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## **Typing of KIR genes in unrelated hematopoietic stem cell transplants: influence of the donor KIR genotype on the outcome of the transplant.**

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CICLO XXIX

ANNO CONSEGUIMENTO TITOLO 2017

*Dedicata a mio Padre  
che mi ha sempre sostenuto*

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## **Abstract**

*In recent years, the anti-leukemic potential of Natural Killer (NK) cells and their role in hematologic malignancies and in Hematopoietic Stem Cell Transplants (HSCT) has attracted greater interest and a recent study by Cooley S. et al. showed a better clinical outcome when patients with Acute Myeloid Leukemia received a transplant from unrelated Group B KIR haplotypes donors. As a consequence of these results, an algorithm called "KIR B-content score" was formulated, based on the number of centromeric and telomeric Group B KIR haplotypes gene-content motifs. The KIR B-content score defines three categories of donors: Neutral donors have none or one KIR B motifs, Better donors have two or more B motifs without KIR Cen B/B and Best donors have two or more B motifs with KIR Cen B/B. The aim of our research is a retrospective analysis of HSC unrelated transplants performed in our center to analyze the effect of the donor KIR B status on the clinical-outcome. Our results showed a better overall survival-rate in the AML recipients, HLA mismatched with the donor, when the donor KIR B content status is Best/Better (37% vs 18% at three years  $P=0,028$ ), whereas there is no beneficial effect for recipients with other haematological malignancies. Moreover, we observed that AML recipients, whose Donors KIR B status was Best/Better, had more incidence of aGvHD grade I II than patients whose Donors KIR B status was Neutral (70% vs 26%) and also a lower rate of relapse (36% vs 58%) and a better Disease Free Survival-rate (58% vs 38% at three years  $P=0,1$ ) because of a better GvL effect.*

**Keywords:** UDHSCT, NK Cells, Donor KIR B Status, AML, GvL effect.

## **1. Introduction**

### **1.1 Hematopoietic stem cell transplantation (HSCT)**

Hematopoietic stem cell transplantation (HSCT) has emerged as one of the most useful therapeutic strategies in the treatment of hematologic malignancies (acute or chronic myeloid or lymphoid leukemia) or hereditary (Thalassemia Major) for which conventional therapies offer little or no chance of cure. HSCT consists in the replacement of the diseased or non-functional bone marrow with healthy stem cells capable of regenerating all blood cells and restoring the normal hematologic and immunologic functions. The source of Hematopoietic Stem Cells (HSC) can be Bone Marrow (BMT), Peripheral Blood after appropriate stimulation (PBHSCT) or Cord Blood (CBHSCT). The transplant can be autologous (the patient's own HSC transplant after suitable treatment) or allogeneic (HSC transplant from a healthy donor). In the latter case it is essential to find a donor with genetic characteristics similar to those of the receiver because one of the most important variables that influence the success of HSCT is the compatibility of the genes of the HLA system. The Class I and II HLA matching between donor and recipient is crucial to prevent alloreactivity and, consequently, rejection, graft failure and, above all, the graft versus host disease (GVHD), because the transplanted cells from the donor are immunologically competent and can attack cells and tissues of the recipient [1-3].

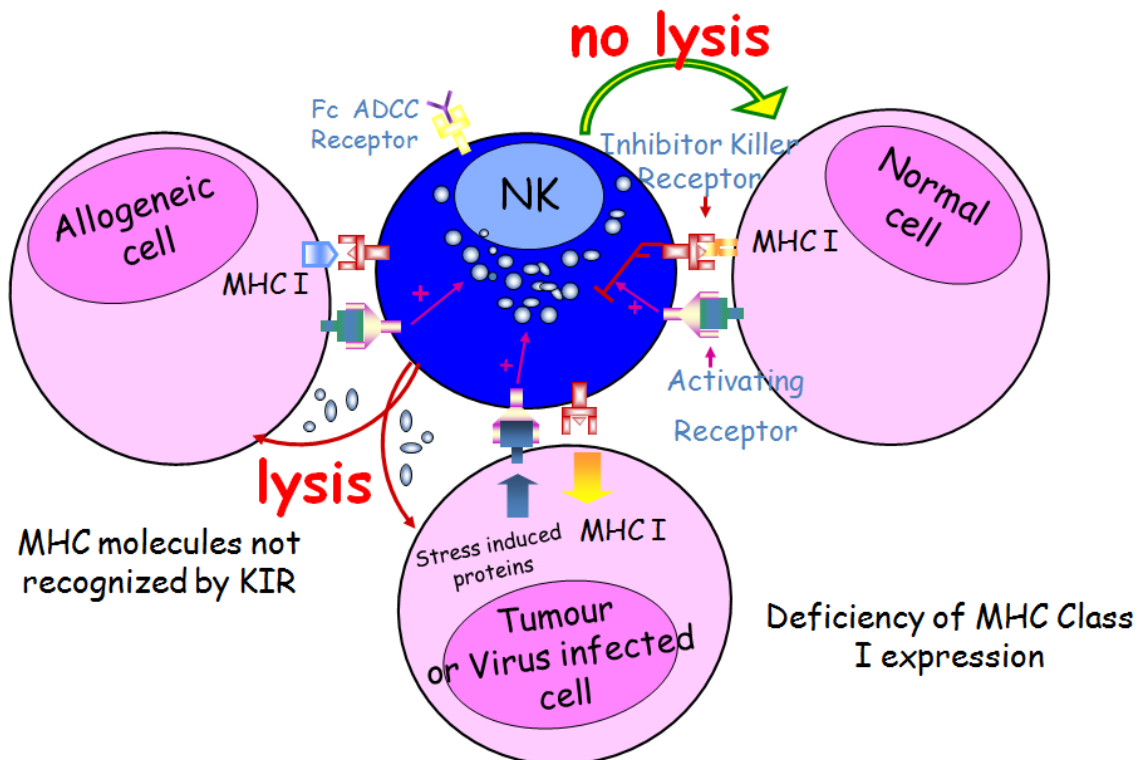
A very important aspect of the HSC transplant is the Graft versus Leukemia effect (GvL), which is a GvHD directed toward the leukemic cells: donors' T lymphocytes attack the cells of the tissues causing GvHD but they can also attack and eliminate residual leukemic cells [4]. If the donor-recipient HLA compatibility is high,

the GvL effect is low, consequently, the possibility of relapse is high; on the contrary, when the HLA compatibility is low, the GvHD reaction will be great. A major challenge in improving the success of allogeneic hematopoietic stem cells in the treatment of leukemia is to minimize GvH reactions and simultaneously preserve and optimize GvL reactions. It has also been demonstrated that the GvL effect may be mediated by NK cells [5].

It has been shown that only 25% of patients who need a HSC transplant find an HLA-identical sibling donor; the remaining 75% do not have an HLA-identical donor in their own family; in this case the alternative is to look for a HLA-matched, unrelated volunteer donor (MUD Matched Unrelated Donor) in the worldwide register of bone marrow donors. If there is no compatible donor, the choice of the haploidentical donor is a viable alternative, because there might often be half-identical donors such as parents or siblings [6]. Recently, the number of so-called alternative donor transplants, MUD and haploidentical transplants with one or more HLA mismatches, has significantly increased. This is due to the rise in the average age of the patients and therefore the impossibility of finding a compatible donor in their own family. For this reason research has steered toward the study of factors other than HLA matching, which could have a positive influence on the outcome of the transplants, including the role of Natural Killer (NK) cells in hematopoietic stem cell transplants and, consequently, the study of KIR genes.

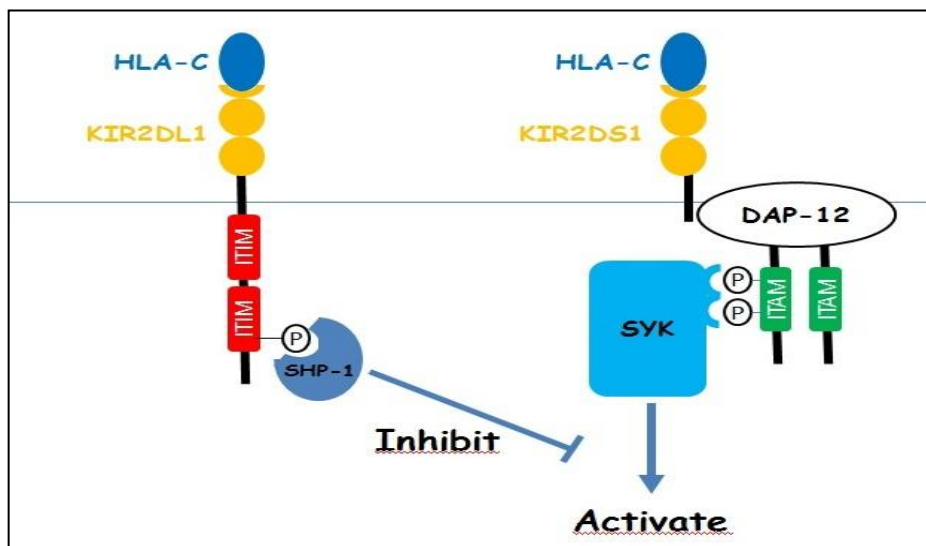
## 1.2 Natural Killer Cells and KIR receptors

The human NK cells are a part of the immune system with an important role in the host's defense against infections from pathogens and in "immune surveillance" against cancer cells. Their function depends on several families of activating and inhibitory receptors including KIR receptors (killer-cell immunoglobulin-like receptors). The "missing-self" concept [7, 8], put forward by Karré and colleagues in the 1980s, formed the basis for understanding the mechanisms of the action of NK cells (Figure 1). According to this hypothesis the function of NK cells is to recognize and kill autologous cells which are deficient in MHC class I expression, a frequent event in tumour or virus-infected cells.



**Figure 1.** "Missing-self hypothesis" explains the mechanism of action of NK Cells.

KIRs belong to the immunoglobulin super-family and are structurally characterized by two or three extracellular immunoglobulin-like domains. KIRs recognize the MHC class I molecules such as HLA-A, HLA-B and HLA-C. There are two distinct groups of KIRs: inhibitors and activators. The family of KIR genes, located on chromosome 19q13.4, includes 15 different loci [9]. Receptors are monomeric (single chain) with two (KIR2D) or three (KIR3D) immunoglobulin domains, which can be further divided into those with a long (Long) cytoplasmic tail (KIR2DL and KIR3DL) and those with a short (Short) one (KIR2DS and KIR3DS). The short tail generates an activation signal, while the long one generates an inhibition signal (Figure 2). Each group has the same extracellular domain, and consequently each group binds the same ligands. However, due to differences in their transmembrane and intracellular domains, a group of KIRs determines an inhibitory response, while the other group determines an activating response [10]. KIR receptors and their ligands are summarized in Table 1.



**Figure 2.** The inhibitory signal results from the presence of the long cytoplasmic tail containing tyrosine inhibitors motifs (ITIM: "immunoreceptor Tyrosine-based Inhibition Motifs"); the activating signal results from the presence of the short cytoplasmic tail containing tyrosine activation motifs (ITAM: "Immunoreceptor Tyrosine-based Activation Motifs"). Figure based on [10].



**Table 1.** KIR Receptors and their Ligands.

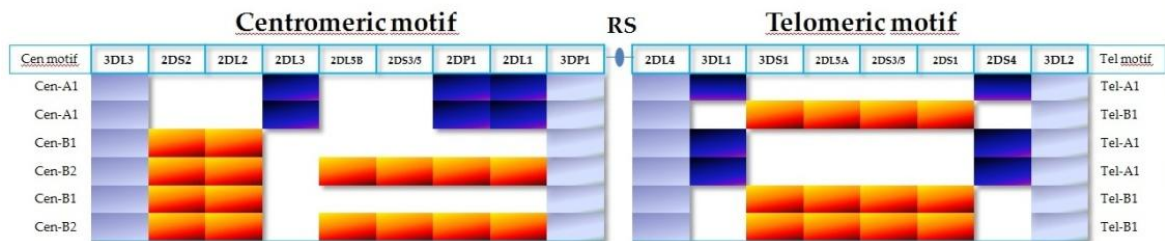
<b>KIR Receptors</b>	<b>KIR Ligands</b>
KIR2DL1 KIR2DS1	<b>Group 2</b> HLA-C Asn77 Lys80 (w2, w4, w5, w6)
KIR2DL2 KIR2DL3 KIR2DS2	<b>Group 1</b> HLA-C Ser77 Asn80 (w1, w3, w7, w8)
KIR3DL1	HLA-Bw4
KIR3DL2	HLA-A3,-A11
KIR2DL4	HLA-G
KIR2DL5 KIR3DL7 KIR2DS4 KIR2DS5 KIR3DS1	<u>Unknown</u>

In normal cells the expression of MHC class I molecules and their binding to NK inhibitory receptors inhibits the lysis, while in the virus-infected or cancer cells the lack of expression of self MHC molecules determines their susceptibility to lysis mediated by NK cells. In tumour or virus infected cells, stress-induced molecules are expressed on the cell surface and are recognized by the activating receptors that enhance the lysis. NK cells can also lyse allogeneic cells expressing HLA class I antigens that are not recognized by their inhibitory NK receptors.

The KIR genes are polymorphic in humans and there are different allelic variants; each individual possesses his own KIRs repertoire depending on the allelic variants possessed. Different haplotypes contain a different number of KIR genes; some have only one or a few activating receptors, others have more activating receptors. In humans, two groups of KIR haplotypes have been identified, A and B, based on the content of different KIR genes. Haplotypes of group A are present in the entire population and consist of six KIR inhibitory genes KIR3DL3, KIR2DL3, KIR2DL1,

KIR2DL4, KIR3DL1, KIR3DL2 and one activator KIR2DS4, which is often present in a null allele variant that is not expressed on the cell surface. Group B comprises haplotypes with a different genetic content including genes KIR2DS2, KIR2DL2, KIR2DL5, KIR2DS3, KIR2DS1, KIR2DS5, and KIR3DS1, which are not part of the haplotypes of Group A. As a consequence, many haplotypes in Group B encode for a greater number of activating receptors compared to haplotypes in Group A (Figure 3).

All individuals can be classified on the basis of the two KIR genotypes possessed: A/A, which is homozygous for the haplotypes of Group A, B/x, which contains one (heterozygous A/B) or two haplotypes of Group B (homozygous B/B). Individuals B/x possessing a larger number of activating receptors theoretically should have a better response against cancer or virus-infected cells.



**Figure 3.** The organization of genes in the KIR locus. The centromeric and telomeric regions are separated by a unique recombination site (RS) sequence that can function to reassort the centromeric and telomeric gene motifs. 3DL3, 3DP1, 2DL4 and 3DL2 are conserved framework genes. B haplotype genes are orange, and A haplotype genes are blue. Figure based on [14].

### 1.3 Role of NK Cells in HSCT

In recent years, the anti-leukemic potential of Natural Killer (NK) cells and their role in hematologic malignancies and in HSCT has been attracting ever greater interest; however, it has not yet been clearly established whether the typing of KIR genes may be a useful tool in the selection of an HSC donor to promote a better outcome of the transplant [11].

The beneficial GvL effect, promoted by allo-reactive NK cells derived from the donor, was demonstrated for the first time in the HSC haploidentical transplants in patients with acute myeloid leukemia (AML); the best donor is NK mismatched with the recipient and therefore the donor's KIRs do not recognize HLA class I molecules expressed by the host. In this way the donor's NK cells are responsive to the direction GvH and this results in a better anti-leukemic effect with a better overall survival-rate and a lower rate of relapse of leukemia [12].

More recently, Cooley S. et al. [13] have demonstrated that AML patients who received HSC transplants from unrelated Group B KIR haplotype donors, had a better outcome after transplant, whereas recipient KIR genotype had no effect. The clinical outcome was better when the donors had one or two KIR B haplotypes (KIR B/x donors) rather than donors who had two KIR A haplotypes (KIR A/A donors); with a KIR B/x donor, relapse was reduced and the leukemia-free survival-rate (LFS) was increased. This is because the haplotypes of Group B have a larger number of activating receptors, and this translates into better anti-leukemic activity. A subsequent study [14] sought to determine whether the protective effect of KIR B could be mapped to either the centromeric or the telomeric region of the KIR locus. The centromeric region

contains genes encoding the inhibitory receptors for the C1 and C2 epitopes of HLA-C, whereas the telomeric region contains genes encoding the inhibitory receptors for the Bw4 and A3/11 epitopes and the activating C2 receptor (Table 2). It was found that both the centromeric and telomeric regions of KIR B correlated with protective effect, but the much stronger association was with the centromeric region. As a consequence of these results, an algorithm was formulated, called "KIR B-content score", based on the number of centromeric and telomeric Group B KIR haplotypes gene-content motifs. The KIR B-content score defines three categories of donors: Neutral donors have none or one KIR B motifs, Better donors have two or more B motifs without KIR Cen B/B and Best donors have two or more B motifs with KIR Cen B/B (Table 3). A calculator for classification of the donor KIR B status (Best, Better, Neutral) may be found at <http://www.ebi.ac.uk/ipd/kir/>.

Recently, these results have been confirmed in HSCT from HLA-identical sibling donor [15]. Together, these results highlight the need for further studies of KIR polymorphisms, possibly at allelic level, to determine whether the typing of the donor KIR genes may be useful in donor selection for HSCT.

**Table 2.** Groups used for the Cen and Tel analysis on the basis of the content of the inhibitory (L-long) or activating (S-short) KIR genes. Table based on [14]

<u>Centromeric (2DS2, 2DL2, 2DL3)</u>	
<b>Cen-A/A</b>	2DL3 only
<b>Cen-A/B</b>	2DL3 with 2DS2 and/or 2DL2
<b>Cen-B/B</b>	2DS2 and/or 2DL2; no 2DL3
<u>Telomeric (3DL1, 3DS1, 2DS1, 2DS4)</u>	
<b>Tel-A/A</b>	3DL1 and 2DS4 only
<b>Tel-A/B</b>	3DL1 and 2DS4 with 3DS1 and/or 2DS1
<b>Tel-B/B</b>	Lacking 3DL1 and/or 2DS4

**Table 3.** KIR B-content score. The KIR B-content score is calculated by adding the number of Cen-B and/or Tel-B motifs in each genotype. Table based on [14]

Donor KIR Genotype	B content score	<u>Cen</u>	Tel	Donor KIR B Status
A/A	0	A/A	A/A	Neutral
B/x	1	A/A	A/B	
		A/B	A/A	
	2	A/A	B/B	Best/Better
		A/B	A/B	
	3	B/B	A/A	
		A/B	B/B	
4	B/B	A/B		
		B/B	B/B	

## **2. Research aims**

The aim of our research is a retrospective analysis of HSC transplants performed at the Bone Marrow Transplant Unit (UTMO) of the “V. Cervello” Hospital in Palermo. The category of HSC transplants to be studied is the one from MUD.

The objectives of this study are the following:

1. Typing of the donors' KIR genes of the transplants performed;
2. Donors classification in accordance with the KIR B Content Score in three categories: Neutral, Better, Best;
3. Correlation of the Donor KIR B status with the clinical outcome of the transplants considering overall survival, the degrees of Graft versus Host Disease (GvHD) Graft versus Leukemia (GvL), Relapse and Disease Free Survival (DFS).

### 3. Materials and Methods

#### 3.1 Patient cohort

We analyzed no. 89 patients, who received a URD HSCT between 1996 and 2013. The median age of the 89 patients was 43 years (14-65). The transplanted patients had different hematological malignancies: 33 Acute Myeloid Leukemia (AML), 19 Acute Lymphoblastic Leukemia (ALL), 9 *Myelodysplastic Syndromes* (MDS), 6 Non Hodgkin Lymphoma (NHL), 6 Multiple Myeloma (MM), 5 Hodgkin Lymphoma (HL), 4 Chronic Myeloid Leukemia (CML), 3 Chronic Lymphocytic Leukemia (CLL), 1 Aplastic Anemia Syndrome (AAS), 1 Fanconi Syndrome, 1 Ematodermic Neoplasia, 1 Biphenotypic Acute Leukemia (BAL). The HSC source was: Bone Marrow no. 19, Peripheral Blood no. 70. Complete high-resolution, allele-level HLA-A, B, C, DRB1 and DQB1 typing of the 89 pairs of recipients and donors was previously performed: 32 pairs were 10/10 matched, 33 pairs were 9/10 and 24 were  $\leq 8/10$ . Patient and transplant characteristics are summarized in Tables 4 and 5.

**Table 4.** Patient characteristics

<b>Median age (range)</b>	43(14-65)
<b>Diagnosis</b>	
AML	33
ALL	19
MDS	9
NHL	6
MM	6
LH	5
CML	4
CLL	3
AAS	1
Fanconi Syndrome	1
Ematodermic Neoplasia	1
BAL	1



**Table 5.** Transplants characteristics

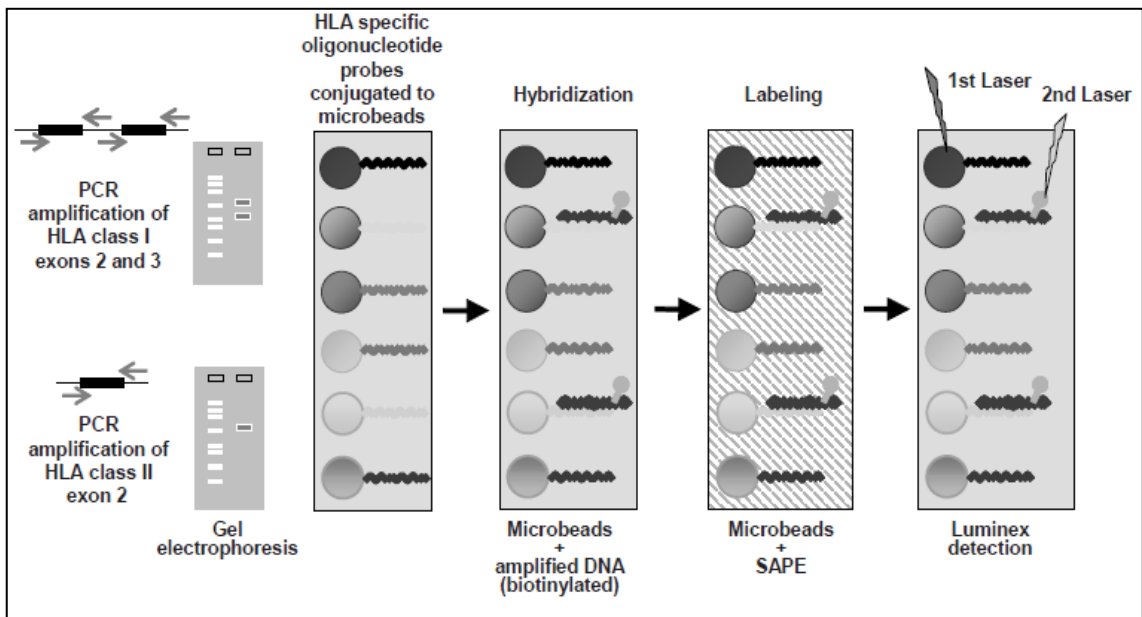
<b>Graft Type</b>	
Bone Marrow	19
Peripheral Blood Progenitor Cells	70
<b>Conditioning</b>	
Busilvex-Ciclofosfamide	15
Busilvex-Fludarabine	15
Fludarabine-Melphalan	4
Thiotepa + others	7
Ciclofosfamide + others	14
Endoxan	4
Unknown	32
TBI	6
ATG	12
<b>Donor/ Recipient HLA-allele matching at A, B, C, DRB1 and DQB1</b>	
10/10	32
9/10	33
≤8/10	24

### 3.2 KIR genotyping

The donors' DNA, which has already been typed for HLA, has been preserved in our bio-bank; about 80% of the samples is still available. The DNA was extracted from buffy coat or whole blood by the automatic extractor Maxwell 16 (Promega) or by the salting out method.

Typing of KIR genes was performed with PCR-SSO Luminex, the same method already used in our laboratory to type the HLA genes. The kit used for KIR genes typing was the SSO KIR Genotyping Test (One Lambda Inc., Canoga Park, CA) and the method was performed in accordance with the manufacturer's instructions. Luminex technology is based on the ability to soundly measure multiple analytes simultaneously in a single reaction and this is done through the use of plastic microbeads (microspheres), which are color-coded with two fluorescent dyes so as to emit two different wave lengths in red and infrared. In this way a set of over 100 different microspheres can be created. The microspheres represent the solid support of oligonucleotide probes which can bind amplified DNA, which is labeled with a fluorescent labeled reporter. Subsequently, the microbeads are acquired by the Luminex machine through a precision sheath fluid system, based on classical flow cytometry, which aligns them in single file, where they pass through two lasers: the red laser excites the colours inside the microbeads to identify which microbead is currently being read while the green laser excites the colour on the microbead surface, i.e. the labeled reporter tag. Finally, the color signals are detected by an advanced optical system, and the signals are processed into data for each reaction [16].

The PCR reaction using specific primers for the KIR genes is first conducted with a specific amplification program via the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The amplification products are tested by electrophoresis on 2% agarose gel and then observed and photographed under a UV trans illuminator. The PCR product is biotinylated, which allows it to be detected by streptavidin-conjugated with PE (SAPE). Subsequently, the PCR products are denatured facilitating rehybridization to complementary DNA probes bound to the microbeads with a specific sequence (SSO) to recognize the polymorphic sites within KIR genes. The amplified products which have undergone a hybridization reaction are labeled with streptavidin conjugated with R- Phycoerythrin and then acquired with the flow cytometry system LABScan™ 100 Luminex (Figure 5), which measures the fluorescence intensity of phycoerythrin on each microsphere (The steps of PCR-SSO method are summarized in Figure 4). The data are then analyzed with the software HLA –Fusion (Figure 6).



**Figure 4.** The steps of PCR-SSO (Sequence Specific Oligonucleotide).



**Figure 5.** The Luminex machine: the microbeads are transported into the Luminex analyzer by using a precision sheath fluid stream to align them in single file, where they pass through two lasers, similarly to classical flow cytometry. The red laser excites the colours inside the microbeads to identify which microbead is currently being read. The green laser excites the colour on the microbead surface, i.e. the labeled reporter tag. Finally, the color signals are detected by an advanced optical system, and the signals are processed into data for each reaction.

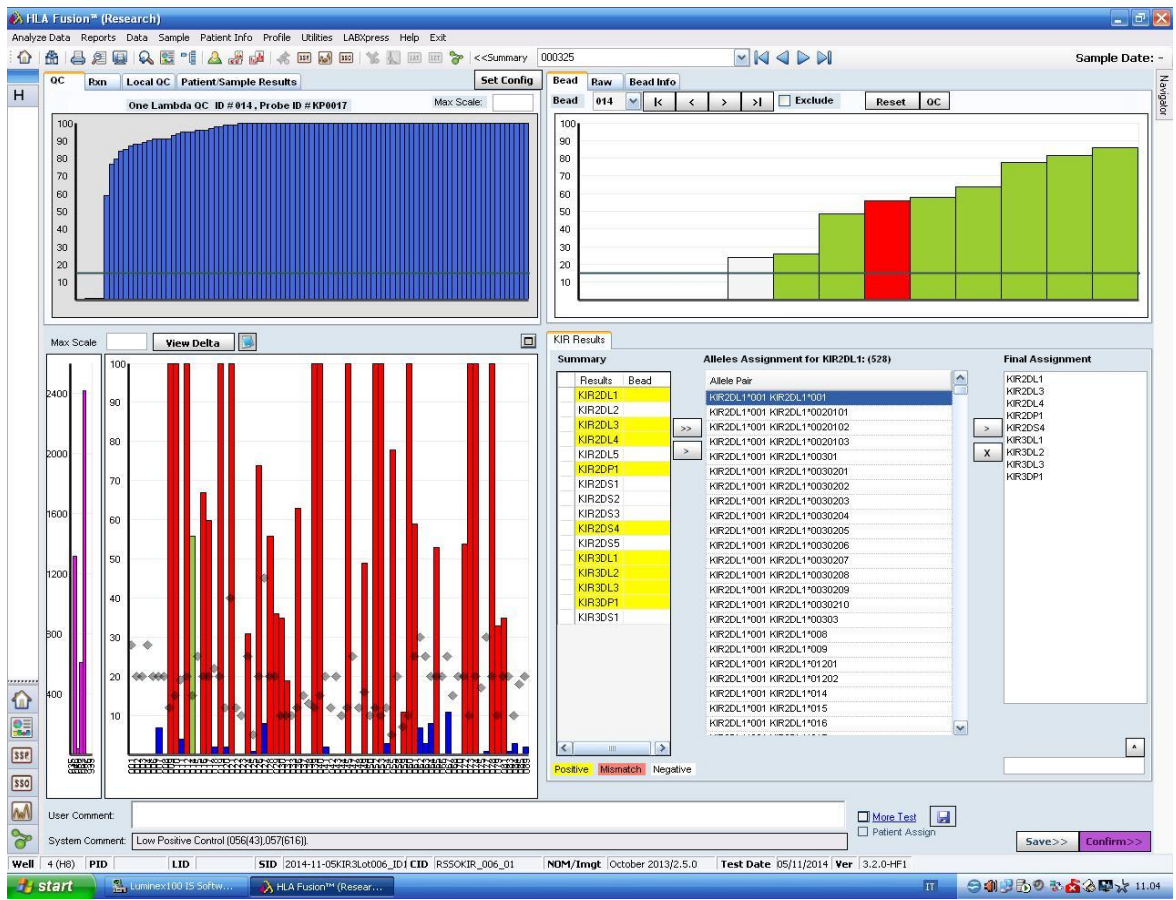


Figure 6. Fusion Software is used to analyze results of PCR-SSO Luminex.

## 4. RESULTS

### 4.1 Donors classification

We typed KIR genes of the 89 selected donors of the transplanted patients and we classified them on the basis of the KIR haplotypes possessed.

Figure 7, 8 and 9 are reports of three donors KIR typing: in the first case there is KIR2DS4 and so it is Group A Haplotype and the donor KIR B status is Neutral; in the second one there are KIR2DS1 and KIR2DS3 and so it is Group B/x Haplotype and the donor KIR B status is Better; in the third there is KIR2DS2 and so it is Group B/x Haplotype and the donor KIR B status is Best.

KIR gene frequency among the donor population was similar to that of published data [17, 18]: no. 30 (33,7%) donors were A/A and no. 59 (66,3 %) were B/x.

Then we classified them using the Donor KIR B Content Score Calculator at <http://www.ebi.ac.uk/ipd/kir/>: no. 63 (70,8%) donors were neutral, no. 18 (20,2%) Better and no. 8 (9%) Best. In our analysis we combined the Better and Best groups to form the KIR Better/Best donor group (with two or more B motifs). The classification of the donors in accordance with the KIR B content score is summarized in Table 6.

**KIR-SSO**

---

Lab Name: Tipizzazione HLA Molecolare  
 Institute: A. O. Cervello  
 Street: Via Trabucco, 180  
 Lab City: Palermo  
 Region:  
 Country:  
 Notes:

Lab Code:  
 Contact: Dr.ssa V. Cappuzzo  
 Email:  
 Phone:  
 Fax:

---

Patient ID:  
 Molecular Typing:  
 Serology Typing:  
 Sample ID: **00046002**  
 Sample Date:  
 Test Date: ago 21, 2014

Allele Code/No Code: NMDP  
 Saved By: Ingrassia, Francesco  
 More Testing Needed  
 Comment

Code Update Date:  
 Date: ago 21, 2014  
 False Rxns exist

Nom. Date: October 2013  
 Confirmed By: Cappuzzo, valentina  
 Ambiguity Exists

Imgt Ver: 2.5.0  
 Date: ago 21, 2014


---

Approved By:  
 Date:

---

**Interpretation**

Assigned Allele Code:  
 Possible Allele Code:  
 Assigned Allele Pairs:  
 KIR2DL1  
 KIR2DL3  
 KIR2DL4



KIR3DL2  
 KIR3DL3  
 KIR3DP1

Other Assignment:  
 Force False:  
 Close Reaction Beads:

**KIR Haplotypes: A/A**

**Donor KIR B status: Neutral**

**Figure 7.** A report of a donor KIR typing: in this case there is KIR2DS4 and so it is Group A Haplotype and the donor KIR B status is Neutral.

**KIR-SSO**

---

Lab Name: Tipizzazione HLA Molecolare  
 Institute: A. O. Cervello  
 Street: Via Trabucco, 180  
 Lab City: Palermo  
 Region:  
 Country:  
 Notes:

Lab Code:  
 Contact: Dr.ssa V. Cappuzzo  
 Email:  
 Phone:  
 Fax:

---

Patient ID: Name:  
 Molecular Typing:  
 Serology Typing:  
 Sample ID: 00041565 Local ID:  
 Sample Date: Test Date: ago 21, 2014

---

Allele Code/No Code: NMDP Code Update Date: Nom. Date: October 2013 Imgt Ver: 2.5.0  
 Saved By: Ingrassia, Francesco Date: ago 21, 2014 Confirmed By: Cappuzzo, valentina Date: ago 21, 2014  
 More Testing Needed  False Rxns exist  Ambiguity Exists  
 Comment Low Positive Control (056(916))

---

Approved By: Date:

---

**Interpretation**

Assigned Allele Code:  
 Possible Allele Code:  
 Assigned Allele Pairs:  
 KIR2DL1  
 KIR2DL3  
 KIR2DL4  
 KIR2DS1  
 KIR2DS3  
 KIR2DL2  
 KIR3DL3  
 KIR3DP1  
 KIR3DS1

Other Assignment:  
 Force False:  
 Close Reaction Beads:

**KIR Haplotypes: B/x  
 Donor KIR B status: Better**

**Figure 8.** A report of a donor KIR typing; in this sample there are KIR2DS1 and KIR2DS3 and so it is Group B/x Haplotype and the donor KIR B status is Better.



**KIR-SSO**

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**Lab Name:** Tipizzazione HLA Molecolare **Lab Code:**  
**Institute:** A. O. Cervello  
**Street:** Via Trabucco, 180 **Contact:** Dr.ssa V. Cappuzzo  
**Lab City:** Palermo **State/Province:** **Email:**  
**Region:** **Zip/Postal Code:** **Phone:**  
**Country:** **Fax:**  
**Notes:**

---

**Patient ID:** **Name:**  
**Molecular Typing:**  
**Serology Typing:**  
**Sample ID:** 00079493 **Local ID:**  
**Sample Date:** **Test Date:** ago 5, 2014

---

**Allele Code/No Code:** NMDP **Code Update Date:** **Nom. Date:** October 2013 **Imgt Ver:** 2.5.0  
**Saved By:** Ingrassia, Francesco **Date:** ago 8, 2014 **Confirmed By:** Cappuzzo, valentina **Date:** ago 8, 2014  
 More Testing Needed  False Rxns exist  Ambiguity Exists  
**Comment:** Low Positive Control (056(565)).

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**Approved By:** **Date:**

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**Interpretation**

**Assigned Allele Code:**  
**Possible Allele Code:**  
**Assigned Allele Pairs:**  
 KIR2DL1                      KIR2DP1                      KIR2DS4                      KIR3DL3  
 KIR2DL2                      KIR2DS2                      KIR3DL1                      KIR3DP1  
 KIR2DL4                      KIR2DS3                      KIR3DL2

**Other Assignment:**  
**Force False:**  
**Close Reaction Beads:**

KIR Haplotypes: B/x  
 Donor KIR B status: Best

**Figure 9.** A report of a donor KIR typing: in this sample there is KIR2DS2 and so it is Group B/x Haplotype and the donor KIR B status is Best.

**Table 6.** Donors classification according to the KIR B content score

Donor/ Recipient HLA- allele matching at A, B, C, DRB1 and DQB1	Total n=89				AML Recipients n=33				ALL Recipients n=19				Other Hematological Malignancies n=37			
	Donor KIR B Status Neutral		Donor KIR B Status Best/Better		Donor KIR B Status Neutral		Donor KIR B Status Best/Better		Donor KIR B Status Neutral		Donor KIR B Status Best/Better		Donor KIR B Status Neutral		Donor KIR B Status Best/Better	
	Tot	63	26	33	21	12	19	14	5	28	9	11	11	9	28	9
10/10	32	24	8	10	9	1	7	4	3	11	4	15	11	4	11	4
9/10	33	22	11	14	7	7	8	7	1	8	3	11	8	3	8	3
≤8/10	24	17	7	9	5	4	4	3	1	4	1	11	9	2	9	2

## 4.2 Clinical outcome analysis

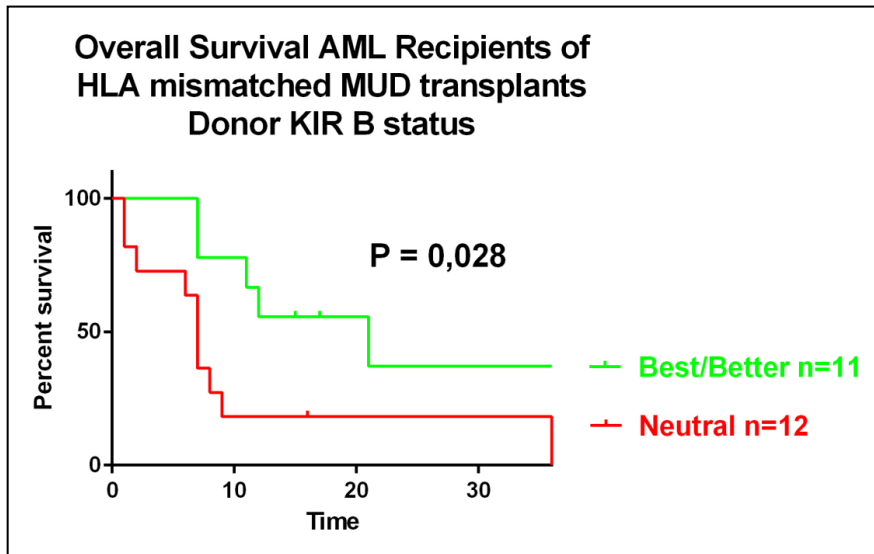
### 4.2.1 Overall Survival, Relapse and Disease Free Survival

The overall survival-rate was analyzed generating the Kaplan-Meyer curves, using the GraphPad6 Demo software; the data were compared using the Log-Rank Test 95%CI.

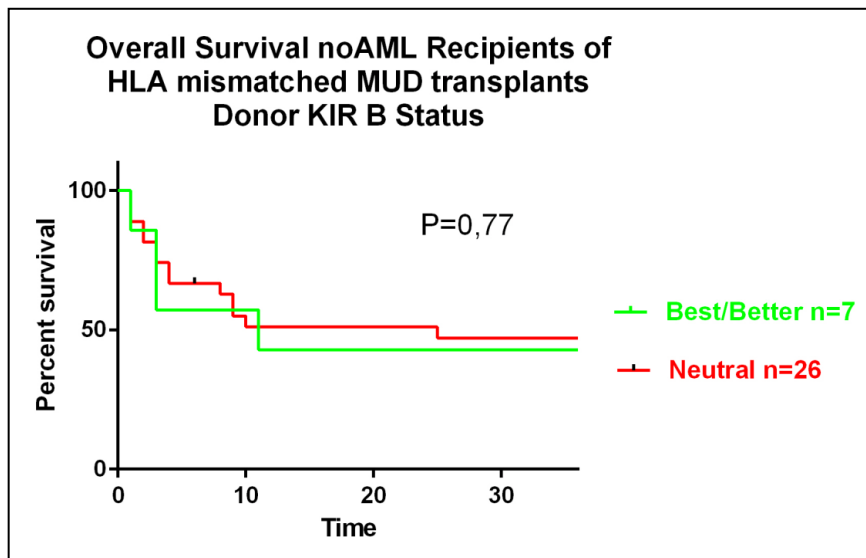
We observed no significant difference in the overall survival-rate of those patients transplanted from A/A donor and those from B/x donor, while we found a better overall survival-rate in the AML recipients, HLA mismatched with the donor, when the donor KIR B content status is Best/Better (37% vs 18% at three years Log-Rank-Test  $P=0,028$ ) (Figure 10), whereas there is no beneficial effect in recipients with other haematological malignancies (Figure 11).

Moreover, we observed that AML recipients, whose Donors KIR B status was Best/Better, had a lower incidence of relapse than patients whose Donors KIR B status was Neutral (36% vs 58%) (Figure 12 and 13) and a better Disease Free Survival-rate (58% vs 38% at three years  $P=0,1$ ) (Figure 14).

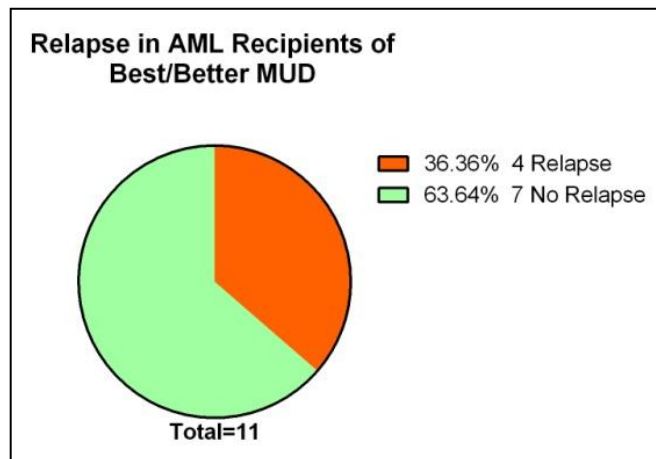
We have not been able to make a comparison of the 10/10 HLA matched AML recipients because only one donor was Best/Better.



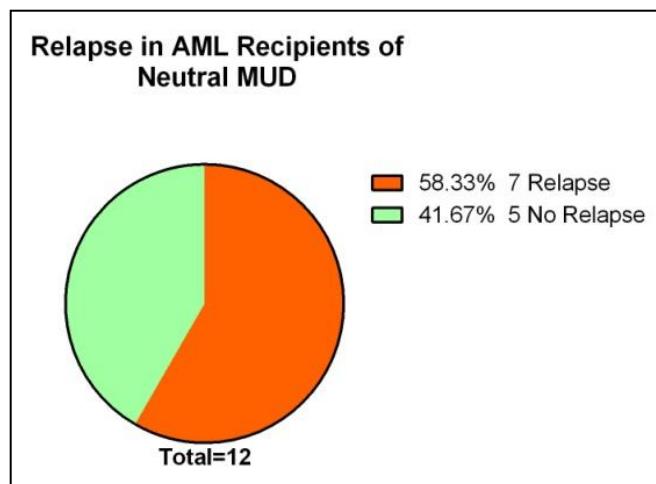
**Figure 10.** Overall Survival of AML recipients of HLA mismatched MUD transplants.



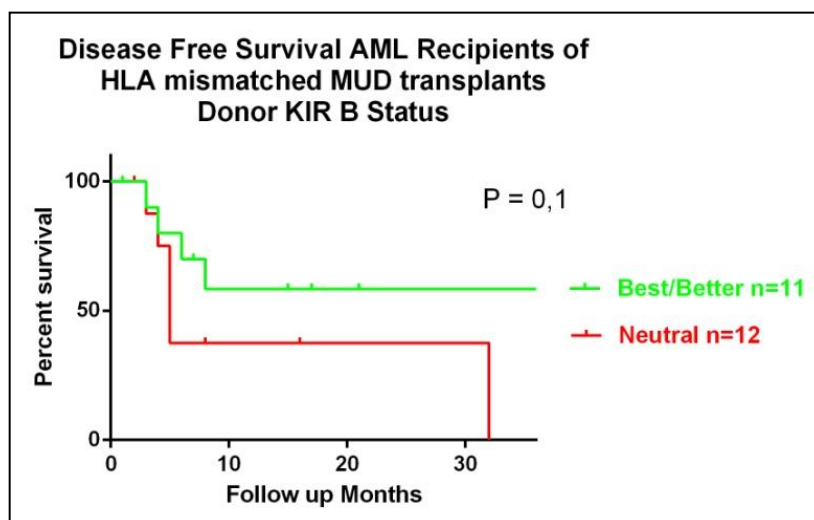
**Figure 11.** Overall Survival of recipients with other hematologic malignancies of HLA mismatched MUD transplants.



**Figure 12.** Disease Free Survival-rate of AML recipients of HLA mismatched MUD transplants.



**Figure 13.** Incidence of Relapse in AML Recipients of Best/Better MUD.



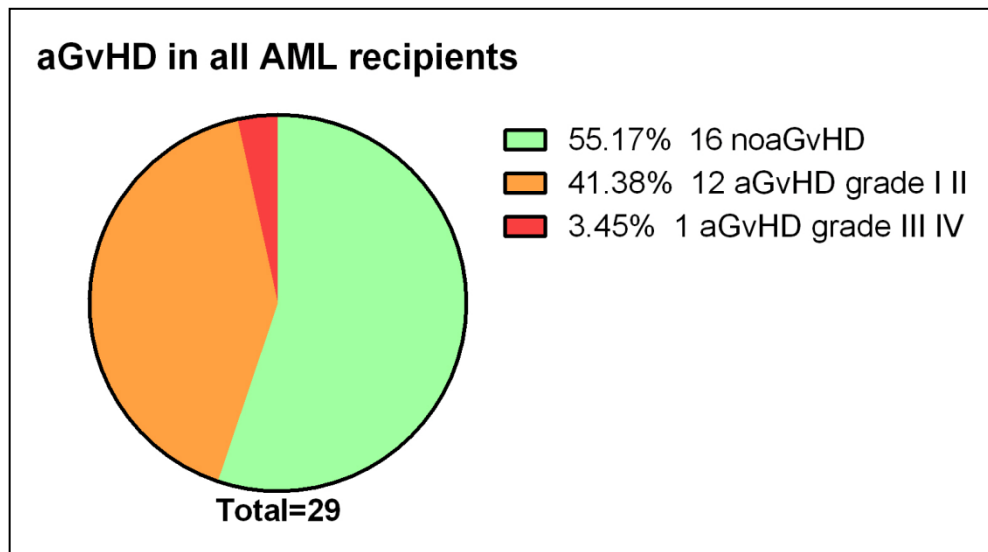
**Figure 14.** Disease Free Survival-rate of AML recipients of HLA mismatched MUD transplants.

#### 4.2.2 aGvHD

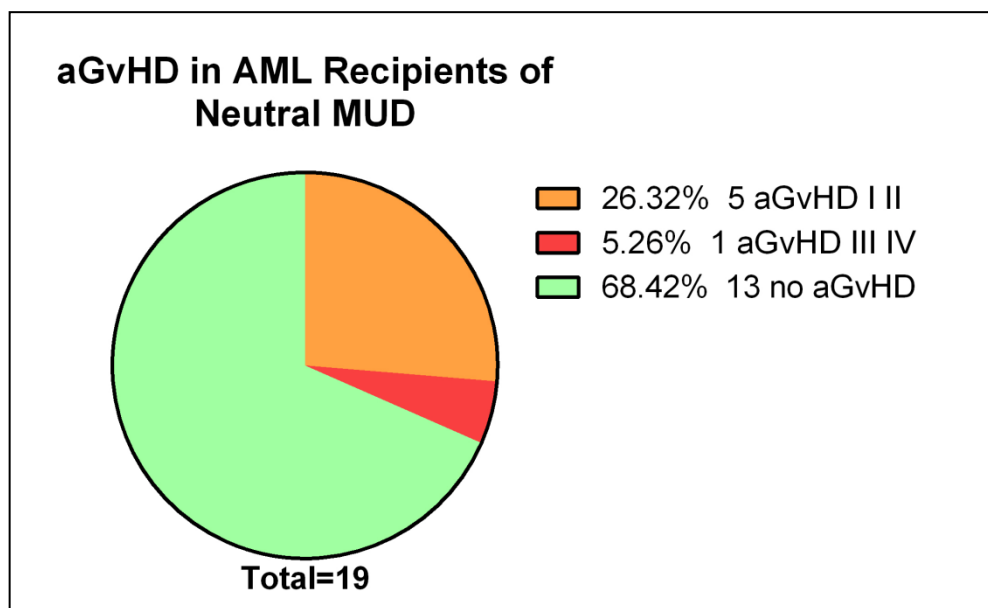
The incidence of aGvHD in AML patients was: 55,17% (no. 16/29) of no aGvHD, 41,38% (no. 12/29) of aGvHD grade I II, 3,45% (no. 1/29) of aGvHD grade III IV (Figure 15).

We observed that AML recipients, whose Donors KIR B status was Best/Better, had more incidence of aGvHD grade I II than patients whose Donors KIR B status was Neutral: 70% vs 26% (Figure 16 and 17).

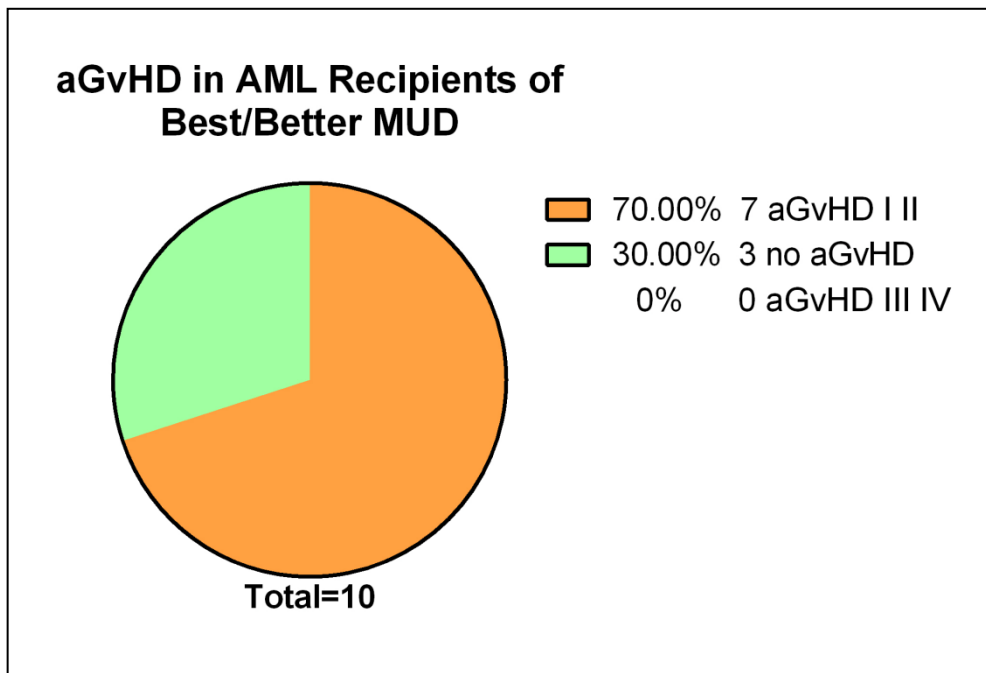
Actually, as regards patients who have aGvHD grade I II, this results in a beneficial effect on the overall survival-rate. In fact, we observed that patients with aGvHD grade I II had a significantly better overall survival-rate than patients with noGvHD (51,6% vs 38% at three years  $P=0,04$ ) (Figure 18). This agrees with those studies [4] showing that patients with aGvHD I II have a better overall survival-rate and a lower rate of relapse due to a GvL effect.



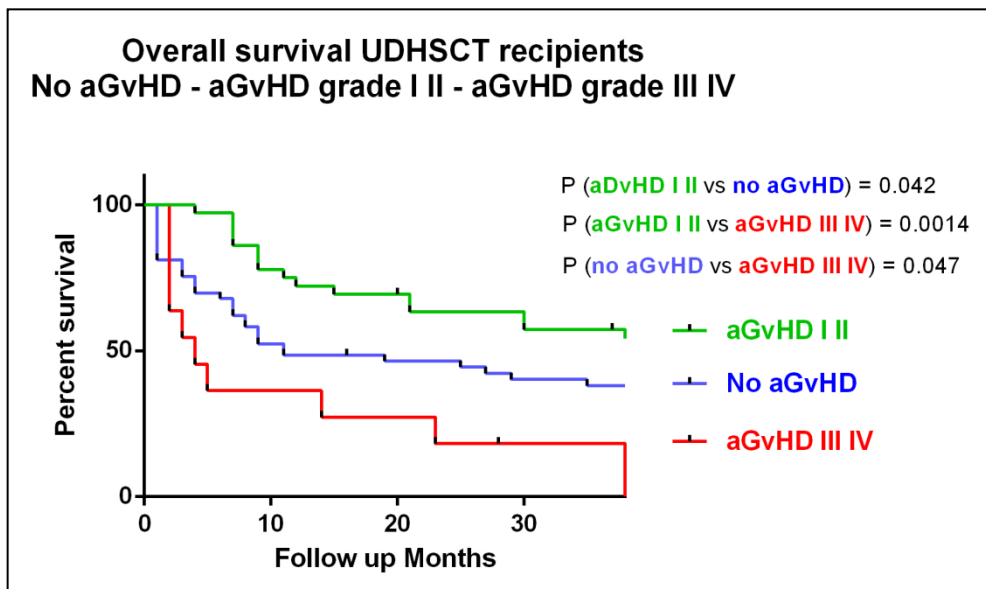
**Figure 15.** The incidence of aGvHD in AML patients



**Figure 16.** Incidence of aGvHD in AML Recipients of Neutral MUD.



**Figure 17.** Incidence of aGvHD in AML Recipients of Best/Better MUD.



**Figure 18.** Patients with aGvHD grade I II had a significantly better overall survival than patients with noGvHD (51,6% vs 38% at three years P=0,04)



## 5. Discussion

In recent years, various studies have shown that the beneficial GvL effect promoted by allo-reactive NK cells derived from the donor can improve the outcome of the HSCT from Haploidentical, Unrelated and HLA identical sibling donors. In particular Cooley S. et al. [12] have demonstrated that AML patients who received HSC transplants from unrelated Group B KIR haplotypes donors, had a better outcome after transplant because the haplotypes of Group B have a larger number of activating receptors, and this translates into better anti-leukemic activity. In a subsequent study [13], they found that some particular KIR receptors had a stronger effect and they defined three categories of donors: Neutral (none or one KIR B motifs), Better (two or more B motifs without KIR Cen B/B) and Best (two or more B motifs with KIR Cen B/B).

In our study we analyzed the impact of the donor KIR B status on the outcome of the unrelated HSC transplants performed in our center. We observed no significant difference in overall survival of those patients transplanted from A/A donor and those from B/x donor, probably because we do not have a large enough cohort of transplanted patients, and we were not able to make a meaningful comparison; on the other hand, despite this small cohort of patients, we have been able to observe the powerful effect of the Best/Better Donor KIR B status in the AML recipients HLA mismatched with the donor. They had a significantly better overall survival-rate if the donor KIR B content status was Best/Better and they also had a higher incidence of aGvHD grade I and II, and had a lower rate of relapse due to GvL effects.

Even though our cohort was small, our results confirmed that the presence of more activator KIR genes in donors can improve the outcome of UDHSC HLA mismatched transplants in AML recipients whereas there is no benefit in recipients with other hematologic malignancies. Our next target is to increase the number of cases by adding the other transplants performed and to study the effect of GvL in relapse in the same patients.

In any case, our results confirm that NK cells derived from donors with two or more KIR B motifs, have an improved ability to kill residual leukemic blasts in AML recipients of UDHSCT. As a consequence of this, the KIR genotype of the donor is another important criterion to be taken into account for the choice of the best possible donor in unrelated, sibling and haploidentical HSC transplant settings, together with all the other important factors, such as HLA matching, CMV status, blood group, age and gender. Future studies should be aimed at finding the right alloreactive subsets among donor NK cell repertoires, opening up the possibility for successful NK cell-based immunotherapy. Antileukemic NK cells, either allogeneic or unlicensed autologous NK cells, emerge as a feasible therapy option and might improve the clinical outcome in myeloid leukemia.

Note: This work has been accepted for publication in the book “Hematopoietic Stem Cells” with the Title "Impact of the Donor KIR Genotype on the Clinical Outcome of Hematopoietic Stem Cell Unrelated Transplants: a single center experience", ISBN 978-953-51-4937-8.

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