UNIVERSITY OF INSUBRIA
School of Medicine
PhD program in Experimental Medicine and Oncology
XXVI Course

ARRAY-CGH IN THE INVESTIGATION OF KARYOTYPE
CHANGES OF CD34+ HAEMATOPOIETIC STEM CELLS IN
LYMPHOMA AND MULTIPLE MYELOMA PATIENTS WHO
UNDERWENT AUTOLOGOUS TRANSPLANTATION

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Academic year: 2012-2013
ABSTRACT

Hematopoietic stem cell transplantation (HSCT) represents an effective treatment strategy for a variety of hematologic and not hematologic malignancies.

In particular, autologous transplantation of haematopoietic stem cells (ASCT) from bone marrow of patients with hematologic malignancies is feasible and has low treatment-related mortality (Gribben JG, 2009). However, literature assessed late mortality in 29.4% of individuals who had survived 2 or more years after autologous haematopoietic cell transplantation (Burns, L.J., 2009; Bhatia S. and al, 2005). This could be due to the purified stem cells which might carry a mutation on a chromosome predisposing to the disease and lead to the risk of pathology recurrence.

Lymphoma and myeloma are perfect candidates for autologous transplantation after G-CSF stimulation and bone marrow ablation trough chemotherapy. However, in this case too, pathology relapse or secondary malignancies are found in a high percentage of patients.

The aim of this project is to verify the existence of detectable imbalanced chromosome anomalies in stem cells before any ablative treatment for HSCT or developed after G-CSF stimulation or chemotherapy.

A cohort of 24 lymphoma and myeloma patients have been analyzed trough array-CGH to identify significant imbalanced chromosome anomalies also present in low
percentage of mosaicism. The result showed anomalies in 8/24 patients: one patient affected by Hodgkin Lymphoma (HL) revealed a deletion of chromosome 2 in p16.1, where the REL gene is located and in part deleted; the amplification of chromosome 11 in q12.2q13.4 containing CCND1 gene (this patient was investigated both before and after transplantation) was found in one patient with multiple myeloma (MM); alterations of chromosome 14 in q32.31-33, where genes for variable chain of immune globulin are located, were found in five patients with Hodgkin and non Hodgkin lymphomas (HL/NHL). FISH on interphase nuclei has been used to confirm a-CGH data.

A short-time (36 months) clinical and haematological follow-up examination did not show a different trend between patients with chromosome imbalances and without but a long-term follow-up is needed to definitely correlate the imbalances with the clinical evolution and to have the indications of global survival of the considered population.

Work in progress is the extension of clinical and haematological observation to obtain evidence of a difference statistically significant and to reach the final goal of suggesting a possible protocol to candidate patients to purging treatments before the CD34+ cells re-infusion.
INTRODUCTION

Hematopoiesis

Hematopoiesis is the process by which new blood cells are produced each day to maintain physiologic functions. The most important players in this process are pluripotent hematopoietic stem cells (HSCs) that act as a reservoir that self-renew and differentiate on demand to replace senescent and damaged cells during embryonic and post-natal life (Marks-Bluth, J and al, 2012).

During the embryogenesis, HSCs emerge from the aorta-gonad-mesonephros (AGM) and placenta, and expand in the fetal liver where they ensure the development of the blood system. These cells are called Fetal HSCs and have high proliferative and self-renewal ability.

Adults develop two subsets of hematopoietic organs, one devoted to the production of the myeloid cells and the other to lymphoid cells.

Bone marrow is the most important myeloid organ and it is designated to the formation of erythrocytes (erythropoiesis), granulocytes (granulocytopoiesis), monocytes (monocytopoiesis) and platelets (megakaryocytopoiesis). Thymus and bone marrow are the two primary organs in charge of formation of B and T lymphocytes (lymphocytopoiesis) whereas secondary organs are spleen and
mucosa-associated lymphoid tissue (MALT). The spleen has also hemochateresis function during which old erythrocytes and platelets are removed. MALT is a diffused lymphoid organ and it includes gut-, bronchial-, nasopharinx-, larynx-, eye, vascular- and skin-associated lymphoid tissues. Function of MALT is to ensure a complete immune response after local stimulation using both, B and T lymphocytes.

Another function of bone marrow is to supply a specific microenvironment for proliferation, differentiation and release of blood cells. Indeed the bone marrow contains cells of haematopoiesis system and also stromal cells with their mature elements (fibroblasts, osteoclasts, chondrocytes and adipocytes). These cells generate the microenvironment (niche) that let blood cells to grow (Rosati P, et al 2006). This heterogeneous composition of cells create a structural support, synthesizes growth factors and promotes interaction between cells and microenviroment. Those interactions are bidirectional: niche regulates stem cell self-renewal and cell fate decisions, in turn stem cells modulate the nurturing microenvironments in which they reside (Psaila B et al, 2012).

According to their differentiation potency stem cells can be classified in totipotent, pluripotent, multipotent and unipotent (monopotent). During the differentiation, cells are subjected to changes in their phenotype and this peculiarity has been investigated by the cytofluorometry and FACS. These technologies allowing to isolate stem cells have revolutionized stem cell research and clinical practice.
Furthermore, they have helped to clarify the hierarchical organization of hematopoietic differentiation.

A great improvement has been generated by the characterization of CD34 antigen. Hematopoietic stem cells are contained within the CD34+ compartment and CD34 antigen is lost during differentiation.

Most stem cell divisions are asymmetrical, yielding one stem cell keeping the quiescent state and a more differentiated cell, which has a limited self-renewal ability. Extrinsic instructions provided by unique microenvironments (niches) regulate the fate of individual HSCs and progenitors. Specifically, stem cell activation is initiated by primary lineage determinants which can ‘transcriptionally prime’ multipotential progenitors at a sub-threshold level, establishing a low-level expression of a mixed lineage pattern of gene expression. Upon reaching a critical threshold of activity, the primary determinants will differentiate the multi-potential progenitors along a particular cell-fate through secondary regulators. These molecules are transcription factors and cytokines that cooperate or antagonize with primary determinants and act ‘locking in’ the decision by repressing the alternate lineage and promoting the uni-lineage choice (Burda P et al, 2010). The orientation toward erythroid lineage is mediated by GATA-1, whereas Pu.1 acts promoting the lymph-myeloid-one. Further, differentiation derives from added cytokines and growth factors activities (Epo, TPO, interleukins, GM- and G-CSF each influencing a single lineage) (Fig 1).
Other players in this process are chemokines. Based on their molecular structure, these proteins are divided into two major subgroups CCL and CXCL that bind CCR and CXCR receptors, respectively. These mediators are important for regulation of cell viability, proliferation, differentiation, migration and homing (the ability to find destination, or niche).

Recent studies demonstrated that stem cells are gifted of plasticity and that cells partially differentiated could be reprogrammed upon particular conditions and converted in different hematopoietic lines. For this reason, a lymphoid cell could
be converted in a myeloid cell. In pathologic conditions, HSCs may differentiate into non-hematological cells according to the need of the body. For example, in ischemic brain, transplanted BM cells could differentiate into neurons and astrocytes (Woodbury D et al, 2000).

Furthermore, similar to most organs and tissues, the hematopoietic system shows evidence of aging, which is associated with increased incidence of myeloid malignancies, myelodysplasia, myeloproliferative neoplasms, chronic anaemia and multifactorial immune dysfunction (Rossi DJ, 2008).

Hematological diseases generally reflect inappropriate regulation or alterations of the genetic program controlling complex processes during hematopoiesis. These diseases range from disruption of blood cell production leading to absence of one or more cellular components (bone marrow failure syndromes) to faulty maturation processes such as disordered globin chain switching (hemoglobinopathies) to excessive hematopoietic cell production (leukemias).

Therefore understanding the mechanisms which regulate the haematopoiesis and the immunity system re-establishment could lead to the decrement of mortality caused by hematological diseases and by treatments as allogenic and autogenous transplantations.

**Blood malignancies**

The hematologic malignancies can be divided into lymphoid and myeloid disorders depending on which system is injured.
**Lymphoid disorders:**

Lymphoid leukaemia is characterized by a diffuse involvement of lymphoid organs and also of bone marrow, often associated with a large number of neoplastic cells in the peripheral blood; lymphomas are solid lymphoid proliferations presenting often with several lymphoid masses. However, the limit between leukemia and lymphoma is not always very well defined and leukaemia may be a lymphoma progression. Indeed, neoplastic lymphocytes keep their ability to circulate in the blood and the cancer can be spread in lymphatic and haematic ways. When the latter happens may be impossible to distinguish between lymphoma and leukaemia.

Lymphoma is a neoplasm presenting clonal heterogeneity and resulting from clonal evolution (Masao Seto, 2013). Genome profile analysis demonstrated that genomic alterations may have a hierarchy regarding occurrence. Early and late genetic events may exist and some conditions may predispose to lymphoma (as acquired immunodeficiency states consecutive to transplantations or AIDS) but the pathogenic pathways are not fully understood.

Lymphomas can be classified in Hodgkin and Non-Hodgkin lymphomas (Fig2). Hodgkin’s Disease malignant cells usually remain localized in one lymph node or a surrounding chain in the neck, shoulder, and chest. Non-Hodgkin lymphoma do not contain Reed-Sternberg cells, typical of HL, and tend to develop in peripheral lymph nodes and spread throughout the body.
Clinic manifestations, diagnosis and staging are similar in both categories. Most frequent symptom is the enlargement of lymph-nodes in neck, underarm and groin regions. Sometimes fever, asthenia, itch and weight loss could be present; in that case lymphoma is indicated as type A, otherwise as type B. The next paragraphs describe more in detail the biological aspects of the two kinds of lymphoma.
Hodgkin Disease (Hodgkin Lymphoma, HL):

It is one of the most frequent lymphomas whose incidence is 3 new cases per 100000 persons per year in the Western countries. In particular, in 2012, approximately 9060 individuals were diagnosed with Hodgkin lymphoma (HL) in the United States (US) with about 1190 deaths (American Cancer Society. http://www.cancer.org/acs/groups/content/@epidemiologysurveillance/documents/document/acspc-031941.pdf; 2012. [Accessed March 21, 2012]). Incidence of HL increases to 4.5 cases per 100000 in individuals between the ages of 20 and 25, decreases slightly through ages 35 and 60, and then increases again to over 4 cases per 100000 individuals above the age of 70.

Epstein–Barr virus (EBV) is believed to play a causal role for one third of cases. This kind of tumour tends to arise in different members of the same family also of far generations. The risk to develop HL increases until 7 times between brothers and the probability is even bigger between twins. Anticipation phenomenon is observed analyzing tumours of parents and sons: this may have some genetic cause. Clinically, HL involves peripheral lymph nodes and, at a later stage, organs such as liver, lung, and bone marrow. It is characterized by the proliferation of particular mononucleated giant cells (20-60 μm) called Hodgkin cells and multinucleated giant cells called Reed-Sternberg (HRS) which constitute a small fraction (0.1-5%) of neoplastic tissue, feature that hampers their molecular study (Fig.3). Recent analysis of immunoglobulin (Ig) genes revealed that these cells are usually clonal B cells since presenting identical rearrangements of Ig heavy and/
or light chain genes. Then somatic hypermutations occur in B lymphocytes of germinal centre but HRS cells escape from apoptosis.

**Fig.3: Reed-Steinberg cells. Source Licthman MA et al**

Due to reprogramming of gene expression, these HRS cells lose the expression of most B-cell specific genes, acquire expression of multiple genes typical of other haematopoietic cells and attract various cells of immune system into lymphoma tissue resulting in an inflammatory microenvironment. Figure 4 summarizes all cellular interactions of HRS cells.

Indeed, HRS cells show not only constitutive activity of signalling pathways normally transiently activated in B cells (such as NF-κB, jak–Stat and PI3K), but also activation of signalling molecules and pathways not normally activated in B cells (such as NOTCH 1 and multiple receptor tyrosine kinases).

Furthermore, there is evidence that the recruitment of inflammatory cells into the lymphoma microenvironment is essential for HRS cells survival (Küppers, 2009).
Fig. 4: Cellular interactions in HRS cells microenvironment.

Particularly, HRS cells secrete a variety of cytokines and chemokines for T-helper 2 (Th2) cells including thymus and activation regulated chemokine (TARC) which attract CD4+ cells (Van den Berg et al. 1999). Th2 secretes cytokines such as IL-13 which directly promote HRS survival since these cells express IL-13 receptors and signal through the STAT-6 pathway. Subpopulations of CD4+ cells secrete interleukin 10 (IL-10), transform growth factor β (TGFβ), inhibit cytotoxic T-cell function and protect HRS cells from apoptosis (Marshall et al. 2004). Multiple other cytokines secreted by infiltrating cells appear to provide survival signals to the HRS cells including CD30, CD40 and NOTCH1, all of which act through TNF
family receptors of pro-survival receptors which activate signaling pathways including PI3-kinase/Akt/mTOR, ERK/MAPK and NF-κB. The ability to establish an inflammatory microenvironment is the essential key to HL pathogenesis.

From a cytogenetic point of view no chromosome abnormality has been demonstrated in HL as isolated change. HRS cells show abnormal karyotypes, in fact conventional cytogenetics find chromosome loss or gain in until 92%of metaphases.

In HL, conventional comparative genomic hybridization (CGH) studies have shown many chromosome imbalances as recurrent gain of 2p16.1 (including REL oncogene which encodes c-Rel, a member of Rel/NFκB transcription factors family) and gain of 9p23.24 (including JAK2 which is a member of JAK/STAT pathway and promotes cells growth and proliferation).

Alterations have been found also in 8q24.21 (including PVT1. The PVT1 locus encodes several micro-RNAs thought to be important in T lymphomagenesis and T-cell activation. Co-activation of c-Myc and PVT1 has been shown in a variety of human tumors as well as prostate, breast, colorectal and prostate cancers).

ATM (11q22-23) is also mutated in HL (ATM is a tumour suppressor gene that regulates the balance between DNA repair and apoptosis in response to DNA damage. Mutations of ATM lead to genetically instable diseases as Ataxia Telangiectasia). Furthermore two SNPs have been found in 10p149p encompassing the transcription factor and putative tumor suppressor gene GATA3 which encodes the GATA binding protein 3 isoform 2. Expression of GATA3 is
important in hematopoietic and lymphoid cell development, acting as a master transcription factor for differentiation of Th2 cells. A high proportion of the reactive infiltrate in classical Hodgkin lymphoma (cHL) is composed of Th2-like cells with a T\text{regulatory} phenotype, which can influence EBV-positive cHL cell growth, depending on EBV antigenic presentation by MHC molecules (Enciso-Mora V, 2010). HRS show also mutations in IKB and BCL-6 genes (which act as a sequence-specific repressor of transcription).

In spite of the absence of a constant genetic pattern able to explain the disease pathogenetic mechanisms, the high frequency of HL among family members and the phenomenon of anticipation suggest involvement of genetic factors in its etiology. In that sense, human leukocyte antigen (HLA) haplotypes might be involved. In fact, Epstein Barr virus (EBV) is a well-established causal factor in a subset of HD patients and genetic variation in host anti-viral immune responses related to HLA polymorphisms might be an important contributor to the development of virally induced malignancies. Study of 934 HL patients shows that HLA-A1 was associated with an increased risk for EBV+ HL, whereas HLA-A2 was associated with a decreased risk for EBV+ HL (Hjalgrim, 2012).

-Classification-

HL is divided in two groups which differ substantially in their histopathologic pattern and clinical course: classic HL (cHL) and lymphocyte-predominant HL (LPHL).
**cHL** is characterized by only a few malignant cells and an abundance of inflammatory cells. Hodgkin and Reed–Sternberg (HRS) cells are surrounded by T and B cells admixed with plasma cells, macrophages, eosinophils and mast cells. These non-malignant cells, recruited and/or induced to proliferate by tumour cells, produce soluble or membrane-bound molecules involved in tumour cell growth and survival. In particular, no other B-lymphoid malignancy shows such a dramatic loss of the B-cell phenotype.

**LPHL** is a rare form of HL (5%) and presents two morphologic patterns, nodular and diffuse. In lymph nodes, the nodular pattern is characterized by the presence of atypical “lymphocytic and histiocytic” (L&H) or “popcorn” cells, embedded in a nonneoplastic nodular background composed mostly of small B lymphocytes. In the diffuse pattern, the L&H cells are set against a diffuse background of reactive T cells. It shows very good prognosis, especially in early stages without risk factors (Nogovà L, 2006).

**-Clinical staging and prognosis-**

An accurate evaluation of the malignancy extension is essential for prognosis and planning the therapeutic strategy. To this purpose Ann Arbor stage (AAS) is used.

**Stage I.**  
Involvement of a single lymph node region (I) or a single extra-lymphatic organ or site (IE).

**Stage II.**  
Involvement of 2 or more lymph node regions on the same side of the diaphragm (II) or localized involvement of
extra-lymphatic organ or site and of 1 or more lymph node regions on the same side of the diaphragm (IIE). An optional recommendation is that the numbers of node regions involved be indicated by a subscript [e.g., II3].

Stage III. Involvement of lymph node regions on both sides of the diaphragm (III), which may also be accompanied by localized involvement of extra-lymphatic organ or site (IIIE) or by involvement of the spleen (IIIS), or both (IHSE).

Stage IV. Diffuse or disseminated involvement of 1 or more extra-lymphatic organs or tissues with or without associated lymph node enlargement.

The elapse of the illness depends also from the number of area involved from the disease, the possible bone marrow or liver involvement and from mediastinal participation.

-Treatment-

In most of cases HL is a curable disease trough the first line treatment (chemotherapy and/or radiotherapy). However, 15% to 25% of patients relapse or do not respond. For these patients, the standard of care is based on autologous stem cell transplantation (ASCT). Patients who relapse after ASCT or who are refractory to chemotherapy have a very poor prognosis. Allogenic stem cell
transplantation (alloSCT) is a treatment option for such patients with controversial results (Marcais A, 2013).

-Hodgkin lymphoma has become one of the most easily curable malignancies in oncology. However, severe, life-threatening treatment-related side effects occur, which include organ toxicity and secondary malignancies.

In particular, death caused by HL has the strongest impact within 15 years after treatment. After 18 years the mortality rate of second cancers (SCs) exceeds the one of HL. The high incidence of SCs after HL recovery induced to hypothesize the presence of germ-line mutations which can predispose to the development of them. Many studies show the correlation between therapy and SCs. Particularly, the probability to develop a SC is 10.1% after 15 years and 14.9% after 20 years of HL treatment. Radiotherapy seems to be the more important suspected cause of solid tumors (especially lung 23%, breast 12% and gastrointestinal 12%); In particular, the risk of breast cancer in women treated by irradiation before the age of 30 is increased to a level higher than that in 50-year-old women in the general population; on the other hand chemotherapy is correlated to secondary laeukemias and NHL. Thyroid function disorders are also frequent and may be found in up to 80% of patients, depending on study conditions, the form of treatment, and the type of test used (Hodgson DC, et al.,2007), (Ng AK, et
al., 2008). SCs risk increases with age (5.7% for patients under 28 years, 25.6% for patients above 44 years) and with gender (18% vs 8.9% of male vs female).

Non-Hodgkin Lymphoma (NHL)

90% of all lymphomas are referred to as non-Hodgkin lymphoma and more than two-thirds of patients are above 60 years of age. Malignant non-Hodgkin lymphoma (NHL) has been increasing in incidence worldwide and a study involving a North American adult population between 1999 and 2003 demonstrated a population-based incidence rate of NHL of 1.73 per 100,000, higher than in other parts of the western world (JM Howell et al., 2012).

Non-Hodgkin lymphomas consist in a heterogeneous group of cancers. There are now approximately 60 distinct subtypes of NHL listed in the 2008 World Health Organization (WHO) classification. 85–90% of the group arise from B lymphocytes; the remainder derive from T lymphocytes or NK lymphocytes. This diverse group of malignancies usually develops in the lymph nodes, but can occur in almost any tissue. Any organ can be the primary site of NHL. However, the gastrointestinal tract is the most frequent extranodal site in non-Hodgkin lymphoma, and the stomach is the most frequently implicated part of the gastrointestinal tract. Immunosuppression is the most well-established risk factor for the development of NHL. Patients with HIV, organ-transplant recipients, patients who have had high-dose chemotherapy with stem-cell transplantation, and those with inherited
immunodeficiency syndromes or autoimmune disease present an increased risk of developing NHL.

Escape from apoptosis is the way by which lymphomas could develop. Indeed during B-cell maturation, cells spring up from the central lymphoid tissues where recombination of V, D, and J gene segments results in the assembly of immunoglobulin heavy-chain and light-chain genes.

In this process, two enzymes (RAG1 and RAG2) cause breaks in double-stranded DNA. In normal cases, DNA repair processes are activated to resolve them. However, these strand breaks can contribute to chromosome translocations which typically result in proto-oncogene activation in lymphoma (Shankland KR, 2012).

For example, the genetic hallmark of follicular lymphoma is the t(14;18) chromosome translocation, which juxtaposes BCL2 gene on chromosome 18 to the transcription active immunoglobulin heavy-chain region on chromosome 14. This translocation can be detected in 80–90% of cases, and up-regulates BCL2, which prevents programmed cell death. Anyway, none of these chromosome translocation is self sufficient to cause the tumour. It is necessary the accumulation of other genetic damages. Unfortunately, the identity of additional damages and therefore the mechanism of tumour progression is still unknown.

-Classification-

The more common types of lymphoma are listed below according to whether they are B-cell or T-cell lymphomas.
B-cell lymphomas represent most (about 85%) of non-Hodgkin lymphomas in the United States and are:

- Diffuse large B-cell lymphoma (DLBCL): it is a fast growing lymphoma, but it often responds well to treatment.

- Follicular lymphoma: About 1 out of 5 lymphomas. The term follicular means that cells tend to grow in a circular pattern in lymph nodes. The average age for people with this lymphoma is about 60. Follicular lymphomas are often slow-growing and respond well to treatment.

- Mantle cell lymphoma: Only about 5% of lymphomas are of this type. The cells are small to medium in size. It afflicts men most often. The average age of patients is in the early 60s.

- Burkitt lymphoma: This type makes up about 1% to 2% of all lymphomas. Close to 90% of patients are male, and the average age in the US is about 30. It is named after the doctor who first described this disease in African children and young adults. The cells are medium sized. This is a very fast-growing lymphoma but more than half of patients can be cured by intensive chemotherapy.

- Lymphoplasmacytic lymphoma: This type is not common, accounting for 1% to 2% of lymphomas. The lymphoma cells are small and found mainly in the bone marrow, lymph nodes, and spleen.
Among T-cell lymphoma there are:

- Precursor T-lymphoblastic lymphoma/leukemia: This disease accounts for about 1% of all lymphomas. The cancer cells are small-to-medium sized, immature T-cells. Patients are most often young adults, with males being affected more often than females. This lymphoma is fast-growing, but usually not involving bone marrow. When it is first diagnosed the chance of cure with chemotherapy is quite good.

- Peripheral T-cell lymphomas: These types of lymphomas develop from more mature forms of T cells. They are rare.

Table 1 lists the different types of B-cell NHL with their translocations and the molecular consequences.

<table>
<thead>
<tr>
<th>NHL-B</th>
<th>ANOMALY</th>
<th>(%of cases)</th>
<th>GENE/PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoplasmacytoid</td>
<td>t(9;14)</td>
<td>(50%)</td>
<td>PAX-5/transcription factor</td>
</tr>
<tr>
<td>Follicular</td>
<td>t(14;18)</td>
<td>(70-90%)</td>
<td>BCL-2/Anti-apoptosis</td>
</tr>
<tr>
<td>Diffuse large B-cell</td>
<td>t(3;many)</td>
<td>(30-40%)</td>
<td>BCL-6/transcription factor</td>
</tr>
<tr>
<td></td>
<td>t(11;18)</td>
<td>(30%)</td>
<td>BCL-2/Anti-apoptosis</td>
</tr>
<tr>
<td>Mantle-cell</td>
<td>t(11;14)</td>
<td>(100%)</td>
<td>BCL-1/Cyclin D1</td>
</tr>
<tr>
<td>Burkitt</td>
<td>t(8;14), t(8;22), t(2;8)</td>
<td>(100%)</td>
<td>c-MYC/transcription factor</td>
</tr>
<tr>
<td>MALT</td>
<td>t(1;14)</td>
<td>(?)</td>
<td>BCL-10/Anti-apoptosis</td>
</tr>
</tbody>
</table>

Table 1: Distribution of principal chromosome translocations in different NHL-B subtypes, frequency, involved proto-oncogenes and codified proteins.
-Clinical staging and prognosis-

Ann Arbor classification is used in NHL but only 7-10% of patients begin at the I stage whereas 70-80% are identified at III-IV stage.

At the opposite of HL, clinical staging has not prognostic and therapeutic impact.

The International Prognostic Index (IPI) is the most widely used prognostic model for patients with non-Hodgkin lymphoma. Considering as risk factors age ( <60 years), lactate dehydrogenase concentration, Eastern Cooperative Oncology Group performance status, Ann Arbor stage, and number of extranodal sites implicated, four risk groups were delineated: low risk (zero to one clinical feature), low-intermediate risk (two features), high-intermediate risk (three features), and high risk (four to five features). When applied to 2031 patients, these risk groups had 5-year survivals of 73%, 51%, 43%, and 26%, respectively.

-Treatment-

Surgery has an important role because biopsy of the suspected lymph-node is fundamental to the diagnosis of lymphoma, the histotype definition and the consequent choice of therapy.

Radiotherapy is the treatment for I-II stage localized and for I stage aggressive lymphomas. Also it is indicated for advanced stages after chemotherapy as consolidation.
Chemotherapy is used to eradicate non-aggressive lymphomas or to contain the disease in the indolent ones.

-Survival-

NHL can be divided into aggressive (fast-growing) and indolent (slow-growing) types.

- Highly aggressive lymphomas: The complete remission is superior to 70%
  The long-term survival is 30-60% depending on age, stage, LDH and leukemic phase

- Aggressive lymphomas: The complete remission is 60-80%
  The long-term survival is 40-50%

- Mild aggressive lymphomas: The complete remission is not superior to 40%

- Indolent lymphomas: The complete remission is 60-70%

Not responding patients and relapse: Patients not responding to initial therapy constitute 5-10% of cases and have bad prognosis at short time. Patients partially responding and patients with premature relapse can have a complete remission in 20-35% of cases.
Multiple Myeloma (MM)

Multiple myeloma (MM) is a B-cell/plasma cell neoplasm which represents 0.8% of cancer cases worldwide and comprises about 13% of hematological malignancies (Ferlay et al. 2010; Kyle and Rajkumar, 2004). MM is principally a disease of older patients. Surveillance Epidemiology and End Results (SEER) data indicate that, in the United States, the median age at diagnosis of MM is 69 years, with an age-adjusted incidence of 5.8 cases per 100,000 persons per year. About 75% of MM cases are diagnosed in persons over 50 years of age. The incidence of MM varies widely, ranging from 0.4 to 5 cases per 100,000 persons, with the highest rates in Australia, New Zealand, North America and parts of Europe, and the lowest rates in Asia (Parkin et al., 2005).

The disease is characterized by infiltration of bone marrow, bones and sometimes other tissues by malignant plasma cells, which typically produce a monoclonal paraprotein. Multiple myeloma (MM) is characterized by multi-stage accumulation of genetic abnormalities deregulating different pathways. It can evolve from a monoclonal gammopathy of undetermined clinical significance (usually known as MGUS) which is present in about 3% of the general population over 50 years, and carries a risk of progression to MM of about 1% of cases per year (Kyle et al. 2006). Initially, multiple myeloma is confined to the bone marrow (intramedullary), but later the tumour can be evident in extramedullary locations (such as blood, pleural fluid and skin) (Fig.5). Common clinical manifestations include lytic bony
lesions, renal impairment, anemia, hypercalcemia and immune dysfunction.

**Fig 5: Initiation and progression of myeloma.** MGUS is an indolent, asymptomatic condition that transforms to myeloma in 1% of cases per annum. Smouldering myeloma lacks clinical features; by contrast, symptomatic myeloma has various clinical features which provide an indication that require treatment. Later in the disease progression, the myeloma plasma cells can be found at extramedullary sites and as circulating leukaemic cells. The initial deregulated cell belongs to the MGUS clone; however, subsequent to the development of sufficient genetic abnormalities, it acquires a clonal advantage, expands and evolves. At the end of this evolutionary process, at the stage of plasma cell leukaemia (PCL), the clone is proliferative and no longer confined to the bone marrow; the clone expands rapidly and leads to patient death. Cells at this stage mostly show chromosome anomalies and the precursor subclones will be present at low levels because of competition for access to the stromal niches in the bone marrow: these clones may be eradicated by more aggressive clones.
Imbalanced bone remodeling in the myeloma bone marrow is caused by increased osteoclast activity, together with reduced osteoblast function. Myeloma cells cause an increased production of osteoclast activating factors and cytokines that inhibit osteoblast differentiation. The unopposed osteolysis is also responsible for hypercalcemia. While the causes of MM remain poorly understood, factors affecting risk for development of the disease include age, gender and ethnic background, underlying immunodeficiency, exposure to radiation and family history of MM and other hematolymphoid neoplasms (Alexander et al. 2007).

Several lines of direct and indirect evidence also suggest the existence of inherited factors, which may predispose individuals to development of MM, MGUS and other related cancers.

Multiple Myeloma arises because of acquired genetic changes that occur during the terminal differentiation of B lymphocytes into plasma cells. The majority of MM cells appear to be mature, quiescent, and terminally differentiated consequently without long-term proliferative potential. This raises questions about which cells in MM patients are clonogenic and capable of proliferation. Usually, immature B lymphocytes after differentiation in the bone marrow, migrate as naïve B lymphocytes to the secondary lymphoid tissue where antigen stimulation leads to their proliferation. At this stage, somatic hypermutation in the IgH and light-chain genes gives rise to the selection of B-cell clones expressing high-affinity Ig.

Then, cells may either leave the secondary lymphoid organs and circulate as memory B cells or differentiate into post-follicular plasmablasts following a switch
in Ig class from IgM to IgG, IgA, IgD, or IgE. Plasmablasts migrate back to the bone marrow for the terminal differentiation into plasma cells.

MM cells show extensive somatic hypermutations of rearranged Ig genes which happen after the switch. They express markers associated with plasma cells and also markers associated with natural killer (NK) cells (CD56/NCAM) and T cells (CD28). Definitively, the exact phenotype of the clonogenic cells in MM remains to be established but some studies have suggested that they may be resistant to chemotherapy and that may persist following treatment (Kiel, 1999).

From a genetic point of view, the majority of MM cases is characterized by complex chromosome abnormalities (Fig. 6).

On the basis of alterations in chromosome number, patients can be divided into 3 groups:

- hypodiploid/hypotetraploid (less than 46 chromosomes/75-90 chromosomes)
- pseudodiploid (46 chromosomes with structure alterations)
- hyperdiploid (more than 46 chromosomes)

In particular, in around half of cases, trisomies of several odd numbered chromosomes occurs (in particular of chr.3, 5, 7, 9, 11, 15 and 19); remaining cases are characterized by a chromosome translocation, which places an oncogene into the immunoglobulin heavy chain gene on chromosome 14 (IgH translocation). The result is oncogene overexpression and dysregulated cell proliferation. This subtype encompasses hypodiploid and pseudodiploid MM
composed of high level of IgH rearrangements (localized on locus 14q32) generally associated with more aggressive clinical features and shorter survival.

**Fig 6. Disease stages and timing of oncogenic events.** The earliest oncogenic changes are present in monoclonal gammopathy of undetermined significance (MGUS) and involve two minimally overlapping pathways, primary IgH translocations (black triangle) and multiple trisomies (white triangle), each of which can include a del13 pathway (grey triangle). Other karyotypic abnormalities and epigenetic changes can occur at all stages.

Activating mutations of MAF (which upregulates cyclin D2, a promoter of cell cycle progression) appear to mark, if not cause, the MGUS to multiple myeloma (MM) transition in some cases, but sometimes occur during subsequent progression of MM. It has been proposed that dysregulation of cyclin D gene provides a unifying, early oncogenic event in MGUS and MM Late oncogenic events that occur at a time when tumours are becoming more aggressive include MYC dysregulation, bi-allelic deletion of p18, inactivation of Rb, and loss or mutation of p53.

The IgH (14q32) translocations can involve many different partners and often cause exchanges involving oncogenes. The most frequent IgH translocations in myeloma are \( t(4;14)(p16;q32) \), \( t(11;14)(q13;q32) \), \( t(6;14)(p21;q32) \), \( t(14;16)(q32;q23) \), and \( t(14;20)(q32;q12) \).
The t(4;14) chromosome translocation is the genetic event in MM with the most important clinical significance and occurs in about 15% of patients. Two genes located in 4p16 are dysregulated by t(4;14): MMSET (over-expressed in all tumours with a t(4;14)) and FGFR3 (a fibroblast growth factor receptor, over-expressed in about 20% of these tumours). MMSET is a chromatin-remodelling factor and has a role in DNA repair. Indeed, following DNA damage MMSET is recruited to sites of double strand breaks (DSB) and required for recruitment of p53-binding protein. Approximately half of the translocation breakpoints in t(4;14) MM results in a truncated MMSET and then in a loss of the normal DNA damage response pathway. Also, MMSET has been shown to post-transcriptionally enhance the expression of MYC and to mediate constitutive NF-kB activation. As to FGFR3, the translocation appears unbalanced in up to 25% of cases, losing the derivative chromosome 14, which is associated with the loss of FGFR3 expression.

The t(11;14) is found in about 15% of patients and associated with iperexpression of cyclin D1; t(6;14) is associated with iperexpression of cyclin D3; t(14;16) is found in about 5-10% of cases and associated with iperexpression of MAF (transcription factor controlling the expression of interleukin-4 (IL-4) and the adhesion of molecules able to enhance the ability of tumour cells to interact with the BM microenvironment). The t(14;20) occurs in about 2% of patients with iperexpression of MAFB.
With the exception perhaps of FGFR3, it is interesting to note that none of the primary translocations causes dysregulation of strong oncogenes.

A large number of secondary chromosome aberrations are found during tumour progression. These aberrations include translocations of MYC, deletions of chromosome 13, deletions and/or amplifications of chromosome 1, and deletion of chromosome 17p13, as follows:

- A majority of MYC translocations involve an Ig locus.

The MYC translocations mostly are nonreciprocal translocations or insertions, often with the involvement of three chromosomes and sometimes with associated duplication, amplification, inversion, and other associated chromosome abnormalities.

- An other anomaly concerns chromosome 13 (85% of cases as monosomy, 15% as deletion). In particular monosomy of 13 is associated with poor prognosis: RB1 gene is located in the lacking segment and its haploinsufficiency is though to promote tumourigenesis. A recent genome wide sequencing study identified mutations of DIS3 (gene of unknown function on 13q), in about 10% of MM. 13q deletion is associated with poor prognosis only if other cytogenetic abnormalities, such as t(4;14) and deletion of 17p13, are present.

- Gain of chromosome 1q and loss of 1p frequently occur together in MM, and each of them is associated with a poor prognosis. MCL1 gene with anti-apoptotic function has been suggested as a potential driver of the adverse survival. On two regions of 1p potential targets associated with a poor prognosis are: CDKN2C
(p18INK4c) at 1p32.3 and FAM46C at 1p12. Homozygous deletion of CDKN2C gene (cell cycle regulator) is present in about 30% of HMCL and about 5% of untreated MM.

-Deletion of chromosome 17p13, is the most important cytogenetic factor because it involves the tumour suppressor gene p53 and it is associated with negative treatment outcomes. Table 3 illustrates the correlations between chromosome anomalies and risk of MM.

In general, genetic abnormalities alter the expression of adhesion molecules on myeloma cells, as well as responses to growth stimuli in the microenvironment. The adhesion of myeloma cells to hematopoietic and stromal cells induces the secretion of cytokines and growth factors, including interleukin-6, vascular endothelial growth factor (VEGF), insulin-like growth factor 1, members of the super-family of tumour necrosis factor, increasing tumour growth, survival, migration, and drug resistance (Palumbo A, 2011).

<table>
<thead>
<tr>
<th>HIGH RISK</th>
<th>STANDARD RISK</th>
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<tr>
<td>Hypodiploidy</td>
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<td>Del 17p</td>
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<tr>
<td>Del 13</td>
<td>t(6;14)</td>
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<td>t(4;14)</td>
<td>Other anomalies</td>
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<td>t(14;16)</td>
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Table 3: Correlations between the most frequent chromosome anomalies and risk of MM
- Classification-

Myeloma is classified into three categories. Individuals in the first two categories are considered asymptomatic and do not have to receive anti-myeloma treatment immediately.

- **MGUS**: Considered a precursor to myeloma. Bone marrow plasma cells <10% and no evidence of other B-cell disorders. No related organ or tissue impairment. Risk of progression to malignancy: 1% per year (about 20%-25% of individuals during their lifetime).

- **Smoldering or asymptomatic myeloma**: Bone marrow plasma cells >10% and no related organ or tissue impairment. Risk of progression to malignancy: 10% per year.

- **Symptomatic myeloma**: Bone marrow plasma cells or plasmacytoma and related organ or tissue impairment.

- Clinical staging and prognosis-

It is possible to distinguish 4 stages of the disease on the basis of bone marrow morphology:

1) Mature myeloma (formed by more than 10% of mature plasmacells and less than 12% of immature plasmacells).

2) Intermediate myeloma (all myelomas which not reenter in the other subtypes)
3) Immature myeloma (formed by more than 12% of immature plasmacells and less than 10% of mature plasmacells).

4) Plasmoblastic myeloma (formed by more than 2% of plasmoblasts)

Although the introduction of novel therapeutic agents has transformed the outlook for many patients, myeloma remains a heterogeneous disease. Some patients will live for more than eight years after diagnosis, whereas a subset with high risk disease will die within 24 months.

-Treatment-

Patients with MGUS and asymptomatic myeloma are observed but not treated until they develop symptomatic myeloma.

No intervention has been found to delay or prevent the progression of MGUS to myeloma.

Most of MM patients respond to initial treatment and enter a period of disease stability, which is generally associated with good quality of life. Because of the lack of curative treatment, relapse is inevitable, but at least half of patients respond to chemotherapy a second time, using similar or different drugs. Subsequent relapses become increasingly less responsive to treatment, until refractory end stage disease ensues, sometimes with extramedullary manifestations and cytopenias. The approach to treating newly diagnosed symptomatic myeloma depends on age and comorbidities. Initial chemotherapy regimens aim to achieve the deepest response with the lowest toxicity, and for patients who are young
(generally <65 years) and fit enough, this is consolidated with high dose chemotherapy and autologous stem cell transplantation.

Older patients, or those with serious comorbidity, who not fit enough to undergo autologous transplantation are treated with chemotherapy only. A second autologous stem cell transplant may be considered in patients who are young and fit enough and who achieved a long (≥18 months) remission after first transplant.

-Survival-

Multiple myeloma is a disease not eradicable. The survival can vary between some months and some years with a median of 2.5-3 years for patients treated with conventional therapy. The latest increasing use of high doses therapy and the introduction of new drugs have produced a significant improvement in survival and in free-disease time.

**Genomic instability and cancer**

Genomic alterations are important causes of cancer development. Typical clonal expansion of tumour is the result of progressive accumulation of genomic changes. This results in the loss of tumour suppressor functions, in the activation of oncogenes and/or in the generation of fusion genes with oncogenic potential and they lastly lead to the phenotypes of malignant cancer cells.

Changes which could occur are simple mutations, genomic rearrangements and number and structure chromosome anomalies.
In the last few years a new phenomenon, termed chromothripsis has been revealed by using combination of next-generation DNA sequencing, single nucleotide polymorphism array analyses and bioinformatics methods. Chromothripsis indicates chromosomes carrying dozens to hundreds of clustered rearrangements acquired in the most of cases in a single catastrophic event in a cancer cell (Stephen PJ et al, Cell 2011). These rearrangements derived from distinct chromosomes or chromosome regions fragmented into many segments some of which joined together by DNA repair mechanisms in ways that did not necessarily relate to their original order or orientation in the progenitor chromosome. The stamp of chromothripsis can be described in at least 2%–3% of all cancers, across many subtypes, including hematopoietic malignancies like chronic lymphocytic leukaemia, lymphoma and multiple myeloma (Fig7). The agent of this physical chromosome damage is unknown. We wonder if this phenomenon may be found also in NHL and MM patients.
Catastrophic chromosome breakage

Non-homologous end-joining

Lost to cell

Fig 7: Example of chromosome catastrophe might break the chromosome into many pieces joined back together haphazardly

Hematopoietic stem cell transplantation

The discovery of CD34 as a stem cell marker, absent in more differentiated cells, has simplified the haematopoiesis study, revolutionized the assessment of progenitor cells present in the peripheral blood stem cell graft and improved the parameters to perform hematopoietic stem cell transplantation. Hematopoietic stem cell transplantation (HSCT) is the transplantation of multipotent hematopoietic stem cells representing an effective treatment strategy for a variety of hematologic malignancies. In fact, the vast majority of allogeneic HSCT (70%) are performed for hematologic diseases and in particular: acute myeloid leukaemia (AML, 32%) and acute lymphoblastic leukaemia (ALL 16%), myelodysplastic syndromes (MDS 16%) and non Hodgkin lymphoma (NHL 9%). In particular, only
diseases with poor prognosis (<50% survival at 5yrs with conventional treatment) are currently indications for allogeneic transplantation. On the other hand, the most frequent indications for autologous HSCT (ASCT) in Europe are plasma cell myeloma (PCM), HL and the majority of patients with NHL. In particular HL patients receive this treatment once they have relapsed after chemotherapy.

Sources of hematopoietic stem cells for transplantation are different:

- Bone marrow: the major source of cells for allogenic transplant.
- Peripheral blood: now the most common source of stem cells for HSCT. They are collected from the blood of patient (in the case of autogenous transplantation ) /donor (in the case of allogenic transplantation) through a process known as apheresis.

Autologous transplantation (ASCT)

The collection of adequate numbers of HSCs is a prerequisite for proceeding to autologous transplantation; however, the optimal mobilization regimen remains unknown for moving and procurement of autologous HSCs from the bone marrow to the peripheral blood. A variety of mobilization strategies are currently used, including growth factors alone or in combination with chemotherapy. The most used growth factors are the recombinant granulocyte-colony stimulating factor (G-CSF) analogs. The harvest of peripheral HSC is made during the process of apheresis through cells separators and then cryopreserved. Counting of CD34+ cells in peripheral blood allows to estimate the number of collected stem cells.
To eradicate the disease and to create space in the bone marrow cavity for stem cell proliferation all patients are exposed to the conditioning regimens. Indeed chemotherapy is added to improve CD34+ cell yield and for in vivo purging of mobilized tumour cells to reduce tumour burden (although there are limited supportive data). This is high doses chemotherapy after which normal hematopoiesis is irreversibly destroyed. The aplastic phase lasts 10-15 days during which the patient cannot produce erythrocytes, platelets and leucocytes and he is exposed to the infection risk. After 24-48 hours from the conditioning regimens the collected stem cells are re-infused to the patient through a normal blood transfusion. Using their migration ability, stem cells reach the depleted niches and there take root to build up the physiologic bone marrow.

For ASCT, risks include the short and long term toxicities of the high dose treatment during the preparative regimen. Consequences may be organ damage and risks of infection and bleeding associated with the marrow aplasia.

**Indications for autologous transplantation:**

Hematologic neoplasms treated with autologous stem cells are:

- Acute and chronic leukaemia (in acute myeloid leukaemias which present high risk of relapse disease, the autologous transplantation could be used as consolidation therapy for patients in first complete remission. In acute lymphoid leukaemia it is used after relapse)
Introduction

- Lymphoma (in which it is employed as first therapy but also after relapse after complete remission)

- Multiple Myeloma (in which it is used in first line of treatment)

<table>
<thead>
<tr>
<th>Illness</th>
<th>Disease</th>
<th>Risk group</th>
<th>Stage of the disease</th>
<th>Allogeneic transplant</th>
<th>Autologous transplant</th>
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<tbody>
<tr>
<td>Aggressive NHL: from the peripheral cells TPTCL and diffused BDCL</td>
<td>CR1, risk indicator aIPI2–3</td>
<td>From siblings</td>
<td>From unrelated donor</td>
<td>CO</td>
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<td>CR1</td>
<td>NR</td>
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</table>

After progression CR >1, PR, high risk

**Table 4. Indications for transplantation of hematopoietic stem cells in adults based on the EBMT recommendations.** Abbreviations: aIPI – international risk indicator adjusted for age, CR >1 – consecutive remissions achieved after relapses, S – standard indication based on clinical trials, CO – justified indication, but requiring confirmation in clinical studies, CR1 – completely first remission, D – experimental use in clinical studies, NR – generally not recommended (Holowiecki, J, 2008)

Limitations, consequences and perspectives:

The autologous transplantation is performed in patients up to 70 years to reduce the risk of rejection and mortality presented by the allogenic transplantation.

Major limitation of this kind of transplantation consists in the relapse of disease which can be either caused by residual therapy-resistant cancer niches in the patient or by contaminating cancer cells within re-infuses stem cells (Rahman M, 2010). In order to eliminate contaminating cancer cells and to protect the normal HSPCs necessary for subsequent hematopoietic reconstitution, several cancer cell
“purging” strategies have been tested over the last three decades. Results are still conflicting. It is still not possible to know which purging technique could be used with good results (Yang et al., 2011).

Also, it has been hypothesized that the use of growth factors to mobilize cells from bone marrow to peripheral blood could promote the mobilization of cancer cells also (Moreb et al., 2011).

Factors affecting the outcome of autologous transplantation are age of patient, stage of disease and number of previous treatments.

Encouraging results are obtained in acute myeloid leukaemia patients.

In acute lymphoid leukaemia, results are more disappointing since an high percentage of relapse is observed after the transplantation.

In myeloproliferative and lymphoproliferative syndromes, relapse is inevitable; in that cases the goal of ASCT is the survival extension and the enhancement of life quality.

Studies are on going to associate autologous and allogenic transplantation in absence of myeloablative conditioning to cure multiple myeloma and chronic lymphatic leukaemia.
AIM OF THE STUDY

The therapy of various hematologic diseases consists of high dose of chemotherapy/radiotherapy associated with autologous stem cells transplantation. The aim of this study is to correlate chromosome alterations with the pathology progress and to highlight significant regions for prognosis and transplantation. The hypothesis to confirm is that growth factor stimulation in combination with chemotherapy could influence the chromosome stability of hematopoietic precursors; this could cause the isolation of pathologic clones then re-infused during the transplantation.

Diseases analyzed in this study are lymphoma and multiple myeloma. All evaluated patients have been submitted to chemotherapy, G-CSF mobilization, apheresis and transplantation.

Stem cells have been purified and frozen at -196C to preserve vitality and stability during the time. In this phase samples have been collected to perform genetic analysis: CD34+ cells have been isolated and DNA extracted from them. a-CGH analysis have been performed and the observed imbalanced chromosome anomalies confirmed by FISH. Furthermore a clinical follow-up study has been accomplished in all patients considering the haematic recovery after stem cells re-infusion, the survival without disease and the appearance of second neoplasms.
Future research consists in the extension of clinical records to obtain statistical relevance.
MATERIALS AND METHODS

Patients affected by hematopoietic diseases following treatments at the medical oncology department of Ospedale di Circolo Fondazione Macchi of Varese and offering material for this study are:

<table>
<thead>
<tr>
<th>Patient</th>
<th>Year of birth</th>
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<tbody>
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Material and methods

Since 2009 37 mobilizations of hematopoietic stem cells have been realized and an aliquot of 1.5 ml of CD34+ has been collected to the research. Between them 16 are NHL, 13 are MM, 7 are HL and 1 is a malign therathome. 18/37 patients have not been re-infused.

-Stimulation protocol-

Patients have been treated with induction-chemotherapy and mobilized by G-CSF 5ug/Kg twice per day. To determine the recovery of bone marrow, leucocytes concentration has been estimated by hemocromo-cytometric analysis using SYSMEX XT-2000i. Also, to establish the number of stem cells ISHAGE cytometric assay has been applied by taking advantage of membrane proteins expression as CD34 and CD45.

The data were strongly heterogeneous.

-Apheresis-

Strategy of stem cell collection from peripheral blood consists in:

- Accurate evaluation of patient through examinations as blood count, coagulation test, thorax radiography, electrocardiogram and echocardiogram.
- Estimation of stem cell number by using cytometric analysis to individuate when to accomplish the apheresis.
- Monitoring arterial pressure and cardiac frequency
- Use of systems to limit platelets, erythrocytes and lymphocytes contaminations in the final product which could cause a lesser engraftment of transplantation.
In particular, apheresis procedures consist in the processing of two haematic volumes calculated considering 65 ml per Kg of patient as standard.

Patients have been connected to the cells separator Fresenius COM.TEC (Fresenius kabi).

Cells have been centrifuged and stem cells were found in the middle phase of the gradient.

Number of cycles for each patient depends on blood volume and it can vary between 16 and 30, giving different cell properties at final product.

-Cells Freezing-

-Weigh sacs and collect an aliquot to determine leucocytes and stem cells concentrations

-Add equivalent volume of physiologic/ albumin 7%

-Centrifuge sac 1100RPM , 25min, 20degree

-Prepare solution of recipient plasma 20%DMSO on ice

-Calculate final volume of sac considering leucocytes and stem cells

-Eliminate supernatant and add equivalent volume of plasma and 20%DMSO

-Freeze cells by decreasing 1 degree per minute until -7C, plateau for 5 minutes at -7C, decreasing 3 degrees per minute until -30C and fast decreasing of 5 degrees per min until -100C.

-Preserve cells in liquid nitrogen
-Cells Thawing -

-Slowly thaw cells at 37C

-Add 40ul heparin and 10ml of RPMI1640

-Centrifuge at 2000 RPM for 10 minutes

-Take out supernatant and add 10 ml RPMI1640

-Centrifuge at 2000 RPM for 10 minutes

-Take out supernatant and add 6ml of RPMI1640/ 2% pen-strept/10% FCS

-Labelling-

- Add mAb CD45/CD34 and 7AAD

- Incubate 25min, at dark, at RT

**Sorting with BD FACS ARIA II**

- Set up stream: DROP 1 ~180; 7 GAP; frequency ~88; nozzle 70 µm;

- Use ISHAGE methods to acquire;

- Set up threshold for debris as 50.000 on SSC channel;

- Define stem cell populations according to these characteristics:
  dimensions of lympho monocytes on SSC/FSC channels, intermediate expression of CD45, strong expression of CD34;

- Set up flow rate not exceeding 20.000 events per second;

- Sorting stem cells with purity above of 90 %.
Array comparative genomic hybridization (a-CGH)

The aCGH is a high-resolution detection of DNA copy number aberration.

The assay consists in sample and reference DNA extraction, quantification labelling and mixing in the hybridization, then in washing and signal detection.

Using an image-processing software, chromosome regions with an abnormal sample: reference ratio are found (with loss or gain of DNA sequences).

-DNA extraction-

DNA of CD34+ cells sorted by FACS has been extracted by using Qiagen Flexigene Kit #51204 (Qiagen Inc., Valencia, CA – USA) with some specific expedients.

-300.000-1.000.000 cells of each FACS separation have been centrifuged at 300g for min.;
- Supernatant has been eliminated and cells re-suspended in FG1 buffer;
- Lysis buffer (mix of FG2 buffer and Protease) has been added;
- Sample has been vortex 3 times for 5sec. and incubated for 15min at 65°C;
- DNA precipitation has been carried out by using TR Iso-propanol;
- Sample has been centrifuged for 60min, 4°C at 20.000 x g;
-After supernatant elimination, samples were washed in 70% ethanol and centrifuged for 15 min, 4°C at 20.000 x g;
- Pellet has been dried for 5-10 min;
- DNA has been re-suspended in FG3 buffer and incubated for 15 min at 65°C;
-DNA quantification-

DNA has been quantified by using Qbit assay (Invitrogen # Q32857, Invitrogen Ltd (European Headquarters), Paisley PA4 9RF, UK) and BR (#Q32850) based on the average of 3 readings of each sample. The usual DNA concentration was between 25 e 35 ng/μl. For lower concentrations DNA has been amplified through WGA2 KIT (Sigma-Aldrich Corporate - St. Louis, MO – USA).

-DNA quality control-

The quality of extracted DNA has been verified through electrophoresis on 1,2% agarose/ TAE gel and spectrophotometric method by using Nanodrop ND-1000 (Thermo Scientific – Nanodrop division, Wilmington, DE – USA).

Best parameters of Absorption for 260/280 are 1,8-2,0 and for 260/230 are 1,9-2,2.

-DNA Labelling-

DNA of reference and patient have been prepared for a-CGH hybridization following Agilent Enzymatic Genomic Labelling protocol version 6.2. Briefly:

-500 ng of each DNA have been digested with AluI e RsaI restriction enzymes (Promega Corporation Woods Hollow Road Madison, WI – USA) for 2h at 37°C;

-The enzymes have been inactivated at 65°C for 20’;

-Obtained fragments (100-500bp) have been labelled in random priming with Agilent Genomic DNA Enzymatic labelling KIT #5190-0449 (Agilent
Technologies, Inc, Santa Clara CA – USA), then denatured at 95°C for 3min and incubated for 5min on ice;

-Labeling mix has been prepared. Patient DNA has been labeled by using dCTP-Cy5 and reference DNA by using dCTP-Cy3;
- The labeled mix has been added to DNA
- The reaction has been incubated at 37°C for 2h and inactivated at 65°C for 10 min;
- The purification of labeled fragments has been performed through columns Kit Amicon Ultra 0.5 #UFC503096 (Millipore Corporate Headquarters, Billerica, MA – USA). After TE addition and centrifugation at 14.000g for 10min, the eluted was eliminated (twice);
- Labeled purified DNA has been recovered by centrifugation at 1.000g for 1min;
- The efficiency of labeling reaction has been checked through Nanodrop spectrophotometer;
- Reference and patient labeled DNAs have been mixed and added with blocking solution, hybridization solution (Agilent #5188-5220) and Cot-1 DNA (Invitrogen Ltd - European Headquarters, Paisley PA4 9RF, UK).
- The hybridization mix has been denatured at 95C for 3min, placed at 37°C for 30min for pre-annealing reaction and then placed with oligonucleotides spots whole genome 244K Agilent G4411B slide in a hybridization chamber at 65°C for 40h;
Material and methods

-The array slide has been washed 5 min in the solution 1 at room temperature and 1 min in the solution 2 at 37°C (Both the solutions are from Agilent).

-The array was scanned by using Agilent scanner with a resolution of 5μm.

Fluorescence analysis was performed by Agilent Feature Extraction 9.6 software;

a-CGH profile has been interpreted by using Agilent Genomic Workbench 5.0 software and ADM2 algorithm (threshold 6.0).

Computer analysis

Data analysis has been performed comparing all patients with benign copy number variants (CNVs) present in http://projects.tcag.ca/variation/ site. This site consists of all identified sequences with altered number of copies in healthy subjects. Since compared regions were copious, an house software was made to easily permit the alignment. The data allowed to exclude sequences which do not represent pathologic markers since already present in literature and highlighted as normal polymorphic alterations (bCNV).

Fluorescence in situ hybridization (FISH)

The Fluorescence in situ hybridization allows the localisation of a specific sequence of DNA on fixed chromosomes, nuclei or tissue samples obtained from fresh or cryopreserved biologic material as blood, biopsy and amniotic fluid.
The assay is based on the reversible denaturation DNA property and it consists in the binding of a fluorescence probe and the DNA complementary sequence of the sample; in this way the chromosome region of interest results easily identifiable at fluorescence microscopy.

FISH technique consists in:

- Ageing of samples: low salty solutions increase the probe accessibility to targets sites.
- Denaturation of probe and target: conditions change with probe and sample. The correct one can be found acting on temperatures (between 70 and 80 °C) and denaturation time (between 2 and 5 minutes). It is frequent the use of denaturation solutions containing formamide (to reduce the double chain heat stability) and don’t resort too high temperatures.
- Hybridization: Conditions for probe-target heteroduplex formation are important and in particular the probability to maintain heteroduplex containing wrong couples is lower when conditions are stronger (low salts concentration, high temperature, denaturation agents).
- Wash: the fluorescent probe can bind incompletely sequences with lesser homology. The resulting less stable hybrids can be dissociated intervening on temperature, time and solution composition of washing.

Samples can be observed with a fluorescence microscope (LeicaDMRA).
The BACs (Bacterial artificial chromosome) probe used in this study are RP11-596C4 and CTD-2130L6, specific for a small region in chr2 p16.1 and produced in our laboratory by using BAC Phase Prep Kit (Sigma).

Once isolated, BAC has been labelled by Nick Translation.

-Nick translation-

This assay is based on DNase I ability to introduce nicks on single chain of DNA and on E. Coli DNA Polymerase exonuclease 5’-3’ ability to repair nicks, re-polymerizing DNA partially. Therefore, digoxigen-11-dUTP labelled nucleotides (Roche Biochemicals) in the buffer are incorporated during the reaction. For Nick Translation reaction NICK TRANSLATION KIT (Roche Biochemicals) has been used. After incubation for 2h at 15 C, Cot-1 DNA (Roche Biochemicals) and Salmon Sperm DNA have been added in excess to saturate repetitive sequences in the probe.

Then DNA has been precipitated and re-suspended in formammide, SSC and Dextran sulphate solution.

-Preparation of slides-

Part of sorted cells has been re-suspended in fixative solution and gently dropped on slides. For good FISH results is important to obtain intact nuclei in a good concentration. Slides have been treated with 2XSSC solution for 30’ at 37 C and dehydrated in alcohol 70%-85%-100% at RT.
Material and methods

-Denaturation, hybridation and signal amplification.-

BACs have been denatured at 70°C for 10’, then pre-annealed at 37°C for 1h 30’. Meanwhile slides have been denatured at 72°C for 5’ in 70% formammide/2XSSC solution and dehydrated in alcohol 70%-85%-100% at RT. Hybridization was performed at 37°C O/N. Three cycles of amplification have been done by using anti-DIG for 60’, anti-Mouse for 45’ and anti-Rabbit for 45’again at 37°C. Nuclei have been contrasted by DAPI and Propide Iodide. Slides have been observed with fluorescence microscope (Leica DMRA) and analysis performed by using Q-FISH Leica Imaging System Ltd software.

Cytogenetics and molecular genetics data

Cytogenetic analysis have been realized in multiple myeloma, mantel and follicular lymphoma.

About multiple myeloma, in particular at the onset when the percentage of plasmacells is not high, it is important to select the clone to be analyzed by FISH. The selection is accomplished by immunomagnetic separation on column by using CD-138 as antigen. FISH have been performed to check:

-rerarrangements in 14q32
-t(11;14)(q13;32) and the consequent increase of CCND1 expression
-t(4;14)(p16.3;q32) which involves MMSET (multiple myeloma set domain) and FGFR3 (fibroblast growth factor receptor 3)
-monosomy of 13 and deletion of 13q who represent the majority of cytogenetic alteration in MM
-17p13 deletion which involves p53 deletion

**Clinical follow-up**

Since 2009 mobilized patients have been 37; between them 18 have not been re-infused and consequently excluded from this study. Left patients have been analyzed considering: the immediate effect of stem cells reinfusion, the values of leucocytes, platelets, haemoglobin and haematocrit at 1,6,12 months after reinfusion. Also, disease free survival (DFS= time between the transplantation and the progression of pathology) and overall survival (OS) have been evaluated.

Patients subjected to pre-transplantation cytogenetic analysis have been studied in the same way after the ASCT to verify the presence of old or new mutations. Data of follow-up have been compared to cytogenetic analysis.
RESULTS

Apheresis optimization

All patients have been subjected to chemotherapy and to one or more apheresis procedures.

As indicated in fig 8, first effect of chemotherapy is the aplastic state which reaches the lowest level at day 10 in peripheral blood. Later, G-CSF causes stem cells mobilization at day 14 and the retake of normal haematopoiesis.

Fig 8: Scheme of bone marrow aplasia and stem cells mobilization after chemotherapy and G-CSF administration.

As indicated in material and method section, final product of apheresis could have different cell properties.

Indeed, volumes of apheresis resulted included between 510 mL and 55 mL with a mean of 255 mL.

Furthermore, concentration of white-cells in the peripheral blood at the
moment of apheresis caused different concentrations of leukocytes in the apheresis product included between 257,000,000 cells/mL and 36,500,000 cells/mL with a mean of 158,780,000 cells/mL and a median of 152,000,000 cells/mL. Freezing, thawing and labelling stem cells optimization

Cells obtained by apheresis are stocked at -196 C.

To this aim cells are transferred in a sac with physiologic solution, 7% albumin and centrifuged. Obtained pellet is re-suspended in 10% DMSO. Concentration of leukocytes can’t exceed the limit of 200,000,000 cells/mL to maintain cell vitality during freezing procedure.

The data were included between 255,000,000 cells/mL and 28,700,000 cells/mL with a mean of 169,100,000 cells/mL and a median of 175,800,000 cells/mL. So in the case of 255,000,000 cells/mL the limit was overstepped determining a decreasing of vitality at 77% against the mean of 90%.

Also the concentration of stem cells in the collected samples presents different values due to the variability in the number of cells in the peripheral blood at the moment of the draw and included between 21,000,000 cells/Kg and 1,400,000 cells/Kg with a mean of 6,800,000 cells/Kg and a median of 5,400,000 cell/Kg.

Using these data a thawing protocol was developed to permit a maximum cell yield necessary for a-CGH experiments (for which 500ug of DNA and 300000 nuclei approximately are essential).

Cells have been thawed by using RPMI-1640. Heparin has been added to prevent aggregated formation at a final concentration of 4%.
After washed twice cells have been re-suspended in RPMI 1640 medium/serum 5%/ pen/strept 2%. This expedient permitted to higher the efficiency of sorting obtaining a purity major of 90% and in some cases collecting a great number of cells passing the million of CD34+cells.

This study leaded to the selection and the quantification of CD34+ cells (Fig 9).

**Fig 9: ISHAGE methods to define stem cell concentration**

Obtained data manifest high heterogeneity in leukocyte counting and in stem cell concentration. In effect, analyzed patients show values of CD34+cells/µl
between 26 and 385. This variability confirms the bone marrow compromission after chemotherapy and mobilization (Table 5).

<table>
<thead>
<tr>
<th>Purity%</th>
<th>Number of sorted CD34+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>91.00</td>
</tr>
<tr>
<td>Patient 2</td>
<td>90.00</td>
</tr>
<tr>
<td>Patient 3</td>
<td>96.00</td>
</tr>
<tr>
<td>Patient 4</td>
<td>95.00</td>
</tr>
<tr>
<td>Patient 5</td>
<td>89.00</td>
</tr>
<tr>
<td>Patient 6</td>
<td>94.00</td>
</tr>
<tr>
<td>Patient 7</td>
<td>96.00</td>
</tr>
<tr>
<td>Patient 8</td>
<td>98.00</td>
</tr>
<tr>
<td>Patient 9</td>
<td>95.00</td>
</tr>
<tr>
<td>Patient 10</td>
<td>92.00</td>
</tr>
<tr>
<td>Patient 11</td>
<td>92.00</td>
</tr>
<tr>
<td>Patient 12</td>
<td>92.00</td>
</tr>
<tr>
<td>Patient 13</td>
<td>nd</td>
</tr>
<tr>
<td>Patient 15</td>
<td>94.00</td>
</tr>
<tr>
<td>Patient 16</td>
<td>93.00</td>
</tr>
<tr>
<td>Patient 18</td>
<td>89.00</td>
</tr>
<tr>
<td>Patient 21</td>
<td>93.00</td>
</tr>
<tr>
<td>Patient 27</td>
<td>98.00</td>
</tr>
<tr>
<td>Patient 28</td>
<td>92.00</td>
</tr>
<tr>
<td>Patient 29</td>
<td>89.00</td>
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<tr>
<td>Patient 30</td>
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</tr>
<tr>
<td>Patient 31</td>
<td>91.00</td>
</tr>
<tr>
<td>Patient 35</td>
<td>89.00</td>
</tr>
<tr>
<td>Patient 36</td>
<td>91.00</td>
</tr>
</tbody>
</table>

- **MAX**: 98.00 1,065,338
- **MIN**: 89.00 268,219
- **MEAN**: 92.52 643,779

**Table 5**: Recapitulatory data of sorting.
Array-CGH (a-CGH)

The DNA from CD34+ cells sorted was extracted.

The DNA and the optimal labeling of hybridization quality have been evaluated through the red and green fluorescence ratio (Fig. 10)

Results allow to consider as significant all the alterations that have been found.

**Fig 10: Intensity of signal distribution on green and red channels (Agilent Genomic Workbench 5.0)**

The data from a-CGH have been filtered by ADM2 algorithm which confers a statistic significance to each present probe in the slide based on the gap from the zero line. In order to evidence the aberrations, the fixed parameter was of 5 consecutive probes (around 30000 bp of mean) with significant deviation for ADM2. Furthermore, the alteration has to be not inferior to 10% of cells in the sample (Valli R. et al, 2011). Fig.11 shows an example of segmental loss of chromosome 18 in 36.4% of cells.
Fig. 11: Example of imbalance found by a-CGH: chromosome 18 loss in 36.4% of cells.

Out of 37 patients, 24 have been processed for a-CGH.

Obtained a-CGH data showed a clear difference in the number of found imbalances. Indeed the majority of patients had a mean of 20.3 alterations but 3 patients have more than 100 imbalances. Overall, data showed a maximum of 525 altered regions and a minimum of 15 with a mean of 65.53 and a median of 27.

The hypothesis is that alterations predisposing to the pathology could be present in HSCs. Therefore, common altered sequences have been searched among the recurring alterations of patients affected by the same disease. Patients have been divided in two groups, those affected by MM and those affected by Lymphoma (HL
and NHL). 453 sequences of patients with lymphoma and 661 sequences of patients with myeloma have been analyzed. The comparison between different patients showed copy number polymorphisms and interesting regions not mapping in the database but altered in at least two patients.

Found data showed 26 imbalances in a single patient which need in-depth analysis and the same imbalances not mapping in CNV database in 2/8 Multiple Myeloma patients (see Appendix).

In the case of Lymphomas, obtained data showed 17 imbalances in a single patient and common imbalances region in 10/16 patients on chromosome17 as expected for the pathology (Chui DT et al, 2003).

The most significant alterations were:

In patient (2) a deletion of 4.34MB in 14.5% of cells has been found on chromosome 2 in p16.1 (start: 56.586.512, end: 60.932.053 bp) and including part of REL oncogene associated to rare forms of myelomas and lymphomas (Gilmore and Gerondakis, 2011) (Curry et al., 2009) (Fig.12).

Patient (3) presents a duplication of 16.08Mb on chromosome 11 between q12.2 (start: 60.517.445 bp) and q13.4 (end: 76.600.804 bp) bands in 12.38% of cells and including CCND1 gene often rearranged or duplicated in myelomas (Fig.13).

Patients (8), (9), (10), (27) and (30) showed imbalances (duplications or deletions) in q32.31-q32.33 region of chromosome 14, containing heavy chain genes of immune globulin cluster often rearranged in lymphomas and myelomas (Fig.14).
All these patients are affected by lymphoma and in particular (8), (9), (10) are HL, (27) and (30) are NHL.

**Fig12:** a-CGH results of patient (2). (a) Deletion of short arms of chromosome 2 at band p16.1 is highlighted (blue square); (b) part of REL gene is included in the deletion region (blue square).
Fig13: aCGH results of patient (3)
(a) Amplification of long arms of chromosome 11 at band q12.2 and q13.4 is highlighted (grey square); (b) part of CCND1 gene is included in the amplification region (grey square).
Fig14: aCGH results of patients (8, 9, 10, 27 and 30).
(a) Imbalances of long arms of chromosome 14 in q32.31-q32.33 region is highlighted (grey square). (b) Enlargement of q32.31-q32.33 region. (Patient 8: blue line, patient 9: pink line, patient 10: yellow line, patient 27: brown line, patient 30: green line).
Results

Fig15: aCGH results of patient (1): example of the high number of imbalances found.
(a) Ideogram of chromosome 1; (b) Ideogram of chromosome 11. Numerous picks corresponding to imbalances with variable dimensions are present and highlighted (blue square).

In patients (1), (8) and (9) the number of imbalances found by a-CGH was much bigger than others with a number of alterations of 525 in patient (1) (see fig 15), 128 in patient (9) and 103 in patient (8) where the mean for the other patients was 25.57 (min value: 15 in patient 10; max value:37 in patient 29).

In patients 4, 5, 6, 7, 11, 12, 16, 28, 29 and 31 no imbalances have been found through a-CGH
For patients 13, 15, 18, 21, 35 and 36, the quality of material was not good for a-CGH analysis.

To confirm a-CGH data, FISH or real-time PCR would be used.

Since in this study imbalances are at low clonality (around 10%), the former is the best technique to consider.

In patients (8), (9), (10), (27) and (30), presenting imbalances on chr14, was not possible perform FISH because of the presence of a large duplicon in 14q32.31-q32.33 region which could produce false positive. Indeed, duplicons are chromosome-specific low-copy repeats whose occur in multiple regions of the human genome.

It is known that homologous recombination between different duplicon copies by the process of “Non Allelic Homologous Recombination (NAHR)” leads to chromosome rearrangements, such as deletions, duplications, inversions, and inverted duplications (Ji Y. et al, 2000). These recombinations bring to human genetic disorders and can be implied in translocation events in heavy chains of IgH found in some of lymphoma and myeloma. Furthermore, it is known that apparently balanced events as translocations could carry genomic imbalances (Baptista J. et al, 2008).

Therefore, the presence of imbalances found on chr14 by a-CGH could be correlated to this kind of translocations.
In patient (2), presenting imbalances on chr2, FISH was first performed by using RP11-596C4 probe, specific for p16.1 region. Because of background this BAC was not optimal. On the other hand, the second probe CTD-2130L6 confirmed aCGH data showing deletion of 2 p16.1 in 12% of cells (Fig.16).

For patient (3) not enough material was available the number of cells was not enough to carry out the FISH.

![Image](image_url)

**Fig 16: Deletion of 2 p16.1 region shown by FISH analysis**

**Cytogenetic analysis**

Cytogenetic analysis and molecular cytogenetic on pathologic lymph node biopsy have been performed in the Laboratory of Cytogenetic at the Department of Clinical Pathology of Ospedale del Circolo, Fondazione Macchi in Varese (table 6)

12 patients (10 affected by MM, 1 by NHL and 1 by HL) have been analyzed.

7/10 patients with MM presented normal karyotype 46,XX/XY.

3/10 patients with MM presented chromosome imbalances and in particular

Patient 11: 47,XY/46,XY,del(6)(q22qter);

Patient 6: 46,XY/45,XY,del(6)(q23q25),-17;

Patient 3: 46,XY,del(6)(q23q25);
The more frequent alteration is the monosomy of chromosome 13 presents in 4 patients, then the amplification of CCND1 in three patients, the loss of p53 and the amplification of IgH gene in one patient.

The patient affected by mantle-cell NHL showed normal karyotype with translocation t (11;14) in 14% of nuclei, loss of ATM gene in q22-23 region of chromosome11 in 52% of nuclei.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Karyotype</th>
<th>FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>MM 46,XY</td>
<td>Monosomy 13 (total or partial)</td>
</tr>
<tr>
<td></td>
<td>MM 46,XY</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>MM 46,XY</td>
<td>Neg</td>
</tr>
<tr>
<td>Patient 3</td>
<td>MM 46,XY</td>
<td>IgH rearrangement (3%)</td>
</tr>
<tr>
<td></td>
<td>MM 46,XY</td>
<td>IgH/CCND1 rearrangement (3,3%)</td>
</tr>
<tr>
<td>After transpl.</td>
<td>MM 46,XY,q23/q25</td>
<td>IgH rearrangement (3,2%)</td>
</tr>
<tr>
<td></td>
<td>MM 46,XY,q23/qter</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>MM 46,XY</td>
<td>CCND1 amplification (83%)</td>
</tr>
<tr>
<td></td>
<td>MM 46,XY</td>
<td>CCND1 amplification (16%)</td>
</tr>
<tr>
<td></td>
<td>MM 46,XY</td>
<td>Monosomy 13</td>
</tr>
<tr>
<td>Patient 6</td>
<td>MM 46,XY</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>MM nd</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>46,XY/45,XY,del(6)(q23q25)</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>MM 17</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>MM 46,XY</td>
<td>Neg</td>
</tr>
<tr>
<td>Patient 11</td>
<td>MM 46,XY,del(6)(q22/qter)</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>MM 46,XY</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
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<tr>
<td>--------</td>
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<td>-----------</td>
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<tr>
<td>Patient 13</td>
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<td>Patient 15</td>
<td>MM</td>
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</tr>
<tr>
<td></td>
<td>MM</td>
<td>46,XX</td>
</tr>
<tr>
<td>Patient 16</td>
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<td>46,XY</td>
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<tr>
<td></td>
<td>MM</td>
<td>46,XY</td>
</tr>
<tr>
<td>Patient 18</td>
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<td>46,XX</td>
</tr>
<tr>
<td></td>
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<td>46,XX</td>
</tr>
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<td></td>
<td>MM</td>
<td>46,XX</td>
</tr>
<tr>
<td>Patient 19</td>
<td>HL</td>
<td>nd</td>
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<tr>
<td>Patient 24</td>
<td>MM</td>
<td>46,XY</td>
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<td>46,XY</td>
</tr>
<tr>
<td></td>
<td>MM</td>
<td>46,XY</td>
</tr>
<tr>
<td>Patient 25</td>
<td>MM</td>
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</tr>
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<td></td>
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<td>46,XX</td>
</tr>
<tr>
<td></td>
<td>MM</td>
<td>46,XX</td>
</tr>
<tr>
<td>Patient 26</td>
<td>MM</td>
<td>46,XY</td>
</tr>
<tr>
<td></td>
<td>MM</td>
<td>46,XY</td>
</tr>
</tbody>
</table>

Table 6: Results of cytogenetic analysis
**Clinical follow-up**

The distribution of different diseases of the 37 mobilized patients is shown in fig 17.

![Diagram showing the distribution of diseases](image)

Fig 17: Distribution of diseases of evaluated patients in the follow-up; MM: Multiple Myeloma, HL: Hodgkin Lymphoma, NHL: Non Hodgkin Lymphoma, Theratoma.

The recovery day of haematic crasis after transplantation, the number of transfusions received from patients, the disease-free survival (DFS) and the overall survival (OS) have been evaluated.

The haematic crasis is the day after stem cells re-infusion in which value of leucocytes is major than 1000 and platelets major than 20000 for 2 consecutive days. The average value of haematic crasis achievement has been the day 11 (maximum value=19 in patient 14; minimum value=8 in patient 10). Not clear differences between diseases have been observed.
About the number of transfusions received from patients the mean has been 2.23 transfusions of red irradiate cells (max value=12 in patient 20; minimum value =0 in patients 3,6,10,11,15,16,18,19,21,24,25). Again, not differences between pathologies have been observed. As to disease-free survival (DFS), 13/19 patients (57.7%) relapsed after transplantation and the time free from re-infusion to the relapse diagnosis is 2.8 months (min value= 1month in patients 2, 8,14; max value=18 months in patient 6). After a mean follow-up of months 33.8, 14 are alive patients (6/14 are completely recovered and 8/14 relapsed) and 5 are death (Fig.18). Survival is under study with a minimum value of 3 days (patient 1) and a maximum value of 52 months (patient 3).

![Fig 18: Clinical follow-up at 33.8 months after transplantation. D: death, R: relapse, CR: complete remission](image)
Furthermore, it is important to report the appearance of hyperthyroidism in patient 3 and hypogonadism in patient 21. Also, secondary neoplasms occurred in two patients and in particular prostatic cancer in patient 11 and melanoma in patient 7. The figure 19 shows the correlation between array-CGH results and clinical follow-up.

Fig 19: Correlation between array-CGH results and clinical follow-up. D/R: death or relapse; CR: complete remission. No significant differences between the two groups were revealed.

In particular,

-Patient 2 (affected by HL with deletion of 2p16.1 region) showed progression of disease one month after the autologous transplantation. Because of the young age, the patient has been submitted to bone marrow allogenic transplantation after 4 months from CD34+ cells re-infusion and he expired due to pulmonary infection.
Patient 3 (MM with duplication of chr11 between q12.2 and q13.4 bands in 12.38% of cells and including CCND1 gene) relapsed after 7 months from transplantation. Cytogenetic analysis have been performed before and after transplantation. Results were interesting, since before the transplantation the rearrangement of IgH gene was present in 3.3% of nuclei and a deletion of chromosome 6 (at q23q25 level); analysis performed after the transplantation revealed the deletion of chromosome 6 (but in a different region: q23qter) and later the reappearance and the amplification of CCND1.

Last cytogenetic control revealed a deletion of chr 13. The patient is followed in Day Hospital Oncology and his disease is in progression.

- Patients 8, 9, 10, 27 and 30 showed imbalances of chromosome 14 where IGH gene is present.

Patient 27 and 30 have not accomplished CD34+ re-infusion therefore the follow-up is performed only for patients 8, 9 and 10 (affected by HL).

Patients 9 and 10 are completely in remission at 34 and 38 months from transplantation.

Patient 8 relapsed. Particularly, he had one month of disease free time from and died one year after transplantation.

- Patient 1 showed a number of imbalances higher than others and had a bad progression: he died 3 days after CD34+ cells re-infusion.

-In patients 4, 5, 6, 7, 11, 12, 16, 28, 29 and 31 no imbalances have been found by a-CGH.
Because of the disease progression, patients 28, 29 and 31 have not been re-infused and therefore excluded from follow-up analysis. In the 7 analyzed patients, the mean follow-up obtained has been 40 months (min value: 31 months in patient 6; max value: 49 months in patient 12).

Patients 7 and 12 died at 10 and 5 months from transplantation: patient 7 because affected by a secondary neoplasm (melanoma) and patient 12 because of the disease progression.

Patient 4 relapsed at two months from re-infusion and he is in treatment at the Oncology Department.

At patient 11 diagnosis of prostatic cancer has been performed after 24 months from re-infusion.

Patient 6 relapsed at 31 months from transplantation and he is in treatment at the Oncology Department.

Patients 5 and 16 are in completely remission at 34 and 22 months from transplantation.

-For patients 13, 15, 18, 21, 35 and 36 the material was not good for a-CGH analysis. Patients 35 and 36 are in complete remission.

On the other hand, patient 13 was in remission but 18 months after transplantation showed nodules at thyroid level.

Patients 15, 18 and 21 relapsed.
The role of hematopoietic stem cell transplantation (HSCT) in NHL, HL and MM is well established (Calderón-Cabrera C et al, 2013), but a complete evaluation of its relevance in the clinical story of the disease and in long-term course is still under debate.

Indeed there is not enough evidence regarding whether transplantation is the best therapeutic approach in NHL and HL. Moreover, published data on long-term follow-up of high-risk lymphoma patients treated with HSCT are poor.

The aim of this study was to evaluate the chromosome imbalances of stem cells collected for HSCT compared with clinic follow-up of lymphoma and myeloma patients who underwent to auto transplantation.

Copious regions of imbalances have been found, also at a low clonality.

These regions have been analyzed and compared to sequences present in http://projects.tcag.ca/variation/ site that reports all variations not directly linked with pathologies. In some of our patients altered regions were unique and not already known as CNPs.

Among chromosome imbalances detected, some indicate patients scientifically interesting as to proportion of the anomalous clone and gene content.
Patient (2) reveals in 14.5% of cells a deletion of chromosome 2 in p16.1 region where the REL oncogene is located (Curry et al, 2009); (Gilmore, 2011): as a consequence it was partially deleted. Alterations of REL oncogene are associated with numerous neoplasms among which Hodgkin lymphoma (Enciso-Mora et al, 2010). The clinic follow up of the patient showed a relapse after one month from the transplantation and an overall survival of 4 months. In particular, cause of death was septic shock during the aplastic phase after the transplantation.

Patient (3) shows in 12.38% of cells a duplication of chromosome 11 between q12.2 and q13.4 bands where CCND1 is located. In particular according to FISH nuclei, CCND1 was amplified in 3% of nuclei before the transplantation and in 86% of nuclei after the transplantation. This patient showed also a deletion of chromosome 13 appeared at 2 years from transplantation. Clinically he relapsed 7 months from transplantation and actually, still alive, he is still followed at the Oncology Department.

Analysis of these results and clinic outcome of patients suggest that haematopoietic stem cells re-infusion can be a possible risk factor when the chromosome alteration compromises significant genes.

These two cases indicate that the techniques used in this study could found a clinical application for a more accurate investigation of patients, and for their prognostic evaluation.

Patients 8, 9, 10, 27 and 30 showed altered portion of chromosome 14 in q32.31-q32.33 region where IgH gene is present and often rearranged in lymphomas and
myelomas. This region is also characterized by numerous benign CNVs so it is possible that array-CGH results are due to them. Translocations involving the IgH gene are frequent in lymphoma and, although cytogenetic information is lacking in these specific patients, we might speculate of imbalances associated with these translocations.

Clinically, patient 8 relapsed, patients 9 and 10 are in complete remission at 34 and 38 months from transplantation.

Patients (1), (8) and (9) affected with MM and HL were significantly different from all the other patients taken into account, at a-CGH level. As depicted in results they showed a considerable number of imbalances, very far from other patients. Particularly patient (1) revealed 525 imbalances, patient (9) 128 and patient (8) 103. In the first two cases the clinic course has been notably negative. Indeed, patient (1) expired after 3 days and patient (8) after 12 months from stem cells reinfusion. Patient (9) is in complete remission at 34 months from transplantation. It is possible to hypothesize that the alterations revealed from array-CGH could be due to chemotherapy treatment at which patients were exposed before of stem cells mobilization, but one should remake that the same would apply also to all other patients. The phenomenon called chromotripsis was mentioned in Introduction and could be at the basis of the karyotypic disaster of these patients.

In patients 4, 5, 6, 7, 11, 12, 16, 28, 29 and 31 no imbalances were found through a-CGH, although a deletion of chromosome 6 long arms was detected by cytogenetic studies in patients 6 and 11.
Because of the disease progression, patients 28, 29 and 31 have not been re-infused. Patients 5 and 16 are in completely remission at 34 and 22 months from transplantation. Patients 4 and 6 relapsed at 2 and 31 months from re-infusion.

To patients 7 and 11 have been diagnosed a secondary neoplasm after 1 month for patient 7 and 24 months for patient 11. Patients 7 died for melanoma at 10 months after transplantation. Patient 12 relapsed 2 months from the re-infusion and died at 5 months after transplantation.

At 36 months, the correlation between array-CGH results and clinical follow-up shows not significant differences between patients with and without imbalances.

Also, it is important to highlight patient treatment consequences. Indeed, a cyto-reducing treatment precedes the therapy for autotransplantation with the goal to eliminate cancer cells. Furthermore, in HSCT suitable MM patients, the treatment is performed in first line, while in HL and NHL patients this treatment is performed in second line or in case of high risk disease. This imply that submitted patients to transplantation obtained one or more lines of chemotherapy which could modify cytogenetic evidences.

One of future steps of this study would be the correlation between array CGH and cytogenetic data during different phases of disease with the aim to monitor a cytogenetic profile. Indeed, cytogenetic data work as prognostic and therapeutic factors. For example, deletion of chromosome 13 in MM patients is a known negative prognostic factor which refers to more aggressive treatment. Therefore,
this kind of monitoring which implies a constant cytogenetic follow up could define new clinic-prognostic correlations.

Different experimental data (Scott and Reece, 2011); (Fenk et al., 2012) show higher risk of secondary neoplasm after HSCT and strong incidence of relapse in spite of ASCT; therefore the identification of alterations before the re infusion could allow the use of therapies to purify the apheretic pool.

Again, new information about pathogenesis of different tumours (Chen et al, 2007) could derive from the study of cancer stem cells or more significantly of a clone provided of characteristics similar to stem cells (auto-maintenance and migration ability). In this case would be important to extend the clinical observation.

Finally, indications of global survival of the population considered may come from a continuous cytogenetic and clinic follow up during the time.
## APPENDIX

<table>
<thead>
<tr>
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Table A1: Found alterations in patient 1 (MM)
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**Table A2: Common alterations found in patients 3 and 6 (MM)**

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<td>4</td>
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<tr>
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**Table A3: Found alterations in patient 2 (HD)**

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<td>17</td>
<td>q21.2 - q21.32</td>
<td>37,114,755</td>
<td>42,396,127</td>
<td>54.57% DUP</td>
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<td>37,146,898</td>
<td>40,794,930</td>
<td>34.24% DUP</td>
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</tbody>
</table>

**Table A4: Common found alterations in patients 5, 7, 8, 9, 10, 12, 27, 28, 30 and 31 (HD and NHL)**


Precancerous stem cells have the potential for both benign and malignant differentiation.

Chui DT, Hammond D, Baird M, Shield L, Jackson R, Jarrett RF
Classical Hodgkin lymphoma is associated with frequent gains of 17q.

Curry CV, Ewton AA, Olsen RJ, Logan BR, Preti HA, Liu YC, Perkins SL, Chang CC.
Prognostic impact of C-REL expression in diffuse large B-cell lymphoma.

A genome-wide association study of Hodgkin's lymphoma identifies new susceptibility loci at 2p16.1 (REL), 8q24.21 and 10p14 (GATA3).

Secondary primary malignancies in patients with multiple myeloma treated with high-dose chemotherapy and autologous blood stem cell transplantation.
Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM.
Int J Cancer. 2010 Dec 15;127(12):2893-917

Gilmore TD, Gerondakis S.

Gribben JG
Stem cell transplantation in chronic lymphocytic leukemia.

Increasing incidence rates, distribution and histological characteristics of primary gastrointestinal non-Hodgkin lymphoma in a North American population.

Hjalgrim H.

Hodgson DC, Koh ES, Tran TH, et al.

Ji Y, Eichler EE, Schwartz S, Nicholls RD.

Kiel K, Cremer FW, Rottenburger C, Kallmeyer C, Ehrbrecht E, Atzberger A, Hegenbart U, Goldschmidt H, Moos M.
Analysis of circulating tumor cells in patients with multiple myeloma during the course of high-dose therapy with peripheral blood stem cell transplantation.
Bone Marrow Transplant, 1999 May;23(10):1019-27.
Kyle RA, Rajkumar SV.

Prevalence of monoclonal gammopathy of undetermined significance.

Küppers R.

Marçais A, Suarez F, Sibon D, Frenzel L, Hermine O, Bazarbachi A.

Marks-Bluth J and Pimanda JE.
Cell signalling pathways that mediate haematopoietic stem cell specification.

Marshall NA, Christie LE, Munro LR, Culligan DJ, Johnston PW, Barker RN, Vickers MA.

Ng AK, Li S, Neuberg D, et al.
A prospective study of pulmonary function in Hodgkin’s lymphoma patients.

Nogová L, Rudiger T, Engert A.
Palumbo A, Anderson K.

Psaila B, Lyden D, Roberts I.
Megakaryocytes, malignancy and bone marrow vascular niches.


Rossi DJ, Jamieson CH, Weissman IL.
Stems cells and the pathways to aging and cancer.

Scott E, Reece D
What is the benefit of maintenance therapy with lenalidomide or bortezomib after autologous stem cell transplantation in multiple myeloma and what is the risk of developing a secondary primary malignancy?

Seto M.
Malignant lymphoma as a consequence of clonal evolution.

Shankland KR, Armitage JO, Hancock BW


