# Diagnosis and management of newly diagnosed childhood autoimmune haemolytic anaemia. Recommendations from the Red Cell Study Group of the Paediatric Haemato-Oncology Italian Association

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## Abstract

Autoimmune haemolytic anaemia is an uncommon disorder to which paediatric haematology centres take a variety of diagnostic and therapeutic approaches. The Red Cell Working Group of the Italian Association of Paediatric Onco-haematology (*Associazione Italiana di Ematologia ed Oncologia Pediatrica*, AIEOP) developed this document in order to collate expert opinions on the management of newly diagnosed childhood autoimmune haemolytic anaemia.

The diagnostic process includes the direct and indirect antiglobulin tests; recommendations are given regarding further diagnostic tests, specifically in the cases that the direct and indirect antiglobulin tests are negative. Clear-cut definitions of clinical response are stated. Specific recommendations for treatment include: dosage of steroid therapy and tapering modality for warm autoimmune haemolytic anaemia; the choice of rituximab as first-line therapy for the rare primary transfusion-dependent cold autoimmune haemolytic anaemia; the indications for supportive therapy; the need for switching to second-line therapy. Each statement is provided with a score expressing the level of appropriateness and the agreement among participants.

**Keywords**: autoimmune haemolytic anaemia, child, DAT, diagnosis therapy.

## Introduction

Autoimmune haemolytic anaemia (AIHA) in childhood is an uncommon condition caused by the presence of auto-antibodies directed against antigens on the surface of red blood cells, leading to premature destruction of the cells[1-3]. The overall annual incidence is reported to be 1-3 cases/100,000 people and approximately 0.2 cases/1,000,000 individuals under 20 years of age[4-8], although these figures are probably underestimates, partly because of the lack of understanding of the diagnostic tools. Nevertheless, AIHA is an important issue for many paediatricians, who have to deal with the diagnosis and treatment of critically ill patients in the absence of consistent support from the literature. On this background, the Red Cell Working Group of the Italian Association of Paediatric Onco-haematology (*Associazione Italiana di Ematologia ed Oncologia Pediatrica*, AIEOP) developed this document with the aim of providing shared recommendations for paediatricians.

These consensus recommendations are not intended as standards or fixed rules, but as an instrument to support paediatricians in the diagnostic work-up and initial treatment of newly diagnosed patients with AIHA.

## Materials and methods

The design and methodology implemented to draw up these recommendations were similar to those adopted for the AIEOP Consensus Guidelines on

childhood aplastic anaemia, congenital and acquired neutropenias, and immune thrombocytopenias[9-11] using a procedure validated by the AIEOP board. In particular, representatives from 20 AIEOP centres and 10 other non-AIEOP centres participated in the AIHA Committee. Issues to be addressed in the Recommendations were identified by the whole Committee; each topic was developed by a subgroup in a single document, which included a brief description of the state-of-the-art knowledge, followed by specific recommendations.

In order to draw up the pre-guideline documents, the authors extracted evidence from the literature included in the Medline database (initially searched from January 1,1991 to December 31, 2015, and then updated in February 2016 during the compilation of the final draft). Search terms included: autoimmune haemolytic anaemia (AIHA), warm autoimmune haemolytic anaemia, warm antibodies, cold autoimmune haemolytic anaemia, direct antiglobulin test (DAT), indirect antiglobulin test (IAT), therapy, diagnosis, paediatric, children. The MEDLINE® search yielded 326 articles that were examined, 76 of which were selected as they offered data related to the topics of the present paper. The search was also extended to older papers, specifically retrieved following cited references, and to haematology textbooks. All the evidence collected was attributed a strength that was scored using the level of evidence criteria reported in Table I.

**Table I -** Levels of evidence for studies evaluating the diagnosis and therapy of AIHA in children.

| Level of evidence | Study design |
|---|---|
| I (strongest) | Prospective randomised trial with high statistical value |
| II | Prospective randomised trial with lower statistical value |
| III | Non-randomised study with concurrent control group |
| IV | Non-randomised study with historical control group |
| V | Case report(s) with no control group |

Each draft was reviewed by the entire Committee and modified accordingly after exhaustive discussion. The Committee prepared statements that were then subjected to validation during the Consensus Conference, during which 44 participants scored the final items.

The strength of consensus was quantified on a 1-9 scale where 1 represented no consensus and 9 represented full consensus regarding the appropriateness and necessity of the practice. For each statement a mean score was calculated. Mean scores from 1 to 3 indicated an inappropriate practice, mean scores from 3.1 to 6.9 indicated a practice of uncertain appropriateness and mean scores from 7 to 9 signified an appropriate/necessary practice. The level of agreement among participants, indicating the rate of consensus, was also graded by evaluating the distribution of the standard deviations (SD) within each statement and then dividing the level of agreement into four categories:

A: strong agreement (variation more than 1 SD below the average of the variances, on a logarithmic scale);
B: moderate agreement (variation less than 1 SD below the average of the variances);
C: moderate disagreement (variation less than 1 SD above the average of the variances);
D: strong disagreement (variation more than 1 SD above the average of the variances).

## Diagnosis of autoimmune haemolytic anaemia

### *Classification and initial evaluation*

According to pathogenesis, AIHA can be defined as primary, with no other underlying condition (37%), post-infective (10%), or secondary (53%); in this last case, AIHA is part of a more complex disease, usually of immunological, infective or neoplastic nature[1-4,12-25]. AIHA can be classified based on both the thermal properties of the antibodies involved and the immunoglobulin class, as reported in Table II.

The panel of experts outlined the importance of the initial evaluation, including history and physical examination, based on the first-level tests listed

**Table II -** Characteristics of the various forms of AIHA.

| Clinical form | Frequency (%) | DAT | Ig class | Thermal optimum (°C) | Avidity and ability to fix complement | Antigen specificity | Site of haemolysis |
|---|---|---|---|---|---|---|---|
| Warm antibodies | 60-70 | IgG+ or IgG+/C3d+ | IgG | 34-37 | –/+ | Anti-Rh | Extravascular |
| Cold antibodies | 20-25 | Neg. or C3d+ | IgM | 4-27 | +++ | Anti-I | Extravascular and intravascular |
| Cold paroxysmal haemoglobinuria | 6-12 | Neg. or C3d+ | IgG Biphasic | Fixing 4-27 Lysis 34–37 | +++ | Anti-P | Intravascular |
| Mixed AEA | < 5 | IgG+ or IgG+/C3d+ or C3d+ | IgG/IgM | IgG 34-37 IgM 4-27 | ++ | Anti-Rh Anti-I | Extravascular and intravascular |

AIHA: autoimmune haemolytic anaemia; DAT: direct antiglobulin test; Ig: immunoglobulin; AEA: anti-erythrocyte autoantibodies; Neg.: negative.

**Table III -** First level tests.

| |
|---|
| WBC count, red cell morphology on peripheral smear |
| Reticulocyte count |
| Indices of haemolysis (haptoglobin, indirect bilirubin, LDH) |
| DAT and IAT |
| Blood group |
| Liver and kidney function |
| Urinanalysis |

WBC: white blood cell; LDH: lactate dehydrogenase; DAT: direct antiglobulin test; IAT: indirect antiglobulin test.

in Table III; the consensus on the appropriateness of these tests was **8.9-A**. Whole blood count, together with reticulocyte count, haptoglobin, lactate dehydrogenase (LDH) and bilirubin are the parameters to define haemolytic anaemia. The reticulocyte count is typically high, as a consequence of increased erythropoiesis; nevertheless, in up to 39% of children, AIHA can be associated with initial reticulocytopenia[1,4], due to antibodies against erythroid precursors, induction of apoptosis of bone marrow erythroblasts, or a concomitant viral infection, e.g. Parvovirus B19[26-30]. The search for auto-antibodies, which clarifies the immune mechanism involved in the haemolytic anaemia, is discussed below. Haemoglobinuria is found in the case of intravascular haemolysis and may be manifested be the emission of overtly dark urine. Table IV reports a list of additional tests, some of which may be appropriate, according to the patient's particular situation, in order to identify underlying disorders in secondary forms of AIHA **(8.3-B)**.

**Table IV -** Second-level tests.

| |
|---|
| Extensive red blood cell typing in anticipation of possible transfusion |
| Further immune-haematological investigations: C3, C4, CH50 |
| Auto-antibodies (ANA, anti DNA), antiphospholipid antibodies, RA test |
| - Thyroid function and anti-thyroid antibodies (anti-TG, anti-TPO) |
| - Lymphocyte subpopulations (CD3, CD4, CD8, CD19, CD16, CD56) |
| - Double-negative T cells: CD3+, CD4-, CD8-, TCR alpha/beta+ |
| Hepatitis B and C, markers and HIV serology |
| Coagulation screen blood test |
| Serum total protein and protein electrophoresis |
| Immunoglobulin class quantification |
| C-reactive protein |
| EBV, CMV, Parvovirus B19, HSV serology |
| Other assessments for infectious diseases, as clinically appropriate |

C3: complement component 3; C4: complement component 4; CH50: total component activity; ANA: antinuclear antibodies; RA test: rheumatoid factor test; TG: thyroglobulin antibody; TPO: thyroid peroxidase antibody; HIV: human immunodeficiency virus; EBV: Epstein-Barr virus; CMV: cytomegalovirus; HSV: herpes simplex virus.

Bone marrow aspiration is not usually required: this investigation is only recommended in the presence of another associated cytopenia or persistent reticulocytopenia or on suspicion of neoplasia or myelodysplasia **(8.7-B)**. The evaluation of bone marrow usually highlights maturing erythroid hyperplasia, sometimes with mild dyserythropoiesis and no abnormality of the remaining haematopoietic series[1].

### *Immune-haematological diagnosis: the direct antiglobulin test*

Laboratory diagnosis of AIHA is based on the demonstration of auto-antibodies adhering to autologous erythrocytes (the direct antiglobulin test [DAT] or direct Coombs' test) and free in the serum (the indirect antiglobulin test [IAT] or indirect Coombs' test)[2].

The DAT is performed first with polyspecific sera, capable of identifying both IgG and C3 on red blood cells, using various methods characterised by different sensitivities and specificities. If the screening DAT performed with polyspecific sera is positive, the use of monospecific anti-IgG and anti-C3 antiserum is recommended **(8.8-A)**.

The search for antibodies adhering to red blood cells (DAT) must be combined with a search for irregular antibodies in the serum (IAT) and, if found, their identification **(8.7-A)**.

The IAT can be positive even in the presence of auto-antibodies, detectable in 34% of cases of AIHA[31-36], in addition to various other conditions of alloimmunisation (prior transfusion, pregnancy, etc.). Finally, in the case of a negative IAT, it is appropriate to repeat the search and the possible identification on the eluate of the red blood cells. If serum/eluate IAT are both negative, it is appropriate to consider a drug-induced AIHA **(8.8-A)**.

In the case of "verified or suspected" AIHA with intravascular haemolysis, regardless of the result of the DAT (negative DAT and/or DAT positive for IgG and/or C3d), a search for the Donath-Landsteiner auto-antibody is recommended **(8.8-A)**. This IgG auto-antibody, also known as biphasic haemolysin, cleaves complement at low temperatures and causes haemolysis at 37 °C and allows the diagnosis of paroxysmal cold haemoglobunuria.

### *Direct antiglobulin test-negative autoimmune haemolytic anaemia*

Some cases of AIHA are not diagnosed by the commonly used DAT. The reported frequency of these DAT-negative AIHA is about 10%[2,37]. The diagnosis of DAT-negative AIHA, after the exclusion of other non-immune-mediated causes of haemolysis (listed in Table V), requires additional tests **(8.7-B)** with monospecific anti-IgA sera, since the content of anti-IgA

**Table V -** Non-immune-mediated haemolytic anaemias.

| | |
|---|---|
| Congenital forms | Spherocytosis and other defects of the:<br>- erythrocyte membrane proteins;<br>- erythrocyte enzyme deficiency;<br>- dyserythropoietic anaemia;<br>- haemoglobinopathies;<br>- Wilson's disease. |
| Haemolytic anaemia from mechanical causes | Synthetic heart valves;<br>march haemoglobinuria;<br>cardiopulmonary bypass. |
| Haemolytic anaemia due to vascular injury | Microangiopathic anaemia;<br>thrombotic thrombocytopenic purpura;<br>haemolytic-uraemic syndrome;<br>disseminated intravascular coagulation;<br>arterio-venous malformations. |
| Haemolytic anaemia due to thermal damage | Extensive burns. |
| Haemolytic anaemia from chemical causes | Chemicals solvents;<br>methyl chloride;<br>lead;<br>arsenic and hydrogen;<br>snake venom. |
| Haemolytic anaemia due to infectious agents | Bacteria (*Mycoplasma pneumoniae*, *Clostridium welchii*);<br>viruses (cytomegalovirus, herpes virus);<br>protozoa (*Plasmodium spp.*). |

immunoglobulin in polyspecific sera may be inadequate to recognise the presence of IgA antibodies[2,38], and low ionic strength solutions (LISS) and/or polyethylene glycol (PEG) in order to detect low-affinity antibodies that can be washed out with routine methods[35,39,40].

If the DAT is still negative, it is appropriate to use more sensitive methods, capable of detecting a smaller number of IgG adhering to red cells **(8.3-B)**.These methods are: flow cytometry, able to detect even 30-40 molecules of IgG/red cell[41], immunoenzymatic[42-44] and immunoradiometric tests[45], a complement-fixing antibody consumption test[46], and tests after mitogenic stimulation in culture (MS-DAT)[47].

***"Warm" IgM autoimmune haemolytic anaemia***

On rare occasions, haemolysis is due to atypical warm IgM antibodies, which are capable of inducing spontaneous red cell agglutination *in vivo*, resulting in more pronounced haemolysis and a very severe clinical picture[48]. Recognition of such antibodies may be difficult since the DAT may be positive for C3, or less frequently IgG and C3, so this form is often misdiagnosed as a cold or mixed AIHA. Positivity for C3 in a warm AIHA should lead to a suspicion of IgM involvement. Furthermore, some cases can be negative with the usual DAT. Therefore, a double DAT (dual direct antiglobulin test, DDAT), capable of revealing the presence of weak or non-agglutinating "warm" IgM auto-antibodies[49], is recommended in severe cases with signs of *in vivo* agglutination **(8.3-B)**.

***Drug-induced autoimmune haemolytic anaemia***

Drug-induced antibodies are "drug-independent" if they can be identified with the common techniques of antibody research (IAT), and "drug-dependent" if they need the presence of the drug in the reaction system (immune complex mechanism, DAT positive for complement), or pre-treatment of erythrocytes with drugs (hapten/neoantigen formation, DAT positive for IgG)[2-16].

In the case of DAT-positive and serum/eluate IAT-negative AIHA, associated with recent administration of drugs, consideration of drug-induced AIHA is recommended **(8.8-A)**.

A comprehensive summary of the diagnostic work up is reported in Figure 1.

**Treatment of autoimmune haemolytic anaemia**

Preliminary to the discussion of therapy, the response criteria were defined. A complete response was defined as the achievement of a haemoglobin (Hb) concentration greater than or equal to the lower normal limit for age, with no signs of haemolysis, i.e. normal reticulocyte count and bilirubin concentration **(8.9-A)**. A partial response was defined as an increase of Hb of ≥2 g/dL, without the Hb concentration reaching a normal value for the patient's age **(8.5-B)**. No response was defined as an increase of Hb <2 g/dL and/or dependence on transfusions **(8.4-B)**.

**Warm autoimmune haemolytic anaemia**

***Pharmacological treatment***

Steroids are the first-choice treatment in all cases of warm-type AIHA[50-54]. Initial treatment involves the use of oral prednisone at a dose of 1-2 mg/kg/day **(8.6-B)**; in the case of poor compliance to oral administration, intravenous methylprednisolone can be used (0.8-1.6 mg/kg/day) **(8.6-B)**; in severe cases, a higher initial dose may be indicated, i.e. intravenous methylprednisolone 1-2 mg/kg every 6-8 hours for 1-3 days **(7.9-C)**. Routine use of high-dose steroids is not recommended **(7.1-D)**.

Intravenous immunoglobulins have been used in AIHA in addition to steroids[1,55-61]. In a review of 73 cases of AIHA, Flores *et al*. concluded that treatment with intravenous immunoglobulins (0.4-0.5 g/kg for 5 days) was effective in 39.7% of patients, with a higher efficacy (54.5%) in children[55]. Intravenous immunoglobulins may, therefore, be indicated as adjunctive therapy to steroids, in more severe cases **(7.8-B).**

A comprehensive therapeutic algorithm is proposed in Figure 2. Steroid tapering should always be slow, in order to extend the treatment for at least 6 months **(7.8-C)**: a gradual and sustained reduction of the steroid dose correlates with a lower incidence of relapse[62].

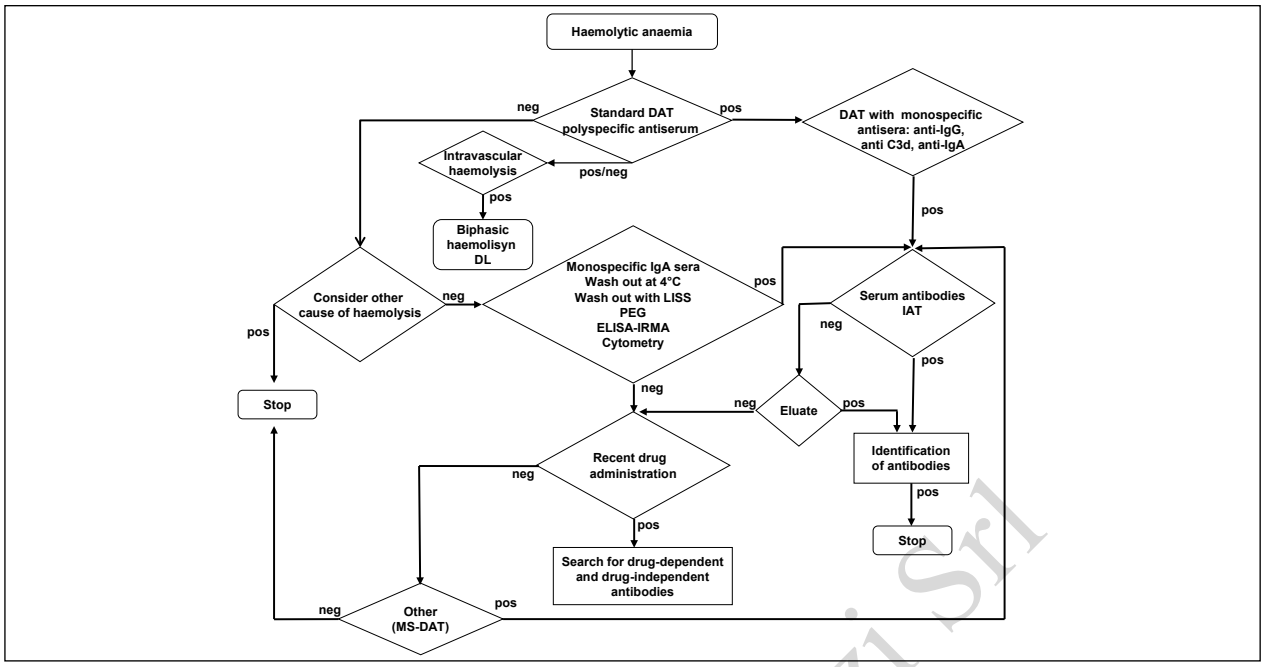


**Figure 1 -** Diagnostic work up.
neg: negative; DAT: direct antiglobulin test; pos: positive; DL: biphasic haemolysins of Donath-Landsteiner; IAT: indirect antiglobulin test; LISS: low ionic strength; PEG: polyethylene glycol; ELISA: enzyme-linked immunosorbent assay; IRMA: immunoradiometric tests; MS-DAT: direct antiglobulin test after mitogenic stimulation.

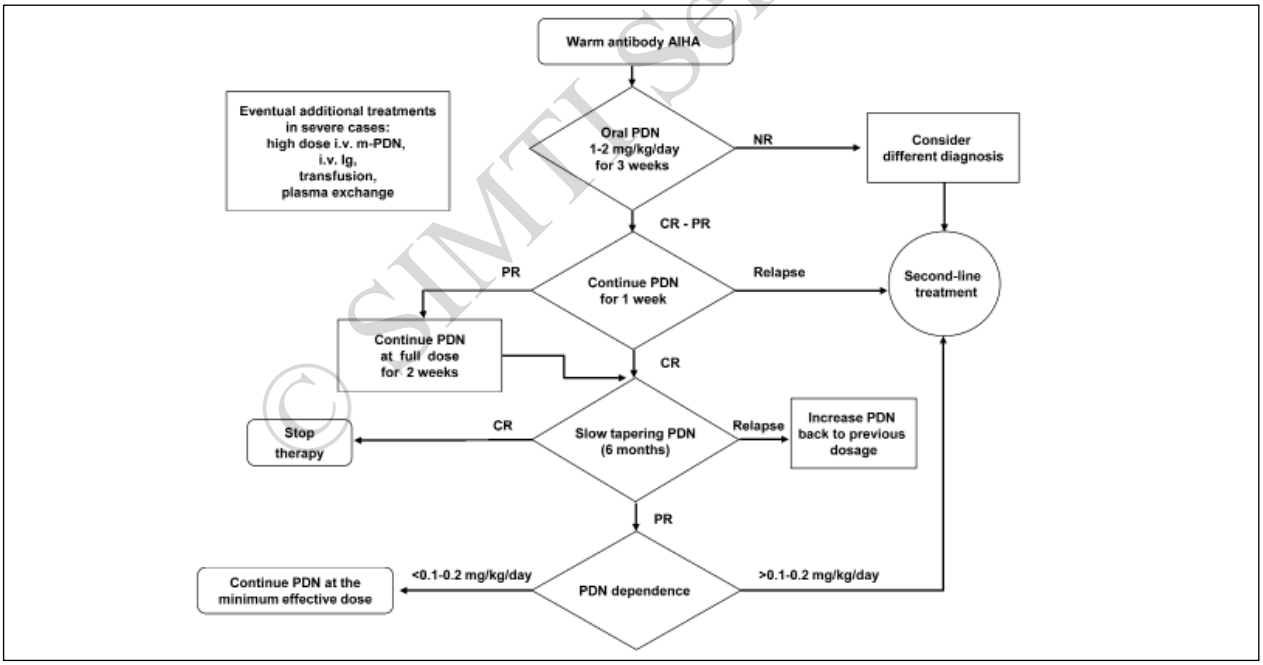


**Figure 2 -** First-line therapy of warm antibody AIHA.
If steroid induces no response at 3 weeks, having excluded a different diagnosis, the patient is shifted to second line treatment (8.6-A). For responsive patients, initial steroid therapy should last at least 4 weeks (8.6-B); in the case of CR, steroid can be tapered (8.8-B); in the case of PR, the full dosage should be continued for 2 more weeks. In any case, after 6 weeks, steroid must be tapered (8.4-B). Tapering schedule: the full dosage is reduced by 25-50% over 4 weeks, thereafter, the reduction must be gradual, in order to extend the treatment for at least 6 months (7.8-C); if a relapse or exacerbation of the hemolysis is observed, during the tapering process, the dosage should be brought back at the previous level (8.2-C).
AHIA: autoimmune haemolytic anaemia; PDN: prednisone; Ig: immunoglobulin; NR: no response, CR: complete response, PR: partial response.

The indications to start a second-line therapy are no response to the first-line treatment **(9-A)**, or steroid dependence, with a prednisone dosage of 0.1-0.2 mg/kg/day, the Consensus being uncertain on whether to fix the dosage threshold at 0.1 **(5.3-D)** or 0.2 **(6.3-D)** mg/kg/day.

***Transfusion therapy***

Transfusion of packed red blood cells is not a routine treatment for several reasons: often it is difficult to find red cell units matched to the recipient, both because of the auto-antibodies reacting with the donor's red blood cells and because of the possible simultaneous presence of allo-antibodies. Auto- and allo-antibodies may be responsible for the destruction of transfused red cells, with exacerbation of the haemolytic process[63]. Transfusion should, therefore, be reserved to cases of very severe anaemia, in patients with impairment of vital signs **(8.5-C)**. It is recommended that the patient's blood samples are tested for a timely and complete definition of red cell phenotype and detection of possible allo-antibodies masked by auto-antibodies **(8.5-B)**. Extensive red cell antigen typing is recommended with the goal of improving the transfusion performance; the typing should preferably be performed using molecular methods, including at least: C, c, D, E, e, K, Jka, Jkb, Fya, Fyb, S, and s **(8.6-B)**. It is recommended that only quantities sufficient to improve symptoms (approximately 3-5 mL/kg) are transfused, in order to minimise the complications of overload and incompatibility **(8.6-B)**. Packed red blood cells for transfusion in this context must be leucodepleted, preferably prior to storage **(8.8-A)**. the transfusion must be performed slowly, under careful supervision and within the maximum time allowed of 4 hours (**8.5-B**).

***Plasma exchange***

Thorough systematic studies regarding plasma exchange in paediatric AIHA are lacking and clinical data are limited to case reports[64-66]. The rationale of using plasma exchange is to remove circulating immune complexes, complement activated components and circulating auto-antibodies. It is estimated that each cycle can remove up to 65% of the circulating auto-antibodies, so, it is frequently necessary to repeat the procedure.

Plasma exchange is an option that should be considered only for extremely severe cases of AIHA, with no response to either transfusion or pharmacological therapy **(8.0-C)**. It should be taken into account as an alternative to splenectomy in the case of emergency **(7.2-C)**. Given its indication for use in critically ill patients, it should be performed in centres with solid paediatric experience.

**Cold autoimmune haemolytic anaemia**

Cold antibody AIHA is usually secondary, mostly associated with bacterial or viral infections (*Mycoplasma pneumoniae*, Epstein-Barr virus, varicella, hepatitis C, rubella, parvovirus, mumps, cytomegalovirus)[1]; therapy is, therefore, based on control of the underlying disease. Useful measures include: keeping the patient warm, maintaining adequate hydration, and monitoring urine output in the presence of significant intravascular haemolysis. If red cell transfusion is required, it is advised that the erythrocytes are pre-heated during transfusion with a heat generator inside the tubing. Exchange transfusion can be a valid treatment option in the acute phase of AIHA because both the sensitised red blood cells and the circulating auto-antibodies can be removed simultaneously. Plasma exchange may be considered, with AIHA mediated by IgM having a better response to this procedure than that mediated by IgG, probably because of the different dimensions of the two types of molecules and the prevalent distribution of IgM within the circulation[67].

Pharmacological treatment is indicated only for the rare, primary transfusion-dependent forms **(8.2 -B)**. Steroids induce a limited clinical response in less than 15% of adults affected by primary forms of cold AIHA[67,68]. There is anecdotal evidence regarding beneficial effects of high-dose intravenous steroid therapy[69-71]. The recommendation to use steroids is of uncertain appropriateness **(6.9-D)** and the administration of alpha-interferon or immunosuppressants had disappointing results[67,68,72,73]. Rituximab, a humanised chimeric monoclonal antibody directed against the CD20 antigen expressed by B lymphocytes, proved to be effective and well-tolerated: two prospective trials in adults, although neither controlled nor randomised, showed that rituximab induced a response in 45-60% of cases[74,75]; this percentage rose with the addition of fludarabine[76]. Rituximab can, therefore, be considered the first choice of treatment for primary forms of cold AIHA **(8.0-C)**. The average dose is 375 mg/m$^2$/week for 4 weeks; the response is independent of age, previous treatment, and association with other drugs.

## Conclusions

The present report represents a valuable effort to support paediatricians by providing a tool for streamlining diagnosis and managing first-line therapy of childhood AIHA. Notwithstanding the limitations of opinion-based recommendations, the Committee achieved a broad consensus on issues related to how to treat children newly diagnosed with AIHA, yielding a comprehensive review of all relevant clinical aspects.

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Where possible, "A" is added for studies conducted in adults, and "P" for those conducted in paediatric patients and the level of evidence (I-V), as described in Table I, is stated.

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