

# **Aplastic anemia - a population-based study of epidemiology, treatment, and prognostic factors**

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Cover illustration: Bone marrow in aplastic anemia (magnification: ~40x)

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## **Abstract**

**Background and aims.** Aplastic anemia (AA) is a rare but life-threatening disease. The introduction of immunosuppressive treatment (IST) and hematopoietic stem cell transplantation (HSCT) has considerably improved the outcome of patients with AA. However, modern-day population-based data are limited. This thesis aimed to retrospectively analyze the incidence, treatment modalities, survival, and immune markers in the bone marrow of patients diagnosed with AA in Sweden from 2000–2011.

**Patients and methods.** Patients were included via the National Patient Registry and diagnosed according to the Camitta criteria. All data were collected from medical charts. In paper IV, immunohistochemistry was used to obtain data on regulatory T cells and macrophages in the bone marrow.

**Results and conclusions.** We identified 257 confirmed cases, with an overall incidence of 2.35 cases per million inhabitants per year. The 5-year overall survival (OS) was >90% in patients aged up to 39 years but 38.1% in patients aged  $\geq 60$  years. Multivariate analysis showed that age  $\geq 40$  years, very severe AA, and no specific therapy were independent risk factors for inferior survival. First-line IST treated patients (n=158) showed a 47% response rate with no difference regarding the age groups or anti-thymocyte globulin (ATG) formulation. The response was significantly associated with the severity grade at the time of treatment initiation, and very severe AA patients exhibited a response rate of 22%. Sixty-eight patients underwent HSCT with a 5-year OS of 86.8%. The graft-versus-host-disease-free, relapse/rejection-free survival at 5 years was 69.1%. Patients aged  $\geq 40$  years had higher transplant-related mortality that translated into a lower 5-year OS. In paper IV, we found lower numbers of FOXP3-positive regulatory T cells in AA patients without predictive value for IST response and that patients with a higher number of CD163-positive macrophages had a better 5-year OS, but this benefit was only observed in the non-severe AA group. In conclusion, younger patients have very good long-term survival regardless of the choice of therapy, whereas the outcome for patients  $\geq 60$  years remains poor. Very severe AA patients respond poorly to ATG, which indicates the need for a different treatment approach.

**Keywords:** aplastic anemia, real-world data, ATG, HSCT, regulatory T cells  
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## Sammanfattning på svenska

Aplastisk anemi (AA) är en sällsynt men mycket allvarlig blodsjukdom. Den orsakar låga blodvärden och cellfattig benmärg. AA har i de flesta fall en autoimmun orsak och svarar ofta på immunhämmande behandling (IST) med antithymocytglobulin (ATG). Sjukdomen har olika svårighetsgrader, som beror på nivån av vita blodkroppar i blodet. Man har kunnat påvisa en minskad förekomst av regulatoriska T-celler (Tregs) och ökad förekomst av makrofager hos AA-patienter. Vi har retrospektivt studerat incidens, överlevnad, behandlingsregimer och immunologiska markörer i ett stort befolkningsbaserat, oselektat patientmaterial med AA som insjuknade i Sverige under åren 2000-2011. Vi observerade att incidensen på AA inte har ändrat sig de senaste 30 åren: i Sverige insjuknar 2,35 personer per miljon invånare och år. Vi identifierade sammanlagt 257 AA patienter med en medianålder på 60 år. Primär behandling i form av IST gavs till 63%, 10% benmärgstransplanterades, och 27% fick palliativ eller ingen behandling alls. Vi såg att 5-års-överlevnaden tydligt påverkades av ålder och att ålder >40 år, mycket svår AA (VSAA) och palliativ behandling/ingen behandling alls var oberoende riskfaktorer för sämre överlevnad. Vidare har vi analyserat behandling med ATG (n=158) och sett att knappt 50% svarar på sådan behandling. Behandlingssvaret var tydligt beroende av svårighetsgraden: patienter med VSAA svarade sämst. Benmärgstransplanterade patienter (n=68) hade en 5-års-överlevnad på 86,8% och överlevnaden påverkades av patienternas ålder: 92% för patienter <40 år och 71% för patienter ≥40. Transplantat-mot-mottagare sjukdom (GvHD) kan vara en mycket allvarlig komplikation efter transplantation. Vi analyserade därför det nya kombinerade utfallsmåttet *GvHD och avstötning/återfallsfri överlevnad* (GRFS) vilket tidigare inte gjorts vid aplastisk anemi och såg en 5-års-GRFS på 69%. Patienter över 40 år hade en sämre GRFS (53%). Till slut analyserade vi immunmarkörer (Tregs och makrofager) i benmärgen på AA patienter och friska kontroller. Vi fann mycket låga Tregs hos AA patienter utan samband med behandlingssvar. Samtidigt såg vi att patienter med högre antal makrofager hade bättre överlevnad, i synnerhet icke-svåra AA patienter.

Vi kan konkludera att unga patienter klarar sig bra, oavsett primär behandling och att den största utmaningen idag är förbättra behandlingen för lite äldre patienter och patienter med mycket svår aplastisk anemi.



# List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Vaht K, Göransson M, Carlson K, Isaksson C, Lenhoff S, Sandstedt A, Uggla B, Winiarski J, Ljungman P, Brune M, Andersson P-O. Incidence and outcome of acquired aplastic anemia: real-world data from patients diagnosed in Sweden from 2000-2011. *Haematologica*. 2017;102(10):1683-1690.
- II. Vaht K, Göransson M, Carlson K, Isaksson C, Lenhoff S, Sandstedt A, Uggla B, Winiarski J, Ljungman P, Brune M, Andersson P-O. Low response rate to ATG-based immunosuppressive therapy in very severe aplastic anaemia - A Swedish nationwide cohort study. *European J Haematology*. 2018;100(6):613-620.
- III. Vaht K, Göransson M, Carlson K, Isaksson C, Lenhoff S, Sandstedt A, Uggla B, Winiarski J, Ljungman P, Andersson P-O, Brune M. High Graft-versus-Host Disease-Free, Relapse/Rejection-Free Survival and Similar Outcome of Related and Unrelated Allogeneic Stem Cell Transplantation for Aplastic Anemia: A Nationwide Swedish Cohort Study. *Biology of Blood and Marrow Transplantation*. 2019;25(10):1970-1974.
- IV. Vaht K, Brenner J, Bram Ednersson S, Ljungman P, Brune M and Andersson P-O. Bone marrow expression of CD68/CD163 macrophages, IL-17 and FOXP3 cells in aplastic anemia and their relation to prognosis. Manuscript

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# Abbreviations

AA	Aplastic anemia
ALC	Absolute lymphocyte count
AML	Acute myeloid leukemia
ARC	Absolute reticulocyte count
ATG	Anti-thymocyte globulin
BM	Bone marrow
cGvHD	Chronic Graft-versus-Host disease
CR	Complete remission
CsA	Cyclosporine A
DC	Dyskeratosis congenita
FA	Fanconi anemia
GPI	Glycosylphosphatidylinositol
GRFS	Graft-versus-host disease free, rejection-free survival
GvHD	Graft-versus-Host disease
hATG	Horse anti-thymocyte globulin
HLA	Human leukocyte antigen
hMDS	Hypoplastic myelodysplastic syndrome
HSCT	Hematopoietic stem cell transplantation
HPF	High power field
IAAS	The International Agranulocytosis and Aplastic Anemia Study
IBMFS	Inherited bone marrow failure syndromes
IL-17	Interleukin 17

IFN- $\gamma$	Interferon- $\gamma$
IST	Immunosuppressive treatment
MDS	Myelodysplastic syndrome
M $\Phi$	Macrophage
NSAA	Non-severe aplastic anemia
OR	Overall response
OS	Overall survival
PBSC	Peripheral blood stem cells
PNH	Paroxysmal nocturnal hemoglobinuria
PR	Partial remission
rATG	Rabbit anti-thymocyte globulin
RNA	Ribonucleic acid, a biological macromolecule
SAA	Severe aplastic anemia
TRM	Transplant related mortality
Tregs	Regulatory T cells
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
URD	Unrelated donor
VSAA	Very severe aplastic anemia

## Definitions in short

Severe AA	Bone marrow cellularity <25%, or 25%-50% with <30% residual hematopoietic cells and 2/3 of the following: neutrophil count <0.5×10 <sup>9</sup> /l; platelet count <20×10 <sup>9</sup> /l; reticulocyte count <20×10 <sup>9</sup> /l (Camitta, 1975)
Very severe AA	As for severe AA but neutrophils <0.2×10 <sup>9</sup> /l (Bacigalupo 1988).
Non-severe AA	Not fulfilling the criteria for severe or very severe aplastic anemia (Camitta, 1975)
Complete remission	Hemoglobin normal for age; neutrophil count >1.5×10 <sup>9</sup> /l; platelet count >150×10 <sup>9</sup> /l (Camitta, 2000)
Partial remission	Transfusion independent, no longer meeting criteria for severe disease (Camitta, 2000)



# 1 Introduction

Aplastic anemia (AA) is a rare and potentially lethal disease. It is characterized by empty bone marrow and pancytopenia. More than 100 years ago (1888), Paul Ehrlich described the autopsy of a young woman who had a short fatal illness characterized by anemia, bleeding, and infection accompanied by yellow instead of red bone marrow in her femur<sup>1</sup>. The disease was first named *pernicious* to reflect its fatal course. In 1904, Vaquez and Aubertin described another fatal case in which histological examination revealed fatty bone marrow with few lymphocytes, and the term AA was used for the first time<sup>2</sup>. Clinical disease features were described by Richard C. Cabot and other pathologists in the early 20<sup>th</sup> century<sup>3</sup>. Factors responsible for AA development have changed over time. At the beginning of the 1900s, the industrial exposure of benzene and subsequent development of AA were reported among Swedish bicycle factory workers and Newark (USA) leatherworkers<sup>4,5</sup>. In the 1960s, several AA patients were diagnosed during the introduction of chloramphenicol,<sup>6,7</sup> and the direct toxic effect was assumed to be responsible for the occurrence of AA. Since the 1970s, after the first bone marrow transplantation and the use of anti-lymphocyte globulin (ATG) to treat AA, evolving evidence for a possible immunological mechanism contributing to AA has been reported. Today, immunological bone marrow destruction is considered the main underlying mechanism in AA.



VII.

Ueber einen Fall von Anämie mit Bemerkungen über  
regenerative Veränderungen des Knochenmarks.

Von

Professor Dr. **P. Ehrlich**,  
Assistent der II. medicinischen Klinik.

## 1.1 Definition and diagnostic criteria

AA is sometimes referred to as idiopathic, acquired, or immune. It is defined as pancytopenia with hypocellular bone marrow in the absence of fibrosis or an abnormal infiltrate. For the diagnosis of AA, at least two of the three following criteria must be present: hemoglobin  $<100$  g/l, platelet count  $<50 \times 10^9/l$ , or neutrophil count  $<1.5 \times 10^9/l$ <sup>8,9</sup>.

To assess AA severity, modified Camitta criteria are used<sup>8,10</sup>:

Severe AA (SAA) is defined as bone marrow cellularity  $<25\%$  or  $25\%–50\%$  with  $<30\%$  residual hematopoietic cells and two of the following three criteria: neutrophil count  $<0.5 \times 10^9/l$ , platelet count  $<20 \times 10^9/l$ , or reticulocyte count  $<20 \times 10^9/l$ . Very severe AA (VSAA) is similar to SAA but with a neutrophil count  $<0.2 \times 10^9/l$ . Non-severe AA (NSAA) does not fulfill the criteria for SAA or VSAA.

## 1.2 Epidemiology

The incidence of AA varies considerably in different parts of the world and has also changed over the years. Historically, reports from Europe and the United States in 1960–70 showed diverse results; incidence rates of up to 6–10 AA cases per million people per year. In some of these studies, an association with toxic agriculture substances was shown<sup>11–13</sup>. However, different diagnostic criteria were used, and some cases represented other diagnoses<sup>11</sup>. The International Agranulocytosis and Aplastic Anemia Study (IAAS) involving several regions in Europe and Israel was published in 1987 and established a well-accepted AA incidence of  $\sim 2$  cases per million people per year<sup>9</sup>. Several other studies from the UK<sup>14</sup>, France<sup>15</sup>, Spain<sup>16</sup>, and Brazil<sup>17</sup> have shown similar results. However, recent studies from Asia show a much higher incidence of up to 7 cases per million people per year,<sup>18–22</sup> which are mostly associated with agricultural substances<sup>19,21</sup>. It is also well known that the incidence of AA has two peaks, one between 15–30 years of age and another after 60 years of age<sup>15,16</sup>. The incidence for men and women appears to be similar but has been reported to be slightly higher among women in some studies<sup>9,14</sup>.

## 1.3 Pathophysiology

Most cases of AA can be pathophysiologically characterized by the T cell-mediated destruction of bone marrow hematopoietic cells<sup>23</sup>. However, hematopoietic failure can also result from direct physical or chemical damage to the marrow. Radiation and cytostatic drugs used to treat cancer often cause aplasia in the bone marrow, but other treatments, such as nonsteroidal anti-inflammatory drugs, antibiotics (sulfonamides), antithyroid drugs, antiepileptics, psychotropics, gold, penicillamine, and allopurinol, have also been associated with AA development<sup>24,25</sup>. Childhood AA may follow viral exposure. For example, the Epstein-Barr virus and seronegative hepatitis can cause SAA<sup>26,27</sup>, likely by activating the immune system. In addition, other immunological conditions, such as eosinophilic fasciitis<sup>28</sup>, thymoma,<sup>29</sup> and Graft-versus-Host disease (GvHD)<sup>30</sup>, can be complicated with AA.

### 1.3.1 Immunological mechanisms

The idea that immunological mechanisms are responsible for the development of AA was assumed many years ago (1960–1970s) based on the first bone marrow transplantation results. The conditioning regimen with an anti-lymphocytic serum that enhanced the transplantation results (reduced acute secondary disease)<sup>31</sup> and failed attempts of the syngeneic transplants without conditioning showed that the substitution of bone marrow/stem cells alone could not repopulate the bone marrow<sup>32</sup>. Even if some immunological mechanisms contributing to the development of AA have been identified, the responsiveness to immunosuppressive treatment (IST) remains the most convincing evidence of an underlying immune pathophysiology<sup>33</sup>. Already in the 1990s, a deficit of early progenitor cells was found, proposing that less than 10% of healthy stem cells are left at the time of the clinical presentation of AA<sup>23,34</sup>. Moreover, research has focused on cytotoxic T cells, which appear to be functionally and phenotypically activated<sup>35-40</sup>, producing interferon- $\gamma$  (IFN- $\gamma$ )<sup>41,42</sup> and stimulating apoptosis through the Fas/Fas ligand pathway<sup>3,43</sup>. Recently, it was

shown that macrophages (MΦs) play a central role in IFN- $\gamma$ -related stem cell destruction. Specifically, they produce tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), engage cytotoxic T cells, and consequently enhance the production of IFN- $\gamma$ , forming an interactive loop<sup>44-46</sup>. Another important aspect involves regulatory T cells (Tregs), which are decreased in AA. There are some indications that the number of Tregs or a unique subtype of Tregs is correlated with the response to ATG treatment, and Tregs appear to increase with the response to treatment<sup>47-49</sup>. Interleukin (IL)-17-producing CD4<sup>+</sup> cells (Th17) play a pivotal role in autoimmunity<sup>50</sup>, and although AA does not share many similarities with autoimmune diseases, Th17 cells are increased (SAA) and seem to play a role during the early stage of AA<sup>51,52</sup>.

### 1.3.2 Telomeres

Even though immune destruction of hematopoietic cells is the leading cause of AA, up to 1/3 of patients have short telomeres, and in some cases, these are caused by mutations in the telomerase gene complex<sup>53,54</sup>. Telomeres are nucleotide repeats at the ends of chromosomes, which protect them from damage. Telomere maintenance requires the telomerase ribonucleoprotein complex, which consists of telomerase reverse transcriptase (TERT) and its RNA template (TERC)<sup>55,56</sup>. Originally, telomere shortening was thought to be the result of a higher than normal number of progenitor cell divisions to produce a mature cell<sup>53,57</sup>. Nevertheless, discoveries in congenital AAs, such as dyskeratosis congenita (DC), Fanconi anemia (FA), and Shwachman-Diamond syndrome, helped to connect genetic lesions and telomere shortening of hematopoietic cells<sup>58,59</sup>. Mutations in *TERT* and *TERC* genes have been found in 4%–10% of AA patients<sup>53,60</sup>. AA patients with short telomeres appear to have dismal overall survival (OS) after IST and a significantly higher risk for relapse and clonal evolution<sup>54</sup>.



### 1.3.3 Clonality

Although AA is a non-malignant disease, it has a well-accepted association with clonal hematopoiesis<sup>61,62</sup>. The most used phrase is clonal evolution, which can occur many years after the initial diagnosis and describes when AA transforms to a myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). In different studies, the long-term risk for developing AML or MDS ranged between 5%–20%, and it often involves the loss of all or a portion of chromosome 7<sup>63-68</sup>. However, it is not uncommon for AA patients to have chromosomal abnormalities at the time of diagnosis or acquire them during the course of the disease<sup>69,70</sup> without further development of malignancy<sup>3,68</sup>. The responses to IST and survival appear to be similar between patients with and without cytogenetic aberrations<sup>71</sup>, but patients with monosomy 7 respond poorly to IST<sup>70</sup>.

Next-generation sequencing has identified several mutations in specific genes, adding evidence of the clonal nature of AA. Most of the mutations are known from myeloid malignancies, but AA differs from malignancies in its limited set of mutations and clone size<sup>72,73</sup>. Previous studies have shown that clonal populations are present in 19% to 47% of AA patients, and the common somatic mutations are *DNMT3A*, *ASXL1*, *BCOR*, *BCORL1*, and *PIGA*<sup>72,73</sup>. These mutations have also been associated with the clinical outcome and prognosis; patients with *PIGA*, *BCOR*, and *BCORL1* respond well to IST and have better OS compared with patients with *DNMT3A* and *ASXL1* mutations<sup>72</sup>. In another study, the presence of *DNMT3A* and *ASXL1* increased the risk for MDS development<sup>73</sup>.

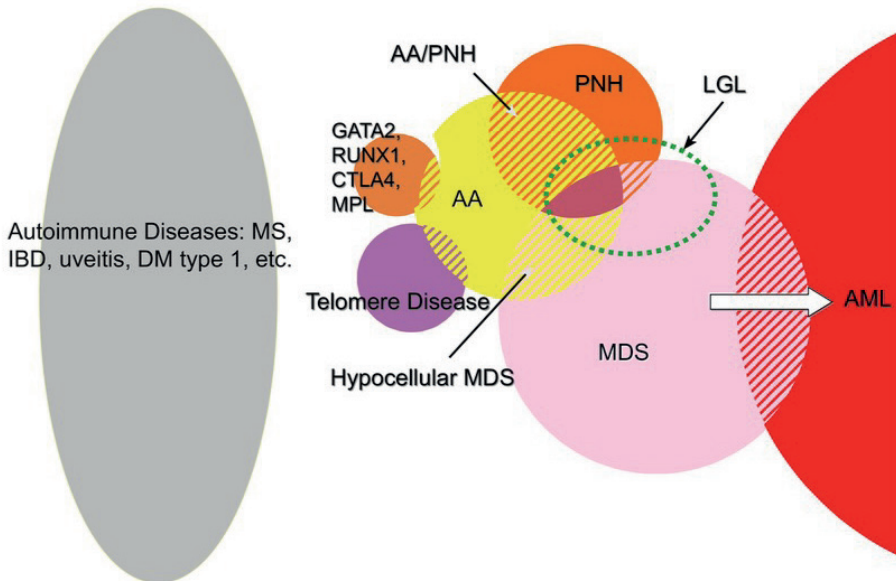
Paroxysmal nocturnal hemoglobinuria (PNH) provides historical and considerable evidence of benign clonal hematopoiesis<sup>61</sup>. PNH is caused by mutations in the X-linked gene *PIGA*, which encodes phosphatidylinositol N-acetylglucosaminyltransferase subunit A (*PIGA*) involved in the synthesis of glycosylphosphatidylinositol (GPI),

making the cell surface extremely sensitive to complement attack<sup>74,75</sup>. PNH is strongly associated with bone marrow failure. It is believed that the cell-mediated autoimmune attack that causes AA may spare selectively GPI-negative hematopoietic stem cells, providing a growth advantage for the *PIGA*-mutant GPI-negative clone<sup>74</sup>. More than 50% of AA patients have a PNH clone, most of which have a clone size of <10%, and the development to a symptomatic PNH disease occurs in 2%–19% of AA cases<sup>76-80</sup>. Nevertheless, the progression to acute leukemia in patients with PNH and from PNH clones in patients with marrow failure is rare<sup>61,81</sup>.

AA development has also been associated with specific human leukocyte antigens (HLA)<sup>82</sup>. Class I HLA molecules, such as HLA A02:01, A02:06, A31:01, and B4:02, can act as auto-antigens and are targeted by cytotoxic T cells<sup>83</sup>. A substantial proportion of AA patients have clonal hematopoiesis characterized by the loss of specific HLA alleles as a result of acquired copy number-neutral loss of heterozygosity (CNN-LOH) on the short arm of chromosome 6 (6pLOH)<sup>83</sup>. 6pLOH(+) clones have been detected in 11%–13% of AA patients. Reduced HLA auto-antigen expression gives stem cells an opportunity for clonal outgrowth as an “escape” from the autoimmune attack<sup>82-86</sup>. This may be the mechanism by which hematopoiesis is maintained for years in AA patients<sup>83</sup>.

## 1.4 Differential diagnosis

AA is a diagnosis of exclusion, and there is no single test that reliably establishes an AA diagnosis (**Figure 1**). Other causes of pancytopenia (e.g., drugs or viruses), hypoplastic bone marrow, and inherited bone marrow failure syndromes (IBMFS) should be excluded.



**Figure 1.** Differential diagnosis of aplastic anemia. Reproduced with permission from (Young, N; *Aplastic anemia*; *N Engl J Med* 2018;379;1643-1656). Copyright Massachusetts Medical Society.

### 1.4.1 Inherited bone marrow failure syndromes

IBMFS are traditionally considered pediatric disorders, but they can also be diagnosed in adults. IBMFS are rare disorders, but the most common ones to develop to AA and evolve into MDS and AML are FA, DC, and *GATA2* spectrum disorders<sup>87</sup>. Patients with IBMFS often have both physical and hematologic findings, but depending on the disease, they may not have any physical abnormalities.

## **Fanconi anemia**

FA is the most common inherited cause of bone marrow failure. Genes that are mutated in FA patients are termed *FANC*, and more than 20 have been identified; *FANCA*, *FANCC*, *FANCG*, and *FANCD2* are the most common<sup>88,89</sup>. FA patients have a cumulative hematopoietic dysfunction likely related to an excess of genetic instability, cellular stress, p53 activation, and cell death<sup>90</sup>. Patients often present with congenital abnormalities, such as a short stature, microphthalmia, thumb and radius deformities, skin hyperpigmentation, and other signs, but approximately 25% have no physical signs, and the disease can be missed during childhood. Up to 80% of FA patients have been reported to develop bone marrow failure, and it usually occurs during the first or second decades of life<sup>90,91</sup>. MDS or AML is diagnosed later on in the disease course, and the risk to develop solid tumors increases with age<sup>92</sup>.

## **Dyskeratosis congenita**

DC is a disease in which many patients reach adulthood before diagnosis. Possible physical signs increase with age, and more than half of the patients are diagnosed later than 15 years of age<sup>87</sup>. Dysplastic nails, oral leukoplakia, hypogonadism, lacrimal duct stenosis, early greying, and liver and pulmonary fibrosis can all be symptoms of DC. Since 1998, eleven DC genes have been identified<sup>89</sup>. Several of them are essential in telomere maintenance, which becomes defective and leads to very short telomeres. DC can have different inheritance patterns. *DKC1* is responsible for X-linked DC and often found in children<sup>89</sup>. Autosomal dominant DC is heterogeneous, and three mutations, including *TERC*, *TERT*, and *TINF2*, have been recognized<sup>89</sup>. Autosomal-recessive DC has been associated with *NOP10* and *NHP2* mutations<sup>93</sup>. Bone marrow failure is a frequent complication in DC (in up to 80% of patients), and it often develops in the second or third decade but can develop from any time after birth to the seventh decade of life<sup>87,93</sup>. DC patients generally have a 4-fold higher risk for developing malignancies, but a substantially higher risk is noted for

some types of tumors, including head and neck carcinomas (~70-fold), MDS (~500-fold), and AML (~70-fold)<sup>94</sup>.

### **GATA2 spectrum disorders**

GATA2 is a transcription factor essential for hematopoiesis by maintaining the pool of hematopoietic stem cells<sup>95</sup>. GATA2 deficiency has a broad clinical spectrum that includes viral and bacterial infections, lymphedema, deafness, alveolar proteinosis, monocytopenia, and low B, T, and NK cells, and it is associated with bone marrow failure, MDS, and AML<sup>96</sup>. *GATA2* mutations associated with bone marrow failure result in loss of function and *GATA2* haploinsufficiency<sup>89</sup>. Patients with GATA2 deficiency have a high risk of developing MDS and leukemia<sup>97</sup>, and MDS is typically hypocellular. Additionally, the most common cytogenetic aberration is monosomy 7, but other abnormalities, such as trisomy 8 and trisomy 21, have also been described<sup>95</sup>. Flow cytometry analysis of GATA2 patient bone marrow samples showed a concomitant reduction in monocytes, B cells, and NK cells, which were significantly lower than in AA patients<sup>98</sup>.

### **1.4.2 Hypoplastic myelodysplastic syndrome**

The diagnosis of a MDS requires morphological dysplasia in 10% of one or more myeloid lineages<sup>99</sup>, and most patients have a hypercellular or normocellular marrow. Still, 10%–20% of MDS patients have cellularity under 30%, having a hypoplastic MDS (hMDS)<sup>100,101</sup>. Distinguishing hMDS from AA can be difficult; using only morphological alterations is challenging because of the low number of assessable cells. The detection of clonal markers as a chromosomal aberration or molecular marker can be helpful, but some overlapping with AA occurs<sup>102</sup>. However, dysplastic megakaryocytes, a normal or increased number of CD34 cells, and some mutations are not consistent with AA<sup>103-106</sup>. Spliceosome genes mutations and multiple mutated genes are characteristic for MDS but not AA<sup>72,107</sup>. Patients with hMDS

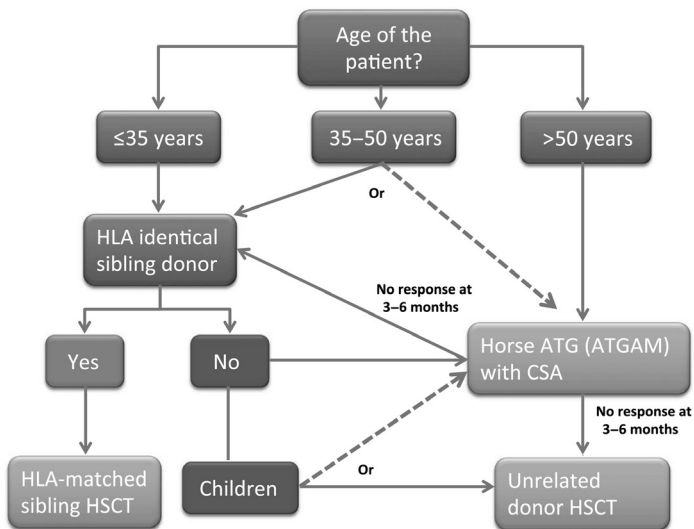
Aplastic anemia - a population-based study of epidemiology, treatment, and prognostic factors

have a higher risk of developing leukemia and shorter OS compared with AA<sup>106,108</sup>.

Differentiating between pediatric bone marrow failure syndromes is challenging. The most common form of pediatric MDS is refractory cytopenia characterized by a hypocellular bone marrow with a normal karyotype<sup>109,110</sup>. Somatic mutations and inherited constitutional mutations should be analyzed for correct diagnosis and treatment<sup>89,110</sup>.

## 1.5 Treatment

Patients with SAA or VSAA have a direct indication of treatment. NSAA has a different clinical course of the disease as they can develop SAA or be stable for years. Spontaneous recoveries occur but are rare<sup>111,112</sup>. NSAA patients can be monitored with blood tests and treated when they become dependent on transfusions or have a neutrophil count  $<0.5 \times 10^9/l$ <sup>113</sup>. Current treatment recommendations stratify patients according to age and the availability of an HLA-matched sibling donor (**Figure 2**)<sup>3,113,114</sup>. However, the choice between hematopoietic stem cell transplantation (HSCT) and IST is not always clear-cut, and both comorbidities and the severity of AA should be taken into account<sup>115</sup>.



EBMT SAAWP, Sureda et al, 2015

**Figure 2.** Treatment of acquired aplastic anemia; Reprinted from, *Brit. J. Haematol*;172(2), Killick, B.S, et al; *Guidelines for the diagnosis and management of adult aplastic anaemia*, Pages No 187-207, Copyright (2016), with permission from Elsevier.

## 1.5.1 Hematopoietic stem cell transplantation

The replacement of failing bone marrow with a healthy one is a curative treatment for AA patients. HSCT has several complications, and GvHD remains one of the most feared reactions. It is the major cause of non-relapse mortality after HSCT. Patients with severe chronic (c)GvHD have decreased quality of life, and the long-term mortality rate increases to 50%<sup>116,117</sup>.

The choice of primary treatment depends on the availability of an HLA-matched sibling donor, and patients <40 (-50) years of age with a sibling donor should undergo transplantation as the first-line treatment<sup>113</sup>. These recommendations have been changed over the years. As early as 1988, it was established that patients <20 years of age had better OS with an HLA-matched sibling donor HSCT than IST (66% vs. 56%); this was especially true for patients with VSAA (64% vs. 38%,  $p=0.01$ ). Older patients with VSAA responded poorly to both treatments (OS 44% HSCT vs. 43% IST), but SAA patients had better survival with IST (82%) than HSCT (62%)<sup>10</sup>. In 1995, HSCT was also suggested for patients 20–45 years of age<sup>27</sup>. However, even though studies still show exceptionally promising HSCT results for younger patients, patients >40 years of age have a considerably inferior outcome. A European Group for Blood and Marrow Transplantation (EBMT) study from the year 2000 showed better results for young (<20 years of age) patients with low neutrophil counts ( $<0.2 \times 10^9/l$ ) receiving HSCT<sup>118</sup>. A follow-up study from 2016 that compared the findings from before and after 1999 confirmed the superior results for the younger group, in which the OS after HSCT was 86%, and patients 21–40 years of age also had a high OS with HSCT (76% compared with 65% for IST,  $p=0.6$ ). Nevertheless, patients >40 years of age showed a worse OS with HSCT (IST 58% and HSCT 56%,  $p=0.006$ )<sup>119</sup>. Patients >40 years of age had a declining OS: 40–49 years 67%, 50–59 years 58%, and >60 years 48% ( $p<0.0001$ )<sup>120</sup>. It is well-known that older patients have higher transplant-related mortality (TRM), and a low performance score pre-transplant has been



shown to increase the mortality risk<sup>121</sup>. As infections, GvHD, and toxicity are the main reasons for death, questions of the most appropriate conditioning regimen and the optimal GvHD prophylaxis have been raised<sup>120,122,123</sup>.

Bone marrow (BM) has been widely used as a stem cell source for HSCT in AA patients. At the beginning of the 21st century, when peripheral blood stem cells (PBSCs) were being increasingly used, a switch from BM to PBSC occurred. This also led to studies comparing the transplant results using BM or PBSCs. A large study combining data from EBMT and the Center for International Blood and Marrow Transplant Research (CIBMTR) was published in 2007 and included 692 AA patients. A clear advantage for BM was observed in the <20 years of age group, with a better OS (85% vs. 73%), as well as a lower risk for cGvHD<sup>124</sup>. In another study, the survival of patients receiving BM was 84% compared with 68% for PBSCs ( $p<0.0001$ ), and this advantage was present in all age groups (<20 years: 90% vs. 76% and >20 years: 74% vs. 64%, respectively)<sup>125</sup>. As a result, BM is currently the preferred stem cell source for transplantation in AA<sup>113</sup>, even though some studies reported no differences in OS or GvHD using PBSCs instead of BM<sup>120</sup>.

As only a few patients have an HLA-matched sibling donor, the first attempts to use unrelated donors (URDs) was made in the 1980s. The probability of long-term survival after an URD transplantation in AA ranged from 29% to 50%<sup>126-129</sup>. Since then, the results have improved with the introduction of high-resolution HLA typing and the use of fludarabine-based conditioning, low dose total body irradiation, and the CD52-antibody alemtuzumab<sup>130-133</sup>. An EBMT study on SAA patients transplanted between 1990 and 2005 found that cohorts transplanted before and after 1998 significantly differed in outcomes. The 5-year survival increased from 32% before 1998 to 57% for those transplanted after ( $p<0.0001$ ), and the improved survival was associated with less graft failure and reduced acute and chronic GvHD<sup>134</sup>. Another EBMT

analysis of 1448 AA patients transplanted between 2005 and 2009 included 508 URD transplants, which showed significantly more acute grade II–IV (25% vs 14%) and chronic GvHD (26% vs 14%). In survival analyses, URD compared with an HLA-matched sibling donor graft was not a statistically significant predictor<sup>135</sup>. The OS after an URD graft is currently >70% for adult patients (up to 40 years of age) and >90% for children (<18 years of age)<sup>136-139</sup>. Therefore, URD transplantation is recommended as a second-line treatment for adults but can be used as a first-line treatment for children<sup>137</sup>.

In addition to well-defined transplantation options with an HLA-matched sibling donor or an URD, alternative stem cell sources, such as cord blood or a haploidentical donor, have been used. These may also be curative, but the risk of graft rejection, infectious complications, and GvHD are higher<sup>140</sup>. A large cohort study of unrelated cord blood transplantation between 1996 and 2009 showed an estimated 3-year OS of 38%. The major cause of death was graft failure and infections<sup>141</sup>. More recently, an increasingly used option is haploidentical transplantation. Almost 400 AA patients have been transplanted, and encouraging results have been reported mainly in the pediatric population and young adults<sup>142</sup>. The median overall engraftment rate was 92%, and the median risk of grade 2–4 acute GvHD was 12%, with a median 1-year OS of 85%<sup>143</sup>.

Apart from developing acute or chronic GvHD, HSCT is associated with other potential complications. Rejections are observed in 5%–15% of AA patients and can be reduced with the use of ATG, low-dose irradiation, fludarabine-based conditioning for URD grafts, and using a marrow cell dose higher than  $2 \times 10^8/\text{kg}$ <sup>114</sup>. Late complications include the development of solid tumors, the incidence of which has been reported to be 12%, and the most prominent locations were the skin, cervix, head and neck. The conditioning regimen, especially irradiation, and cGvHD were risk factors. Another late complication is

osteonecrosis, with an incidence of 15%–20%, and the risk factors were age, previous IST, and treatment with steroids<sup>144-146</sup>.

### 1.5.2 Immunosuppressive treatment

Today, the standard IST is horse anti-thymocyte globulin (hATG) and cyclosporine A (CsA), with hematologic recovery in 50% to 70% of cases<sup>77,114,147-151</sup>. Adding CsA to hATG considerably improved the response rate at three months (65% in the hATG/CsA group vs. 39% in the hATG group) and six months (70% vs. 46%, respectively)<sup>147</sup>. However, later attempts to enhance hATG/CsA treatment by adding an additional immunosuppressive drug have been unsuccessful. Mycophenolate mofetil (MMF) inhibits the proliferation of activated lymphocytes and was anticipated to favor the induction of tolerance. The OR rate at six months was 62% with a 37% relapse rate, and more than half of the relapses occurred during MMF administration, suggesting that this drug was not effective in preventing relapses among responders<sup>152</sup>. Sirolimus blocks CsA-resistant pathways and inhibits T cell activation. A study in which sirolimus was added to hATG + CsA showed no differences in the OR rate at either three or six months (37% and 51% for the hATG/CsA/sirolimus group and 50% and 62% for the hATG/CsA group, respectively)<sup>153</sup>. Alternative treatment regimens, such as alemtuzumab and cyclophosphamide, have also been used for AA treatment. As first-line treatment, alemtuzumab achieved only a 19% response rate<sup>154</sup> and is not currently recommended. Cyclophosphamide administered at moderate or high (30–200 mg/kg) doses is reported to be too toxic, causing prolonged neutropenia and a high incidence of fungal infections and mortality<sup>155,156</sup>.

IST is offered as a first-line treatment to patients without an HLA-matched sibling donor or those >40 years of age<sup>113</sup>. There are several ATG products available with different efficacies. All ATG products have a lymphocyte-depleting effect, but rabbit ATG (rATG) causes more severe depletion of CD4+ T cells and Tregs<sup>157</sup>. However, this

biological effect does not translate into a better clinical outcome. Several studies comparing hATG with rATG have shown both an inferior hematological response (68%–79% for hATG vs. 37%–53% for rATG)<sup>157-160</sup> and OS (96% for hATG vs. 76% for rATG)<sup>157</sup>. In contrast, some studies have found a similar response rate between different ATG formulations<sup>158,161</sup>. However, the only randomized study<sup>157</sup> has shown the superiority of hATG, which is considered to be more effective and the preferred first-line treatment.

The OS after ATG treatment is age-dependent. An EBMT study of 192 patients treated with hATG and CsA with or without granulocyte colony stimulating factor showed that the OS at 15 years was 89%±12% for patients >20 years, 81%±13% for patients 20–39 years, 55%±15% for patients 40–59 years, and 32%±16% for patients ≥60 years<sup>67</sup>. Similar results of decreased OS for patients >40 years have been reported in several studies<sup>115,159,162-164</sup>. Recently, a study involving 955 patients receiving Thymoglobulin® treatment showed similar 10-year survival numbers: patients <20 years 80%, 21–40 years 70%, 41–60 years 49%, and over 60 years 38%<sup>165</sup>. Instead, children treated with IST have an excellent outcome in most studies, and the survival comparison with HSCT does not differ considerably, but event-free/failure-free survival has been significantly inferior in patients receiving IST (33%–64%)<sup>166-168</sup>.

The response and survival after ATG treatment additionally depend on the AA severity grade. For NSAA, ATG + CsA compared with CsA alone resulted in significantly higher response rates (74% vs. 46%, respectively)<sup>169</sup>. VSAA patients have a similar response to treatment as SAA patients depending on the ATG type (30%–50%)<sup>160,170,171</sup>, except for children with VSAA who have been reported to achieve more complete responses (CRs) than SAA patients (68% vs. 45%)<sup>172</sup>. Patients with VSAA have higher infection rates (44%) during the first 90 days compared with SAA patients (22%)<sup>79</sup>, and higher mortality. The 5-year survival is lower for VSAA (76%) compared with SAA (98%)

patients<sup>173</sup>. As an exception, children with VSAA appear to have better survival compared with adults (83% vs. 62%)<sup>115</sup>.

In addition to the AA severity grade, other predictive factors of patient response and survival have been identified based on hATG and CsA treatment. The response to ATG treatment is slow, and evaluation at six months after IST is recommended. In multivariate analysis, Scheinberg and coworkers found that younger age, higher baseline absolute reticulocyte counts (ARCs), and absolute lymphocyte counts (ALCs) were highly predictive of the response at six months. Patients with  $ARC \geq 25 \times 10^9/l$  and  $ALC \geq 1 \times 10^9/l$  had a higher probability of response compared with patients with lower ARCs and ALCs (83% vs. 41%, respectively), which translated to a longer 5-year survival (92% vs. 53%)<sup>170</sup>.

Several complications are associated with IST, such as relapse, refractory disease, and clonal evolution. Up to 35% of patients experience relapse after IST<sup>78,174</sup>, and the second course of ATG results in a 55%–65% response rate, where responding patients have a good survival rate<sup>175,176</sup>. Refractory disease is more challenging to treat, and the response to the second course of ATG is around 30% (up to 70%)<sup>177-179</sup>. In these cases, HSCT should be considered instead, especially for younger patients<sup>180,181</sup>. More than 40% of patients with refractory disease die from bleeding or infections within 5 years after their diagnosis<sup>182</sup>. Alemtuzumab monotherapy is equivalently effective as second-line ATG, with a hematologic response observed in 30%–40% of patients<sup>154</sup>. The risk of clonal evolution to MDS/AML is ~15%, and the development of hemolytic PNH occurs in 10%<sup>78</sup>.

Eltrombopag was presented as a new treatment option in 2012. Eltrombopag is a thrombopoietin mimetic that binds to the receptor c-MPL expressed on megakaryocytes, hematopoietic stem cells, and progenitor cells<sup>183</sup>. In the first study conducted by the National Institutes of Health (NIH), eltrombopag was used to treat refractory patients.

Twenty-five patients were treated, with a 44% hematologic response in at least one lineage<sup>183</sup>. In the second cohort, an additional 18 refractory patients were included, and the overall response rate was 40%. Five of the responding patients discontinued eltrombopag and continued to have normal marrow function. However, eight patients developed clonal cytogenetic abnormality during the treatment<sup>184</sup>. A new phase 2 study included 40 patients who had previously failed at least one ATG treatment. Targeted deep and whole-exome sequencing was performed. Fifty percent of the patients achieved a hematologic response, and 18% of the patients developed clonal evolution. Clonal evolution was an early event in 87% of the patients and occurred within six months of eltrombopag initiation, suggesting a direct link between eltrombopag treatment and clonal evolution, and thus close monitoring with bone marrow samples is warranted<sup>185</sup>. Eltrombopag has also been used for untreated patients in combination with hATG/CsA. Eltrombopag was given as a 150 mg daily dose and at different time points depending on the cohort. Promising results were observed, especially in the third cohort who received eltrombopag from days 1 to 180. The overall response rate at six months was 94%, and the survival rate at 2 years for all patients was 97%. Clonal evolution occurred early and in a total of 7 (7.6%) patients<sup>186</sup>.

## 1.6 Population-based studies

Although randomized clinical studies are the most suitable to compare different treatments, a population-based study on a cohort representing a defined population (e.g., geographic boundaries) offers some advantages, especially in external validity. Randomized trials usually represent a selected cohort of patients; several patients are excluded because of age, co-morbidities, or patient refusal<sup>187</sup>. Other studies, such as registry and single-center studies, can be biased depending on the reporting activity and type of patients treated in a specific center. Transplant centers usually treat younger patients and report their results

to the transplant registries. Patients treated in smaller hospitals can be lost to follow-up, and their long-term outcomes not reported. Real population-based studies in AA are difficult to perform because of the lack of nationwide AA registries collecting information on treatments and outcomes.

Most AA studies have been performed on selected patient cohorts, such as randomized trials, transplant registry studies, and single-center reports, in which most included patients were also young. Moreover, numerous epidemiological studies on AA from Europe, the United States, South America, and Asia from 1970 until the 1990s have reported outcome data with short follow-up periods<sup>11,14,17</sup>. The latest epidemiological study in Scandinavia was issued in 1996 and focused on pediatric population<sup>188</sup>. The importance of complete longitudinal population-based data was underlined at the International Working Group of Severe Aplastic Anemia meeting in 2010, where the foundation of a population-based registry for the longitudinal collection of data on AA patients from diagnosis to after treatment was proposed<sup>189</sup>. In Sweden, where disease codes of all patients are centrally registered, there exists an excellent opportunity to identify patients with AA and collect data on incidence, treatment, and outcomes of the entire population.

## 2 Aims

The overall aim was to conduct a retrospective population-based study in patients with AA diagnosed in Sweden during the years 2000–2011, with emphasis on incidence, treatment, and survival.

### **Paper I**

To determine the incidence of AA, type of treatment, and survival in the entire cohort.

### **Paper II**

To analyze the AA patient cohort receiving ATG treatment, with a special focus on the following: (a) response rate and duration after first- and second-line ATG therapy, (b) response rate to different ATG formulations (horse and rabbit), (c) predictive clinical factors for patient response, and (d) survival.

### **Paper III**

To analyze the allografted AA patient cohort, focusing on the effects of disease severity, stem cell source, and age and transplantation timing on patient survival, as well as causes of death and the new composite endpoint graft-versus-host disease-free, rejection-free survival (GRFS).

### **Paper IV**

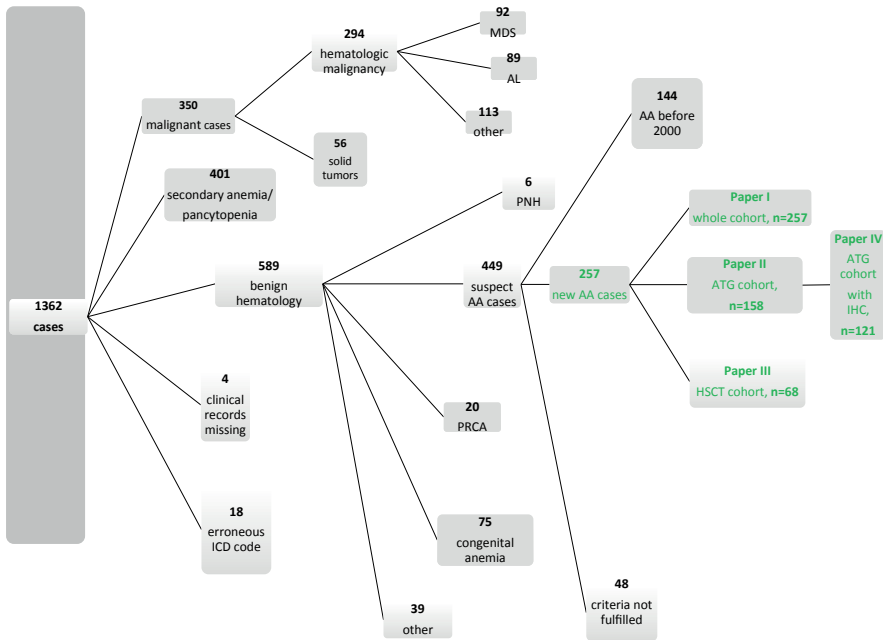
To determine the possible relationship between Tregs and MΦs and AA severity and responses to IST in the ATG-treated AA patient cohort by performing immunohistochemical staining in bone marrow biopsies from the time of diagnosis.



### 3 Patients and methods

#### Identification of AA cases (paper I–IV)

The Swedish National Patient Registry held by the Swedish National Board of Health and Welfare was used to identify new AA cases (both children and adults) diagnosed from 2000–2011 in Sweden. The registry includes data on all patients treated as in- or out-patients in the national health care system. The 10<sup>th</sup> version of the International Classification of Diseases (ICD) was used for the registry search, and the disease codes D61.0–D61.9 were applied. The initial evaluation showed more than 5,700 cases. Because of the suspiciously high number of potential new cases, we performed a pilot study at the Sahlgrenska University Hospital in Gothenburg to evaluate the registry and establish new search criteria. Seven-hundred and twenty cases were found at the Sahlgrenska University Hospital, and all medical charts were evaluated. Fifty-seven valid AA cases were identified. The other patients did not fulfill the diagnostic criteria, had a false diagnosis code (clerical error), had secondary anemia because of other diseases (e.g., cancer or rheumatic diseases), or had congenital AA. A common finding was that all genuine cases of AA were diagnosed at the departments of internal medicine, hematology, infectious diseases, or pediatrics. Additionally, all AA patients had more than one medical contact with a D61.0–D61.9 diagnosis code. When the cut-off level was set as at least two medical contacts and a diagnosis code of D61.0–D61.9, only one genuine AA case was missed. Based on the pilot study, the following new search criteria for the registry were established: 1) one of the codes from D61.0–D61.9, 2) at least two medical contacts with this diagnosis code, and 3) the diagnosis was made in the internal medicine, hematology, infectious diseases, or pediatrics department. A new national search revealed 1,362 unique patients with a potential diagnosis of AA, and all medical charts were thoroughly analyzed (**Figure 3**). Data were collected from the treating hospitals (university hospital, n=7; regional and county hospitals, n=29) in case report forms, and unclear cases were



**Figure 3.** Identification of cases from the Swedish National Patients Registry and allocation between papers I-IV. AL-acute leukemia; MDS-myelodysplastic syndrome; PNH-paroxysmal nocturnal hemoglobinuria; PRCA-pure red cell anemia

re-evaluated. Follow-up information was acquired from medical charts and the Swedish Cause of Death Registry.

The diagnosis of AA and AA severity grade was confirmed according to the Camitta criteria. Patients with congenital disease, pancytopenia without a marrow biopsy performed, marrow fibrosis, or other signs of malignancy or dysplasia were excluded. Mild dysplasia in erythropoiesis was accepted. The patient allocation between papers I–IV is shown in **Figure 3**.

### Immunohistochemistry (paper IV)

Paraffin-embedded bone marrow tissue blocks were used to produce whole tissue sections with a thickness of 4 microns. After routine deparaffinization, sections were stained with CD68 (Agilent, Ca, United States), CD163 and FOXP3 (BioCare, CA, United States), and IL-17

(Abcam, Cambridge, UK). The PT Link/PT200 HIER, Heat-Induced Epitope Retrieval, and Autostainer Link 48 were used. The choice of antibodies was based on the following: CD68 (for MΦs subtype M1) and CD163 (for subtype M2) are proteins expressed on monocyte-macrophage lineage cells, IL-17 is a marker of Th17 cells, and forkhead box protein 3 (FOXP3) is a well-recognized marker of Tregs. The counting of positively stained cells was performed digitally using NDP.view2 software from Hamamatsu, Japan. Cells with positive staining, nuclear (FOXP3) or cytoplasmatic (IL-17) for lymphocytes, and cytoplasmatic (CD68, CD163) in the large white cells with abundant cytoplasm with or without filopodia and detectable medium to large nuclei were counted in ten consecutive high-power fields (HPF). Microscope Zeiss Axioscope 2 Plus with the optical field of view (FOV) 23 was used as a standard for digital HPFs, giving a calculated area of 0.26 mm<sup>2</sup> per HPF.

### **Statistical methods**

Rates and proportions were compared using Pearson's chi-squared test. The Kaplan–Meier method was used to estimate overall survival, and comparisons were based on the log-rank test. Uni- and multi-variable Cox regression proportional hazards were used to analyze factors influencing survival and a logistic regression analysis for predictive factors for response to treatment. The statistical analyses were performed either by SPSS versions 23–26 or Stata for Macintosh version 13.1, and the relative survival ratio was calculated using the str module.

### **Ethical aspects**

The studies were approved by the Regional Ethical Review Board in Gothenburg (615-12). For healthy individuals included in paper IV, the Swedish Ethical Review Authority approved the study (2019-04900), and they all provided informed written consent.

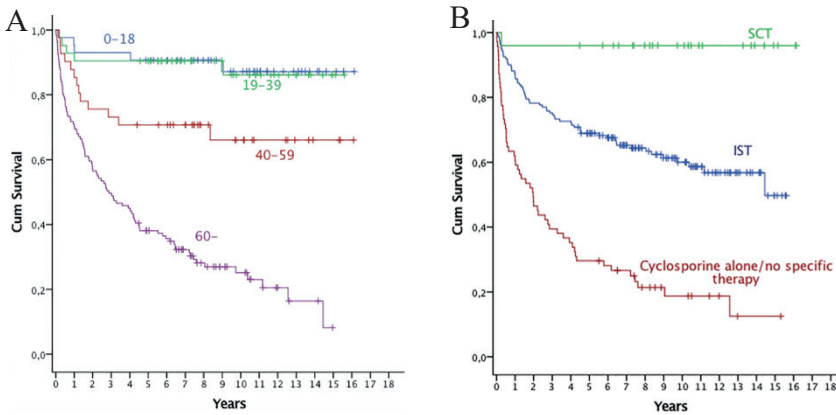
## 4 Results

### Paper I

Of 1,362 potential cases, we identified 257 new AA cases diagnosed between 2000 and 2011 (**Figure 3**). The overall incidence was 2.35 cases per million inhabitants per year. Biphasic age distribution was observed with one peak in patients aged 15–20 years (2.89) and one in patients >60 years old (4.36). The median age at diagnosis was 60 years, and 52% of the patients were female. At diagnosis, 38% had NSAA, 38% had SAA, and 24% had VSAA. First-line treatments included IST (63%), 10% underwent HSCT, and 27% were treated with single-agent CsA or no specific therapy. The median follow-up was 76 months, and 47% died during follow-up. Almost 2/3 of the patients (n=74) died within 24 months, most frequently due to infections (n=41) or bleeding (n=14).

The 5-year survival for all patients with AA was 60.7%, and it was influenced by age as follows: 90.7% in 0–18 years, 90.5% in 19–39 years, 70.7% in 40–59 years, and 38.1% in the  $\geq 60$  years age group (**Figure 4A**). Grouping patients according to disease severity showed a lower 5-year survival in patients with VSAA compared with SAA but not NSAA. According to treatment modality, the 5-year survival was 96% in HSCT patients, 68.9% in the IST group, and 29.6% in patients who received CsA alone/no specific therapy (**Figure 4B**).

The Cox regression analysis revealed that age (40–59 and  $\geq 60$  groups), VSAA, and treatment with CsA alone/no therapy were independent risk factors for inferior survival. The relative 5-year survival for all patients was 65.4%, and according to the median age at diagnosis, the relative 5-year survival was 84.6% in patients less than 60 years but significantly worse for patients  $\geq 60$  years (45.3%).



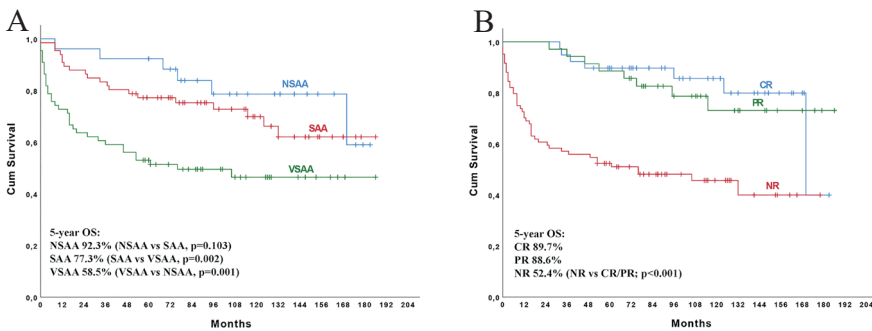
**Figure 4.** *A. Overall survival by age group; B. Overall survival according to primary treatment. SCT- hematopoietic stem cell transplantation (HSCT). Reproduced with permission from (Vaht K, et al; Incidence and outcome of acquired aplastic anemia: real-world data from patients diagnosed in Sweden from 2000-2011. Haematologica. 2017;102(10):1683-1690), Copyright Ferrata Storti Foundation.*

## Paper II

A total of 158 patients treated with first-line ATG were included in this study. The median age was 53 years, and most of the patients (64%) had SAA or VSAA at diagnosis, which increased to 84% at the time of treatment initiation. Different ATG formulations were used, including rATG in 128 patients and hATG in 27 patients.

The best cumulative overall response (OR) was 47%, 25% reached CR, and 22% achieved partial response (PR). The OR rate was similar among the different age groups. The AA severity grade, especially at the time of the start of treatment, influenced the response rate (VSAA 22.7%, SAA 54.5%, and NSAA 88.5%). No significant difference in the response rate was observed between hATG (51.9%) and rATG (45.3%).

The 5-year OS for all patients was 69.6%, and it was age-related. Patients in the age group of 0–18 years had an OS of 86.2%; 19–39, 90%; 40–59, 71.9%; 60–69, 56.1%; and  $\geq 70$ , 46.2%. Disease severity at the time of ATG treatment initiation affected survival. Specifically, the OS rates for the VSAA, SAA, and NSAA groups were 53%, 77.3%, and 92.3%, respectively (**Figure 5A**). Patients responding with CR and PR had a comparable 5-year OS of 89.7%, and 88.6%, respectively, but non-responding patients showed an inferior outcome with an OS of 52.4% (**Figure 5B**).



**Figure 5.** *A. Overall survival by disease severity at the initiation of the treatment; B. Overall survival by response to the treatment. Reproduced with permission from (Vaht K, et al; Low response rate to ATG-based immunosuppressive therapy in very severe aplastic anaemia - A Swedish nationwide cohort study. Eur J Haematol. 2018;100(6):613-620), Copyright John Wiley and Sons. License 4822541059368.*

Considering the predictive factors, univariable logistic regression analysis showed that the well-known risk factor  $ARC < 25 \times 10^9/l$  at diagnosis was highly predictive for a poor response; the same was true for both SAA and VSAA at the time of treatment initiation. In a multivariable analysis, only SAA and VSAA were independent factors

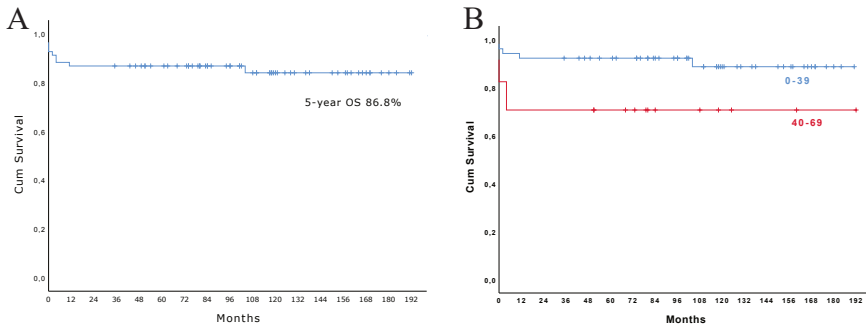
for an inferior response. A logistic model for response prediction containing ARC  $<25 \times 10^9/l$  or  $\geq 25 \times 10^9/l$  at diagnosis and disease severity at the time of treatment initiation revealed that the response probability for patients with VSAA and ARC  $<25 \times 10^9/l$  was only 19%, whereas patients with NSAA and ARC  $\geq 25 \times 10^9/l$  had a response probability of 90%.

### Paper III

Sixty-eight patients who underwent HSCT were included. The median age at transplantation was 22 years, seventeen patients were  $\geq 40$  years of age, and 27 patients were children  $\leq 18$  years old. Sixty-three percent of patients had been previously treated with one or more courses of IST. In more than 50% of cases, the donors were unrelated, and the most common stem cell graft was BM (n=54). According to the type of donor, different conditioning regimes were exploited and included combinations between cyclophosphamide, fludarabine, ATG, and irradiation. Immunosuppression was given with calcineurin inhibitors (in most cases CsA) in combination with methotrexate or other drugs. The median treatment duration with calcineurin inhibitors for patients surviving  $\geq 4$  weeks was 436 days. Graft failure was observed in eight patients.

Twenty-five patients developed acute GvHD, with 16% showing grades III–IV. Eleven patients developed cGvHD, and six patients had moderate or severe cGvHD.

The 5-year OS for all patients was 86.8% (**Figure 6A**). TRM of the entire cohort was 13.2%, which was significantly higher for  $\geq 40$ -year-old patients (29.4%) than in younger patients (7.8%). Patients aged  $<40$  years had a better 5-year OS of 92.2% compared with patients aged  $\geq 40$  years (70.6%) (**Figure 6B**).



**Figure 6.** *A. Overall survival all patients; B. Overall survival according to age groups. Reprinted from, Biol Blood Marrow Transplant; 25(10); Vaht K et al; High Graft-versus-Host Disease-Free, Relapse/Rejection-Free Survival and Similar Outcome of Related and Unrelated Allogeneic Stem Cell Transplantation for Aplastic Anemia: A Nationwide Swedish Cohort Study; pages no1970-1974, Copyright (2019), with permission from Elsevier.*

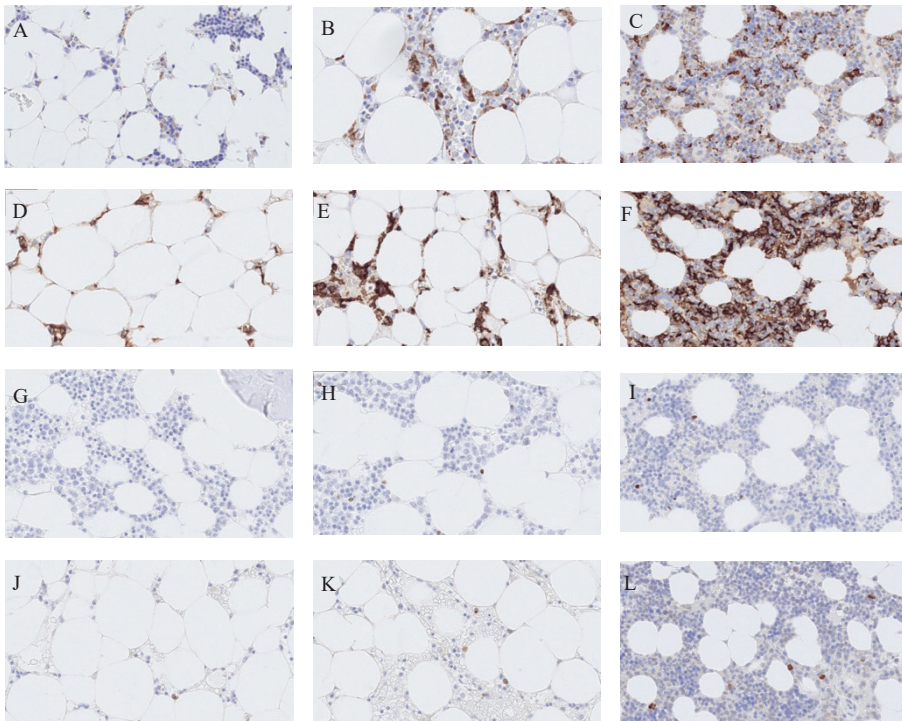
We found no survival difference between HLA-matched sibling donor and URD recipients regarding all patients. Comparing children with adults, we divided patients into  $\leq 18$  and  $\geq 19$  years of age and found a trend for better survival in children with 5-year OS rates of 96.3% and 80.5%, respectively. However, no difference was observed when comparing young adults (19–39 years of age) with children. Further, when analyzing OS according to the stem cell source, we found no difference between BM (85.2%) and PBSCs (100%).

In the entire patient cohort, GRFS at 1 and 5 years was 73.5% and 69.1%, respectively. When dividing patients into age groups of  $< 40$  and  $\geq 40$  years, GRFS at 1 and 5 years was 80.4% vs. 52.9% and 74.5% vs. 52.9%, respectively.



## Paper IV

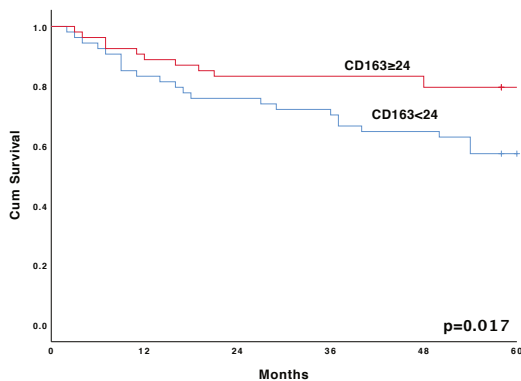
Patients treated with ATG therapy and available bio-banked bone marrow biopsy samples at the time of diagnosis (n=121) and healthy age- and sex-matched volunteers as the control group (n=14) were included in this study. We found that the median number of positive cells per HPF was 0 for FOXP3, 0 for IL-17, 13 for CD68, and 24 for CD163. FOXP3, IL-17, CD68, and CD163 values were all lower in AA patients compared with healthy controls (**Figure 7A-L**).



**Figure 7.** Immunohistochemical staining of CD68, CD163, FOXP3, and IL-17 protein in BM samples of aplastic anemia patients (magnification: ~40x) A. CD68 low; B. CD68 high; C. CD68 healthy control; D. CD163 low; E. CD163 high; F. CD163 healthy control; G. FOXP3 low; H. FOXP3 high; I. FOXP3 healthy control; J. IL-17 low; K. IL-17 high; L. IL-17 healthy control.

We found no differences in FOXP3, IL-17, CD68, or CD163 numbers between AA subgroups. Regarding the response, 48.8% of patients responded to first-line ATG treatment. We found no significant differences in the response when dividing patients into two groups according to the median values of FOXP3, IL-17, CD68, and CD163 positive cells. When analyzing the response according to ARC and ALC, we found that patients with  $ARC \geq 25$  had a better response (65.9% vs. 32.7%) than the  $<25$  group. For ALC, we found no differences in response.

The 5-year OS for all patients was 67.8%. When the cohort was divided according to the FOXP3, IL-17, and CD68 median values, no differences were found in OS. However, when analyzing survival according to the CD163 median subgroups, we found a survival benefit for patients with above-median numbers of CD163-positive cells with an OS of 79.6% vs. 57.4% (**Figure 8**). When dividing patients according to AA severity, we only found a significant difference between CD163 median subgroups in NSAA patients (5-year OS 89.5% vs. 57.9%).



**Figure 8.** The 5-year survival according to median value of CD163.

## 5 Discussion

In this population-based study on AA patients diagnosed from 2000–2011 in Sweden, we found that the incidence was 2.35 per million inhabitants per year with a biphasic distribution. Although early studies showed broad variation in incidence, the IAAS study established a well-accepted overall incidence of ~2 cases per million people<sup>9</sup>. A follow-up study by the Spanish group confirmed these results, showing an incidence of 2.34 cases per million per year<sup>16</sup>. Our incidence data correspond with the previously mentioned studies and show that the AA incidence has not changed since the 1980s.

The 5-year survival in younger patients up to 40 years of age was around 90%, and we found no difference in survival when comparing IST and HSCT as the primary treatment. Our survival data are consistent with other clinical trials reporting ATG and HSCT registry data<sup>119,130,157,158</sup>. Patients  $\geq 60$  years had a 5-year survival  $< 40\%$  and relative 5-year survival of 45%, showing a significant increase in mortality. These older patients had a higher risk of early death than younger ones; 16% were deceased three months after diagnosis. We also observed that patients  $\geq 60$  years treated with IST had a 5-year survival of ~50%, which is similar to the results reported from EBMT AA registry data<sup>164</sup>, implying that a higher percentage of older patients should be treated with IST.

When analyzing patients treated with first-line ATG, we found that the OR rate was 47%, and there were no age-related differences in response rates. Several studies have shown that younger patients respond better to ATG<sup>153,164</sup>, but different ATG formulations were used compared with our data, and their patient cohorts were younger. Previous studies reported diverse response rates between 35% and 80%, depending on the ATG formulation<sup>157-159,190</sup>. Only one prospective randomized study has compared rATG and hATG treatments and found a better response rate (68% vs. 37%) and 3-year survival (96% vs. 76%) in the hATG group<sup>157</sup>. In our study, hATG and rATG treatment resulted in an equal

response rate, which may have been influenced by the disparity in patient numbers between the groups (27 vs. 128, respectively).

The disease severity at the time of treatment initiation significantly influenced the response to ATG. Only 22.7% of VSAA patients responded to treatment, and VSAA patients had only a 19% probability of responding according to a logistic regression prediction model that combined the pre-treatment stage and low ARC. In earlier studies, the response rates among VSAA patients were found to vary between 30% and 81%<sup>66,78,160,170</sup>, and hATG achieved a higher (50%–80%)<sup>78</sup> response rate than rATG (30%–57%)<sup>160</sup>. Most of our patients received rATG (84.4%), and thus our data should be interpreted with caution. However, VSAA patients had a very good 5-year survival after HSCT of almost 90% and 75% for patients >40 years, suggesting that selected VSAA patients, regardless of age, should be counseled for a HSCT as a first-line therapy.

Absolute reticulocyte count and ALC, together with disease severity, are important predictive factors for the response to ATG<sup>170</sup>. Nevertheless, alterations in the immune system have also been reported to be related to the response<sup>48,52</sup>. We found that AA patients had a decreased number of Tregs (FOXP3-positive cells) compared with healthy controls, but no difference in the number of Tregs between AA severity groups or correlation between Tregs and the response or survival was observed. Tregs have reported to be decreased in AA, both in the peripheral blood and bone marrow, compared with healthy controls<sup>52,191,192</sup>. Moreover, the likelihood to respond to treatment appeared to be higher for patients with an increased number of Tregs at diagnosis<sup>49,52</sup>. However, our data indicate that the number of Tregs in the bone marrow of AA patients is not predictive for the response to ATG therapy. Regarding MΦs, and in contrast to the study by Sun et al<sup>44</sup>, we found that AA patients had fewer MΦs, both of the CD68 and CD163 subtypes, than healthy controls. In an AA mouse model, MΦs produced TNF- $\alpha$  and through the TNF- $\alpha$  receptor engaged effector T cells and increased the production of IFN-

$\gamma^{44}$ . Additionally, in another mouse AA model, IFN- $\gamma$ -dependent HSC loss required M $\Phi$ s. IFN- $\gamma$  was necessary for the selective maintenance of bone marrow M $\Phi$ s, and depleting M $\Phi$ s rescued stem cells but did not reduce T cell activation or IFN- $\gamma$  production<sup>193</sup>. In our study, patients with a higher number of CD163-positive M $\Phi$ s (for the M2 subtype) had better survival, and this potential benefit appeared to be limited to the NSAA group. M $\Phi$ s polarization between M1 and M2 subtypes is unclear in AA immunology, but enhanced M2 M $\Phi$  activity was reported to be correlated with hematopoietic stem cell protection in an SAA mouse model<sup>193</sup>. We cannot explain the potential survival benefit of higher CD163/M2 M $\Phi$ s mainly observed in the NSAA group by clinical characteristics, as we did not find any differences in the response to ATG, age, or transplantation rate between the high and low CD163 patient groups.

Transplanted patients had a very good 5-year survival of 87% and GRFS of 69.1%. Reasons for this promising outcome may include the use of ATG in conditioning together with long-term immunosuppression after transplantation. The positive effect of ATG on survival was demonstrated in the EBMT Severe Aplastic Anemia Working Party (SAAWP) study published 2015<sup>135</sup> and the Latin American population-based study, showing a superior event-free survival in the ATG group (79% vs. 61%)<sup>194</sup>. Age greatly influences survival after HSCT<sup>120</sup>. In the EBMT SAAWP study from 2015, negative predictors of survival apart from no ATG in the conditioning regimen included the use of PBSCs, a time lag from diagnosis to transplantation of >180 days, and patient age of >20 years. In our study, patients aged 0–19 and 20–39 years had similar outcomes, whereas patients aged over 40 had worse survival, mostly due to a higher TRM. Survival of older patients could be influenced by a more extended time from diagnosis to transplantation than younger patients; indeed, 4/5 patients >40 years transplanted as first-line treatment were long term survivors. Nevertheless, the outcome for older group appears to be unchanged during recent years<sup>120</sup>.

## 6 Conclusion

Our population-based study included all identifiable patients with AA diagnosed in Sweden between 2000 and 2011 and should reflect the true incidence and survival. We can conclude that age is still a significant risk factor for inferior survival. We found that younger patients, regardless of the initial therapy, experienced a very good long-term survival. However, for patients  $\geq 60$  years, increased mortality was still considerable. In the transplant setting, age  $\geq 40$  years was already associated with unsatisfactory outcomes. Further, we found that only up to 50% of patients responded to ATG treatment, and in particular, the VSAA subgroup had a low possibility of response. Surprisingly, in ATG treated patients, the number of Tregs did not predict the response to treatment, and the number of M2 M $\Phi$ s appeared to have a positive impact on survival, especially in NSAA patients, suggesting that M2 M $\Phi$ s could have possible prognostic importance in AA patients.

In conclusion, the management of older patients with AA should be improved, and combination therapy of ATG with eltrombopag or earlier HSCT for selected patients (especially those with VSAA) may hopefully improve the outcome.

## 7 Future perspectives

Survival after AA diagnosis is now considerably better than 50 years ago. However, older patients and patients with VSAA require a change in their treatment approach. Thrombopoietin (TPO) mimetics, which stimulate the stem cell pool via the c-MPL receptor, are the only available targeted therapy for AA. Eltrombopag has been used as a complementary treatment to IST, and promising results have been reported<sup>186</sup>. Further studies including treatment naïve AA patients are ongoing (NCT01623167, NCT02404025, and NCT04304820). In these studies, combination therapy with different ATG formulations or oral therapy with CsA is being investigated. The TPO-mimetic romiplostim is also being evaluated (NCT03957694, NCT04095936, and NCT02773290) in patients who are refractory and not suitable for IST or as a combination treatment with ATG and CsA. Hopefully, an oral combination of eltrombopag and CsA will be shown to be effective and can be used for older patients in the future.

Another aspect is the direction towards earlier stem cell transplantation. However, as in our patient material, the lack of HLA-matched sibling or unrelated donors, which is the most common cause for repeated IST courses, is one obstacle. Haploidentical transplantation with posttransplant cyclophosphamide has shown promising results with a low incidence of acute and chronic GvHD<sup>195</sup>. In AA, patients who have undergone haploidentical HSCT were mostly children and young adults, with a 1-year survival of 80%–90%<sup>142</sup>. Furthermore, questions on the optimal conditioning and GvHD prophylaxis in older patients remain, and more data should become available before haplotransplantation can be considered as a standard treatment. Nevertheless, haploidentical transplantation appears to be an option for some refractory younger patients, and hopefully, data on older patients will soon be available.

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