ORIGINAL RESEARCH PAPER

Pathology

KEY WORDS: Hematopoietic stem cell transplantation (HSCT), Anti-HLA, antibodies Allogenic, Bone marrow transplant (BMT), Major Histocompatibility Complex (MHC), Acute leukemia

ROLE OF HLA ANTIBODIES IN ALLOGENIC STEM CELL TRANSPLANTATION-PRE TRANSPLANT ASSESSMENT AND POST TRANSPLANT MONITORING

Dr Saurabh Moond	Senior Resident, Dept of Pathology, VMM College & Safdarjung Hospital, Delhi, India
Dr Kavita Sahai	Professor, Office of DGAFMS, Ministry of Defence, Delhi, India
Dr GPS Gahlot*	Associate Professor, Dept of Lab & Mol Med, Army Hospital (R & R) Delhi, India *Corresponding Author
Dr S Agarwal	Associate Professor, Dept of Pathology Command Hospital (Central Command) Lucknow India
Dr Pankaj Pandey	Senior Resident, Dept of Pathology, All India Institute of Medical Sciences, Rishikesh, Uttarankhand, India
Dr Chetan Agarwal	Senior Resident, Dept of Pathology, Sir Ganga Ram Hospital, Delhi, India
Dr Murari Lal Dhanetwal	Assistant Professor, Dept of Pathology, Jaipur National University Institute for Medical Sciences and Research Centre, Jaipur India

ABSTRACT

Background: Hematopoietic stem cell transplantation is a curative therapy for hematologic malignancies/ hematological immune disorders. It involves the transplantation of autologous or allogenic multipotent hematopoietic stem cells derived from bone marrow, peripheral blood or umbilical cord blood.

Aims: To study the prevalence of HLA antibodies in allogeneic HSCT recipients & monitor their post-transplant development of anti-HLA antibodies at different time points with their pre/post transplant correlation to engraftment or graft failure or other transplant outcome

Study design: We carried out the prospective observational study in fifteen cases of allogeneic HSCT over a period of 18 months in a Tertiary Care Superspeciality Hospital of Northern India

Subjects & methods: Flowcytometry based crossmatch & flow PRA (Class I/Class II) was used for pre-transplant & post-transplant HLA class I and class II cases. Antidonor antibodies were detected by complement dependent lymphocytoxicity (CDC) cross match and multiple short tandem repeat (STR) amplification methods using fluorescence labeled PCR combined with capillary electrophoresis.

Statistical Analysis Used: Mean+SD, Mcnemar's Chi-square tests were used for seeing the agreement between pre & post-transplant at different time points. P value <0.05 was considered significant and statistical analyses were performed using SPSS version 21.0.

Results: During the post transplantation follow up for at least 90 days, 4 out of 15 patients succumbed. A total of 13 patients (86.7 %) achieved sustained myeloid engraftment. The cumulative incidence of primary GF was 13.3 % and included GR (N =2). The three months overall survival (OS) was 80% with 95% CI of 306.74 - 520.11 and a mean of 413.43 days as shown in Kaplan Meier curve. It is proposed that sensitized patients who possess anti-HLA antibodies before or after the transplantation could benefit from modification of conditioning and immunosuppressive therapeutic approaches in the future.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is the transplantation of multipotent hematopoietic stem cells (HSC), usually derived from bone marrow, peripheral blood or umbilical cord blood. The era of HSCT began after first atomic bomb explosion in which radiation led to non-functionality of bone marrow. The first allogeneic HSCT was pioneered by E. Donnall Thomas in 1957. In 1979, Thomas reported a cure rate of 50% in acute myeloid leukemia (AML) patients transplanted in first remission. Newer HSCT's indications are congenital disorders of the hematopoietic system, Wiscott Aldrich syndrome, thalassemia, aplastic anemia, metabolic disorders and autoimmune disease.[1] In India's first successful allogeneic bone marrow transplantation (BMT) was done on 20th March 1983 at Tata Memorial Hospital in a nine-year-old girl suffering from AML.

Allogeneic HSCT appears to improve chances for cure or long-term remission after resolution of the immediate transplant-related complications. Allogeneic HSCT involves two people i.e. the donor and the recipient. The selection of optimal donor is based on suitable Human Leukocyte Antigen (HLA) typing and matching. The Major Histocompatibility Complex (MHC) contains >200 genes; situated on the short arm of chromosome 6 at 6p21.3. It is divided into three main regions: HLA class I (HLA-A, B, and C genes), class II (HLA-DR, DQ, and DP genes), and class III regions.[2] HLA molecules provides peptides to CD4 & CD8 T cells, enabling them to recognize, eliminate "foreign" particles and to prevent the recognition of "self" as foreign. The participation of humoral arm of immunity to HLA antigens is important to understand the enormous polymorphism, immunogenicity and heterogeneity of HLA genes.[2] Anti-HLA antibodies present in healthy individuals get sensitized by transfusions, pregnancy, failed previous grafts or alloimmunization in hematological disorders. [3] Advances in HLA matching and understanding of donor selection factors are therefore important to improve outcomes of unrelated donor (UD) and haploidentical HSCT. On the basis of origin, HSCT can be autologous (haematopoietic stem cells from the patient) or allogeneic (HLA matched donor). Myeloablative allogeneic

transplants comprises of chemotherapy or irradiation given immediately prior to a transplant is called the conditioning regimen, the purpose of which is to eradicate the patient's disease prior to the infusion of HSC and to suppress immune reactions. Non-myeloablative allogeneic transplants uses lower doses of chemotherapy and radiation, and has lower risks of serious infections and transplant-related mortality while relying upon the graft versus tumor effect to resist the inherent increased risk of cancer relapse [4]. In general, mismatches of the Class-I genes (i.e. HLA-A, HLA-B, or HLA-C) increase the risk of graft rejection whereas a mismatch of an HLA Class II gene (i.e. HLA-DR, or HLA-DQB1) increases the risk of graft-versus-host disease. HLA mismatches may occur at antigenic or allelic level due to recognition by immunocompetent T-cells that may lead to graft failure, delayed immune reconstitution, graft-versus-host disease (GVHD) and mortality. [5] GVHD; is T-cell mediated inflammatory disease that is unique to allogeneic transplantation. In GVHD, white blood cells of the graft recognize the recipient as "foreign" and then attack the host's body cells resulting in tissue damage and subsequent manifestation. [6] GVHD can also occur after a non-irradiated/non-leucodepleted blood transfusion. The acute or fulminant form (10-80%) of GVHD presents within first 100 days post-transplant mainly affect immune system, skin, liver and intestine whereas the chronic form (30-60%) presents after 100 days that occurred either as an extension of acute GVHD (progressive), after a disease free interval (quiescent), or with no precedent (de novo) [7].

In view of lack of HLA-matched donors, recent research is focused on the identification of permissible HLA mismatches i.e. partially matched donor. [6]In post-transplant cases, anti-HLA antibodies appear due to HLA disparity between the donor & recipient. Important clinical events as engraftment, relapse, and the effects of post-transplant therapies in allogenic bone marrow transplantation (BMT) can be monitored on a molecular level by detecting relative amounts of donor & recipient WBC in the peripheral blood of recipient in the post transplant period.

Haploidentical transplantation (donor; parent, sibling, or child) matched to the patient at least in one haplotype. Haploidentical transplantation using T cell depleted (TCD) grafts with a "mega dose" of stem cells (median of 13.8 million stem cells/kg than a median of 5 million stem cells/kg needed for matched related donor (MRD) transplantation) led to improvement in engraftment rates. Howeverhaploidentical transplantation is limited due to itshigher rate of non-engraftment as compared to MRD. Nowadays, almost 80% of allogeneic transplantations in adult patients >20 years are performed using peripheral blood stem cells.The HLA compatible suitable donor graft is analyzedand administered as an infusion to the patientfollowed by a monitored treatment period at home according to the home care program [8, 9].

The phenomenon of co-existence of cells from two different organisms (evolved from two different zygotes) in one body is called chimerism. Once donor and recipient hematopoiesis could coexist after allo-SCT in the recipientis called mixed chimerism, which might end in an 'autologous recovery'. Post transplant, if all hematopoietic cells are of donor origin, the patient is called a 'complete chimera.' and patients with complete chimerism can later develop a state of 'mixed chimerism' or vice versa. In mixed chimerism recipient cells could be normal hematopoietic cells or leukemic cells. The presence of these small numbers of hematopoietic malignant cells of host origin is associated with minimal residual disease (MRD).

The basic principle in the detection of chimerism is the utilization of the differences between donor and recipient polymorphic genetic markers or their products through methods like cytogenetics red cell phenotyping restriction fragment length polymorphism analysis (RFLP) and fluorescence in situ hybridization of sex chromosomes [10]. Newer faster method as polymerase chain reaction (PCR) technique for investigation of chimerism is preferred. Fluorescent labeling of the primers and resolution of PCR products with capillary electrophoresis allowed accurate quantification of the degree of mixed chimerism. Semiautomated PCR analyses using the appropriate hardware allowed moreover a high sample throughput [11]. PCR-based amplification of a highly polymorphic STR/VNTR system is considered to be the most informative and sensitive technique. Therefore the present study was undertaken to assess the prevalence of anti-HLA antibodies and their influence on the graft outcome in recipients undergoing allogeneic HSCT.

MATERIALS AND METHODS

This prospective study on 15 patients undergoing allogeneic bone marrow transplantation was performed from October 2015 to March 2017 at a tertiary care superspeciality hospital of Northern India. Detailed clinical, demographic information were recorded as per the designed performa containing HLA compatibility status, relationship of patient with the donor, record of the post–transplant immunosuppression, transplant outcome. Flowcytometry based crossmatch & flow PRA (Class I/Class II) was used for pre-transplant & post-transplant, antidonor antibodies detected by complement dependent lymphocytoxicity (CDC) cross match and multiple short tandem repeat (STR) amplification was done using fluorescence labeling PCR combined with capillary electrophoresis as per protocols provided with the kit. These patients were followed up regularly in the transplant clinic at D+30 and D+90 after transplant and there after once in three months. During the post-transplant period, a strict follow up of the following parameters (engraftment, graft failure, GVHD, overall event free survival) was maintained.

Table 2: Summary of various techniques employed

Sr No.	Sample	Technique	Aim
1.	EDTA anticoagulated whole blood	Kit Qiagen- QIAamp DNA blood mini kit(50)	DNA extraction
2.	DNA	PCR-SSP	HLA typing
3.	Recipient; Serum Donor: Heparinised whole blood	Complement dependent lymphocytotoxicity	CDC crossmatch
4.	Recipient: Serum Donor: Heparinised whole blood	Colour flow cytometer	Flow crossmatch
5.	Serum	Kit (ONE LAMBDA)	Flow Panel Reactive Antibodies (PRA)
6.	DNA	STR	Chimerism

Chimerism was calculated by observed peak areas of the informative markers. Standardization of the calculation procedure was done by noting the length of the donor and recipient alleles of each marker on the donor and

recipient DNA isolated before transplant. All markers were scored for their allele lengths. The relative positions of the donor and recipient alleles of a given marker determined their suitability for chimerism calculation. The possibilities were grouped in three allelic distribution types: (a) Type I: The alleles not shared by donor and recipient were used to calculate chimerism. Percentage of donor chimerism was

calculated by dividing the peak areas of donor allele(s) (Ad) by the sum of peak areas of donor (Ad) and recipient alleles (Ar). (b) Type II: One shared and one unshared allele had to be used to calculate chimerism. Schematic representation of complete (Figure 1) and mixed chimerism(Figure 2) has been depicted.

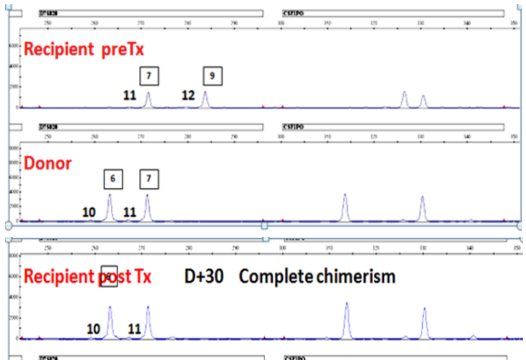

Figure 1: Schematic representation of assessment of Complete chimerism

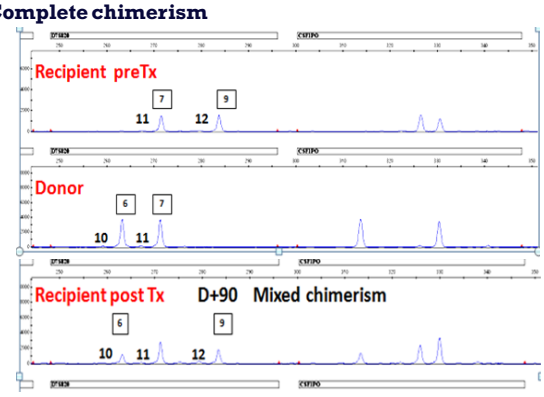

Figure 2: Schematic representation of assessment of mixed chimerism

Mean+SD for continuous variables and Mcnemar's Chi-square for categorical variables were used for seeing the agreement between Pre-transplant and Post-transplant at different timepoints. Non-parametric Wilcoxon signed rank test to evaluate the difference in the median values of **P**re-**t**ransplant **a**ssessment &**P**ost-**t**ransplant **m**onitoring anti HLA antibodies were used. Level of significance of 5%(P value <0.05) was used for statistical significance. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 21.0.

RESULTS

A total of fifteen consecutive cases (nine ABO compatible & six ABO incompatible grafts) of HSCT performed for 18 months duration were followed for a period of three months post transplantation. he median age of recipients was 11 years (age range 5 months-29 years) while of donors was 33 years (age range 8-58 years) & gender-wise 9 were male, 6 females (2 multiparous, 4 nulliparous) . Of the 15 recipient- donor pairs, six pairs had gender mismatch while nine had same gender. The median infused CD34+ cell doses were 5.00×10^6/kg (range, $0.39\text{-}16\times10^6$ /kg). Three of them developed severe GVHD & one relapsed led to succumb to their illness. The remaining twelve had well functioning grafts throughout the follow up period.

Primary indication for alloHSCT of recipients include **S**evere **A**plastic **A**nemia(SAA; 5), **A**cute **L**ymphoblastic **L**eukemia (ALL; 4), **A**cute **M**yeloid **L**eukemia (AML; 2), **W**iskott- **A**ldrich **S**yndrome (WAS; 2) followed by **C**hronic **M**yeloid **L**eukemia (CML; 1) and Familial **H**emophagocytic **L**ympho**h**istiocytosis (HLH; 1). Eight out of 15 recipients had anti-HLA antibodies, 3 had class I HLA antibodies and 5 had both class I & II HLA antibodies at pre-transplantation. Four out of eight antibodies positive & 7 other antibody negative cases were engrafted. Three of these eight patients were females and all three were having anti-HLA antibodies, two suffering from SAA and one from ALL. Two of three females were multiparous and both had anti-HLA antibodies indicating their association with parity. Anti-HLA antibodies in nulliparous female could be due to previous blood transfusion.

Post transplantation 13out of 15 patients survived at D+30 days and 12 at D+90. All the patients were assessed for anti-HLA antibodies, GVHD and chimerism post transplantation. Two patients who had graft failure and died of sepsis on D+12 had presence of anti-HLA antibodies during pre-transplantation screening. Though, six patients engrafted even after the presence of anti-HLA antibodies in pre-transplantation screening.

A total of 8/13 (61.6%) patients were having anti-HLA antibodies at D+30. One patient had only class I anti-HLA antibodies while remaining 7 had both class I and II PRA antibodies. Out of these 8 patients, three patients developed de novo anti-HLA antibodies post-transplantation whereas other fivepatients had antibodies at pre-transplantationas well as post-transplantation. One patient who was positive for anti HLA antibody during pre transplant assessment turned negative at D+30.

This study suggests that anti-HLA antibodies may develop post-transplantation also or may remain present even after radiotherapy and myelosuppressive regimens. Factors responsible for decrease/disappearance of anti-HLA antibodies are not understood; therefore more studies with large sample size and longer follow up are required to establish these findings. Chimerism: Out of 13 patients who survived at D+30, 12 had complete chimerism and one had mixed chimerism (92%) at D+30 whereas at D+90 all evaluable patients had complete chimerism.

Out of 13 patients at D+30 days after transplant, the cumulative incidence of grade 2 to 4 acute GVHD was 33.3%. At D+90 days after transplant, out of 12 evaluable patients, the cumulative incidence of grade 2 to 4 acute GVHD was 53.36%. Development of class I and class II anti-HLA antibodies pre-transplantation and post transplantation, D + 30 as well as at D + 90 days did not show any statistical significance with graft relapse, survival or GVHD.

Pre transplant as well as post transplant cross match was negative in all the cases. During the post transplantation follow up for at least 90 days, 4 out of 15 patients succumbed. A total of 13 patients (86.7 %) achieved sustained myeloid engraftment. The cumulative incidence of primary GF was 13.3 % and included GR (N =2). The three months overall survival (OS) was 80%with 95% CI of 306.74 - 520.11 and a mean of 413.43 days as shown in Kaplan Meier curve.

DISCUSSION

Almost 30-40% of all HSCTs performed worldwide comprises of allogeneic transplantation. As selection of donors is crucial so as to avoid/minimize post-allogeneic stem cell transplant complications, therefore anti-HLA antibodies screening should be performed in all HSCT settings of non-identical HLA donors.

This study was performed to evaluate the prevalence of HLA-antibodies in patients undergoing allogeneic stem cell transplantation and to monitor the post transplant development of anti-HLA antibodies at D+30 and D+90 days. We analyzed prospectively the correlation between the presence of pre-transplant and post-transplant donor specific anti-HLA antibodies and development of primary graft failure (PGF) in 15 patients undergoing allogeneic HSCT at a single institution.

In the present study, 8/15 (53.3%) of our patients showed donor specific anti HLA antibodies whereas similar results of 20-86.6% have been observed in other studies [12-14]. Two females (2/6) were parous with >2 pregnancies each and 4 were nulliparous. Both parous females had donor specific anti-HLA antibodies suggesting their stronger association with parity. 5 males and 3 females developed anti-HLA antibodies with history heavily transfusion prior to transplant due to their primary hematologic disease of all 5 males suggesting blood transfusion as a major cause of alloimmunization similar to study done by Ciurea et al. [15]. Humoral rejection, through preformed antibodies appeared to be the primary mechanism responsible for graft failure than cellular immunity.

2/8 (25%) of four DSA positive patients developed graft failure while none of 7 DSA negative group experienced graft failure. [14] Moreover, only two patients (from DSA positive group) out of a total cohort of 15 patients succumbed to complications, despite all patients received similar median numbers of CD34+ cells thereby negating the bias of stem cell dose. [12-13, 15-17]

The details of our cases succumbed to illness are; (a) A 2 year old boy of severe aplastic anemia with pre-transplant class I PRA of 21%, negative for class II PRA & CDC crossmatch underwent haploidentical ABO compatible bone marrow transplant from mother as donor. (b) An 8 year old boy with severe aplastic anemia & pre-transplant class I& II PRA of 39% & 32% and negative CDC crossmatch. He had ABO compatible haploidentical bone marrow transplant against negative CDC crossmatch. (c) An 8 yr old boy with severe aplastic anemia & pre-transplant class I & II PRA of 14% & 92%, with negative CDC cross match underwent ABO compatible haploidentical bone marrow transplant. (d) A 6 month old boy with Familial HLH pre-transplant class I& II PRA of 27% & 40%, against negative CDC crossmatch underwent ABO compatible haploidentical bone marrow transplant with mother as a donor. All patients received standard treatment thereafter, however succumbed to illness either due to sepsis or graft failure.

In our study, 3 out of total 5 patients with aplastic anemia had adverse outcome as compared to none out of six leukemia cases (p=0.05). This may be attributed to greater number of blood transfusions in aplastic anemia patients leading to highly sensitized patients with post transplant increase in antibody levels.

CONCLUSION

Our results indicate that preformed anti-HLA antibodies can be detected before and may also appear after transplant in allo-HSCT recipients. (8/15) 53% of patients showed prevalence of anti HLA antibodies as evidenced by presence of class I/II PRA in allogeneic sensitized population. In 5 patients anti-HLA antibodies were detected pre & post transplantation; may signify that these antibodies were not destroyed during myeloablative conditioning treatment or by standard immunosuppressive therapy.

The patients with a pregnancy or blood transfusion history had a higher anti-HLA antibody positive rate and thus associated with graft rejection. As the number of haploidentical HLA matched/partially matched/mismatched transplantations has been rising in present era therefore more multicentric studies with large sample size, longer follow-up period & with allele specific anti-HLA antibodies are required to reach a statistically significant data. It is possible that sensitized patients who possess anti-HLA antibodies before or after the transplantation could benefitfrom modification of conditioning and immunosuppressive therapeutic approaches in the future.

REFERENCES

1. Shah: Haematopoetic stem cell transplantation at apollo group hospitals. Molecular Cytogenetics, 2014;7(Suppl 1):I60.
2. Koclega A, Markiewicz M, Siekiera U, Dobrowolska A, Sylwia M, Dzierzak-Mietla M et al.The presence of Anti-HLA antibodies before and after Allogeneic hematopoietic stem cells transplantation from HLA-mismatched unrelated donors. Bone Marrow Res. 2012;24:539825.
3. L. E. Morales-Buenrostro, P. I. Terasaki, L. A. Marino- V´azquez, J. H. Lee, N. El-Awar, and J. Alber ´ u, "Natural human leukocyte antigen antibodies found in nonalloimmunized healthy males," Transplantation, 2008;86(8):1111-15.
4. Alyea EP, Kim HT, Ho V, et al. "Impact of conditioning regimen intensity on outcome of allogeneic hematopoietic cell transplantation for advanced acute myelogenous leukemia and myelodysplastic syndrome". Biol. Blood Marrow Transplant 2006;12(10):1047-55.
5. J. Apperley, E. Carreras, E. Gluckman, and T. Masszi, Haematopoietic Stem Cell transplantation-The EBMT-ESH Handbook;6th edition; 2012.
6. Park M, Seo JJ. Role of HLA in hematopoietic stem cell transplantation. Bone Marrow Res. 2012; 680841. DOI:10.1155/2012/680841.
7. Sullivan KM, Agura E, Anasetti C, et al "Chronic graft-versus-host disease and other late complications of bone marrow transplantation". Semin Hematol. 1991;28:250.
8. Ringden O, Remberger M, Holmberg K, Edeskog C, Wikstrom M, Eriksson B, et al. Many days at home during neutropenia after allogeneic hematopoietic stem cell transplantation correlates with low incidence of acute graft-versus-host disease. Biol Blood Marrow Transplant. 2013;19(2):314-20.
9. Ringden O, Remberger M, Torlen J, Engstrom M, Fjaertoft G, Mattsson J, et al. Home care during neutropenia after allogeneic hematopoietic stem cell transplantation in children and adolescents is safe and may be more advantageous than isolation in hospital. Pediatric transplantation. 2014;18(4):398-404.
10. Seong CM, Giralt S, Kantarjian H et al. Early detection of relapse by hypermetaphase fluorescence in situ hybridization after allogeneic bone marrow transplantation for chronic myeloid leukemia. J Clin Oncol. 2000;18:1831-36.
11. Chalandon Y, Vischer S, Helg C et al. Quantitative analysis of chimerism after allogeneic stem cell transplantation by PCR amplification of microsatellite markers and capillary electrophoresis with fluorescence detection: the Geneva experience. Leukemia. 2003;17:228-31.
12. S. O. Ciurea, P. F. Thall, X. Wang et al., "Donor-specific anti-HLA Abs and graft failure in matched unrelated donor hematopoietic stem cell transplantation," Blood. 2011;118(22):5957-64.
13. S. Yoshihara, E. Maruya, K. Taniguchi et al., "Risk and prevention of graft failure in patients with preexisting donor specific HLA antibodies undergoing unmanipulated haploidentical SCT," Bone Marrow Transplantation. 2012;47(4):508-15.
14. Y. J. Chang, X. Y. Zhao, L. P. Xu et al., "Donor-specific anti-human leukocyte antigen antibodies were associated with primary graft failure after unmanipulated haploidentical blood and marrow transplantation: a prospective study with randomly assigned training and validation sets," Journal of Hematology & Oncology. 2015;8(84).
15. S. O. Ciurea, M. de Lima, P. Cano et al., "High risk of graft failure in patients with anti-HLA antibodies undergoing haploidentical stem-cell transplantation," Transplantation. 2009;88(8):1019-24.
16. C. Cutler, H. T. Kim, L. Sun et al., "Donor-specific anti-HLA antibodies predict outcome in double umbilical cord blood transplantation," Blood. 2011;118(25):6691-97.
17. A. Ruggeri, V. Rocha, E. Masson et al., "Impact of donor specific anti-HLA antibodies on graft failure and survival after reduced intensity conditioning-unrelated cord blood transplantation: a Eurocord, Soci´et´e Francophone d'Histocompatibilit´e et d'Immunog´en´etique (SFHI) and Soci´et´ eFranc¸aise de Greffe de Moelle et de Th´erapieCellulaire (SFGM-TC) analysis," Haematologica, 2013;98(7):1154-60.