Reticular Dysgenesis: International Survey on Clinical Presentation, Transplantation and Outcome

Short Title: International Survey on Reticular Dysgenesis

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Compared to other SCID entities patients with RD have an earlier presentation with bacterial rather than opportunistic infections

Myeloablative agents before transplantation ~~are crucial~~ support reliable ~~for long term~~ myeloid engraftment and long term cure in patients with RD

Abstract

Reticular Dysgenesis (RD) is a rare congenital disorder defined clinically by the combination of severe combined immunodeficiency (SCID), agranulocytosis and sensorineural deafness. Mutations in the gene encoding Adenylate Kinase 2 (*AK2*) were identified to cause the disorder. Hematopoietic stem cell transplantation (HSCT) is the only option to cure this otherwise fatal disease. Retrospective data on clinical presentation, genetics, and outcome of HSCT were collected from centers in Europe, Asia and North America for a total of 32 patients born between 1982 and 2011. Age at presentation was less than 4 weeks in 30/32 patients (94%). Grafts originated from mismatched family donors in 17 patients (55%), from matched family donors in 6 (19%) and from unrelated marrow or umbilical cord blood donors in 8 patients (26%). 13 patients received secondary or tertiary transplants. After transplantation 21/31 patients are reported alive at a mean follow up of 7.9 years (range 0.6-23.6 years). All patients who died beyond 6 months after HSCT had persistent or recurrent agranulocytosis due to failure of donor myeloid engraftment. In the absence of conditioning HSCT was ineffective to overcome agranulocytosis, and inclusion of myeloablative components in the conditioning regimens was required to achieve stable lympho-myeloid engraftment.

In comparison to other SCID entities considerable differences were noted regarding age at presentation, onset and type of infectious complications, and the requirement of conditioning prior to HSCT. Although long-term survival is possible in the presence of mixed chimerism, high-level donor myeloid engraftment should be targeted in order to avoid post-transplant neutropenia.

Introduction

In 1959 De Vaal and Seynhaeve[1] suggested the term "reticular dysgenesia" for a condition which they observed in newborn male twins who had neither lymphocytes nor granulocytes in the peripheral blood and who both died within the first week of life from suspected bacterial infections. Lymph nodes, spleen and thymus were devoid of lymphocytes and the bone marrow showed a failure of myeloid maturation with a developmental arrest at the pro-myelocytic stage. With reticular cells being abundantly present in these tissues the authors hypothesized that the disease originated from a failure of a "multipotent primitive reticular cell" to "develop into the mother cells of the myeloid series, or into lymphocytes and monocytes." With a proportion of less than 2%, RD is a very rare SCID entity and since its first description about 20 patients have been reported in small series or in single case reports.[2] Besides the typical combination of $T^-B^-NK^-$ SCID and agranulocytosis, patients with RD were noted to suffer from a profound sensorineural hearing deficit.[3] Early hematopoietic stem cell transplantation (HSCT) was described as the only curative therapeutic option in this condition.[3-6] In 2009, mutations in the gene encoding Adenylate Kinase 2 (*AK2*) were identified as the molecular basis of the disease by two independent groups.[7,8] Adenylate Kinases are phosphotransferases, which are involved in the intracellular energy transport by providing a shuttle system for high energy phosphorylated adenine nucleotides. AK2 is a protein involved in the energy transfer in the mitochondrial intermembrane space which catalyzes the reaction ATP + AMP $\rightleftharpoons$ 2ADP.

Patients with Reticular Dysgenesis (RD) develop life-threatening infections very early in life, usually within the first days after birth. Immediate diagnosis and decisive therapeutic interventions are mandatory to offer a curative option in this otherwise fatal disease.

This survey was performed in order to comprehensively collect data on clinical presentation, transplantation and outcome to create an objective basis for therapeutic decisions in this very rare disease.

Patients and methods

Patient data were collected in an international survey between November 2010 and November 2015. Participating centers were recruited from repeat presentations of the project in annual meetings of the Inborn Errors Working Party (IEWP) of the European Group for Blood and Marrow Transplantation (EBMT) as well as the biannual meetings of the European Society for Immunodeficiency (ESID). In addition, major transplant centers in North America were contacted directly. Criteria to be included in the study were diagnostic findings consistent with severe combined immunodeficiency in combination with primary agranulocytosis. A questionnaire was completed by participating centers. Furthermore, if not already done, sequencing of *AK2* was offered at the Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, Germany. Written informed consent was obtained from all families according to the regulations of local review boards.

Graft failure was defined as a situation in which neutrophil counts remained below 500/µl after transplantation (primary) or fell below this threshold after initial recovery (secondary) irrespective of donor T-cell engraftment. ~~no cells of donor origin could be detected in the recipient. Failure of myeloid engraftment was defined as absence of donor granulocytes (primary) or loss of donor granulocytes after initial recovery (secondary).~~ Re-transplantation included either a further transplant procedure ~~but~~ with conditioning or a change in the donor. A stem cell transfusion from the same donor and without chemotherapy was not defined as repeat transplantation but as boost.

Methods for chimerism analysis included phenotypic (HLA-flow cytometry, red blood cell flow cytometry, XY-FISH analysis) and genetic (STR-analysis of full blood or sorted subpopulations) methods.

Statistical analysis of overall survival was performed with a Kaplan-Meier survival analysis with 95% pointwise confidence intervals. Statistical significance of differences in survival of defined groups was tested with a log-rank test. The significance level was set at 5%.

Results

Clinical presentation before HSCT

We received complete data sets of 32 patients originating from 29 independent families, who were treated in 15 centers in 11 countries in Europe, Asia and North America (table 1). The ratio male/female was 17/15. Premature birth was reported in more than one third of patients with neonatal data available (11/29; 38%) and almost two thirds of patients (18/29; 62%) were found to be small for gestational age.

Three patients with a positive family history of RD were identified with lymphopenia and agranulocytosis at birth. Two of these three patients developed bacterial sepsis in spite of early diagnosis. Bacterial sepsis was the most frequent infectious complication and developed in 17 of the remaining 29 patients (59%), followed by omphalitis (5/29; 17%). The infections present at diagnosis in the remaining patients are given in table 1. One patient (pt 15) developed typical features of Omenn Syndrome (OS) (generalized erythrodermia, lymphadenopathy, diarrhea) and lymphocytosis due to an oligoclonal expansion of T cells at the age of 8 weeks.[9]

Infectious agents were isolated from blood cultures in 9/19 patients presenting with clinical signs of sepsis and included *Staphylococcus aureus* in three patients, Group B Streptococci (GBS), *Staphylococcus epidermidis*, *Escherichia coli*, *Streptococcus viridans*, *Pseudomonas aeruginosa* and *Candida albicans* in one patient respectively. Non-invasive candidiasis was reported in two children. The age at presentation was within the first month in 27/29 patients (93%), and in 20 of these 27 cases it was within the first week of life. The two late presenting patients (pts 6 and 14) were diagnosed at the age of 2.5 months with whooping cough and positive PCR ~~to~~ for *Bordetella pertussis* in the nasopharyngeal aspirate, the other at the age of

1.8 months with a history of recurrent otitis (*Klebsiella pneumoniae* and *Staphylococcus aureus*).

Laboratory findings at presentation

All patients presented with lymphopenia and with persistent agranulocytosis (see table 2 and ~~supplementary~~ figures S1-~~6~~S8). T-cell numbers were below normal limits in all patients.[10] Maternal T cells were detected in 13/23 patients tested (57%) and an exanthema consistent with an allo-reaction was present in 4/13 (31%). NK-cell and B-cell numbers were within the normal range in merely 2/24 and 4/25 patients tested respectively. [11]

In addition to lymphopenia and agranulocytosis, almost half of the patients exhibited other hematological abnormalities. Hemoglobin levels were ~~found~~ below the normal range in 14/32 patients (44%).[12] In one patient (pt 22) an umbilical blood sample was taken for prenatal diagnosis, which revealed a hemoglobin level of 5.8 g/dl and a platelet count of 48,000/µl without clinical signs of anemia. Thrombocytopenia was observed in 14/31 (45%) patients.[13]

Bone marrow aspirates were reported in 26/32 patients and revealed hypoplasia in 9/26 and hyperplasia in 5/26 patients. The most common finding reported in 22/26 patients was an arrest of myeloid differentiation at the promyelocytic stage (figure 1 and table S1). In 9/26 aspirates, dysmorphic lymphopoiesis was described, which in some cases was difficult to distinguish from malignant disease (figure 1).

***AK2* mutations**

For 30 patients originating from 27 independent families 22 different mutations in *AK2* were reported. In two patients no material was available to perform retrospective genetic analysis. Mutations in 14 patients (table 2 and figure 2) have been previously described.[7-9] Deletions (1

to 5000 nucleotides), missense, nonsense and splice-site mutations were detected in *AK2* sequences, leading to *in silico* predictions of single amino acid missense mutations or premature stop codons with truncated proteins.

Of 23 patients with homozygous mutations, 16 reported a consanguineous background while seven did not. As expected, identical homozygous mutations were found in 3 pairs of siblings (pts 2 and 24, pts 1 and 22, pts 9 and 15). Common mutations were detected in patients originating from the same (pts 25 and 31 from Cape Verde; pts 2, 24 and 7 from Turkey; pts 6, 9, 15 from the Arabic peninsula) as well as from different geographical areas (pts 10 and 17; pts 20 and 32).

Transplantation

With the exception of one patient (pt 1), who died before transplantation from Candida sepsis, the other 31 patients underwent HSCT and received a total of 47 transplantations. The mean age at first HSCT was 3.5 months (range 0.5-11.1 months, median 2.4 months). Thirteen patients required a second HSCT, either because of engraftment failure without any donor cells detectable (pts 2, 18, 20, 21, 29) or because of persistence or recurrence of agranulocytosis (pts 6, 9, 11, 16, 19, 26, 30, 31) (table 3). Secondary transplants were performed more frequently in patients who received non-myeloablative conditioning regimens or in those who were transplanted with a T-cell depleted graft (~~table~~ figure 3A and 3B and ~~figures~~ table S2~~9a and 9b~~).

Overall survival (OS) after HSCT was 68% (21/31). Seven deaths occurred within the first 6 months after transplantation (pts 15, 16, 17, 20, 24, 25, 29) and were either related to infections (encephalitis, respiratory infection of unknown origin, pulmonary aspergillosis, systemic adenovirus infection) or, in one patient, to severe acute GvHD in combination with

veno-occlusive disease. Three late deaths at more than 6 months after transplantation occurred in patients (pts 19, 26 and 30) in whom permanent myeloid engraftment had failed in spite of the presence of donor lymphocytes (figure 4, table S2).

Patients transplanted ~~from~~ with T-cell replete grafts ~~HLA-compatible donors~~

OS in patients transplanted with T-cell replete grafts donated from ~~HLA-identical,~~ HLA-~~compatible~~identical and HLA-mismatched donors was 93% (13/14) (~~table 3,~~ figure 3A, figure 4 and ~~ementary figure 9a~~ table S2).

Six patients had an HLA-identical family donor available and all of these survived. One of these achieved long-term cure after two transplant attempts without conditioning. In the other five patients conditioning was used. Busulfan based conditioning was successful in 2/2 patients (pts 4 and 5). Alternative regimens used in three patients (pts 3, 6 and 7) were sufficient to allow long-term engraftment in two of these. Patient 7 developed mixed chimerism after conditioning with treosulfan only but has normal neutrophil counts at 2 years after transplantation. Patient 6 in contrast experienced a gradual recurrence of neutropenia and ~~necessitated~~ required a second transplant, which was successful.

Transplants from unrelated donors were used in 8 patients. Overall survival in this group was 88% (7/8). In six out of eight patients, unrelated cord blood grafts with variable numbers of mismatches were used (table S2~~table 3~~). Busulfan-based conditioning was given to 4 of these with successful long-term engraftment in all of them (pts 10, 12, 13, 14). Two patients received alternative conditioning (pts 9 and 11), without long-term myeloid engraftment. Both developed secondary neutropenia and were successfully retransplanted receiving a busulfan-based regimen after 2.9 years and 7 months respectively.

Two patients were transplanted with bone marrow grafts from unrelated adult donors (pts 8 and 15). Both received a busulfan-based conditioning. One died from veno-occlusive disease and multi-organ failure[9] while the other is well and alive.

Patients transplanted with T-cell depleted grafts from HLA-haploidentical donors

Seventeen patients (55%) were transplanted with T-cell depleted grafts from HLA-haplo-identical donors (pts 16-32). OS in this group was 8/17 (47%). In five patients (pts 16-20) the initial HSCT was performed without conditioning and resulted in primary graft failure in all of them. One patient died after the first transplant and four patients received repeat transplants after conditioning 1.2 to 8.4 months after the first HSCT. Among them, only one patient (pt 18) survived (~~table~~ figure 3B, ~~figure 3~~ and ~~supplementary figure 9b~~ table S2).

In 12 of 17 patients, conditioning was used prior to the initial haploidentical HSCT (pts 21-32 ~~table 3~~). In 11 patients conditioning was based on busulfan, either alone (pt 22) or in combination with cyclophosphamide (pts 23-31) or fludarabine (pt 32). ~~Transplantation following conditioning with busulfan was~~These regimen were successful and led to engraftment and permanent cure in 6/11 patients. Two patients died following this procedure, one of GvHD (pt 24), the other of interstitial pneumonia (pt 25). Three patients required a second transplant because of primary (pt 29) or secondary (pts 26 and 30) graft failure. All three patients died from adenovirus infection or MDS as detailed elsewhere.[14]

Conditioning with cyclophosphamide alone in one patient (pt 21) was not successful but engraftment was achieved with a re-transplantation following conditioning with busulfan and cyclophosphamide 6 weeks later.

GvHD

If GvHD prophylaxis was given, either cyclosporin A or tacrolimus were included in all cases (~~table 3~~table S2). Steroids, methotrexate (MTX) or mycophenolate mofetil (MMF) were given as additional components. Serotherapy prior to HSCT with either ATG or alemtuzumab was used in 16/31 first transplants. Following haploidentical T-cell depleted HSCT, CSA was given to a minority of 4/17 patients (pts 19, 28, 29, 32). The overall incidence of GvHD was 52% (16/31 patients~~) (52%)~~ and developed *de novo* after transplantation in 11/31 patients (35%) and was preexisting due to engraftment of maternal T cells in 4/31 patients (13%). Only five patients (16%) developed aGvHD of grade III or IV. GvHD was the cause of death in only one patient (pt 24).

Lympho-hematopoietic reconstitution and long term follow up

Twenty one (66%) of the 31 patients are ~~reported~~ alive after HSCT (table 3 and table S2) with a mean follow up of 7.9 years (range 6 months to 23.6 years). ~~None of the patients was reported to suffer from~~No serious or life threatening infectious problems were reported after HSCT. ~~One single patient (pt 5) suffers from CNS-disease (seizures and agenesis of the corpus callosum) which seems not to be related to RD, transplantation or infection.~~ Regarding growth and development, 10/17 patients reported remained below the 10th percentile with their body weight, 7/16 patients with their height and 4/16 patients were reported with learning disabilities (table S3). In a cohort with a high incidence of premature birth, infections and repetitive exposure to chemotherapy and the majority (12/16) being reported to have normal development, it is difficult to characterize developmental delay as a component of RD. Mixed chimerism was found in seven patients (pts 2, 5, 7, 14, 23, 27, 28). Variable proportions of autologous cells were detected in the lymphoid, erythroid and/ or myeloid compartment. T cells were of complete donor origin in all but one patient (pt 14 with 4% of autologous T

cells). For neutrophils, complete donor chimerism was reported in all but two patients (pts 5 and 14). Chimerism for B cells was selectively tested in 5 patients (pts 3, 5, 7, 14, 27) and was found mixed in three with autologous proportions ranging from 5% to 67%. Chimerism of CD34+ cells was ~~checked~~ investigated in three patients. Autologous CD34+ cells were detected in two of three patients tested with an autologous proportion of 90% (pt 2) and 20% (pt 28) (table 4). Red-cell chimerism was found mixed in three patients with an autologous proportion of 50-94%.

Absolute neutrophil counts below 1500/µl were found only in patients with mixed chimerism (pts 2, 7, 27). T-cell counts were >1000/µl in all patients except two (pts 27 and 31), explained by a history of GvHD in one and a short follow up of 0.5 years in the other. The percentage of naïve T cells (here defined as CD45RA+) was >30% in 12/13 analyzed cases indicating good thymic function and capacity of *de novo* T-cell formation. Three patients remained on immunoglobulin substitution. All patients tested had positive antibody responses to vaccinations.

Nineteen out of the 21 long term survivors were reported to suffer from persistent hearing deficiencies and were supported by either conventional hearing aids (n=10) or cochlear implants (n=9). For the remaining two patients this information was not available.

Discussion

In this retrospective international study on patients with RD we have identified a number of particular features with regard to the clinical presentation as well as the therapeutic requirements of the disorder, which are distinct from other variants of SCID.

In contrast to other SCID patients who usually do not develop serious infections before 2 months of age,[15] patients with RD present much earlier with a predominance of invasive bacterial infections due to the associated agranulocytosis. Opportunistic infections, such as respiratory failure due to *Pneumocystis jirovecii* or systemic infections with cytomegalovirus, which are typical for other SCID entities, ~~have~~were not ~~been~~ observed in this series of patients.

Another unique observation in this cohort is the high proportion of patients who are born prematurely and and/or small for gestational age. Since AK2 is a ubiquitously expressed protein involved in basic mechanisms of cellular energy supply, this finding suggests that its deficiency may cause fetal stress leading to premature birth.

Moreover, about half of the patients presented with additional hematological abnormalities affecting erythropoiesis and thrombopoiesis. As these were also present in the absence of systemic infection or before birth, primary involvement can be suspected (figures S1 and S2). These findings implicate that the enzyme deficiency may have a broader although variable effect on hematopoiesis hitherto not recognized.

One could suspect that AK2 deficiency remains unrecognized in a proportion of patients dying from severe fulminant bacterial infections in early infancy. RD has to be considered and ruled out in any neonate with unexplained leukopenia. Absent hearing or the typical abnormalities in a bone marrow aspirate as described above can provide ~~be~~ helpful additional ~~hints~~ clues. However, b~~B~~oth ~~though~~ are difficult to perform and potentially

misleading in a seriously sick newborn. Finally, sequencing of *AK2* will confirm the diagnosis. Newborn screening for SCID, which at present is routinely performed in the majority of states in the USA [16], will most probably identify these patients easily due to the absence of T cells and therefore low to absent TREC levels. Some children though might have experienced their first life threatening infection before these results are available and the benefit for this group in comparison to other SCID entities might therefore be lower.

We were able to identify genetic defects in *AK2* in all patients in whom this gene was sequenced (30/32 patients). The mutations included missense, nonsense and splice-site mutations, large and small deletions and affected each of the 7 coding exons. The missense mutation c.524G>A was the most frequently detected genetic change and affected three independent families originating from the Arabic peninsula, which could indicate a higher frequency of this allele and possibly a common founder. In two of these patients the mutation was associated with a leaky phenotype of SCID. One patient (pt 15) presented with an Omenn phenotype and a T-cell count of almost 2000/µl. The second patient with the same mutation presented with a high B-cell number (1850/µl, pt 6), and abundant B-cell precursors in the marrow. This finding was initially misinterpreted as B-cell leukemia but polyclonality was demonstrated (data not shown). The third patient (pt 9) with this same homozygous mutation had “classical” aleukocytosis (350/µl), which indicates that there is no reliable genotype-phenotype correlation.

In contrast to other SCID patients, permanent cure in patients with RD requires the correction of the myeloid lineage in addition to lymphoid reconstitution. According to the experience presented here, stable engraftment of hematopoietic stem cells is a crucial prerequisite to achieve normal myeloid function in patients with RD. Conditioning and alloreactive donor T cells are important factors, which promote stem cell engraftment. Thus, in T-cell depleted

haplo-identical transplantation, donor cell engraftment is dependent on the conditioning regimen. Accordingly, reduced intensity conditioning regimens without a myeloablative agent (n=1, pt 16) or HSCT without any conditioning (n=5; pts 17-21) were not successful in T-cell depleted transplants. Even after conditioning with busulfan only 6/11 patients (55%) engrafted and four of the remaining patients experienced primary or secondary graft failure. In contrast, after conditioning with busulfan no graft failure was reported with T-cell replete grafts. In this latter group engraftment was not reliably achieved with less intense conditioning regimens: three out of four patients needed a second transplant because of either primary or secondary graft failure. Without any conditioning, T-cell replete transplantation led to stable engraftment in a single patient after two attempts with matched sibling graft. The assessment of the effectiveness of busulfan for myeloablation in this study is based on the combination with cyclophosphamide. Whether the combination with fludarabine, which was administered to one patient only, is equally effective in this disease remains unclear. The same applies for busulfan alone without additional components.

This necessity for conditioning was unknown in the early patients reported here, who were mostly transplanted from haplo-identical donors. This fact at least partially contributes to the inferior results of T-cell depleted transplantations as depicted in figure 3. Due to the limited number of patients per group, survival data have to be interpreted very cautiously. In principle~~. Nevertheless,~~ haplo-identical T-cell depleted transplantation ~~in principle~~ makes sense for patients with RD if no well-matched donors are available, particularly utilizing newer haploidentical approaches. None of the patients in this cohort suffered from viral infections before transplant. Other SCID entities most frequently present with T-cell dependent viral or opportunistic infections. For them, rapid T-cell reconstitution is clearly beneficial in order to clear these infections and delayed T-cell reconstitution after haploidentical transplantation is

a major disadvantage. RD patients, who most frequently present with bacterial infections, need rapid neutrophil engraftment to overcome their infections. Neutrophil reconstitution is not delayed after haplo-identical transplantation and haplo-identical parental donors are readily available for the vast majority of patients.

Complete donor chimerism prevents secondary autologous reconstitution, which in some patients causes post-transplant neutropenia. Prolonged stimulation with GCSF may lead to the development of MDS as reported for two patients.[14] In the case of a loss of myeloid engraftment and post-transplant neutropenia, re-transplantation seems inevitable and should be considered early. Why neutropenia develops in some but not all patients with partial autologous reconstitution remains unclear.

In recent publications new insight was gained into the molecular pathophysiology of AK2 deficiency.[17] In a zebrafish model, Rissone et al[18] were able to demonstrate that AK2 deficiency ~~causes an increase in the AMP/ADP ratio as a sign of a severely disturbed cellular energy metabolism which leads to oxidative stress with elevated levels for reactive oxygen species and finally apoptosis of affected cells. In this model~~ equally affects the myeloid lineage as well as hematopoietic stem and precursor cells ~~are equally affected by the loss of function of AK2~~. In an *in vitro* model for proliferation and differentiation of human bone marrow cells, Six et al[19] demonstrate ~~the link between AK2 deficiency and the oxidative phosphorylation required during the differentiation process.~~that ~~l~~lineage negative CD34+ stem cells reside in hypoxic bone marrow niches and are not affected by AK2 deficiency as their energy needs are covered by anaerobic glycolysis.[20] With proliferation and differentiation, cells switch to aerobic metabolism and become dependent on mitochondrial function and AK2. ~~Pannicke et al.[8] describe the presence of AK1 in cell populations such as erythrocytes, platelets and fibroblasts, which are not thought to be affected by the absence~~

~~of AK2 function suggesting that AK1 may compensate for AK2 deficiency. In monocytes, which are present in some patients with AK2 deficiency, Pannicke et al.[8] and Six et al.[19] find AK1 to be absent suggesting that other metabolic pathways are involved in monocyte differentiation.[21]~~

The clinical data from our cohort are mostly in accordance with data generated in the zebrafish model and the *in vitro* assays on human bone marrow. Long-term survivors with stable mixed chimerism in their CD34+ compartment demonstrate that donor cells do not have any selective advantage in the competition with AK2 deficient lineage negative cells. However, these autologous CD34+ cells do not contribute to granulopoiesis as shown for patients 2 and 28 in table 4.

There is some conflicting data regarding hematopoiesis. Rissone et al[18] demonstrate in their zebrafish model that AK2 deficiency affects proliferation and maturation of red cell precursors. This is confirmed by the same group in a human *in vitro* differentiation model with induced pluripotent stem cells (iPSC) originating from a patient with AK2 deficiency, which can only poorly be driven to red cell maturation. While almost half of the patients reported in our cohort (44%) suffer from anemia at presentation (suggesting that AK2 deficiency negatively affects red cell maturation), autologous CD34+ cells seem to significantly contribute to the peripheral red cell pool in long-term survivors with stable mixed chimerism. These conflicting results from animal models, *in vitro* assays and patient data cannot be explained. ~~It has to be considered that f~~Functional ~~data~~ assays on mutated AK2 proteins might be helpful to better explain ~~are currently unavailable. For~~ the initial p~~henotype~~~~resentation~~ as well as ~~for~~ post-transplant chimerism.~~,~~ ~~r~~Residual function of AK2 in autologous cells or even somatic mosaicism due to genetic reversions in hematopoietic lineages could contribute to “leaky” phenotypes. These factors are not considered in current

models. ~~A functional assay for mutated AK2 patient proteins is therefore needed to be able to include genotype-phenotype correlations in these hypotheses.~~

From a clinical point of view, the sensorineural hearing disability has a strong impact on the development of these children after successful HSCT. The molecular pathology leading to this non-hematological manifestation of RD is unclear and animal models were unable to clarify the mechanism. An AK2-deficient mouse model is not yet established, as a complete knock out of AK2 seems to be lethal in early embryonic development (personal communication K. Schwarz). Cochlear implants are chosen by the majority of patients diagnosed and treated in recent years to correct the hearing disability. Older patients get along with conventional non-invasive hearing aids, which indicates some residual hearing capacity.

In summary, this international initiative has put together clinical and genetic data on the largest cohort of patients with RD so far. We demonstrated the necessity for early diagnosis, rapid and decisive therapeutic measures, the superiority of T-cell replete grafts and the need for conditioning to reliably achieve myeloid engraftment (table 4).

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Authorship contributions:

M.H., U.P., K.S. and W.F. designed and performed the study and prepared the initial draft of the manuscript in close cooperation with C.L-P. and M.C.. M.H., C. L-P., U.P., L.D.N., F.P., A.R.G., M.S., M.J.C., P.S., H.Al-M., D.Al-Z., S-Y.P., W.Al-H., H.B.G., P.V., K.O., K.I., H.Y., L.M.N., N.W., P.S.P., K-W.S., H.M., M.Al-H., K-M.D., C.S., D.M., E-M.J., A.S.S., K.S., A.F., W.F.and M.C. were responsible for patient care and/ or provided clinical, genetic or laboratory data. All authors contributed to the discussion and the preparation of the draft, have read and approved the final version of the manuscript.

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Table 1: Clinical presentation of 32 patients with Reticular Dysgenesis

patient number	year of birth	sex	gesta-tional age	weight at birth		age at presen-tation	infection/problem at presentation
			weeks	~~k~~grams	perc.	days	
1	2006	f	31	930	3-10	1	neonatal sepsis (*C.albicans*), died before HSCT
2	1988	f	33	1150	<3	1	neonatal sepsis
3	2011	f	n.r.	n.r.	n.r.	4	diarrhea
4	2008	m	27	1135	75-90	1	neonatal sepsis (*S.epidermidis*)
5	2008	m	37	2270	<3	19	neonatal sepsis (*S.aureus*)
6	2010	m	40	3000	10-25	53	chronic otomastoiditis (*K.~~lebsiella~~ pneumoniae, S.aureus*)
7	2011	m	39	2470	<3	3	neonatal sepsis
8	2005	f	36	2515	25-50	16	fever, weight loss
9	2005	f	37	2400	3-10	1	neonatal sepsis (*S.agalactiae*)
10	2003	m	36	1910	<3	2	mild respiratory distress, abdominal distension
11	2004	f	33	1100	<3	7	neonatal sepsis
12	2006	m	39	2616	<3	7	neonatal sepsis
13	2007	f	39	2900	10-25	5	omphalitis, pneumatosis intestinalis
14	2009	f	37	2000	<3	75	whooping cough (*B.pertussis*)
15	2011	m	39	2430	<3	1*	neonatal sepsis, Omenn syndrome (8 weeks)
16	1982	m	38	2400	<3	11	neonatal sepsis
17	1987	m	42	2550	<3	1	neonatal sepsis, diarrhea, failure to thrive
18	1987	f	40	3684	50-75	1	neonatal sepsis (*S.viridans*), UTI (*C.albicans*)
19	1993	m	31	1870	75-90	1	ascites, hepato-/splenomegaly, cholestatic liver disease
20	1998	f	36	2300	10-25	7	neonatal sepsis (*E.coli*), pneumonitis, omphalitis
21	1993	f	38	1310	<3	19	neonatal sepsis, omphalitis
22	2009	f	36	2000	3-10	1*	neonatal sepsis
23	1986	m	40	2850	3-10	1	neonatal sepsis
24	1991	m	37	2100	<3	1*	no clinical signs or symptoms
25	1995	m	37	1920	<3	1	prenatal anemia, neonatal sepsis (*P.aeruginosa*)
26	1997	f	n.r.	n.r.	n.r.	18	n.r.
27	1999	m	36	3000	50-75	7	neonatal sepsis (*S.aureus*), omphalitis
28	2000	m	31	1500	25-50	1	ileus due to intestinal dilatation (terminal ileum) of unknown origin
29	2000	f	37	1750	<3	3	neonatal sepsis (*S. aureus*)
30	2001	f	37	2770	25-50	14	prenatal anemia
31	2002	m	n.r.	3350	n.r.	7	petechiae/ thrombocytopenia
32	2003	m	40	3165	10-25	8	omphalitis

Abbreviations: m: male; f: female; n.r.: not reported; perc.: percentile; UTI: urinary tract infection; 1*: positive family history with diagnosis immediately after birth.

Table 2: Blood count, immunophenotype, geographic origin, consanguinity and genotype of 32 patients with Reticular Dysgenesis.

patient number	age at blood analysis	hemo-globin	platelet count	neutro-phils	mono-cytes	lympho-cytes	MFT	exan-thema	T cells	B cells	NK cells	geographic origin	parental consang-uinity	Mutation AK2
	days	g/dl	x 10^9/ml	/µl	/µl	/µl			/µl	/µl	/µl			
1	1	3.7	43	0	40	60	+	-	50	6	6	Turkish	+	c.[498+1G>A];[498+1G>A]; p.[?];[?]
2	10	11.5	101	0	15	0	-	-	n.d.	n.d.	n.d.	Turkish	+	c.[453delC];[453delC]; p.[Tyr152Thrfs*12];[Tyr152Thrfs*12]
3	4	13.1	82	0	0	200	n.r.	-	n.d.	n.d.	n.d.	Japanese	n.r.	c.[409C>T];[307C>T]; p.[Arg137*];[Arg103Trp]
4	120	10.7	410	60	40	480	+	+	150	280	60	Arabic	-	c.[524G>C];[524G>C]; p.[Arg175Pro];[Arg175Pro]
5	21	15.6	62	6	30	564	-	-	59	255	288	Italian	n.r.	c.[94-?_219+?del];[94-?_219+?del]; p.[?];[?]
6	53	9.6	806	70	0	900	+	-	233	1842	204	Arabic	+	c.[524G>A];[524G>A]; p.[Arg175Gln];[Arg175Gln]
7	2	11.6	425	0	20	100	+	-	25	54	3	Turkish	+	c.[453del];[453del]; p.[Tyr152Thrfs*12];[Tyr152Thrfs*12]
8	39	11.0	1201	40	7	440	-	-	10	221	69	Italian	n.r.	c.[229G>A];?; p.[Gly100Asp];?
9	133	8.8	38	0	200	80	n.r.	-	n.d.	n.d.	n.d.	Arabic	+	c.[524G>A];[524G>A]; p.[Arg175Gln];[Arg175Gln]
10	2	13.6	150	30	110	390	n.r.	-	19	175	36	Caucasian	-	c.[636_*2601del];[636_*2601del]; p.[Ser213Aspfs*21];[Ser213Aspfs*21]
11	32	10.8	112	40	10	30	-	-	n.d.	n.d.	n.d.	Caucasian	-	c.[633del5kb];[633del5kb]; p.[Lys233*];[Lys233*]
12	1	15.8	304	6	92	102	n.r.	-	13	114	19	Japanese	n.r.	c.[139G>C];[409C>T]; p.[Gly47Arg];[Arg137*]
13	5	14.6	200	13	34	323	+	+	22	310	35	USA, Hispanic	+	c.[25G>T];[25G>T]; p.[Glu9*];[Glu9*]
14	75	8.5	1069	443	65	2790	-	-	404	2495	58	Caucasian	+	c.[1A>T];[1A>T]; p.[?];[?]
15	48	8.0	1111	0	34	3160	-	+	1918	703	36	Arabic	+	c.[524G>A];[524G>A]; p.[Arg175Gln];[Arg175Gln]
16	11	18.1	204	30	10	200	+	+	200	0	0	German	-	n.d.
17	2	17.3	30	42	300	1850	+	-	255	13	0	German	-	c.[636_*2601del];[636_*2601del]; p.[Ser213Aspfs*21];[Ser213Aspfs*21]
18	36	14.9	101	0	0	0	n.r.	-	n.r.	n.r.	n.r.	USA, Hispanic	n.r.	n.d.
19	1	8.7	28	0	10	190	+	-	n.d.	n.d.	n.d.	German	-	c.[118delT];[1A>G]; p.[Cys40Valfs*5];[Met1Val]
20	199	9	23	150	400	700	-	-	100	500	50	Portugese	-	c.[307C>T];[307C>T]; p.[Arg103Trp];[Arg103Trp]
21	19	8.6	330	30	10	40	n.r.	-	0	n.d.	n.d.	USA (white/ non Hispanic)	n.r.	c.[614-615del];[614-615del]; p.[Gly205Aspfs*92];[Gly205Aspfs*92]
22	1	5.8	41	0	0	100	+	-	100	0	0	Turkish	+	c.[498+1G>A];[498+1G>A]; p.[?];[?]
23	1	16.4	104	0	0	500	+	+	100	60	100	German	-	c.[94-2287_219+542del2956];[94-2287_219+542del2956]; p.[Ala32_Leu73del];[Ala32_Leu73del]

24	2	18.3	126	0	12	150	+	-	5	15	8	Turkish	+	c.[453delC];[453delC]; p.[Tyr152Thrfs*12];[Tyr152Thrfs*12]
25	1	8	n.d.	0	200	300	n.r.	-	270	0	17	Cape Verde	+	c.[494A>G];[494A>G]; p.[Asp165Gly];[Asp165Gly]
26	17	15	442	150	550	900	-	-	90	0	90	French	-	c.[556C>T];[94_219del]; p.[Arg186Cys];[Ala32_Leu73del]
27	15	14.9	296	76	45	207	-	-	4	14	33	Italian	+	c.[556C>T];[556C>T]; p.[Arg186Cys];[Arg186Cys]
28	1	15.2	460	85	12	940	+	+	30	22	5	German	+	c.[331-1G>A];[331-1G>A]; p.[?];[?]
29	3	7.5	16	0	400	0	+	-	0	0	0	Caucasian	-	c.[400-401del];[614-615del]; p.[Leu134Alafs*32];[Gly205Aspfs*29]
30	14	10	100	0	150	260	-	n.r.	10	165	8	Turkish	+	c.[473del];[473del]; p.[Pro158Leufs*6];[Pro158Leufs*6]
31	103	15	62	30	555	670	n.r.	n.r.	70	556	53	Cape Verde	+	c.[494A>G];[494A>G]; p.[Asp165Gly];[Asp165Gly]
32	8	14.3	293	100	200	300	n.r.	-	2	60	n.d.	Palestinian	+	c.[307C>T];[307C>T]; p.[Arg103Trp];[Arg103Trp]

Abbreviations: n.d.: not determined; n.r.: not reported; MFT: materno fetal transfusion. Mutations are indicated according to reference sequences NM_013411.4 for *AK2* cDNA sequence (isoform B) and NP_037543 for AK2 protein.

Table 3: Chimerism and immunological reconstitution after transplantation in 21 survivors after HSCT

		CHIMERISM			IMMUNOLOGICAL RECONSTITUTION						
pt	Follow up	Full blood	T cells	other leukocyte subpopulations	neutrophils	T cells	B cells	NK cells	T cells naive	IVIG	specific antibodies
no.	years	% donor	% donor	% donor	/ µl	/ µl	/ µl	/ µl	%		
2	23.6	mixed	100	non-T cells: >90, PMN >95; CD34+: <10; red cells: 6	1148	1270	109	109	34	no	Tet/Diph/HiB/Pneu
3	7.9	n.d.	100	B cells: 100	4100	n.d.	n.d.	n.d.	n.d.	no	n.d.
4	3.0	100	n.d.	CD34+: 100	1760	5350	1110	490	58	no	Tet/Diph/HiB/MMR
5	8.0	n.d.	100	B cells: 90, PMN: 90	2410	1553	263	174	66	no	Tet/Diph/HiB/Pneu/MMR
6	2.7	100	100	PMN: 100	1600	3620	860	240	54	no	Tet/Diph/HiB/Pneu
7	2.1	87	n.d.	n.d.	400	1138	121	67	n.d.	no	n.d.
8	11.0	100	100	B cells: 100, PMN: 100	1780	2487	324	367	68	no	Tet/Diph/HiB/Pneu/MMR
9	4.1	100	n.d.	n.d.	1530	1060	620	70	70	no	Tet/Diph/HiB/Pneu
10	7.4	100	100	PMN: 99	2260	3290	870	786	52	no	Tet/Diph/HiB/Pneu/MMR
11	6.5	100	n.d.	n.d.	2400	3000	1300	807	n.d.	no	Tet/Diph/HiB/Pneu/MMR
12	5.0	100	100	B cells: 100; NK cells: 100, PMN: 100	1776	1520	830	200	n.d.	no	MMR
13	3.1	100	n.d.	n.d.	4563	2084	322	275	66	yes	unknown
14	3.4	n.d.	96	B cells: 33, PMN: 75	2170	1262	177	397	n.d.	yes	n.d.
18	1.1	100	n.d.	n.d.	3000	1580	200	360	n.d.	unknown	n.d.
21	16.6	100	n.d.	n.d.	3700	n.d.	n.d.	n.d.	n.d.	no	Tet/Diph
22	4.4	100	100	n.d.	2110	3410	810	270	51	no	Tet/Diph/HiB/Pneu
23	17.1	100	100	red cells: 14	2960	1930	210	115	39	no	Tet/Diph/HiB/Pneu
27	17.0	n.d.	100	B cells: 95, PMN: 100	900	461	77	13	65	no	Tet/Diph/HiB/Pneu/MMR
28	12.9	mixed	100	CD34+: 80, non-T cells: >95, PMN: 100; red cells: 50	1672	1315	307	68	49	no	Tet/Diph/HiB/Pneu
31	0.6	100	n.d.	n.d.	3946	480	70	50	0	yes	n.d.
32	9.1	100	n.d.	n.d.	3400	2870	350	140	n.d.	no	VZV

Abbreviations: n.d.: not determined; PMN: polymorphonuclear cells; IVIG: intravenous substitution of immunoglobulins; Tet: Tetanus; Diph: Diphtheria; HiB: Hemophilus influenzae Type B; Pneu: Streptococcus pneumoniae; MMR: mumps, measles and rubella; VZV: Varizella zoster virus.

Table 4: Diagnostic and therapeutic recommendations for patients with RD

Diagnosis	Consider RD in any newborn with **unexplained neutropenia**
	Confirmation by • immunophenotyping • hearing test • bone marrow morphology • genetic analysis of *AK2*
Therapy	**Myeloid engraftment** is crucial for permanent cure
	Myeloid engraftment is supported by • conditioning with myeloablative agents • transplantation with T-cell replete transplants
	In case of primary or secondary graft failure, early retransplantation is recommended

Figure Legends

Figure 1: May-Grunwald-Giemsa stain of a bone marrow aspirate from a patient with RD showing immature lymphoid cells (hematogones) with slim cytoplasm as well as myeloblasts and a dysplastic promyelocyte with nuclear and cytoplasm~~at~~ic vacuoles. In the work up of the bone marrow with immunostaining and flow cytometry immature lymphocytes were identified as polyclonal B-cell precursors (flow cytometry of the bone marrow revealed a population of 46% with markers of pre-B cells CD10+/ CD19+/ cyIgM+/ TdT+/ CD34-, clonality was tested in a multiplex PCR for the rearranged BCR heavy chains).

Figure 2: Genomic map of *AK2* isoform B with mutations identified in 29 patients (GenBank RefSeq cDNA for *AK2*, isoform B: NM_013411.4). The numbers in brackets indicate the frequency of the mutations and whether they were identified as part of a homozygous mutation (indicated with *) or a compound heterozygous mutation (indicated with #).

Figure 3A: Tree diagram for 14 patients transplanted with T-cell replete grafts.

Figure 3B: Tree diagram for 17 patients transplanted with T-cell depleted grafts.

Figure 4: Kaplan-Meier survival estimation with 95% pointwise confidence intervals

Figures

Figure 1

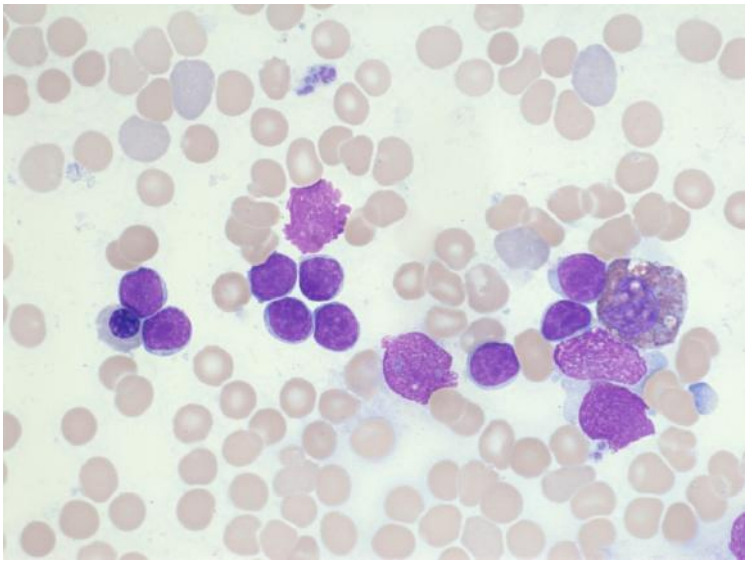


Figure 2

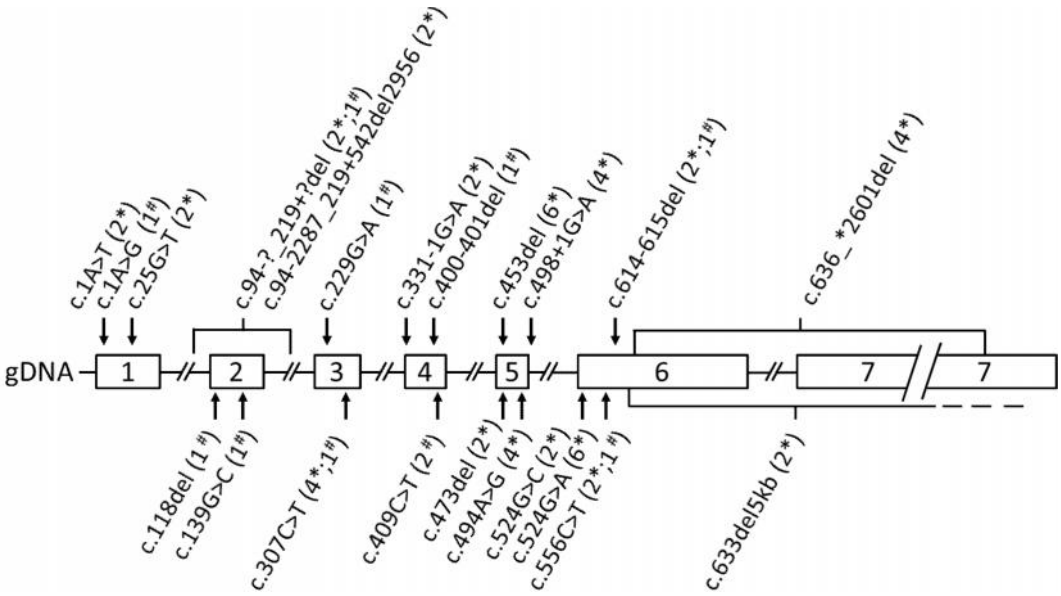


Figure 3A

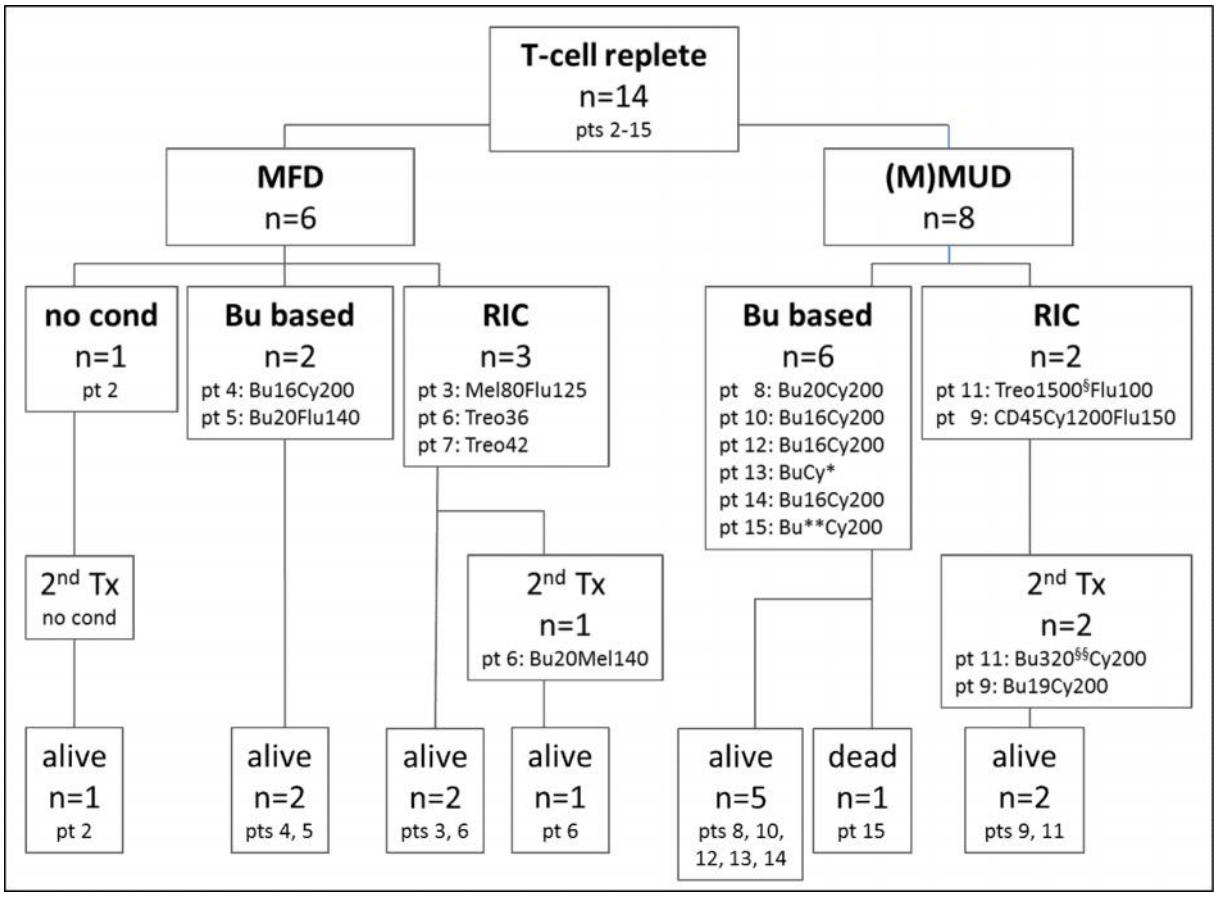
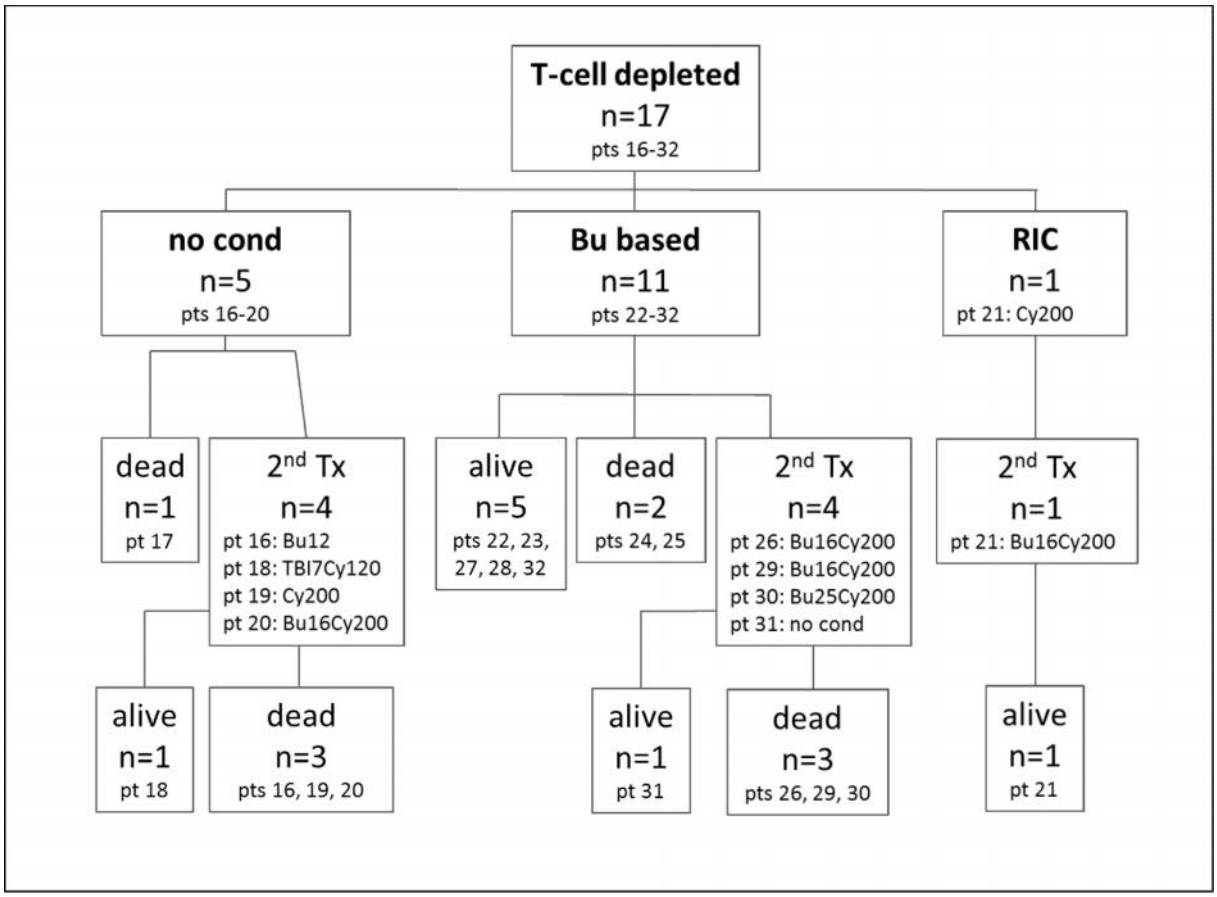
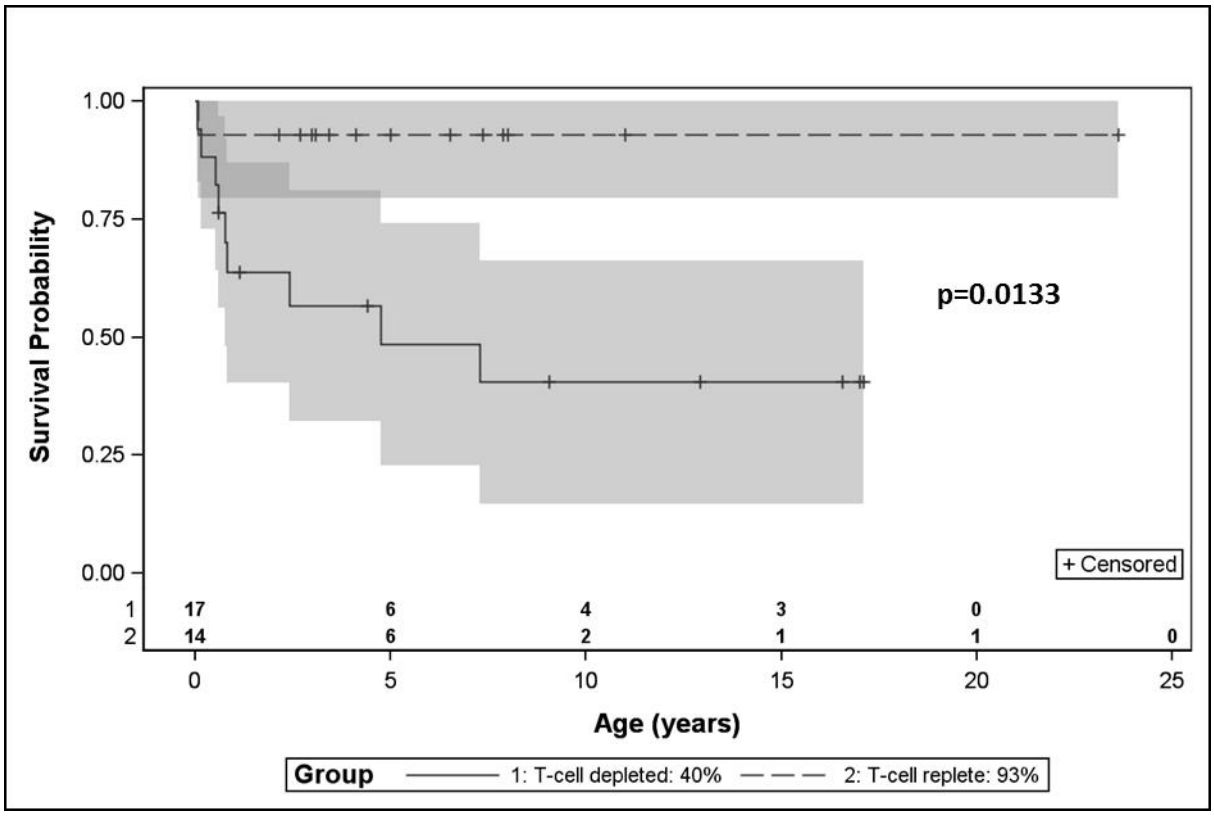

T-cell replete
n=14
pts 2-15
MFD
n=6
(M)MUD
n=8
no cond
n=1
pt 2
Bu based
n=2
pt 4: Bu16Cy200
pt 5: Bu20Flu140
RIC
n=3
pt 3: Mel80Flu125
pt 6: Treo36
pt 7: Treo42
Bu based
n=6
pt 8: Bu20Cy200
pt 10: Bu16Cy200
pt 12: Bu16Cy200
pt 13: BuCy*
pt 14: Bu16Cy200
pt 15: Bu**Cy200
RIC
n=2
pt 11: Treo1500§Flu100
pt 9: CD45Cy1200Flu150
2nd Tx
no cond
2nd Tx
n=1
pt 6: Bu20Mel140
2nd Tx
n=2
pt 11: Bu320§§Cy200
pt 9: Bu19Cy200
alive
n=1
pt 2
alive
n=2
pts 4, 5
alive
n=2
pts 3, 6
alive
n=1
pt 6
alive
n=5
pts 8, 10, 12, 13, 14
dead
n=1
pt 15
alive
n=2
pts 9, 11

Figure 3B



Abbreviations: MFD: matched family donor; MUD: matched unrelated donor; MMUD: mismatched unrelated donor; no cond: no conditioning; Bu based: conditioning regimen contains busulfan; RIC: reduced intensity conditioning; 2^{nd} Tx: second transplant; TBI: total body irradiation dosage given in Gy; CD45: anti-CD45 antibody; Bu: busulfan dosage given in mg/kg; Cy: cyclophosphamide dosage given in mg/kg; Flu: fludarabine dosage given in mg/m^2; Treo: treosulfan dosage given in g/m^2; Mel: melphalan dose given in mg/m^2; **dosage not reported; ***targeted Bu 800-1200 µmol*min/L; §treosulfan dosage given in mg/kg; §§busulfan dosage given in mg/m^2;

Figure 4


1.00
0.75
0.50
0.25
0.00
Survival Probability
p=0.0133
+ Censored
1 17 6 4 3 0
2 14 6 2 1 1 0
0 5 10 15 20 25
Age (years)
Group 1: T-cell depleted: 40% 2: T-cell replete: 93%