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HEMATOPOIETIC STEM CELL TRANSPLANTATION IN CHILDREN WITH NON-MALIGNANT DISORDERS

INDICAZIONI AL TRAPIANTO DI CELLULE STAMINALI EMATOPOIETICHE IN PAZIENTI PEDIATRICI NON ONCOLOGICI

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Abstract

Hematopoietic Stem Cell Transplantation (HSCT) is a curative option for specific non-malignant disorders in childhood, including hemoglobinopathies and primary immune deficiencies. Despite promising results in the recent years, many issues regarding timing of HSCT, donor selection, conditioning regimen and post-transplant care are currently open.

Here, a cohort of 11 patients with Sickle Cell Disease transplanted after a Treosulfan-based conditioning regimen is described showing that this approach is suitable also when alternative donors are employed.

Optimal modalities for HSCT in patients with Hemophagocytic Lymphohistiocytosis (HLH) are investigated through the analysis of outcomes of a 109 transplanted children. We demonstrate that active HLH should not preclude transplantation and that haploidentical HSCT is associated with dismal outcomes.

Finally, data regarding supportive measure and psychological consequences of HSCT in children are presented.

Riassunto

Il trapianto di cellule staminali ematopoietiche (TCSE) è un trattamento curativo per specifiche patologie non oncologiche in età pediatrica, tra cui le immunodeficienze primitive e le emoglobinopatie. Negli ultimi anni, sono stati ottenuti risultati promettenti, ma non è ancora chiaramente definito in quale fase di malattia debba essere effettuato il TCSE, come selezionare il donatore, quale regime di condizionamento e quale terapia di supporto sia più efficace. In questo lavoro, è stata descritta una coorte di 11 pazienti con Drepanocitosi trapiantati impiegando un regime di condizionamento basato sul Treosulfano, dimostrando ottimi risultati anche con l'impiego di donatori alternativi. Sono state analizzate le modalità ottimali del TCSE per pazienti con Linfoistiocitosi Emofagocitica valutando una coorte di 109 pazienti trapiantati in Italia. È stato dimostrato che la malattia attiva al momento del trapianto non preclude il TCSE mentre il trapianto aploidentico è un fattore prognostico sfavorevole. Infine, vengono presentati dati riguardanti le terapie di supporto e le conseguenze psicologiche a lungo termine del TCSE in età pediatrica.

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Introduction

Hematopoietic stem cell transplantation (HSCT) is a curative treatment for malignant and non-malignant disorders but is complex and associated with significant morbidity and mortality.[1] Critical elements include timing of HSCT, donor selection, conditioning regimen and strategies aimed at the prevention of Graft versus Host Disease (GvHD), organ damage and infections. These topics have been addressed by large studies involving patients with malignant disorders (e.g. acute leukemia), but few data are available for patients with non-malignant disorders such as immune deficiencies or hemoglobinopathies.[2] This is due to the relative rarity of these disorders and their heterogeneity which requires specific data for each individual disorder.

The aim of this thesis is to provide further data to optimize the modalities of HSCT for patients with non-malignant disorders, with a particular focus on Sickle Cell Disease (SCD) and Hemophagocytic Lymphohistiocytosis (HLH).

Chapter 1 provides an overview of the rational for HSCT for children with non-malignant disorders. The indications, principles of conditioning regimen and main complications are discussed. The second part of the chapter describes the selection of donor with a focus on strategies employed to manage the risk of GvHD, graft rejection and delayed immune reconstitution after a haploidentical HSCT.

Chapter 2 describes the use of a recently introduced Treosulfan based conditioning regimen in a cohort of 11 patient with SCD transplanted at the Pediatric Hematology-Oncology Clinic, Department of Women's and Children's Health, Padua University Hospital. The second part of the chapter focuses on a single patient for whom both cerebral vasculopathy related to SCD and

autoimmune hepatitis were cured with HSCT, suggesting that occurrence of an autoimmune disorder in a patient with SCD may represent an indication for HSCT.

Chapter 3 describes the outcomes of children with HLH receiving allogeneic Hematopoietic Stem Cell Transplantation in Italy between 2000 and 2014. The analysis of this cohort shows that active disease should not preclude transplantation, which should be performed preferably using either bone marrow or cord blood cells. Since HLA-haploidentical HSCT in patients with HLH is currently associated with unsatisfactory outcomes, innovative approaches are warranted.

Chapter 4 briefly describes other aspects of supportive therapy, quality of life and psychological consequences of HSCT investigated as part of the research contribution of this thesis.

Chapter 1

1.1 Hematopoietic stem cell transplantation for non-malignant disorders

Hematopoietic stem cell transplantation is a curative option for a wide range of acquired and inherited malignant and non-malignant disorders in children and adults.[1] It consists in the infusion of Hematopoietic Stem Cells (HSCs) and progenitor cells aimed at establishing marrow and immune function. The phases of HSCT include:

1. Planning of the procedure and confirmation of the suitability of recipient and donor
2. Conditioning regimen
3. Infusion of hematopoietic stem cells
4. Management of complications
5. Engraftment and immune reconstitution

The HSCs might derive either from the patient itself (autologous HSCT) or from a healthy donor (allogenic HSCT). In children with non-malignant disorders, autologous HSCT is rarely performed and for the scope of this thesis we will focus on allogenic HSCT.

1.1.1 Indications for HSCT

Non-malignant disorders associated with a defect of the HSCs or their progeny are very heterogeneous and comprise primary immune deficiencies, autoimmune diseases, defects of the HSC itself (e.g. inherited bone marrow failure syndrome) and disorders of red blood cells (e.g. hemoglobinopathies), platelets or metabolism.[3] For these conditions, HSCT is a curative option, but

it is associated with a risk of transplant-associated morbidity and mortality. Thus, the selection of the subset of patients eligible for HSCT is a critical point in order to minimize the adverse effects. Eligibility criteria should be determined for every single condition based on the knowledge of the natural history of the disease and the expected benefit of the HSCT. Broadly, conditions to be met for HSCT in non-malignant disorders include clear and confirmed diagnosis, severe phenotype, suitable general condition of the patient and the availability of a donor.[2] For chronic non-malignant disorders for which an alternative treatment is available, also the timing of HSCT is critical. If the HSCT procedure is performed too early, patients with a milder phenotype might be included. Conversely, if HSCT is performed too late, the disease might progress and impede the HSCT.[4] The treatment preceding HSCT should be aimed at reducing the organ damage associated with the underlying disorder and controlling its manifestations in order to reduce the risks associated with HSCT. Finally, indications for HSCT in a specific disorder vary over time as new alternative treatments become available or advances in the HSCT procedure allow to improve the outcomes.[5,6] All these data should be generated and reviewed over time for every single disorder since intrinsic differences in the pathophysiology influence largely the modalities and outcomes of HSCT.

1.1.2 Conditioning regimen

The conditioning regimen acts both on the recipient HSCs and immune system to allow the engraftment of the infused HSCs. The conditioning regimen needs to provide enough myelosuppression to eradicate the recipient HSCs and "create space" within the hematopoietic niche for donor HSC. The second objective is to eliminate the immune response of the recipient in order to prevent the immune-mediated rejection of the graft. These objectives are accomplished by the administration of chemotherapeutic and immuno-modulating agents and/or radiation therapy in the weeks immediately preceding the infusion of the HSCs. In order to clarify the reasons why specific conditioning regimens are currently employed for non-malignant disorders, a historical perspective is useful. The first HSCT were performed to rescue patients from long term bone marrow failure secondary to the accidental exposure to ionizing radiation.[1] Given its cytotoxic effect on the tumor cells, total body irradiation was employed for HSCT in patients with leukemia. Subsequently, agents mimicking the effect of radiation on human cells (e.g. busulfan) were developed and employed for HSCT. When first patients with non-malignant disorders were transplanted, the conditioning regimens were adapted from the established regimens for leukemia patients. In the recent years, development of innovative conditioning regimens were based on the understanding of the specificities of each disorder.[7]

The agents employed in conditioning regimens have a myeloablative and/or immunosuppressive effect, but are also associated with significant toxicities on other organs. This issue is especially

important for patients with non-malignant disorders due to the significant organ damage caused by the underlying disease. To mitigate the regimen related toxicity, reduced intensity conditioning regimens (RIC) have been designed and employed.[8] However, as the toxicity is reduced, also the myeloablative and immunosuppressive potential might be affected, possibly leading to a higher risk of graft rejection or to coexistence of donor and recipient hematopoiesis. As the ultimate goal of HSCT is the cure of the patient more than the establishment of a donor derived hematopoietic system, the presence of recipient-derived HSCs alongside with the donor-derived HSCs might be tolerated as long as the "healthy" cells allow for the remission of the manifestation of the disorder (mixed chimerism).[7,9,10]

The most frequently employed drugs for conditioning in non-malignant disorders include alkylating agents (Busulfan, Treosulfan, Cyclophosphamide, Melphalan and Thiotepa), antimetabolites (Fludarabine) and epipodophyllotoxins (Etoposide). Monoclonal antibodies against lymphocytes such as antithymoglobulin (ATG) or anti-CD52 antibody (Alemtuzumab) are widely employed to prevent graft rejection and GvHD.[11] Historically, the first established conditioning regimen included busulfan, which is associated with significant toxicity and new approaches have been implemented. The unpredictable pharmacokinetics of busulfan was managed with a therapeutic drug monitoring approach which ensures a targeted dose of busulfan for each patient.[12,13] More recently, treosulfan has been reported as a valid alternative to busulfan, due to its more predictable pharmacokinetic profile and the reduced risk of complications.[14,15] Another approach proposed is the use of a less intense conditioning regimen with fludarabine and melphalan which is highly immunosuppressive and less myeloablative. However this approach was associated with a significant risk of rejection and GvHD, especially in patients with sickle cell disease and hemophagocytic lymphohistiocytosis.[16–18]

1.1.3 Complications after HSCT

HSCT is associated with a wide range of adverse events and complications that are responsible for the transplant related morbidity and mortality. A frequent complication limiting the employment of allogenic HSCT is Graft versus Host Disease (GvHD). GvHD arises when the lymphocytes derived from the donor mount an immune response against to the tissues of the recipient which are not recognized as self.[19] The most important proteins responsible for the recognition of the foreign antigens are human leukocyte antigens (HLA), which are expressed on the surface of nucleated cells with a higher frequency on immune cells and on epithelia. GvHD is classically divided in an acute form occurring in the first 100 days after HSCT and involving especially the skin, gut and liver, and a chronic form, occurring later than 100 days post HSCT and involving almost all organs

and tissues. The mainstay of prevention and treatment of GvHD is the use of immunosuppressive agents such as calcineurin inhibitors (Cyclosporine A and Tacrolimus) and steroids.[20]
Other HSCT complications might be due to regimen related toxicity, prolonged aplasia, delayed immune reconstitution or failure of infused HSCs. Early post HSCT complications include infections and regimen related toxicity involving all major organs. Infections occurring post transplant are frequent and diverse, including also agents that cause disease only in the immunocompromised host. Frequent etiological agents include bacteria (both Gram-positive and Gram-negative), fungi (e.g. *Aspergillus* spp and *Candida* spp) and virus (e.g. EBV, CMV, Adenovirus).[21] Regimen related toxicity frequently include risk of bleeding, damage of the mucosae (mucositis), liver and renal impairment, and more rare manifestation such as veno-occlusive disease, thrombotic microangiopathy, cardiac or neurological toxicity.[2] Late effects post HSCT are defined as occurring more than 3 months after HSCT and provoking health restriction for long term survivor. The presence of a late effect might impair the quality of life of the patient, hampering the complete cure of the disease. Almost all organs and systems can be affected depending on the type of transplant and drug employed. The most often observed late effects include ocular disorders (cataracts), thyroid dysfunction, sterility and metabolic disorders.[22]

1.2 Donor choice and Haploidentical Stem Cell Transplantation

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The selection of the donor for allogenic HSCT is an important step in the preparation of the transplant. The most important factor for the donor choice is the compatibility between donor and recipient in order to reduce the risk of graft rejection and GvHD. The donor-recipient compatibility is genetically determined and requires the matching for a very polymorphic locus which encodes for proteins known as human leucocyte antigen (HLA). Also other loci (minor histocompatibility antigens) have a role in the risk for rejection and GvHD, but are not usually taken into consideration when a donor is selected. The preferred donor for HSCT is a healthy HLA-matched sibling, who shares not only the same alleles encoding for HLA but is also more likely to share other minor histocompatibility antigens. Unfortunately, less than one-third of patients in need for HSCT has an HLA-matched sibling donor. Alternative donors such as matched unrelated matched (MUD) or mismatched cord blood units, or three-loci mismatched haploidentical family donors are increasingly used as sources of hematopoietic stem cells. The probability of identifying a MUD in worldwide donor registries is dependent on the diversity of HLA antigens within a population and on the ethnical background of the patient. In addition, a MUD donor search is time-consuming and the time from start of a donor search to the actual transplant is around four to six months. A significant number of patients will experience disease progression or even die within the time of donor search, and the potential life-saving allogeneic HSCT will no longer be a therapeutic option. HLA-haploidentical donors share with the patient only one allele containing the HLA loci and are available for nearly every individual. The haploidentical donor could be any healthy parent, approximately half of all siblings and potentially even more distant relatives with a shared haplotype. In pediatric patients the parents are usually employed, but haploidentical relatives such as aunts or uncles can also be chosen in the rare cases when no parental donor is available or the parent is medically unfit for donation.

1.2.1 HLA complex and HLA-haploidentical transplantation

The HLA locus encodes proteins responsible for cell surface antigen presentation. There are two main classes of HLA proteins: class I (A, B and C locus) and class II (DP, DQ and DR locus). HLA class I genes are constitutively expressed by most cell types. HLA class I molecules present intracellular peptides that are processed in proteasomes and direct CD8+ cytotoxic T-cells towards the elimination of infected cells or cells expressing aberrant peptides. HLA class II genes are constitutively expressed on hematopoietic cells involved in antigen presentation. Their molecules present peptides derived from the fusion of endocytic vesicles with lysosomes and direct CD4+ T cells towards extracellular epitopes. HLA molecules are expressed on every cell and can elicit a strong immune response. Therefore host HLA molecules not present in the donor are a major determinant of the graft-versus-host reaction. Conversely, alloreactivity occurring in the opposite direction is responsible of graft rejection.

The multigene HLA family has at least 12 polymorphic loci with a strong linkage disequilibrium among the alleles. DNA-amplification methods for typing class I and II HLA alleles have confirmed the extensive polymorphism of the HLA system initially documented in serologic studies and have defined alleles that could be identified serologically. In the late 1990s, the Japan Marrow Donor Program (JMDP) demonstrated for the first time the effect on acute GvHD of matching not only HLA class I antigens (serologically) but also alleles (gene sequencing) and the importance of HLA-A and -B allele matching for survival.[23] Analysis of a large cohort in the United States also indicated that HLA allele mismatching is a significant risk factor for severe acute GVHD and mortality.[24] Subsequent extensive analysis of the JMDP, US National Marrow Donor Program (NMDP), European registries and the International Histocompatibility Workshop Group (IHWG) provided strong evidence that HLA allele compatibility, HLA haplotype, HLA epitope and amino acid substitution at peptide-binding pockets of HLA class I molecules are also significantly associated with clinical outcomes such as severe GvHD and mortality.[25–38]

In the setting of haploidentical HSCT, only one of the two HLA-haplotypes is shared: the unshared haplotype encodes allogeneic HLA molecules that strongly activate the immune system leading to graft rejection or GvHD.

1.2.2 Graft Manipulation Strategies

Until 1990s, haploidentical HSCT was performed only in extreme clinical conditions due to high incidence of graft rejection or severe GvHD. These events are mediated by T cells that recognize major class I or II HLA disparities between donor and recipient. To overcome these issues, strategies directed towards the modulation of T cell response have been developed. Two main approaches have been proposed and gained approval: *ex-vivo* T-cell depletion (TCD) with

"megadose" CD34+ cells, and T-cell replete unmanipulated grafts with novel immunosuppression strategies for GvHD prophylaxis.

1.2.2.1 T-cell depletion with "megadose" CD34+ grafts

Since 1976, many techniques were developed to remove T cells from the graft before infusion. Soybean agglutinin and erythrocyte-rosetting with sheep erythrocytes was first used at the Memorian Sloan Kattering Cancer Center. This method can separate T cells from B cells and hematopoietic stem cells. No lethal GvHD was observed in murine models and in the clinical setting this procedure resulted in sustained engraftment without GvHD.[39–41] Unfortunately, anti-donor T-cells in the host could destroy the infused cell product resulting in graft rejection in nearly 20% of the cases.[42–47]

A novel transplantation platform was created in Perugia in order to improve engraftment following a T cell depleted haploidentical transplantation.[48] Recipient T cells were destroyed by the intensification of conditioning regimen, adding alkylating agents or Thiotepa, total body irradiation (TBI) or booster splenic irradiation, and anti-thymocyte globulin (ATG).[49–53] Secondly, a very high stem cell dose ("megadose") obtained using a combination of donor bone marrow and GCSF-mobilized peripheral blood stem cells (PBSCs) resulted in a "veto effect": the large number of CD34+ cells directly inhibited T-cell alloreactivity.[50,54–56] Early clinical results in patients affected by acute leukemia demonstrated that intensive immunosuppressive conditioning with a T-Cell depleted "megadose" allograft could reduce the rates of graft failure and GvHD.[57,58] The complete T cell-depletion in the graft eliminated also the need of post transplant immunosuppression as GvHD prophylaxis.

In the following years other approaches have been proposed. The use of positive selection of CD34 positive cells from PBSCs showed a low rate of graft failure and acute and chronic GvHD. However, there was a significant delay of the immune reconstitution, especially for CD4+ T cells. This caused a 40% non-relapse mortality (NRM), with 65-71% of deaths due to infections.[58,59]

To overcome the high mortality due to infection in patients receiving haploidentical grafts containing only highly purified CD34+ cells, more sophisticated graft manipulation strategies were envisaged. Based on the capability of monoclonal antibodies to discriminate among different population of immune cells, new platforms incorporate the possibility to infuse only selected subsets of B and T cells. The aim is to obtain the "perfect graft" which would lead to no GvHD while preserving the capabilities of the infused cells to respond to infectious agents, kill malignant cells and destroy residual host T cells.

The first experience was based on eliminating from the graft CD3+ T cells (comprising T helper and T cytotoxic cells) and CD19+ B cells. B cell depletion was done in order to reduce both the risk

of post-transplant EBV-related lymphoprolipherative disease and the incidence of chronic GvHD. Compared to the CD34+ positive selection, this approach allowed to infuse in the host a larger number of myeloid cells that could control infection after the transplant. The obtained results were promising but overall EFS remained about 50-60%. The main reason is that this platform was mainly offered to high risk patients after the second relapse or with refractory malignant disease.[60,61]

Further technological advancement came from the knowledge about how different subpopulation of immune cells could modulate the occurrence of GvHD, post-transplant infections and the destruction of malignant cells. In particular, TCRαβ positive T cells are implicated in the GvHD pathogenesis while TCRγδ positive T cells do not mediate any immune response against the host, protect from infections and destroy leukemic cells.[62] This observation led to the development of a transplantation platform in which TCRαβ positive and CD19+ cells are depleted from the graft.[63] The results of this technique are extremely promising showing a disease free survival above 80% in patient with non-malignant disease, similar to matched unrelated donor transplantation.[64,65] Recently data regarding children with advanced hematological malignancies have confirmed these excellent results.[66]

1.2.2.2 T-cell replete unmanipulated grafts with novel immunosuppression strategies for GvHD prophylaxis

Graft manipulation techniques are costly and require adequate facility and expertise to be performed. The observation that proliferating alloreactive T cells are more sensible to high dose cyclophosphamide than stem cells, resting T cells or T regulatory cells has prompted the use of this drug for the prevention of GvHD and graft rejection.[67] Luznik and the Baltimore group have designed a protocol in which high dose cyclophosphamide (50 mg/kg) is given on day 3 and day 4 after infusion of unmanipulated haploidentical bone marrow cells. In addition, patients receive a two drug GvHD prophylaxis (Mycophenolate and Cyclosporine) and G-CSF. Luznik et al. demonstrated that this approach is feasible and efficacious in the preclinical model, in patients with hematologic malignancies and in patients with non-malignant disorders.[68–70] Furthermore, the wide availability of cyclophosphamide and the absence of stem cell manipulation renders this protocol applicable in many low resource countries.[71] Usually this type of haploidentical transplant includes a reduced intensity conditioning and this could explain why it has been mainly used in adults, even above 65 years of age. A recent large prospective trial comparing double umbilical cord blood transplant with this type of haploidentical transplant has shown comparable result between the two platforms. For haploidentical transplantation, the incidence of grade II-IV acute GvHD was 32% and the primary graft failure rate was as low as 4%.[72]

1.2.3 Eligibility and advantages of haploidentical transplantation

Traditionally, haploidentical HSCT was reserved only for high risk patients with no other curative options like relapsed or refractory hematopoietic malignancy or severe congenital immunodeficiency (SCID), in which the high mortality makes transplant an "urgent" procedure. Since the introduction of innovative techniques in the field of haploidentical transplantation, transplant related mortality is comparable with other alternative stem cell sources such as cord blood or matched unrelated donor.[66]

Haploidentical HSCT has some biological and practical aspects that could make it preferable to other alternative stem cell sources:

1. Immediate availability of the donor at the time of transplant: haploidentical donor is immediately available for the patients in urgent need (such as high risk leukemia or severely compromised SCID patients)
2. Possibility to collect a large number of donor cells, either for a "megadose" transplant or for stem cell manipulation
3. Anti-infectious and anti-tumor activity mediated by alloreactive NK or γδT cells, if specific manipulation strategies are employed
4. Prompt donor availability after the HSCT to collect or generate additional cells and enhance antitumor effects or improve immune reconstitution.

1.2.4 Conclusion

HLA-haploidentical transplantation has historically been associated with poor outcomes, owing to high rates of graft failure and graft-versus-host disease (GVHD). In more recent years, several transplantation platforms have been developed in order to overcome these barriers, with different results. No standard-of-care currently exists and randomized prospective studies are warranted to compare these different techniques. The haploidentical donor as a source for stem cells needs also to be compared with other alternative cell source like umbilical cord blood or matched unrelated donor. These comparisons may yield different results in settings differing in the proportion of malignant vs non-malignant disorders, experience of the treating center or resource availability.

Chapter 2

2.1 Treosulfan-Based Conditioning Regimen for Sibling and Alternative Donor HSCT for Children with Sickle Cell Disease.

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Abstract

Background and objectives Lack of suitable donors and regimen related toxicity are major barriers for hematopoietic stem cell transplantation in patients with sickle cell disease. The aim of the study is the assessment of efficacy and toxicity of Treosulfan-based conditioning regimen for SCD also when alternative donors such as mismatched unrelated donor and haploidentical donor are employed.

Methods We report our single-center experience: 11 patients with sickle cell disease received HSCT with a Treosulfan/Thiotepa/Fludarabine/Anti-thymoglobulin conditioning regimen between 2010 and 2015. The donor was a matched sibling donor (n= 7), a haploidentical parent (n= 2), a matched unrelated donor (n= 1) or a mismatched unrelated donor (n=1). The haploidentical and mismatched unrelated donor grafts were manipulated by removing TCRαβ and CD19 positive cells.

Results All patients survived the procedure and achieved stable engraftment. Stable mixed chimerism but no SCD manifestation was observed in 5/11 patients. Grade III-IV regimen related toxicity was limited to mucositis and no grade III-IV graft-versus-host disease (GvHD) was observed. Organ function evaluation showed no pulmonary, cardiac or renal toxicity; cerebral vasculopathy improved in 3/5 evaluable patients. Gonadal failure was observed in 1/4 evaluable patients.

Conclusion Our data suggest that Treosulfan is associated with low toxicity and may be employed also for unrelated and haploidentical donor HSCT.

2.1.1 Introduction

Sickle cell disease is the most frequent haemoglobinopathy worldwide. A point mutation in the beta-globulin gene alters the hemoglobin structure and results in chronic hemolytic anemia and increased blood viscosity. This leads to an heterogeneous phenotypic spectrum: increased morbidity and mortality is due to a higher susceptibility to infections, intermittent vaso-occlusive events and ischemic tissue injury with progressive organ dysfunction.[73] Hematopoietic stem cell transplantation is the most consolidated curative treatment.[74] When considering HSCT for SCD patients, expected SCD morbidity should be balanced against the risk of transplant related mortality and morbidity, keeping in mind that SCD is a non-neoplastic disease with a life expectancy of over 50 years with contemporary treatment.[75]

More than 200 patients transplanted with a matched sibling donor (MSD) after a myeloablative conditioning regimen based on Busulfan and Cyclophosphamide. The limitations associated with this strategy are the significant regimen related toxicity and the risk of graft failure. The transplant associated mortality is around 2-8% and graft failure is observed in around 10-15% patients.[76–83] Although better outcomes have been recently reported with the use of targeted Busulfan therapy, the use of Busulfan-based conditioning regimen is established only in the setting of MSD transplant but only few SCD patients in need of a HSCT have a matched sibling available.[84–87] The use of alternative donors such as matched unrelated donor (MUD), mismatched unrelated donor (MMUD), unrelated umbilical cord blood (UCB) and haploidentical family members is associated with a higher mortality and morbidity, due to pre-existing organ dysfunction, alloimmunisation and risk of GvHD.[74,88] Moreover, suitable matched unrelated donors are difficult to find and experience is limited for umbilical cord blood or haploidentical donor HSCT.[89–93] In order to overcome the limits of the Busulfan-based conditioning regimen and allow the use of alternative donors, different conditioning strategies have been proposed.[17,94] Recently, Treosulfan became attractive to substitute Busulfan due to its lower toxicity and good immune suppressive and myeloablative potential.[14,95–98] This led to the use of Treosulfan in patients with pre-existent morbidity (mainly primary immune deficiency or β-thalassemia) or receiving a second transplantation for malignant disorders.[95,96,99–105] For these reasons, since 2010 at our institution Busulfan was substituted with Treosulfan as standard conditioning regimen for SCD patients.

We present our single-Centre experience of HSCT performed for SCD patients employing Treosulfan-based conditioning regimen also in haploidentical and MUD HSCT .

2.1.2 Methods

From April 2010 to December 2015, all SCD patients undergoing HSCT received a Treosulfan-based conditioning regimen and are described in this retrospective cohort study. Eligibility for HSCT was determined on the basis of published guidelines and criteria included cerebral vasculopathy, recurrent episodes of acute chest syndrome (ACS) or vaso-occlusive crises despite hydroxycarbamide treatment.[106] Donors were chosen on the basis of availability and considered in the following order: MSD, MUD, Haploidentical donor and MMUD. The preferred stem cell source was bone marrow or umbilical stem cells for MSD, bone marrow for MUD and T-depleted peripheral blood stem cell for haploidentical donors and MMUD. The use of a combination of umbilical cord blood and bone marrow was employed in MSD HSCT if the cellularity of the umbilical cord graft was low.[92] Before T-cell depleted HSCT or MUD, autologous bone marrow stem cells were harvested and cryopreserved in order to be re-infused in case of graft rejection, due to the higher risk of this event after T-cell depletion.[107] Before HSCT, all patients received either red cell exchange transfusion or simple transfusion the day before the start of conditioning regimen in order to obtain a proportion of HbS < 30% and a Hb level ≥ 100g/L.

The haploidentical donors underwent PBSC collection after mobilization with subcutaneous Filgrastim 10 µg/Kg twice daily from day -5 to day -1 and once on the day of collection. PBSC were collected using a COBE® Spectra Apheresis System (BCT Terumo, Lakewood, CO). T-cell depletion was performed by removing TCR alpha/beta positive and CD19 positive cells through immuno-magnetic selection (CliniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany).

All patients received Thiotepa (8 mg/kg or 10 mg/kg in 2 doses on day -7), Treosulfan (14 g/m2/day for 3 days from day -6 to day -4) and Fludarabine (40 mg/m2/day for 4 days from day -6 to day-3).[99] Fresenius® anti-thymocyte globulin (ATG) at the dose of 20 mg/kg/day were administered for 3 days in MSD and MUD transplants and 5 mg/kg/day for 4 days in haploidentical transplants. Patient 8 and patient 11 received Rituximab (200 mg/m2) at day -1 to reduce the risk of EBV reactivation and GvHD after HSCT.[64] Graft-vs-Host Disease (GvHD) prophylaxis for T-replete transplants consisted in Cyclosporine 1 mg/kg/day on days -7 to -2, short term Methotrexate (10mg/kg for 4 doses) and Cyclosporine aiming at a pre-dose level of 100-200 µg/L for 6 months. No GvHD prophylaxis was given for T-deplete transplants . The supportive measures, diagnosis and treatment strategy for acute GvHD employed at our institution were recently described.[108]

Engraftment was serially tracked after HSCT. Samples were obtained every 2-3 weeks up to day +100 and monthly thereafter up to 18 months post transplant in patient with complete donor chimerism. Follow up was longer for patients with mixed chimerism. Chimerism analysis was performed by PCR testing for informative short tandem repeats. Adverse events were graded

according to common terminology criteria for adverse events (CTCAE) v4. Neutrophil and platelet recovery were defined as an neutrophil count ≥ 0.5×10^9/L for 3 consecutive days and as a platelet count ≥ 50×10^9/L independently of platelet transfusions for 7 consecutive days.
Organ function was assessed pre-transplant and every year post-transplant by pulmonary function testing, echocardiography, growth and puberty evaluation and hormonal dosage (estradiol/testosterone, FSH, LH, T4 and TSH levels). Transcranial Doppler Ultrasonography (TCD) was performed for all patients prior to the initiation of chronic transfusion and pre-HSCT; data were evaluated according to the criteria defined by the STOP trial.[109] Brain magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA) were performed pre-transplant, and repeated one year post transplant and every two years thereafter if lesions were detected. Immune reconstitution was evaluated by total lymphocyte count, CD4+ cell count and immunoglobulin dosage. Data were recorded at day +30, +90, +180 and +365.

2.1.3 Results

2.1.3.1 Patients

Eleven consecutive children affected by SCD (7 females, 4 males) were transplanted in the Pediatric Hematology and Oncology Department of Padova University Hospital. The origin of patients was African (n = 6), Caucasian (n = 4) or Caribbean (n = 1). Patient and transplant characteristics are summarized in Table 1. Seven patients were diagnosed at birth due to family history, the remaining at their first disease manifestation, between 7 month and 5 years of age. The Hb genotype was HbSS (n = 10) or HbSβ0 (n = 1, P7). Before transplant patients were treated with chronic transfusion (n = 7), monthly red cell exchange transfusion (n = 2) and/or hydroxycarbamide (n = 5). Patients were eligible for HSCT due to cerebral vasculopathy (n=7), recurrent episodes of acute chest syndrome (ACS) or vaso-occlusive crises despite hydroxycarbamide treatment (n = 4). Patient 7 suffered also recurrent splenic sequestration.[106] The median age at transplant was 6.5 years (range: 4 – 16.3 years). At the time of transplant, only two patients had significant comorbidities: relapsing autoimmune hepatitis (P8) and an association of Chiari I malformation and syringomyelia (P11). Donor source was a HbS/A MSD (n = 5), a HbA/A MSD (n = 2), a haploidentical HbS/A parent (mother, n = 1 and father, n = 1), HbA/A MUD (n = 1) or HbA/A MMUD (n=1, 8/12 HLA loci donor/recipient matching). Stem cell source were bone marrow (n=6, median total nucleated cells, TNC = 4,9 x 10^8/kg), combined bone marrow and umbilical cord blood (n=2, median TNC for bone marrow = 2,67 x 10^8/kg; median TNC for cord blood = 2,44 x 10^7/kg) or peripheral blood stem cells (PBSC) (n=3, two haploidentical grafts and one MUD graft, median CD34+ cells = 14,3 x 10^6/kg). One apheresis was sufficient to reach the target dose of CD34+cell (10 x 10^6/kg

recipient) in both haploidentical donors. Both donors complained only grade I-II myalgia and fatigue.

2.1.3.2 Transplant-related outcomes

All patients achieved neutrophil and platelets engraftment at a median of 20 days (range: 15-34 days) and 22 days (range: 12-31 days) from HSC infusion, respectively. No patient experienced primary graft failure. All patients experienced Grade III anemia, grade IV thrombocytopenia and grade IV neutropenia. Grade III-IV non hematological toxicity occurred in 2 patients and consisted in grade IV stomatitis in one patient and acute disseminated encephalomyelitis in one patient. All toxicity resolved completely. Grade I-II gastrointestinal acute GvHD was diagnosed in 3 patients (haploidentical transplant, n = 1; MUD transplant, n = 1, MMUD transplant, n=1). These patients were successfully treated with calcineurin inhibitors (n = 3), steroids (n = 1) and extracorporeal photochemotherapy (n = 3) as per institutional protocol.[108] No grade III-IV acute GvHD or chronic GvHD were observed. No secondary graft failure was observed.

In 6/11 patients a full donor chimerism was demonstrated. Stable mixed chimerism was observed in 5 patients (45%). Donor hematopoiesis ranged from 37% to 90%, but did not affect the HbS proportion: HbS was absent after a transplant from HbA/A MUD (n=1) or compatible with HbS carrier (median 40.5%, range 40.3-41,4%) in patients transplanted from a HbS/A MSD (n=4).

The lymphocyte count reached normal values for age at day +180 in all patients except P11. Median time to reach a CD3+CD4+ cell count higher than 400/µL was 148 days for T-replete grafts (range 57-203 days) and 245 days (range 237-253 days) for T-deplete grafts. Immunoglobulin replacement was necessary only in one patient (P11) who experienced EBV reactivation and received one dose of rituximab. Despite serial monitoring no viral reactivations were documented in any other patient.

Table 1 Patient characteristics and outcomes. Abbreviations: ATG = antithymoglobulin; FLU = fludarabine in mg/m2; RITUX = rituximab; TREO = Treosulfan in g/m2; TT = Thiotepa in mg/kg.

n	sex	age at transplant (years)	HSCT indication	Conditioning	Treatment pre HSCT	Donor	Donor genotype	Stem cell source	Grade III-IV non hematological complication	GvHD	Chimerism	HbS proportion	Follow up (years)
1	M	3.9	Recurrent ACS/VOC	TREO42 TT8 FLU160 ATG	Hydroxycarbamide	MSD	HbAA	BM			100% donor	0	6.5
2	F	9.1	Cerebrovascular disease	TREO42 TT8 FLU160 ATG	Chronic transfusion	MSD	HbSA	BM			41% donor	40.4	5.6
3	F	6.5	Cerebrovascular disease	TREO42 TT8 FLU160 ATG	Chronic transfusion	MSD	HbSA	BM			53% donor	40.3	5.6
4	F	6.5	Cerebrovascular disease	TREO42 TT8 FLU160 ATG	Chronic transfusion + hydroxycarbamide	MSD	HbSA	BM + UCB			100% donor	38.8	4.3
5	F	13.8	Cerebrovascular disease	TREO42 TT8 FLU160 ATG	Monthly red cell exchange transfusion	Haploidentical father	HbSA	TCRαβ/CD19 depleted PBSC			88% donor	40.6	3.9
6	M	4.1	Recurrent ACS/VOC	TREO42 TT8 FLU160 ATG	Hydroxycarbamide	MSD	HbAA	BM + UCB			100% donor	0	2.3
7	M	4.3	Recurrent ACS/VOC	TREO42 TT8 FLU160 ATG	Chronic transfusion	MSD	$HbA\beta^0$	BM	Acute disseminated encephalomyelitis		100% donor	0	2.2
8	F	16.3	Cerebrovascular disease	TREO42 TT10 FLU160 ATG RITUX	Monthly red cell exchange transfusion	Haploidentical mother	HbSA	TCRαβ/CD19 depleted PBSC	Grade IV mucositis	Grade II gastrointesinal aGvHD	100% donor	39.8	1.5
9	F	10.5	Recurrent ACS/VOC	TREO42 TT8 FLU160 ATG	Chronic transfusion + hydroxycarbamide	MUD	HbAA	BM		Grade II gastrointesinal aGvHD	100% donor	0	1.1
10	F	4.1	Cerebrovascular disease	TREO42 TT8 FLU160 ATG	Chronic transfusion + hydroxycarbamide	MSD	HbSA	BM			37% donor	41,4	1
11	M	6.5	Cerebrovascular disease	TREO42 TT10 FLU160 ATG RITUX	Chronic transfusion	MMUD	HbAA	TCRαβ/CD19 depleted PBSC	EBV reactivation	Grade II gastrointesinal aGvHD	61% donor	0	0.8

Table 2 Brain imaging before and after HSCT for patients with cerebral vasculopathy. WHM: white matter hyperintensity; n/a: not available

	Magnetic resonance angiography		White matter changes	
patient	**Before HSCT**	**After HSCT**	**Before HSCT**	**After HSCT**
1	Moderate stenosis	Significant reduction of stenosis	Mild WHM in the hippocampus and temporal cortex	normal
2	Severe stenosis	Stable	WHM in the periventricular region	gliosis of affected region
3	Bilateral severe stenosis	Resolution of stenosis	WHM in the semioval centers	gliosis of affected region
4	Bilateral mild stenosis	n/a	Absent	n/a
5	Bilateral moderate stenosis	Significant reduction of stenosis	Minimal WHM in the left subcortical temporal region	Stable
8	Bilateral moderate stenosis	n/a	Absent	n/a
10	Bilateral severe stenosis	stable	Absent	Absent
11	Bilateral severe stenosis	n/a	Bilateral WHM in the semioval centers	n/a

2.1.3.3 Organ damage and SCD-related outcomes

The median follow-up was 2.35 years (range: 0.8-6.5 years). All patients are alive and well. No episode compatible with acute chest syndrome, stroke or other sickle cell disease manifestation occurred. No patient experienced renal or hepatic dysfunction following transplantation. TCD was normal for patients without cerebral vasculopathy. Data for patients with cerebral vasculopathy are reported in Table 2. Brain MRI and MRA data are available for 10 patients. Three patients had normal pre-transplant brain imaging. Two patient had cerebral vasculopathy before transplantation, but no post transplant imaging. Five patients had alteration on the pre transplant MRI and evaluable data on follow-up (Table 3). Resolution or improvement of the

Table 3 Transcranial Doppler Ultrasonography for patients with cerebral vasculopathy. Results were categorized as normal, conditional or abnormal according to the STOP trail criteria.[109] n/a: not available.

Patient	Before initiation of chronic transfusion	Before HSCT	After HSCT
1	The patient did not receive chronic transfusion	Abnormal	Normal
2	Abnormal	Normal	n/a
3	Abnormal	Normal	n/a
4	n/a	Normal	n/a
5	Abnormal	Normal	n/a
8	Abnormal	Normal	n/a
10	Abnormal	Conditional	n/a
11	Abnormal	Normal	n/a

vascular stenosis was detected in 3/5 patients. The last post-transplant evaluation was performed after a median of 1315 days (range: 268-1417).

Pre-transplant organ function was within normal limits for all patients. Post-transplant lung function evaluation was performed in 7 patients (P1-3 and P5-8) and was normal for all of them after a median of 1104 days from transplant (range 369-2304 days). Hormonal function was evaluated 7 patients (P1-3 and P5-8) after a median of 804 days from transplant (range 205-1518 days). Height and weight growth and thyroid function was normal for all explored patients. Three patients were pre-pubertal at last assessment. Puberty was evaluable in 4 patients (P1, P2, P5 and P8: 1 male and 3 females) and they all had normal pubertal development. Follow up echocardiography (data available for 7 patients) and eye examination (data available for 5 patients) were normal.

2.1.4 Discussion

We report a retrospective case series of 11 SCD patients who received HSCT after a Treosulfan-based conditioning regimen. Sustained engraftment was observed in all patients. Stable mixed chimerism was detected in a significant proportion of patients (45%), did not change after the discontinuation of immunosuppressive treatment and resulted in a cure of SCD for all patients. Previous experiences have demonstrated that full donor chimerism is not needed to cure SCD due to the survival advantage for donor red cell in peripheral blood: pulmonary, gonadal and central nervous system status can be significantly ameliorated also when stable mixed chimerism is obtained.[110–114] Indeed, no clinical manifestation correlated with SCD occurred after HSCT in our cohort. Since cerebral vasculopathy was the cause for transplant in 7 patients, we focused our attention on the evaluation of TCD and brain MRI and MRA. Chronic transfusions resulted in normalization of pre-transplant TCD in all patients that received this treatment; however cerebral artery stenosis were visible on pre-HSCT MRA for all 7 patients. Post-HSCT MRA data were available for 5 patients and showed either a stabilization of the stenosis or an amelioration, as judged by an experienced radiologist. The evidence of improvement in vascular stenosis compares favorably with previous reports and was reported in patients treated with chronic transfusion, hydroxycarbamide or HSCT.[107,115–118] These satisfactory outcomes could be possibly due to the screening program for cerebral vasculopathy performed at our center and to the fact that all patients were transplanted before any clinically evident stroke.[119]

The safety profile of Treosulfan conditioning regimen was excellent and incidence of adverse events was comparable to previous reports: no transplant-related mortality was observed and grade III-IV non hematological toxicity was limited to mucositis which resolved completely

without sequelae.[95,96] The neurological event in our case series cannot be attributed with certainty to the Treosulfan conditioning. This toxicity profile is similar to results obtained in adult patients transplanted after a non-myeloablative conditioning.[120–123] Grade I-II acute GvHD was observed in 3/11 patients in our cohort (27%) with no grade III-IV acute GvHD or chronic GvHD. The GvHD cases were all among patients receiving an alternative donor transplant and no GvHD was observed among the 7 patients receiving a MSD HSCT and all the patients experiencing GvHD responded rapidly to first line treatment. To the best of our knowledge, organ damage related to HSCT has not been previously reported for SCD patients undergoing HSCT after a Treosulfan-based conditioning regimen. In our cohort, the decline in pulmonary and renal function observed after Busulfan-based conditioning regimen was not present and growth, thyroid, gonadal and cardiac function were preserved after HSCT. [107,110]

Current knowledge about outcomes of Treosulfan based conditioning regimen in SCD is limited to a single-center experience reporting 15 patients who received a MSD or MUD HSCT. [96] We have nearly doubled the number of reported patients and we have described the use of Treosulfan-based conditioning regimen for MMUD or haploidentical donor HSCT, which are considered investigational approaches in SCD. [70,88,107] To mitigate the risk of rejection and GvHD, TCRαβ+ and CD19+ cell depletion was performed on PBSC.[64] In the MMUD setting, this approach was reported as safe and efficacious for patients with acute myeloid leukemia or Hurler syndrome but no SCD patient has been described yet.[124,125] If further investigations will confirm its feasibility and efficacy, haploidentical or MMUD HSCT in SCD may open the possibility of cure for many patients without a MSD or MUD donor available.[89–91] When employing alternative donors, a higher risk of GvHD and delayed immune reconstitution should be taken into account; however, in our experience, these drawbacks can be managed by supportive therapy and are outweighed by the satisfactory outcomes. Although PBSC mobilization with G-CSF in HbS heterozygous parents is often perceived as risky, no significant adverse events were reported and, in our experience, both the haploidentical donors underwent PBSC mobilization and collection safely.[126,127]

The main limitation of our study is its retrospective nature and the report of a single center experience; sample size was also limited and warrants further confirmation. Moreover, in order to perform the TCRαβ/CD19 depletion, a facility with experience in stem cell manipulation is needed.

In conclusion, our data show that HSCT after Treosulfan based conditioning regimen for SCD patients is effective and associated with low toxicity. End organ damage may be halted or even

ameliorated as shown by the regression of cerebral vessel stenosis and white matter changes. This strategy is suitable also for alternative donor transplants and, if our data is confirmed in larger cohorts, could pave the way for expanding the access to HSCT to SCD patients lacking a matched sibling or matched unrelated donor.

2.2 Haploidentical Hematopoietic Stem cell Transplantation cures autoimmune hepatitis and cerebrovascular disease in a patient with Sickle Cell Disease

Submitted for publication

Sickle Cell Disease (SCD) may lead to several acute and chronic complications and the end-organ damage may be aggravated by co-existing conditions.[128] Among SCD patients, autoimmune disorders, such as autoimmune hepatitis (AIH) or systemic lupus erythematosus, are not infrequent and are difficult to treat due to the high risk of adverse events associated with steroids or other immunosuppressive treatments.[129–131] Hematopoietic Stem Cell Transplantation is the most consolidated curative therapy for patients with SCD, but benefits and risks in both the short and long term must be carefully considered, taking into consideration disease variability and the recent appearance of new drugs. Despite the progressive broadening of indications for HSCT,[106] the majority of pediatric hematologists still offers it only to patients with a severe phenotype: cerebrovascular disease, recurrent vaso-occlusive crises (VOC) or acute chest syndrome not responsive to Hydroxycarbamide, or sickle nephropathy.[119,132] HSCT is also an option for the management of severe autoimmune disorders such as multiple sclerosis, systemic sclerosis and rheumatoid arthritis, but it has not been previously reported for autoimmune hepatitis.[133] We report a patient with SCD and AIH, both cured after HSCT.

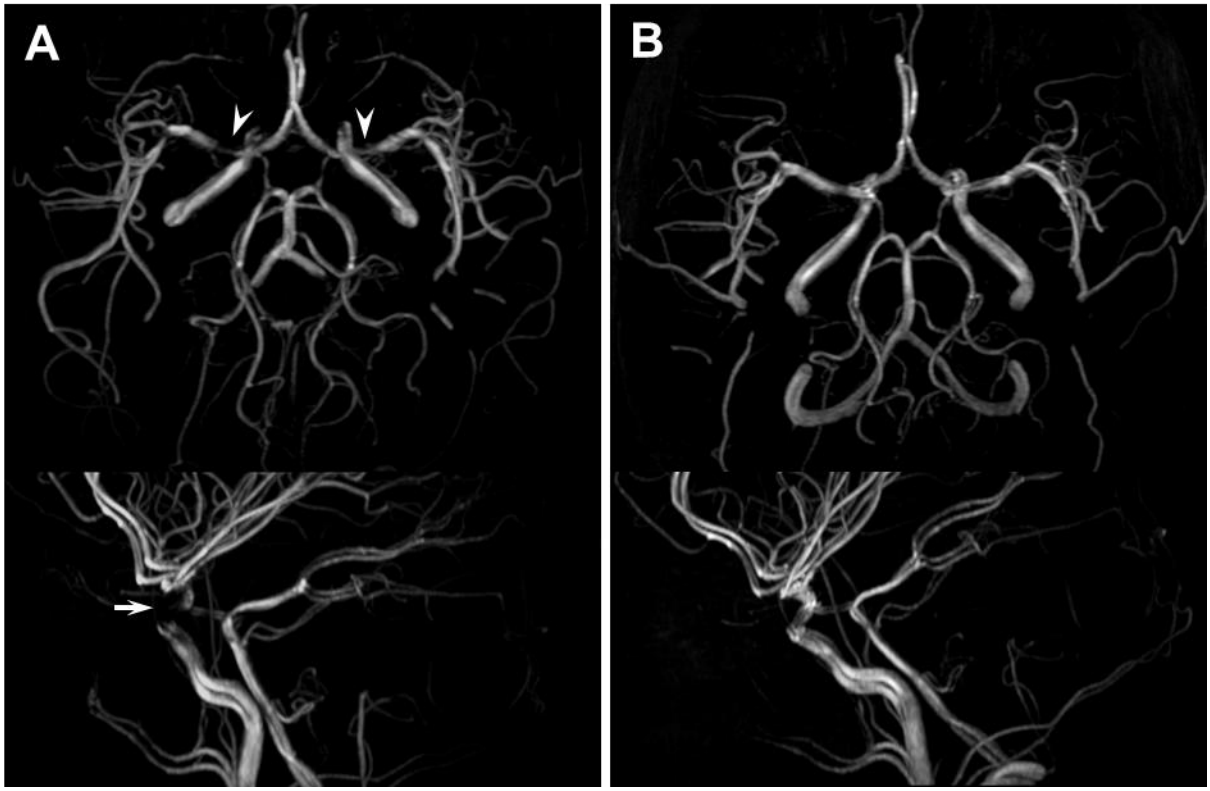

Figure 1 MRA of intracranial arteries pre and post-HSCT.

A. MRA performed at the age of 11 years showing bilateral severe stenosis of the distal internal carotid arteries (arrows), proximal middle cerebral arteries (arrow heads) and their branches.

B. One year after HSCT, the intracranial arteries appears almost normal.

Our patient was diagnosed with HbSS SCD at the age of 4 years when she was hospitalized for severe hemolytic anemia. At the age of 7 years, Trans-Cranial Doppler (TCD) showed a velocity > 200 cm/sec in both the middle cerebral arteries and the basilar artery with a normal brain Magnetic Resonance Imaging (MRI) and Magnetic Resonance Angiography (MRA). The patient began a regular blood transfusion program for stroke prevention, which was changed to monthly red cell exchange transfusions (ECA) at the age of 8 years when adequate venous lines could be placed. At the age of 11 years, severe stenosis of the carotid syphon and middle cerebral arteries and moderate stenosis of the anterior cerebral arteries seen on MRA (Figure 1A) prompted ECA to be scheduled every three weeks to maintain HbS under 30%. At the age of 13 years, MRI/MRA showed a minimal reduction of arterial stenosis with a concomitant normalization of TCD, which was confirmed two years later. No clinical or subclinical stroke occurred.

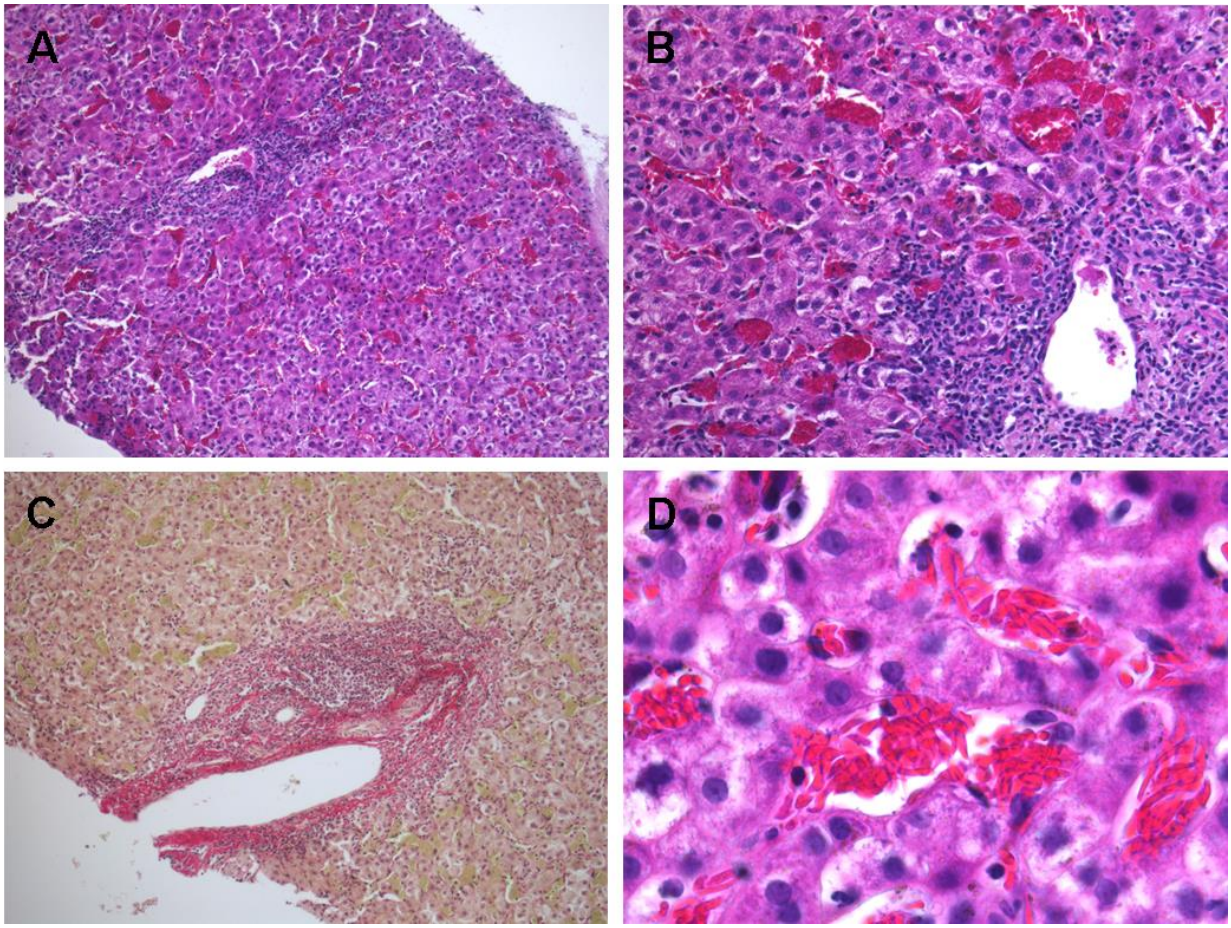

Figure 2 Representative histological features of the liver biopsy.
Histological examination of the liver biopsy showed a high-grade lymphocytic and plasma cell infiltrate within the portal tracts (A), associated with interface hepatitis (B). Some portal tracts were moderately enlarged by portal/periportal fibrosis (C). The histological features were consistent with the diagnosis of chronic active hepatitis and compatible with an autoimmune etiology. (D) Red blood cells within the liver sinusoids disclosed the characteristic sickle cell shape. (H&E and Van Gieson stain; original magnification 10x and 40x).

At the age of 13 years the patient was hospitalized for cholestatic jaundice (total bilirubin 277,1 µmol/L, conjugated bilirubin 171,2 µmol/L) and elevation of liver enzymes (AST 1188 U/L, ALT 974 U/L and GGT 188 U/L). Liver infection, liver sequestration and disorders of the biliary tree were excluded by appropriate investigations including Magnetic Resonance Cholangio-Pancreatography. Anti-nuclear and anti-smooth muscle antibodies resulted positive at a titer of 1:320 and >1:80 respectively, and hypergammaglobulinemia was noticed (IgG 25 g/L). The diagnosis of type I AIH was confirmed by liver histology, which showed portal and peri-portal fibrosis, portal inflammatory infiltrates (mostly constituted of lymphocytes and plasmacells) and focal areas of necrosis (Figure 2). Treatment with prednisolone was initiated with prompt normalization of liver enzymes and resolution of cholestasis, and one month later azathioprine was added after having excluded *TPMT* variants. Steroid treatment was tapered and discontinued 5 months after the diagnosis of AIH but was resumed one month later due to disease relapse. In

the following four years, the patient experienced two relapses of AIH, demonstrating the absolute need of constant immunosuppression with steroids and azathioprine. The anti-nuclear and anti-smooth muscle autoantibodies remained persistently positive during follow-up, with a fluctuating titer corresponding to disease activity. Due to appearance of recurrent and severe VOCs while assuming steroids, exchange transfusions were performed every 18-20 days.
At the age of 16 years, the patient underwent a HSCT after conditioning with Treosulfan, Fludarabine, Thiotepa, Rituximab and Graphalon® anti-thymocyte globulins. The intensive transfusion program required to control the cerebrovascular disease as well as the recurrent VOCs constituted the main indication for HSCT. Due to the lack of a matched sibling donor or a matched unrelated donor, her HbSA haploidentical father was employed as a donor and an ex-vivo TCRαβ and CD19 depletion on PBSC (Peripheral Blood Stem Cell) was performed.[134] The regimen-related toxicity included grade 4 thrombocytopenia, grade 3 anemia, grade 3 febrile neutropenia and grade 3 mucositis, all resolving completely. No post-transplant immune suppression was given and all treatments for AIH were interrupted before the beginning of conditioning. On day + 35 post HSCT, the patient presented with fever, abdominal pain and anorexia; a gastric and rectal biopsy showed histological signs of Graft-versus-Host disease (GvHD) and the patient was diagnosed with grade II acute GvHD. She received treatment with cyclosporine and extracorporeal photochemotherapy, which resulted in complete remission of symptoms within one week. Cyclosporine was continued for one month and extracorporeal photochemotherapy for 4 months. At two years post-HSCT, chimerism analysis showed a full donor hematopoiesis, no SCD manifestation occurred, cerebral vasculopathy resolved almost completely (Figure 1B) and the patient was without any medical treatment. The patient did not experience any relapse of AIH and anti-nuclear and anti-smooth muscle autoantibodies were found negative two months after HSCT and thereafter.
AIH is a liver disease with a wide spectrum of manifestations, ranging from asymptomatic hypertransaminasemia to decompensated cirrhosis and acute liver failure. Its treatment usually consists of long-term immune suppression with steroids and azathioprine; however, unresponsive and severe cases may require intensive treatment including liver transplantation.[135] Our report shows that allogenic HSCT may be a cure for AIH and this finding is especially important for patients with AIH and a concomitant hematological disorder. The autoimmune disorders in patients with SCD pose diagnostic and treatment dilemmas and lead to a significant worsening in the overall health and quality of life. The Treosulfan-based conditioning regime was well tolerated without any liver toxicity and resulted in a stable engraftment notwithstanding the

employment of a non-conventional haploidentical donor. In particular, the haploidentical HSCT for our patient was performed with a TCRαβ and CD19 deplete PBSC graft without post transplant immune suppression, demonstrating that the HSCT itself was responsible for the cure of AIH. Moreover the benefits of this platform include the almost universal donor availability permits to reduce the toxicity associated with the use of calcineurin inhibitors or other immune-suppressive agents. In conclusion, we suggest that the diagnosis of an autoimmune disorder in a patient with SCD might be an indication for HSCT. Further prospective trials are necessary to confirm on larger cohorts the observed benefits of HSCT in terms of morbidity, organ damage and need for medical treatment.

Chapter 3

Outcomes of children with Hemophagocytic Lymphohistiocytosis given allogeneic Hematopoietic Stem Cell Transplantation in Italy

Presented as Oral presentation:

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Abstract

Patients undergoing allogeneic Hematopoietic Stem Cell Transplantation (HSCT) for Hemophagocytic Lymphohistiocytosis (HLH) are a population with specific peculiarities, warranting special considerations about timing of HSCT, donor choice and conditioning regimen. We report here the largest cohort of HLH patients undergoing HSCT. Included in the analysis were 109 patients undergoing 126 transplant procedures between 2000 and 2014 in centers associated with the Italian Pediatric Hematology Oncology Association (AIEOP). Genetic diagnosis was *FHL2* (32%), *FHL3* (33%) or other defined disorders known to cause HLH (20%). Donor for first transplant was an HLA-matched sibling for 25 patients (23%), an unrelated donor for 73 patients (67%) and a partially matched family donor for 11 patients (10%). Conditioning regimen was busulfan-based for 61 patients (56%), treosulfan-based for 21 patients (20%) and fludarabine-based for 26 patients (24%). The 5-year probability of overall and event-free survival were 71% and 60% respectively. Death was mainly due to transplant-related mortality (TRM), while 12 out of 14 patients undergoing a subsequent transplant for rejection/relapse were salvaged. Use of HLA-partially-matched family donor and use of peripheral blood stem cells were associated with adverse outcome in univariate analysis, while only the former variable remained significant in multivariate analysis. Active disease at transplantation did not significantly affect prognosis. These data suggest that active disease should not preclude transplantation, which should be performed preferably using either bone marrow or cord blood cells. Since HLA-haploidentical HSCT in patients with HLH is currently associated with unsatisfactory outcomes, innovative approaches are warranted.

3.1 Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening, hyper-inflammatory syndrome, characterized by cytopenia, fever, hepatosplenomegaly and multi-organ dysfunction. It affects children and adolescents with a higher incidence in the first years of life. HLH can be secondary to infection, autoimmune disease or cancer. In one third of cases, primary immune deficiency resulting in impaired killing of infected cells by T cells or natural killer (NK) cells is present (familiar HLH, fHLH).[136] The genetic defect underlying fHLH results in impaired formation and release of cytotoxic granules, and is caused by genes directly implicated in the secretory lysosome-dependent exocytosis pathway (*PRF1* in FHL2, *UNC13D* in FHL3, *STX11* in FHL4, *STXBP2* in FHL5).[137] HLH can also be part of clinical syndromes with other associated manifestations, such as Chédiak-Higashi syndrome, Griscelli syndrome type 2, Hermansky-Pudlak syndrome type 2, X-linked lymphoproliferative disease type 1 and 2.[138] Approximately, 70% of fHLH in Southern Europe are caused by *PRF1* and *UNC13D* mutation.[139]
Chemo-immunotherapy with dexamethasone, etoposide and cyclosporine-A (CsA) can control the inflammatory manifestation in around 60-80% of the cases.[140,141] However, for patients with fHLH or relapsed/refractory HLH, allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative treatment.[142]
HSCT in a patient with HLH was first reported in 1986 and many case series have since been described.[143] Significant-transplant related mortality (TRM) was reported in earlier experiences with an overall survival of 45-65%.[144–146] This observation has prompted the use of conditioning regimens less toxic than the traditional Busulfan-based myeloablative regimen. The use of Fludarabine or Treosulfan permitted to gradually reduce TRM with better outcomes.[147,148] The major drawbacks related to the use of less toxic regimens including fludarabine/treosulfan are a relevant incidence of mixed chimerism and overt rejection. [102,149]
In this study, we present the outcomes of a cohort of 109 patients affected by HLH who underwent HSCT in centers affiliated with the Italian Pediatric Hematology Oncology Association (AIEOP) network between 2000 and 2014.

3.2 Methods

In this study, we collected data reported to the AIEOP Stem Cell Transplantation Registry and selected patients according to the following criteria: 1) diagnosis of a) fHLH b) a genetic disorder predisposing to HLH c) clinical HLH without genetic markers not responding to chemo-

immuno-treatment or relapsing after treatment 2) HSCT performed in one of the centers participating in the AIEOP network 3) transplantation date comprised between January 1st, 2000 and December 31st, 2014. [16] Whenever needed, centers were contacted for further information about patient status before HSCT, details of the procedure and outcomes. We excluded patients without adequate data available. Forty-two patients included in this cohort have been previously reported.[150]

Patients or legal guardians signed written informed consent for collection, analysis and publication of relevant data. Genetic diagnosis was centrally performed at Meyer Children Hospital in Firenze, Italy, as previously described.[136]

Central Nervous System (CNS) involvement was considered present if a patient had any of the following findings: elevated cerebrospinal fluid (CSF) white cell count, clinical symptoms consistent with CNS involvement (such as seizures or focal or global neurologic deficit), Magnetic Resonance Imaging (MRI) abnormalities consistent with CNS involvement.

Patient status before HSCT was defined according to the following criteria: 1) Complete Response: normalization of all diagnostic clinical and laboratory abnormalities associated with HLH; 2) Partial Response: sustained normalization of three or more of the diagnostic parameters previously validated and no apparent progression of other parameters;[151] 3) Non-Response: normalization of less than two diagnostic parameters or clear progression of other aspects of HLH disease.

After HSCT, disease relapse was defined as recurrence of symptoms typical of HLH with re-establishment of recipient hematopoiesis; rejection was defined as immunologically-mediated graft failure.

3.2.1 Definitions and statistical analysis

The primary endpoint was event-free survival (EFS), defined as the probability of being alive and in continuous CR at last follow-up. In order to determine EFS, death from any cause, relapse or graft failure were considered events. Occurrence of stable mixed chimerism without signs and symptoms of HLH was not considered an event. Full donor chimerism was defined as presence of ≥95% leucocytes of donor origin in peripheral blood or bone marrow. Secondary endpoints were overall survival (OS), time to neutrophil and platelet recovery, incidence of relapse (RI), TRM, acute and chronic GvHD. Probabilities were calculated from date of transplantation until the event or last follow-up.

Neutrophil engraftment was defined as achieving an absolute neutrophil count $\geq 0.5 \times 10^9$/L for three consecutive days with no evidence of autologous recovery (i.e. <5% leucocytes of donor

origin in peripheral blood or marrow). Platelet engraftment was defined as achieving a platelet count $\geq 20x10^9$/L unsupported through platelet transfusions for 7 days. Acute GvHD occurrence was evaluated in all patients with myeloid engraftment, while chronic GvHD was evaluated only in patients surviving beyond day +100 after HSCT. Acute and chronic GvHD were graded according to previously published criteria.[152,153]

Quantitative variables were reported as median value and range, while categorical variables were expressed as absolute value and percentage. Probabilities of EFS and OS were calculated using the Kaplan-Meier estimates. Cumulative incidence functions (CIF) were used to estimate RI and TRM in a competing risks setting, as death and relapse compete with each other. To estimate acute and chronic GvHD incidences, relapse and death were considered as competing events.

A comparison with two sided p-value of less than 0.05 was considered statistically significant. Variables reaching a p-value of less than 0.10 in univariate analysis were included in Cox proportional hazard regression models using a backwards stepwise selection. Statistical analysis was performed using NCSS [NCSS 10 Statistical Software (2015). NCSS, LLC. Kaysville, UT, www.ncss.com/software/ncss] and R 2.5.0 software package (http://www.R-project.org). [154,155] Analysis used January 31st, 2016 as reference date.

3.3 Results

3.3.1 Patient population

One hundred twelve patients with HLH who underwent 129 transplant procedures have been reported the AIEOP HSCT registry. Three patients were not evaluable for this study due to lack of data; thus, the final analysis includes 109 patients and 126 transplant procedures performed in 16 AIEOP centers. Sixty-five patients (60%) were male and 44 (40%) were female. Median age at diagnosis was 1 year (range 27 days-18 years), while median age at first transplantation was 2 years (range 4 months -20 years). Mean time interval between diagnosis and first HSCT was 289 days (range 26-1844 days). Patient and HSCT characteristics are summarized in Table 4.

Genetic testing was performed for 96 (88%) out of 109 patients. Mutation of *PRF1* was found in 31 patients (32%), of *UNC13D* in 32 patients (33%), of *STXBP2* in 2 patients (2%), of *RAB27A* in 6 patients (6%), of *SH2D1A* in 5 patients (5%), of *BIRC4* in 2 patients (2%) and of *LYST* in 1 patient (1%). No known gene abnormality was found in 15 patients (15%).

CNS involvement at diagnosis was recorded for 79 patients (72% of the overall population) and was present in 30 patients (38%): 17 (22%) had elevated CSF white cell count, 20 (25%) had clinical symptoms consistent with HLH and 7 (9%) had MRI abnormalities consistent with HLH.

Table 4 Patient and transplant characteristics.

	N.	%
Gender		
Male	65	60%
Female	44	40%
Genetic diagnosis		
FHL2	31	28%
FHL3	32	29%
Griscelli Syndrome	6	6%
XLP1	5	5%
Other	7	6%
No known genetic defect	15	14%
Study not performed	13	12%
Age at diagnosis, median (range)	1 y (27d – 18y)	
Age at transplant, median (range)	2 y (4m – 20 y)	
CNS involvement		
Present	30	28%
Absent	49	45%
Data not available	30	27%
Treatment before transplant		
HLH-1994 protocol	9	8%
HLH-2004 protocol	41	38%
Euro-HIT-HLH protocol	3	3%
Other	7	6%
Unknown	49	45%
Disease status at first transplant		
First complete remission	24	22%
More advanced complete remission	6	5%
Partial response	17	16%
No response	54	50%
Pre-emptive	2	2%
Unknown	6	5%

	N.	%
Conditioning regimen		
Busulfan-based conditioning	61	56%
Busulfan-Cyclophosphamide	10	9%
Busulfan-Etoposide	18	17%
Busulfan-Fludarabine	6	6%
Busulfan-Thiotepa	22	20%
Other Busulfan-based conditioning	5	4%
Fludarabine based conditioning	26	24%
Fludarabine-Melphalan	12	11%
Fludarabine-Melphalan-Thiotepa	9	8%
Other Fludarabine- based	5	5%
Treosulfan-based conditioning	21	20%
Treosulfan-Fludarabine-Thiotepa	15	14%
Treosulfan-Fludarabine	5	4%
Treosulfan-Fludarabine-Cyclophosphamide	1	1%
Other conditioning regimen	1	1%
Donor type		
Matched sibling donor	25	23%
Matched unrelated donor	73	67%
Partially matched family donor	11	10%
Stem cell source		
Bone marrow	70	64%
Peripheral blood stem cells	18	17%
Umbilical Blood Graft	21	19%
Serotherapy		
ATG	76	70%
Alemtuzumab	7	6%
No Serotherapy	26	24
T-Cell depletion		
Ex vivo T-cell depletion	8	7%

At diagnosis, 9 patients were enrolled in the HLH-94 protocol,[5] 41 patients were enrolled in the HLH-04 trial,[17] 3 in the Euro-HIT-HLH trial [EudraCT#2011-002052-14], 4 received personalized treatment, 2 patients were transplanted without any other treatment due to diagnosis of *BIRC4* mutation before developing clinical HLH and for 49 patients data on the frontline treatment are not available. Two patients received anti-IFNɣ monoclonal antibody in the context of a clinical trial [EudraCT#2012-003632-23, #NCT01818492].[156] Multiple intrathecal injections of methotrexate were employed for preventing/treating neurological involvement.

3.3.2 Transplant procedure

Ninety-five patients received one transplant, while 14 received more than one HSCT because of rejection in 8 patients or disease relapse in 6 patients (preceded by rejection in 1 case): 2 transplants were performed in 12 cases, 3 transplants in 1 case and 4 transplants in 1 case. Thirty HSCT were performed between 2000 and 2004, 42 between 2005 and 2009, and 54 between 2010 and 2014.

Disease status at first HSCT was known for 102 (94%) out of the 109 patients; 71 patients had active disease (no response, n= 54; partial response, n= 17), 24 were in first complete remission and 5 were in a later complete remission. Two patients received HSCT due to diagnosis of *BIRC4* mutation before developing clinical HLH. Conditioning regimen was Busulfan-based for 61 patients, Treosulfan-based for 21 patients, Fludarabine-based for 26 patients and melphalan-etoposide for 1 patient (see also Table 4 and Table 5 for further details).

The donor for the first transplant was an HLA-matched sibling donor for 25 patients, an unrelated volunteer selected using high-resolution HLA typing for 73 patients and an HLA-partially-matched family donor for 11 patients. Seventy patients were transplanted with bone marrow-derived stem cells, 18 with peripheral blood stem cells (PBSC), while 21 children received umbilical cord blood transplantation. The mean dose of mononuclear cells was 6.4 cells x 10^8/kg for bone marrow grafts (range=2.5-27.3 x 10^8/kg) and 11.2 cells x 107/kg (range= 2-29.2 x 10^7/kg) for cord blood grafts. The mean dose of CD34 positive cells for PBSC grafts was 15.9 cells x 10^6/kg (range 2-24.8 x 10^6/kg).

Considering the 109 first transplants, GvHD prophylaxis consisted of Cyclosporine A in 25 cases, of the combination of Cyclosporine A and short-term methotrexate in 55 cases, of the combination of CsA and steroids in 20 cases. Post-transplant high-dose cyclophosphamide was used in 1 case and *in vitro* T-cell depletion was used in 8 allografts.

3.3.3 Engraftment and chimerism

Neutrophil engraftment after first HSCT was obtained in 100/109 (92%) procedures at a median time of 18 days (range 9-57 days). Platelet engraftment after first HSCT was obtained in 87/109 (80%) cases at a median interval of 24 days (range 9-105 days).

Stable mixed chimerism with good graft function and clinical remission of HLH was recorded in 6 patients. Donor contribution to hematopoiesis ranged from 5 to 97%, without evidence of the underlying disease. Fourteen patients received a second transplant. The reason for second HSCT was disease relapse (n=11) or graft failure (n=3). Subsequent transplants (n=17) were performed with Busulfan-based conditioning (n=3), Fludarabine-based (n=5), Treosulfan-based (n=5) or other conditioning regimen (n=4). The donor was a matched sibling donor for 2 procedures, a partially-matched family donor for 6 procedures and an unrelated donor for 9 procedures. Stem cell source was bone marrow and PBSC in 8 transplants each and cord blood for the last allograft.

Neutrophil engraftment after second transplant was obtained in 12/14 (86%) procedures at a median time of 17 days (range 11-34 days). Platelet engraftment after second transplant was obtained in 11/14 (79%) transplants at a median time of 24 days (range 11-55 days).

Out of the 14 patients that received a second transplant, 12 (86%) are alive and well at last follow-up; however, 1 of them required two further transplant procedures to achieve a good graft function. One patient died of viral infection after the second transplant and another of respiratory failure after the third transplant.

Acute GvHD was evaluated among the 115 transplants that resulted in donor engraftment. Grade II and grade III-IV acute GvHD occurred in 29 (25%) and11 (9%) transplants, respectively. Among 95 HSCT after which the patient did not die or was transplanted again before day 100, chronic GvHD was observed in 18 cases (19%): it was of limited severity in 9 cases (9%) and extensive in other 9 cases (9%).

3.3.4 Clinical outcome

The median observation time for surviving patients is 5.2 years (range 0.9-14.9 years), while it was 54 days (range, 7 days-3.8 years) for those who died.

At time of the last follow-up, 78 patients (72%) are alive, with a 5-year OS for the whole study population of 71% (95% CI, 62-79, see also Figure 1).

A total of 26 patients (24%) died to transplant-related causes at a median of 53 days after HSTC; TRM was preceded by graft rejection in 4 cases. The cumulative TRM incidence was 25% (95% CI, 18-35). The number of fatal events according to the type of conditioning regimen employed

is shown in Table 5, while Table 6 summarizes the causes of death of the whole study population; veno-occlusive disease, lung aspergillosis and multi-organ failure were the most frequent cause of death. Graft failure was observed in 14 patients (5%) at a median of 20 days after HSCT (range, 8-51).

Table 5 Comparison of outcome among Busulfan-based, Treosulfan-based and Fludarabine-based conditioning regimens.

	Busulfan based (n=61)		**Treosulfan based (n=21)**		**Fludarabine based (n=26)**		**Chi square P**
	n	(%)	n	(%)	n	(%)	
Active disease at HSCT	38	(62%)	13	(62%)	20	(74%)	N.S.
TRM	16	(26%)	3	(14%)	7	(26%)	N.S.
VOD	4	(7%)	0	(0%)	3	(11%)	N.S.
Rejection	7	(11%)	1	(5%)	6	(22%)	N.S.
Relapse	4	(7%)	2	(10%)	4	(15%)	N.S.
Alive	43	(70%)	18	(86%)	17	(63%)	N.S.
Alive & disease-free	37	(61%)	15	(71%)	14	(52%)	N.S.

The cumulative incidence of graft failure was 13% (8-21). Four of the 14 patients who rejected the transplant died due to transplant-related causes (after a second HSCT in 1 case), while 3 subsequently developed an overt disease recurrence: 2 died due to disease progression, while 1 was rescued by a second HSCT. The remaining 7 patients who rejected are alive and disease-free after a second transplant. A disease relapse was observed in 10 patients (9%) at a median of 163 days after HSCT (range, 41-585 days) and was preceded by a primary rejection in 3 cases. The cumulative incidence of relapse was 9% (96% CI, 5-17). Eight out of the 10 patients who relapsed received a second HSCT; 5 of them are alive and disease-free.

Table 6 Causes of death.

	Number of transplant		
	First HSCT	Subsequent HSCT	***Total***
Disease progression	4	1	***5***
Veno-occlusive disease	7	0	***7***
Lung aspergillosis	5	0	***5***
Multi-organ failure	4	1	***5***
Viral infection (Adenovirus/Cytomegalovirus)	3	0	***3***
Chronic GVHD	2	0	***2***
Acute GVHD	1	0	***1***
Cerebral hemorrhage	0	1	***1***
Thrombotic microangiopathy	1	0	***1***
Unknown	1	0	***1***
Total	***28***	***3***	***31***

Sixty-six patients are alive and disease-free after the first HSCT at time of last follow-up, the 5-year probability of EFS being 60% (95% CI, 50-69). The variables found to be statistically associated, in univariate analysis, with EFS were donor type and stem cell source. Patients transplanted from partially-matched family donors had a significantly worse EFS (9%; 95% CI, 0-26%) than recipients of a matched family donor transplant (73%; 95% CI, 54-92%) or a matched unrelated donor allograft (63%; 95% CI, 52-74%) (P< 0.001, see also Figure 2). Patients given PBSC transplantation had a significantly lower EFS probability (39%; 95% CI, 16-61) as compared to bone marrow recipients (60%; 95% CI, 48-72%) or cord blood recipients (76%; 95% CI, 58-94) (P = 0.0185). Children who received the transplant within 6 months from diagnosis had a better EFS as compared to those transplanted later than 6 months from diagnosis (69%; 95% CI, 56-81 vs. 50%; 95% CI 37-64), but the difference was not statistically significant (P = 0.069). Details on univariate analysis of variables potentially influencing outcome are shown in detail in Table 7.

In multivariate analysis (Table 8), only the use of a partially matched family donor confirmed its statistically significant association with a worse EFS probability, with a relative risk of 12.26 (95% CI, 2.82-53.35) (P = 0.0008).

Table 7 Univariate analysis of factors influencing event-free survival (except for *, data were considered for first HSCT only).

	N.	Events	EFS (%)	(95% CI)	P-value
All patients	**109**	**43**	**60%**	**(50-69)**	**- -**
Genetic diagnosis					
PRF1 mutation	31	10	67%	(50-84)	N.S.
UNC13D mutation	32	14	55%	(38-73)	
Other diagnosis	18	5	72%	(52-93)	
No genetic diagnosis	15	7	53%	(28-79)	
Study not performed	13	7	45%	(17-73)	
CNS involvement at diagnosis					
Present	37	16	57%	(41-73)	N.S.
Absent	72	27	61%	(49-72)	
Years of transplant					
2000-2004	27	13	52%	(33-71)	N.S.
2005-2009	36	15	58%	(42-74)	
2010-2014	46	15	67%	(54-81)	
Time from diagnosis to HSCT					
< 6 months	56	17	69%	(56-81)	0.0699
≥ 6 months	53	26	50%	(37-64)	
Disease status					
Active disease (NR or PR)	71	31	56%	(44-68)	N.S.
CR or pre-emptive	31	10	67%	(50-84)	
Missing information	7	2	69%	(32-100)	

	N.	Events	EFS (%)	(95% CI)	P-value
Conditioning Regimen					
Busulfan-based	61	24	60%	(47-72)	N.S.
Fludarabine-based	27	13	51%	(32-70)	
Treosulfan-based	21	6	70%	(50-90)	
Donor					
MFD	25	6	73%	(54-92)	< 0.001
MUD	73	27	63%	(52-74)	
PMFD	11	10	9%	(0-26)	
Stem cell source					
BM	70	27	60%	(48-72)	0.0185
PBSC	18	11	39%	(16-61)	
UCB	21	5	76%	(58-94)	
No. of HSCT (*)					
First HSCT	109	43	60%	(50-69)	N.S.
Second HSCT	14	4	71%	(48-95)	

MFD: matched family donor; MUD: matched unrelated donor; PMFD: partially matched family donor. BM: bone marrow; PBSC: peripheral blood stem cells; CB: cord blood.

Table 8 Multivariate analysis of factors influencing event-free survival (data were considered for first HSCT only).

Variable	Relative Risk	(95% CI)	P
Interval Diagnosis - HSCT (months)			
> 6 months vs. < 6 months	1.15	(0.59-2.24)	0.68
Donor			
MUD vs. MFD	2.16	(0.85-5.49)	0.11
PMFD vs. MFD	12.26	(2.82-53.35)	0.0008
Stem cell source			
Cord blood vs. Bone marrow	0.48	(0.18-1.28)	0.14
Peripheral blood vs. Bone marrow	0.63	(0.21-1.87)	0.41

MFD: matched family donor; MUD: matched unrelated donor; PMFD: partially matched family donor.

3.4 Discussion

To the best of our knowledge, the cohort presented here is the largest ever reported of HLH patients receiving HSCT. Included were mainly patients with genetic diagnosis of fHLH. The most frequent genetic lesions involved *PRF1* and *UNC13D*, reflecting the epidemiology in Southern Europe.[136] Twenty-eight patients (25%) without a genetic diagnosis but fulfilling the internationally accepted HLH criteria were transplanted for refractory or relapsed HLH.

We show that allogeneic HSCT is a therapeutic option capable of curing a large proportion of patients irrespectively of the genetic defect responsible for the disease, especially if a suitable HLA-matched donor is available. The optimal timing for performing HSCT in HLH patients is a matter of debate, especially in cases with relapsed or refractory disease. In particular, it is unclear whether for relapsed or refractory disease aggressive second-line chemo-immuno-therapy aimed at reaching CR before transplant is warranted. Some case series suggest that active disease at transplantation might be a risk factor, especially when an HLA-haploidentical donor is used; [144,146] however, other data indicate that initial response to treatment (CR after two month of treatment) could be more informative about the prognosis.[145,150,157] Moreover, in the published experiences, around 30-60% of patients have been transplanted with active disease, indicating that CR is difficult to obtain in many patients with HLH.[102,145–147,150,157] Our data could shed further light on this issue: active disease at transplantation was not statistically associated with adverse outcomes, but patients had a tendency for a worse outcomes if the interval between diagnosis and transplantation was longer than 6 months. We thus speculate that active disease at transplantation could be indicative of a more aggressive form of HLH that would not adequately respond to further treatment. The objective should probably be the achievement of the best possible response, without postponing HSCT more than 6 months. Treating patients for a longer time, aiming at obtaining CR before transplantation, could result in deterioration of the general status and make outcomes of transplant worse. Maybe new approaches to HLH immunotherapy, such as that based on the use of an anti-IFNγ monoclonal antibody (EudraCT#2012-003632-23, #NCT01818492), could lead to better rates of CR at time of transplantation in refractory patients.[156]

Donor availability plays an important role in deciding when to perform a transplant. Our data indicate that, although a matched sibling donor is the donor of choice, an unrelated donor selected using high-resolution molecular typing of HLA loci can be used with comparable patient's outcome. Our study confirms also that the use of umbilical cord blood is a feasible option. [150,158] In particular, 21 patients (19%) who received a cord blood allograft had outcomes

comparable with those of patients given BM cells. Probably, young age at HSCT for HLH patients with a consequent favorable ratio of number of cells infused per Kg of recipient body weight makes this kind of procedure more appropriate than in other clinical settings.

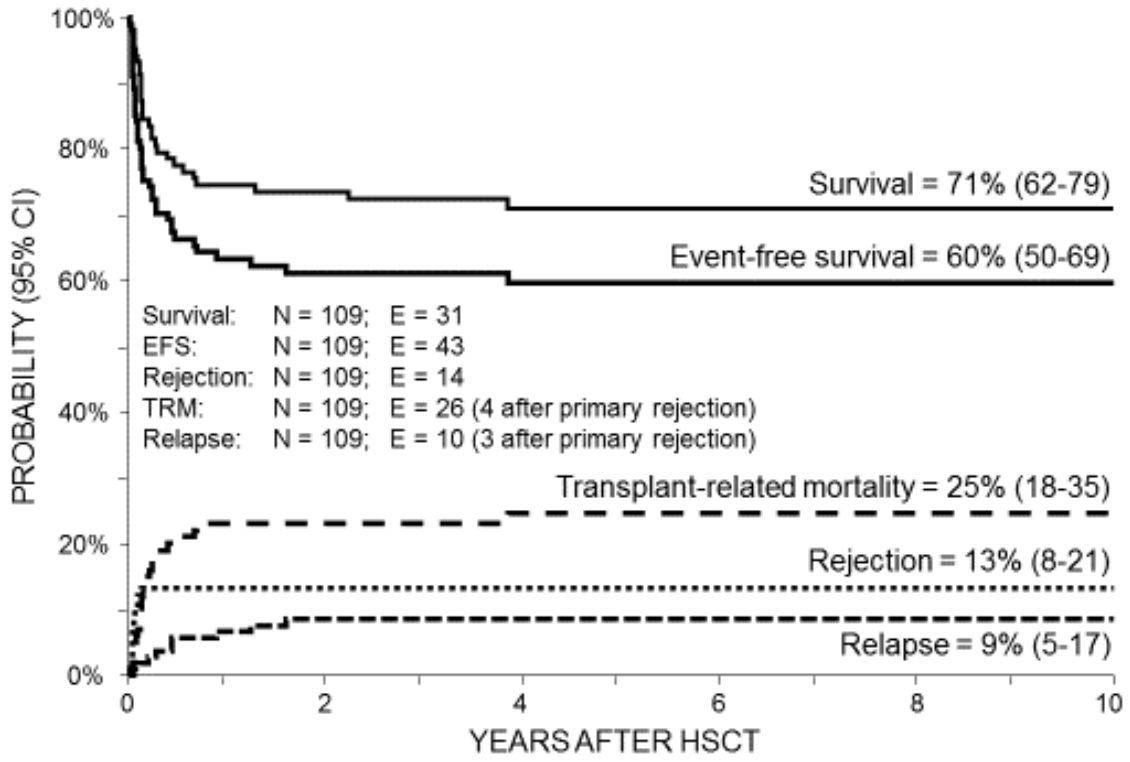

Figure 3 Survival, Event-free survival (EFS), Transplant-related mortality (TRM), Rejection and Relapse for the 109 patients after the first HSCT.

Our data indicate that, so far, the use of HLA-partially matched family donors is associated with a dismal outcome. The investigation of new approaches to HLA-haploidentical transplantation, such as that based on depletion of TCR αβ T cell/CD19+ cells, is urgently needed in order to improve patient's outcome and to offer a timely transplant also to patients lacking a matched donor.[64]

The main causes of death in our cohort were complications related to HSCT, namely veno-occlusive disease, lung aspergillosis and multi-organ failure; HLH relapse accounted for 5 deaths only. Indeed, busulfan-based myeloablative conditioning for HLH patients has been reported to be associated with a high rate of infections, veno-occlusive disease and possibly a higher incidence of pulmonary complications. [144–146,159–161] To overcome these issues, in the mid-2000s,

the use of the fludarabine-melphalan conditioning regimen was introduced, leading to less TRM and better outcomes, at the expense of higher mixed chimerism and relapse rates. [16,147,149,162,163]

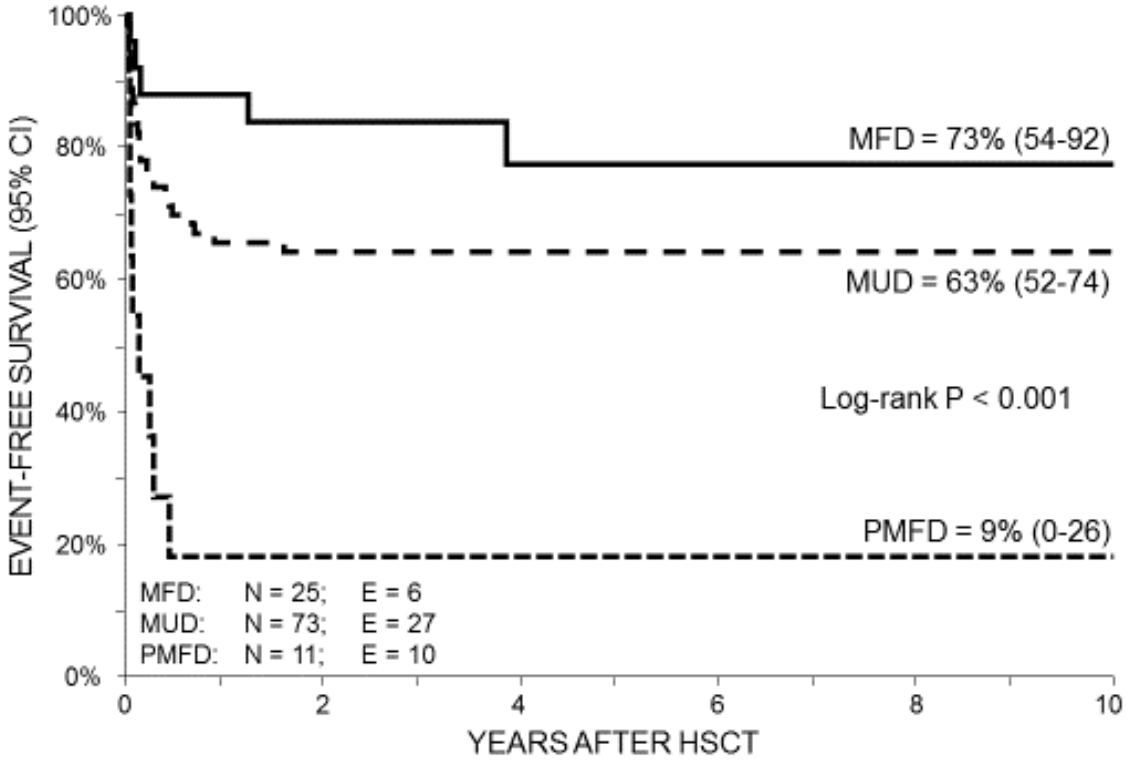

Figure 4 Event-free survival (EFS) according to the type of donor. MFD: matched family donor; MUD: matched unrelated donor; PMFD: partially matched family donor.

Recent experiences report excellent results with the use of treosulfan-based conditioning regimens, which seem to be associated with less extramedullary toxicity.[148,102] Our cohort is the only one in which the three above conditioning regimens have been used in a significant proportion of patients and outcomes could be directly compared (see also Table 2). Although no statistically significant differences were observed, a trend towards better OS and EFS after treosulfan-based conditioning was evident. In our experience, the high TRM (26%) observed with busulfan was not significantly different from that with fludarabine-based regimens. Moreover, fludarabine-based conditioning exposed patients to a higher risk of relapse and need of a second transplant (see also Table 2). On the other hand, treosulfan-based conditioning resulted in lower TRM (14%) with acceptable rates of graft failure (5%) or relapse (10%), this translating into a remarkably high rate of cured patients.

Importantly, patients receiving a second transplant did not have worse outcomes than patients transplanted only once. This finding suggests that when a patient does not suffer from significant

end-organ damage either related to the original disease or to a first HSCT, it is reasonable to perform a second allograft in case of relapse or rejection. Future strategies for HSCT in HLH patients should probably aim more at mitigating TRM than at reducing relapse or rejection, events which could be salvaged by a second procedure.

The multicenter and retrospective design of our study may have some limitations, such as that of lacking information on pre-transplant treatment in a relevant proportion of patients, but describes well the current practice in HSCT for HLH. Our results on probabilities of 5-year OS and EFS survival obtained in a larger cohort of children were slightly better as compared with other similar cooperative studies.[145,158,160,164,165] These data might be explained by the higher proportion of patients receiving fludarabine- or treosulfan-based conditioning in our cohort or by the fact that we included only patients transplanted after 2000.

In conclusion, our data suggest that in patients with HLH allogeneic HSCT is able to definitively cure two thirds of patients, restoring normal immune response towards pathogens and abrogating the hyper-inflammatory state typical of HLH. Haploidentical HSCT in patients with HLH is currently associated with unsatisfactory outcomes and new approaches are needed to ameliorate the outcomes. The use of PBSC should be discouraged, while active disease does not preclude transplantation, which should be ideally performed within 6 month from diagnosis. Finally, the use of treosulfan-based conditioning could be an attractive option to reduce TRM.

Chapter 4

Supportive treatment and psychological consequences of HSCT

In this chapter are summarized other studies in which the PhD candidate was involved and that are focused on supportive care before and after HSCT, and psychological consequences of HSCT in children.

4.1 Eltrombopag use in a patient with Wiskott-Aldrich syndrome

Published as: Gabelli M., Marzollo A., Notarangelo LD., Basso G., Putti MC. **Eltrombopag use in a patient with Wiskott-Aldrich syndrome.** *Pediatr Blood Cancer*. June 2017. doi:10.1002/pbc.26692.

Wiskott-Aldrich syndrome (WAS) is an inherited X-linked recessive disorder characterized by microthrombocytopenia, immunodeficiency and eczema. Affected individuals have an increased risk of autoimmune and neoplastic disorders; mortality is high during childhood and adolescence due to infections, bleeding or malignancies. Although immunodeficiency is variable in presentation and severity, thrombocytopenia is a constant feature, is responsible for fatal bleeding in 25% of patients and a very low platelet count is currently considered an indication

for definitive treatment.[166–168] HSCT from a matched sibling or a matched unrelated donor is the treatment of choice and should be performed in children under the age of 5 years.[169] HSCT or gene therapy may require several months to be performed and, in the meantime, other treatment option include splenectomy and platelet transfusion. [170] However, splenectomy increases the risk of severe infections, particularly after HSCT and platelet transfusions are short-lived, may lead to alloimmunisation and may not be effective in all patients.[167,171] Eltrombopag, a thrombopoietin (TPO) receptor agonist, is a new efficient treatment for immune thrombocytopenia in both adults and children and may also be used for inherited form of thrombocytopenia. [172–176] A male with WAS, profound thrombocytopenia, and bleeding diathesis successfully was successfully managed with Eltrombopag before HSCT. Eltrombopag was given for 32 weeks obtaining a stable platelet count without any platelet transfusion. The patient did not experience any bleeding symptom. This treatment reduced bleeding symptoms and completely eliminated the requirement for platelet transfusion. Our case adds to the cumulative knowledge regarding the efficacy of Eltrombopag in patients with WAS as an effective measure to manage profound thrombocytopenia and recurrent bleeding while awaiting definitive treatment.[174] In particular, we suggest that it may be suitable for children under the age of 2 for whom bleeding is the first severe manifestation of WAS and may be fatal.[167,168] Eltrombopag treatment was very well tolerated and its oral formulation is suitable also for smaller children. Moreover, also a relatively small platelet rise due to treatment may be sufficient to prevent severe bleeding.

4.2 Risk Factors and Outcomes Related to Pediatric Intensive Care Unit Admission after Hematopoietic Stem Cell Transplantation: A Single-Center Experience

Published as: Pillon M., Amigoni A., Contin A., Cattelan M., Carraro E., Campagnano E., Tumino M., Calore E., Marzollo A., Mainardi C., Boaro MP., Nizzero M., Pettenazzo A., Basso G., Messina C. **Risk Factors and Outcomes Related to Pediatric Intensive Care Unit Admission after Hematopoietic Stem Cell Transplantation: A Single-Center Experience.** *Biol Blood Marrow Transplant*. 2017;23(8):1335-1341. doi:10.1016/j.bbmt.2017.04.016.

To describe incidence, causes, and outcomes related to pediatric intensive care unit (PICU) admission for patients undergoing hematopoietic stem cell transplantation (HSCT),we investigated the risk factors predisposing to PICU admission and prognostic factors in terms of patient survival. From October 1998 to April 2015, 496 children and young adults (0 to 23 years)

underwent transplantation in the HSCT unit. Among them, 70 (14.1%) were admitted to PICU. The 3-year cumulative incidence of PICU admission was 14.3%. The main causes of PICU admission were respiratory failure (36%), multiple organ failure (16%), and septic shock (13%). The overall 90-day cumulative probability of survival after PICU admission was 34.3% (95% confidence interval, 24.8% to 47.4%). In multivariate analysis, risk factors predisposing to PICU admission were allogeneic HSCT (versus autologous HSCT, P = .030) and second or third HSCT (P = .018). Characteristics significantly associated with mortality were mismatched HSCT (P = .011), relapse of underlying disease before PICU admission (P < .001), acute respiratory distress syndrome at admission (P = .012), hepatic failure at admission (P = .021), and need for invasive ventilation during PICU course (P < .001). Our data indicate which patients have a high risk for PICU admission after HSCT and for dismal outcomes after PICU stay. These findings may provide support for the clinical decision-making process on the opportunity of PICU admission for severely compromised patients after HSCT.

4.3 Quality of Life and Psychopathology in Adults Who Underwent Hematopoietic Stem Cell Transplantation in Childhood: A Qualitative and Quantitative Analysis

Published as: Sinatora F., Traverso A., Zanato S., Di Florio N., Porreca A., Tremolada M., Boscolo V., Marzollo A., Mainardi C., Calore E., Pillon M., Cattelan C., Basso G., Messina C. **Quality of Life and Psychopathology in Adults Who Underwent Hematopoietic Stem Cell Transplantation (HSCT) in Childhood: A Qualitative and Quantitative Analysis.** *Front Psychol*. 2017;8(AUG):1316. doi:10.3389/fpsyg.2017.01316.

Patients who undergo pediatric Hematopoietic Stem Cell Transplantation (HSCT) may experience long-term psychological sequelae and poor Quality of Life (QoL) in adulthood. This study aimed to investigate subjective illness experience, QoL, and psychopathology in young adults who have survived pediatric HSCT. The study involved patients treated with HSCT in the Hematology-Oncology Department between 1984 and 2007. Psychopathology and QoL were investigated using the SCL-90-R and SF-36. Socio-demographic and medical information was also collected. Finally, participants were asked to write a brief composition about their experiences of illness and care. Qualitative analysis of the texts was performed using T-LAB, an instrument for text analysis that allows the user to highlight the occurrences and co-occurrences of lemma. Quantitative analyses were performed using non-parametric tests (Spearman correlations, Kruskal-Wallis and Mann-Whitney tests). Twenty-one patients (9 males)

participated in the study. No significant distress was found on the SCL-90 Global Severity Index, but it was found on specific scales. On the SF-36, lower scores were reported on scales referring to bodily pain, general health, and physical and social functioning. All the measures were significantly ($p < 0.05$) associated with specific socio-demographic and medical variables (gender, type of pathology, type of HSCT, time elapsed between communication of the need to transplant and effective transplantation, and days of hospitalization). With regard to the narrative analyses, males focused on expressions related to the body and medical therapies, while females focused on people they met during treatment, family members, and donors. Low general health and treatment with autologous HSCT were associated with memories about chemotherapy, radiotherapy, and the body parts involved, while high general health was associated with expressions focused on gratitude (V-Test ± 1.96). In conclusion, pediatric HSCT survivors are more likely to experience psychological distress and low QoL in adulthood compared with the general population. These aspects, along with subjective illness experience of the survivor, show differences according to specific medical and socio-demographic variables. Studies are needed in order to improve the care and long-term follow-up of these families.

4.4 Psychopathological Aspects in Childhood Hematopoietic Stem Cell Transplantation: The Perception of Parents and Adolescents.

Published as: Zanato S., Traverso A., Tremolada M., Sinatora F., Porreca A., Pozziani G., Di Florio N., Capello F., Marzollo A., Tumino M., Cattelan C., Basso G., Messina C. **Psychopathological Aspects in Childhood Hematopoietic Stem Cell Transplantation (HSCT): The Perception of Parents and Adolescents.** *Front Psychol*. 2017;8(April):272. doi:10.3389/fpsyg.2017.00272.

Data about psychosocial sequelae of HSCT in children are limited and the association with a specific donor type or other medical factors is largely unknown. The aim of the present study was to compare the psychological aspects of pediatric HSCT survivors with healthy peers. A secondary aim was to detect whether parents and children differed in the perception of mental health status. The influence of medical factors on psychological status was also examined. Thirty seven HSCT survivors (23 males) with a mean age of 14.4 years (SD = 3.03; range 8.16–18.33) were recruited. Twenty-six patients underwent an allogenic HSCT (matched unrelated donor, n = 20; matched sibling donor, n = 6) and 11 patients received an autologous HSCT. The children

psychological aspects were assessed using the Youth Self Report (YSR) and compared to a group of matched healthy peers. At the same time, parents were requested to complete the Child Behavior Checklist 6–18. Medical and socio-demographic data were also collected. HSCT survivors reported significantly higher levels of somatic complains ($t27 = 3.14$; $p = 0.004$; mean = 3.1) when compared to healthy peers (mean = 1.5). The parent CBCL scores on "child total competence" exceeded the normative clinical cutoff in 48.6% cases. Inter-rater agreement between parent and patient reports was present only in three scales: total competence score ($K = 0.06$, $p = 0.002$), somatic complaints ($K = 0.21$, $p=0.003$) and attention problems ($k = 0.13$; $p=0.02$). According to Ancova models, internalizing problems were more frequent in HSCT from family donors ($F2 = 3.13$; $p = 0.06$) or in the presence of acute complications ($F1 = 11.95$; $p = 0.003$). In contrast to the perception of parents, pediatric HSCT survivors reported good psychological health. However, they complained about more somatic problems as compared with healthy peers. Medical aspects such as donor source and the presence of acute complications should be taken into consideration for the psychological approach in order to improve pediatric HSCT survivor care.

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Abbreviations

ATG: Antithymoglobulin

CsA: Cyclosporine A

EFS: Event Free Survival

GvHD: Graft versus Host Disease

HLA: Human Leukocyte Antigens

HSCs: Hematopoietic Stem Cells

HSCT: Hematopoietic Stem Cell Transplantation

HLH: Hemophagocytic Lymphohistiocytosis

MMUD: Mismatched Unrelated Donor

MUD: Matched Unrelated Donor

MSD: Matched Sibling Donor

OS: Overall Survival

RIC: Reduced Intensity Conditioning

SCD: Sickle cell disease

SCID: Severe Combined Immune Deficiency

TCD: T-cell depletion

TPO: Thrombopoietin

UCB: Umbilical Cord Blood

WAS: Wiskott-Aldrich syndrome

List of publications

In the following, a list of publications during the PhD program is given:

1. Amigoni A., Vettore E., Brugnolaro V., Brugnaro L., Gaffo D., Masola M., Marzollo A., Pettenazzo A., **High doses of benzodiazepine predict analgesic and sedative drug withdrawal syndrome in paediatric intensive care patients.** *Acta Paediatr*. 2014;103(12):e538–e543. doi:10.1111/apa.12777. IF (2014): 1,674

2. Tumino M., Serafin V., Accordi B., Spadini S., Forest C., Cortese G., Lissandron V., Marzollo A., Basso G., Messina C., **Interleukin-22 in the diagnosis of active chronic graft-versus-host disease in pediatric patients,** *Br. J. Haematol*. 2015;168(1):142–145. doi: 10.1111/bjh.13068 IF (2015): 4,971

3. Tumino M., Marzollo A., Gazzola M., Calore E., Mainardi C., Pillon M., Destro R., Gabelli M., Strano A., Barioni M., Messina C. **Cell Manipulation in Pediatric Haploidentical Stem Cell Transplantation: State of the Art.** *J Pediatr Biochem.* 2016;05(4):115–119. doi: 10.1055/s-0036-1572322 SJR: 0.147

4. Marzollo A., Calore E., Tumino M., Pillon M., Gazzola MV., Destro R., Colombatti R., Marson P., Tison T., Colpo A., Mainardi C., Gabelli M., Boaro MP., Rossin S., Strano A., Quaglia N., Menzato F., Basso G., Sainati L., Messina C. **Treosulfan-Based**

Conditioning Regimen in Sibling and Alternative Donor Hematopoietic Stem Cell Transplantation for Children with Sickle Cell Disease. *Mediterr J Hematol Infect Dis*. 2017;9(1):e2017014. doi:10.4084/MJHID.2017.014.

5. Zanato S., Traverso A., Tremolada M., Sinatora F., Porreca A., Pozziani G., Di Florio N., Capello F., Marzollo A., Tumino M., Cattelan C., Basso G., Messina C. **Psychopathological Aspects in Childhood Hematopoietic Stem Cell Transplantation (HSCT): The Perception of Parents and Adolescents.** *Front Psychol*. 2017;8(April):272. doi:10.3389/fpsyg.2017.00272.

6. Gabelli M., Marzollo A., Notarangelo LD., Basso G., Putti MC. **Eltrombopag use in a patient with Wiskott-Aldrich syndrome.** *Pediatr Blood Cancer*. June 2017. doi:10.1002/pbc.26692.

7. Sinatora F., Traverso A., Zanato S., Di Florio N., Porreca A., Tremolada M., Boscolo V., Marzollo A., Mainardi C., Calore E., Pillon M., Cattelan C., Basso G., Messina C. **Quality of Life and Psychopathology in Adults Who Underwent Hematopoietic Stem Cell Transplantation (HSCT) in Childhood: A Qualitative and Quantitative Analysis.** *Front Psychol*. 2017;8(AUG):1316. doi:10.3389/fpsyg.2017.01316.

8. Pillon M., Amigoni A., Contin A., Cattelan M., Carraro E., Campagnano E., Tumino M., Calore E., Marzollo A., Mainardi C., Boaro MP., Nizzero M., Pettenazzo A., Basso G., Messina C. **Risk Factors and Outcomes Related to Pediatric Intensive Care Unit Admission after Hematopoietic Stem Cell Transplantation: A Single-Center Experience.** *Biol Blood Marrow Transplant*. 2017;23(8):1335-1341. doi:10.1016/j.bbmt.2017.04.016.

9. Marzollo A., Bustamante J., Putti MC., Stronati M., Bendavid M., Casanova J., Basso G., Borghesi A. **Difetti immunitari non convenzionali in pazienti con infezione grave**. *Medico e Bambino*. 2017; 8:503-510

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