Universitat de Girona
Facultat de Medicina

LAG-3 GENOTYPE IN HAEMATOPOIETIC STEM CELL TRANSPLANT FROM HLA IDENTICAL SIBLING DONOR

DEGREE FINAL PROJECT

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1. LIST OF ABBREVIATION

CD34: transmembrane protein in haematopoietic stem cells

HSCT: haematopoietic stem cell transplant

HSC: haematopoietic stem cell

RT: radiotherapy

GVHD: graft-versus-host-disease

GVL: graft-versus-leukaemia

MHC: major histocompatibility complex

HLA: human leukocyte antigen

TBI: total body irradiation

mHa: minor histocompatibility antigens

DCs: dendritic cells

GM-CSF: granulocyte-macrophage colony stimulating factor

APCs: antigen presenting cells

TNF: tumor necrosis factor

INF: interferon

IL: interleukins

TGF: tumor growth factor

ECP: extracorporeal photopheresis

TCR: T-cell antigen receptor

MIC: MHC class I polypeptide-related sequence A

IEC: intestinal epithelial cells

CTLA-4: cytotoxic T-lymphocyte molecule associated to protein 4

PD-1: programmed cell death 1

LAG-3: lymphocyte activation gene 3

ITM: immunoreceptor tyrosine-based inhibition motif

ITSM: immunoreceptor tyrosine-based switch motif

LSECtin: liver sinusoidal endothelial cell lectin

ADAM: disintegrin and metalloproteinase domain-containing proteins

MUD: matched unrelated donor

OS: overall survival

DFS: disease-free survival

TRM: transplant related mortality

2. ABSTRACT

BACKGROUND: Allogeneic Haematopoietic Stem Cell Transplant is the treatment of choice in various malignant hemopathies. Graft-versus-host-disease is the main complication, but a lack of clarity exists considering genetic differences between donor and recipient, or the mechanisms that modulate the immune outcome. LAG-3 (CD223) is a surface protein belonging to an immunoglobulin superfamily and its function is to inhibit the immune response of T-lymphocytes. Researchers does not discuss whether the LAG-3 genotype conditions are related the post-allogeneic Haematopoietic Stem Cell Transplant complications.

HYPOTESIS AND OBJECTIVE: The aim of this retrospective multicenter single cohort study is to analyse the association of allogeneic Haematopoietic Stem Cell Transplant with incidence of acute Graft-versus-host-disease based on the donor's LAG-3 genotype in 16 hospitals from Spanish Group of Haematopoietic Transplant (GETH) between 2000 and 2014, coordinated by ICO Girona.

METHODS: A registry of 797 patients with an allogenic Haematopoietic Stem Cell Transplant from an HLA-identical sibling donor was described and analysed all the data collected. Polymorphism rs870849 C>T of the LAG-3 gene was analyzed in donors. Clinical events of patients whose donor was homozygous for allele C were compared against homozygous for the allele T or heterozygous C/T. This was done to determine polymorphism with Allelic discrimination being performed.

RESULTS: No statistically significant differences were found for the incidence of acute Graft-versus-host-disease between homozygous genotypes for allele C with respect to homozygous for allele C or heterozygous C/T. In contrast, statistically significant differences were found in Disease-free-survival (55.3% vs 41%, p-0.02). Overall Survival was also worse in patients whose donor had CT/TT (57% vs 43.9%, p-0.003). Additionally, there is a higher increase in relapses for CC (29.4% vs 38.5, p-0.074), and also in transplant-related mortality in the same group (20.2% vs 31.7%, p-0.002).

CONCLUSIONS: The donor's LAG-3 genotype is associated with differences in OS after an allogeneic Haematopoietic Stem Cell Transplant from an HLA-identical sibling donor. Therefore, it is associated with an increase in relapse and TRM. However, there are no significant differences in the incidence of acute Graft-versus-host-disease.

KEYWORDS:

LAG-3, allogeneic HSCT, acute GVHD, HLA-identical sibling donor, leukaemia, myelodysplastic syndromes.

3. INTRODUCTION

3.1. HEAMATOPOIESIS

The haematopoiesis is a biological process that gives rise to the three types of blood cells: erythrocytes, leukocytes and thrombocytes. The life of these cells is short, so to maintain steady levels for viable life, constant renewal is necessary to meet peripheral needs (1).

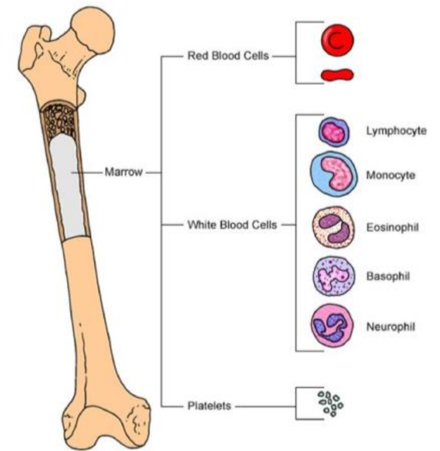

Figure 1. Blood cell linage (1)

The average life of each type of cell is:

- Erythrocytes: 120 days
- Thrombocytes: 8 – 10 days
- Leukocytes: it depends on the type of leukocyte
    - Granulocytes: between 8 and 10 hours in the bloodstream, and 1 or 2 days on the tissue
    - Lymphocytes: some years.

In a human body, the haematopoiesis has different anatomical locations throughout the embryonic development. Blood cell's production starts in the yolk sac during first weeks of gestation, with stem cells added and creating blood islets (1).

The production of haematopoietic cells in the foetus between the second and the seventh month of pregnancy, takes place in the liver and in a smaller degree in the spleen, lymph node and thymus, which are the most important production sites (1).

From the seventh month, the bone marrow, which is the tissue located between the bone trabeculae, turns into the main haematopoietic organ until birth. At the end, it is the only haematopoiesis focus in usual conditions (1).

In addition, in individual aged 5 to 20 years, the long bones slowly lose their ability to produce hematic cells. The haematopoietic tissue is found in the vertebrae, sternum, ribs and pelvis. Either the liver or the spleen maintains a residual ability to produce blood cells, which will reassume their functions only when pathological circumstances occur. It is called extramedullary haematopoiesis (1).

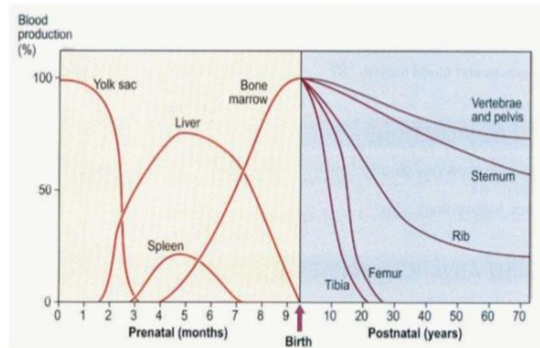

Figure 2. Sites of haemopoiesis during life (1)

For the bone marrow to be the main producer of haematopoiesis it's important that it creates a microenvironment where nesting, proliferation and differentiation of haematopoietic stem cell is feasible. At a certain point in the maturation of the stem cell, it produces the differentiated haematopoietic cells that pass from the spinal cord to the peripheral blood through the sinusoidal wall of the bone (1).

This step is very important for the pathogeny of different hematologic diseases, due to the fact that the blood cell's path through the exit has to produce openings in the endothelial cells, which is supposed to be a first selective barrier. In certain pathological processes such as neoplastic infiltration and fibrosis, the sinusoidal wall structure is disrupted, an action that eases the immature cells path to the peripheral blood (1).

3.1.1. STEM CELLS

Stem cells are defined by their ability to autorenovate, so as to produce other stem cells, and their ability to differentiate into one or more mature differentiated cell lineages.
There are three groups of stem cells:

- Totipotential stem cell: which are defined by their ability to produce any cell that the body contains, including the extraembryonic tissues.
- Pluripotential stem cell: which are defined by their ability to produce cells from the three germ layers: endoderm, mesoderm and ectoderm. Also, they can create any foetal cells or adult, but they can't produce extraembryonic tissue such as the placenta.
- Multipotencial stem cell: which are defined by their ability to produce specific cells from the same marginal layer (endoderm, mesoderm and ectoderm). They are located in the tissues in small quantities and they are in charge of replacing the destroyed cells from the same tissue.

Due to the types of stem cell, the haematopoietic stem cell is a multipotential stem cell. The HSC generates all blood cells and the immune system during the whole life (1).

The haematopoietic stem cell has determined characteristics which make it different from other cells' populations. It is small, nucleated and unrecognizable for an optical microscope; therefore, it is needed in vitro culture techniques, immunological studies and ultrastructural. They are located in the bone marrow and in a smaller portion in the peripheral blood, where they significantly increase after chemotherapeutic process or the use of recombinant haematopoietic growth factors (1).

The haemopoietic stem cell express the CD34 antigen on cell's surface, allowing its identification by flow cytometry.

3.1.2. STEM CELL DIFERENTATION

Differentiation of haematopoietic stem cells develops in several stages. However, it must be taken into account that this is a hierarchical process. The stem cells are located at the apex in a resting state and therefore have an indefinite capacity for self-renewal (1).

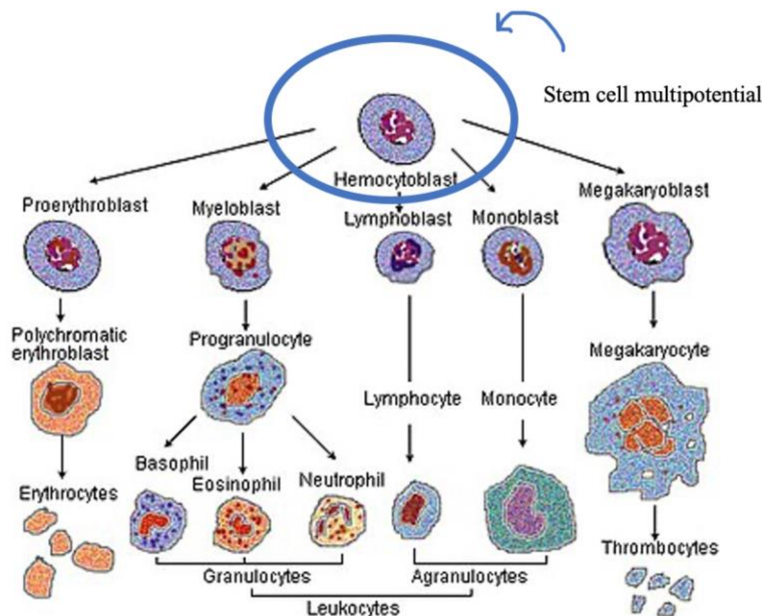

Figure 3. Serial cell differentiation

For the maturation of these stem cells, the cell cycle is necessary in which the cells will differentiate into each cell lineage. The differentiation in each blood cell series depends on the growth factor concentration and the signals produced by the cells located at the bone marrow stroma (1). HSCs produce all the elements of blood: lymphoid, myeloid and mononuclear cells.

During this process, there is an increase of the proliferative index that persists up to the most distinguished precursors. Moreover, some morphological changes occur at the same time as they differentiate (1).
Morphologically, stem cells with nuclei are large and the cytoplasm are scare due to their immaturity. As they mature, the nucleus decreases and the cytoplasm increases.

3.2. HAEMATOPOIETIC STEM CELL TRANSPLANT

HSCT is an optional treatment for a few patients with haematological neoplasia, bone marrow failure disease and other congenital diseases. It consists of the haematopoietic stem cells infusion so as to restore the core function after administering a conditioning treatment.

The first mention of HSCT in the literature dates from late 19th century, Brown-Sequard introduced the idea of administering the animal's spleen to anaemic patients (1).
Later, the consolidated idea of HSCT came from the air raids of Hiroshima and Nagasaki in 1945. The attacks caused different hematologic effects produced by exposure to the radiation from the bombs (2).
Finally, the first successful HSCT were carried out in the late 60's and early 70's (1).

3.2.1. TYPES OF HSCT

There are different types of HSCT depending on the relationship between the donor and the recipient, the source of stem cells used, the type of patient conditioning and the handling of the cells before infusion (2).

3.2.1.1. CELL SOURCE

Depending on the cell source:

- **Bone marrow**: HSCs are collected from the bone marrow by the bone marrow aspiration technique which consists of perforating the posterior superior iliac crest or sternum where marrow blood is obtained from the marrow. It must be done in an operating room and under general anaesthesia (3).
  This procedure takes between 2 to 3 hours. Normally, it is necessary to aspirate up to 1 litre of blood in order to have a sufficient quantity of cells. Moreover, an autotransfusion is necessary during the procedure in order to restore the lost blood volume. Therefore between 15 to 20 days before the process, a blood donation should be made so that can be transfused on the day of the medullary aspiration (4).

- **Peripheral blood**: HSCs are collected through the leukapheresis after mobilisation with haematopoietic growth factors (G-CSF) in order to pass the from the bone marrow to the peripheral blood. Although peripheral blood collection achieves more lymphocytes than bone marrow collection, it has a higher risk of chronic GVHD (3).
  In the case of auto-HSCT mobilisation, can carried out after one of chemotherapies cycles administration. This process is done without anaesthesia and hospitalisation is not necessary. At present, obtaining HSC from peripheral blood is used most frequently as it is easy to obtain and bone marrow recovery is faster (4).

- **Umbilical cord**: HSCs are obtained from the umbilical cord blood after the labour. As they are immature, HLA mismatches between patient and donor are acceptable. The main problem is that the obtained volume is not enough for an adult receptor with a high corporal volume. Hence, it may only be useful in children and in some adults (3).
  The HSC are stored in umbilical cord blood banks until they are required. Presently, Spain is ranked in the fifth position of countries that have more stored umbilical cord blood units (4).

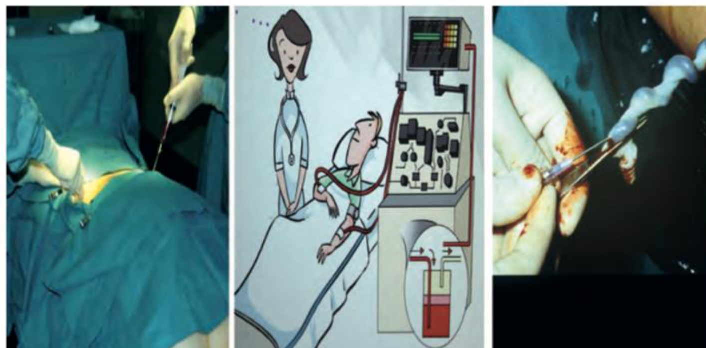

Figure 4. Different cell source for HSCT: bone marrow, peripheral blood and umbilical cord (2).

3.2.1.2. HSCT DONOR

Depending on the donor:

- **Autologous transplant**

Haematopoietic progenitors are obtained from the patient with the aim of giving a high-dose chemotherapy (conditioning regime) when the patient is in remission of the disease. The limit age for this type of transplant is 70 years old. In this model GVHD is not developed (4).

Nowadays, the autogenic transplant approach is also used for some autoimmune and inborn metabolic diseases. As well as in some haematological malignancies such as lymphoma, multiple myeloma, histiocytosis and amyloidosis (2).

- **Allogenic transplant**

HSCs are obtained from an identical HLA sibling donor or unrelated donor. The graft is free of neoplastic cells, but as donor and recipient are genetically different, the donor cells can be recognised and rejected by the recipient. Furthermore, the donor's T-lymphocytes can cause serious complications, such as GVHD. To prevent this complication, an immunosuppressive treatment is required (4).

Although it has more serious complications than autologous transplant, it also has beneficial effects, such as the graft-versus-tumour effect, in which the T-lymphocytes are able to recognise tumour receptor antigens and eliminate residual neoplastic cells (4).

Allogenic stem cell transplant is indicated as the first line of neoplasms with complete remission: in acute myeloid leukaemia and in myelodysplastic syndromes. It can also be understood as second line treatment: in acute lymphoblastic leukaemia, relapsed lymphomas and multiple myeloma (2).

- **Syngeneic transplant**

Syngeneic transplants are allogeneic transplants that come from identical twin (4).

3.2.1.3. TYPE OF CONDITIONING

Depending on the patient's preparation for HSCT it is necessary to eliminate the haematopoietic and tumour cells, create a medullary space for the transplant cells and immunosuppress the patient to avoid rejection of the cells (2).

- **Myeloablative conditioning**

This is the classic treatment. It produces an irreversible injury of the haematopoietic cells of the bone marrow, generating an intense pancytopenia.

Moreover, it creates a space in the bone marrow for the new transplanted cells and prevents their rejection by the residual lymphocytes of the recipient. The marrow function can be only recovered after infusion of HSC. One or more high-dose chemotherapy drugs are usually administered and, in some cases, may be combined to total body irradiation (TBI) (4).

- **Non-myeloablative or reduced intensity conditioning**

  The treatment is weaker than conventional condition regimen, less myelosuppressive but more immunosuppressive. Not all haematopoietic cells are destroyed, and pancytopenia lasts less. The transplanted donor T-lymphocytes are able to displace the patient's haematopoiesis and make the donor's haematopoiesis predominant (4).

  It is a two-stage process: in the first stage there is a mixed chimera, which mean that there is HSC from donor and from the recipient. In the second stage, the donor's T-lymphocytes are capable of removing all the patient's haematopoietic cells, having a complete chimera (2).

3.2.2. HSCT PHASES

As we have seen in the last chapter, there are different types of transplants, although the procedure is similar in each transplant. However, in any HSCT there are different phases, which are identical (**Annex I**):

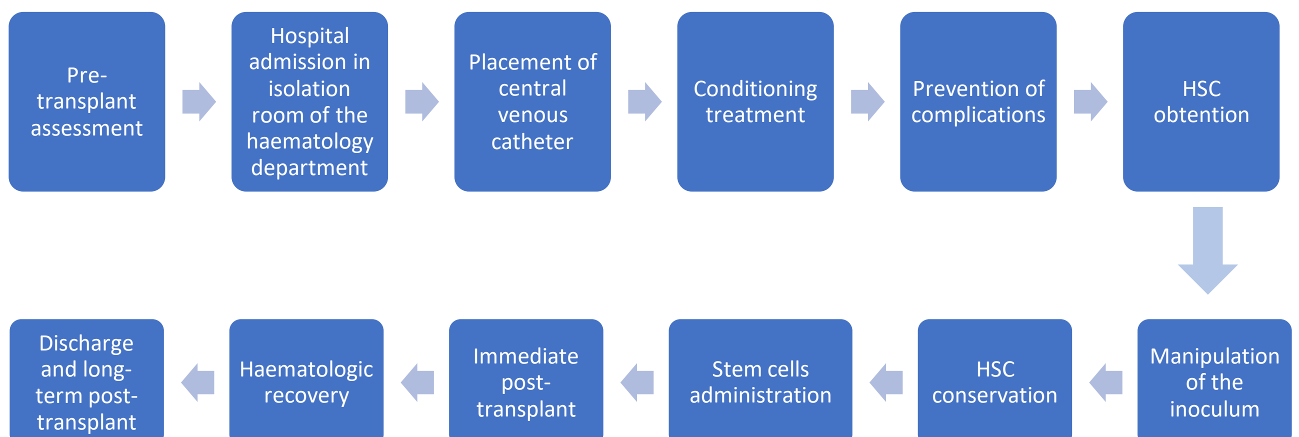

Figure 5. Itinerary to follow in the HSCT (4)

3.1. GRAFT-VERSUS-HOST-DISEASE

This is a complication of allogeneic HSCT that is produced by the reaction generated by the recognition of donor T-lymphocytes as a foreign antigens (4).

This complication is typical of allogeneic HSCT because the donor is not completely identical to the recipient, despite a very HLA compatible donor is being sought. In contrast, in auto-HSCT there is no such complication because the donor is the patient himself (4).

GVHD is one of the most important and serious complications that occurs after the allogenic transplant. It is the main cause of death and morbidity related to this procedure (2).

3.3.1. PATHOPYSIOLOGIC MECANISM GVHD

The main cause for GVHD is competent T-cells. The graft contains immunologically competent cells and on the other hand, the receptor expresses surface antigens that are not present in the transplant graft. Furthermore, the receptor must be unable to generate an effective response to eliminate the transplanted cells because otherwise rejection can happened (5).

There are three phases in the understanding of the pathophysiology of acute GVHD:

Phase I

Donor T-cells are stimulated by dendritic cells of the host. This interaction occurs through mHa recognition by CD4s. In addition, CD4 cells are stimulated by the HLA class II of the DCs complexes (6).

The activation of the antigen-presenting in the host is due to the pre-transplantation conditioning. This accelerates the stimulation of the T helpers cells and increases the risk of acute GVHD (7).

The activation of APCs and inflammatory cytokines stimulates the T helpers’ cells, while at the same time stimulating INF-γ and GM-CSF. The stimulation of these factors creates a cycle called the cytokine storm (6,8).

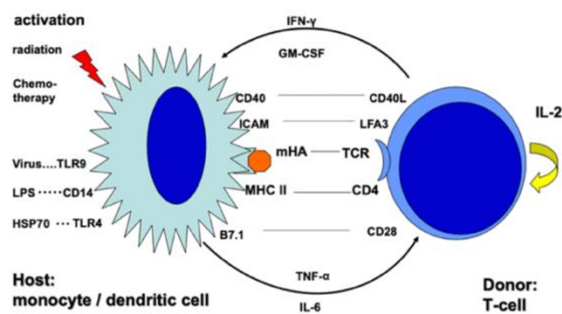

Figure 6. Pathophysiology of GVHD: the donor T cell is encountering a host antigen-presenting cell (6).

<u>Phase II</u>

In addition to stimulating CD4 cells when DCs are activated, CD8 are also stimulated by the presentation of the HLA class I. As a consequence of the activation of cytotoxic T-cells ($CD8^{+}$) in the transplant, there are able to attack epithelial or haematopoietic through mHA (6).

In order for this attack of cytotoxic T-cells to be generated, the expansion of the MHC class II through APCs and the stimulation of the CD4s is necessary. Moreover, the danger signals produced in phase I by the costimulatory molecules are also evident (7). To initiate this attack, it is not suficient that only the host's DCs are involved. Therefore, it is be necessary for graft Dcs to express MHC class I to maximize the effect of GVHD.

Nowadays it has been shown that in animals experiments, if regulatory T-cells are added to the donor's graft, that this would result in suppression of T-cells proliferation and prevent the occurrence of GVHD. Regulatory T-cells secrete inflammatory cytokines IL-10 and TGF-β. NK cells from the donor or the recipient have also been shown to modulate acute GVHD (7,8).

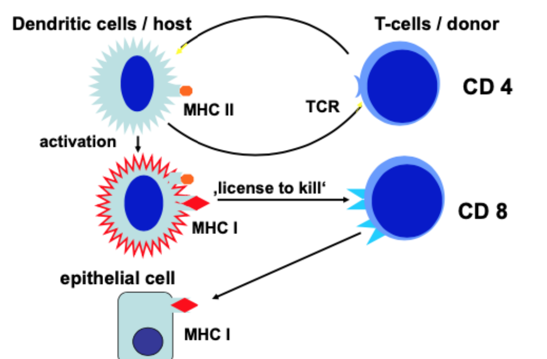

Figure 7. Pathophysiology of GVHD: presentation HLA to T cells of the donor (6).

Phase lll

The stimulated Dcs generate TNF-α and IL-1 which produce tissue damage, in addition to recruiting lymphocytes and neutrophiles.

TNF-α is an effector molecule in the skin and lymphoid tissues, which activates DCs, recruiting lymphocytes, neutrophils and monocytes from the target organ. This promotes the presentation of alloantigen's, causing direct damage by apoptosis and necrosis. Moreover, IL-1 acts in a synergistically with TNF-α (7).

3.3.2. ACUTE GVHD

It is a complication that occurs during the first three months after the transplant. It usually affects 50% of the patients who have had allo-HCT. The greater the difference between the donor's and the recipient's HLA systems, the greater the impact of GVHD. Other risk factors are age, a poor GVHD prophylaxis and the patient's cytomegalovirus seropositivity (9).

The target organs of the acute GVHD are the liver, skin and bowel. Also, the haematopoietic tissue and the immune system it is expressed as pancytopenia. In addition, it produces deep immunosuppression that can reactivate latent viruses (2).

Skin

The main organ affected is the skin. It starts with a maculopapular rash on the hand palm and soles of the feet, which can cover entire body surface. The injuries can coalesce, covering more areas and can be confused with toxic epidermal necrolysis. In severe cases it can create blisters (2).

Liver

The clinical manifestations of this organ begin with jaundice and the analytics shows a bilirubin increase. It produces a pattern intrahepatic cholestasis. It is necessary to make a differentiated diagnosis with other causes of hepatitis (2).

Digestive tract

The main consequence is at the intestinal level with strong diarrhoea that can be even 10 L/day, which exudative and bulky traits. Also, when it affects the upper digestive tract, it can produce anorexia, nausea and gastric juices. The effect may be present in all parts of digestive tract (9).

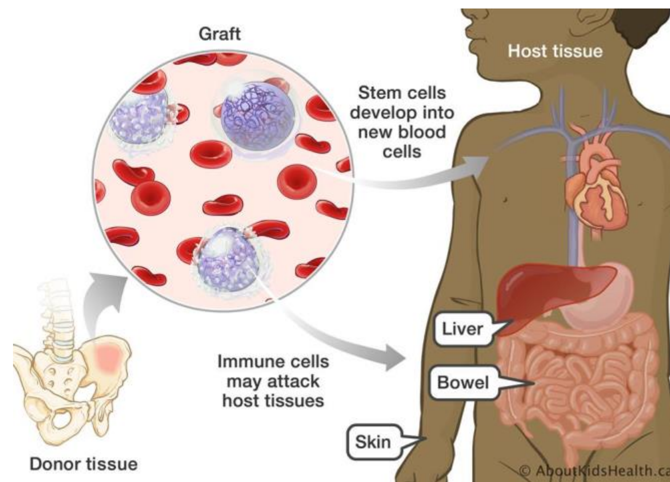

Figure 8. mechanism of attack on host tissues (29)

<u>Diagnosis</u>

The diagnosis is based on the clinical manifestations and with a biopsy the affected organ. The skin is the easiest organ to biopsy.

As it is a disease with of high incidence, morbidity and mortality, prophylaxis is administrated for six to nine months with cyclosporine A and a short course of methotrexate. Another option is depleting T-lymphocytes, but there is a higher risk of graft rejection, relapse or infection. Hence, this option is only used in specific cases (2).

<u>Gradation</u>

There are different levels depending on the number of affected organs and the severity. These stages allow us to estimate a long-term prediction. For example, stages III-IV (very severe) are associated with poor survival rate (between 5 and 25%) (9).

Table 1. Clinical grading of GVHD against the acute recipient adapted from Pregrado.

Stage	**Skin/maculopapular rash**	**Liver/bilirubin (µmol/L)**	**Gastrointestine/diarrhea**
+	<25% of body surface	34-50	>500 mL
++	25-50% of body surface	51-102	>100 mL
+++	Generalized erythroderma	103-255	>1500 mL
++++	Bullae formation and desquamation	>255	Severe abdominal pain with or without ileus

Treatment

The basic treatment is immunosuppression. Corticoids are administered topically or systemically, depending on the need. Prophylaxis is maintained with cyclosporine A.

The response to treatment is poor in severe cases, so it is necessary to administer monoclonal antibodies such as anti-CD3, anti-IL-2 or anti-TNF; mycophenolate mofetil, ruxolitinib or others rescue therapies (7).

3.3.3. CHRONIC GVHD

Chronic GVHD is the leading cause of late non-relapse death after HSCT. It is distinguished from the acute form by the distribution of the affected organs like and by its clinical presentation (4).

As we have explained before, the pathophysiology mechanism is different from the acute mechanism, because in the chronic form what is produced is a loss of tolerance to its own cells. This occurs in 50% of patients with allo-HCT. Its incidence increases in elderly patients, in patients with peripheral blood HCT or with an unrelated donor. Moreover, it has a higher risk when patients have previously suffered an acute GVHD (9).

Symptoms and signs

Its presentation is similar to any other autoimmune disease. This form of GVHD can affect one organ or several organs.

- Skin and mucosa: most patients have cutaneous affectations. They usually show erythematosus injuries in the form of papules or patches. It can affect the entire mucosa with lichenoid injuries and dryness. Alopecia can also occur in patches and brittle nails.
- Lungs: usually presents as an obstructive pneumopathy debuting as obliterative bronchiolitis. Lung involvement is related to a poor prognosis of the disease.
- Liver: it is affected in the form of chronic liver disease with increased cholestatic signs on analysis. An 80% of patients with chronic GVHD have liver involvement at different levels.
- Musculoskeletal system: it usually manifests as arthritis. Also, as joint stiffness, fasciitis or myositis.
- Humoral and cellular immunodeficiency; chronic GVHD is associated delayed immune recovery. This results in a higher risk of infections and the activation of a latent pathogen (5).

Diagnosis

By presenting a diagnostic criterion of those already mentioned in the symptoms and signs, it can then be diagnosed. It's not necessary to confirm this with a biopsy (7).

Treatment

Usually, it is treated with several immunosuppressive drugs. The result of treatment cannot be predicted, as there may be several outcomes in different organs in the same patient.
Corticoids are the standard treatment in patients with chronic GVHD. Other immunosuppressive therapy such as mycophenolate mofetil or the tacrolimus can be used. ECP can also be used. When one organ is affected at a more minor level, at times it may be untreated (7).

3.4. ALLORECOGNITION. CONCEPT AND PATHWAY.

3.4.1. BACKGROUND

Allorecognition means the ability of an organism to distinguish its own cells from those that are foreign. To recognise foreign cells, of the donor T-cells are used through the antigen expressed by the HLA molecule on the surface of the recipient APCs (10).

After allogeneic transplant, the donor's T-cells recognise the recipient's antigens as foreign tissue and produce an immune response. As it has been explained before, activation of immune cells leads to GVHD in bone marrow transplant patients or graft rejection (10).

The cells involved in allorecognition are called T-receptor cells, which recognise alloantigens in three different pathways: direct, indirect and semi-direct. The direct pathway starts with the donor's APCs that provide a higher allogenic major histocompatibility complex to the recipient's T-cells (10). In contrast, the indirect pathway occurs with the presentation of the recipient's APC and processing of allopeptids when self-MHC class ll is detected. Finally, the semi-direct pathway is a mixture of the direct and indirect pathways (10).

3.4.2. ANTIGEN PRESENTATION

T-cells' function is very important for the control of pathogens; whether intracellular or extracellular. T-cells that express $CD4^+$ on their surface are responsible for attacking extracellular organisms through the production of cytokines. However, T-cells expressing $CD8^+$ lysate cells are responsible for lysing cells infected by intracellular organisms (11).

Pathogenic or no pathogenic damage when it occurs, and the innate defence barrier fails, the adaptive immune response starts antigen recognition by extremely variable molecules such as immunoglobins and T-cells receptors. The recognition of antigens on cells surface is through the HLA molecule (12).

The cells mainly responsible for presenting the antigens are the APCs and they do this by MHC to the T-lymphocytes. These cells are responsible for capturing the antigens and processing them in order to present them. Thus, an antigen-presenting cell is one that contains HLA molecules on its surface. Once the T-cells recognise a specific membrane antigen, its transduction begins through the T-cell antigen receptor (TCR). The binding of MHC complexes and TCR produces different biochemical processes that end in the activation of the T-cells. The activation of each type of T-cell depends on the protein presented in the MHC to the TCR (12).

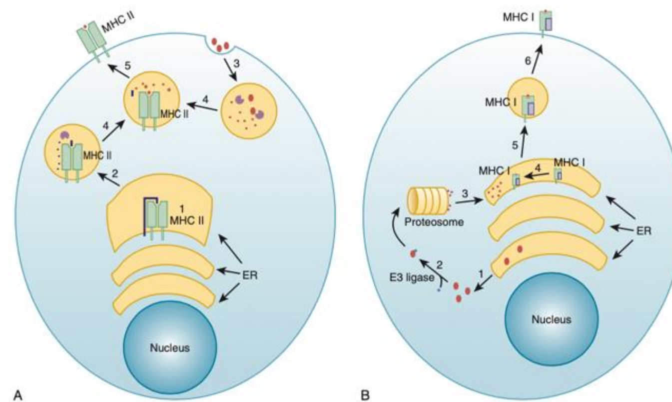

Figure 9. Presentation of peptides by MHC class I and class II (12)

In order to understand this mechanism, it is necessary to know the MHC's structure. The MHC are molecules that present their peptides on the cell surface that serve as markers of identity recognition. In humans these molecules are known as HLA. There are three types of regions or classes: I class belongs to the distal region of the complex and contains the classical and non-classical genes, including MICA and MICB. They are found in all the nucleated cells on their surface and are recognised by $CD8^{+}$ T-lymphocytes (13).

HLA class II is found in a more centromeric portion of the MHC region and is responsible for controlling the immune response. The genes code for molecules are HLA-DR, HLA-DQ, HLA-DP, HLA-DM and HLA-DO. Specifically, they are expressed in the APCs as macrophages, B-lymphocytes, dendritic cells, Langerhans and Kupffer cells. Once the antigen is processed by these cells, the degradation enzymes bind to the class II MHC proteins to be recognised by $CD4^{+}$ T-cells. The class II MHC proteins bind to the TCR (12).

Finally, III type is located between the previously described regions and is responsible of a huge number of genes that as associate with immune functions. Like the complement genes, a group of tumour necrosis factor and heat shock protein 70 (13).

3.4.3. ANTIGEN PROCESSING

Antigen processing is responsible for converting native proteins into peptides that associated with MHC molecules. The antigen is processed through proteolysis mechanisms within the APCs. In the case of extracellular proteins, they enter the cell by endocytosis in vesicular compartments. Once, the proteins are in the endosomes, the proteins are divided into peptides thanks to the enzymes and an acid pH. Class II molecules of CPH are synthesised in the endoplasmic reticulum (11).

A protein known as Ii (protease) is associated with MHC class II, which is responsible for occupying the cleft where the antigenic peptide will bind (11). Indeed Kaisser-lian et al. have demonstrated that murine IECc can function as APCs to DCs cells. In the class II pathway in several IEC lines, it is shown that these cells can process and deliver antigen to $CD4^+$ T-cells in a pathway modulated by the expression of Ii and HLA-DM (14).

On the other hand, there is also antigenic processing in the nucleated cells. Peptides associated with class l MHC are produced by proteolytic degradation of cytosolic proteins. The proteolysis mechanism in nucleated cells is done by the proteosome found in the cell's cytoplasm. The resulting peptides in the cytosol are transported to the endoplasmic reticulum. Once there, a binding protein attach to the class l MHC molecules. It is then released to be exocytosed to the cell surface (11).

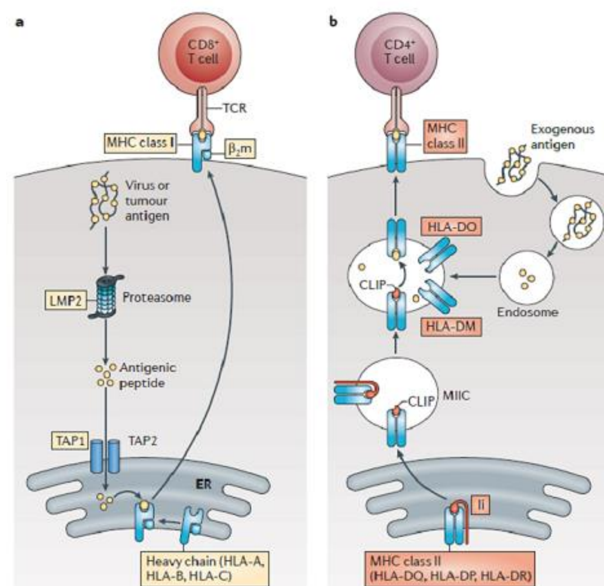

Figure 10. Antigen processing in their receptor (30)

3.4.4. ANTIGEN RECOGNITION

The T-lymphocytes activation involves a multi-step process that begins with antigen recognition by MHC proteins and results to the expression of specific immune functions such as the secretion of regulatory factors or the expression of cytolytic activity (15).

Under normal conditions, primitive T-cells travel through the blood and the lymphoid organs. During these circuits, they are in contact with thousands of APCs. The T-lymphocyte's activation is unleashed when the complexes MHC-peptides on the target cell surface are recognised by the TCR receptor on the T-cells. Strong adhesion to the target cell will be generated and effectors molecules will be released into the target cell. Depending on the proteins that are present through the MHC l o ll determines which subpopulations of T-lymphocytes will be activated (16).

Apart from this merger that has been explained, so as to generate the lymphocytes T activation is necessary to have a second costimulatory signal that has to be released by the APC joined to the lymphocyte T (16).

The most studied co-stimulator molecules are the B7 molecules on the surface of the T-lymphocyte activating cells, which the CD28 as their binding agent. The fusion of these costimulatory cells allows for the activation of T-cells, and depending on the number of proteins that are expressed, or maintain or modify their signal, this ends up generating clonal expansion and differentiation (16).

3.4.5. LYMPHOCYTE INHIBITION

The immune responses are self-regulating or self-limiting, otherwise they would get out of control and the cells would attack healthy tissues. A certain group of molecules are needed to inhibit the immune response. This group includes CTLA-4, PD-1 and LAG-3.

3.4.5.1. CTLA-4

The cytotoxic T-lymphocyte molecule associated with protein 4 is a strong in vitro inhibitor of lymphocyte function that requires the collaboration of T and B cell (17). CTLA-4 is mainly expressed in activated lymphocytes and is carried out with cytotoxicity mediated T-cells in inducible models of this process (18).
This molecule shares many similarities with CD28, as they belong to the same lg superfamily and also have the same gene chromosomal region in common (2q33-34) (17).

B7 is the ligand by which CTLA-4 and CD28 fuse to APCs', therefore, these two molecules compete with each other. However, CTLA-4 has greater affinity for B7 than CD28. Normally, there is an increase in CTLA-4 levels after lymphocytic activation in order to antagonize costimulation signals. The levels of CTLA-4 are also modulated according to the number of ligands expressed by the APCs. If there is a high number of ligands, CTLA-4 will be saturated. On the other hand, if there are fewer ligands expressed on the surface of the APCs, the binding of CTLA-4 will predominate. (17).

The molecule that binds to B7 determines whether the T-lymphocyte will have a reactive or anergized outcome. If the binding is CD28-B7, a reactive response will be generated and consequently, more T-lymphocytes will be activated. However, if the binding is between CTLA-4-B7, an anergized response will occur in the T-lymphocytes. (17).

3.4.5.2. PD-1

PD1 means programmed cell death 1. PD1 is a molecule belonging to the CD28/CTLA-4 family and is expressed on the surface of activated T cells, B cells and macrophages. It is co-localized with LAG-3 in activated $CD8^+$ T cells. Its association with LAG-3 contributes to a faster immunological synapsis, generating an inhibitory synergy response on T-cell signalling (19).

It is important to be aware of PD-1´s structure, the cytoplasmic domain as it contains an immunosuppressant tyrosine-based inhibition motif (ITM) and immunoreceptor tyrosine-based switch motif (ITSM) through which it will perform its functions.PD1 is responsible for restricting the T-cells activation by recruiting tyrosinase phosphatases through ITIM and ITSM. This action generates an attenuation of T-cell receptor signalling and inhibits cytokines production (19).

PD1 expression has high levels in chronically activated T-cells. In addition, it is transiently up regulated on activated T cells and neutralises their function (20).

On the cytoplasmic domain of PD1 contains immunosuppressant tyrosine-based inhibition motif (ITM) and immunoreceptor tyrosine-based switch motif (ITSM). PD1 restricts the T cells activation recruiting tyrosinase phosphatases through the ITIM and ITSM, this action generates an attenuation of the signposting of the recipient T cells and inhibits the cytokines production (19).

3.4.5.3. LAG-3

LAG-3 (CD223), known as lymphocyte activation gene 3, is a fusion protein which acts as a potential immunotherapeutic target for cancer because of its negative regulatory role on T cells and its ability in combination with PD1, to mediate a state of exhaustion in the cells. LAG-3 was discovered in 1990 by Triebel and colleagues as a novel transmembrane 498- amino acid type I protein identified in human activated NK cells and T-cell (21).

Its gene is located next to CD4 gene on chromosome 12 in human's genome. It is placed exactly on the distal part of the short arm of chromosome 12 (band p13.3 for LAG3) (22).

Its structure is made up of four extracellular immunoglobulins superfamily domains (D1-D4). In the D1 domain it has an extra loop chain that differentiates it from CD4. In addition, the D2 domain is essential for the binding LAG3-MHC class II (21).

LAG-3 binds to the monomorphic residues of MHC class II with a higher affinity than CD4 immunoglobulin, due to its structure with extra loop chain in D1. It is also able to blocking CD4/MHC class II interaction, as they compete for the same receptor (21,23).

Other alternative ligands for LAG-3 may be Galectin-3. At the moment, LAG-3 has also been shown to be essential for Galectin-3-mediated suppression of $CD8^+$ T cell-secreted INFγ in vitro. It has been associated to other ligands like the liver sinusoidal endothelial cell lectin (LSECtin), This molecule is also been identified in human melanoma tissues where it promotes the cancer's growth by the inhibition of T-cell dependent anti-tumour responses.

LAG-3 is a negative regulator of T-cell activation and function, as it has been shown that increased proliferation of cells with high production of IL-2, IL-4, INFγ and TNFα is generated when LAG-3 is blocked in human CD4 clones (24).
LAG-3 can mediate bidirectional signalling in the APCs it interacts with. It has been shown that the binding of MHC class II to the T_{regs} that express LAG-3 inhibits DCs activation, thus supressing its maturation (21).

Multivalent anti-receptor antibody induced LAG-3 signalling leads to lack of response to TCR stimulation with inhibition of both proliferation and cytokine secretion, and down-regulation of TCR expression (24).

Metalloproteases are necessary in order to regulate expansion and function of T-cells. LAG-3 cleavage is mediated by two transmembrane metalloproteases ADAM10 and ADAM17, with activity is modulated by two different mechanisms dependent on TCR signalling (25).

LAG-3 must be cleaved from the cell surface to allow for normal T-cell activation, as noncleavable LAG-3 mutants prevent proliferation and cytokine production (25).

Finally, LAG-3 limits autoimmunity and sustained co-expression with other IRs. Studies show that in LAG-3 and PD-1 combinatorial blockade synergistically enhanced antitumor immunity is found in several tumours such as ovarian. Dual targeting increased $CD4^+$ and $CD8^+$ T-cell infiltration, as well as increasing the frequency of INFγ and TNFα. This combination has been demonstrated to be efective in the long-term survival of 80% of mice, compared to 40% of mice receiving anti-PD1 alone (25).

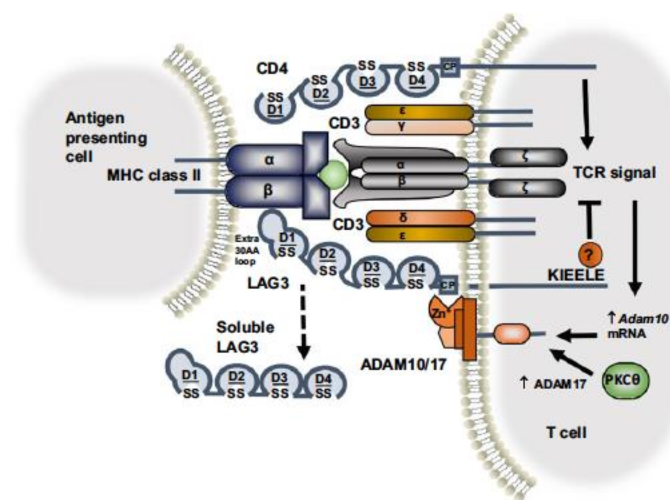

Figure 11. LAG-3 structure

4. JUSTIFICATION

Currently, allogenic HSCT is the choice treatment in patients with acute myeloid leukaemia and myelodysplastic syndromes, which reduces the cancer progression and responds to an answer to the chemotherapy treatment or attempts to replace a faulty haematopoiesis. Around 25.000 allogenic HSCT are produced annually in Occident as stated by Ferrara et al (8).

As it has been mentioned in the introduction, the most common complication is acute GVHD. There is an influence of systemic acute GVHD on identical sibling donors of 40% of recipient's HLA-identical grafts.

A complication such as acute GVHD produces a high mortality and morbidity rate for the patient. The refractory cases there can be up to 85% of the mortality rate.

Moreover, it is necessary to consider the need for more hospital admissions, days hospitalized and the use of either treatments or interventions, as they generate a huge economic impact on the health system.

The LAG-3 is a protein that acts as an inhibitor of the T-cells response. The genotype detection would help us to prevent allogenic responses of these donor's cells infused and therefore, a lower incidence in acute GHVD on the patients with allogenic HSCT.

For these reasons we have decided to explore this association between the donor's LAG-3 genotype and acute GVHD incidence rate in a retrospective cohort.

5. HYPOTHESIS AND OBJECTIVES

5.1. HYPOTHESIS

Our hypothesis that donor's LAG-3 genotype would be associated with acute GVHD incidence after allogeneic HSCT from an identical HLA sibling donor. This will be realised through a multicenter study and coordinated by ICO Girona.

5.2. OBJECTIVES

Primary objectives

- To analyse the association of allogeneic HSCT with incidence of acute GVHD based on the donor's LAG-3 genotype.

Secondary objectives

- To describe the impact on overall (OS) and disease-free survival (DFS) in allogeneic HSCT patients from an identical HLA sibling donor.
- To determine if transplant related mortality (TRM) is associated with LAG-3 genotype of the donor in patients with allogeneic HSCT from an identical HLA sibling donor.

6. PATIENTS AND METHODS

6.1. STUDY DESIGN

It has been designed a retrospective multicenter single cohort study coordinated by ICO Girona.

6.2. STUDY POPULATION

The study population was based on patients receiving an allogenic HSCT from an identical HLA sibling donor.

6.2.1. INCLUSION CRITERIA

- Patients receiving an allogenic HSCT from an HLA-identical sibling donor in hospitals from Spanish Group of Haematopoietic Transplant (GETH) between years 2000-2014.
- Available DNA sample of patient and donor.

6.2.2. EXCLUSION CRITERIA

- Matched unrelated donor (MUD)
- DNA sample of the donor unavailable
- Exclusion transplant criteria: age under eighteen, non-controlled arterial hypertension, advanced heart or lung disease.

6.3. PATIENTS

The study will include DNA samples from 797 patients with an allogenic HSCT from an HLA-identical sibling donor collected by Spanish transplant teams of Spanish Group of Haematopoietic Transplant (GETH) between 2000-2014. Samples and data from patients included in this study were provided by the Biobank of the Biomedical Research Institute of Girona (IDIBGI), joined in the Spanish National Biobanks Network and they were processed following standard operating procedures with the appropriate approval of the Ethics and Scientific Committees.

Before DNA storage, all patients and donors signed the informed consent, and the study met with the recommendations of the Helsinki declaration.

Table 2. Population characteristics of the population included in the LAG-3 study

Population characteristics		
Gender	Man	61.2%
	Woman	38.8%
Diagnosis	Acute lymphoblastic leukaemia	16.8%
	Acute myeloblastic leukaemia	35%
	Chronic myeloid leukaemia	13.3%
	Medullary aplasia	4.3%
	Lymphoma	18.9%
	Myelodysplastic syndrome	10.4%
	Others	1.2%
Stage disease	Early	62.3%
	Advanced	37.7%
HSCT conditioning	Myeloablative	69.5%
	Non myeloablative	30.5%
Cell source	Peripherally blood	75.1%
	Bone marrow	24.9%
Mismatch sex	Yes	24.1%
	No	75.9%

6.4. STUDY VARIABLES

Independent variable

The independent variable in this study is the donor's genotype LAG-3 in patients with allogenic HSCT.

Dependent variable

- **Having an acute GVHD**. Having or not the graft-versus-host-disease in our patients with allogeneic HSCT from an identical-HLA sibling donor it was analysed.
- **Rate of recurrence of the base disease**. We considered death without recurrence of the base disease.
- **Transplant-related mortality**. Patients with allogeneic HSCT have died in relation with the transplant procedure.

- **Disease-free survival.** Time our patients have survived without signs of the disease after allogeneic HSCT.
- **Overall survival**. Percentage of patients who are still alive a period of 10 years after allogeneic HSCT.

Co-Variables

- Patient's and donor's age: in years old, measured as a quantitative continuous variable.
- Patient's and donor's gender: man or woman. It was analysed as a categorical dichotomous variable.
- Diagnosis: Data was categorized in eight groups; acute myeloid leukaemia, Acute lymphoblastic leukaemia, Myelodysplastic syndrome, Chronic myeloid leukaemia, Medullary aplasia, Lymphoma and others.
- Cell source: peripherally blood or bone marrow. It was analysed as a categorical dichotomous variable.
- Disease stage: early or advanced. It was analysed as a categorical dichotomous variable.
- TBI: yes or not. It was analysed as a categorical dichotomous variable.

6.5. DATA ACQUISITION

All the biological data was obtained thanks to a rigorous method and following up all the security protocols.

In order to extract DNA samples from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, GmbH, Hikden, Germany). Moreover, following on behind the manufacturer's instructions and stored at -80 ºC until use. As you can see in **annex IV**, which I did an intership in IDIBGI.

We decided to study rs870849 C>T polymorphism within the exon 8 of LAG-3 due to the T-allele relation with higher incidence of multiple sclerosis. Therefore, there are some studies suggesting this association with LAG-3 and multiple sclerosis in chromosome 12, so we used C-allele (26).

The genotype of the donor for these polymorphisms was determined via allelic discrimination plots on the Applied Biosystems 7500 Fast Real-Time PCR system by using TaqMan real time PCR primers and probes obtained as commercially available AB Assay on Demand reagents (Life Technologies, Carlsbad, CA).

The Assay on Demand reagents included both necessary primers and fluorescently labeled (FAM and VIC) TaqMan MGB probes to amplify and detect each polymorphism.

The PCR cyclin conditions were the following: first of all, the denaturation at 95ºC for 10 minutes. Secondarily, were 40 cycles of denaturation at 95º for 15 seconds. Finally, the annealing/extension at 60ºC during 60 second.

As a result of comparing the relative end-point fluorescence created by the degradation of each fluorescently labelled TaqMan prove, we could define the presence of wild-type and variant alleles. All this process, according to the manufacturer's instructions.

6.6. STATISTICAL ANALYSIS

The statistical analysis was carried out using SPSS and R software packages. A p-value ≤ 0.05 was considered statistically significant.

For the univariate analysis, the allele frequencies, genotype and haplotypes were designed by direct counting.

Therefore, homogeneity between genotype groups was executed using by the chi-square test or Fisher's exact test for qualitative variables. In other hand, for continuous variables was used Student's T-test.

As we have said in the page before, our main study objective was to detect differences in the acute GVHD incidence according to the LAG-3 genotype of the donor. So, cumulative incidence estimates were used to explore differences in acute GVHD. A competitive risk for acute GVHD was determined death without signs of GVHD.

Our secondary objectives were overall survival (OS), relapse incidence, disease-free survival (DFS) and transplant-related mortality (TRM). The association of LAG3 with the rate of recurrence of the underlying disease (competitive risk: death without recurrence) and with transplant-related mortality will also be assessed using cumulative incidence. Kaplan-Meier curves were derived to determinate overall survival and disease-free survival (DFS), and curves were compared by means of the log-rank test.

Multivariate analysis was performed using the Cox regression model. All the variables with a P-value ≤ 0.2 in the univariate analysis were included in the multivariate analysis. Results were expressed as hazards ratios, and 95% confidence intervals (95% CI).

7. ETHICAL CONSIDERATIONS

This project complies with ethical principles of the *Declaration of Helsinki* about researching involving human subjects established by World Medical Association. Before beginning our study, the correspond project was evaluated by the Clinical Research Ethical Committee (CEIC) of Hospital Universitari Josep Trueta, in Girona.

Due to the participation of humans, the design and the treatment of patient's personal data will be carried out respecting the Basic Ethical Principles, and, moreover the study will respect the criteria established by the Nuremberg Code, the Belmont Report and Oviedo Convention.

It was followed the Law 41/2002 of 14 November, that regulates the autonomy of the patient and their right to information and clinical documentation and *Royal Decree 1720/20007 of December 21* that regulates the security of files containing patient data.

According to the legal framework of human rights and data confidentiality specified in Organ *Law 15/1999* on the Protection of Personal Data (LOPD), data was registered and analysed anonymously and under non-identifying numeric codes. The author didn't have access to any confidential information of the patient which was only used for the purpose of the research.

After receiving the appropriate information, the patients voluntarily signed an informed consent for LAG-3 genotype, information contained in the *Law 14/2007* for invasive procedures.

Because obtaining the LAG-3 genotype of the DNA is considered a genetic test, this process is protected under the Law on research of biological samples (Law 14/2007 and Royal Decree 1716/2011).

Moreover, Royal Decree 1716/2001, which establishes the basic requirements for the authorisation and operation of biobanks for biomedical research and the treatment of biological samples of human origin. This Royal Decree is useful in this study, as these institutions are required to store the samples.

Once the patients have signed in their informed consent, the study is allowed to begin.

This study does not have any commercial bias or interests.

8. RESULTS

ACUTE GVHD

We defined two groups of patients according to the LAG-3 rs870849 genotype of their donors: patients receiving grafts from donors homozygous for the rs870849 C allele would be expected to have a less active LAG-3 function when compared with those with CT/TT genotypes. The homogeneity study demonstrated no clinically significant differences between the two groups of LAG-3.

The cumulative incidence of grades II-IV (34,9% vs 32,8%; p: 0.555) and III-IV (14,6% vs 14%; p: 0.763) was compared of patients who had received allogeneic HSCT from an identical HLA sibling donor and no significant differences were found (Figure 12). Moreover, statistical analysis demonstrated no significant differences in chronic GVHD incidence.

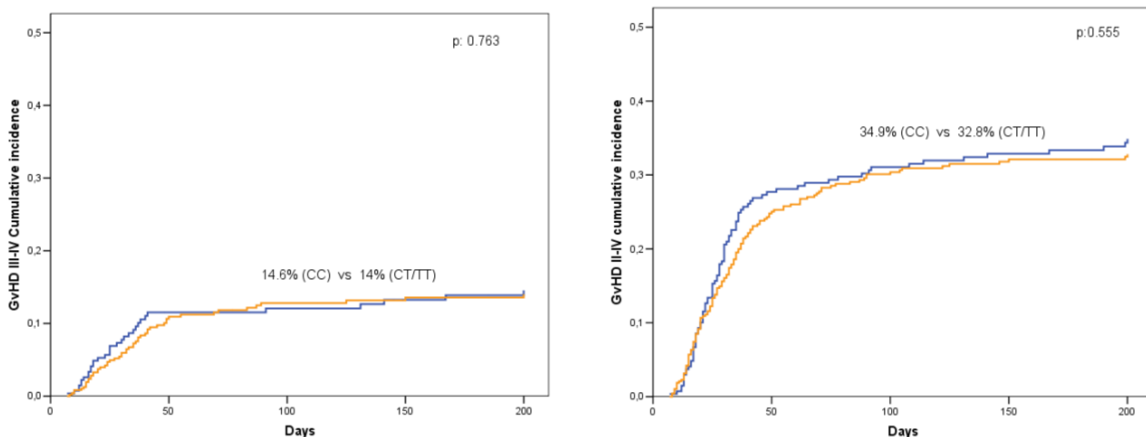

Figure 12: acute GVHD III-IV cumulative incidence according to LAG-3 genotype of the donor and post-transplant days (A). Cumulative incidence of II-IV acute GVHD according to LAG-3 genotype of the donor and post-transplants days (B).

OVERALL SURVIVAL

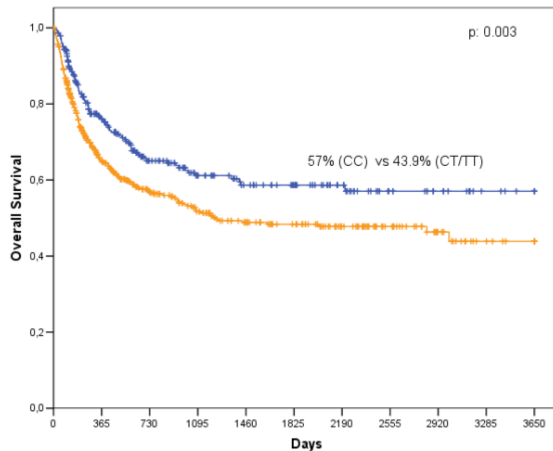

Figure 13. Overall survival according to the LAG-3 genotype of the donor.

In reference to overall survival, the comparison between patients with rs870849 homozygous C donor had a better overall survival and those who received grafts from donors with rs870849 CT/TT genotypes (represented in yellow, in figure 13) (57% vs 43.9%; p: 0.003). Multivariate analysis confirmed this association (Table 3).

Table 3. Multivariate analysis (Cox regression model) of OS.

Variable	p-value	Hazard ratio	IC 95%
Age	< 0.001	1.02	1.01-1.03
Diagnosis	< 0.001	1.65	1.26-2.17
Stage disease	< 0.001	2.47	1.91-3.20
Cell source	0.062	0.74	0.55-1.01
LAG-3 genotype	0.022	1.38	1.05-1.81

DISEASE-FREE SURVIVAL

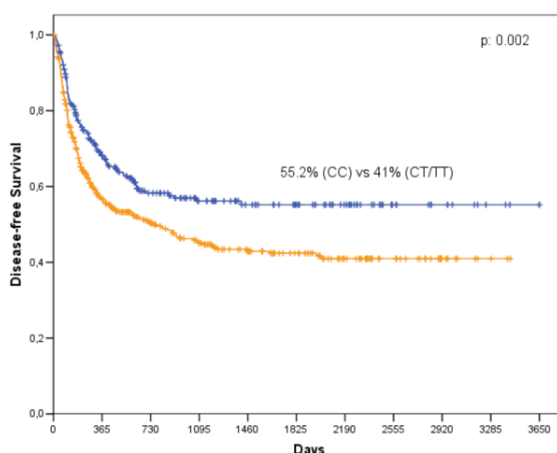

Figure 14. Disease free survival according to LAG-3 genotype of the donor.

Similarity, significant differences in disease-free survival were detected, showing a better DFS those patients receiving grafts from donors homozygous for the C allele (represented in blue, in Figure 14) (55.2% vs 41%; p: 0.002). This fact was confirmed by multivariate analysis (Table 4).

Table 4. Multivariate analysis (Cox regression model) of DFS.

Variable	p-value	Hazard ratio	IC 95%
Age	0.001	1.02	1.01-1.03
Diagnosis	0.001	1.60	1.20-2.10
Stage disease	< 0.001	2.01	1.56-2.59
TBI	0.011	1.43	1.09-1.89
LAG-3 genotype	0.001	1.61	1.22-2.12

RELAPSE

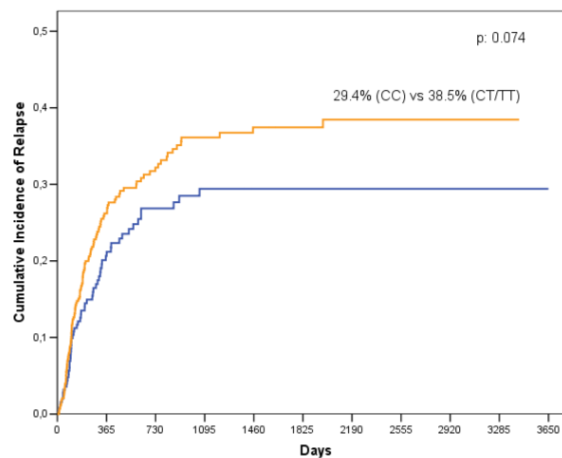

Figure 15. Cumulative incidence of relapse according to donor's LAG-3 genotype.

Univariate analysis showed an increased incidence of relapses in patients whose donor carried at least one T allele when compared with those with a homozygous CC donor (29.4% vs 38.5%; p: 0.074) (Figure 15). Again, multivariate analysis is statistically significant (Table 5).

Table 5. Multivariate analysis (Cox regression model) of relapse.

Variable	p-value	Hazard ratio	IC 95%
Diagnosis	0.004	1.79	1.21-2.64
Stage disease	< 0.001	2.31	1.66-3.23
TBI	0.014	1.54	1.08-2.24
LAG-3 genotype	0.017	1.56	1.09-2.17

TRANSPLANT RELATED MORTALITY

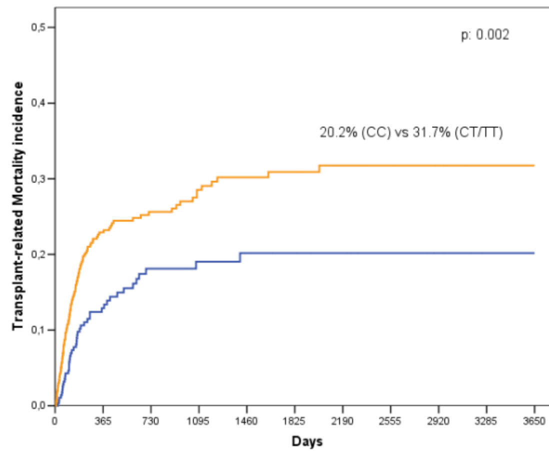

Figure 16. Transplant-related mortality incidence according to donor's LAG-3 genotype.

Finally, patients receiving grafts from donors with rs870849 CT/TT genotypes (represented by yellow line, in Figure 16) had higher TRM than those whose donor was homozygous for the C allele (20.2% vs 31.7%; p: 0.002). This was confirmed in multivariate analyse (Table 6).

Table 6. Multivariate analysis (Cox regression model) of TRM.

Variable	p-value	Hazard ratio	IC 95%
Age	< 0.001	1.03	1.02-1.05
Stage disease	0.001	1.80	1.28-2.45
Gender	0.077	1.37	0.96-1.95
LAG-3 genotype	0.002	1.82	1.24-2.67

9. DISCUSSION

The purpose of our study was to analyse the association of allogeneic HSCT with incidence of acute GVHD based on the donor's LAG-3 genotype. The results of this study highlight the importance of the regulatory molecule LAG-3 in the outcome for patients who receive allogeneic HSCT from an HLA-identical sibling for the treatment of several haematologic malignancies.

Whereas LAG-3 polymorphism (rs870849 C>T) has been correlated with the clinical outcome after allogeneic HSCT from an HLA-identical sibling donor, no statistically significant differences are observed for the incidence of acute GVHD.

Our main finding in the study is that the donor LAG-3 genotype is associated with difference in overall survival after an allogeneic HSCT from an identical sibling HLA donor. We also found statistically significant differences in disease-free survival.

These differences in survival were associated with a decreased risk of relapse when donor had LAG-3 rs870849 CC genotype, which may be due to the fact that a lower functionality of LAG-3 associated with this genotype may lead to a lower inhibition of the immune response. It is very interesting that this effect offers a graft-versus-leukaemia effect without increasing the incidence of graft-versus-host disease.

Increased transplant related mortality in patients receiving grafts from CT/TT homozygous donors, may be due to a higher incidence of infections during the procedure that result in the patient's death due to a higher T-cell inhibition.

This study gives us a prediction of how our patients with an allogeneic HSCT will evolve. Therefore, it allows to be able to select donors when more than one donor is available or to monitor more closely those patients receiving grafts from CT/TT donors, due to its worst overall survival.

The study by Emanuela et al., from Stanford University, confirms that with low doses of conventional T-cells which normally induces only mild GVHD, if T-cells are lacking LAG-3, they induce aggressive GVHD resulting in 100% mortality in their animal studies (27).

Our data is consistent with studies from the Blazar group that are dedicated to studying other inhibitory molecules such as PD-1 and CTLA-4 in the limitation of alloresponse in GVHD. Since they have shown that by co-locking CTLA-4:B7 and PD-1:PD-1L, GVHD is additively accelerated (28).

As stated in Andrews et al., there are currently four LAG-3 modulating agents that have just come out as an anti-cancer therapist. There are also several studies in preclinical phases. There are three different LAG3-specific mAbs developed as cancer treatment drugs. These are BMS-986016 (Bristol-Myers Squibb, fully human IgG4), LAG525 (Novartis, humanized IgG4), and MK-4280 (Merck). This relates to our study, as we would be able to extend the overall survivals of patients with a certain LAG-3 genotype (21).

Therefore, more effective treatments can be used to address the complications of allogeneic HSCT, allows for optimization of resources. It also provides an improved quality of life for the patients, thus extending their survival. In addition, it would also allow us to know, from the time the allogeneic HSCT is performed, that patients may have a worse survival and therefore, be able to monitor them more closely.

10. LIMITATIONS AND STRENGTHS

LIMITATIONS

There are some potential limitations that should be considered:

- It is a multicenter study, so most variables are collected in hospitals where allogeneic HSCT is performed and there may be information bias. To avoid this, information is collected using standardised instruments (Glucksberg scale, which assesses acute GVHD).
- This study should be compared with other research groups in different European countries to see if the same results are obtained. If the same results are attained, these could be extrapolated to the Caucasian population.
- It should be validated at an international level; as we do not know if it is beneficial for other ethnic groups. Therefore, it should be compared with studies made in other countries, for example, Japan.
- Extrapolation of HSCT from an unrelated donor should also occur, as this type of donor is increasingly used.

STRENGTHS

There are some strengths of this study:

- As it is a multicenter study linked to the Spanish Group of Haematologic Transplants (GETH), the results can be extrapolated to the Spanish population.
- As it is a retrospective study, the data has already been collected, so there was no loss of patients.
- Our results can be modified because there are different covariates. Therefore, this confounding bias is minimised by using multivariate analysis to adjust results to confounding factors.

11. CONCLUSIONS

After completing this work, it can be concluded:

1. The donor's LAG-3 genotype is associated with differences in overall survival after allogeneic HSCT from HLA-identical sibling donor.

2. Higher rate of relapse is correlated with different outcomes in overall survival of our patients.

3. An increased transplant-related mortality has also been observed, being higher for patients in which the LAG-3 donor was heterozygote or homozygote for the T allele.

4. However, no differences have been found in the incidence of acute GVHD.

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ANNEX I: Explanation of HSCT phases

1. Pre-transplant assessment: from the patient and the donor. It has to be proved that the illness is in the best conditions for a transplant. In good conditions it would be a patient in complete remission, so it would be more successful. It has to be confirmed that the patient will be able to resist the conditioning treatment, so the associated comorbidities have to be evaluated. In case that the patient have them, a reduced intensity conditioning regimen has to be contemplated. The patient and the family have to be well informed, moreover they have to know the side effects. More tests have to be done on the patient or the donor (2).

2. Hospital admission in an isolation room of the haematology department: Depending on the type of transplant, patient and conditioning type, there will be more or less risk of infections. Depending on the risk, the patient will be isolated (2).

3. Placement of a central venous catheter: It can be placed in the jugular vein or subclavian vein. Through the catheter, the patient receives all the medication, transfusion and the blood is extracted for analysis. The catheter has to be attached with stiches or tunnelling. To tunnel it, a tunnel has to be made under the skin so 5 to 10 centimetres of catheter can stay inside the skin. This procedure is beneficial for long treatments, as they can last months or years. The catheter needs very rigorous care since infections are very frequent, and also obstructions, breaks, etc (4).

4. Conditioning treatment: depending on the patients' traits, illness, compatibility with the donor and the stem cells source. This treatments' objective is to eliminate the maximum number of neoplastic cells. A part from to preparing the organism for receive and tolerate the donor's cells (2).

5. Prevention of complications: such as nausea, vomiting and mucositis, also from infections by endogenous bacteria or pathogens in a latent state and after allogenic transplantation prevent GVHD (2).

6. As previously mentioned HSC obtention can come from umbilical cord, peripheral blood or bone marrow (4).

7. Manipulation of the inoculum: may be performed in more cases to eliminate red blood cells if there is also incompatibility, eliminate neoplastic cells or remove T-lymphocytes (4).

8. HSC conservation: may be stored in fresh or after cryopreservation (4).

9. Stem cells administration: after the conditioning treatment, the donor's stem cells are infused on day zero. During the infusion, the vital signs from the patient and the appearance of side effects are monitored. The procedure lasts between 15 minutes and an hour. In case that complications appear Complications can appear, due to the fact that infused cells had been frozen, *stored with DMGO* and might cause fever, vomits, chills, dark urine and strange smell (2).

10. Immediate post-transplant: it is a phase of aplasia between 10 and 16 days after the transplant, when side effects might appear. Its intensity is related to the conditioning treatment's intensity. Also, immune complications between the donor and the recipient might appear.

    The side effects are nausea, vomiting, oral mucositis, diarrhoea, alopecia and medullar insufficiency. Also, there are other less common side effects such as implant failure, haemorrhagic cystitis, hepatic veno-occlusive disease, diffuse alveolar haemorrhage, idiopathic pneumonia and thrombotic microangiopathy.

    Finally, if there is a stem cell implant, some side effects may appear such as the engraftment syndrome and acute GVHD, which will be explained later (2).

11. Hematologic recovery: recover analytical levels of normal haematopoietic function (4).

12. Discharge and long-term post-transplant follow up: basically, control complications (4).

ANNEX II: CEIC's permission

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Marta Riera Juncà, Secretària del Comitè d'Ètica d'Investigació CEI GIRONA, amb domicili a l'Hospital Universitari de Girona Dr. Josep Trueta Avinguda de França s/n 17007 Girona

CERTIFICA

Que el Comitè d'Ètica d'Investigació CEI GIRONA, segons consta en l'acta de la reunió celebrada el dia 27/03/2018 ha avaluat el projecte: **Modulación de respuesta inmune en trasplante alogénico de progenitores hematopoyéticos por el genotipo de moléculas inhibidoras de checkpoint. Biobanco español de aloTHP** amb el Dr. DAVID GALLARDO GIRALT com a investigador principal.

Que els documents s'ajusten a les normes ètiques essencials i per tant, ha decidit la seva aprovació.

I, perquè consti, expedeixo aquest certificat.

Hospital Universitari de Girona Doctor Josep Trueta Comitè Ètic d'Investigació Clínica Institut Català de la Salut

Girona, a 28/06/2018

55002196

ANNEX III: Informed consent

HOJA DE INFORMACIÓN AL PACIENTE

OBTENCIÓN Y UTILIZACIÓN DE MUESTRAS BIOLÓGICAS Y DATOS CLÍNICOS POR INVESTIGACIÓN MÉDICA Y CONSERVACIÓN EN UN BIOBANCO

En la mayoría de hospitales, además de la asistencia a los pacientes, se realiza investigación biomédica. La finalidad de esta investigación es progresar en el conocimiento de las enfermedades y en su prevención, diagnóstico y tratamiento. Esta investigación biomédica requiere recoger datos clínicos y muestras biológicas de pacientes y donantes sanos para analizarlos y obtener conclusiones con el objetivo de conocer mejor las enfermedades y avanzar hacia su diagnóstico y/o tratamiento.
Las muestras y datos clínicos obtenidos para el diagnóstico o control de las enfermedades, una vez utilizadas para dicha finalidad, resultan también útiles y necesarias para la investigación. De hecho, muchos de los avances científicos obtenidos en estos últimos años en medicina son fruto de este tipo de estudios.

Solicitamos su autorización para la obtención de una muestra biológica adicional que se le extraerá en su hospital de referencia, con el fin de depositarla en la colección GET (DNAteca del Grupo Español de Trasplante Hematopoyético y Terapia Celular (GETH) del Biobanc IDIBGI, así como su autorización para utilizar la información clínica asociada a este material biológico para proseguir con la investigación biomédica.

Siguiendo lo que establece la Ley 14/2007, de Investigación Biomédica, y Reglamento (UE) 2016/679, del Parlamento Europeo y del Consejo de 27 de abril de 2016 relativo a la protección de las personas físicas, le solicitamos que lea detenidamente este documento de información y el consentimiento informado que se le adjunta al final para su firma, si está de acuerdo al participar en esta propuesta.

Un Biobanco es una institución regulada por leyes específicas que facilita la investigación biomédica, es decir, aquella destinada a promover la salud de las personas. Las muestras incluidas en un Biobanco pueden ser cedidas para la investigación en medicina, siempre bajo la supervisión de un comité científico y otro de ética. Las muestras se cederán generalmente sin información personal asociada, aunque a veces podrá ser necesario el acceso a la historia clínica o al resultados otras pruebas para completar la investigación.

FINALIDAD DE LA INVESTIGACIÓN: progresar en el conocimiento de las enfermedades.

La finalidad de la investigación es mejorar nuestro conocimiento de las enfermedades. Las muestras, los datos clínicos y analíticos y las pruebas de imagen se utilizarán para la investigación biomédica. Todo esto permitirá progresar en el conocimiento de la prevención, diagnóstico, pronóstico y/o tratamiento de las enfermedades.

CONSIDERACIONES ESPECÍFICAS DE LA COLECCIÓN:

El ADN es una molécula que contiene toda la información genética necesaria para las funciones celulares. El código genético da lugar a las características heredadas individualmente, tales como el color de los ojos y el grupo sanguíneo. El motivo por el cual el Grupo Español de Trasplante Hematopoyético quiere desarrollar un banco de ADN de receptores y donantes es para identificar cuáles son las diferencias genéticas que se asocian a complicaciones post-trasplante. Una vez identificadas, esta información puede ser muy valiosa para la selección de futuros donantes.
Esta colección contribuirá a los avances en el conocimiento del trasplante de células madre hematopoyéticas, así como a mejorar el éxito de tales trasplantes en el futuro. Se pretende identificar diferencias genéticas entre pacientes y donantes que supongan un riesgo aumentado para desarrollar complicaciones post-trasplante, de tal manera que puedan desarrollarse estrategias individualizadas para cada paciente según su riesgo individual.
Para participar en esta colección se requiere una única donación de una muestra de sangre, que será extraída en el mismo centro donde se realizará el trasplante, por personal cualificado. Los riesgos de participación en dicha colección son exclusivamente las inherentes a la propia extracción de sangre.
La colección GET incluye la participación de todos los centros españoles donde se realizan trasplantes alogénicos (a partir de un donante) que así lo deseen. Una vez obtenida la sangre de paciente y donante, ésta será transportada hasta Girona, dónde se halla el nodo responsable de extraer el ADN para almacenarlo en el Biobanco IDIBGI. Los datos clínicos en cuanto al desarrollo del trasplante y sus complicaciones (si es que aparecieran) serán recogidos por el equipo que realiza el trasplante y reportados al centro de referencia.

MUESTRAS BIOLÓGICAS E INFORMACIÓN ASOCIADA: les muestras obtenidas se custodiaran y conservaran en el Biobanc IDIBGI hasta su extinción.

Se guardará y dispondrá de la muestra biológica adicional de *aprox. 6 ml de sangre* para realizar estudios de investigación biomédica, sin que este hecho le cause molestias adicionales.
De forma paralela a la recogida de muestras se procederá a la recogida de datos antropométricos y clínicos, que se asociarán a sus muestras biológicas.
El GETH cuenta con distintos centros que participan en el reclutamiento de muestras. Una vez obtenida la sangre, se envía a Girona para su procesamiento y almacenamiento en el Biobanc IDIBGI. Los datos clínicos recogidos son introducidos a la aplicación informática del BIOBANC IDIBGI.
La donación de estas muestras cedidas al Biobanc IDIBGI no impedirá que usted o su familia puedan usarlas, cuando sea necesario por motivos de salud. Las muestras y la información asociada a estas se custodiarán y conservarán en el Biobanc (banco de muestras biológicas) IDIBGI, hasta su extinción.
Este Biobanco es un establecimiento sin ánimo de lucro e inscrito en el *Registro Nacional de Biobancos* dependiente del *Instituto de Salud Carlos III* con la referencia B.0000872, que acoge colecciones organizadas de muestras biológicas e información asociada en las condiciones y garantías de seguridad que exige la legislación anteriormente referida y los códigos de conducta aprobados por los comités de ética. Las mencionadas muestras y su información asociada quedan disponibles por aquellos investigadores que lo soliciten al Biobanco.
Cualquier estudio de investigación para el cual se solicite la utilización de estos datos o muestras tendrá que disponer siempre de la aprobación del Comité de Ética de la Investigación Clínica (CEIC) competente, que velará para que los investigadores desarrollen sus estudios siguiendo siempre las más estrictas normas éticas y legales. Además, el comité científico del Biobanco garantizará que los proyectos sean de excelencia científica. La investigación biomédica es actualmente un fenómeno global, de forma que ocasionalmente estas muestras podrán ser cedidas a grupos de investigación fuera de España, siempre que cumplan los requisitos de la legislación española y lo aprueben los correspondientes comités.
A partir de las muestras dadas, en los casos en que la investigación lo requiera, se realizarán estudios genéticos, y a partir de ellos se puede obtener información sobre su salud y la de sus familiares. Siempre se actuará velando por la protección de esta información (apartado protección de datos).
Por este consentimiento, los responsables del Biobanc IDIBGI podrán consultar su historial clínico, sólo en el supuesto de que esto sea imprescindible para la investigación del proyecto para el cual se solicitan las muestras y previa autorización por parte del comité de ética correspondiente.
En el caso de ser necesaria alguna muestra adicional, la institución sanitaria se podría poner en contacto con usted para solicitarle nuevamente su colaboración. En este caso se le informará de los motivos y se le solicitará de nuevo su consentimiento.

PROTECCIÓN DE DATOS Y CONFIDENCIALIDAD: las muestras se conservaran codificadas.

Los datos personales que se recojan serán obtenidos, tratados y almacenados cumpliendo en todo momento el deber del secreto, de acuerdo con la legislación vigente en materia de protección de datos de carácter personal.
La identificación de las muestras biológicas del Biobanco será sometida a un proceso de codificación. A cada muestra se le asigna un código de identificación, que será el utilizado por los investigadores. Sólo el personal autorizado por el Biobanco podrá relacionar su identidad con los citados códigos. Mediante este proceso los investigadores que soliciten muestras al Biobanco no podrán conocer ningún dato que revele su identidad. Del mismo modo, aunque los resultados obtenidos de la investigación realizada con sus muestras se publiquen en revistas científicas, su identidad no será facilitada. En aquellos estudios en los cuales no se prevean resultados potencialmente útiles para su salud, y de acuerdo con el correspondiente Comité de Ética, las muestras y datos podrán ser anonimizadas, es decir, no habrá ninguna posibilidad de volver a asociar la muestra con su identidad.
Sus muestras y datos clínicos asociados a las mismas pasarán a formar parte del fichero del Biobanco, inscrito en la Agencia de Protección de Datos bajo la responsabilidad del Instituto de Investigación Biomédica de Girona (IDIBGI), mientras no ejerza su derecho de oposición.
Usted podrá ejercer sus derechos de acceso, rectificación, cancelación y objeción, así como obtener información sobre el uso de sus muestras y datos asociados, dirigiéndose a:

DIRECCIÓN DEL BIOBANC IDIBGI	Parc hospitalari Martí i Julià, c/Dr. Castany s/n
Institut d'Investigació Biomèdica de Girona Dr. Josep Trueta	17190 Salt
biobanc@idibgi.org	Tel 872987087

CARÁCTER ALTRUISTA DE LA DONACIÓN: la cesión de muestras biológicas que usted realiza al Biobanc IDIBGI es gratuita.

La donación tiene por disposición legal carácter altruista, por lo cual usted no obtendrá ni ahora ni en el futuro ningún beneficio económico de la misma, ni tendrá derechos sobre posibles beneficios comerciales de los descubrimientos que se puedan conseguir como resultados de la investigación biomédica.

PARTICIPACIÓN VOLUNTARIA: su negativa NO repercutirá en su asistencia médica, presente o futura.

Su participación es totalmente voluntaria. Si firma el consentimiento informado, confirmará que desea participar. Puede negarse a participar o retirar su consentimiento en cualquier momento posterior a la firma sin tener que explicar los motivos y que esto repercuta en su asistencia médica, presente o futura.

COSTE Y RIESGOS ASOCIADOS: su donación no le supondrá ningún coste.

La extracción de la muestra no le supondrá a usted ningún coste económico. En el caso de una extracción de sangre, el riesgo para su salud es muy pequeño, pero puede incluir las molestias habituales de una extracción de sangre: dolor de poca importancia, piel contusionada, sangrado por donde entra la aguja o la ansiedad frente las agujas. Se tomarán precauciones para evitar estos inconvenientes.

REVOCACIÓN DEL CONSENTIMENTO: si usted decide firmar este consentimiento podrá también cancelarlo libremente. Esto comportará la destrucción de sus muestras.

Si en un futuro usted quisiera anular su consentimiento, sus muestras biológicas serían destruidas y los datos asociados a las mismas serán retirados del Biobanco. También podría solicitar la anonimización de las muestras, de forma que en este caso se eliminaría la relación entre sus datos personales (que revelan su identidad) y sus muestras biológicas y datos clínicos asociadas. Los efectos de esta cancelación o anonimización no se podrían extender a la investigación que ya se haya realizado. Si deseara cancelar el consentimiento, lo tendría que solicitar por escrito a la Dirección del Biobanc IDIBGI, a la dirección anteriormente mencionada.

INFORMACIÓN SOBRE LOS RESULTADOS DE LA INVESTIGACIÓN: se le proporcionará información si usted la desea recibir.

En el supuesto de que usted lo pida expresamente, el Biobanco podrá proporcionar información sobre cuáles son las investigaciones en que se han utilizado sus muestras y de los resultados globales de estas investigaciones, excepto en el caso de cancelación o anonimización.
Los métodos utilizados en investigación biomédica suelen ser diferentes de los aprobados para la práctica clínica, por el que no tienen que ser considerados con valor clínico para usted. A pesar de esto, en el supuesto de que estas investigaciones proporcionen datos que pudieran ser clínica o genéticamente relevantes para usted e interesar a su salud o a su familia, le serán comunicados si así lo estima oportuno. Así mismo, podría darse el caso de obtenerse información relevante para su familia. En este supuesto, le corresponderá a usted decidir si quiere o no que esta información le sea comunicada. En caso afirmativo, tiene que consignarlo a la casilla que aparece al final de este documento.
Si usted no desea esta información, tenga en cuenta que la ley establece que, cuando la información obtenida sea necesaria para evitar un grave perjuicio para la salud de sus familiares biológicos, un Comité de expertos estudiará el caso y tendrá que decidir si es conveniente informar a los afectados o en sus representantes legales.

Por favor, pregunte al personal sanitario que le ha comunicado esta información sobre cualquier duda que pueda tener, ahora o en el futuro, en relación a este consentimiento. Así mismo, puede comentar sus dudas a su médico, que le pondrá en contacto con el personal sanitario autorizado.

Muchas gracias por su colaboración.

BIOBANC IDIBGI

Le agradecemos su desinteresada colaboración con el avance de la ciencia y la medicina.
De esta manera está colaborando para vencer las enfermedades y ayudar a multitud de enfermos actuales y futuros.

Ejemplar DONANTE

CONSENTIMENTO INFORMADO

OBTENCIÓN Y UTILIZACIÓN DE MUESTRAS BIOLÓGICAS Y DATOS CLÍNICOS PARA INVESTIGACIÓN MÉDICA I CONSERVACIÓN EN UN BIOBANCO

Si ha comprendido la información que se le ha proporcionado en el documento informativo, resuelto cualquier duda que pudiera tener y decide colaborar con el Biobanc IDIBGI en los términos antes explicados, por favor, lea y firme a continuación esta hoja:

Quién firma el presente documento autoriza a su centro hospitalario de referencia ...., a obtener la muestra biológica adicional de *aprox. 6 ml de sangre* para que puedan ser incorporada al Biobanc IDIBGI, el cual podrá almacenar y utilizar científicamente tanto la información clínica y asistencial de su historial médico como las pruebas de imagen y las muestras biológicas obtenidas, con el fin de desarrollar proyectos de investigación biomédica, siempre que estos cuenten con la obligada aprobación del Comité de Ética de Investigación competente.
Esta autorización la concede después de haber sido informado verbalmente y haber leído la información adjunta sobre el consentimiento informado para la recogida de datos clínicos, analíticas, pruebas de imagen y muestras biológicas para investigación biomédica.

Confirmo que:

1. Autorizo que la muestra biológica cedida y la información clínica asociada se utilice en investigaciones:
   Nacionales: ☐ SI ☐ NO Internacionales: ☐ SI ☐ NO

2. Deseo que se me comunique la información derivada de la investigación que realmente sea relevante y aplicable para mi salud o la de mi familia:
   ☐ SI ☐ NO Teléfono o email de contacto.................................

3. Autorizo a ser contactado en el caso de necesitar más información o muestras biológicas adicionales:
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4. He expresado mi deseo de que se me respeten las siguientes excepciones respecto al objetivo y métodos de las investigaciones:
   ....
   ....

PACIENTE/DONANTE	PERSONA QUE INFORMA	☐ TESTIGO (1) / ☐ TUTOR (2)
Nombre *Apellidos* *DNI* *Edad* *Signatura*	*Nombre* *Apellidos* *DNI* *Edad* *Signatura*	*Nombre* *Apellidos* *DNI* *Edad* *Signatura*

(1) Autorizado por el donante
(2) Representante legal

A rellenar en el hospital de origen

☐ PACIENTE
☐ DONANTE

A ...., a ....de.................de........

Llegada la mayoría de edad, el donante tiene derecho a la anulación del consentimiento. En caso de que no lo ejerza, se considerará que el actual documento de consentimiento continúa vigente.

Ejemplar HOSPITAL

CONSENTIMENTO INFORMADO

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Ejemplar BIOBANCO

CONSENTIMENTO INFORMADO

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ANNEX IV: Practices at the research laboratory

EXTRACCIÓ DE CÈL·LULES MONONUCLEARS DE SANG PERIFÈRICA

La tècnica del FICOLL és una tècnica de centrifugació per gradient de densitat per separar cèl·lules mononuclears de sang perifèrica d'altres cèl·lules de la sang. Durant la centrifugació es van separant les diferents capes degut a la diferència de densitat. El sediment estarà format principalment per granulòcits i eritròcits que han migrat a través el medi per tenir una major densitats que el FICOLL i per la tendència de les cèl·lules a formar agrupacions (en el cas del granulòcits). A sobre es troba la capa del FICOLL que és menys densa que el sediment i sobre aquesta una altra capa fina constituïda per les cèl·lules mononucleades (limfòcits i monòcits) que tenen encara una menor densitat. La part superior del falcon seria on hi trobaríem una altra capa on estarien les plaquetes i el plasma. Una vegada realitzada la centrifugació, és recollirà la capa que conté les cèl·lules mononucleades.

Material i reactius

- Tubs falcon de 15mL/50mL
- FICOLL PAQUE
- Suero Fisiològic
- Pipetes Pasteur de vidre i plàstic
- Gradetes
- Centrífuga
- Cambra de Neubauer
- Trypan Blue solution 0.4%, liquid, sterile-filtered (PEL recompte cel·lular)

Procediment

1. Temperar el FICOLL (ha d'estar a TºA) a la campana de cultius.
2. Diluir la mostra de SP en PBS (volum/volum).
3. Preparar un falcon amb 10mL de FICOLL (la meitat del volum de la sang+PBS).
4. Afegir lentament sobre el FICOLL i **sense barrejar les fases.**

Figura 1. Tapa de polipropileno y extracción de anillo de plata

Figura 2. Retirada de medios Ficoll-Paque

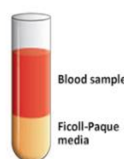
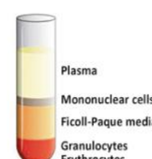
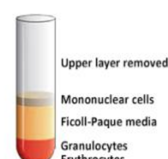


Figura 3 . Muestra de sangre en capas sobre medios Ficoll-Paque



La Figura 4 . Antes de la eliminación de la capa superior.



La Figura 5 . Después de la capa superior (plasma) eliminado.

5. Centrifugar durant 20 minuts a 2000rpm (o 1200g) i desaccelerar la centrífuga.
6. Recollir l'anell de separació.
7. Fer el recompte cel·lular amb la cambra de Neubauer.

EXTRACCIÓ DE DNA (KIT QIAGEN)

- Engegar termobloc a 56 °C
- Posar en un criotub nou 20 µl de proteïnasa (que s'ha de descongelar prèviament)
- Afegir 200 µl de mostra (150ul si es tracta de MO)
- Afegir 200 µl de buffer AL (150 ul si es tracta de MO)
- Vortejar 15 segons
- Incubar a 56ºC durant 10 minuts
- Pols de centrífuga
- Afegir 200 µl d'etanol absolut (150 ul si es tracta de MO)
- Vòrtex uns 15''
- Pols de centrífuga
- Transvasar-ho tot a l'spincolumn, en un tub col·lector net i centrifugar 1 min a 8000 rpm
- Posar la columna en un nou tub collector i afegir 500 µl de buffer AW1
- Centrifugar 1 min a 8000 rpm
- Canviar la columna a un nou tub col·lector i afegir 500µl de buffer AW2.
- Centrifugar 3 min a 8000 rpm + 0.30 min a 14000 rpm
- Canviar la columna a un col·lector nou, i afegir 100 µl de buffer AE. Deixar 1 (5) minut a Room Temperature, i centrifugar 1 (2-3) min a 8000rpm.
- Sense canviar la columna a un col·lector nou, afegir 100 µl de buffer AE. Deixar 1 (5) minut a Room Temperature, i centrifugar 1 (2-3) min a 8000rpm
- Llençar spincolumn, i quedar-nos amb l'eluït. Canviar-ho a un criotub, etiquetat correctament.

→ Guardar tubs en caixa de DNATeca i omplir registre

→ La concentració esperada en els 200 ul d'eluït és de 30ng/ul

PCR REAL-TIME DE GENOTIPACIÓ

Material

- QuantStudioTM 6 and 7 Flex Real-Time PCR System (Applied Biosystem®)
- MicroAmp™ Optical 384-Well Reaction Plate with Barcode; ref: 4309849 (ThermoFisher Scientific)
- MicroAmp™ Optical Adhesive Film; ref: 4311971 (ThermoFisher Scientific)
- Pipetes de 2µl, 10µl, 200µl i 1000µl (Eppendorf®)
- Tubs eppendorfs de 5ml (Eppendorf®)

Reactius

- TaqMan® SNPmGenotyping Assay LAG-3; **rs870849**; ref: 4351379 (Applied Biosystem®)
- TAQPATH ProAmps W/Rox, 50ml, GPR; ref: A30867 (ThermoFisher Scientific)
- Nuclease-free water 10x50ml; ref: AM9937 (Ambion ™)

Procediment

1- **Tria de mostres, segons l'estudi que es realitza** (mostres del GETH, Grupo Español de trasplante Hematopoyético y terapia Celular.

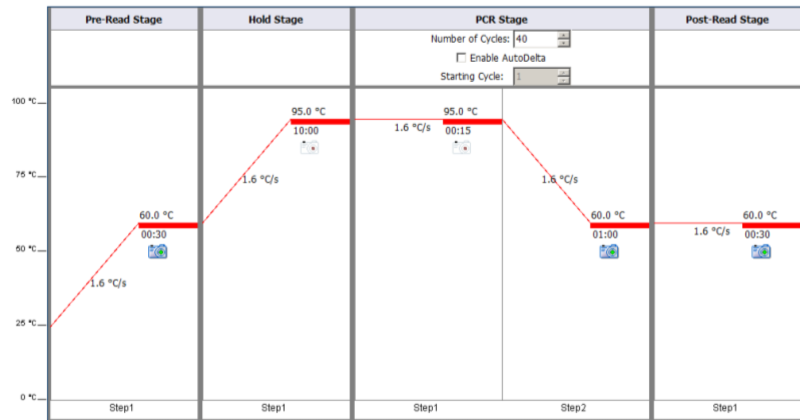
2- **Cada pou és una mostra**. En total a la placa es poden carregar 384 mostres diferents, però també posem els BLANCS (reacció igual de la real-time, però enlloc de mostra, es posa aigua). A més hi afegim els controls endògens: mostres que **JA** sabem prèviament quin és el seu genotip per l'rs que volem analitzar (en aquest cas LAG-3) i que ens ajuda a l'hora d'analitzar la placa.

3- Es realitzen els càlculs per fer la mix de reacció (taqman+assay+aigua):

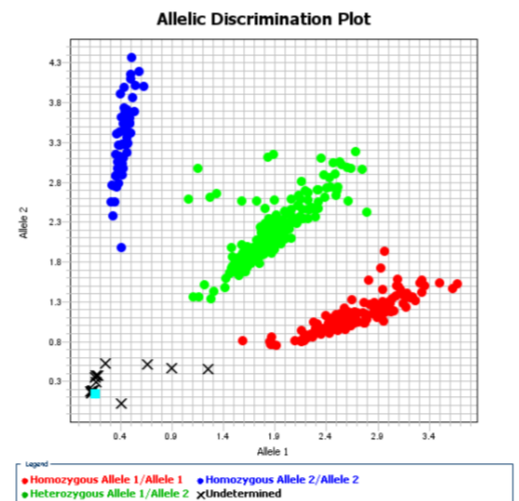
	x1 mostra	x384mostres
DNA mostra	0,4µl	
TAQPATH ProAmps W/Rox, 50ml	2,5µl	163,2µl
TaqMan® SNPmGenotyping Assay LAG-3	0,125µl	960µl
Nuclease-free water	1,975µl	758,4µl
	Vf:5ml	

4- A cada pou, on prèviament hem carregat cadascuna de les mostres que s'analitzen, **afegirem 4,6µl de la mix de reacció.**

5- Col·locarem la placa a la màquina i esperarem que es realitzi l'anàlisi (aproximadament 1:30min)



6- Un exemple de resultat seria la imatge que adjuntem a baix, on es pot veure ben diferenciat les tres poblacions possibles: homozigot per **l'al·lel 1 o 2 i** homozigot. Les mostres que no hi ha genotip (les "x", degut segurament a què hi ha poca quantitat de DNA) i el requadre blau seria el BLANC (punt de referencia "0" que té la màquina per fer l'anàlisi).



El que es mostra a continuació, és el que ens va sortir quan vam realitzar-lo al laboratori.