

Clinical Trial Protocol

EWOG-MDS RC 06

**TCR V β Repertoire Analysis and PNH Clones
in Children with Refractory Cytopenia (RC)
An open non-randomised multi-center prospective study**

Version

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List of Abbreviations

AA	<i>Aplastic Anemia</i>
ANC	<i>Absolute Neutrophil Count</i>
ATG	<i>Antithymocyte Globuline</i>
BM	<i>Bone Marrow</i>
CBC	<i>Complete Blood Count</i>
CI	<i>Coordinating Investigator</i>
CR	<i>Complete Response</i>
CRF	<i>Case Report Form</i>
CSA	<i>Cyclosporine A</i>
CSC	<i>Coordinating Study Center</i>
DC	<i>Dyskeratosis Congenita</i>
EWOG-MDS	<i>European Working-Group of MDS in Childhood</i>
FA	<i>Fanconi Anemia</i>
FFS	<i>Failure Free Survival</i>
G-CSF	<i>Granulocyte-Colony Stimulating Factor</i>
GPI	<i>Glycophosphatidylinositol</i>
GCP	<i>Good Clinical Practice</i>
HLA	<i>Human Leukocyte Histocompatibility Antigen</i>
HSCT	<i>Hematopoietic Stem Cell Transplantation</i>
IEC	<i>Independent Ethics Committee</i>
ICH-GCP	<i>International Conference on Harmonisation- Good Clinical Practice</i>
IST	<i>Immunosuppressive Therapy</i>
IV	<i>Intravenously</i>
JMML	<i>Juvenile Myelomonocytic Leukemia</i>
mCR	<i>Molecular Complete Resonse</i>
MDS	<i>Myelodysplastic Syndromes</i>
MFD	<i>Matched Familiy Donor</i>
MUD	<i>Matched Unrelated Donor</i>
mPR	<i>Molecular Partial Response</i>
mNR	<i>Molecular Non Response</i>
NR	<i>Non Response</i>

<i>OS</i>	<i>Overall Survival</i>
<i>PB</i>	<i>Peripheral Blood</i>
<i>PCR</i>	<i>Polymerase Chain Reaktion</i>
<i>PNH</i>	<i>Paroxysmal Nocturnal Hemoglobinuria</i>
<i>PNH-sc</i>	<i>Subclinical Paroxysmal Nocturnal Hemoglobinuria</i>
<i>PR</i>	<i>Partial Response</i>
<i>RC</i>	<i>Refractory Cytopenia</i>
<i>SAA</i>	<i>Severe Aplastic Anemia</i>
<i>SC</i>	<i>Subcutaneously</i>
<i>SCT</i>	<i>Stem Cell Transplantation</i>
<i>SD</i>	<i>Standard Deviation</i>
<i>TCR</i>	<i>T-Cell Receptor</i>
<i>WBC</i>	<i>White Blood Cells</i>
<i>WHO</i>	<i>World Health Organisation</i>

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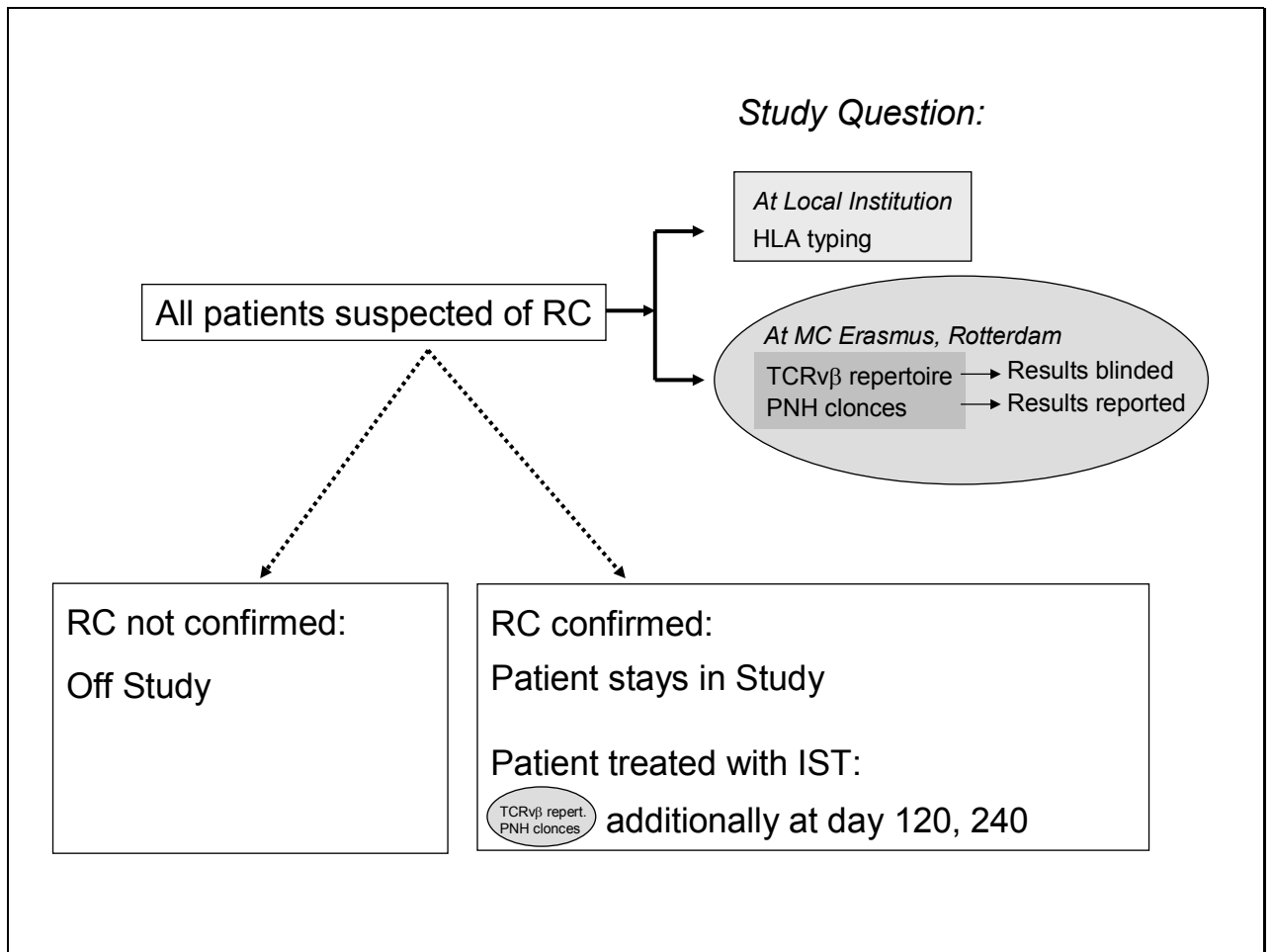
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Synopsis

TITLE OF THE STUDY: <i>TCR Vβ Repertoire and PNH clones in Children with Refractory Cytopenia (RC). An open non-randomised multi-center prospective study</i>
Protocol No.: EWOG-MDS RC 06
Objectives: Primary: <ul style="list-style-type: none">• To evaluate the value of TCR Vβ repertoire analysis for the determination of autoimmunity in RC.• To evaluate which immunophenotypic hematopoietic subclones are associated with oligoclonal T-cell expansion in RC.• To evaluate the presence of PNH clones in RC. Secondary: <ul style="list-style-type: none">• To compare the molecular response with the hematologic response in patients with RC after treatment with immunosuppressive therapy (IST).• To compare the molecular response with HLA expression in patients with RC after treatment with IST.
Design: Prospective open non-randomised study
Planned Study Duration: The total study duration is 5 years. The study ends 12 months after enrollment of the last patient (total study end). Study duration for each patient is a minimum of 12 months (from inclusion) to a maximum of 6 years.
Study Population: A total of 100-125 RC patients are expected to be enrolled.
Inclusion Criteria: <ul style="list-style-type: none">• Written informed consent by the caretakers and whenever possible the patient's assent.• Age: age less than 18 years• All children with RC included in EWOG-MDS 2006
Exclusion Criteria: <ul style="list-style-type: none">• Denied informed consent and/or assent by caretakers/patient• Previous therapy with IST for RC
Endpoints Primary endpoints: <ul style="list-style-type: none">• Number of patients with TCR Vβ oligoclonality at diagnosis• The immunophenotype of patients with oligoclonal T-cell expansion• Number of patients with GPI deficient clones Secondary endpoints: <ul style="list-style-type: none">• Number of patients with molecular response as compared to hematological response after IST• Number of patients with HLA-DR15 antigen expression and molecular response as compared to number of patients with other HLA-DR antigens and molecular response
Methodology: TCR V β repertoire and PNH clone analysis of mononuclear PB and BM cells of RC patients using PCR heteroduplex analysis, and immunophenotyping (flowcytometry)
Statistical Methods: An interim analysis will be performed in the first quarter of 2008. The frequency of PNH clones will be analyzed in all patients enrolled. For patients treated with IST the frequency of PNH clones will be correlated to the response to IST and survival. The final analysis will be conducted in the third quarter 2012. For patients treated with IST the association between PNH clones and TCRV β T-cell oligoclonality and response to IST, failure-free survival (FFS) and overall survival (OS) will be studied

Timetable: Start of Study: 01.09.2006 Enrollment: 60 months End of Study: 31.08.2012 Data available: Third quarter 2012 Study report: End of 2012
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Flow Chart



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2 Introduction

2.1 Studies of the European Working Group of MDS in Childhood

The first prospective diagnostic study of the European Working Group of Myelodysplastic Syndrome (MDS) in Childhood (EWOG-MDS) was initiated in 1998 (EWOG-MDS 98). In 2006, the follow-up study EWOG-MDS 2006 was implemented. EWOG-MDS 2006 is a prospective multi-center study for epidemiology and characterization of MDS and juvenile myelomonocytic leukemia (JMML) in childhood. Patients with MDS of the subtype of refractory cytopenia (RC) will also be enrolled in the study described here, EWOG-MDS RC 06, which will evaluate the implications of oligoclonality of T-cell subtypes and PNH clones. In case of hematopoietic stem cell transplantation (HSCT), patients with RC can also be enrolled in study EWOG-MDS SCT RC RIC 06 or study EWOG-MDS SCT MDS 06. An overview of the current EWOG-MDS studies is given in figure 1.

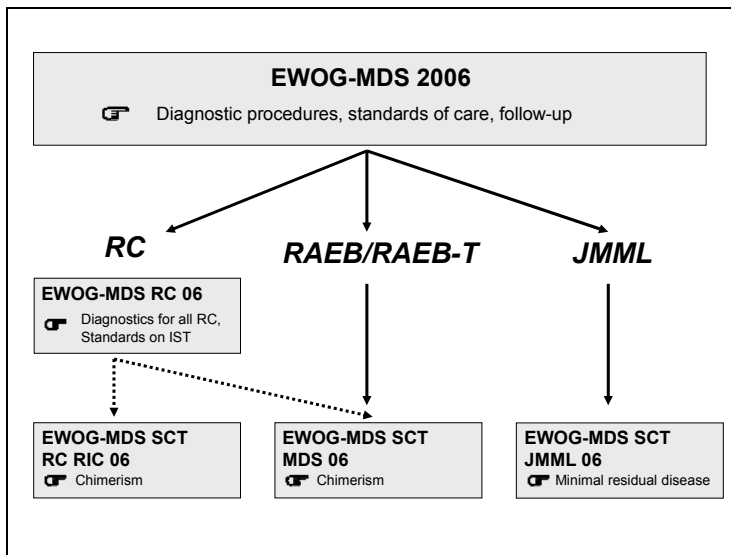


Figure 1: Overview on EWOG-MDS Studies

2.2 Diagnosis of Refractory Cytopenia

RC is a subtype of childhood MDS characterized by less than 2% blasts in the peripheral blood (PB) and less than 5% blasts in the bone marrow (BM) aspirate and biopsy. RC may be difficult to diagnose, because the cellularity is often decreased, cell lines can be absent and dysplasia may be subtle. Moreover, dysplasia can be found frequently in non-MDS conditions like in congenital or acquired BM diseases, but also as a non-specific feature concomitant with infections, metabolic disorders, and deficiencies. Therefore, the differential diagnosis of RC includes severe aplastic anemia (SAA), Fanconi anemia, Shwachman Diamond syndrome, dyskeratosis congenita, Pearson syndrome etc.

Details on the diagnostic procedure and epidemiology of RC are described in the study protocol EWOG-MDS 2006. All patients included in this study are protocol patients of EWOG-MDS 2006.

2.3 Overview on Therapy in Refractory Cytopenia

The therapeutic aim in children with MDS is cure and not palliation. If cytopenia necessitates treatment, current therapy options include HSCT with either myeloablative or reduced intensity preparative therapies. For some patients with hypoplastic RC and normal karyotype or trisomy 8, immunosuppressive therapy (IST) can be an option (see Appendix 1). The current guidelines for therapy for RC as outlined in study EWOG-MDS 2006 are summarized below.

Current guidelines for therapy of RC

Therapy options in RC are dependent on karyotype, peripheral blood counts and bone marrow cellularity.

1. Patients with **monosomy 7, 7q-, or complex karyotypes** should be transplanted soon after the diagnosis is established (generally within 3 months). The recommended preparative regimen is myeloablative consisting of busulfan, cyclophosphamid and melphalan (details see EWOG-MDS SCT MDS 06)
2. Patients with all **other karyotypes** can be followed according to a watch and wait strategy if their ANC is $> 1000/\mu\text{L}$ and there is no need for transfusions.
3. Patients with karyotypes other than monosomy 7 or complex abnormalities who have an ANC $< 1000 / \mu\text{L}$ or are transfusion dependent will require therapy.
 - 3.a. In case of hypocellularity of the BM
 - HSCT with reduced intensity from a sibling or unrelated HLA matched donor (8/8 or 1 allelic disparity) is recommended (details see study EWOG-MDS SCT RC RIC 06).
 - Therapy with IST can be a treatment option for patients with normal karyotype or trisomy 8. For patients on IST who are non responders on day 120, an unrelated donor search is to be initiated
 - In the presence of non-response it is advised to transplant the patient as soon as a suitable donor is identified.
 - 3.b. In case of normocellular or hypercellular BM, the patient is not a candidate for IST, therapy consists of HSCT following a myeloablative preparative regimen (see study EWOG-MDS SCT MDS 06).

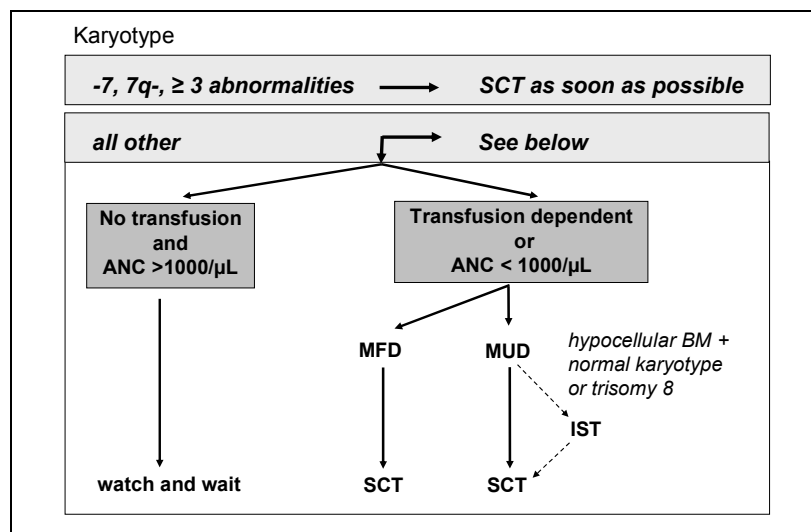


Figure 2: Stratification of therapy in children with RC included in EWOG-MDS 2006

3 Investigational Plan

3.1 Research Questions

Research questions from two areas will be explored.

3.1.1 TCRV β Repertoire Analysis in Refractory Cytopenia

Hypoplastic MDS is very difficult to differentiate from SAA based on histology. Diagnostic tools to investigate the autoimmune character in SAA have become available to study the associated oligoclonality of T-cell subtypes using T-cell receptor V β repertoire analysis. In SAA this provides a patient-specific signature of auto-immunity and these characteristics can be used to monitor molecular response after treatment with IST (1-3). In a pilot study in 35 pediatric SAA and 32 MDS patients we found oligoclonality in 60% of the SAA patients but also in 60% of the RC and advanced MDS patients, suggesting that this may be a valuable diagnostic tool in the future for patients with RC too, specifically for selecting those patients that might benefit from IST and for molecular monitoring of response. Until now, it is not clear which immunophenotype of the malignant clone and/or the involved T-cells are associated with the TCR oligoclonality found in RC patients.

Studies in adults demonstrated that 34% - 50% of MDS patients have clinically relevant responses to IST (4;5). In a pilot study of EWOG-MDS, IST according to the German study SAA 94 was applied [ATG (0.75ml/kg/day for 8 days), prednisolone (1mg/kg/day, day 1-14, then taper till day 28), CSA (5 mg/kg/day for > 6 months), and G-CSF (5 μ g/kg/day depending on neutrophil count)]. In an interim analysis (October 2005), 18 of 29 patients (69%) responded to IST (currently complete response: n=9, partial response: n=10, toxic death n=1). Response was generally observed within 3-6 months. Currently, prospective pediatric studies with TCR V β repertoire analysis in correlation with clinical and molecular response to IST are not available.

Because some autoimmune diseases show HLA restriction, several investigators studied tissue types in AA and found a high frequency of HLA-DR15 in patient cohorts of different ethnic origins. In addition, HLA-DR15 overrepresentation was recently demonstrated in MDS responding to IST (6). There are currently no data on HLA type and response to IST in the pediatric patient population.

3.1.2 Analysis of PNH Clones in Refractory Cytopenia

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by intravascular hemolysis, nocturnal hemoglobinuria, thrombotic events, serious infections and bone marrow failure. This acquired disease, caused by a deficiency of glycosphosphatidylinositol (GPI) anchored proteins (CD16, CD24, CD52, CD55, CD59, CD58, CD66b/67, CD73, CD87, CD90, CD108) on the hematopoietic cells, is rare in children (7-9). The deficiency follows a somatic mutation in the hematopoietic stem cell affecting the *glycosphosphatidylinositol (GPI) complementation class A (PIG-A)* gene. The original name of the disorder is derived from the unusual susceptibility of PNH erythrocytes, caused by deficiency-driven complement-mediated intravascular hemolysis, which results in intermittent hemoglobinuria. Currently PNH is classified in 3 categories, i.e. 1) classical PNH, 2) overt PNH in the setting of a bone marrow failure and 3) alternatively subclinical PNH (PNH-sc) (10). According to this recently published classification by the international PNH Interest Group, patients with classic PNH have clinical evidence of intravascular hemolysis but no evidence of a defined bone marrow abnormality. Patients with class 2 PNH have clinical

and laboratory evidence of hemolysis but also have concomitantly, or have had a history of, a defined underlying marrow abnormality. Bone marrow analysis and cytogenetics are used to determine if PNH arose in association with aplastic anemia, MDS, or other myelopathy (eg, myelofibrosis). Patients with subclinical PNH (PNH-sc) have no clinical or laboratory evidence of hemolysis. Small populations of GPI-anchorage protein–deficient hematopoietic cells (PB erythrocytes, granulocytes, or both) are detected by very sensitive flow cytometric analysis. In childhood about 40 cases with PNH clones have been described so far, which illustrate that most often class 3 PNH is observed, i.e. PNH-sc, in association with bone marrow failure syndromes, particularly aplastic anemia (AA) and RC (8-10).

In most reported SAA and MDS cases PNH clones develop as a late hematologic complication. In the absence of clinical signs, PNH clones can be noted by flowcytometry in 10% - 28% of adults with MDS at diagnosis and during follow-up. These PNH clones are more frequently observed in refractory anemia than in high grade MDS. Prospective cohorts of children with RC have not been studied for the presence of PNH clones.

3.2 Study Design

This is a prospective, non-randomized study to evaluate the TCRV β repertoire and the presence of PNH clones in all children with RC irrespective of karyotype, bone marrow cellularity or therapy intended.

3.3. Time Points for Analysis of TCRV β Repertoire and PNH Clones

All RC patients are eligible for TCRV β repertoire, PNH analysis and HLA typing (molecular typing for HLA A, B, C and DRB1) at diagnosis irrespective of karyotype, peripheral blood counts, BM cellularity or therapy intended.

- Patients with PNH clones will be followed for the persistence of these clones on an individual basis.
- Patients treated with IST will be investigated for TCRV β repertoire analysis at additional time points in order to determine molecular response. Obligatory time points for analysis of TCRV β repertoire are day 120 (BM and PB), day 240 (BM and PB) after initiation of IST.

3.4 Participating Centers

The center of Freiburg, Germany, is the Coordinating Study Center (CSC) for the study EWOG-MDS 2006 and this study. Patients for this study are also study patients of EWOG-MDS 2006. They are recruited in European centers which are located in the following countries: Austria, Belgium, Czech Republic, Denmark, Finland, Germany, Iceland, Italy, the Netherlands, Norway, Poland, Sweden and Switzerland. Centers and the locally responsible investigators are listed in Appendix 3. In every region a EWOG-MDS Regional Coordinator is responsible for forwarding the national data to the CSC.

The EWOG-MDS Regional Coordinators are solely responsible for conducting the study in their country/study group. Data are collected regionally by the Regional Coordinator and transferred on a 3-monthly schedule to the CSC.

3.5 Number of Patients

It is expected that within the next 5 years 100-125 patients will be enrolled in this study. Based on the EWOG-MDS 98 study it can be calculated that 40-50 RC patients will be eligible for IST.

3.6 Central Laboratory

Studies on the TCRV β repertoire, analysis of GPI clones and immunophenotyping will be performed in the Immunology department of the Erasmus MC, Rotterdam, The Netherlands. Therefore the study will be conducted in close collaboration with Dr. A W. Langerak, who will (together with MvdHE) be responsible for the analysis of the TCRV β repertoire analysis and Dr.V.H.J. van der Velden who will (together with MvdHE) be responsible for the immunophenotyping and reporting of real-time results of the PNH screening. The overall accountability, financial responsibility and the coordination of the laboratory study will be conducted by the Pediatric Oncology/Hematology Department of the Erasmus MC-Sophia Children's Hospital laboratory, Rotterdam (Head: Prof. Dr.R. Pieters, Cl.: M.M. van den Heuvel-Eibrink).

4 Study Population

4.1 Study Population

Patients will only be allowed to enter the trial if they or their caretakers provide written informed consent about their participation (following full explanation of the trial) and if the physician has verified that the patient meets all of the Inclusion Criteria and none of the Exclusion Criteria.

4.2 Inclusion Criteria

RC patients enrolled in this study are to meet the following Inclusion Criteria:

- All RC Patients included in the EWOG-MDS 2006 protocol irrespective of therapy
- Written informed consent by the caretakers and whenever possible the patient's assent
- Age less than 18 years

The caretakers will have given their written informed consent to participate in the study. Consent will be documented by the caretaker's dated signature which will be also signed and dated by the investigator in the participating center. If the patient is able to understand the meaning and consequences of the study and its procedures his/her written informed assent is also needed. Written informed consent has to be obtained prior to enrollment into the study.

4.3 Exclusion Criteria

Patients who do not fulfill the Inclusion Criteria may not be included into study. Specific Exclusion Criteria are:

- Denied informed consent and/or assent by caretakers/patient
- Previous therapy with IST for RC

5 Enrollment and Patient Registration

5.1 Time and Mode of Enrollment

It is planned to start enrollment 01.09.2006. Enrollment is planned to be finished 31.08.2011. RC patients included in this study are all registered in the EWOG-MDS 2006 study.

6 Objectives and Endpoints of the Study

6.1 Objectives

Primary objectives:

- To evaluate the value of TCRV β repertoire analysis for the determination of autoimmunity in RC.
- To evaluate which immunophenotypic hematopoietic subclones are associated with oligoclonal T-cell expansion in RC.
- To evaluate the presence of PNH clones in RC.

Secondary objectives:

- To compare the molecular response with the hematologic response in patients with RC after treatment with IST.
- To compare the molecular response with HLA expression in patients with RC after treatment with IST.

6.2 Endpoints

Primary endpoints:

- Number of patients with RC with TCRV β oligoclonality at diagnosis
- The immunophenotype of patients with oligoclonal T-cell expansion in RC
- The number of patients with GPI deficient clones

Secondary endpoints:

- Number of RC patients with molecular response who also reach a hematological response after IST
- Number of patients with HLA-DR15 antigen expression and molecular response as compared to number of patients with other HLA-DRB1 antigens and molecular response

7 Methodology

7.1 Use of Patients' Material

PB, BM aspirates and BM biopsies are retrieved from the patient for diagnostic procedures as described in the diagnostic protocol EWOG-MDS 2006. At the time of the diagnostic procedure, an extra 5 ml of PB and BM for TCRV β repertoire and PNH analysis are obtained of this study. PB for HLA typing (5-20 ml depending on blood counts) can be obtained at any time point during the clinical course with routine blood drawing.

Remaining material may be partially stored for future research purposes, for which consent has to be obtained from the patient/caretakers. Every patient caretaker may state if he wants to be informed about the research results. No patient is undergoing an additional invasive procedure just to gain material for research. It has to be stated that the material will only be used for doing research on the disease and that molecular analysis will only concern the biology of the disease. The research is not commercial. The material will be stored in the Erasmus MC laboratory (see section 3.6). Research performed on this stored material beyond the research questions outlined in this protocol will also require prior consent of the regional coordinators.

7.2 Storage of Patients' Material

According to the diagnostic protocol EWOG-MDS 2006 material from PB and BM will be retrieved with informed consent during the diagnostic process, and thereby from all cases with suspected RC. This assures that material will be obtained before the start of IST or HSCT. The material remaining after analysis of PNH clones will be stored for TCRV β analysis

Molecular response of TCRV β repertoire to IST will be measured in patients treated with IST on day 120 (BM and PB) and day 240 (BM and PB). In patients with PNH clones the influence of IST on the clones will be measured at the same time points (PB only).

For TCRV β and PNH analyses the following material is required:

- 5-10 ml of fresh heparinized PB
- 5 ml of fresh heparinized BM

The BM and PB at will be send fresh to the Department of Immunology, Erasmus MC, Rotterdam (see Appendix 7).

7.3 Method of TCRV β Repertoire Analysis

For TCRV β repertoire analysis at diagnosis lymphocytes will be isolated after Ficoll separation from 5 ml of heparinized PB and BM ($3-6 \times 10^9$ lymphocytes). Immunophenotyping will be performed using specific monoclonal antibodies by flowcytometry (2). Part of the material will be used to isolate RNA (min. 5 μ g) for heteroduplex PCR analysis as described previously (11). In case of treatment with IST, TCR V β repertoire analysis will be performed at diagnosis, and at time of evaluating response (days 120 and 240)(1-3). The results will be collected and blinded from clinical data.

7.4 Flowcytometry for Detection of GPI Deficient Clones and Immunophenotyping

For analyzing GPI deficient clones full blood will be analyzed by phenotyping using flowcytometry. For that purpose CD14, CD16 and CD24 expression will be evaluated in CD45 positive cells. Erythroid cells will be evaluated for CD55 and CD59 expression searching for clear populations with a lack of GPI-linked molecules. In addition, immunophenotyping using flowcytometry will be performed to evaluate which differentiation stages of the major hematopoietic lineages in BM and PB are associated with TCRV β repertoire skewing. Comparison between BM and PB will identify which is the optimal compartment to analyze the responsible hematopoietic clones.

7.5 HLA Typing

All patients will be HLA typed at the local institution. For the purpose of this study serological typing of HLA class I is sufficient, HLA class II will be typed molecularly.

8 Data Handling and Reporting

All children included in this study are registered in EWOG-MDS 2006 (for details on data handling and reporting see study protocol EWOG-MDS 2006).

The CI will inform the treating physician, regional coordinator and the Coordinating Study Center (CSC) via E-mail on incoming samples for the study (date of arrival, material being sent, initials of the patients, date of birth and local center).

The results of the PNH analyses will be sent within 3 weeks by regular mail to the Regional Coordinator and to the EWOG-MDS Study Coordinating Center (CSC) in Freiburg. It is the responsibility of the Regional Coordinator to communicate the results with the treating physician.

The results of the TCRV β analysis and immunophenotyping will be blinded from clinical data and kept at the Erasmus MC. The correlation with clinical data will only be performed after closure of the study. Interim analyses without the correlation to clinical response data are permitted.

The results of the PNH analyses will be added to the EWOG-MDS data base at the Study Coordinating Center (CSC) in Freiburg as soon as the report from the Erasmus MC is available, the results of the TCRV β analyses and immunophenotyping after the completion of the study.

9 Quality Assurance

In the framework of the clinical trial quality-control and quality-assurance will be guaranteed by a data supervision board, a steering committee and an authorized supervision. The details on this procedure as listed in protocol EWOG-MDS 2006.

9.1 Data Supervision Board

The Data Supervision Board consists of all Regional Coordinators and two independent members. Their duty is to supervise the Coordinating Study Center (CSC) at least every two years. Correct data handling from the CRF to the database is their main area of monitoring. They will have access to all study documents including the trial master file and the standard operating procedures.

9.2 Steering Committee

All Regional Coordinators are part of the Steering Committee in their role as coordinating investigators for their countries/ regions. The Committee meets once a year in one of the member countries. Study problems and interim data and analysis are discussed. The Committee guarantees the scientific value and actuality of the study.

9.3 Authorized Supervision

During the course of the study, the study assistant located at the Coordinating Study Center (CSC) will have the duty of an authorized supervisor together with the study coordinator and the coordinating investigator.

The authorized supervisor will stay in regular contact with the study centers to get information about the compliance with the study protocol requirements, consensus of the data in the CRF and the originals, the updated patient identification lists, and the archiving system. The contacts will be mostly done by e-mail and telephone and are supposed to control the progress of the trial, realize problems early and potentially solve them.

The authorized supervisor will review the case report forms of the patients in the study to make certain that the items have been completed and that the data provided are plausible and obtained in the manner specified in the protocol.

The authorized supervisor signs to handle all data that are under professional secrecy or show the patient's identity confidentially and will use the data only for the purpose the patient gave informed consent for. No data disclosing the identity of patients should leave the study center as a result of the monitoring procedure.

9.4 Data Verification

There will not be a source data verification in the sense of controlling the recorded data of the CRFs in regard to correctness and completeness compared to the original data.

There will be an inherent plausibility check contained in the principal data set. If primary data is suspected to be incorrect a query form is going to be generated. The local investigator has to respond to the query and provide written information as soon as possible. The authorized supervisor will also generate written queries if data entered into the data base does not seem plausible. A query trail will document all changes to the data set.

The regional coordinator is responsible for visiting the major national centers on a regular schedule.

9.5 Auditing Procedures

In addition to the quality assurance procedures outlined above, audits can be done in the framework of the auditing system according to the ICH-GCP-guidelines. It can be an inspection initialized by authorities (even after the study has been completed).

In the context of an audit it will be checked if planning, conduction and analysis of a clinical trial are in agreement with the law and the requirements of the ICH-GCP-guidelines.

This includes controlling of the data keeping and organization of the study center as well as controlling of laboratories and the original documents. The aim of auditing is to assure that all results and conclusions written in the final report can be drawn from the raw data.

All persons who are auditing are obliged to sign to handle data that are professional secrecy or show the patient's identity confidentially and use the data only for the purpose the patient gave informed consent for.

The investigator will be informed in time about planned audits. The investigator is required to inform the Coordinating Study Center (CSC) immediately of an inspection requested by a regulatory authority.

10 Trial Design, Definitions and Statistics

10.1 Trial Design

This is an open not randomized multicenter prospective study in children and adolescents below the age of 18 years. The primary and secondary objectives are defined in chapter 6.1.

10.2 Oligoclonality and Molecular Response Definitions

The results of the TCRV β repertoire analysis will be evaluated via a scoring system taking into account the number of TCRV β family primer combinations showing oligoclonality as well as the intensity of expression of skewed TCRV β family combinations in individual patients. A complete molecular response (mCR) is defined as a complete decrease of oligoclonality, resulting in full polyclonality, whereas a partial molecular response (mPR) is defined as any clear decrease in the number and/or intensity of skewed

TCRV β family combinations. No molecular response (mNR) is diagnosed when neither mPR nor mCR criteria are fulfilled.

10.3 Definitions of PNH Clones

PNH clones are defined as (sub)populations of hematopoietic cells that clearly lack surface membrane expression of particular GPI-linked molecules (that are expressed by normal cells). For analyzing GPI deficient clones, PB will be analyzed by flowcytometric immunophenotyping and analysis will be focused on granulocytes, monocytes, and erythrocytes.

10.4 Statistics

An interim analysis will be performed in the first quarter of 2008. It will include an analysis on the frequency of PNH clones and response to IST, overall survival (OS) and failure free survival (FFS).

The final analysis will be conducted in the third quarter 2012. For patients treated with IST the association between PNH clones and TCRV β T-cell oligoclonality and response to IST, FFS and OS will be studied. The relationship between the response rate to IST and the presence of PNH clones or TCRV β T-cell oligoclonality will be studied by calculating chi-square values testing the hypothesis of independence. In the multivariate analyses, logistic regression will be used to evaluate the effect of PNH clones, TCRV β T-cell oligoclonality, and confounding potential risk factors for non response on the response to IST.

Probability of OS and FFS will be analyzed by the Kaplan-Meier method and comparisons between probabilities in different patient groups will be performed using the log-rank test. Death, acquisition of chromosomal aberration, progression to advanced MDS, second course of IST, HSCT, non response (NR) at 6 months, and conversion to NR from partial response (PR) or complete response (CR) ("relapse") are considered treatment failure. All p-values were 2-sided and values less than 0.05 were considered statistically significant.

11 Conditions for Protocol Amendments

11.1 Changes in Protocol

Any change or addition to this protocol requires a written protocol amendment. No change to the protocol may be made without the joint agreement of the Coordinating Investigator, the Study Coordinator and the Regional Coordinators. Any amendment has to be signed by all parties before the change of or addition to the final protocol is effective.

If an amendment significantly affects the safety of the patients, the scope of the investigation or the scientific quality of the study, it should be formally approved by the Ethics Committee, and communicated to the regulatory authority, as required by local law.

After approval, an amendment becomes an integral part of the protocol. All Regional Coordinators will be informed immediately after approval by the Coordinating Study Center (CSC) in Freiburg.

The Coordinating Investigator is authorized to decide the discontinuation of the study due to relevant medical or administrative reasons.

The above-mentioned requirements do not preclude any immediate action taken by the investigator in the interests of the patient's safety. In the case where such an immediate change to the protocol is implemented and the Coordinating Investigator should be notified immediately.

12 Ethical and Legal Considerations

The study will be conducted in accordance with the Declaration of Helsinki (Appendix 4), the current revision of ICH Topic E6 (Appendix 5), Guideline for GCP: "Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95), and the legal requirements of each participating country in its valid version. It is mandatory that all considerations regarding the protection of the patients be carried out in accordance with the Declaration of Helsinki. The data protection will be granted according to the local law.

To ensure compliance the investigator agrees, by written consent to this protocol, to fully cooperate with compliance checks by allowing access to all documentation by authorized individuals.

12.1 Patient Information and Informed Consent

All patients must sign and personally date an approved Informed Consent Form after receiving detailed written and verbal information about the reason, the nature and the methods of the study. The information comprises also information about the patient insurance and the conditions subsequent to this policy. The Informed Consent complies with regulatory requirements.

The written informed consent must be obtained before the entry of the patient into the study!

Furthermore, the patient must be notified that participation is voluntary and that he/she may withdraw from the study at any time and that withdrawal of consent will not affect his/her right to the most appropriate medical treatment or affect the doctor/patient relationship. A written patient information leaflet will be handed to the patient, whose contents have to be discussed with the patient by the treating physician. The investigator will provide the patient ample time and opportunity to inquire about details of the study and to decide whether or not to participate in the study. All questions about the trial will be answered to the satisfaction of the patient. The patient should be given sufficient time to read and understand the statement him/herself before signing his/her consent and dating the document. Neither the investigator nor the trial staff will coerce or unduly influence a patient to participate or to continue to participate in the trial.

Personal information will be treated as strictly confidential and will not be publicly available.

The patient will receive a copy of the written informed consent once signed, and the original version of the informed consent has to be kept in the investigator file.

12.1.1 Patient Withdrawal

A patient may withdraw from the study at any time, at his or her own request, for any reason, specified or unspecified, and without penalty or loss of benefits to which the patient is otherwise entitled. Patients who are withdrawn from the study will not be allowed to re-enter later.

Date of discontinuance, all recorded results at this time and, if known the reasons for discontinuance are to be documented in the CRF. If possible a final examination has to be done.

12.2 Disclosure and Confidentiality

Throughout this study all data will be treated confidentially.

Throughout the whole data-recording and -analysis patients will be identified only by a patient identification number and medication number - never by their full name, initials and date of birth. The legal provisions by the respective Laws will be heeded.

Patient data are only accessible to specialists for scientific research and are used only for purpose of scientific research. Personal data may only be published if the patient has given consent.

The investigator is responsible for keeping sufficient information for every patient (initials, date of birth, internal clinic number, EWOG-patient identification number, gender, informed consent), in order to identify the patient. According to the ICH-GCP-guidelines these documents (Patient Identification List) have to be archived for at least 15 years. This list is on file at the regional coordinating center and at the coordinating study center in Freiburg.

Patients' samples are identified by name.

By conducting this study, the investigator agrees that he and his staff will maintain all information in strict confidence. The professional discretion applies for the study. The investigator is requested to insist on similar confidentiality for this information from other bodies such as the Hospital Scientific Committees and Ethic Committees/Institutional Review Boards that have been consulted by the investigator. Study documents provided by (protocols, CRFs and other material) will be stored appropriately to further ensure their confidentiality. It is understood that the confidential information provided to the investigator will not be disclosed to others without direct written authorization from the patient. Such information will not be communicated by telephone to potential or enrolled patients or to any other individual.

The evaluation of the study will be done by the Coordinating Study Center (CSC) in Freiburg exclusively. Scores, CRFs and all other documents used are the property of the study group and may not be used differently or passed on without permission.

12.3 Independent Ethics Committee (IEC) / Institutional Review Board

Prior to implementation of this study, the protocol, Patient Information Sheet and the proposed Informed Consent must be reviewed and approved by the Ethics Committee. Signed and dated approval by the Ethics Committee must be obtained by prior to study initiation and patient enrollment.

The investigator is committed in accordance with local requirements to inform the IEC of any emergent problem and/or protocol amendments.

12.4 General Disclosure Duty

Before starting the clinical trial authorization from the national authorities has to be obtained. All regional authorities will be informed.

According to the law of participating centers in other countries the corresponding authorities will be informed correctly as well.

12.5 Insurance

The aim of this study is the evaluation of TCR β repertoire and presence of PNH clones in patients with RC and not the investigation of clinical or pharmacological properties of drugs. The study is therefore exempt from clinical trials insurance coverage according to law. Patients are covered by the public liability insurance of their hospitals.

13 Study Documents and Archiving of Records

13.1 Investigator's File

The investigator's file contains all essential and relevant documents (e.g. regulatory and study documents, correspondence with ethics committee and general information). The investigator's file has to be accessible during audits and authorized inspections. After finishing the trial the investigator's file is to be kept within the study center according to the ICH-GCP-guidelines and legal regulations at least for 15 years.

13.2 Documentation of Patient Data

13.2.1 Case Report Form (CRF)

The investigator or his representatives (according to the signature form) document the data currently and continuously on the trial relevant CRF. If possible, documentation should be made immediately. No section of the CRF is to be left blank without an appropriate explanation by the Investigator.

All data have to be entered in an accurate, plausible and complete way by the investigator or a person authorized by him.

13.2.2 Documentation of data in the patient's file

The investigator documents in the patient's file the participation to the study, the frequency of the trial visits, all relevant data of disease, all examinations and diagnostic evaluations and concomitant treatment.

13.2.3 Patient Identification List

For this study, according to the ICH-GCP-guidelines the investigator has to keep a patient identification list which allows an accurate relation of the patient's identity to his/her enrollment into the trial.

The following information will be recorded on the patient identification list:

- Patient's initials
- date of birth
- EWOG-MDS identification number
- gender
- Inclusion and Exclusion Criteria of the study fulfilled yes/no

13.3 Archiving of Records

According to the German law, all investigational records must be retained at the investigational site for a minimum of 15 years. Patient files and other source documents must be kept for the maximum period of time permitted by the hospital/institution, but for not less than 15 years.

Originals of all documentation and copies of outgoing correspondence concerning the study will be stored and retained in a safe area in the Master File at the Coordinating Study Center (CSC) in Freiburg.

14 Administrative Considerations

14.1 Financing

The overall accountability, financial responsibility of this laboratory study will be conducted by the Pediatric Oncology/Hematology department of the Erasmus MC-Sophia Children's Hospital laboratory, Rotterdam (Head: Prof.Dr.R.Pieters, CI: M.M. van den Heuvel-Eibrink). The coordination will be accomplished by the Coordinating Study Center (CSC). The EWOG-MDS study and registration is supported by public grants. There is no support from the industry.

14.2 Final Report

The final report of the trial will be written by the responsible study coordinators together with the Coordinating Study Center (CSC) in Freiburg in collaboration with the Regional Coordinators.

Except for cogent reasons nobody will pass on data to third persons unless all parties agreed upon the analysis and interpretation of the results.

14.3 Publication of Study Results

Any formal presentation or publication of data collected as a direct or indirect result of this trial will be considered as a joint publication by the investigators. It requires the agreement of the Coordinating Investigator and all Regional Coordinators. Authorship will be determined by mutual agreement and based on contribution.

The results of the study may be presented during scientific symposia or published in a scientific journal only after review and written approval by the Coordinating Investigator and all regional coordinators.

Investigators participating in multicenter studies must agree not to engage in presentations based on data gathered individually or by a subgroup of centers before publication of the first main publication, unless this has been agreed otherwise by all other investigators.

Every clinical study should be published to avoid the problem of 'Publication Bias'.

At least within 1 year of termination of the study, a manuscript for publication has to be jointly finalized.

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16 Protocol Approval

Coordinating Investigator



Signature

21.11.2006
Date

Study Coordination



Signature

21.11.2006
Date

Statistician



Signature

21.11.2006
Date

The original of this page is to be filed in the Central Trial Master File.

17 Investigator Statement

Regional Coordinator:

**Trial center and
country:**

COMMITMENTS

By signing this document, I agree to conduct the trial as outlined in the protocol and in accordance with the Declaration of Helsinki (Appendix 4) as well as all applicable government regulations and GCP. I declare:

1. I am well qualified by scientific training and experience to conduct investigational studies in the clinical area of the proposed trial and I am affiliated with a recognized medical school or with an independent institution recognized for its excellence.
2. I shall provide information to all staff members involved in the trial about their obligations as described in this document.
3. I shall submit the protocol, Informed Consent form/Patient Information Sheet and other required documentation to the EC for review and approval.
4. I shall make no changes to the protocol without formal amendment (signed by the coordinating investigator and submitted to the EC for notification/approval), except when necessary to protect the safety, the rights or welfare of patients. In this last case I will inform the coordinating investigator of the change.
5. I shall require Informed Consent from each patient prior to enrollment into the study. The Informed Consent shall be documented by use of a written consent form approved by the national authority and the EC.
6. I shall complete the Study's Case Report Form (CRF) in a timely and legible manner.
7. I shall maintain accurate source records (hospital or other institutional records), which will support the data entered into Case Report Forms and I shall maintain these as specified by the protocol.
8. I shall allow monitoring visits by representatives of the supervising boards as needed.
9. I shall allow any Regulatory Authorities to inspect the facilities and pertinent records at reasonable times and in a manner which ensure patients confidentiality.

Following completion of the study, the data may be considered for reporting at a scientific meeting and/or for publication in a scientific journal. A copy of the manuscript or abstract will be provided to all main regional co-investigators for review before submission to a scientific journal for publication and/or a scientific meeting selection committee for oral or poster presentation.

Investigator Signature

Date

The original of this page is to be filed in the Central Trial Master File. A copy is kept by the Investigator.

Appendix 1 Guidelines for Immunosuppressive Therapy (IST) in Refractory Cytopenia

1. Introduction

Most children with RC have a hypoplastic bone marrow and differentiating RC from aplastic anemia (AA) may be a challenge. In fact, an overlap between acquired AA and RC has been suggested. In patients with AA autoimmunity has been shown to play an important role in pathogenesis. Consequently, IST is currently standard therapy for adult and pediatric patients with severe aplastic anemia (SAA), if a matched family donor is not available. Standard regimens for SAA include a combination of cyclosporin A (CSA) and antithymocyte globuline (ATG). Some studies administer G-CSF depending on the leukocyte count (12-16). IST with 1 or 2 courses has been proven successful in about 60-70% of all cases. In the German study SAA 94 response to IST was observed in 77% of the 114 SAA cases, resulting in an EFS of 54% (17). Recently, in a cohort of 213 pediatric AA patients a CR was reached in 68% in very severe SAA (VSAA) compared to 45% in SAA patients and 5 year survival was respectively 93 % and 81%, indicating that a more severe disease predicted for better outcome in patients treated with IST (18). A Japanese study of 119 children resulted in 71% response (CR+PR) after 6 months (15). Similar results were found in a combined study of 100 adults and pediatric patients showing a response rate (CR+PR) of 77% (12). In a study of 30 adults and children, 77% of the patients who were IST-unresponsive patients, a response was obtained (CR+PR) with rabbit ATG (12).

In 4-10% of SAA cases evolution into MDS within a few years has been reported (5;14;19-21). Concern has been raised that in SAA the addition of G-CSF to IST can increase the risk of evolution to MDS. In contrast, 2 recent reports did not show a difference in clonal evolution for patients treated with or without G-CSF (16;22;23). On the other hand there is some evidence that the number of days of G-CSF administration does seem to increase the risk of disease evolution. In a recent study, 9 of the eleven children who developed MDS/AML had been treated with G-CSF for periods over 1 year of which 6 had received dosages of G-CSF exceeding 10 µg per kg per day (16;22;23). In a study from Kojima *et al.*, 113 children with AA with normal cytogenetics were treated with IST and danazole (with or without G-CSF) 12/113 developed MDS (15). The number of days of G-CSF administration was one of the risk factors for progression in a multivariate analysis. Most recently, IST has been applied in elderly patients with refractory anemia not eligible for HSCT (5;16;19;21;22;24-26). About 35% - 50% of adult RC patients have a favorable response to IST, with the highest response rate noted in patients with a hypoplastic bone marrow, normal karyotype and younger age (27). Shimamoto *et al.* found a drastic hematological response on IST in 60% of adult patients (n=50), i.e. mainly for the erythroid lineage. In the responders TCR repertoire analysis was performed which showed marked skewing TCR patterns (26). Tamayose *et al.* have described disappearance of chromosomal abnormalities and recovery of hematopoiesis after IST, 1 of them had trisomy 8 (28). Also 2 responding patients with AA with trisomy 8 have been reported by Geary *et al* (29).

2. Previous EWOG-MDS Experience on Immunosuppressive Therapy (IST) for Children with Refractory Cytopenia

In a recent pilot study of EWOG-MDS, IST was used for a selected pediatric RC cohort with recent onset pancytopenia, hypocellular BM a normal karyotype. IST was delivered according to the German SAA study SAA 94 [horse-ATG (0.75ml/kg/day for 8 days), prednisolone (1mg/kg/day, day 1-14, then taper till

day 28), CSA (5 mg/kg/day for > 6 months), and G-CSF (5µg/kg/day depending on neutrophil count)]. In an interim analysis (October 2005), 18 of 29 patients (69%) responded to IST (currently complete response: n=9, partial response: n=10, toxic death n=1). Response was generally observed within 3-6 months. There was no obvious correlation between the degree of neutropenia (ANC < 200/µl [n=6], 200 - 500/µl [n=9] or > 500/µl [n=14]) and response to IST. Data on long term follow-up are not yet available, and it is currently unknown whether IST can result in sustained responses in a substantial number of children with RC.

The EWOG-SAA study group was forced to change the previous IST guidelines as horse-ATG production was stopped by Genzyme in the first quarter of 2007. As there are no data available on other ATG products for SAA in children, the group agreed to the use of rabbit ATG (thymoglobuline Genzyme) instead.

3. Description of the Consensus on Immunosuppressive Therapy (IST) for Children with Refractory Cytopenia

It is currently consensus for IST treatment in RC to follow the guidelines for therapy of children with SAA treated with CSA and ATG (Figure 3). Some of guidelines like tapering of CSA dosage are based on the experience in the German SAA study (18).

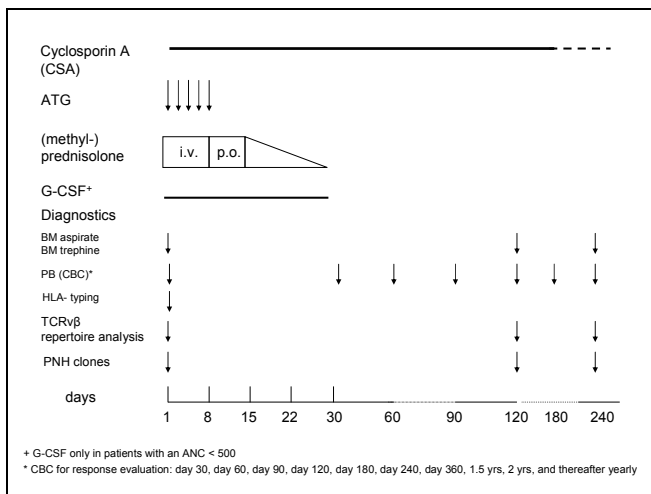


Figure 3: Schedule for treatment with IST and response measurement of IST in RC

+ G-CSF only if indicated (see page 35)

* Complete blood counts (CBCs) to measure response to IST are obtained at days 1, 30, 60, 90, 120, 180, 240, 360, 18 months, 2 years and later yearly after start treatment with IST.

CSA:

Starts on the first day of the ATG administration at 5 mg/kg/day orally. Levels (through) should be aimed to be 200-400 ng/ml (polyclonal assay) or 100-200 ng/ml (monoclonal assay). CSA should be continued until day 180.

In cases of CR at day 180, CSA should be tapered slowly (10% per month) under regular monitoring of blood counts (every other week).

In cases of PR at day 180, CSA should be continued for another 2 months to allow further improvement of blood counts.

If a CR can be reached at day 240, CSA should be tapered as indicated above. If only PR is achieved day 240, further management (continue CSA or proceed to HSCT) will depend on the quality of PR and the availability of an HLA-matched donor. Irrespective of response CSA tapering should be started at latest on day 360.

ATG: Rabbit ATG (Thymoglobuline, Genzyme), 3.75 mg/kg/day in 6-8 hours i.v., day 1-5, administered in 6-8 hours.

(Methyl-)prednisolone: (Methyl-)prednisolone should be added iv or orally to prevent serum sickness, 1 mg/kg/day* starting as a bolus injection 30 minutes prior to the first dose of ATG, this will be repeated for 5 days. Thereafter orally prednisolone (equivalent dose) will be administered in a dose of 1 mg/kg/day divided over 3 dosages, until day 14. In the following 14 days prednisolone will be tapered down by reducing the dose by 50% every 5 days until it stops at day 29.

* An additional dose of 1-2 mg/kg methylprednisolone may be necessary to treat severe adverse reactions during ATG therapy.

G-CSF: **G-CSF only in patients with an ANC < 500.** If indicated an initial dose of 5 µg/kg/day sc or iv (according to manufacturer's directions) will be administered until day 28. If there is a response (ANC > 500/µl) the dose is tapered by giving it every second day, every third day etc. If the WBC decreases again to $0.5 \times 10^9/l$, the original dose of 5 µg/kg should be restarted. G-CSF therapy in these cases should be finished by day 100. If at day 28 there is no response (ANC < 500/µl) the G-CSF dose should not be increased but continued with 5 µg/day for another 32 days. If there again is no response (ANC < 500/µl), G-CSF should be stopped at day 60. In responders tapering should be handled as described above.

Supportive care: Selective decontamination of bowel, trimethoprim-sulfamethoxazol prophylaxis of pneumocystis carinii, antihistaminics as prophylaxis before administration of ATG. In cases transfusions are required, blood products should be irradiated.

4. Consensus for Measurement of Response to IST

Time points:

To evaluate (continuous) hematologic response:

CBC at day 30, 60, 90, 120, 180, 240 and 360, 18 months, 2 years and later yearly after start of IST.

BM aspirate and biopsy at day 120 (4 months), day 240 (8 months) and in case of relapse (= NR following PR or CR)

Definitions of response:

The criteria for response are defined according to the International consensus on evaluation of IST in SAA. The criteria for red cell response were specified according to age adjusted normal ranges and their lower limits (-2SD). The definition of CR, PR and NR is indicated in table 2. The dates of achieving hematological PR and CR following initiation of IST are reported. The date of PR is the date of first CBC indicating PR 4 weeks after the last platelet or red cell transfusion. PR and CR should sustain for a minimum of 3 consecutive blood counts over a period of at least 4 weeks.

Complete remission (CR):	<p>All the criteria below have to be fulfilled:</p> <ul style="list-style-type: none"> • ANC $\geq 1.5 \times 10^9/l$ • Hemoglobin \geq age adjusted cut-off value <ul style="list-style-type: none"> 0.5 -2 years: 10.5 g/dl 2-14 years: 11,5 g/dl 15-18 years: 12 g/dl(girls), 13.0 g/dl(boys) • Platelet count $\geq 150 \times 10^9/l$.
Poor Partial Response (PPR):	<p>All criteria below have to be fulfilled:</p> <ul style="list-style-type: none"> • ANC $\geq 0.5 \times 10^9/l$ • Platelet count 20 - 50 $\times 10^9/l$ • Hemoglobin ≥ 6.0 g/dl • No platelet or red cell transfusion
Good Partial Response (GPR):	<p>All criteria below have to be fulfilled:</p> <ul style="list-style-type: none"> • ANC $\geq 1.0 \times 10^9/l$ • Platelet count $\geq 50 \times 10^9/l$ • Hemoglobin ≥ 6.0 g/dl • No platelet or red cell transfusion
Non-response (NR):	Neither PR nor CR is reached

Table 2. Definitions of hematological response after IST

5. Consensus on Therapy according to Response to IST at Day 120

At day 120 of IST:

- NR: proceed to HSCT

- PR: continue CSA, if relapse or progression to advanced MDS proceed to HSCT
- CR: wait and see, discontinue CSA after day 180 (see 3.)

6. For Patients off Cyclosporine: Consensus on Early Resumption of Therapy with Cyclosporine

In the absence of another cause of decrease in platelet count it is recommended to start CSA again

- Patients with prior CR: as soon as the platelet count falls below $100 \times 10^9/l$, documented in 2 consecutive CBC's within 1-2 weeks.
- Patients with prior GPR: as soon as the counts deteriorate to PPR, documented in 2 consecutive CBC's within 1-2 weeks.

Application of CSA should be limited to a maximum of two years.

7. Consensus on HSCT following IST

- NR day 120
- NR at any time point following PR or CR
- PPR at any time point following CR or GPR in the presence of a 9/10 identical donor (high resolution molecular typing)

Appendix 2 List of Regional Reference Centers

Appendix 3 List of Participating Study Centers

Appendix 4 Declaration of Helsinki

<http://www.wma.net/e/policy/b3.htm>

Appendix 5 ICH-GCP Guidelines

<http://www.ich.org>

- E6 Good Clinical Practice: Consolidated Guideline
- E11 Clinical Investigations of Medicinal Products in the Pediatric Population

Appendix 6 CRFs

Appendix 7 Patient Information/ Informed Consent

Appendix 8 Invoice Forms

Appendix 9

Approval by the Ethics Committee

Appendix 10 Format report form PNH results

Appendix 11 Flow Charts