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APLASTIC ANEMIA - FROM PATHOPHYSIOLOGY TO DIAGNOSIS, MANAGEMENT AND TREATMENT

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“HAPINESS IS ONLY REAL WHEN SHARED”

Jon Krakauer, *Into the Wild*

Dedicated to my parents and my brother.

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Abstract

Aplastic anemia (AA) is a rare hematopoietic disease characterized by a pancytopenia and a hypoplastic bone marrow. AA can be congenital (CAA) or acquired (AAA). Acquired AA comprises those cases where a causative factor is identified (Secondary AA) and also idiopathic cases (Idiopathic AA). There was a marked improvement on treatment options in the last years that had resulted on increased overall survival rates. It is known that a correct management of this entity is directly related with an efficient diagnostic investigation, and for that, it is fundamental to be aware of the most effective strategies or techniques available nowadays.

Therefore, the aim of this review is to make a state of art of the most recent available data concerning this disorder, particularly IAA, including all the sub-topics inherent: etiology, pathophysiology mechanisms, differential diagnosis, management and treatment options.

Key-words: Aplastic Anemia, Idiopathic Aplastic Anemia, Congenital Aplastic Anemia, Autoimmunity, Hypocellular Bone Marrow, Pancytopenia, Immunosuppressive therapy, Hematopoietic Stem Cell Transplantation, Paroxysmal Nocturnal Hemoglobinuria.

Resumo

A Anemia Aplásica (AA) é uma doença hematopoiética rara caracterizada por pancitopenia e hipocelularidade da medula óssea. A AA pode ser congénita ou adquirida. A AA Adquirida inclui os casos em que é possível identificar um fator causal (AA Secundária) e também os casos idiopáticos (AA Idiopática). Nos últimos anos tem havido uma melhoria notável nas opções de tratamento o que tem conduzido a melhores taxas de sobrevivência. É também um facto estabelecido que uma orientação clínica correta está diretamente relacionada com uma investigação diagnóstica eficiente, o que por sua vez exige um conhecimento sobre as estratégias e técnicas mais eficazes disponíveis na atualidade.

O objetivo desta revisão é fazer um estado da arte das publicações e dados mais recentes relacionados com esta doença, especificamente em relação à Anemia Aplásica Idiopática, incluindo assim todos os subtópicos inerentes: etiologia, mecanismos fisiopatológicos, diagnóstico diferencial, conduta e opções de tratamento.

Palavras-chave: Anemia Aplásica, Anemia Aplásica Idiopática, Anemia Aplásica Congénita, Auto-imunidade, Medula Óssea Hipocelular, Pancitopenia, Terapia Imunossupressora, Transplante de células estaminais hematopoiéticas, Hemoglobinúria Nocturna Paroxística.

Abbreviations

AA - Aplastic Anemia

AAA - Acquired Aplastic Anemia

AL - Acute Leukemia

ALL - Acute Lymphoblastic Leukemia

ALZ - Alemtuzumab

AML - Acute Myeloid Leukemia

ATG - Antithymocyte Globulin

BM – Bone Marrow

BM-MSC - BM Mesenchymal Stem Cells

BMT - BM Transplant

CAA - Congenital Aplastic Anemia

CAMT - Congenital Amegakaryocytic Thrombocytopenia

CsA - Cyclosporine

CTL - Cytotoxic T Cells

Cys - Cyclophosphamide

DBA - Diamond-Blackfan Anemia

DC - Dyskeratosis Congenita

DEB - Diexpoxybutane

DRS-1- Diazepam-Binding Inhibitor–Related Sequence 1

EBV - Epstein–Barr virus

ELA2- Neutrophil Elastase Gene

FA - Fanconi Anemia

FBC - Full Blood Count

FLU - Fluradabine

G-CSF - Granulocyte Colony-Stimulating Factor

GPI - Glycosylphosphatidylinositol

GVHD - Graft-Versus-Host Disease

h-ATG - Horse Antithymocyte Globulin

Hb - Hemoglobin

HbF - Fetal hemoglobin

HGF - Hematopoietic Growth Factors

HIV- human immunodeficiency virus

HLA- Human Leukocyte Antigen

HSC - Hematopoietic Stem Cells

HSCT - Hematopoietic Stem Cell Transplantation

IAA - Idiopathic Aplastic Anemia

INF-γ - Interferon gamma

IST- Immunosuppressive Therapy

LDH - Lactate Dehydrogenase

LGLL- Large Granular Lymphocyte Leukemia

MDS- Myelodysplastic Syndromes

MMC - Mitomycin C

MRI - Magnetic Resonance Imaging

NIH - National Institutes of Health (U.S. Department of Health and Human Services)

N-SAA – Non Severe Aplastic Anemia

OS - Overall Survival

PNH - Paroxysmal Nocturnal Hemoglobinuria

r-ATG - Rabbit Antithymocyte Globulin

SAA – Severe Aplastic Anemia

SCN - Severe Congenital Neutropenia

SCT – Stem Cell Transplant

SDS - Shwachman-Diamond Syndrome

TNF-α - Tumor Necrosis Factor Alpha

UD - Unrelated Donor

VSAA - Very Severe AA

Introduction

Aplastic Anemia (AA) is a singular hematological rare disease that combines a blood pancytopenia with a hypocellular bone marrow (BM) – the simplicity of these criteria conferred this clinical condition a reference as the paradigm of BM failure syndromes.[1]

AA can be congenital or acquired. Congenital AA (CAA) comprise the inherited disorders of BM failure that usually presents in the first years of life, being also associated with one or more somatic abnormalities. Acquired AA (AAA) includes the impaired hematopoiesis that can result from secondary causes (like exposure to toxics, drugs, radiation and virus) or it can be idiopathic, where the causative agent is unknown.

Idiopathic AA (IAA) is the aim of this review. Several studies reports that it is an immune-mediated disease, characterized by a T-cell-mediated organ specific destruction of BM hematopoietic cells – and several questions had been introduced since the description of the first case by Ehrlich in 1888: what leads to the immune response against hematopoietic stem cells? Is there any risk factor- environmental or genetic? Can we change the clinical outcome? Why some clear associations with other diseases, like Paroxysmal Nocturnal Hemoglobinuria (PNH) or Myelodysplastic Syndromes (MDS)? What can we offer to the patients?

Some old questions remain unanswered, and the improvement in cellular and biological research combined with the advances on investigation methods lead to new questions, providing a wide collection of publications regarding this clinical entity. Research works are therefore fundamental to the development of more accurate differential diagnostic algorithms (which include phenotypic, clinical laboratory and genetic data) or the most effective treatment options.

In fact, the differential diagnosis should be taken as one crucial step, being the main key of a successful treatment. For example, the presence of a fatty BM on biopsy indicates aplasia;

however, marrow hypocellularity, and the correspondent degree of cytopenias, can occur in several others hematologic diseases. Time between the establishment of a diagnosis and the beginning of treatment is also crucial since it is directly related to outcome regardless of the therapeutic option chosen.

The goal of this review is to make a state of art of this disorder – and therefore, line up a working method. In the presence of a potential case of IAA is fundamental to know what others diseases should be ruled out, which are the best treatment options and what are the possible outcomes. Considering that a correct management is only possible with a correct understanding of the disease, all the remaining sub-items inherent (such as incidence or pathophysiology) will also be explored.

1- Incidence of Acquired Aplastic Anemia

AAA had been the target of several epidemiologic studies – however, the most recent remains a prospective multicenter study between 1980 and 2003, in the metropolitan area of Barcelona[2]. This study shows an overall incidence of AA of 2,34 cases per million per year, which is reported to be similar to data reported on other population-based studies which had taken place in different Western countries (European countries such as France and United Kingdom, and also Israel and Brazil). Asian data and studies shows different incidence rates, being reported as two to three folds higher.[3] This dissemblance is observed and related in several epidemiologic studies. It is supported by different studies that use the same methodology, in opposition to some historical literature that report larger differences between Western and East. These differences are currently considered as exaggerated, and can be explained considering the absence of some diagnostic tools (e.g. the use of marrow biopsies for the AA diagnosis instead of only consider blood counts)[1].

The marked difference between these two global areas (European countries and Asian countries) remains unexplained.[4] However, and without any other recent data, this geographic difference between East and West is currently accepted.

The largest studies such as Barcelona[2] also reports a sex ratio close to 1:1, and two patient age peaks of incidence, translating a biphasic age distribution: one peak among young adults (15-25 years old) and a second peak in the elderly (≥65 years old).

Due the lacking of a clear division in all studies between the cases of Secondary AA and the IAA cases, these incidence data are referent to AAA - the cases diagnosed as CAA were excluded and correctly defined as one exclusion criteria in all the mentioned articles.

2- Pathophysiology

IAA is an immune-mediated disease?

AA is characterized by pancytopenia with a hypocellular BM, caused by the decrease of hematopoietic cells. Hematopoietic stem cells (CD34+) [u1]are markedly diminished, and it is reported a reduction on stem cell pool to 1% of normal at the time of presentation in *in vitro* assays.[8] Literature also refers a predominance of fat tissue, which is believed to result from the replacement of the BM cells.[8]

Any attempts to establish a clear knowledge about the pathological mechanisms involved in AAA are hampered by its nature – it's a rare disorder, as seen by the epidemiological data, as well as a result of its characteristics, namely pancytopenia and hypocellular BM. Therefore, the possible cells of interest had disappeared, increasing substantially all the hindrances of achieving satisfactory answers.

Etiology was firstly linked to possible relationships of AA to environmental factors – like chemicals and medical drug exposures - or to some clinical associations with conditions like pregnancy, seronegative hepatitis or syndromes like eosinophilic fasciitis. A full list of some etiologic agents of AAA can be seen in Table 1.

Table 1 – Classification of Acquired Aplastic Anemia
Some etiologies which had already showed a clear association are also listed.

Secondary	**Radiation**
	Drugs and chemicals **1-Agents that regularly produce marrow depression in commonly employed doses:** Cancer chemotherapy **2-Agents that frequently but not inevitably produce marrow aplasia:** Benzene **3-Agents associated with aplastic anemia but with a relatively low probability:** Chloramphenicol, Insecticides, Antiprotozoals: quinacrine and chloroquine, mepacrine, Nonsteroidal anti-inflammatory drugs, Anticonvulsants (hydantoins, carbamazepine, phenacemide, felbamate), Heavy metals (gold, arsenic, bismuth, mercury), Sulfonamides: some antibiotics, antithyroid drugs (methimazole, methylthiouracil, propylthiouracil), antidiabetes drugs (tolbutamide, chlorpropamide), carbonic anhydrase inhibitors (acetazolamide and methazolamide) Antihistamines (cimetidine, chlorpheniramine), d-Penicillamine, Estrogens (in pregnancy)
	Viruses Epstein-Barr virus, Hepatitis (non-A, non-B, non-C), Parvovirus B19,HIV-1
	Imune diseases Eosinophilic fasciitis, Hyperimmunoglobulinemia, Thymoma/thymic carcinoma,Graft-versus-host disease in Immunodeficiency
	Pregnancy
Idiopathic	---

(Adapted from *Harrison's Principles of Internal Medicine* 18Th ed.[8])

However, even that some relationships appears to be real, it is also reported that neither chemicals or drugs appear to be responsible for the majority of cases and no satisfactory mechanisms were infered.[1]

Nowadays, literature leads us to separate the cases where a triggering condition can be linked, and label it as Secondary AA, from those where a causative agent is unknown, being this classified as Idiopathic AA.

Idiopathic AA is reported as one pathologic entity linked to an immune mechanism.

Considering both the oldest and current reports a major conclusion can arise: even that there are plenty of laboratory data supporting an immune pathophysiology, concrete information about detailed mechanisms are still lacking.[9]

Therefore there are plenty factors believed to be involved in the immune attack, and this immune-mediated disease is widely reported to be a result of several particular roles concretized by each one of them. Considering the latest reports, it is now presented a summary of those most widely referred on current literature, namely the role of lymphocytes, cytokines, autoantibodies and genetic factors.

2.1 – Lymphocytes

Lymphocytes T are widely reported as having a major role in the BM destruction. In 2006, Young published a wide compilation about the pathophysiologic mechanisms in AAA[1]. The author reports that the effector cells of the BM destruction phenomena were the activated cytotoxic T cells (CTL). He also explains that this conclusion resulted from the observation of the improvement of aplastic BMs after removal of lymphocytes and the inhibition of hematopoiesis after their addition to normal BMs *in vitro,* allied with the identification by immunophenotyping techniques.

CTL express Th1 cytokines. Th1 cytokines are the responsible for the production of the pro-inflammatory responses necessary to destroy intracellular parasites and for perpetuating autoimmune responses (see section 2.2).[10]

Young also presented the concept of the oligoclonal expansion of CD8+ CD28- cells in AA patients. The author explained that these clones can be identified by flow cytometry analysis of T cell receptor (TCR) Vβ subfamilies – the method includes spectratyping technology, to detect skewing of CDR3 length and in the end, sequencing the CDR3 region to establish a molecular clonotype[1]. The author reported that the use of these techniques in AA cases allowed the detection of oligoclonal expansions of a few Vβ subfamilies in patients at the time of clinical presentation. It is also mentioned that these same clones diminish or disappear after successful therapy. Therefore, it can be related with the course of the disease by itself, as explained in the Treatment section (section 6) in this document.

The oligoclonal expansion is widely explored in the later publications from different authors and a 2014 review[13] by Dolberg & Levy presented the main conclusion: the finding of these T cell subpopulation supports the hypothesis that one of the mechanisms of AA is an antigen-specific lymphocyte attack against hematopoietic tissue.[13]

2.2- Cytokines

CTL express Th1 cytokines and these play a major role in the development of BM failure. Interferon gamma (INF-γ) is the main Th1 cytokine. The role of INF-γ is also explored by the work of Young (2006) which explained that after using microarray of the scant CD34+ cells from marrow failure patients it was possible to reveal a transcriptome in which genes involved in apoptosis, cell death, and immune regulation were upregulated. It is also stated that this transcriptional signature can be reproduced in normal CD34+ cells exposed to INF-γ.[1]

It is also reported that IFN-γ gene expression is specifically prevalent in the BM of patients with IAA, and disappears with response to immunosuppression.[13]

One of the most mentioned apoptotic ways is the increased expression of FAS antigen on BM CD34+ cells of patients – this FAS antigen is described as being one receptor molecule that mediates signals for programmed cell death. It is enhanced by INF-γ, leading to a hypoplastic BM induction.[13]

Tumor necrosis factor gamma (TNF-α), another Th1 cytokine, is also reported to be related to this apoptotic way, having a similar role to that of IFN-γ.[13]

Besides INF-γ and TNF-α, some other cytokines had been identified and mentioned in literature: IL-17, which is secreted from TH17 cells (a subset of T helper cells), is also a Th1 cytokine and it is stated that the expression of this cytokine is increased in patients with AA.[13] IL-27 is reported to have an immune regulatory function – it can activate the T-bet[u2] transcription (see section 4.2.2), Th1 differentiation can also induce initial CD4+ T cells to differentiate into Th1 cells promoting Th1-type immune responses. Like IL-17, IL-27 levels, are also reported to be increased in IAA patients.[13]

2.3- Autoantibodies

Some autoantibodies were associated with AA. Nakao *et al* (2005) explored extensively two of them: antibodies to kinectin and antibodies to diazepam-binding inhibitor–related sequence 1 (DRS-1).[12] Kinectin is one of the most mentioned in literature. Kinectin is a protein only expressed by a reduced number of human tissues which includes the liver, the brain, the testis, and also BM CD34+ cells. DRS-1 protein is highly expressed by CD34+ cells from healthy individuals. Antibodies directed to each protein were detected in some AA patients in contrast to their absence on healthy individuals.

Autoantibodies to the hematopoietic cell line K562 and to the BM stromal cell line hTS-5 are also mentioned by Dolberg & Levy (2014) as being correlated with IAA.[13]

2.4 – Genetic Factors

Some host genetic characteristics were identified and are currently associated with a higher susceptibility of IAA, as Human Leucocyte Antigen (HLA), T-Cell encoding genes, . cytokines polymorphisms and telomeres.

2.4.1- Human Leukocyte Antigen (HLA)

HLA-DR2 is widely associated with susceptibility to IAA - it was demonstrated and reported that there was an overrepresentation of DR2 in AA patients when compared with their siblings and parents[14]. HLA-DR2 has been split into DR15 and DR16, and it was also demonstrated that susceptibility could be attributed to DR 15 but not DR 16.[15]

Nakao *et al* (2005) also stated that the frequency of HLA-DR15 is significantly higher in AA patients when compared with healthy control populations and the frequency of this allele is also particularly high in Japanese adult patients with IAA aged with more than 40 years old.[12]

HLA association in children has been reported as inconsistent, which may lead to the hypothesis of different causative factors for AA in children from those in the older age groups.[14]

2.4.2 – T-Cell Encoding Genes

T-bet gene belongs to the T-box family of transcription factors, and it is the key regulator of Th1 development and function.[13] It is reported to be found in Th1 but not in Th2 cells.[13]

It was presented by Bacigalupo in 2007 that $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells are deficient in IAA patients, and this deficient regulation of T cells could then lead to an increase

of T-bet protein levels, increasing INF-γ (T-bet transcribes actively the IFN-γ gene) leading, as seen, to stem cell destruction.[6]

2.4.3 – STAT3 Acquired Mutations

A recent publication by Young from 2013[9] introduces some new concepts to those already explored in older publications – the author refers the possible role of acquired mutations in STAT3 in AA patients. These mutations would functionally result in constitutive activation of this signal transducer; Young also states that acquired STAT3 mutations had been reported in others pathological situations like autoimmune diseases. They are also prevalent in Large Granular Lymphocyte Leukemia (LGLL), a clinical entity in which a single T-cell clone dominates and suppresses BM function.[9]

2.4.4 – Cytokines Polymorphisms

Polymorphisms in cytokine genes that are associated with an increased immune response are also reported by Young in 2006 to be more prevalent in AA patients. In the same article, the authors also points out specific examples, like a nucleotide polymorphism in the TNF-α promoter at –308 and also homozygosity for a variable number of dinucleotide repeats in the gene encoding INF-γ.[1]

2.4.5 – Telomeres

This genetic determinant is one of the most recent data presented by literature, with several publications nowadays covering this subject. [9,16,17]

Young (2006) presents some concepts: telomere loss is compensated by telomerase, a telomere repair complex.[1] The complex consists of the TERT enzyme, a reverse transcriptase, and its RNA template, TERC. Telomerophaties was firstly linked to Dyskeratosis Congenita

(DC), a CAA syndrome where the unifying feature of patients with DC is the presence of very short telomeres[27] (see section 5.1). However, Young (2006) pointed some relevant findings like the fact that family members who share mutations in TERT or TERC, and short telomeres also have hypocellular marrows, reduced CD34+ cell counts and poor hematopoietic colony formation. The author also states that all this findings can coexist with normal or near normal blood counts.[1] These data allowed the author to infer that these mutations, and telomere length, confer a quantitatively reduced hematopoietic stem cell compartment that could be qualitatively inadequate to sustain immune mediated damage.[1]

Young (2013) widely explored the role of telomeres and telomeropathies and their possible role on BM failure.[9] On the same article the role of TERT or TERC mutations are mentioned as being risk factors and not precise genetic determinants of BM failure.[9]

Dolberg & Levy (2014) presented a rate of 10 to 20% of patients with AAA as having short telomeres. These patients are also associated with a lower survival rate and higher relapse rates after treatment (see section 6.3- Definitive Treatment) than those with longer telomeres.[13]

As mentioned, information about detailed or concrete mechanisms are lacking: what induces the breakdown of immune tolerance to antigens on hematopoietic cells is still unclear on current literature.

Nakao *et al* (2005) suggested that the primary immune response may be directed not to antigens restricted to hematopoietic stem cells but rather to antigens expressed by immature hematopoietic cells in the BM. The authors explained that immune responses would then lead to the production of inflammatory cytokines capable of inhibiting the growth of hematopoietic stem cells. They also describe a *bystander effect* of these cytokines that would be responsible for the abrupt loss of hematopoietic cells in the BM.[12]

Young (2006) considers that the molecular basis of the aberrant immune response could be represented as a set of susceptibility factors as the ones mentioned above: cytokine gene polymorphisms, abnormalities in the regulatory pathways of INF- γ, and the possible role of HLA-DR2 for antigen recognition. A set of some these would then lead to marrow failure, and the recovery of this stem cell depletion could also be limited for others genetic risk factors like telomerase deficiency or short telomeres.[1]

A recent article by Bueno *et al* published on March of 2014 should be noted – it actually clarifies a question presented by Bacigalup, in 2007 – "Is acquired AA a disease of the seed (HSC) or soil (microenvironment, being this, the stromal cells)?".[6]

Bueno *et al* (2014) described that BM mesenchymal stem cells (BM-MSC) have immunomodulatory and anti-inflammatory properties, being an essential component of the BM hematopoietic microenvironment. They are related to regulation of hematopoiesis homeostasis through the production and secretion of cytokines and extracellular matrix molecules. The results presented in this report states that BM-MSC from AA patients have the same phenotype and differentiation potential as controls from normal BM – the authors therefore conclude that BM- MSC from AA patients do not have impaired functional and immunological properties, being able to support hematopoiesis. They also indicate that considering this, BM-MSC do not contribute to the pathogenesis of the disease.[7] The authors also pointed that the study and the BM samples were collected from elderly patients diagnosed as N-SAA and SAA (see section 6.1 for Classification of Severity) and for that reason larger studies are necessary to unravel whether age at the diagnosis and the disease severity can affect the homeostasis and function of the BM microenvironment. However, these results strongly support that the HSC are the main target of the immune mechanism.

In conclusion, most of literature supports the role of the immune system and the dysfunction in the immune response as the responsible for the HSC cells as the main target[u3]. Nissen &

Stern published in 2009 a new hypothesis that should be mentioned for the possible new insight about the role of immune system in some cases of AA.[18] In the mentioned article, the authors states that autoimmunity can inhibit growth of solid tumors, and that this anti-tumor activity can also be applied to hematologic diseases – raising then the question that malignant altered cells may be preexisting. Autoimmunity against hematopoietic stem cells may reflect an attempt to eradicate these malignant cells, instead of considering the primary event the dysfunction of regulatory T-cells (leading to the sequential BM failure). The same hypothesis is supported by reference to some recent observations – and can also elucidate about some questions concerning the eventual outcomes of IST therapy, like clonal evolution (see section 6.3 – Definitive Treatment).

3- Aplastic Anemia and Paroxysmal Nocturnal Hemoglobinuria

Paroxysmal Nocturnal Hemoglobinuria (PNH) is described as an acquired hemolytic anemia with a classic triad of hemolysis, thrombosis and marrow failure. It is also known to be caused by a clonal expansion of a hematopoietic progenitor cell that has acquired a mutation in the X-linked phosphatidylinositol glycan class A (PIG-A) gene.[19]

The relationship between AA and PNH had been widely documented. Historically AA was described as a condition that evolve to PNH.[20] However, recent publications allowed a different perspective - it is now proved that about 40-50% of AA patients have a PNH clone detected at the time of diagnosis.[21]

3.1- Genetic features of PNH

The clonal expansion of a PNH clone leads to a cluster of cells which are deficient in all proteins linked to the membrane by a glycosylphosphatidylinositol molecule (GPI-anchored proteins).[19] This is reported as being due to somatic mutations in the X-linked *PIG-A* gene.[19] Another particular note is that the localization on the X chromosome in addition to the lionization in female somatic cells, leads to the conclusion that only one mutation is required in either males or females to abolish the expression of GPI-linked proteins.[19]

PNH is a hematologic disease and to date PIG-A gene mutations have only been described in hematopoietic cells. In PNH patients GPI-anchored proteins are lacking from variable proportions in the different hematopoietic cells, and are also consistent with the activity of an aberrant hematopoietic stem cell clone.[20]

3.2 – Pathophysiology

Pathophysiology of PHN is widely studied – Bessler & Hiken directly address and clarifies this subject on a publication in 2008.[19] The authors described the two central pathophysiologic

components as being BM failure and the occurrence of blood cells that are deficient in GPI-anchored proteins. BM failure is reported as being a finding present in all patients with PNH, (even with normal peripheral blood counts and a hypercellular BM). The degree of BM failure however is described as variable.

The authors also explain one of the most characteristic features of PHN – the complement activation. This phenomenon is reported to occur due to the lack of GPI-anchored proteins in PNH blood cells. In normal conditions complement activation should be regulated by specific proteins, present on the surface of normal human cells. These proteins are identified as two GPI-linked inhibitors of complement activation, CD55 (decay accelerating factor, DAF) and CD59 (membrane inhibitor of reactive lysis, MIRL) as well as one membrane cofactor protein (CD46, located at the plasma membrane via a transmembrane domain). Erythrocytes lack CD46 and express only CD55 and CD59, in opposition to platelets or white blood cells which express also CD46. This last fact is described as the reason of the massive intravascular lysis, and the classic hemoglobinuria which named the disease.[19]

3.3- Clinical Features

Clinical features are also described by Bessler & Hiken (2008): PHN is reported here as a rare acquired chronic disorder, with no inherited form described.[19] Affects both gender equally and no differences in incidence are reported concerning variables like socioeconomic status or geographic worldwide distribution. The clinical manifestations described are those of a hemolytic anemia, acquired thrombophilia, and BM failure. The degree to which each contributes to the clinical presentation is reported to be variable between patients and during the course of the disease. [19]

It is also reported that the diagnosis is mostly done in young adults, but there's data reporting that it can be done at any age. Currently, flow cytometric analysis is the preferred laboratory test for diagnosis purposes.[19]

Treatment options are clear in all recent publications – one increased understanding of pathophysiology of this disease in the last years allowed the introduction of complement inhibitors (Eculizumab) which directly improved management and treatment prognosis.[19,20]

3.4- Idiopathic Aplastic Anemia and PNH

Young *et al*, in a 2002 publication, reported that flow cytometry testing indicated that marrow failure and PNH clonal expansion frequently coexisted, this being apparent in 40-50% of patients at the time of diagnosis of AA.[20] These findings justifies the interest of scientific community in the clarification of a possible relationship between both entities, leading to the several publications regarding this subject.

The mechanism responsible for the expansion of PNH cells in AA - even if several hypotheses were already proposed and tested - remains unknown. In 2002, on the cited report, Young *et al* had already included a review of some of them[20]: one hypothesis relay on the fact that PIG-A deficiency could confer on mutant cells an intrinsic growth or survival advantage – however, the authors states that clinical observations showed that PNH as a disease doesn't reveal an invasive character. Most patients are even reported to maintain a stable proportion of PNH cells over the years. To support this, the authors also cited results from studies with chimeric knock-out mice (with PIG-A mutate cells), that, after being cross-pated with analysis of the transcriptome of normal and PNH cells, showed that PIG-A mutation wasn't associated with major changes in the cell's program for growth, differentiation, or death. For last, small PNH clones can also be identified in normal individuals, and the authors refered that this finding doesn't have any clinical significance.

Another hypothesis was formed considering precisely the frequent association of PNH with AA - this circumstance suggested that the hypocellular marrow might be an extrinsic factor to promote clonal expansion of PHN cells. However, even that PNH clones are present in a very large proportion of IAA patients, PNH does not occur after other conditions that could lead to a hypocellular BM like chemotherapy, radiation-induced aplasia or following stem cell transplant. The authors conclude that PNH as a clinical entity does appear to correlate mainly with immunologically mediated marrow failure.

Another relevant fact analyzed by the authors is that IAA and PHN/AA shows the same pathologic mechanisms: using fine analysis of T cell receptors it is reported to be possible to confirm that the immune response is antigen driven in PNH/AA, exactly like IAA. Young *et al*, cited a study of patients with PNH/AA, where was found not only a limited utilization of the Vβ chain of the T cell receptor, but that among different patients the dominant CD4 T cell clones used had identical CDR3 region for antigen binding. It is also reported that this CDR3 region specifically inhibit autologous hematopoiesis.[20]

In other study by Chen *et al*, also cited in the 2002 article by Young *et al*, it was reported a deficiency among the normal cells rather than an advantage for the PIG-A mutant cells. Methodology included the culture of CD34+ cells, which were separated based on the presence or absence of CD59, and cultivated in the presence of growth factors. The authors described that the cells of PNH phenotype behaved normally, without any sign of impaired production, while CD34+ cells of normal phenotype showed little growth.[20] These results are similar with those found in IAA as already mentioned on section 2.2 - where minimal numbers of colonies derived from committed progenitors revealed a transcriptome in which genes involved in apoptosis, cell death and immune regulation were upregulated[1].

Analyzing this results a conclusion is already lined - the difference between GPI deficient cells and normal CD34+ cells can indeed be explained by an immunologic mechanism. The

authors reported that exactly like CD34+ cells for AA, also normal CD34+ from PNH had a raised expression of FAS and concomitant sensitivity to FAS-ligand mediated apoptosis that wasn't seen in the GPI-anchor protein deficient cells. Young *et al* (2002) also have reproduced these results: when comparing CD34+ cells of the PNH phenotype with normal BM cells from the same patient, these last showed lower cloning efficiency, increased FAS receptor expression, and also increased apoptosis.

These results support the most acceptable possible mechanism for clonal expansion in PNH, that is also presented by Young *et al*, in 2002, and still remains as the most likely and still referred in newest publications[19,21,22] - PNH cells may indeed have a proliferative advantage over non-PNH cells, not by intrinsic growth or survival advantage, but by an immune mechanism of selection. Some GPI-anchored proteins are ligands for T cell receptors, including CD58 and CD59, or serve immune functions, as do CDI4 and the Fc III receptor[20]. The absence of these molecules or the ligands for T cells receptors could then result in a failure of PNH cells to be recognized by the immune system – creating a possible path to escape from immune surveillance. Young *et al,* also refers that most experiments to directly test this possibility had been negative – however these authors and others had mentioned, the effect described could be so specific that only manifest under special *in vivo* circumstances. Until now, there aren't any publications to clarify this subject.

For last, in the referred 2002 report, the hypothesis that the PNH cell might have a more primary role in the development of BM failure is also stated. Several mechanisms that could lead to this pathology are also mentioned: first, GPI-anchored proteins might serve as antigens by themselves. The authors also explain that this could happen by two mechanisms: due to molecular mimicry with exogenous peptides, or being directly involved in the process of antigenic spread. Secondly, the PNH clone itself might induce autoimmunity – and this could happen considering the normal physiology of protein trafficking. It is clarified that due to

absence of GPI anchors, GPI-anchorless proteins in the PIG-A- cell be processed by the proteasome and their peptides displayed via class I HLA. In the normal circumstances, the membrane-bound protein degradation should occur in lysosomes, leading to presentation of peptides in the context of class II HLA instead of I HLA.[20]

3.5 – AA/PNH syndrome – a predictive indicator?

As seen, about 40-50% of AA patients have a PNH clone detected at the time of diagnosis[21], and during management of PNH, AA/PNH syndrome is already referred as one clinical subgroup[19] in some publications.

There is no significant difference reported between the different classifications of severity of AA (see section 6.1) and the detection of a PHN expanded clone. Considering this, a major question arises: what means the presence of this expanded clone and what consequences could it bring for patients?

The literature contradicts what could be the first negative conclusion resulting from a introduction of a second diagnosis of another rare disease, that was reported with such potentially serious consequences - in the past, therapy of PNH was often restricted to the treatment and prevention of complications (e.g. red blood cell transfusions for the treatment of anemia or immunosuppression for the treatment of BM failure). PNH was also associated with increased propensity for the development of life-threatening venous thrombosis, being also reported that patients with a large PNH clone were at a higher risk of developing thrombosis than patients with a small PNH clone. However, introduction of complement inhibitors for the treatment of PNH was a major advance in the management and prevention of these complications[19]- eculizumab, a monoclonal antibody directed against the complement protein C5, is reported as effective in blocking intravascular hemolysis and reducing the incidence of thromboembolic events in patients with PNH.[21]

IIt is reported that having a PNH clone at time of AA diagnosis is associated with low morbidity and mortality (considering only the implications that PHN as a clinical entity could bring), and specific measures to clinical management of PNH are seldom required[21] – this conclusion is broadly stated in several reports, and a retrospective analysis by Scheinberg *et al* (2010) is particularly representative of this. In the quoted publication, authors presented the results achieved by quantification of PNH clones by flow cytometry of 207 patients classified as severe aplastic anemia (SAA) (see section 6.1) who had received immunosuppressive therapy (IST) with a horse anti-thymocyte globulin (h-ATG) based-regimen (see section 6.3), concerning the period from 2000 to 2008[u4][u5]. A total of 83 patients (40%) had a PNH detected prior to treatment and the response rate for all patients was 62% - there was no difference in response rate between patients with or without a pre-treatment clone. Therefore, the presence of a PNH clone does not directly interferes with IST response in AA. A summary of the results obtained is presented in Table 2.

Some results, due to their significance should be underline – first, the concept that PNH was a condition evolving from AA is once more widely contradict - the development of a PNH clone in patients who do not have a pre-existent clone prior to IST is uncommon (21%). Secondly, in those with a pre-existing clone, a decrease in clone size is likely

Table 2 – SAA after IST regimen and relation with PHN clone

207 patients (100%) submitted to IST regimen	After IST
124 patients (60%) without PNH clone pre-treatment	a) 26 patients (21%) had a PNH clone b) A large PNH clone (>50%) developed only in one patient – who also had an elevated LDH but no episodes of dark urine, esophageal spasms, abdominal pain or thrombotic events. c) Other patients with smaller clones did not have clinical evidence of hemolysis or thrombosis.
83 patients (40%) with PNH clone pre-treatment	a) 10 patients (12%) didn't had a PNH detected b) The remaining 73 patients (88%): an increased in clone size was noted in 30 patients (25%) two years after IST

IST- Immunosuppressive Therapy, LDH- Lactate Dehydrogenase, PNH- Paroxysmal Nocturnal Hemoglobinuria , SAA – Severe Aplastic Anemia

(Adapted from "Paroxysmal nocturnal hemoglobinuria clones in severe aplastic anemia patients treated with horse anti-thymocyte globulin plus cyclosporine." by Scheinberg et al. *Haematologica* 2010 Jul)

to occur in the years following IST - only about 25% of those with a pre-treatment clone had an expansion.

Clinically, hemolysis was reported as being mild or subclinical in the majority of patients – and symptoms only occurred when the clone size was large (>50%). Authors also states that only less than 5% of patients required specific interventions for PNH.

This study underlines a management strategy widely adopted worldwide - in clinical practice patients with SAA are treated with IST regardless of the presence of a PNH clone. The detection of this PHN clone is even considered as a mandatory step in the management of a potential IAA case. This strategy will be explored further in this document, on section 6.

Scheinberg *et al* (2010) also explained the reason for this strategy - resumption of hematopoiesis must be seen as the primary goal, once that specific therapy for PHN like eculizumab doesn't have a role in improving marrow function.

Concerning AA, even that some others studies reported that the presence of a clone has been reported to be predictive of response to IST, in this study that was not observed.

4- Clinical Features of Idiopathic Aplastic Anemia

Clinical features of IAA are reported to be directly related with the definition of the disease: a clinical entity that includes the presence of pancytopenia (with at least two of the following required: hemoglobin (Hb) <10 g/dL; platelet count <100 $\times 10^9$/L; and neutrophil count <1.5 $\times 10^9$/L) alongside a hypocellular BM (in the absence of an abnormal infiltrate and with no detectable increase in reticulin).[25]

Pancytopenia is widely reported as the first indicator to be unrevealed: it may cause the symptomatology that leads the patient to seek medical attention, or it can be discovered occasionally in asymptomatic patients in the set of procedures like preoperative evaluation, blood donation or any other analytical investigation.

When symptomatic, patients can present the symptoms related to anemia or thrombocytopenia. Neutropenia, even if severe, it is reported as being asymptomatic and infection at presentation as infrequent. [24]

Anemia, can lead to classic symptoms like fatigue, tachycardia, headache, dizziness, leg cramps or insomnia. Physical exam of these patients can reveal findings like a forceful heartbeat, strong peripheral pulses and/or a systolic "flow" murmur. The skin and mucous membranes may be pale when Hb is lesser than 8 to 10 g/dL.[8]

Thrombocytopenia, can lead to findings and symptoms like skin or mucosal hemorrhage, epistaxis, or even visual disturbance (which results from retinal hemorrhage).[8]

Physical findings like lymphadenopathy or hepatosplenomegaly, which could be related with anemia or thrombocytopenia, are not present in AA, and strongly suggest another diagnosis (see section 5).

Literature is objective in this topic – there are no specific symptoms or signs, and no pathognomonic finding is described or reported.

5- Differential Diagnosis

IAA, despite the precise definition criteria, is considered a diagnosis of exclusion – besides the Congenital AA, as there are also a largely collection of other hematologic conditions documented that can lead to pancytopenia or hypocellular BM.

Diagnosis is strongly recommend to be founded on a step-by-step approach (see fig.1, section 5.3), comprising a narrow clinical history, a detailed physical examination alongside one thorough investigation. In the end, all the findings must allow to confirm the diagnosis, exclude other possible causes of pancytopenia with a hypocellular BM, Congenital AA or Secondary AA, assess the severity of the disease and define the best treatment option. This judicious approach is indispensable and substantiated in several publications - treatment and management options may vary with the different causes of pancytopenia and BM failure. There's also a clear relationship documented between time taken from first medical contact and the beginning of treatment in a confirmed IAA case, so all the efforts must be to making a correct diagnosis in the shortest time possible.

The next two sections (5.1 and 5.2) comprise a summary of some clinical entities that literature states as some of the pathologies that should always be present in clinical logic as possible differential diagnosis.

5.1 – Congenital Aplastic Anemia

As mentioned in the sections above, there are several forms of Congenital AA and current literature make aware that some patients may not present the classic signs and findings usually described. These syndromes may become true diagnosis challenges, leading to a generalized recommendation from the majority of authors: every physician who deal with potential IAA cases should be familiarized with some characteristics which may allow a correct differential diagnosis.

There's also a consensus on the fact that labeling of IAA rather than one of the CAA syndromes is more than a semantic division. A correct classification is widely reported as having implications for treatment options, and also allowing the possibility of genetic counseling for other family members.

There are plenty publications regarding CAA and a report by Weinzier & Arber (2013)[25] is particular exhaustive considering these syndromes and others causes of pancytopenias.

Considering that one extensive description of each one of CAA syndromes isn't the aim of this document, Table 3 presents a synthesis of some features concerning few characteristics of the syndromes most representatives in the clinical setting.

Table 3 - Congenital AA syndromes

	Inheritance pattern	Congenital defect	Median age	Clinical findings	Diagnosis
Fanconi Anemia (FA)	AR	16 genes[25] identified	<7 years (literature report ranges of age of diagnosis up to 49 years[26])	**Congenital abnormalities:** skeletal abnormalities, small stature Urogenital, gastrointestinal and neurologic abnormalities **Cancer susceptibility** **Progressive hematologic dysfunction**	test of chromosomal breakage with DEB or MMC
Dyskeratosis Congenita (DC)	XL AD AR	Presence of very short telomeres[27] XL: DKC1[26] AD: TERC and TERT[26] AR: NOP10[26]	skin and nail abnormalities < 10 years old bone marrow failure, by 20 years in 80% of patients[25]	**Classic triad:** of leukoplakia, nail dystrophy, and lacy skin pigmentation 20% of patients also presents pulmonary manifestations of reduced diffusion capacity or restrictive pulmonary disease[25] **Bone marrow hypoplasia** **Predisposition to malignancy**	Genetic testing (a negative test result does not exclude DC - pathologic genetic mutations are uncharacteristic in approximately 50% of DC cases[25])
Diamond-Blackfan Anemia (DBA)	AD	RPS19 and RPS24 genes represent about 25% of known patients.[26]	early infancy	**Reticulocytopenic and NM anemia** **Somatic abnormalities:** short stature and craniofacial, thumb, cardiac and urogenital mal-formations[26,27,28] Predisposition to malignancy [26,27,28]	Bone marrow findings, elevated erythrocyte deaminase activity, macrocytosis, elevated HbF[26]
Shwachman-Diamond Syndrome (SDS)	AR	Mutations in chromosome 7 affecting the Shwachman-Bodian-Diamond syndrome (SBDS) gene[25]	early infancy	**Exocrine pancreatic insufficiency** **Skeletal abnormalities and** **Progression into MDS and AL[28]**	Exocrine pancreatic insufficiency Cytogenetic abnormalities of chromosome 7[25]
Congenital Amegakaryocytic Thrombocytopenia (CAMT)	AR	Mutations in MPL	early infancy	**Classic clinical signs due to thrombocytopenia[25]**	Genetic analysis (a negative result does not exclude the diagnosis)
Severe Congenital Neutropenia (SCN)	AR	Heterozygous mutations in ELA2, HAX1 GFI1 and WASP genes[28]	early infancy	**Early onset neutropenia Pyogenic infections Marrow maturation arrest[26]**	Severe neutropenia on at least three blood counts BM examination

AD- autosomal dominant; AL- Acute Leukemia; AR- autosomal recessive; BM– bone marrow; DEB- diexpoxybutane; HbF- fetal haemoglobin; MDS- Myelodysplastic Syndromes; MMC- mitomycin C ; NM- normochromic macrocytic; XL – X linked;

5.2 – Differential Diagnosis of others Pancytopenias

Besides Congenital AA, there is a substantial number of others diseases that can lead to pancytopenia. A summary is presented in Table 4.

A special attention should be given to those entities that besides pancytopenia also reveal a hypocellular BM, leading to an increased diagnosis dilemma.

Table 4 - Differential Diagnosis of Pancytopenia

Pancytopenia with Cellular Bone Marrow	Primary bone marrow diseases Secondary to systemic diseases Myelodysplasia PHN (see section 3) Myelofibrosis Some aleukemic leukemia Myelophthisis Bone marrow lymphoma Hairy cell leukemia Systemic lupus erythematosus Hypersplenism B12, folate deficiency Overwhelming infection Ostheopaties Alcohol Brucellosis Sarcoidosis Tuberculosis Leishmaniasis
Pancytopenia with Hypocellular Bone Marrow	**AAA** **CAA** (see section 5.1) Hypocellular MDS Rare aleukemic leukemia Some acute lymphoid leukemia Some lymphomas of bone marrow
Hypocellular Bone Marrow ± Cytopenia	Q fever Legionnaires' disease Anorexia nervosa, starvation *Mycobacterium* infection

AAA - Acquired Aplastic Anemia; CAA - Congenital Aplastic Anemia; MDS- M*yelodysplastic* Syndromes; PNH - Paroxysmal Nocturnal Hemoglobinuria

(Adapted from *Harrison's Principles of Internal Medicine* 18a ed.[8])

5.3 – Investigation

As mentioned, several scenarios are reported as potential reasons to lead to the first step of investigation – patient can seek medical attention, due to pancytopenia related symptoms, or blood count abnormalities be revealed during any other clinical routine/practice. Regardless of the reason, it is widely recommend that a proved and documented pancytopenia should always be clarified, and most of all, correlated with the condition underlying it.

On section 5.1 and 5.2 it was presented certain entities that are documented as the ones that the physician should be acquainted with when managing a potential IAA patient. On the other hand, it is also reported that any thorough investigation must be compatible with a period of time that will not compromise the beginning of treatment of the pathology subjacent (IAA or any other). A simplified and outlined protocol may reduce the delay between first medical contact and decision, allowing a standard approach in every potential patient – it is now presented a proposal, that according with recent literature, incorporates all the exams that are vital, and, therefore, must always guide medical practice in these cases. It is widely reported that in most cases a test or exam can't dictate an exact diagnosis, and none permit to allege IAA.

Table 5 – Investigations required for the diagnosis of IAA

1. FBC and reticulocyte count
2. Blood film examination
3. HbF% in children
4. Bone marrow aspirate and trephine biopsy, including cytogenetics
5. Peripheral blood chromosomal breakage analysis to exclude Fanconi anemia
6. Flow cytometry for GPI-anchored proteins
7. Urine hemosiderin if GPI-anchored protein deficiency
8. Vitamin B12 and folate
9. Liver function tests
10. Viral studies: hepatitis A, B and C, EBV, HIV
11. Anti-nuclear antibody and anti-dsDNA
12. Chest X-ray
13. Abdominal ultrasound scan and echocardiogram
14. Peripheral blood gene mutation analysis for DC if clinical features or lack of response to immunosuppressive therapy.

DC - Dyskeratosis Congenita, EBV - Epstein–Barr virus, FBC - Full Blood Count, GPI – Glycosylphosphatidylinositol, HbF - Fetal hemoglobin, HIV- human immunodeficiency virus

(Adapted from "Guidelines for the diagnosis and management of aplastic anemia" by Marsh *et al, British Journal of Haematology,* Oct 2009)

As conclusion, it's then possible to

state that a correct diagnosis it's only possible by an interconnection between clinical features, genetic and biological results.

Figure 1 represents the step by step proposal that may support this strategy and Table 5 the investigations that the majority of literature states as the ones required for the diagnosis of AA.

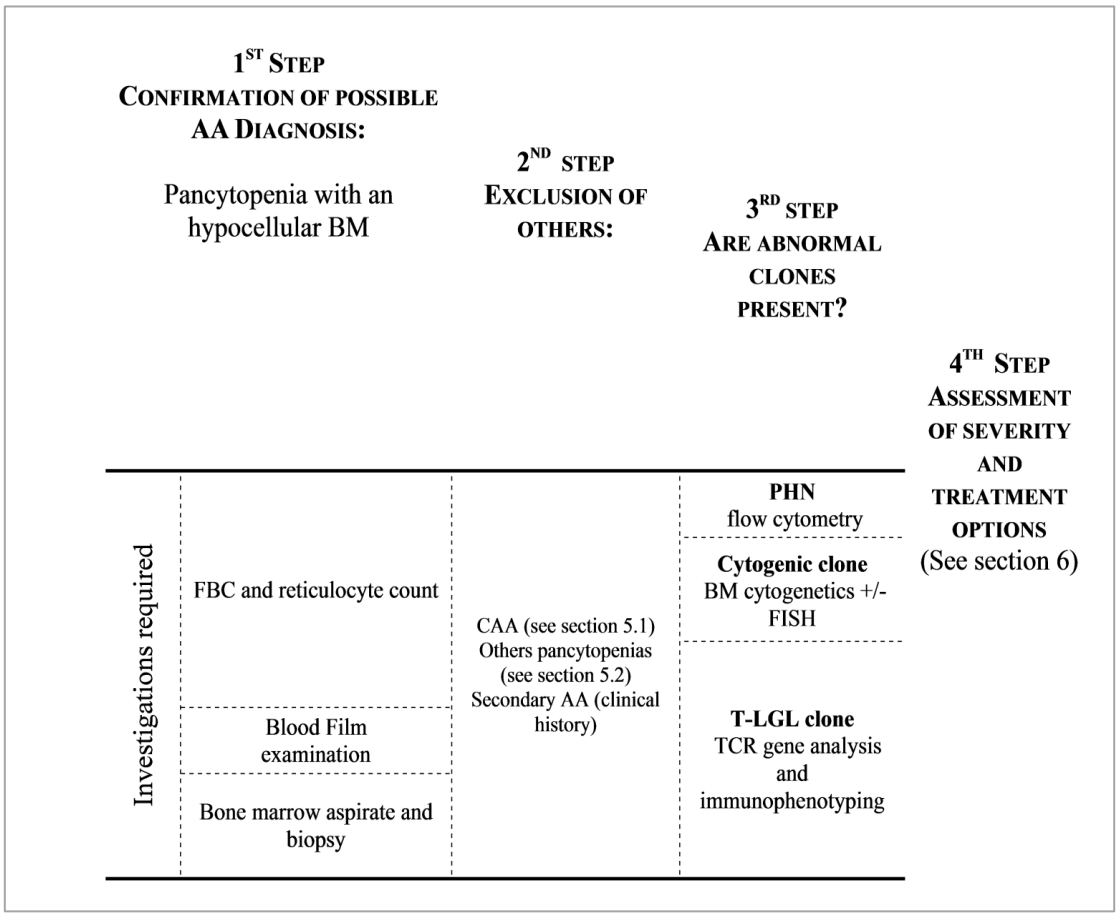

Figure. 1- Step-by-step approach and investigations that should be underlying it.
Step 3 and 4 refers to a confirmed IAA case.

AA- Aplastic Anemia, BM- Bone marrow, CAA- Congenital Aplastic Anemia, FBC- Full blood count, PHN- Paroxysmal Nocturnal Hemoglobinuria, TCR- T cell receptor, T-LGL- Large granular lymphocyte leukemia

5.3.1 – Clinical history

It is consensual that a detailed interview followed by a narrow physical exam is fundamental regardless if the patient seeks medical care for symptomatology or in the setting of occasional analytic findings. It is recommend an effort to reveal a possible link between personal background and exposures to chemicals, pesticides or drugs. Concerning drug history, it is highly remarked that a special awareness should be lead to the ones that could had been consumed during the period that comprises six months before and one month prior[29] to date – ideally, this step could reveal some possible etiologies for Secondary Aplastic Anemia (see Table 1). Even that no test allows ascertainment of causal relationships between exposure and subsequent BM failure[30], it is highly recommended that any possible related chemical exposure should be removed, or any putative drug discontinued or given again to the patient.[29] Medical history and correlation with physical exam could route to a possible Congenital AA (see section 5.1). Age of the patient is also a major clue, and CAA is indeed related to earlier ages. However, all the later presentations reported should alert the clinic – and all this findings should be correctly studied and registered/excluded. Family history is also relevant – some of these disorders are correlated with a considerable phenotypic heterogeneity[28], like, for example, DC. The substantive limitations of the examination in firmly excluding alternative diagnoses previously felt to have pathognomonic findings[30] doesn't allow to prescind the specific diagnostic tests that are recommended to incorporate the next steps of investigation.

Others pathologic backgrounds should also be excluded – a preceding history of jaundice (usually 2-3months before)[29] for a possible post-hepatitic AA, as well as previous viral infections or acquainted immune diseases.

Physical findings like lymphadenopathy or hepatosplenomegaly (in the absence of current infection) are not correlated with IAA[29] – and this observation should lead the practitioner for a different diagnosis.

In conclusion, this topic directly relates to Aplastic Anemia and the subdivision in Congenital or Acquired (Secondary or Idiopathic).

5.3.2 – Investigations required

5.3.2.1 - Confirmation of AA

Full Blood Count, Reticulocyte Count, Blood Film and % Hbf

For an IAA diagnosis, it is consensual that full blood count (FBC) should show pancytopenia, with a preserved lymphocyte count. In very early stages it is reported that the finding of isolated cytopenias may occur.[29] Reticulocyte count should show reticulocytopenia – being this a sign for BM failure. Blood film analyses should focus on what not to expect in IAA: dysplastic neutrophils, abnormal platelets, blasts or any other abnormal cells should lead the investigation to other different entity besides IAA. Common findings are documented to include anisopoikilocytosis and possible toxic granulations in neutrophils.[29] HbF (fetal hemoglobin) can be related to some CAA syndromes[26] (see section 5.1), and it is recommended that it measurement should be done before any blood transfusion.

Bone Marrow Examination

BM examination should include both aspirate and threphine biopsy. Descriptions about findings in aspirate include hypocellular fragments with prominent fat cells and variable amounts of residual hematopoietic cells. It is also clear that erythropoiesis, megakaryocytes and granulocytic cells must be reduced or absent. Lymphocytes, macrophages, plasma cells and mast cells however, are described as prominent. Dysplastic cells aren’t reported as a

feature in IAA, but dyserythropoiesis is considered common and can also be marked.[29] Another finding related to the early stages of the disease, is a possible prominent haemophagocytosis by macrophages, and also background eosinophilic (which may represent interstitial edema). A BM trephine biopsy, of at least 2 cm, is indicated as necessary to allow assessment of the overall cellularity, the morphology of residual hematopoietic cells and also to exclusion of an abnormal infiltrate.[29] Findings including an increase of reticulin or any other abnormal cells are not expected results at IAA and should be directly linked with others possible diagnosis. Increased blasts are a finding that literature recommends that when present should immediately lead to two other possible etiologies: hypocellular MDS or evolution to leukemia.

5.3.2.2 - Exclusion of others etiologies:

Determination of vitamin B12 and folate levels

In order to exclude megaloblastic anemia it is highly recommended that any documented deficiency of B12 or folate should be corrected before a possible final diagnosis of IAA.

Liver function tests

Liver function tests should be performed to detect previous hepatitis.

Viral serology studies

The recommended regular viral studies include hepatitis A antibody, hepatitis B surface antigen, hepatitis C antibody and Epstein–Barr virus (EBV). Cytomegalovirus (CMV) and other viral serology should be included if BMT (see section 6 - Treatment) is being considered. It is also mandatory the exclusion of HIV.

Autoantibody screen

Anti-nuclear antibody and Anti-DNA antibody are recommended to exclude a systemic lupus erythematosus.

Congenital AA syndromes

FA should be screened by chromosomal breakage test with diepoxybutane (DEB) or mitomycin C (MMC). This test is recommended by all literature as screening for FA as regular basis, and mostly to those who are BMT candidates (see section 6). Some authors set an upper age limit for screening, but the reported cases occurring in the fourth or fifth decade makes this topic uncertain. Marsh *et al* (2009) guidelines[29] doesn't include an age limit, and most recent reports set a limit between 40 to 50 years old.

Others syndromes screening remains controversial. Genetic testing has theoretically an undeniable interest and possible use for screening of CAA - however, the generalizable message is that these tests provide incomplete information, considering all of the inherited genetic variants of CAA syndromes that are still unknown .[30]

Therefore, the following tests, even that are potentially associated with some CAA, are not recommended as routine clinical service:

- Identification of mutations in *DKC-1*, *TERC* or *TERT* genes, determination of telomere length alongside information with nomograms of telomere length by age and by cell of origin, if there are a suspicious of Dyskeratosis congenita.[30]
- *SDBS* gene analysis in cases of Scwachmann-Diamond Syndrome[31].

5.3.2.3- Other Investigations

Radiological Investigations

A chest X-ray is recommended for all patients at presentation as a way of exclusion for any infection and to comparison with subsequent films. Abdominal ultrasound should be performed to exclude lymphadenopathy or splenomegaly. Abnormal or anatomically displaced kidneys can also be associated with a possible FA case.

PNH clone

PNH exclusion should be excluded and the presence of a PNH clone should be documented[32] – it should be done using flow cytometry and in the neutrophil and monocyte lineages. FLAER is currently the most sensitive technique.[30]

Cytogenic Investigations

It is reported that approximately 10% of patients with apparent aplastic anemia by all other criteria may have clonal chromosomal abnormalities.[30] The presence of abnormal cytogenetics at presentation in children, especially monosomy 7, can be an indicator to the likelihood of MDS but abnormal cytogenetic clones may also arise during the course of the disease – this situation is explored at section 6.3.

6-Treatment Options

6.1- Assessment of severity

AA is classified according to the severity of the disease – and this classification is commonly used to underline the treatment strategy.

Camitta criteria, published in 1975 by Camitta *et al*, remains the most widely criteria used, and a sub-classification proposed by Bacigalupo *et al* in 1988 is also frequently associated - both can be seen on table 6.

Table 6 – Severity of Aplastic Anemia

Severe AA (SAA) Camitta et al 1975	BM cellularity<25% or 25-50% with <30% residual hematopoietic cells And 2 of 3 following: Neutrophil count <0.5 x 109/l Platelet count <20 x 109/l Reticulocyte count <20 x 109/l
Very Severe AA (VSAA) Bacigalupo et al 1988	As for the SAA, but neutrophils<0.2 x 109/l
Non-Severe AA (N-SAA)	Patients not fulfilling the criteria for SAA or VSAA

(Adapted from "Guidelines for the diagnosis and management of aplastic anemia" by Marsh *et al, British Journal of Haematology,* Oct 2009)

Management of a newly diagnosis IAA patients usually assumes some fundamentals, which had arised from several clinical observations. Therefore, SAA and VSAA patients requires treatment, both supportive and definitive – this is a clear point, and no controversy is underlying it. N-SAA, represents a different case, without consensus on literature. The majority of publications and clinical practices suggest that in these cases, observation can be appropriate, especially if patients are blood transfusions independents. Clinical experience often shows that most of these patients may have stable blood counts for years.[24] The evolution and prognosis however, are not certain - some patients are reported to evolve to more severe pancytopenia, meeting the criteria for SAA or becoming transfusion-dependent, and in this cases, it is recommend the same management as SAA primary cases.[24,13] This

approach is the most commonly reported, and therefore, blood counts of these patients should be routinely monitored.[29] However some recent reports shows that a "watch and wait practice" could be associated with low response rate and poor long-term outcome after IST (see section 6.2 - definitive treatment) and, therefore, further controlled studies are warranted to determine the optimal treatment strategy in these cases.[33]

Other controversial subject is the validity of Camitta Criteria as prognostic significance in the present clinical era – many throwbacks are stated against these criteria, supporting a possible modification. The first topic of discussion is due to the conclusion that routine and more accurate automated reticulocyte counting (available nowadays) will lead to an over-estimation of the level of reticulocyte count used in the cited criteria.[29]

Secondly, other variables had been widely reported as having a direct connection with positive outcomes, like age at diagnosis, telomere length and interval of time between diagnosis and treatment. That leads to infer that considering only blood counts should not be taken as the single factor that could affect overall survival (OS). Therefore, the criteria should ideally be correlated with all the cited variables.

At last, there is also a recent report[34] that focus the fact that no studies have reviewed or validated Camitta criteria, even after these becoming the most widely accepted standard for the diagnosis of AA. The publication also cites another study where it is reported that neutrophil count is indeed one factor that is directly linked with overall survival, and therefore should not be considered an optional criteria.[34]

6.2 - Supportive Care

Before any definitive therapies, it is widely reported and recommended to be assed if the patient requests any supportive measures. Supportive measures alone are not considered as definitive therapies, and it is recommended that definitive options should always be considered in SAA cases. Exceptional cases are described as to be the ones concerning elderly, feeble, or patients with serious comorbidities that may not benefit from the approaches described in section 6.3. As some publications states, those patients may remain stable and maintain quality of life with regular red blood cell transfusions.[24]

The majority of publications and guidelines available reported that the initial immediate management in the majority of patients should consist of blood transfusions, platelet concentrates and treatment or prevention of infection. Each clinical institution may define the protocol to be followed, but, most clinical data reveals some points that should be taken in consideration:

Transfusions

- Blood products should be irradiated to prevent transfusion associated graft-versus-host disease (GVHD) and filtered to reduce the incidence of viral infections and prevent alloimmunization;[35]
- Transfusions from family members should be avoided to decrease sensitization to potential BM donors;[35]
- The initial goal of transfusion therapy for anemia should be to correct or avoid cardiopulmonary complications, and therefore, patients should only be transfused with packed red blood cells when symptomatic[35] - overuse of blood products should be avoided, but so also should inadequate transfusions;[24]

- It is recommended to give prophylactic platelet transfusions when platelets are less than 10 x 10^9/l (or less than 20 x 10^9/l in presence of fever);[24,29,32]
- If the patient is a potential BMT candidate (see section 6.3), CMV negative products should be used until CMV status is known;[29,32]
- Granulocyte transfusions remain controversial[35], but irradiated granulocyte transfusions may be used in life-threatening infections[32];
- Iron chelation therapy should be considered when the serum ferritin is >1000 ng/mL;[29,32]

Growth factors

Measurement of endogenous serum levels of hematopoietic growth factors (HGF) in patients are reported as being markedly elevated[35], predicting that the use of these is of limited value in AA. Therefore, current publications state the following recommendations:

- There are no effective or safe hematopoietic growth factors to support red cell and[u6] platelet counts in AA patients;[29]
- There may be a limited role for granulocyte colony stimulating factor (G-CSF) administration in an attempt to stimulate a neutrophil response in the presence of severe infection[35] but recent guidelines recommends that this should be given as a short course and it should be discontinued after one week if there is no improvement in the neutrophil count[29,32];

Infection

Fungal and bacterial infections are a major cause of death in patients with SAA[35], so it is highly recommended that:

- Prophylactic antibiotics and antifungal drugs should be given to patients with an absolute neutrophil count <0.2 x 10^9/l according to institutional guidelines;[32]
- *Aspergillus* species are responsible for high incidence of infections in AA, but *Pneumocystis jiroveci* infections are rare among patients with SAA, considering that T cells are not defective[35]. There is no indication for routine prophylactic measures against this last agent, or anti-viral prophylaxis in untreated patients with aplastic anemia;[29]
- Patients who are severely neutropenic (ANC <0.2 x 10^9/l) should ideally be nursed in protective isolation;[32]
- Foods that may be contaminated with bacteria or fungal pathogens must be avoided;[29]

Patients at intermediate risk of infection (neutrophil count 0,2-0,5 x 10^9/l) has less clear recommendations – and the decision is best determined on an individual basis according to the frequency and severity of previous infections.[29]

Psychological support

Considering the chronic nature and slow response to any of the options of definitive treatment, patient and family should be closely followed by a multidisciplinary team – besides physicians and other clinical staff, it is highly recommended that it must be also composed by psychologists and others professionals. Patients and relatives should also be offered information about relevant support groups available.

Supportive care is therefore reported as being fundamental at AA management – even when patients are already through definitive treatment modalities (see section 6.2 and 6.3), due to the usual delayed response of blood counts.

6.3 - Definitive Treatment

The aim of definitive treatment is pretty clear: the restoration of hematopoiesis. They are currently two options proven to be effective on this goal: hematopoietic stem cell transplantation (HSCT) or immunosuppressive therapy (IST).

Advances in both techniques are described to be the main reason to the major improvement on survival in SAA when compared with oldest reports. It had led to a very different optimistic vision before a pathologic entity that represented in the past survival rates of 10-20%. Survival rates of SAA are now bruited to be around 80-90%.[36]

Overall long-term survival is reported to be comparable with both treatment modalities – therefore, the decision between both should include the consideration of factors like age, presence of a histocompatible donor and others comorbidities.

It is also widely recommended that any patient should be clinically stabilized before being referred to any of the treatment options, implying the inexistence of any proven infection or uncontrolled bleeding. IST given in these cases has resulted in possible detrimental outcomes and even life threatening complications. The presence of infection is also considered an adverse factor for outcome after HSCT[29]. Exceptional cases are described, in which it may exist the necessity to proceed with HSCT even in the presence of active infection, considering that transplant offers the best chance of early neutrophil recovery. Therefore, it is recommend a special caution in those cases where a delay in definitive treatment may lead to a lethal progression of a possible infection.[29]

It is usual that different institutions adopt different protocols for concrete management – however, there is a worldwide accepted algorithm treatment that, being structured based on several reports, is also recommended by the most recent publications. This algorithm is presented on figure 2.

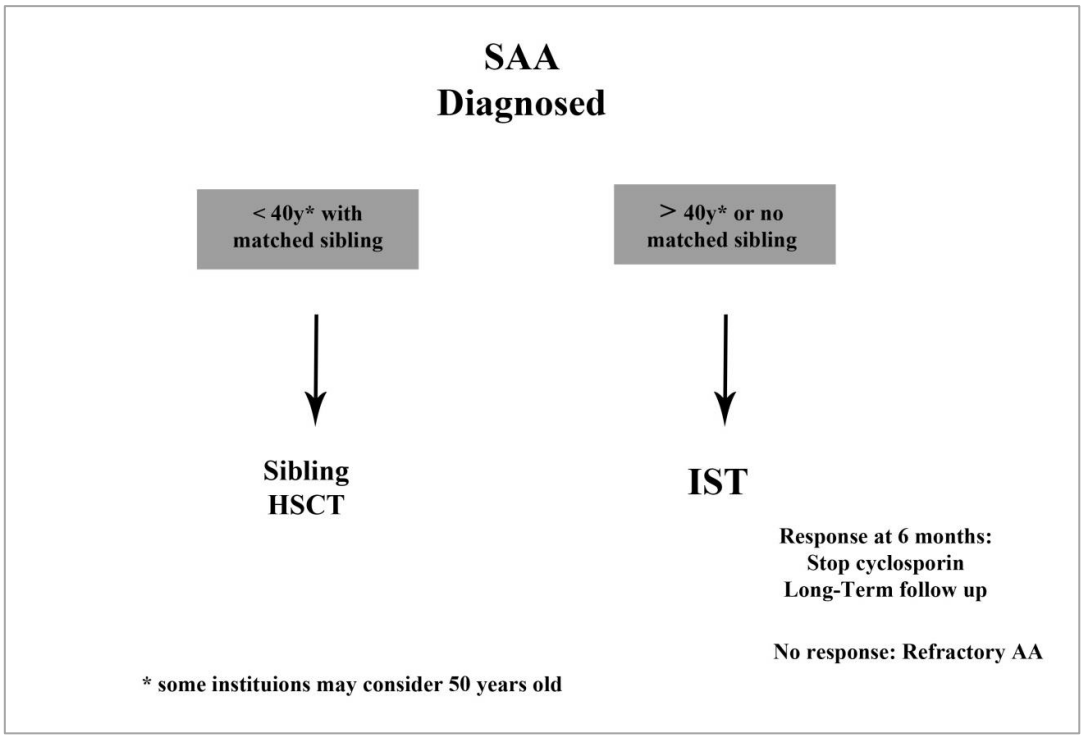

Figure 2 - Treatment algorithm for patients with a recent diagnosis of SAA.
Even that sibling HSCT is the first option at younger patients, at any age the presence of significant comorbidities should favor IST as the initial treatment.

AA-Aplastic Anemia, HSCT - Hematopoietic Stem Cell Transplantation, IST- Immunosuppressive therapy, SAA- Severe Aplastic Anemia

There's a clear recommendation from almost the majority of publications: definitive treatment should not be delayed much beyond the necessary time to clinically stabilize the patient, confirm the diagnosis, assess severity, typing of a potential HLA sibling donor and a management planed discussed.[29] Early spontaneous recovery are reported to occur infrequently, and therefore, watchful waiting can be harmful and completely inappropriate in SAA or VSAA patients.[24]

Corticosteroids are of unproven benefit and inferior to conventional immunosuppression regimens[24], being ineffective as a mean to treat AA – they are also more toxic and can lead to bacterial and fungal colonization or precipitate serious gastrointestinal haemorrhage[29] in the presence of severe thrombocytopenia.

6.3.1- Hematopoietic Stem Cell Transplantation (HSCT)

HSCT from a HLA identical sibling donor is considered the treatment of choice for a newly diagnosed patient if they are under 40 years old (there's some controversy, but most orientations states this as the preferred age limit), who has SAA or VSAA.[29]

This treatment is described in literature as one potential curative option, representing a 75-90% chance of long term cure.[29] The setting of the age limit present in the majority of guidelines can be explained by the correlation of increasing age with the risk of graft-versus-host disease (GVHD).[23] GVHD can lead to significant morbidity and mortality after transplantation, possibly overcoming the potential benefit. Therefore, age of the patient is described as one of the factors that directly determine the decision to choose IST over HSCT as first choice treatment.

BM grafts are recommended to be the preferred stem cell source, since the peripheral blood stem cells grafts had showed inferior positive results, and also a higher rate of complications like GVHD.[23]

It is also highly recommended that all younger patients without a matched sibling donor (being the IST the first choice in those cases) to be screened at the time of initiating IST for a potential HLA-matched unrelated donor.[37]

The most commons complications of transplantation identified are graft failure, acute and chronic GVHD.

Transplantation protocols may differ, but there is a worldwide consensus about the use of cyclophosphamide (Cys) and antithymocyte globulin (ATG) as conditioning and cyclosporine (CsA) and methotrexate as GVHD prophylaxis.[37]

There is also several references to the use of fluradabine (FLU) in addition to Cys plus ATG regimens to enabled engraftment in heavily transfused and sometimes alloimmunized patients.[35,37]

There's also data reporting that with a FLU, Cys and alemtuzumab (ALZ) conditioning regimen for SAA, the rates of extensive chronic graft-versus-host disease and graft rejection are now less than 5%.[38]

Availability of an HLA-matched sibling donor is described as being around 30%.[35]

Unrelated donors and mismatched transplants had been reported to have almost twice the transplant-related mortality and risk of GVHD as matched sibling donor transplants – however, there are publications reporting a major improvement on the outcomes with unrelated donor (UD) HSCT. These same publications directly relate these outcomes with more stringent donor selection facilitated by high-resolution molecular typing, less toxic and more effective conditioning regimens in addition to higher quality transfusion and antimicrobial supportive care.[24]

Experience from larger cohorts reported in the last couple years from the United States, Japan, Korea, and Europe suggests that the outcome with UD HSCT is still not as favorable as that of a matched sibling donor[24] - therefore, BM transplant (BMT) from unrelated or mismatched donors is mostly recommended to be seen as an second option reserved for patients who failed to respond to one or more courses of IST.

Younger patients are currently a target of controversy in this matter – even that some authors state that UD HSCT is not recommend as first therapy for SAA[24], a recent report[38] by Samarasinghe *et al* (2014), doesn't exclude this option as a first treatment choice, considering that compared to IST, transplantation offers a more complete restoration of hematopoiesis, lower relapse rates and better protection against secondary cancers (see section 6.3.2).

The potential drawbacks to this new approach are equally presented by Samarasinghe *et al* (2014). This report mentioned several concern aspects like the difficulties in finding donors, the time from diagnosis to stem cell donation, GVHD risk, donors opting to donate peripheral blood stem cells rather than BM and treatment-related mortality. The authors also mention

that the long-term survival among children who respond to horse antithymocyte globulin (ATG) plus CsA is excellent, approximating 90%, and that an optimal conditioning for UD HSCT is not yet defined.

The delaying time between definitive IST while conducting a search for a nonfamily donor may be dangerous[24], and this is one of the major negative factors. However, the 2014 report presents a new perspective, introducing the results of a study by Yoshimi *et al*: the median time from diagnosis to administration of IST was 60 days while it was 134 days in those who were submitted to upfront matched UD HSCT.[39] Even considering that IST can always be started earlier than a transplant option, the time to cellular recovery following IST is reported as 3-6 months. Cellular recovery following matched UD HSCT is around a month – thus, time to cellular recovery following IST or matched UD HSCT can be considered as similar if a matched UD can be found quickly.

This report concludes recommending that if a matched UD can be found quickly, then transplantation could be considered as a potential first-line option in children who lack a matched sibling donor. The decision should then depend on the preferences of patients, physicians and donor availability until further data become available.[38]

6.3.2 – Immunosuppressive Therapy (IST)

Several large prospective trials in the United States of America, Europe and Japan showed consistent studies establishing the efficacy of IST[37], which is recommended to be the first-line therapy for SAA patients who lack a matched sibling or those who aren't candidates for BMT (due to age or other comorbidities).

The standard regimen remains horse antithymocyte globulin (h-ATG) plus Cyclosporine A (CsA), leading to hematopoietic response rates ranging from 60 to 75%, with children reported to achieved response rates of 75%[37]. The probability of survival at 5 years ranges from 60 to 85%.[35]

There are several publications describing the attempts to improve the standard regimen described above – both as an attempt to improve the response rates achieved, but also as a way to prevent the known complications from this therapeutic modality, like relapse or clonal evolution (explored further in this section). Amendments included the use of rabbit-ATG (which is a more potent immunosuppressant than h-ATG) instead h-ATG, the addition of high-dose corticosteroids[9], mycophenolate mofetil[9], rapamycin[9] or sirolimus[37] to ATG regimen, or the use of high dose of Cys (50mg/Kg/d during 4 days).

Rabbit-ATG (r-ATG), being effective in some patients when used on refractory and relapsed settings as a salvage regimen[9] (see section 6.4), was widely mentioned as possible more effective first-line therapy. However, latest reports including a prospective and randomized study at the National Institutes of Health (NIH) concluded that at 6 months, the rate of hematologic response was markedly inferior with r-ATG (37%) when compared with h-ATG (68%). The difference in response rate resulted in a significant survival difference, with 70% of patients alive at 3 years after rabbit ATG compared with 94% with horse ATG. [37]

The addition of others immunosuppressants (like mycophenolate mofetil or sirolimus) have not improved the outcomes of ATG[37], and in a 2013 report[9], Young states that neither regimen was able to prevents relapse or clonal evolution. High dose Cys is reported to be used at some institutions, but had not been broadly accepted since it is associated with prolonged neutropenia and early mortality due to infection when compared with the ATG plus CsA regimen[9].

Table 7 - Immunosuppression Therapies

	Dose	Notes
ATG (h-ATG)	40 mg/Kg over 4 hours, daily for 4 days* *should be given as an in-patient, with patients nursed in isolation room[32]	In the presence of life-threatening reactions, the ATG infusion is recommended to be slowed or held temporarily until alarming signs and symptoms subside. Common infusion reactions are managed symptomatically: rigors - meperidine, fevers- acetaminophen* rash- diphenhydramine* hypotension- intravenous hydration hypoxemia- supplemental oxygen *recommended also as premedication before each ATG dose[24]
Prednisone	1mg/kg Started on day 1 and continued for 2 weeks	Given as prophylaxis for serum sickness (which can appear on 7-14 days)
Cyclosporine	Started on Day 1 to a target trough level between 200 and 400 ng/mL, starting at a dose of 10 mg/kg per day (in children, 15 mg/kg per day)[24] Treatment should be continued for at least 12 months after a maximal response before starting to taper the drug[13]	**Complications:** **Hypertension** – amlodipine (it has minimal overlap with CsA toxicities) **Bothersome gingival hyperplasia** - short course of azithromycin Monitoring of liver and of kidney function is necessary - creatinine >2 mg/mL may require temporary cessation of CsA with later reintroduction at lower doses[24]
Other therapies: Antibiotic, anti-viral, anti-fungal prophylaxis	As per institutional practice[32]	

This results, and the consideration that despite the use of different h-ATG preparations in different institutions, the rates, time course, and patterns of hematologic recovery have been reported as consistent across studies, leaded to the acceptance of h-ATG plus CsA as the first IST choice.

Even that the therapeutic protocols for immunosuppression may vary, there are some key recommendations that apparent to be worldwide accepted and commonly used – these are described on table 8.

6.3.2.1- Management after IST

IST and its efficiency can be evaluated based on the response rates – and to allow the comparison between several cases, there are also criteria pre-defined for this classification, which were approved in 2000. These are presented in table 9.

Table 8 - Response criteria for severe aplastic anemia

None	Still severe
Partial	Transfusion independent No longer meeting criteria for severe disease
Complete	Hemoglobin normal for age Neutrophil count >1-5 x 10^9/l Platelet count >150 x 10^9/l

(Adapted from "What is the definition of cure for aplastic anemia?" by Camitta, *Acta Haematol.* 2000)

Responses are recommended to be confirmed by two or more blood counts at least 4 weeks apart, and should ideally be measured in patients who are not receiving hematopoietic growth factors.[40] Hematologic improvement is reported to not expected for 2-3 months after ATG, with the majority (90%) of responses described to be occurring within the first 3 months, and with fewer reported to occur between 3-6 months or after[24].

6.3.2.2 - Responder's patients

It is common practice tapering CsA after response - however, there's still some controversy about the best approach. Possible strategies identified may include a CsA tapper at 6 months among responders [24] or given for a minimum of 12 months[32]. Either way, it is widely know that there is a significant risk of relapse with rapid tapering of CsA[13], therefore a very slow tapper is highly recommended.

There's also reference in some reports about the possibility of some patients being CsA dependent – these appear to need a low dose for a long period of time, being also mentioned the possibility of becoming impossible to stop the CsA completely.[32]

Fluctuations in blood counts are reported to be frequent in the weeks after IST, and an over-flow of blood counts analysis is not recommended. It is proposed that these procedures should be adapted to the criteria for response (e.g. assessment at 3 and 6 months)[24]. Continued improvement of blood counts occurring over years[24] is also described and reported.

6.4 - Refractory Patients and Salvage Therapies

Refractory patients had been defined in literature as those cases that 6 months after one course of the standard regimen of IST still present blood counts fulfilling the SAA criteria – thus, there is a lack of response, with persistence of severe pancytopenia.[24]

A 2013 review by Marsh & Kulasekararaj[41] is a particular informative publication about the treatment options currently available for these patients. The authors start by highly recommend that in these cases and before considering any other therapeutic options, some issues must be clarified: first, IAA diagnosis must be reassessed – this includes both exclusion of a hypocellular MDS and also CAA that may be unnoticed at initial presentation. The same review also points some factors that could lead to the lack of response to initial IST, raising the hypothesis that the pathogenic mechanism may not be immune mediated in this cases (representing a CAA case and/or telomere disease) or, that there may be a situation of extreme hematopoietic stem cell exhaustion (being this finding consistent with the results reported in other publications, stating that a third treatment with IST, with the previous two ineffective, is invariable useless in SAA).[41]

Even that there's no preferential strategy defined, considering the 2013 review cited and also several other recent publications it is possible to underline some assumptions: first, it is recommended that the first option should rely on HSCT – both sibling BMT if the patient is older than 40 years (or 50), or UD. The last option, reserved for younger patients, as seen in section 6.3.1, is already a matter of controversy, with a possibly role of a UD HSCT even as first line therapy, instead a first course of IST.

In this concrete question there is a reported unquestionable benefit with UD BMT over a second course of IST – even that the 5-year overall survival was reported as being similar in both strategies, failure-free survival is reported to be significantly higher in the patients

submitted to transplantation. Marsh & Kulasekararaj (2013) reported rates reaching 84% in a Japan study, against 9% from those receiving a second IST course.[41]

The unavailability of a suitable matched donor is not infrequent – and it is directly related with the main difficulty reported in the management of this cases. Treatment options are described as strategies which may include a repeat course of ATG, the use of other agents, immunosuppressive or not, and, nowadays, also a possible alternative donor transplantation (cord blood transplantation or haploidentical HSCT).

A repeated course of ATG, using r-ATG instead of h-ATG is the most reported and used approach to refractory SAA - Marsh & Kulasekararaj (2013) made reference to response rates between 22% to 77%.[41] The authors also refer other options like ALZ, which is a humanized anti-CD52 IgG1 monoclonal antibody used currently in autoimmune cytopenias, allogeneic HSCT conditioning, pure RBC aplasia, and had been also reported and used as a possible salvage therapy of refractory SAA. The response rates and OS are apparently comparable to those obtained with a r-ATG plus CsA regimen, with the advantage of being one CsA free regimen, making it as a possible option for patients with comorbidities that could increase the toxicity of a CsA regimen, or those who experienced poor tolerability to CsA before.[36,41]

High dose Cys, has been used as a first line therapy for SAA in some centers, but, as mentioned, is also associated with early mortality, and therefore, not recommended. Therefore, the same 2013 review recommends that at a refractory SAA setting, the use of Cys should be restricted to clinical trials, considering the currently existence of less toxic drugs.[41]

On example of these drugs also mentioned is Eltrombopag, which is an oral thrombopoietin mimetic reported as one inductor for platelet maturation and release by binding to c-MPL receptors on megakaryocytes.[41] It is been widely mentioned on recent literature due to the results of recent studies where trilineage responses were identified in 24% of studied patients [41]. In other publication by Young in 2013, the author reported results from a pilot study where

more than 40% of AA refractory patients from two dozen evaluated had shown response with a normal cellularity also found in BM at 9 to 12 months – this finding is also characterized by the author as being unusual even after successful IST.[9] There's still no prospective clinical trials about the safety and efficacy of eltrombopag, but the hypothesis that this agent can also have a role as a first line therapy along with ATG plus CsA has arisen in several publications[9,41].

For last, androgens are also mentioned in the 2013 review[41] by Marsh & Kulasekararaj – and are reported to induce responses in patients with telomeropathies who manifest as apparent AAA due to increased telomerase activity via aromatization of estradiol to steroids.[41] However, this agents had lacked efficacy in early randomized studies when combined with ATG, but some centers report to offer a trial of androgen therapy for 3 months to patients who are refractory to IST and lack good HSCT options.[24]

Alternative stem cell sources have been a currently area of intense investigation – unrelated cord blood transplant is one of the options being studied, and some studies had already been reported. Marsh & Kulasekararaj identified the main problem as being engraftment failure, with a cumulative incidence of neutrophil recovery of 51% at 2 months and a 3 year overall survival.[41] The optimal conditioning regimen for this type of transplant is still unknow.[41]

Haploidentical related HSCT is an option that, if successful, allows the possibility of the majority of patients having a possible donor, with a short time between diagnosis and procedure[41] - however the authors conclude that results with this option had not shown to be effective.

A promising combination of haploidentical with cord blood cells are also reported in the recent reports[37,41] , with preliminary data showing promising engraftment of the cord unit and low rates of GVHD [37]. Further studies are currently on course.

To conclude, supportive care, fundamental at all stages of the disease, assumes at the refractory patients a major role, considering the reported longer neutropenia periods at which these patients are submitted. There's also a significant reduction in deaths from infection in the latest years, unquestionably due to the improvements of supportive care in the refractory setting.

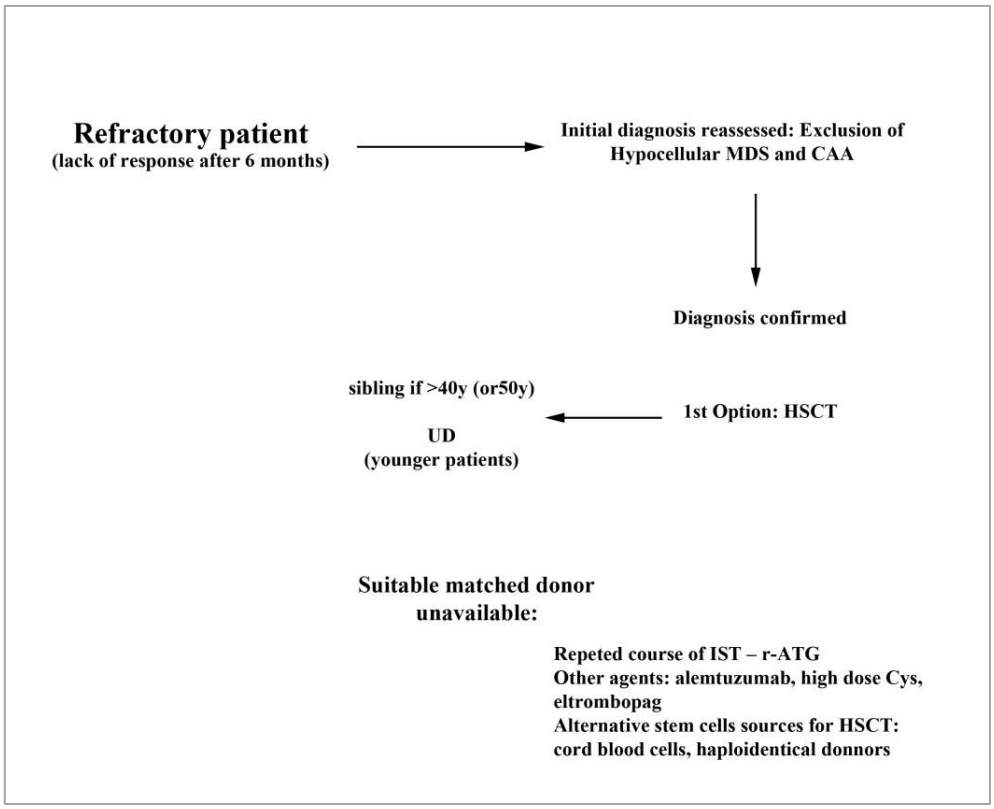

Figure 3 – Summary of options available for refractory AA patients.

AA- Aplastic Anemia, CAA – Congenital Aplastic Anemia, Cys – cyclophosphamide, HSCT - Hematopoietic Stem Cell Transplantation, IST- Immunosuppressive therapy, MDS- Myelodysplastic Syndromes, UD - Unrelated Donor, r-ATG - Rabbit Antithymocyte Globulin, SAA- Severe Aplastic Anemia

6.5- Long Term Management

Long term follow up is a necessity at AA management considering the risk for well documented late complications like hematologic relapse or clonal evolution. There isn't a currently defined protocol described for the best strategy - however it is widely recommended the assessment of BM morphology and karyotype (Scheinberg & Young[24] recommended this to be done at the 6 and 12 months after treatment followed by a yearly evaluation).

Literature is pretty consistent - the finding of a hypocellular BM should not be linked with refractory AA, or even relapse, if blood counts improvement had occurred. Marrow cellularity does not correlate with blood counts, and this last it's recommend as the parameter to guide management.[24]

6.5.1 - Relapse

There isn't a defined concept of relapse - Scheinberg & Young[24] defined this as those cases when reintroduction of IST is required for decreasing blood counts. The authors also state that this usually occur within 2 to 4 years of IST.

It is also mentioned as being necessary the confirmation of a trend, and not a single blood count, since oscillating numbers can occur normally, and most patients are reported to respond to further immunosuppression.

Relapse is recommended to be treated with reintroduction (or dose increase) of CsA for 2 to 3 months, and if CsA alone is ineffective, a second course of r-ATG/CsA is reported to yield responses in approximately 50% to 60% of cases.[24] ALZ monotherapy is also an option mentioned in several publications. UD HSCT on first relapse in younger is not recommend, due to the positive outcomes of further immunosuppression. Relapse alone has not been correlated to worse survival in SAA.[24]

6.5.2 - Clonal evolution

IST and the protocols proposed had result in excellent long-term survival rates, but had also lead to the recognition of possible late clonal complications like clonal evolution to Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML).

It was already referred at section 5.3 that 10% of patients with apparent AA by all other criteria may have clonal chromosomal abnormalities – the most frequently observed are trisomy 8, trisomy 6, 5q- and anomalies of chromosomes 7 and 13.[29]

The true meaning of the presence of these findings is still unknown – and in the absence of morphological evidence of MDS or AML after thorough review of blood smears and BM slides, a diagnosis of AA rather than MDS/AML is usually done by most practices.

In some of these patients, chromosomal abnormalities are reported to be transient, and may even disappear, what is often seen after successful IST.[42]

Marsh *et al* guidelines (2009) even recommend that patients who are not BMT candidates and have an abnormal cytogenetic clone (except monossomy 7) must be managed in the same way as patients who have none.[29]

Monossomy 7 is reported to be related with a high risk to the evolution of MDS – evolution to MDS can occur either early or late in the course of the disease. Afable *et al* (2011) states that some patients who are initially suspected to have IAA may show a rapid progression to AML, but the late evolution to MDS is more typical, and usually seen in refractory cases or in those who didn't achieved a full response.[42]

MDS evolution is described in the majority of publications as one clinical set where worsening blood counts remain unresponsive to immunosuppression, and where it can be found prominent dysplastic cells in the BM and also abnormal cytogenetics. On these cases, treatment with HSCT is reported as the only potentially curative therapy. [24]

Afable *et al* (2011), cited variation rates of the setting of this complication from 1,7% to 57% in the observation period of 5 to 11 years. The authors also mentioned that biomarkers able to predict MDS evolution are currently not known in AA, but there are data that suggest that those AA patients with complete and durable responses to IST are at lower risk of progressing to MDS.[42]

As also mentioned in the 6.4 section, in a case of decreasing counts, a BM examination is reported as being necessary to rule out the possibility of evolution to MDS and to avoid futile therapy with ATG (which is usually ineffective)[42].

Literature presents also another conception: true clonal evolution and progression to MDS or AML - which includes findings of numerical expansion of clonal cells, and therefore, diagnosis of a clonal malignancy - has to be distinguished from pseudoclonality. This concept refers to those analyzes of BM samples that reflect findings which had resulted from clonal abnormalities often transient and with relative expansion in a contracted stem cell pool.[42]

PHN evolution is also possible – and this subject was already presented and explored on section 3. Even that this correlation with AA is still matter of clear discussion, in many aspects PHN evolution can be similar to the evolution of chromosomal abnormalities.[42]

Pathogenesis of clonal evolution is also unclear and in the 2011 report, Afable *et al* also presented some points over this subject: considering the main pathologic process in AA – the contraction of stem cell compartment and the decrease number of available HSC – it is likely that at the peak of the disease, hematopoiesis in AA is oligoclonal or even clonal. As seen, some patients presents chromosomal abnormalities and/or PHN clones at the initial diagnosis of AA - depletion of the stem cell pool may then predispose the recruitment of genetically defective hematopoietic clone, mostly if the genetic defect involves survival pathways. Some other mechanisms may also exist – the presence of excessive short telomeres may represent a risk factor to acquisition of chromosomal damage, and the occurrence of some mutations may

also lead to more rapid evolution of cryptic clones and progression to more advanced disease (identified mutations are reported to be on *TET2*, *CBL*, and *DNMT3A*[u7] genes in several pos-AA monossomy 7 patients). At last, authors present the theory already presented at section 2 - cytogenetically abnormal cells may trigger an immune response first directed to eliminate abnormal cells but also leading to destruction of normal cells[18] – the following selecting pressure would then lead to the generation and escape of mutant clones that would outgrow normal hematopoiesis.

The authors end the article relating the following hypothesis: irrespective of initiating events, the expansion of aberrant clones is likely a result from an immune selection, and clonal progression may be regarded as a form of immunologic escape.[42]

Conclusion

AAA, even being considered a rare hematologic disease, is the target of several research works. To current date, incidence is reported as 2,34 cases per million per year on the Western countries. There's no specific data regarding Portugal, so it's only possible to assume that this is also valid for our population. It is also reported and accepted a biphasic distribution and a sex ratio of 1:1.

There's still plenty difficulties reported to establishment of a clear knowledge about the pathological mechanisms of IAA, but several recent publications support the immune system role on stem cell depletion already sated by the oldest ones. It is worldwide accepted that this disease is most likely an outcome of several results from particular roles concretized by different factors. Factors nowadays reported include lymphocytes, cytokines, autoantibodies and also some genetic factors like HLA, T cell encoding genes, mutations and polymorphisms and telomeres. This last is widely reported and represents now, besides a proven risk factor, a probable prognostic indicator. This fact has led to the currently overflow of reports and studies concerning telomeres roles on AAA. The reason why T cells are activated, leading to the immune attack is still unknown.

The mechanism responsible for the singular relation with PNH remains also unknown, and several hypotheses tested were presented in this document. Literature also states as the most acceptable possible mechanism, the fact that PNH cells may have a proliferative advantage over non-PNH cells, not by intrinsic growth or survival advantage, but by an immune mechanism of selection. Concerning IAA, the true significance of the presence of a PHN clone is unclear – and there is also some unconformity between available studies. Some stated that the presence of a clone has been reported to be predictive of response to IST, while others have not observed this finding. This variation of results should lead to new studies.

Diagnosis of IAA, and the investigations required support the peculiarities of this disease – clinical symptoms and signs are unspecific and none classical or pathognomic sign is reported. Therefore, IAA remains a diagnosis of exclusion. It is also fundamental being familiar with the conditions that must be excluded, and which findings or characteristics must lead the clinic to assume as unlikely to be related to a case of IAA. Literature is consensual that if a thorough investigation is lead, majority of cases will be correctly identified or excluded – leading to a correct management of IAA or other conditions.

After an identified and confirmed IAA case, severity of the disease must be assessed, which will then allow the definition of the best treatment/management option. Severity had been classified using the Camitta criteria – these criteria are worldwide accepted and are directly linked with the treatment management. It is consensual in literature that SAA and VSAA patients require treatment, both supportive and definitive. Non-Severe AA cases, in most publications and clinical practices are suggested to be kept under observation, especially if patients are blood transfusions independents. However some recent data reported a possible prejudice in this "watch and wait practice" – and there is an actual need in further controlled studies. Camitta criteria by itself are also the target of some reports – these last question the validity of the criteria considering the current panorama of clinical practice and the proved other variables linked to IAA that may have a significant role on prognosis, and aren't included in the criteria. However, this classification, and the management that its use implies, are still the practice recommended on recent guidelines.

Supportive measures are seen as fundamental in all cases – however these measures alone are not definitive therapies and they should not replace definitive therapies if these are indicated. It is usual that each institution adopts the protocol to be followed.

Definitive measures incorporate two modalities: BMT and IST. There's clear data about the primordial choice on sibling BMT if possible - this modality, if succeeded, can be curative,

and current approaches allow rates of success with a sibling donor of almost 90%. Complications are related with the technique itself, and not with the disease – that means, graft failure, acute and chronic GVHD, which can occur with any clinical entity that requires BMT as treatment option. The availability of an HLA-matched sibling is the major hurdle to this option – which is reported to be around 30%. Considering that, UD and mismatched transplants, have almost twice the transplant-related mortality and risk of GVHD as matched sibling donor transplants, and also the correlation of increasing age with the risk of GVHD. This option is currently recommend and worldwide accepted to be the first line therapy only if patient and donor fulfills the criteria required (such as an HLA-matched sibling donor and age under 40 years old). However, recent medical advances leads to a new panorama where literature mentions a major improvement on the outcomes UD HSCT – factors like more stringent donor selection, less toxic and more effective conditioning regimens, and also higher quality supportive care are reported to be crucial on this. The positive outcomes of IST still directs current guidelines to consider the BMT from UD or mismatched donors as a second option reserved for patients who failed to respond to one or more courses of IST, but a potential role on the management of younger patients without a sibling donor is already under discussion. There's still no consensus on this matter.

IST is actually comparable to BMT outcomes. The standard regimen remains horse h-ATG plus CsA. Hematopoietic response rates are reported to range from 60 to 75%, with survival at 5 years rates from 60 to 85%.

The current debate sets on what are the options for those patients that don't achieved a complete response after a first IST protocol, the relapsed cases, or how to avoid known complications like the clonal evolution.

Literature focus now in some future goals: a possible improvement in the current IST outcomes, with possible addition of others agents to the current first-line scheme, new salvage

therapies, and what can be done to prevent possible late clonal complications like clonal evolution to MDS and AML.

The ability to identify patients with a higher probability of hematologic response, relapse, clonal evolution and early mortality may allow for a more logical treatment allocation, and then, improve or avoid the actual outcomes.

Optimistic views lead to possible improvements where better knowledge about the pathological ways and specific factors could improve the current outcomes – allowing a panorama similar with the PHN after the introduction of eculizumab. However, it is largely present in the results of recent studies that medical advances concerning IAA may have reached a plateau.

IAA shows the referred singularities that can represent true handicaps on medical investigation, e.g. a rare disease, where the potential cells of interest (HSCs) are lacking. Evidence-based medicine assumes a major role and prospective studies could lead to more concrete information about the best treatment options. However, these last are objected by ethical questions: considering the highly positive outcomes with the known therapies, these should not be denied to a patient in the set of a possible research of others agents. Other factors, like possible different mechanisms on younger patients and older ones, or even between the responders and non-responders also represent a major hurdle to the establishment of protocols based on proven benefits.

It is clear that advances in medical care lead to outstanding positive outcomes, comparing the past survival rates of 10 to 20% to the actual 80 to 90% - but those, had led to different challenges. In the current era of the chronic disease paradigm, the role of IAA patients, the long-term management of these, the possible new therapies or options (like new sources for HSC for BMT) and the options reserved for those that evolve to clonal conditions still remain as questions that not being perfectly defined, still needs to be sorted.

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References

1- Young NS. Pathophysiologic mechanisms in acquired aplastic anemia. Hematology Am Soc Hematol Educ Program [Internet]. 2006 Jan;72–7.

2- Montané E, Ibáñez L, Vidal X, Ballarín E, Puig R, García N, et al. Epidemiology of aplastic anemia: a prospective multicenter study. Haematologica [Internet]. 2008; 93(4):518–23.

3- Fan R, Wang W, Wang X-Q, Lin G-W. Incidence of adult acquired severe aplastic anemia was not increased in Shanghai, China. Ann Hematol [Internet]. 2011 Oct;90(10):1239–40.

4- Maluf E, Hamerschlak N, Cavalcanti AB, Júnior AA, Eluf-Neto J, Falcão RP, et al. Incidence and risk factors of aplastic anemia in Latin American countries: the LATIN case-control study. Haematologica [Internet]. 2009 Sep;94(9):1220–6.

5- Young N, Barrett A. The treatment of severe acquired aplastic anemia. Blood [Internet]. 1995

6- Bacigalupo A. BM Failure Syndromes Aplastic Anemia : Pathogenesis and Treatment. 2007;23–8.

7- Bueno C, Roldan M, Anguita E, Romero-Moya D, Martín-Antonio B, Rosu-Myles M, et al. BM mesenchymal stem cells from patients with aplastic anemia maintain functional and immune properties and do not contribute to the pathogenesis of the disease. Haematologica [Internet]. 2014 Jul;99(7):1168–75.

8- Dan Longo, Anthony Fauci, Dennis Kasper, Stephen Hauser, J. Jameson JL. Harrison's principles of internal medicine 18ª ed. 2011

9- Young NS. Current concepts in the pathophysiology and treatment of aplastic anemia. Hematology Am Soc Hematol Educ Program [Internet]. 2013 Jan;2013:76–81.

10- Berger A. Th1 and Th2 responses: what are they? Bmj [Internet]. 2000;321(August):5500.

11- Hoffbrand, Moss P. Essential Haematology, 6th Edition, March 2011

12- Nakao S, Feng X, Sugimori C. Immune pathophysiology of aplastic anemia. Int J Hematol [Internet]. 2005 Oct [cited 2014 Aug 17];82(3):196–200.

13- Dolberg OJ, Levy Y. Idiopathic aplastic anemia: diagnosis and classification. Autoimmun Rev [Internet]. 2014 [cited 2014 Aug 17];13(4-5):569–73.

14- Dhaliwal JS, Wong L, Kamaluddin MA, Yin LY, Murad S. Susceptibility to aplastic anemia is associated with HLA-DRB1*1501 in an aboriginal population in Sabah, Malaysia. Hum Immunol [Internet]. Elsevier Inc.; 2011 Oct [cited 2014 Aug 16];72(10):889–92.

15- Kapustin SI, Popova TI, Lyschov AA, Togo AV, Abdulkadyrov KM, Blinov MN. HLA-DR2 frequency increase in severe aplastic anemia patients is mainly attributed to the prevalence of DR15 subtype. Pathol Oncol Res 1992;3:106–8

16- Calado RT, Young NS. Review article Telomere maintenance and human BM failure. 2008;111(9):4446–55.

17- Holohan B, Wright WE, Shay JW. Cell biology of disease: Telomeropathies: an emerging spectrum disorder. J Cell Biol [Internet]. 2014 May 12 [cited 2014 Jul 21];205(3):289–99.

18- Nissen C, Stern M. Acquired immune mediated aplastic anemia: is it antineoplastic? Autoimmun Rev [Internet]. Elsevier B.V.; 2009 Sep [cited 2014 Aug 17];9(1):11–6.

19- Bessler M, Hiken J. The pathophysiology of disease in patients with paroxysmal nocturnal hemoglobinuria. Hematology Am Soc Hematol Educ Program [Internet]. 2008 Jan;104–10.

20- Young NS, Maciejewski JP, Sloand E, Chen G, Zeng W, Risitano A, et al. The relationship of aplastic anemia and PNH. Int J Hematol [Internet]. 2002 Aug;76 Suppl 2:168–72.

21- Scheinberg P, Marte M, Nunez O, Young NS. Paroxysmal nocturnal hemoglobinuria clones in severe aplastic anemia patients treated with horse anti-thymocyte globulin plus cyclosporine. Haematologica [Internet]. 2010 Jul [cited 2014 Aug 25];95(7):1075–80.

22- Pu JJ, Hu R, Mukhina GL, Carraway HE, McDevitt M a, Brodsky R a. The small population of PIG-A mutant cells in myelodysplastic syndromes do not arise from multipotent hematopoietic stem cells. Haematologica [Internet]. 2012 Aug [cited 2014 Sep 16];97(8):1225–33.

23- Chen R, Nagarajan S, Prince GM, et al. Impaired growth and elevated FAS receptor expression in PIGA(+) stem cells in primary paroxysmal noctumal hemoglobinuria. J Clin lnvest. 200; 106:689-696

24- Scheinberg P, Young NS. How I treat acquired aplastic anemia. Blood [Internet]. 2012 Aug 9 [cited 2014 Jul 15];120(6):1185–96.

25- Weinzierl EP, Arber D a. The differential diagnosis and BM evaluation of new-onset pancytopenia. Am J Clin Pathol [Internet]. 2013 Jan [cited 2014 Aug 13];139(1):9–29.

26- Alter BP. Diagnosis, genetics, and management of inherited BM failure syndromes. Hematology Am Soc Hematol Educ Program [Internet]. 2007 Jan;29–39.

27- Shimamura A. Inherited BM failure syndromes: molecular features. Hematology Am Soc Hematol Educ Program [Internet]. 2006 Jan;63–71.

28- Ahmed M, Dokal I. Understanding aplastic anaemia/bone-marrow failure syndromes. Paediatr Child Health (Oxford) [Internet]. Elsevier Ltd.; 2009 Aug [cited 2014 Aug 17];19(8):351–7.

29- Marsh JCW, Ball SE, Cavenagh J, Darbyshire P, Dokal I, Gordon-Smith EC, et al. Guidelines for the diagnosis and management of aplastic anaemia. Br J Haematol [Internet]. 2009 Oct [cited 2014 Jul 30];147(1):43–70.

30- Guinan EC. Diagnosis and management of aplastic anemia. Hematology Am Soc Hematol Educ Program [Internet]. 2011 Jan;2011:76–81.

31- Marsh J. Making therapeutic decisions in adults with aplastic anemia. ASH Educ Progr B [Internet]. 2006 [cited 2014 Sep 9];44(208).

32- Nigel P, Hunter A. East Midlands Cancer Network Guidelines for the Management of Aplastic Anaemia in Adults. :1–9.

33- Lisukov I, Ivanova M. Practice For Moderate Aplastic Anemia (MAA) Are Associated With Low Response Rate and Poor Long-Term Outcome After Immunosuppressive Therapy (. Blood [Internet]. 2013 [cited 2014 Sep 30];122(21):12.

34- Yoon HH, Huh SJ, Lee JH, Lee S, Kim S-H, Kwon HC, et al. Should we still use Camitta's criteria for severe aplastic anemia? Korean J Hematol [Internet]. 2012 Jun;47(2):126–30.

35- DeZern A, Brodsky RA. Clinical management of aplastic anemia. 2012;4(2):221–30.

36- Scheinberg P. Aplastic anemia: therapeutic updates in immunosuppression and transplantation. Hematology Am Soc Hematol Educ Program [Internet]. 2012 Jan;2012:292–300.

37- Scheinberg P. Aplastic anemia: therapeutic updates in immunosuppression and transplantation. Hematology Am Soc Hematol Educ Program [Internet]. 2012 Jan;2012:292–300.

38- Samarasinghe S, Marsh J, Dufour C. Immune suppression for childhood acquired aplastic anemia and myelodysplastic syndrome: where next? Haematologica [Internet]. 2014 Apr [cited 2014 Aug 13];99(4):597–9.

39- Yoshimi A, van den Heuvel-Eibrink MM, Baumann I, Schwarz S, Simonitsch-Klupp I, de Paepe P, et al. Comparison of horse and rabbit antithymocyte globulin in immunosuppressive therapy for refractorycytopenia of childhood. Haematologica. 2014;99(4):656-663.

40- Camitta BM. What is the definition of cure for aplastic anemia? Acta Haematol. 2000;103:16–8.

41- Marsh JCW, Kulasekararaj AG. Review Article Management of the refractory aplastic anemia patient : what are the options ? 2013;122(22):3561–7.

42- Afable MG, Tiu R V, Maciejewski JP. Clonal evolution in aplastic anemia. Hematology Am Soc Hematol Educ Program [Internet]. 2011 Jan;2011:90–5.