IgG. A clinical trial (NCT03075878) is investigating its safety in wAIHA.

<u>DLI and second transplant</u>

DLI have been used in AIHA that occurred in association with mixed chimerism, with resolution of AIHA and reversal to full donor chimerism in 3 out of 5 patients.[23]

Second HSCT is a possible option for severe cases not responding to any treatment and in those developing refractory cytopenia,[21,28] although this clearly needs to be balanced against the risk of morbidity and mortality.

Other treatments

IVIG is rarely used alone, it has been used with steroids in the acute setting, with little benefit.[21]

Plasma exchange has been used in a very small proportion of patients, generally in association with other agents. Like in primary AIHA, it should be used in the acute setting as a temporary measure until the response to immunosuppressive therapy is awaited.[57] Similarly, whole blood (plasma and erythroid) exchange has been effective in acute life-threatening episodes of primary AIHA.[58]

Cytotoxic agents such as Cyclophosphamide, 6-mercaptopurine have been used in few cases[16,19,34] but are not recommended in the post-HSCT setting due to the risk of myelotoxicity.

Other treatments (Azathioprine, Danazole, Alemtuzumab, Ofatumumab, Eculizumab) have been used in rare cases without efficacy.

In conclusion, post-HSCT AIHA should be treated in first instance with steroids and Rituximab with the aim of improving efficacy and reducing the steroid burden. Beyond first line, the scarcity of good quality data hampers the possibility to draw evidence-based guidelines. From the available literature, Sirolimus or Bortezomib appear as reasonable options and Daratumumab and Fostamatinib as promising new drugs. There is a clear need for prospective clinical studies to guide clinicians in managing these difficult patients.

Mortality

Post-HSCT AIHA can be very difficult to treat, often because of disease recurrence and needs for long-term immune-suppression. Presence of GVHD, infections and other post-HSCT complications add further difficulty to the clinical management. Patients most often die of infections or of massive haemolysis.[59] Several groups have described an increased mortality in patients who experienced post-HSCT AIC.[19,24,26,31,60] In a large study in adults, Wang *et al* demonstrated that presence of AIHA post HSCT increased the overall mortality and TRM.[5] However, more recent reports have not confirmed this.[20,25,28] Lum *et al* demonstrated that mortality due to AIHA was higher in patients treated before 2011 (25% *vs* 5%), possibly due to the recent different approach with early institution of steroid sparing agents (Rituximab and

Sirolimus in their centre) and better supportive care.[28] The availability of different treatment options has definitely reduced the steroid exposure and the need for splenectomy. These measures may have reduced mortality in this group of patients.

As the leading cause of mortality in post-HSCT AIHA are infections, it is important that transplanted patients on long-term steroids and other immunosuppressants are maintained on broad anti-microbial prophylaxis particularly against fungi, but also against Herpes and Varicella Zoster virus, Pneumocystis and encapsulated bacteria.

Conclusion

AIHA is a well-recognized complication that contributes to the morbidity and the risk of mortality in patients post allogenic HSCT. A timely diagnosis and prompt institution of first line therapy with steroids is essential. Rituximab should be consider early in severe cases, in those unresponsive/dependent to steroids or when a shorter duration of steroid is advisable. Further lines of therapy should be considered for refractory/relapsing cases. Bortezomib and Sirolimus have shown efficacy a good tolerability in this setting, and may have contributed to the reduced mortality in recent reports. Daratumumab may show promise for this difficult to treat condition, and other agents, as Fostamatinib, are in trial for wAIHA.

Given the unique immune *milieu* post HSCT and apparent differences in steroid responsiveness between primary and post-HSCT AIHA, it is not clear how data can be extrapolated from the former and apply to the latter. There is a pressing need for prospective trials evaluating these agents formally in AIHA post HSCT, ideally randomising one agent against another. Because of the incidence of this complication such studies will need to be multicentre and ideally involve international collaboration.

Prospective research into reducing the incidence of this complications post HSCT is equally important. As lympho-depleting agents, particularly Alemtuzumab, seem to have a crucial role, more targeted approaches based on patient characteristics and pharmacokinetics may be beneficial,[21,28] as could development of strategies to accelerate T reg recovery post-HSCT.

Authorship

M.G., C.A., N.C. and P.I.A. wrote, revised and approved the final version of the manuscript.

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Conflict of interest

M.G., C.A. and P.I.A have no competing interest to disclose.

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