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Identification of Outcome-Oriented Cut-Offs for Copy Number Alterations in Multiple Myeloma: Predictive Biomarkers with improved prognostic accuracy

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"La colpa di Eva è stata quella di voler conoscere, sperimentare, indagare con le proprie forze le leggi che regolano l'universo, la terra, il proprio corpo, di rifiutare l'insegnamento calato dall'alto, in una parola Eva rappresenta la curiosità della scienza contro la passiva accettazione della fede."

Margherita Hack

Summary

ABSTRACT

Multiple Myeloma (MM) genomic architecture is complex and includes several Copy Number Alterations (CNAs), commonly employed to stratify patients into prognostic significant subgroups. Due to the sub-clonal composition of MM, CNAs call is not trivial, especially when small clonal frequencies should be discriminated from background.

Aim of the present study has been to develop a statistical approach to define the best cut-.off CNAs calling from genomic data provided by high throughput experiments, able to predict a specific clinical end point.

743 newly diagnosed MM patients with SNPs array-derived genomic data and clinical annotations were included in the study; the endpoint chosen for the study was relapse at 18 months (early relapse). CNAs were called both by a conventional (classic, CL) and an outcome-oriented (OO) method and Progression Free Survival hazard rations of CNAs called by the two approaches were compared. Variable selection was performed for multivariate Cox-Proportional Models by Stepwise Backward selection with AIC.

The OO approach successfully identified patients at higher risk of relapse and the univariate survival analysis showed stronger prognostic effects for OO-defined high-risk alterations, as compared to that defined by CL approach, statistically significant for 12 CNAs.

Multivariate Cox-Proportional analysis confirmed the improved fitness of survival models including OO-defined CNAs.

Overall, 155/743 patients relapsed within 18 months from the start of therapy. A small number of OO-defined CNAs were significantly more recurrent in early-relapsed pts (ER-CNAs), namely amp1q, amp2p, del2p, del12p, del17p, del19p. By stratifying patients according to the presence of at least one of these CNAs, two groups of 249 and 494 patients, either carrying or not ≥1 ER-CNAs were identified, the first one with significantly shorter progression-free (PFS) and overall survivals (OS) (PFS HR 2.15, CI 1.79-2.58 p<0001; OS HR 2.37, CI 1.86-3.02 p<0.0001). The worse prognostic impact was observed even in the absence of both amp1q and del17p, observed in 38/249 (15%) patients (PFS HR 2.57, CI 1.79-3.70 p<00001, OS HR 2.49, CI 1.57-3.94 p=0.0001), compared to 494 patients. The risk of relapse defined by the presence of ≥1 ER-CNAs was independent from those conferred both by R-IIS 3 (HR=1.51 CI 0.17-2.39-2.2; p=0.01) and by low quality (< stable disease) first-line best clinical response (HR=2.59 CI0.33-2.84 p=0.004). Notably, the type of induction therapy was not descriptive in this multivariate model, suggesting that early relapse is strongly related to patients' baseline genomic architecture, and not to induction therapy they were provided to.

In conclusion, the OO- approach employed here allowed to define CNAs-specific dynamic clonality cut-offs, improving the CNAs calls' accuracy to identify MM patients with the highest probability to early relapse. As being outcome-dependent, the OO-approach is dynamic and might be adjusted according to the selected outcome variable of interest (e.g. MRD-negativity) thus providing outcome specific clonality cut-offs.

1. STATE OF THE ART: MULTPLE MYELOMA

Multiple Myeloma (MM) is a B-lineage lymphoproliferative disease, characterized by the accumulation of plasma cells (PCs) in the bone marrow (BM).

Active MM is preceded by a preneoplastic stage, monoclonal gammopathy of undetermined significance (MGUS), and by a stage of indolent disease, smoldering myeloma (SMM). The terminal phase of the disease is marked by the loss of homing of the neoplastic PCs clone(s): this begins to migrate from the marrow to the peripheral blood, where they can be found in higher percentages than the physiological average (Plasma Cell Leukemia).

MM is a genetically complex disease, characterized by the presence of various genetic anomalies such as structural and numerical chromosomal aberrations and point mutations in oncogenes and tumor suppressor genes. [1–4]

The MM clone is characterized by a high intra-clonal heterogeneity, that is, the genomic alterations and/or mutations that are observed in each patient are not necessarily present in all the cells comprising the neoplastic clone. This suggests that several sub-clones may coexist in the same patient, competing for the access to the limited resources available in the marrow.[5]

Remarkable advances in MM therapy have been achieved over the past 15 years due to the progressive availability of novel classes of agents targeting MM sub-clones in their BM microenvironment. These highly active drugs have been combined with each other, resulting in a dramatic increase in the rate and depth of response, up to the level of minimal residual disease (MRD) negativity, tested by next generation flow cytometry or sequencing techniques.

MM is still considered as an incurable chronic disease, and the precise mechanism and/or the actual causes of its development are still unknown. However, several risk factors have been identified that can increase the likelihood of disease developing, predict its clinical course, and provide insight into the patient's prognosis, such as advanced age, male gender, genetic predisposition, and exposure to radiation or certain chemicals.

Clinically, it is well recognized that despite the widespread clinical efficacy of modern treatments and therapeutic combinations for MM, all patients eventually relapse, and with each successive relapse, the probability of response typically decreases, and the disease becomes refractory. One main feature associated with non-response to therapy is the genomic profile: certain genomic alterations are known to be associated with an unfavorable prognosis because they confer a particularly aggressive phenotype to the clone, (i.e., making it hyper-proliferative), and thus able to escape the selective pressure of the therapy used.

Hence, there is considerable clinical interest in early identification, based on the genomic profiles, of patient subgroups that may not respond effectively to therapy. This endeavor seeks to implement a tailored approach to patient care. From a genomic perspective, DNA copy number alterations (i.e., changes in the number of copies of specific regions of DNA in the genomes of myeloma cells - CNAs) not only represent the type of chromosomal aberrations most prevalent in tumor PCs, contributing significantly to the heterogeneity of this pathology, but they are also considered important prognostic factors in MM. The consistent occurrence of specific CNAs enables the identification of patient subgroups with a discernible clinical trajectory.

For MM, several biomarkers have been identified that can predict the clinical course of the disease and provide insight into the patient's prognosis; one of the most important biomarkers is the presence of specific chromosomal abnormalities, such as t(4;14), t(14;16) and del(17p) translocations, and amp(1q). These genetic anomalies can influence the treatment response and patients' overall survival.[4]

The presence of CNAs has traditionally been detected using molecular cytogenetic technologies (particularly Fluorescence in Situ Hybridization, FISH); the recent introduction of high-resolution molecular technologies like SNPs-Array, Next Generation Sequencing (NGS), or Single Cell methods has paved the way for studying CNAs using this high-throughput genomic approach. This led to the challenge of establishing precise threshold levels for calling various alterations detected with high-resolution molecular approaches (SNPs-array and NGS), which could potentially exist in a minor MM sub-clone.

Furthermore, the significance of different sub-clonal alterations remains uncertain, and it is unclear whether the size of the clone harboring specific CNAs can impact the patient's prognosis. Identifying predictive biomarkers for the clinical course of MM is essential for tailoring treatment to the unique needs of each patient. These biomarkers allow clinicians to assess the risk of disease progression, monitor treatment effectiveness, and make precise therapeutic decisions, ultimately enhancing the comprehensive management of MM.

1.1. PATHOLOGY

Multiple Myeloma (MM) is a hematological disease characterized by the proliferation and accumulation in the bone marrow (BM) of B lymphocytes and plasma cells (PCs) that synthesize monoclonal immunoglobulins, either complete or incomplete monoclonal component (MC), detectable in serum and/or urine. [6]

Unlike most hematological diseases, the genome of MM patients is more related to that of patients with solid neoplasms, as it is characterized by numerous structural and numerical chromosomal aberrations, as well as point mutations in oncogenes and tumor suppressor genes, some of which have been linked to the pathogenesis of the disease and its clinical course. [7]

The symptoms of MM can vary greatly, especially in the early stages of the disease. One of the first symptoms associated with the onset of the disease is bone pain, especially in bones rich in hematopoietic marrow (back, hip, and skull). Associated with bone pain is often a greater bone fragility, with frequent fractures, even following minor trauma. In more severe cases, this symptom can also cause nerve-type pain due to the compression of nerves by weak and fractured bones. A characteristic symptom is the presence of anemia, linked to the decrease in the number of red blood cells resulting in fatigue, weakness, and dyspnea. The decrease in white blood cells (leukopenia) and platelets (thrombocytopenia) is manifested instead with infectious morbidity, which is the leading cause of death in these patients.

1.2. EPIDEMIOLOGY

Multiple myeloma (MM) was first described in the second half of the 19th century by an Austrian doctor, Otto Kahler (1849 – 1893), and for this reason, it is also referred to as Kahler's Disease.[8]

The annual incidence is about 3-4 cases per 100,000, although it varies from country to country: from 1 annual case per 100,000 in the East to 4 cases per 100,000 in most Western countries.

The Afro-American population has twice the incidence compared to the Caucasian ethnicity.[8]

MM accounts for approximately 1% of all malignant tumors in Caucasians and 2% in Afro-Americans; 13% of all hematological malignancies in Caucasians and 33% in Afro-Americans.

These differences are maintained even in emigrant countries, suggesting the existence of genetic factors as well.

Men have a higher risk of developing MM: the male/female ratio is 3:2.

The incidence of MM increases with age: the average age at diagnosis is 68 years, although in the last 10 years there has been a decrease in the average age at diagnosis. Still, it rarely (about 2% of cases) drops below 40 years.

Generally, we can state that globally the incidence of MM is 4 individuals per 100.000[8], accounting for roughly 20% of deaths from hematological pathology and about 2% of deaths from cancer.[9]

In our country, every year 9.5 cases are diagnosed per 100,000 men, and 8.1 cases per 100,000 women; estimates speak of just over 2,100 new myeloma cases each year among women and about 2,300 among men. The risk of contracting this tumor in one's lifetime is 3.6 per thousand for women (one in 275 women) and 5.2 per thousand for men (one in 191 men).

Improved diagnostic techniques and the aging of the population can only partly explain the increased incidence in recent decades. Between 1973 and 1990, there was an increase of almost 15% among the subjects, especially in younger patients seen in the last 3-4 years. From 1990 to 2016, the global incidence of MM increased by 126% due to population growth, an aging population and increased age-specific incidence rates.[10]

The etiology of myeloma and epidemiological observations regarding occupational exposure suggest that infectious or chronic inflammatory processes might play a primary role. In the last ten years, following the introduction of innovative effective drugs, including the immunomodulator Thalidomide and the first-generation proteasome inhibitor, Bortezomib,

significant progress has been made in the treatment of MM. Despite improvements in care and novel therapies for the treatment of MM, eventually nearly all patients experience relapse.

1.3. COPY NUMBER ALTERATIONS

Copy number alterations (CNAs) belong to a significant category of somatic alterations found in cancer, called "structural rearrangements". Structural rearrangements are the results of chromosomal fragments dislocation, after the occurrence of genomic breakpoints, and mostly cause copy number changes. If structural aberrations occur in germinal cells, they may lead to congenital defects and mental retardation, increasing the risk of infertility or miscarriage. These structural anomalies either can appear *de novo* or can be inherited directly from parents who are carriers or can be the result of further rearrangements favored by a particular genomic structure present in one of the parents (**Figure 1**)[11]. Overall, structural rearrangements are the consequence of errors during cell division, mostly occurring during crossing-over, and consist in the loss, acquisition or rearrangement of one or more parts of one or more chromosomes.[12]

In general, unequal crossing-over phenomena lead to chromosomal deletions or duplications, while a single break within a chromosome leads a terminal deletion, two breaks in the same chromosome can induce an interstitial deletion, a ring chromosome or an inversion.

Two breaks in two different chromosomes cause structural abnormalities, such as reciprocal and Robertsonian translocations, resulting, in the latter case, in the fusion of two chromosomes at the centromere, with consequent loss of the short arm. Two types of structural chromosomal abnormalities can be observed: balanced (reciprocal translocations, Robertsonian, inversions) and unbalanced (deletions, duplications); in the first case, the chromatin remains quantitatively unchanged, while in the second case, the rearrangement involves a loss or gain of genetic material. Balanced chromosomal anomalies do not imply a pathological phenotype in the carrier subject, unless they interrupt and inactivate a specific gene, thus leading to a disease.

These types of anomalies are instead relevant to reproductive risk, since they can lead to the formation of unbalanced gametes, resulting in the conception of an individual with chromosomal syndrome. Unbalanced chromosomal anomalies, on the other hand, directly cause a pathological phenotype.[13]

Copy number deletion is an example of a “cut and paste” structural variant and copy number duplication is the product of a “cut and paste” structural variant.[14]

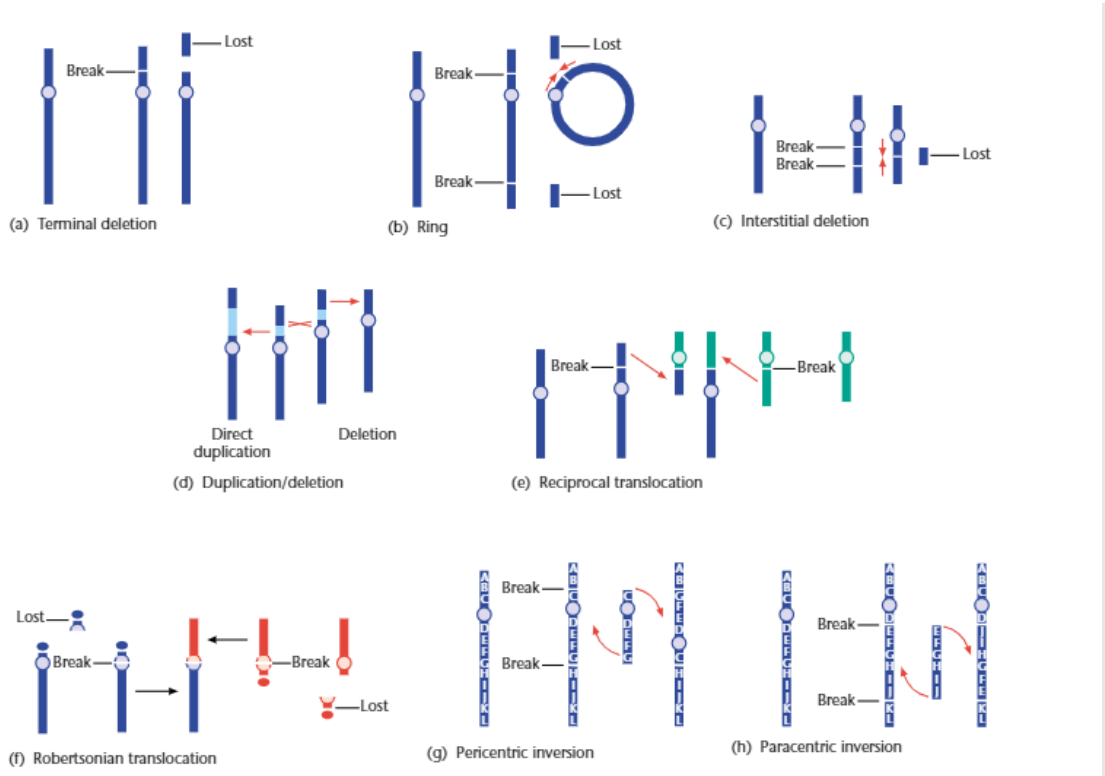


Figura 1 .[11]***Structural rearrangements.*** *(**a**) Terminal deletion: arises from a single chromosome break, with loss of the terminal chromatin segment. (**b**) Ring chromosome: originates from a break in each of the arms of the chromosome, with loss of the terminal segments and reunion of the centromeric one. (**c**) Interstitial deletion: originates from two breaks on the same chromosome arm, with loss of the interstitial segment and reunion of the two remaining segments. (**d**) Duplication/deletion: origin of a direct duplication and a deletion through an unequal crossing-over event. (**e**) Reciprocal translocation: arises from a break in each of the chromosomes and exchange of non-centromeric segments. (**f**) Robertsonian translocation originates from a break at the centromere of each of the chromosomes, union of the long arms and loss of the short arms of the two chromosomes. (**g**) Pericentric inversion originates from a break in each of the two chromosomal arms, with rotation of the centromeric segment by 180° and its reunion with the two terminal segments. (**h**) Paracentric inversion originates from two breaks in the same chromosomal arm, with rotation of the interstitial segment by 180° and reunion of the same at the terminal segments. Moore, C. M. & Best, R. G. Chromosomal Genetic Disease: Structural Aberrations (Wiley, 2001).*

1.4. COPY NUMBER ALTERATION IN MM

The genomic landscape of myeloma is very complex and heterogeneous, with a wide range of translocations, mutations, methylation, microRNA abnormalities and, mostly, CNAs.[4,15–18] (**Figure 2**).[2]

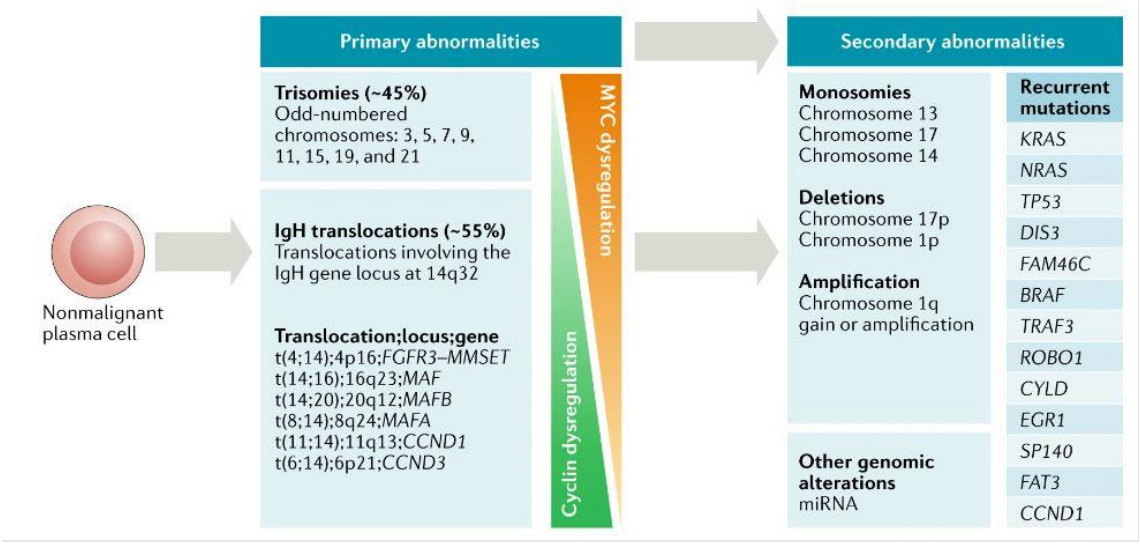


Figure 2. *Cyclin D dysregulation varies based on the underlying primary cytogenetic abnormality and is most prominent in t(6;14) and t(11;14) myeloma, in which cyclin D genes are directly involved in the translocation and is least affected in trisomic multiple myeloma (MM). By contrast, MYC proto-oncogene protein dysregulation is most prominent in trisomic MM. The complexity increases dramatically as each primary cytogenetic type acquires one or more secondary cytogenetic abnormality, including monosomies, deletions, amplifications, or specific gene mutations. IgH, immunoglobulin heavy chain; miRNA, microRNA. Kumar, S. K. & Rajkumar, S. V. The multiple myelomas - current concepts in cytogenetic classification and therapy. Nat Rev Clin Oncol 15, 409–421 (2018).*

Odd-numbered chromosomes hyperdiploidy (HD), which occur in half of MM, and immunoglobulin heavy chain (IgH) locus translocations, both are considered MM primary genomic events. The mutual exclusive presence of either hyperdiploidy or IgH translocations allow the stratification of MM patients in two main subgroups:

- *hyperdiploid MM* (HMM), represent approximately 50% of MM case; hyperdiploidy involve most odd-numbered chromosomes, including 3,5,7,9,11,15,19 and 21, and has been hypothesized to occur during the rapid germinal center proliferation, which occasionally might lead to errors in chromosome segregation;[4,19–21]

- *non-hyperdiploid MM* (NHMM), where trisomies of odd-numbered chromosomes 3, 5, are either absent, or no more than two trisomies are present. Accordingly, MM patients can be further divided into hypodiploid, pseudodiploid, or sub-tetraploid.[4,20,21]

Most of MM primary translocations (>90%) involve the IgH gene locus on chromosome 14(14q32.33) and one of several partner chromosomes, including chromosomes 4, 6, 11, 14, and 20[4,16]. IgH-translocations commonly lead to the de-regulated expression of genes located on partner chromosomes, due to the dislocation of the potent IgH enhancer(s); in particular:

- t(11;14), described in 15-20% of patients, causes the cyclin D1 deregulated expression; patients carrying t(11;14) are considered at standard risk; [4,16,22]
- t(4;14), described in 10-15% of patients, causes the MMSET/FGFR3 deregulated expression; patients carrying t(4;14) are considered at high risk, even though it seems to correlate to good response to early treatment with proteasome inhibitors; [16,23]
- t(14;16), described in 3-5% of patients, causes the MAF deregulated expression; patients carrying t(14;16) are considered at high risk; [16,24]
- t(6;14), described in 1% of patients, causes the cyclin D3 deregulated expression; patients carrying t(6;14) are considered at standard risk; [16]
- t(14;20), described in 1% of patients, causes the MAFB deregulated expression; patients carrying t(14;20) are considered at high risk.[4,15,16,23,25]

The distribution of secondary CNAs differs between HMM and NHMM, in details:

- **deletion of chromosome 13, del(13q)** occurs as clonal alteration in almost 50% of NDMM. The 13q arm includes the well-known tumor suppressor gene (TSG) *RB1*, which prevents cell cycle progression by sequestering *E2F* transcription factors, and *DIS3*, an exosome endoribonuclease. *RB1* is inactivated through a mono-allelic deletion and rarely with a biallelic loss, as opposed to loss of *DIS3* that is biallelic in 75% of MM.[16,17,25,26]
- **Amplification of chromosome arm 1q, amp(1q)** recurs in 40% of MM and patients carry 3 or more copies of 1q. It results from pericentromeric chromosomal instability and leads to the amplification of a several key genes, such as *CKS1B*, which ubiquitinates the cyclin dependent kinase *CDKN1B*.[4,16,24,27,28]
- **Deletion of chromosome arm 1p, del(1p)** recurs in 20-25% of MM and often co-occurs in patients with hypodiploidy (loss of one or more chromosomes). Del(1p) is associated with

the deletion of cyclin dependent kinase *CDKN2C* gene and *FAM46C*, a non-canonical poly(A) polymerase. *FAM46C* inactivation brings a cell survival advantage, and its overexpression produces an unfolded protein eliciting cell death.[4,16,24,29,30]

- **Deletion of chromosome arm 17p, del(17p)** is observed in 10% of NDMM with extramedullary disease and rarely in MGUS. The short arm of chromosome 17 is the site of *TP53* gene, tumor suppressor that controls different cellular activity, such as apoptosis, cell cycle and DNA repair. In 5% of NDMM, del(17p) occurs with *TP53* mutation, suggesting a stepwise progression where the deletion predispose to the biallelic loss of *TP53* by selection of cells with *TP53* mutation.[4,16,24,31,32]

Molecular cytogenetic methodologies such as G-band karyotyping, fluorescence in situ hybridization (FISH), and comparative genomic hybridization (CGH) combined with more advanced genetic techniques, encompassing single nucleotide polymorphism (SNP) arrays and, more recently, next-generation sequencing (NGS)[33] have made it possible to identify the most recurrent chromosomal and genetic alterations in MM, allowing their categorization into chromosomal translocations, CNAs, and point mutations.[33,34] The progressively higher depth of detection of the above-mentioned new technologies, when employed to highlight the presence of CNAs, has shown a wide range of distribution of each detected CNA, suggesting that the MM sub-clonal composition might be highly variable among patients. Moreover, different technologies have different limits of detection, that determine the proper CNAs call. Therefore, the CNAs calls might change, when different techniques are used and the employed cut-off for CNAs calling should be always reported. To date, just FISH, which is the gold-standard approach for the genomic characterization of MM, has well-defined conventional cutoffs, stated according to the technical features of the method; in particular, a generic 10% cutoff is commonly employed, to call any type of chromosomal aberrations (i.e., translocation and CNAs).[35] (**Figure 3**)

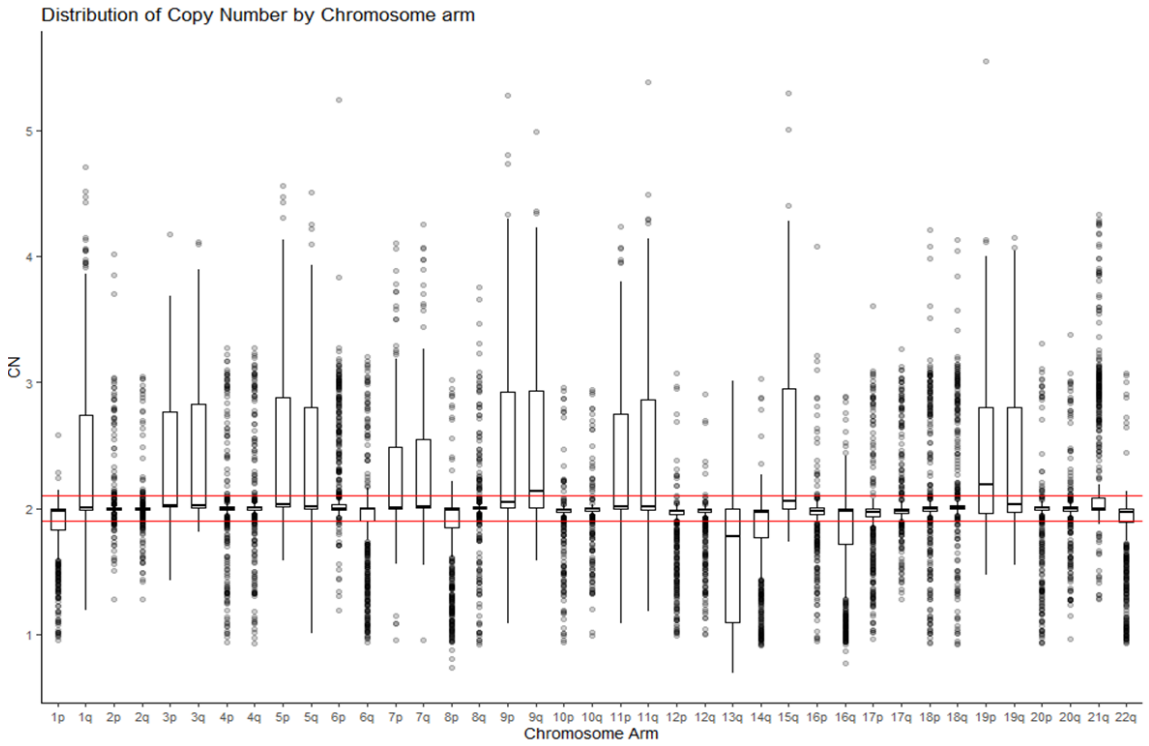


Figura 3. Box-plot of the CNs continuous values for each arm-chromosome. *On the X-axes is shown the chromosom arm of reference and on the Y-axes is found the Copy Number. Quantiles, median and outliers are shown. The red line, set at a value of 10%, in amplification and deletion, shows a generic cutoff to discriminate the presence of alterations above this value.*

1.5. RISK STRATIFICATION IN MM

Over the past 20 years, the introduction of new therapies and the increased use of high dose melphalan and autologous stem cell transplantation (ASCT) have improved the MM patients' overall survival (OS) at 5 and 10 years, across all age groups, races, and ethnicities.[36]

Notably, these benefits remain less pronounced in a small subgroup of patients, commonly referred as *high-risk*, meaning that for these patients' survival is invariably shorter than that of the overall population, independently from the therapy provided and that early disease recurrence is more frequently observed. Overall, approximately 20% of MM patients are at high-risk, and the early identification (possibly at diagnosis) of these patients would be crucial to catch this subgroup of patients and address it to personalized treatments, more intensively designed to target high-risk disease[37,38]. In fact, by accurately assessing the risk at diagnosis, (1) the length of the first remission can be expected longer and (2) an accurate definition of risks classes' definition can be employed for clinical trial enrollment.[37]

However, despite this clinical need, precise and universal recognition of high-risk patients is still missing, and a reliable MM risk definition remains a challenge. In fact, the commonly employed risk stratification systems, while defining categories of high-risk patients, still do not capture, nor describe, all their biological characteristics, thus frequently resulting inaccurate[37]

Most employed risk stratification systems are the following:

- the international staging system (ISS): this is one of the earliest validated risk stratification systems for NDMM patients[39], which distinguishes three stages based on two simple parameters: serum levels of albumin and β2 microglobulin (**Table 1**) [39]:

Stage I	β2 microglobuline < 3.5 mg/L and serum albumin ≥ 3,5 g/dl
Stage II	β2 microglobuline < 3.5 mg/L; serum albumin < 3.5 g/dL; or β2M 3.5 to 5.5 mg/L, irrespective of serum albumin
Stage III	β2 microglobuline > 5.5 mg/L

Table 1. International Staging System (ISS) parameters[39].

A correlation with prognosis has been observed with stage I, II and III, with a median survival of 62, 45 and 29 months, respectively.[37]

- R-ISS (Revised ISS): it integrates the classic ISS (albumin and β2 microglobulin) with two other factors: the serum value of lactate dehydrogenase (LDH) (whose high concentration correlates with a more aggressive disease, a greater proliferative index and can suggest the presence of disease in the extramedullary site) and the possible presence or absence of some high-risk chromosomal abnormalities (CA) such as del(17p13), t(4;14) and t(14;16), evaluated by FISH (**Table 2**) [37,40–42]

R-ISS I	ISS stage I and standard-risk CA by FISH and normal LDH
R-ISS II	Not R-ISS stage I or III
R-ISS III	ISS stage III and either high-risk CA by FISH or high LDH

Table 2. Revised International Staging System (R-ISS) parameters.[39]

The prognostic role of R-ISS staging is evident from the evaluation of outcomes: PFS at 5 years is 55% for R-ISS I, 36% for R-ISS II and 24% for R-ISS III; the 5-year OS is 82% for R-ISS I, 62% for R-ISS II and 40% for R-ISS III.[37,42]

- The IMWG risk assessment: In 2014, the International Myeloma working Group (IMWG) published an updated risk stratification, focusing on differentiating high from standard-risk patients by combining the ISS with certain high-risk iFISH changes including t(4;14), del17p13, and +1q21[36,37,43]

Low Risk	ISS I/II, age<55, and absence of all of t(4;14), del(17p13), and 1q21+
Standard Risk	Not Low or High-Risk
High Risk	ISS II/III and t(4;14) or del(17p13)

Table 3. International Myeloma working Group (IMWG) criteria.[37,43]

This stratification method highlighted that high-risk patients are characterized by an overall survival of 2 years or less, on the contrary, low-risk patients will be those who survive more than 10 years.

- R2-ISS: in 2022, an update of R-ISS, known as R2-ISS, was proposed with the aim of better stratifying high-risk patients by formally incorporating amplification/gain 1q. This update identifies 4 risk groups[44]. Through a rigorous statistical analysis, the features that most impact survival have been identified and assigned a value, the C-index[44], as follows:

Risk feature	**OS HR (95% C.I.)**	**Score Value**[44]
ISS II	1.75 (1.49 to 2.05)	1
ISS III	2.53 (2.13 to 3.01)	1.5
del(17p)	1.82 (1.53 to 2.17)	1
LDH high	1.60 (1.36 to 1.88)	1
t(4;14)	1.53 (1.29 to 1.81)	1
amp/gain 1q	1.47 (1.29 to 1.68)	0.5

The cumulative value of the presence of individual identified features determines membership in one of the 4 identified risk classes [44]:

GROUP	**TOTAL ADDITIVE SCORE**[44]
Low (I)	**0**
Low-Intermedie (II)	**0.5-1**
Intermedie-high (III)	**1.5-2.5**
High (IV)	**3-5**

Overall, these risk score systems share the approach employed for their building, mainly based on statistical considerations, which might be considered a limit, as it does not highlight biological pathways related to the expression of high-risk characteristics. In fact, for instance, despite the significant prognostic value of R-ISS, especially when compared to the previous ISS, there exists a considerable heterogeneity in prognosis, particularly among patients in R-ISS stage II. This variation suggests the need for further and more detailed sub-classifications. [41,45,46] In particular, the evaluation of negative prognostic factors such as low performance status, thrombocytopenia, and hypodiploidy or the presence of del(17p) has been suggested.[47]

Moreover, none of the above-mentioned risk-score systems account for several features, whose role has progressively emerged in recent years and that several studies have validated: the presence of circulating neoplastic PCs[48,49], the presence of extramedullary disease[50,51], the sFLC ratio[52,53], as well as additional cytogenetic alterations and distinctive gene expression patterns.

In the era of risk-adapted therapy, high-risk patients must be identified so that they are not missed and so that they may receive the most powerful therapeutic combinations. However, as research studies utilize newer technologies, such as whole genome sequencing (WGS), more high-risk factors are being identified including mutations of *TP53*, *DIS3*, *BRAF*, and complex structural events. Integration of comprehensive genomic studies into clinical trials will aid in defining the genomic high-risk landscape of MM, which in turn can be transferred to individual patient diagnostics and treatment management.

Although FISH can accurately risk stratify many patients according to their cytogenetic risk, the biological processes in myeloma cells are dependent not only on large structural variations but also on point mutations (not captured by FISH), transcription profiling, proteomics, and the interactions between MM and the microenvironment, among others. Technologies evaluating these elements of myeloma cell biology have been studied and do appear to add prognostic value in many instances. High-risk signatures from the GEP70 and SKY92 gene expression profiles[54] and the presence of chromothripsis and APOBEC mutational signatures detected on whole-genome sequencing appear to be very powerful tools to identify high-risk patients.[55] Furthermore, detection of low-level circulating PCs by flow cytometry can also identify patients with otherwise standard- risk features who have a worse prognosis[56]. Comprehensive immune profiling may also allow for improved risk stratification and/or prediction of outcomes to initial therapy[57]. Integration of these tools into routine risk stratification will help to further characterize the spectrum of risk profiles that can be observed in MM and hopefully will help to clarify which risk factors can be overcome with certain therapies and which will require novel strategies.

1.6. MM EARLY RELAPSE

The survival rate for patients with newly diagnosed multiple myeloma (NDMM) has been on the rise, currently nearing 8 years, due to advancements in novel treatments and enhanced supportive care.[58,59]However, MM remains predominantly untreatable, resulting in an annual deaths' rate of approximately 12,000 patients in the United States and 30,000 in Europe. The primary cause of death is the disease's resistance to the drugs available today and several data suggest that relapse can be associated to the emergence of clones characterized by increasingly higher genomic complexity, possibly explaining the drug resistance phenotype. [60]

The duration of remission is one of the most critical factors impacting patients' prognosis,[61] and it is conditioned by several factors, among which the initial treatment and the genomic landscape are the most critical. A recent study, aimed at the characterization of patients who relapsed within 18 months has been conducted in the CoMMpass IA14 cohort of patients and highlighted a correlation with several baseline features and the risk of early relapse, including ISS stage III, gain(1q), IgL translocations [62] (2p12, kappa o 22q11, lambda), high APOBEC signature [62], high LDH, KRAS mutation, IGLL5 mutation, and TP53 mutation [62].

Overall, remissions shorter than 2 years, observed in approximately 20% of patients [62], typically characterize high-risk disease, that still represents an unmet clinical need. In other studies, early relapse is considered when occurring within 18 months [62] of first-line treatment; this time-frame is chosen, as the time to ASCT is approximately 6 months and most published studies of MM patients with early progression have defined it as "relapse within 12 months of ASCT". However, in contrast with these observations, the most frequently employed risk scores identify different proportions of high-risk patients; for instance, the R-ISS identifies only 10% of high-risk patients.

This highlights the need for better baseline risk stratification systems and the importance of a dynamic prognostic evaluation approach. Compared to FISH, high-resolution genomic characterization's approaches (such as NGS or SNPs arrays) can improve patient stratification at diagnosis and can support the identification of patients at high risk of early relapse; however, specific genomic patterns for a reliable prediction of early relapse have not yet been identified, thus preventing the design of therapeutic approaches specific for at-risk patients.

2. RATIONALE AND AIM OF THE STUDY

The study aimed to use information relating to various aspects of MM, (clinical, biological and molecular), with the purpose of exploring the prognosis in patients with MM, by integrating data obtained from genomic and clinical sources Specifically, the primary aim was to devise a statistical methodology capable of pinpointing distinct genomic biomarkers linked to the survival of MM patients. This would be achieved by discerning distinctive "patterns" within data, reflecting the heterogeneous nature of MM, which directly corresponded to specific clinical prognoses.

CNAs are among the main biomarkers used to define prognosis in MM and are conventionally detected by cytogenetics; more recently, high resolution molecular technologies (SNPs-Array, NGS, single cell) have been also successfully employed to this aim. The reading depth of these new technologies has highlighted that signals defining the presence of CNAs might occur within a wide range of amplitude, suggesting that the dimension of sub-clone(s) carrying the different CNAs might be very different from patient to patient.

Therefore, an important technical issue is currently represented by the definition of the signals' cutoffs, enabling the reliable CNAs calling across different experiments, by avoiding the experimental signal noise that might be confused with sub-clonal CNAs' signals. FISH analysis has commonly accepted consensus cut-offs, employed for the identification of numerical aberrations. It is important to highlight that these thresholds are not the same for all alterations. For instance, in the paper of Palumbo et al. (2015), cut-offs ranged from 8% to 20%. [63] Even for most commonly explored alterations, and even for those defining high risk in MM, a consensus in the definition of CNAs signals' cut-off has not been reached, as observed, for instance, for del(17p)[40,64].

In the context of high-resolution molecular technologies, it has been conventionally established that all signals either above or below 10% around the 2N signal define the presence of either amplifications or deletions, respectively[37] . However, it is worth highlighting that signal cut-offs might also have a clinical meaning, as by shifting up or down any CNA's cut-off, it is possible to define the dimension of the clone carrying that specific CNA. Therefore, the characterization of the genomic landscape is not trivial, as the call of any specific CNAs strictly depends upon the cut-off employed, that in turn can be modified according to specific requirements.

In the present study, the main objective has been to employ an innovative statistical approach to define the optimal cut-offs' thresholds for CNAs calls. This approach was tailored to each chromosomal arm, considering their distinct impact on survival outcomes. In particular, we

employed the early relapse as clinical endpoint, defined as clinical recurrence within 18 months from the commencement of therapy.

The CNAs identified using the newly established "outcome-oriented" thresholds served a dual purpose. Initially, they were employed to delve into the heterogeneity of the disease, aiming for a more refined characterization of the genomic landscape. Subsequently, these findings were integrated, to enhance the stratification of MM patients, building the foundation for the development of a predictive model for early relapse in MM.

3. PATIENTs AND METHODs

3.1. Patients

743 newly diagnosed (ND) MM patients, whose genomic data obtained from SNP Array experiments were available, have been included in the study.

133 patients have been treated at the "L. and A. Seragnoli" Institute of Hematology, in the context of the daily practice; 610 have been previously enrolled in the EMN02/HO95[65] and GIMEMA MM-BO2005[66] clinical trials.

The median follow-up of patients to the Progression Free Survival (PFS) event is 78.23 (IQR: 57.84;94.10); based on the reverse Kaplan-Meier method applied to the censored times reversing the roles of event status and censored.

Clinical and molecular characteristics are reported in **Table 4**.

Characteristic	N	BO2005, N = 133[1]	Clinical Practice, N = 207[1]	EMN02, N = 403[1]	p-value[2]
Old Variable (Age >= 65 years)	**740**				**<0.001**
0		133 (100%)	89 (44%)	393 (98%)	
1		0 (0%)	115 (56%)	10 (2.5%)	
Unknown		0	3	0	
Gender					
F	**743**	49 (37%)	88 (43%)	170 (42%)	0.5
M		84 (63%)	119 (57%)	233 (58%)	
Translocation					
no	**489**	1 (2.4%)	38 (51%)	201 (54%)	**<0.001**
yes		41 (98%)	37 (49%)	171 (46%)	
Unknown		91	132	31	
Haemoglobin<10.5 g/dL					
no	**738**	84 (63%)	121 (60%)	234 (58%)	0.6
yes		49 (37%)	81 (40%)	169 (42%)	
Unknown		0	5	0	
Platelets<150 mL					
no	**735**	118 (89%)	169 (85%)	345 (86%)	0.6
yes		15 (11%)	30 (15%)	58 (14%)	
Unknown		0	8	0	
Plasmacells >60%					
no	**690**	54 (43%)	103 (59%)	163 (42%)	**<0.001**
yes		72 (57%)	71 (41%)	227 (58%)	
Unknown		7	33	13	
LDH (UL)					
no	**517**	40 (43%)	130 (90%)	176 (63%)	**<0.001**
yes		53 (57%)	15 (10%)	103 (37%)	
Unknown		40	62	124	
ISS					
1	**716**	60 (45%)	62 (34%)	153 (38%)	**0.008**
2		50 (38%)	58 (32%)	156 (39%)	
3		22 (17%)	61 (34%)	94 (23%)	
Unknown		1	26	0	
R-ISS					
1	**522**	0 (NA%)	35 (25%)	63 (16%)	0.078
2		0 (NA%)	85 (61%)	265 (69%)	
3		0 (NA%)	20 (14%)	54 (14%)	
Unknown		133	67	21	
Calcium >10.5 mg/dL					
0	**316**	111 (89%)	160 (86%)	4 (100%)	0.5
1		14 (11%)	27 (14%)	0 (0%)	
Unknown		8	20	399	
Induction Therapy					
Immunomodulatory Agents		37 (28%)	45 (22%)	0 (0%)	

Characteristic	N	BO2005, N = 133[1]	Clinical Practice, N = 207[1]	EMN02, N = 403[1]	p-value[2]
Proteasome Inhibitors & Immunomodulatory Agents	743	96 (72%)	98 (47%)	0 (0%)	<0.001
Other Therapy		0 (0%)	13 (6.3%)	0 (0%)	
Proteasome inhibitors		0 (0%)	51 (25%)	403 (100%)	
ASCT					
no	693	9 (6.9%)	86 (45%)	143 (39%)	<0.001
yes		121 (93%)	106 (55%)	228 (61%)	
Unknown		3	15	32	
N. ASCT					
No ASCT	693	9 (6.9%)	86 (45%)	143 (39%)	<0.001
double		99 (76%)	53 (28%)	110 (30%)	
single		22 (17%)	53 (28%)	118 (32%)	
Unknown		3	15	32	
Consolidation					
no	654	34 (27%)	105 (59%)	224 (64%)	<0.001
yes		90 (73%)	73 (41%)	128 (36%)	
Unknown		9	29	51	
Maintenance					
no	652	41 (33%)	94 (53%)	54 (15%)	<0.001
yes		83 (67%)	83 (47%)	297 (85%)	
Unknown		9	30	52	
Progression Events					
no	743	44 (33%)	78 (38%)	170 (42%)	0.2
yes		89 (67%)	129 (62%)	233 (58%)	
Overall Survival Event					
no	743	71 (53%)	131 (63%)	276 (68%)	0.006
yes		62 (47%)	76 (37%)	127 (32%)	

[1] n (%)

[2] Pearson's Chi-squared test

Table 4. *Clinical characteristics of the patients enrolled in the study*.

A total of 155/743 (20.8%) patients experienced early progression free survival (PFS, progression or death event) (defined as Early Relapsed patients ER), while the remaining 588/743 (79.2%) patients were included in the reference population (Not-ER).

Clinical and molecular characteristics are reported in **Table 5**.

Characteristic	N	Overall, N = 743[1]	Not-ER, N = 588[1]	ER pts, N = 155[1]	p-value[2]
Protocol					
BO2005	**743**	133 (18%)	119 (20%)	14(9.0%)	**<0.002**
Clinical Practice		207 (28%)	152 (26%)	55 (35%)	
EMN02		403 (54%)	317 (54%)	86 (55%)	
Gender					
F	**743**	307 (41%)	245 (42%)	111(72%)	0.7
M		436 (59%)	343 (58%)	44 (28%)	
Translocation					
no	**489**	240 (49%)	192 (51%)	48 (42%)	**<0.089**
yes		249 (51%)	183 (49%)	66 (58%)	
Unknown		254	213	31	
Haemoglobin<10.5 mg/L	**738**				**<0.001**
no		439 (59%)	366 (63%)	73 (47%)	
yes		299 (41%)	218 (37%)	81 (53%)	
Unknown		0	4	1	
Platelets<150 mL					
no	**735**	632 (86%)	506 (87%)	126 (82%)	0.15
yes		103 (14%)	76 (13%)	27 (18%)	
Unknown		0	8	0	
Plasmacells >60%					
no	**690**	320 (46%)	254 (46%)	66 (46%)	>0.9
yes		370 (54%)	293 (54%)	77 (55%)	
Unknown		53	33	13	
LDH (UL)					
no	**517**	346 (67%)	294 (69%)	52 (59%)	0.086
yes		171 (33%)	135 (31%)	36 (41%)	
Unknown		226	159	67	
ISS					
1	**716**	275 (38%)	239 (42%)	36 (24%)	**<0.001**
2		264 (37%)	216 (38%)	48 (32%)	
3		177 (25%)	112 (20%)	65 (44%)	
Unknown		27	26	0	
R-ISS					
1	**522**	98 (19%)	86 (21%)	12 (16%)	**<0.001**
2		350 (67%)	272 (68%)	78 (69%)	
3		74 (14%)	44 (11%)	30 (14%)	
Unknown		221	186	35	
Calcium >10.5 mg/dL					
0	**316**	275 (87%)	222 (88%)	53 (83%)	0.3
1		41 (13%)	30 (12%)	11 (17%)	
Unknown		427	336	91	
Induction Therapy					

Characteristic	N	Overall, N = 743[1]	Not-ER, N = 588[1]	ER pts, N = 155[1]	p-value[2]
Immunomodulatory Agents	743	82 (11%)	58 (9.9%)	24 (15%)	<0.001
Proteasome Inhibitors & Immunomodulatory Agents		194 (26%)	174 (30%)	20 (13%)	
Other Therapy		13 (1.7%)	9 (1.5%)	4 (2.6%)	
Proteasome inhibitors		454 (61%)	437 (59%)	107 (69%)	
ASCT	693				<0.001
no		238 (34%)	167 (45%)	71 (39%)	
yes		455 (66%)	412 (55%)	43 (61%)	
Unknown		50	9	41	
N. ASCT	693				<0.001
No ASCT		238 (34%)	167 (29%)	71 (62%)	
double		262 (38%)	243 (42%)	19 (17%)	
single		193 (28%)	169 (29%)	24 (21%)	
Unknown		50	9	41	
Consolidation	654				<0.001
no		363 (56%)	296 (52%)	67 (76%)	
yes		291 (44%)	270 (48%)	21 (24%)	
Unknown		89	22	67	
Maintenance	652				<0.001
no		189 (29%)	136 (24%)	53 (61%)	
yes		463 (71%)	429 (76%)	34 (39%)	
Unknown		91	23	68	
Progression Events	743				0.2
no		292 (39%)	267 (45%)	25 (16%)	
yes		451 (61%)	321 (55%)	130 (84%)	
Overall Survival Event	743				0.006
no		478 (64%)	440 (75%)	38 (25%)	
yes		265 (36%)	148 (25%)	117 (75%)	

[1] n (%)

[2] Pearson's Chi-squared test

Table 5. *Clinical characteristics of patients stratified by achieving a PFS event (progression or death) within 18 months, ER patients, compared to the control population.*

3.2. Methods

3.2.1. Genomic data processing and data availability

For all patients included in the study, the genomic profile of CNAs was obtained through Affymetrix SNP-Array experiments, including Cytoscan HD and GenomeWide 6.0. For each SNPs array experiment, raw CEL files were generated. The genomic segments profiles (SEG files) were generated using Rawcopy[67] R package (version3.0) and CBS algorithm, using the human genome GRCh37/hg19 as reference. The significance threshold for segmentation (alpha parameter of the CBS algorithm) was set at 10−7.

Values of Copy Number (CN) profiles were corrected for ploidy using the R package BoBafit[68], which leverages a list of chromosomes that typically do not undergo alterations in the disease to ensure proper centering of the diploid region. To this aim, the list of normal chromosomes was generated using the ComputeNormalChromosomefunction of the package, with a tolerance rate set at 0.20.

To compare the newly identified cutoffs with the classic 10% cutoff, the dataset was categorized a second time uniformly for all CN calls.

To define the optimal cut-offs for CNAs calls[69,70], specific for any specific alteration (i.e., any chromosomal arm's amplifications and deletions), which are most significantly associated to patient survival, a Receiver Operating Characteristic (ROC) curves analysis [69,70] was performed, resulting in the definition of outcome-oriented [63,64] thresholds, specific for each CNAs[69,70].

As the clinical endpoint of the present study was early relapse (ER), defined as relapse within 18 months from the start of therapy[58], we established CNAs cut-offs that most accurately predicted this clinical outcome.

All analyzes were performed with an R Studio 4.1.2.

3.2.2. Statistical analysis

Pre-processing phase

The dataset was divided into two parts: one for the amplifications (CN> 2) and one for the deletions (CN <2), with chromosomal arms (p and q) used as observational unit. It was necessary to develop specific cut-off for amplifications and deletions, because by considering the CN whole range (0<=CN<2n) would lead to inappropriate management of the type of data. A "background noise" of 4% around the normal number of copies (2N)[71,72] was defined, which led to the division of the CNAs dataset as follows: CN ∈ [1.96; 2.04] = background noise; CN > 2.04 = amplifications; CN < 1.96 = deletions.

ROC analysis[69,70,73] was limited to CNAs with an incidence in the population higher than 4%, this was due to computational reasons that did not allow the package to perform in absence of a minimum number of observations. For these low-incidence alterations, a standard cut-off of 10% was employed due to the inability to define an outcome oriented one, the former will be referred to as “static” or “classical” cut-off (CL).

For chromosomal arms’ CNAs with incidence higher than 2%, the "*surv_cutpoint*" function of the *"survminer"* package was used to define the cut-point that better discriminates on the survival outcome chosen. The function was applied to the continuous biomarkers, in this case on amplifications (CN > 2.04) and deletions (CN < 1.96) for each chromosomal arm, and to their survival information such as early relapse. Early relapse or death was defined as a dichotomous variable: 1, event of relapse or death prior to 18 months, 0 otherwise. Time to the event was expressed in months from diagnosis to the date of occurrence of event if prior to 18 months, otherwise right censoring was performed if the event is not experienced in this timeframe, by assigning 18 months as time and no event (0).

The objective is to discriminate between the two overlapping distributions of CN levels that have a different impact in terms of survival. Analytically, the function considers all possible cut-offs and then chooses the one that maximizes the log-rank statistic with the most significant relation to the outcome, as exemplified in **figure 4**.[32,69,70,73]

The analysis identifies the optimal cut-point for each variable: in this case we show the identification of the cut-point for amp(1q), maximizing the difference in survival curves between the groups of patients divided based on that specific value. This allows the prognostic impact of each factor to be more precisely discerned and to guide more informed clinical decisions.

Algorithm:

1. Split dataset considering a background noise of 4%:
    a. if the CN>2.04 is defined as an amplification and assigned as continuous variable to the dataset for amplification;
    b. If the CN < 1.96 is defined as a deletion and assigned as continuous variable to the dataset for deletions.
2. In for amplification, for each chromosome arm is assessed their relative frequency as: freq=N of patient with alteration/Total N of patients:
    a. If freq <= 0.04 then the classical cut-off of 10% is assigned:
        i. Each alteration is categorized into 0, 1.
    b. Otherwise, will be computed the outcome-oriented cut-off:
        i. For each chromosome arm is built a separate dataset on which:
            1. Missing values are omitted
            2. Surv_cut-point is applied to continuous CN with PFS months with censoring at 18 months as time and PFS event 1 if the event occurred within 18 months and 0 otherwise.
                a. All possible cut-point are tested and the one that maximises the log-rank statistics is chosen.
            3. The CN are categorized with the chosen cut-point
        ii. Separate data for specific amplifications categorized are merged by patient id
    c. Data for outcome-oriented amplifications are merged with data categories for less frequent alterations.
3. Repeat the process for deletions.
4. Merge categorized dataset for amplification and deletion.

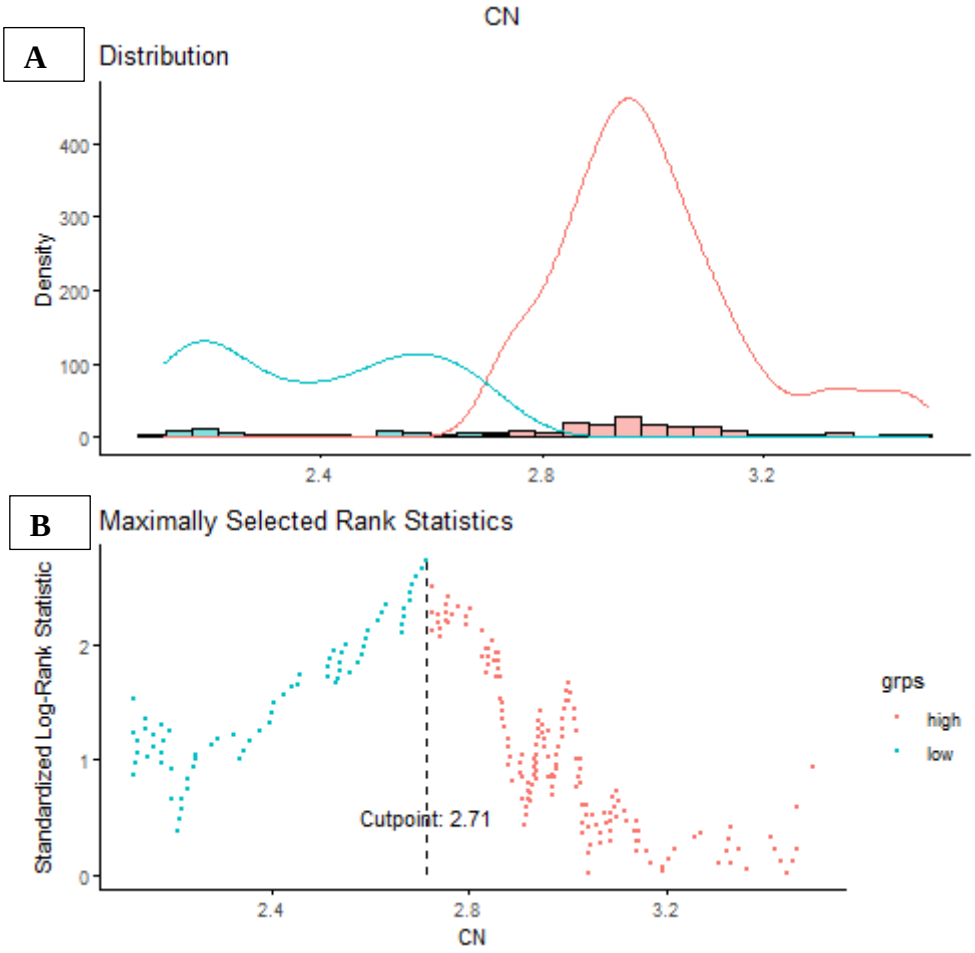


Figure 4. *The plot generated using the R package "surv_cutpoint", shows the analysis of optimal cut-points in relation to patient survival. This plot is relared to the amplification of Chromosome arm 1q. A) It shows the probability density distribustions definded by the specific cut-off value, B) it hows the Standardized Log-Rank Statistics for each possible CN value and defines with the dotted line the cutpoint that maximises the statistics.*

This analysis was performed separately for amplifications and deletions and led to the definition of "dynamic" cut-offs, referred in the following pages as "outcome-oriented" (OO). To classify observations into amplifications or deletions, in terms of presence/absence (0/1), the specific dynamic and where not possible the static cut-offs were used.

The dataset was finally categorized according to the OO cut-offs, [63,64] obtaining a that describes the CNAs profile of 743 patients with two categories for each chromosomal arm in total 78 (amp, del) which can assume values of 0 for absence or 1 for presence.

❖ **Statistical and Exploratory analysis**:

For descriptives analysis the mean, median, range and interquartile range (IQR) were used for describing continuous variables such as CN values by chromosomal arm, and for their visualization have been used boxplots and probability density distributions (PDFs). Clinical characteristics were expressed with the same means if continuous/numerical and though absolute and relative frequencies (percentages, %), if they were characterized by two or more levels (respectively dichotomous and categorical)

Bivariate analysis was performed with parametric and non-parametric tests such as Kruskal Wallis test on medians. For assessing correlation among categorical variable Pearson's Chi-squared test and Fisher's exact test were used as appropriate, and Cramer's V was useful for determining the strength of association. For survival outcomes in univariate were used both Kaplan Meier survival curves and relative Log-rank rest for a non-parametric testing of right-censored data and for a straightforward visualization. The semi-parametric approach of Cox Proportional Hazard Model was used in both univariate and multivariate survival models for quantifying risk differences among groups through Hazard Ratios (HRs).

The prognostic effect of calls made using the classic cutoffs (CL) at 10% and those made using dynamic cutoffs (OO) were evaluated using univariate survival analysis, including Kaplan-Meier curves and the semi-parametric Cox model to obtain HRs. These latter were statistically compared using the T-test for assessing if there were differences among alteration with different threshold of call. Additionally, multivariate survival models were constructed using the Cox Proportional Hazard Model with both sets of cut-offs (classic and OO), firstly including all genomic variables and then performing a stepwise backward selection with the Akaike Information Criterion (AIC). This criterion is used for assessing the goodness of fit for non-nested model, i.e., that have indeed different variables (in fact an alteration called with classic cut-off is different from those defined by the OO cut-off, however the models should be on the same set of data.

Reduced models were obtained through a stepwise backward selection using the ***stepAIC*** function from the MASS package[74], that starting from a full model at each step each iteratively, it is removed the covariate whose removal minimizes the AIC until no other improvement was possible, such that are retained only the most covariates hat improve the fit of the model without increasing much its complexity.

Finally, for further validation of the new cut-offs, logistic models were built to find out the best model among the full and reduced ones with CNA calls according to the classic method

(10%) and the dynamic method (OO), a, with in term of predictivity ability of early relapse patients (0,1).

Model performance comparison (classic cut-offs vs. dynamic cut-offs) was conducted using the Area Under the Receiver Operating Characteristic Curve (AUC), both before and after data balancing, performed with random oversampling using the R package *ROSE*[75,76] for both complete and reduced models.

Data balancing was necessary due to the imbalanced nature of the data (155 ER patients out of 743 total). Additionally, internal validation was performed through bootstrap resampling (n=1000) for assessing the stability of performance indicators. The DeLong test[77] was used to statistically demonstrate the difference between the ROC curves.

After having assessed the performance of the models, an exploratory analysis was conducted using Non-Metric Multidimensional Scaling (NMDS) with the *vegan* r package[78], which allowed us to observe the distribution of each alteration, defined by the novel outcome-oriented threshold, in space carrying out a reduction of data dimensionality.

Subsequently, to measure the degree of association between nominal variables, Cramer's V and the Fisher test or Chi-square test were used to assess the association strength and statistical independence with its significance level. Cramer's V is a measure of association used for nominal variables, that means to quantify the effect size of the association, without giving insight of its direction, one among the interpretations[79–81] it is linked with the degree of freedom defined the minimum between the number of rows minus one and the number of columns minus one. In our case all variables have two levels (0, 1) such that the degrees of freedom are equal to 1. This enabled the exploration of the extent of relationships between alterations.

4.RESULTS

4.1. Identification of new cut-offs for proper CNAs calls

The study included 743 NDMM patients with CNAs data derived from SNPs array experiments.

For the whole dataset, CNAs were defined by a measurement of tendency of the array signals' distribution, as shown in **Table 6**, where both average and median signals values are displayed. Notably, IQRs (Q1-Q3) highlighted a wider ranges of signal distribution for some CNAs, as compared to others, suggesting a huge variability among chromosomes, as shown in **Figure 3** and in **Table 6**.

CNAs	N	Mean	Median	Range	Q1-Q3
CN.1p	743	1.875	1.981	**0.958-2.587**	1.833-1.995
CN.1q	743	2.322	2.006	**1.188-4.706**	1.990-2.738
CN.2q	743	2.003	1.997	**1.280-3.049**	1.990-2.003
CN.2p	743	2.03	1.998	**1.283-4.020**	1.990-2.006
CN.3q	743	2.341	2.022	**1.812-4.116**	2.003-2.832
CN.3p	743	2.305	2.023	**1.428-4.176**	2.010-2.763
CN.4q	743	2.022	2.002	**0.929-3.270**	1.990-2.015
CN.4p	743	2	2	**0.936-3.274**	1.984-2.011
CN.5q	743	2.326	2.013	**1.012-4.509**	2.000-2.804
CN.5p	743	2.383	2.036	**1.584-4.560**	2.010-2.877
CN.6q	743	1.92	1.996	**0.939-3.200**	1.903-2.007
CN.6p	743	2.122	2.001	**1.193-5.244**	1.991-2.027
CN.7q	743	2.272	2.014	**0.960-4.249**	2.002-2.549
CN.7p	743	2.241	2.008	**0.954-4.108**	1.997-2.483
CN.8q	743	2.008	2.003	**0.918-3.752**	1.992-2.015
CN.8p	743	1.855	1.986	**0.734-3.018**	1.850-2.006
CN.9q	743	2.497	2.133	**1.588-4.987**	2.003-2.937
CN.9p	743	2.47	2.052	**1.088-5.276**	2.003-2.922
CN.10q	743	1.976	1.992	**0.991-2.941**	1.980-2.002
CN.10p	743	1.953	1.984	**0.937-2.956**	1.970-1.995
CN.11q	743	2.37	2.014	**1.180-5.381**	1.991-2.860
CN.11p	743	2.292	2.011	**1.085-4.236**	1.997-2.746
CN.12q	743	1.936	1.985	**0.996-2.904**	1.968-1.992
CN.12p	743	1.91	1.979	**0.987-3.072**	1.955-1.991
CN.13q	743	1.593	1.778	**0.697-3.010**	1.098-1.992
CN.14q	743	1.828	1.975	**0.910-3.029**	1.771-1.991
CN.15q	743	2.5	2.06	**1.731-5.292**	1.998-2.949
CN.16q	743	1.805	1.98	**0.770-2.889**	1.714-2.000
CN.16p	743	1.958	1.983	**0.943-4.078**	1.957-2.004
CN.17q	743	2.021	1.98	**1.276-3.261**	1.960-2.001
CN.17p	743	1.955	1.972	**0.968-3.608**	1.938-1.995
CN.18q	743	2.075	2.005	**0.923-4.127**	1.992-2.020
CN.18p	743	2.043	1.997	**0.927-4.211**	1.980-2.010
CN.19q	743	2.365	2.032	**1.554-4.144**	1.973-2.802
CN.19p	743	2.41	2.19	**1.476-5.543**	1.963-2.805
CN.20q	743	1.992	1.995	**0.963-3.378**	1.977-2.010
CN.20p	743	1.956	2.005	**0.926-3.308**	1.986-2.015
CN.21q	743	2.228	2	**1.277-4.331**	1.990-2.082
CN.22q	743	1.867	1.973	**0.934-3.072**	1.891-2.000

Table 6. CNs continuous values for each arm-chromosome. *Mean, Median, Quantiles and range are shown.*

These findings underscored the importance of establishing alteration-specific thresholds for the accurate identification of each CNAs on individual chromosomal arms. This precaution helps mitigate the risk of erroneous calls stemming from low signals, which could potentially overlap with background signal noise. Notably, in some cases, IQRs were very wide, meaning either that 50% of the population was characterized by a large dispersion of CN values, or that the median signal values were far over (or below) the diploid signal [1.90;2.10], suggesting a certain degree of skewness in the signal distribution.

Moreover, by shifting up or down any CNA's cut-off, it has been possible to define the dimension of the clone carrying that specific CNA. Therefore, to implement a clinical meaningful CNAs call, we employed a ROC-Outcome-Oriented (OO) analysis, to determine, for each chromosomal arm's CNAs, the best signal cut-offs, predictive of ER. This approach allowed the identification of OO-cut-offs for 60 out of 78 alterations. As the recurrence of the remaining 18 alterations was <4%, OO-cut-offs couldn't be defined and we decided to employ a conventional 10% cut-off. **Figure 5** shows the density plot of CNAs values for each chromosomal arm, with standard cut-offs depicted in blue and OO-cut-offs in red.

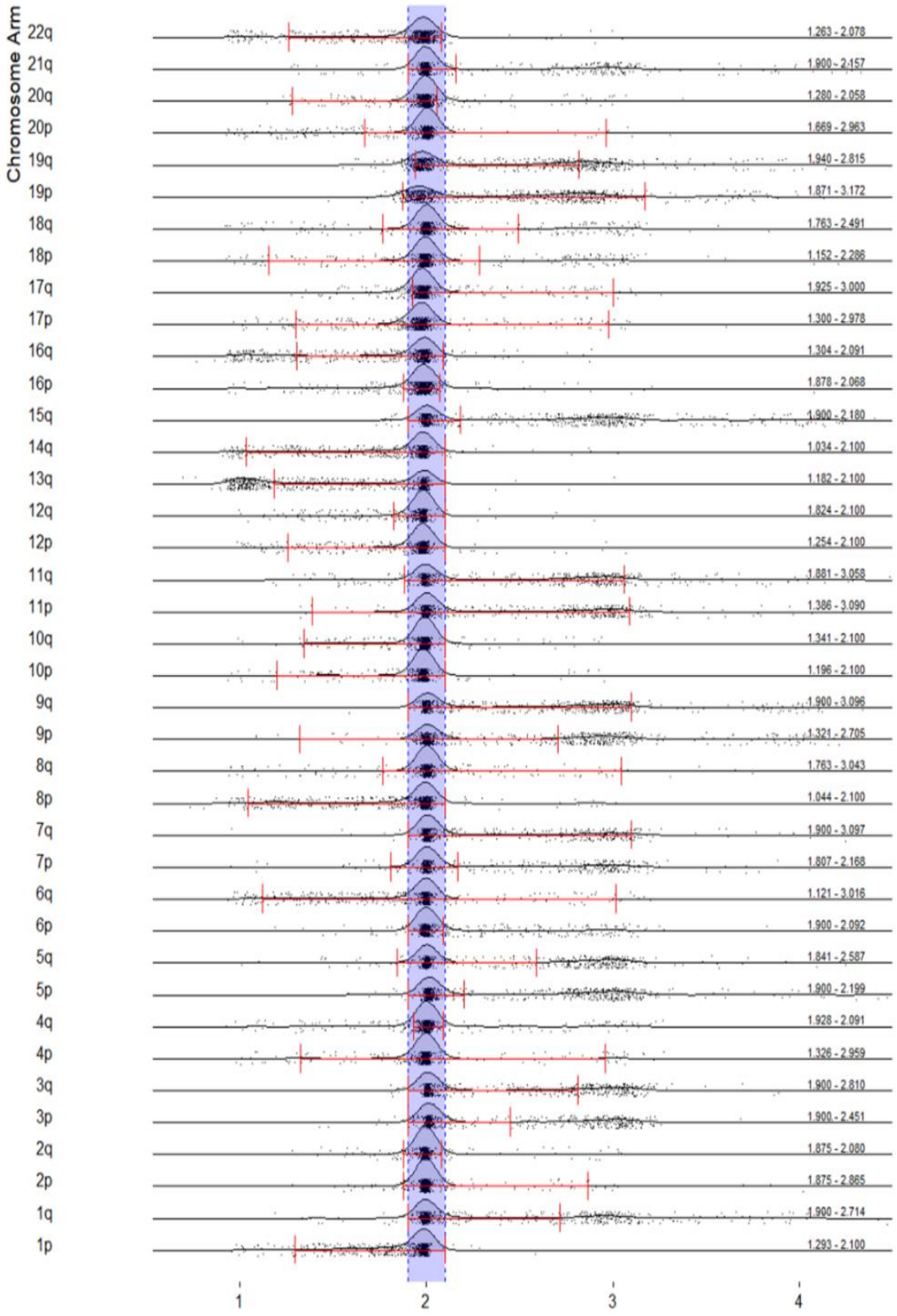
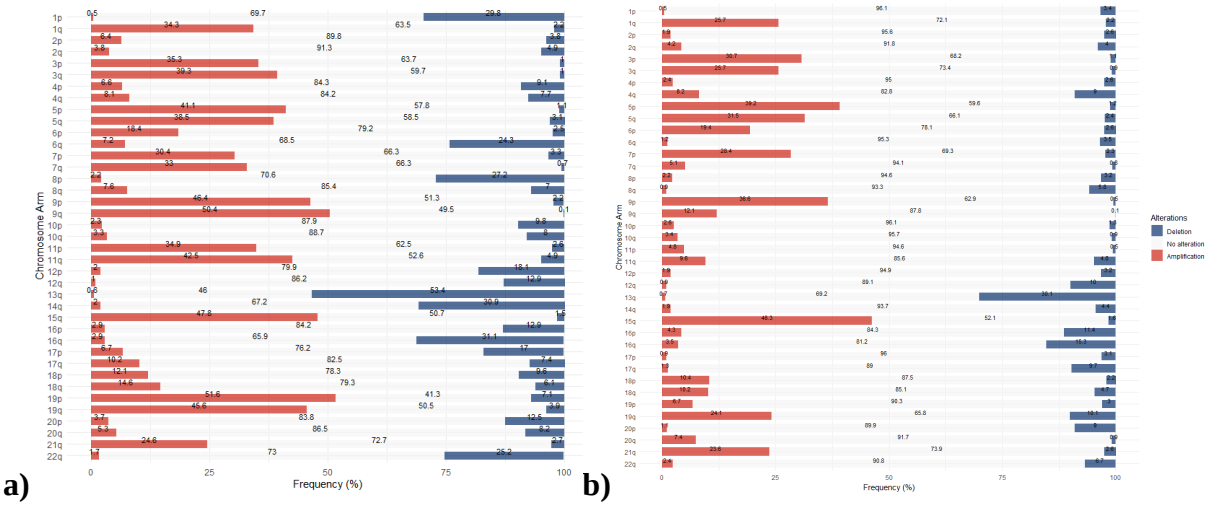


Figure 5. Density plot of CN values per chromosomal arm. *In red, the specific cut-off values for deletions and amplifications obtained from the RoC analysis are represented; values within this range are considered unaltered - normal. On the right are the cut-off values for the chromosomal arm*

The comparison of the whole genome CNAs landscape frequencies, as called either by the standard or by the OO-approaches, is shown in **Figure 6**. In each dataset, each CNAs was assigned a binary value: 0 if it was not detected (absent), and 1 if it was identified (present). Notably, the OO-approach resulted in a lower count of identified CNAs, as compared to the standard approach. This suggests that only a small subset of CNAs, whose signals resulted above the standard threshold, truly influenced the survival outcome. Details of CNAs frequencies defined by the OO-approach are shown in **Figure 6b**.



a) **b)**

Figure 6. Box Plot of the frequencies of CNAs specific to each chromosomal arm. *The amplifications are described in red and the deletions in blue and the non-alteration frequency is specified in white. (**a**) frequencies of the CNAs described of the classical cut-off of 10%; (**b**) frequencies of CNAs described by the OO cut-offs.*

4.1.1. Explorative analysis of genomic landscape

To explore the overall genomic landscape of patients included in the study, including the OO-defined CNAs and IgH translocations t(4;14), t(11;14) and t(14;16) (as identified by FISH), we performed a dimensionality reduction analysis by *Non-Metric Multidimensional Scaling* (NMDS). The positioning of alteration in space aims at representing the distances among alterations, based on their similarity matrix. This analysis aimed to understand the distribution of unsupervised CNAs in space, conditioned by the genomic profile of the patients, by positioning the alterations according to their similarity.

The result of this analysis is summarized in **Figure 7**, showing that along the first dimension (MSD1), CN losses tended to have a behavior similar to that of IgH translocations, whereas CN gains were closer among each other. Notably, among CN gains, chromosome 1q CN gain showed an opposing behavior, as it tended to be closer to CN losses. On the contrary, along the second dimension (MSD2), a group of alterations stood out including the loss of 13q CN and the gain of 1q CN, quite distinctly both from those leading to other CN losses and from those leading to t(11; 14).

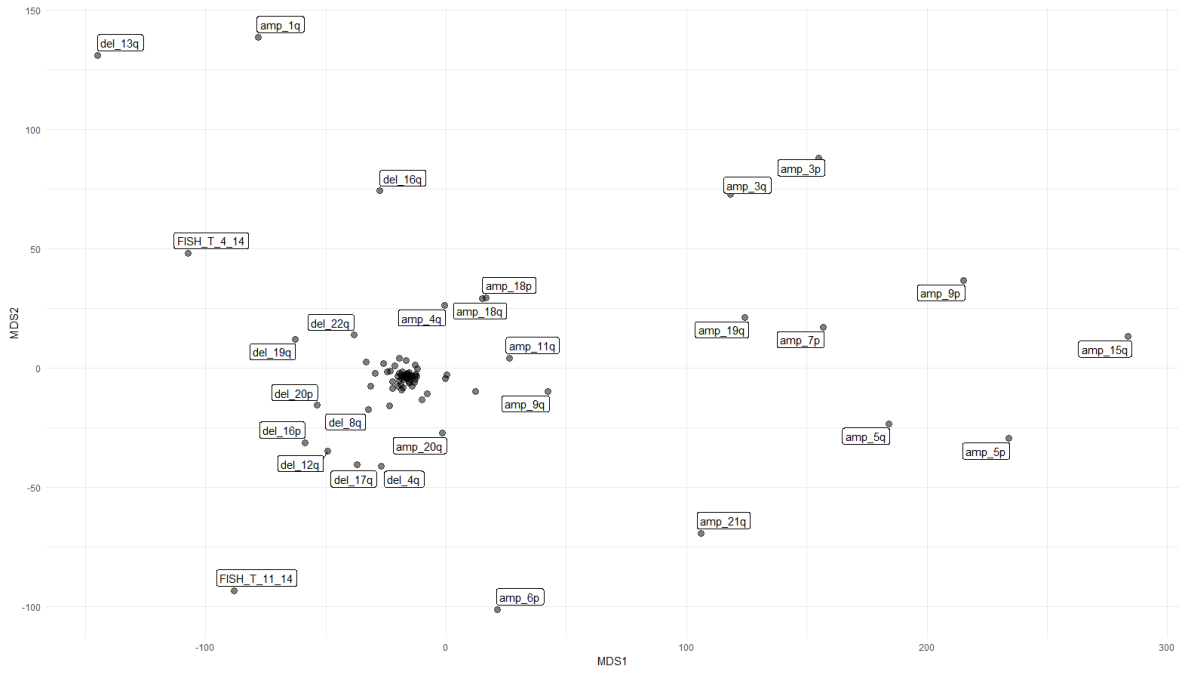


Figure 7. *Non-Metric Multidimensional Scaling (NMDS).*

A similar scenario was highlighted by the Cramer's V, employed to measure the degree of association among CNAs called by OO approach. This analysis resulted in a complex network of interconnections between chromosomal alterations, as shown in **Figure 8**. The network plot showed all possible associations among chromosomal alterations that resulted stronger than 0.30 and highlighted known pattern of relationship among alterations [79–81].

In fact, distinct groups of interconnected alterations can be seen with strong associations.

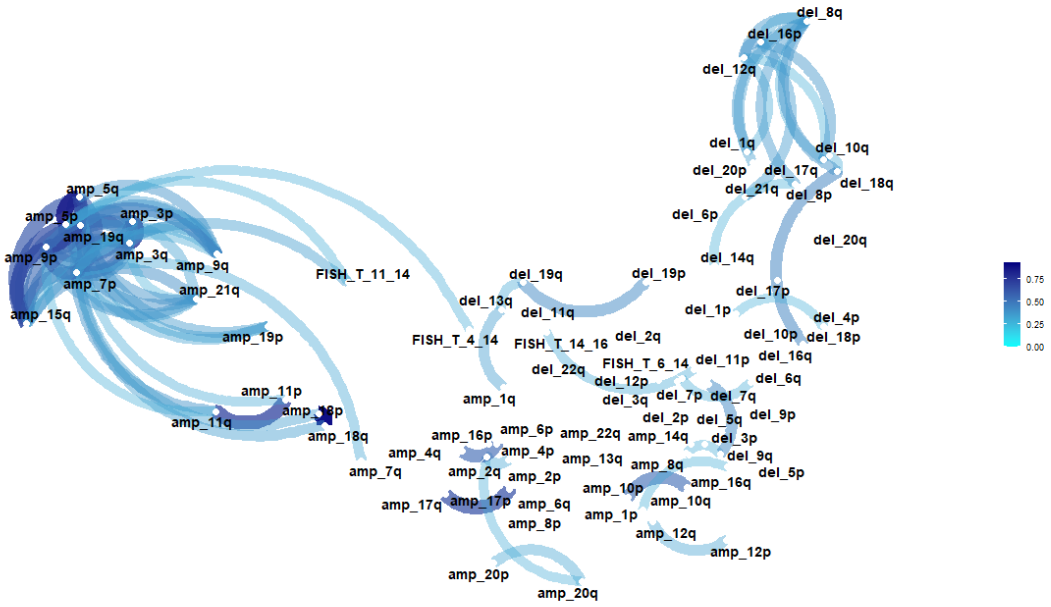


Figure 8. *Network plot of Cramer-V association between variables.*

Therefore, both the NMDS and the network plot confirmed peculiar relationships between OO-defined CNAs, allowing a clear description of the genomic landscape of the disease. For instance, a precise characterization of odd-numbered chromosomes' hyperdiploidy was achieved, showing a close distribution of amp(5p), amp(5q), amp(15q) and amp(9), amp(3p), amp(3q) and amp (7p), located on the far right of figure 7. This distribution was also confirmed by Cramer's test, with values exceeding 0.6, suggesting a very strong association between these amplifications (**Figure 8**). Similarly, other odd-numbered chromosomes hyperdiploidies are strongly associated; for instance, p and q arms of chromosomes 11, 18 amplifications and amp(9q) are associated with each other. Finally, Cramer test confirmed both the strong association (>0.4) of deletions distributed on the first dimension (MDS1) with IgH

translocations, and the co-occurrence of amp(1q) and del(13q), observed along the second dimension (MSD2) (**Figure 7**).

4.2. Validation: comparison between the classic and the Outcome-Oriented approaches

The attainment of a novel CNAs calls landscape based on the OO approach and characterizing the cohort of patients included in the present study was finally studied by performing survival analyses, to confirm the improvement in the reliable prediction of patients' outcome.

To this purpose, we first compared the univariate hazard ratios (HR) provided by the presence of each CNAs detected in our cohort of patients, as called either by the OO or by the conventional approach, by a t-test (**Table 7**).

For 36/60 CNAs, this analysis highlighted a stronger prognostic effect when called by the OO method, as compared to when called by the conventional one.

Moreover, for 12 CNAs, a statistically significant difference between HRs was highlighted, as show in table 7, where these CNAs are show in red and ranked according to *p*-value.

CN Alterations	Outome Oriented (OO) Cut-offs (a)		Classical Cut-offs (b)		p.value t-test (c)
	Hazard Ratio	p-value	Hazard Ratio	p-value	
del(12p)	3.58205	**0.00000**	1.28828	**0.02496**	**0.00000**
amp(7q)	1.31326	0.16071	0.72148	**0.00096**	**0.00071**
amp(9q)	1.02811	0.84309	0.69506	**0.00006**	**0.00214**
del(22q)	1.80914	**0.00020**	1.22423	**0.04264**	**0.00395**
del(2p)	2.24610	**0.00055**	1.56695	**0.03066**	**0.00482**
del(17p)	2.20540	**0.00076**	1.25935	**0.04467**	**0.00617**
del(20q)	3.06455	**0.00205**	1.26509	0.11825	**0.00752**
amp(2p)	2.04768	**0.00932**	1.18893	0.32401	**0.01212**
del(19p)	2.69386	**0.00002**	1.85886	**0.00011**	**0.02418**
del(9p)	3.73378	**0.01495**	1.40113	0.24687	**0.02748**
amp(4p)	1.16317	0.57706	0.77532	0.17650	**0.03201**
del(2q)	1.51504	**0.04590**	1.30007	0.17701	**0.04030**
amp(19p)	0.89963	0.56326	0.67205	**0.00001**	0.05509
amp(11q)	1.05598	0.72028	0.84179	0.06231	0.05936
del(10p)	1.69857	0.13298	1.01648	0.91301	0.06261
amp(1q)	1.86450	**0.00000**	1.70452	**0.00000**	0.07521
del(16q)	1.35296	**0.01332**	1.17884	0.08763	0.07980
amp(9p)	0.78587	**0.01194**	0.72906	**0.00056**	0.09863
del(10q)	1.54679	0.32845	0.92444	0.63851	0.11266
amp(3p)	0.83543	0.07168	0.79188	**0.01470**	0.11900
del(4p)	1.39499	0.21777	1.08708	0.57621	0.14446
amp(11p)	0.94360	0.78594	0.76433	**0.00542**	0.15314
amp(19q)	0.68184	**0.00049**	0.62351	**0.00000**	0.16364

amp(20p)	1.41021	0.40059	1.01312	0.95674	0.16843
amp(3q)	0.85430	0.13165	0.79991	**0.01691**	0.18870
del(18p)	1.34803	0.28665	1.08506	0.57828	0.19432
del(1p)	1.45009	0.11067	1.20026	0.06186	0.20220
del(11q)	1.51629	**0.03149**	1.46775	**0.04075**	0.23908
amp(7p)	0.72194	**0.00162**	0.70749	**0.00064**	0.24776
amp(2q)	0.88045	0.58659	0.84774	0.50342	0.31462
amp(6p)	1.09541	0.41218	1.08738	0.45421	0.34110
del(8p)	1.31503	0.24072	1.21153	0.05287	0.35837
amp(20q)	1.11372	0.52895	1.07732	0.71185	0.38318
amp(5p)	0.72088	**0.00057**	0.71777	**0.00040**	0.43883
del(20p)	1.25936	0.12590	1.24303	0.09023	0.44126
amp(16q)	1.01311	0.95915	1.01131	0.96811	0.49272
amp(22q)	0.58154	0.12385	0.58585	0.18805	0.51228
del(8q)	1.09458	0.61629	1.10173	0.56183	0.53205
del(17q)	1.12221	0.42136	1.13352	0.42834	0.54971
del(13q)	1.44871	**0.00011**	1.46436	**0.00003**	0.55052
del(19q)	1.71576	**0.00006**	1.76661	**0.00709**	0.56698
del(5q)	1.32596	0.35369	1.37813	0.20566	0.59844
amp(6q)	0.72887	0.48003	0.81089	0.22070	0.60149
del(4q)	1.13304	0.40733	1.15352	0.37129	0.62208
del(12q)	1.09459	0.53835	1.13729	0.31395	0.68333
amp(16p)	0.77966	0.25347	0.83636	0.48190	0.69557
amp(17p)	0.52242	0.25404	0.71559	0.08479	0.72015
del(11p)	0.71551	0.63510	1.05006	0.85712	0.72639
amp(17q)	0.62345	0.28887	0.82400	0.21648	0.74606
amp(8q)	0.89482	0.80473	1.23051	0.20857	0.77427
amp(21q)	0.88398	0.25102	0.90383	0.33549	0.78102
amp(5q)	0.68071	**0.00015**	0.71507	**0.00041**	0.81910
amp(15q)	0.74838	**0.00155**	0.76694	**0.00362**	0.84527
del(14q)	0.72331	0.17614	0.97253	0.77454	0.90161
del(18q)	1.00513	0.98015	1.15518	0.41746	0.91151
amp(18q)	1.10885	0.47786	1.26418	0.05639	0.94309
del(6q)	0.83396	0.48829	1.24926	**0.03189**	0.94824
del(7p)	0.77074	0.46404	1.14889	0.59663	0.96896
amp(18p)	1.08887	0.55560	1.21007	0.15513	0.97456
del(16p)	0.92161	0.55721	1.01089	0.93491	0.98818

Table 7. *Univariate Hazard Ratios (HRs) and relative p-values for all CNAs in amplification and deletion, both for the Outcome Oriented (**a**) and Classical Cut-offs (**b**). Pairwise comparison of differences among univariate HRs is performed by t-test (**c**). In red are shown 12 HRs with statistically significant differences.*

In particular, the analysis confirmed the prognostic role of several CNAs, such as 1q amplification and 17p deletion, that are commonly associated to higher risk of early relapse. Moreover, the analysis underlined the importance to accurately assess the clonality of CNAs that impact patients' outcome, as it resulted overall higher for those alterations whose early relapse's HRs were significantly different, when evaluated by the OO approach.

For instance, in the present analysis, deletion of chromosome 17p resulted more significantly correlated to ER when its clonality resulted higher than 70%, (cut-off defined by OO approach), as compared to when its clonality resulted higher than 10% (cut-off defined by the Classical approach), even though the number of patients carrying highly clonal del(17p) is small (35/743 patients, 5%). Indeed, the risk of ER of this small subgroup of patients was approximately two fold higher than that of patients not carrying this aberration (HR 1.912, CI 1.209-3.031, *p*=0.0049), whereas it was just 1.2-fold higher for patients carrying 10% del(17p), (HR 1.259, CI 1.005-1.578, *p*=0.045), as shown in **Figure 9**.

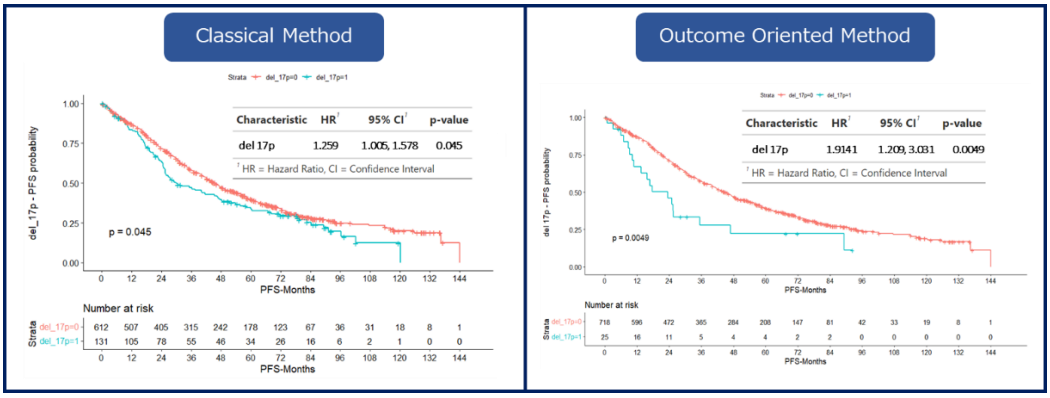


Figure 9. *Univariate analysis in progression free survival of the 17p deletion. Comparison between the CNA call described with the classic method and with the OO method.*

In addition to that, the OO approach has highlighted the role of CNAs that were not particularly known before as being associated with patient prognosis before, such as the deletion of chromosome 12p,[82] whose HR was 3.58 (CI: 2.323 - 5.523, *p*-value < 0.001), when called with a cut-off of 1.254 (thus affecting 24/743 patients, 3.2%), whereas it was 1.288 (CI: 1.032 - 1.609, *p*-value = 0.0254) when called with a cut-off of 1.9 (thus affecting 133/743 patients, 17.9%); these HRs resulted significantly different (t-test. *p*-value<0.001), (**Figure 10**).

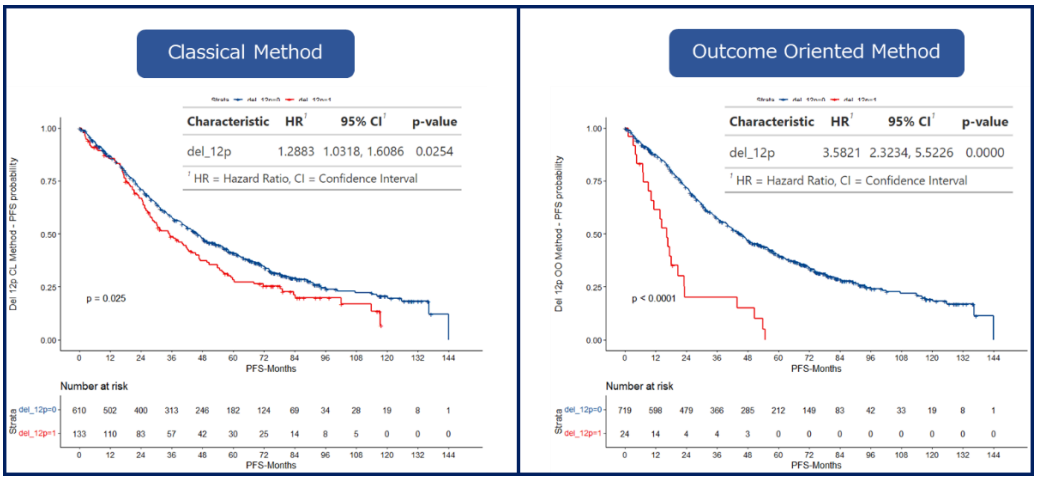


Figure 10. *Univariate analysis in progression free survival of the 12p deletion. Comparison between the CNA call described with the classic method and with the OO method.*

To assess the effect of CNAs calls in a multivariate framework, separate multivariate PFS analysis was therefore performed, including either the variables defined by the OO approach or those defined by the CL model. Multivariate models' comparisons were performed first for the complete sets of alterations and then after a model reduction, obtained by a stepwise algorithm. Models were then compared to assess their fitness in terms of AIC.

Specifications of the multivariate models are as follows:

Full model: $\boldsymbol{H(t) = H_0(t) \cdot \exp[b_i x_i]}$

$$H(t) = H_0(t) \cdot \exp[b_1 amp1q + b_2 del1q + b_3 amp1p + \cdots + b_{77} amp22q + b_{78} del22q]$$

Full model with Outcome oriented cut-off calls with all 78 calls:

$$H(t) = H_0(t) \cdot \exp[b_1 amp1q_{OO} + b_2 del1q_{OO} + b_3 amp1p_{OO} + \cdots + b_{77} amp22q_{OO} + b_{78} del22q_{OO}]$$

Full model with Classical cut-off calls with all 78 calls

$$H(t) = H_0(t) \cdot \exp[b_1 amp1q_{CL} + b_2 del1q_{CL} + b_3 amp1p_{CL} + \cdots + b_{77} amp22q_{CL} + b_{78} del22q_{CL}]$$

After stepwise backward selection, 14 and 25 variables were obtained, for CL and OO approaches, respectively, best implementing the multivariate models. Notably, few OO-defined CNAs included in model are not commonly considered high-risk features, probably underestimating their effect on patients' prognosis.

Specifications of the multivariate models after stepwise backward selection are as follows:

Reduced model with CL cut-offs calls with 14 covariates:

$$\boldsymbol{H(t) = H_0(t) \cdot \exp[b_1 del1p_{CL} + b_2 amp1q_{CL} + b_3 amp4q_{CL} + b_4 del5p_{CL} + b_5 amp9q_{CL} + b_6 del10p_{CL} + b_7 amp11q_{CL} + b_8 del12p_{CL} + b_9 del13q_{CL} + b_{10} del14q_{CL} + b_{11} amp18q_{CL} + b_{12} del19p_{CL} + b_{13} amp19q_{CL} + b_{14} del20p_{CL}]}$$

Reduced model with OO cut-offs calls with 25 covariates:

$$\boldsymbol{H(t) = H_0(t) \cdot \exp[b_1 amp1q_{OO} + b_2 amp2p_{OO} + b_3 del2p_{OO} + b_4 amp4p_{OO} + b_5 amp4q_{OO} + b_6 del4q_{OO} + b_7 amp5q_{OO} + b_8 del6q_{OO} + b_9 amp7q_{OO} + b_{10} amp8p_{OO} + b_{11} del9p_{OO} + b_{12} amp9q_{OO} + b_{13} amp10p_{OO} + b_{14} del10q_{OO} + b_{15} amp11q_{OO} + b_{16} del12p_{OO} + b_{17} del13q_{OO} + b_{18} amp14q_{OO} + b_{19} del16p_{OO} + b_{20} del16q_{OO} + b_{21} amp17q_{OO} + b_{22} del17p_{OO} + b_{23} amp18q_{OO} + b_{24} del19p_{OO} + b_{25} del20q_{OO}]}$$

Results of the overall models' comparison are summarized in table 8, highlighting that the OO model, both including the complete and the reduced sets of variables, had lower AIC, as compared to the CL one, suggesting its improved prediction fitness.

Model	Classical Approach (CL)		Outcome Oriented Approach (OO)	
	df	AIC	df	AIC
Complete Model	78	5749.165	78	5701.13
Reduced Model	14	5656.732	25	5634.72

Table 8. *Summary of model comparison between Classical Approach and Outcome Oriented one for full and reduced models. The degree of freedom (df) indicates the number of variables considered in the model.*

Finally, logistic models were employed to assess the OO and the CL models' performances in predicting early relapse (ER).

To this aim, the AUC values were compared as an evaluation parameter to assess the performance of the models.

The OO model had always significantly higher AUCs, as compared to CL model, thus resulting the fittest model for patients' outcome prediction. The estimates of the AUC were stable after balancing and boostrapping (**Table 9**).

Method	Classical Approach (CL)		Outcome Oriented Approach (OO)		p-value
	AUC	CI	AUC	CI	
Complete Model	0.7327	0.6885 - 0.7769	0.8125	0.7767 - 0.8483	0.0003
Complete Model Boostrap	0.7327	0.6874 - 0.7746	0.8125	0.7775 - 0.8466	-
Complete Model post balancing	0.7469	0.7118 - 0.7820	0.8010	0.7697 - 0.8323	0.0220
Complete Model post balancing Boot	0.7708	0.7364 - 0.8015	0.8010	0.7689 - 0.8315	-
Reduced Model	0.6637	0.6144 - 0.7129	0.7424	0.6995 - 0.7853	0.0034
Reduced Model Bootstrap	0.6636	0.6173 - 0.7121	0.7425	0.7008 - 0.7823	-
Reduced Model post balancing	0.6571	0.6180 - 0.6962	0.7265	0.6904 - 0.7625	0.0080
Reduced Model post balancing Boot	0.6452	0.6061 - 0.6830	0.7265	0.6903 - 0.7609	-

***Table 9**. Summary of Area Under the Roc Curve values for different configuration of the model complete and reduced, before and after dealing with unbalance and with bootstrapping both by a Classical and by an Outcome Oriented approach. For each model it is shown the AUC values with its Confidence Interval (CI) and p-value for testing the statistical difference between AUC values.*

4.3. Identification of biomarker predictive of early relapse (ER)

Once confirmed the improvement in outcome prediction of the occurrence of OO-defined CNAs we sought to implement these features in clinical model of up-front reliable prediction of early relapse.

Overall, 155/743 patients included in the present study relapsed within 18 months from the start of therapy (Table 5).

A correlation analysis was first performed, to assess which CNA-OOs resulted more frequently carried by early relapsing patients. As shown in **table 10**, early relapsing patients carried more frequently amp(1q) and del(17p), both known to confer high risk to MM patients[58] .

CNAs	N	Level	ER pts N=155[1]	Not-ER pts N=588[1]	p. value[2]
1p	743	del	21 (13.5%)	52 (8.8%)	0.21
		no alteration	133 (85.8%)	533 (90.6%)	
		amp	1 (0.6%)	3 (0.5%)	
1q	743	del	1 (0.6%)	15 (2.6%)	**<0.001**
		no alteration	93 (60.0%)	443 (75.3%)	
		amp	61 (39.4%)	130 (22.1%)	
2p	743	del	8 (5.2%)	11 (1.9%)	**0.021**
		no alteration	141 (91.0%)	566 (96.3%)	
		amp	6 (3.9%)	11 (1.9%)	
2q	743	del	5 (3.2%)	5 (0.9%)	**0.042**
		no alteration	143 (92.3%)	566 (96.3%)	
		amp	7 (4.5%)	17 (2.9%)	
3p	743	del	3 (1.9%)	5 (0.9%)	0.144
		no alteration	106 (68.4%)	366 (62.2%)	
		amp	46 (29.7%)	217 (36.9%)	
3q	743	del	1 (0.6%)	6 (1.0%)	0.515
		no alteration	104 (67.1%)	366 (62.2%)	
		amp	50 (32.3%)	216 (36.7%)	
4p	743	del	3 (1.9%)	2 (0.3%)	0.072
		no alteration	150 (96.8%)	582 (99.0%)	
		amp	2 (1.3%)	4 (0.7%)	
4q	743	del	2 (1.3%)	1 (0.2%)	**0.027**
		no alteration	147 (94.8%)	538 (91.5%)	

		amp	6 (3.9%)	49 (8.3%)	
5p	743	del	3 (1.9%)	6 (1.0%)	0.129
		no alteration	98 (63.2%)	328 (55.8%)	
		amp	54 (34.8%)	254 (43.2%)	
5q	743	del	7 (4.5%)	7 (1.2%)	**0.003**
		no alteration	108 (69.7%)	369 (62.8%)	
		amp	40 (25.8%)	212 (36.1%)	
6p	743	del	1 (0.6%)	12 (2.0%)	0.147
		no alteration	153 (98.7%)	560 (95.2%)	
		amp	1 (0.6%)	16 (2.7%)	
6q	743	del	0 (0.0%)	18 (3.1%)	0.057
		no alteration	145 (93.5%)	520 (88.4%)	
		amp	10 (6.5%)	50 (8.5%)	
7p	743	del	8 (5.2%)	21 (3.6%)	0.659
		no alteration	124 (80.0%)	480 (81.6%)	
		amp	23 (14.8%)	87 (14.8%)	
7q	743	del	1 (0.6%)	5 (0.9%)	0.441
		no alteration	143 (92.3%)	556 (94.6%)	
		amp	11 (7.1%)	27 (4.6%)	
8p	743	del	24 (15.5%)	65 (11.1%)	0.317
		no alteration	128 (82.6%)	510 (86.7%)	
		amp	3 (1.9%)	13 (2.2%)	
8q	743	del	2 (1.3%)	10 (1.7%)	0.817
		no alteration	149 (96.1%)	558 (94.9%)	
		amp	4 (2.6%)	20 (3.4%)	
9p	743	del	3 (1.9%)	1 (0.2%)	**0.007**
		no alteration	94 (60.6%)	319 (54.3%)	
		amp	58 (37.4%)	268 (45.6%)	
9q	743	del	0 (0.0%)	1 (0.2%)	0.706
		no alteration	145 (93.5%)	557 (94.7%)	
		amp	10 (6.5%)	30 (5.1%)	
10p	743	del	5 (3.2%)	2 (0.3%)	**0.004**
		no alteration	146 (94.2%)	571 (97.1%)	
		amp	4 (2.6%)	15 (2.6%)	
10q	743	del	10 (6.5%)	57 (9.7%)	0.448
		no alteration	140 (90.3%)	511 (86.9%)	
		amp	5 (3.2%)	20 (3.4%)	
11p	743	del	4 (2.6%)	10 (1.7%)	0.76
		no alteration	130 (83.9%)	501 (85.2%)	
		amp	21 (13.5%)	77 (13.1%)	
11q	743	del	11 (7.1%)	25 (4.3%)	0.188
		no alteration	126 (81.3%)	510 (86.7%)	

		amp	18 (11.6%)	53 (9.0%)	
12p	743	del	15 (9.7%)	9 (1.5%)	**<0.001**
		no alteration	137 (88.4%)	571 (97.1%)	
		amp	3 (1.9%)	8 (1.4%)	
12q	743	del	14 (9.0%)	68 (11.6%)	0.599
		no alteration	139 (89.7%)	515 (87.6%)	
		amp	2 (1.3%)	5 (0.9%)	
13q	743	del	96 (61.9%)	276 (46.9%)	**0.003**
		no alteration	59 (38.1%)	308 (52.4%)	
		amp	0 (0.0%)	4 (0.7%)	
14q	743	del	17 (11.0%)	56 (9.5%)	0.776
		no alteration	136 (87.7%)	521 (88.6%)	
		amp	2 (1.3%)	11 (1.9%)	
15q	743	del	3 (1.9%)	9 (1.5%)	**0.041**
		no alteration	94 (60.6%)	292 (49.7%)	
		amp	58 (37.4%)	287 (48.8%)	
16p	743	del	14 (9.0%)	71 (12.1%)	0.545
		no alteration	138 (89.0%)	508 (86.4%)	
		amp	3 (1.9%)	9 (1.5%)	
16q	743	del	31 (20.0%)	83 (14.1%)	0.139
		no alteration	120 (77.4%)	495 (84.2%)	
		amp	4 (2.6%)	10 (1.7%)	
17p	743	del	12 (7.7%)	13 (2.2%)	**0.002**
		no alteration	138 (89.0%)	544 (92.5%)	
		amp	5 (3.2%)	31 (5.3%)	
17q	743	del	6 (3.9%)	38 (6.5%)	0.322
		no alteration	148 (95.5%)	541 (92.0%)	
		amp	1 (0.6%)	9 (1.5%)	
18p	743	del	7 (4.5%)	9 (1.5%)	0.069
		no alteration	131 (84.5%)	519 (88.3%)	
		amp	17 (11.0%)	60 (10.2%)	
18q	743	del	7 (4.5%)	31 (5.3%)	0.657
		no alteration	127 (81.9%)	492 (83.7%)	
		amp	21 (13.5%)	65 (11.1%)	
19p	743	del	11 (7.1%)	11 (1.9%)	**0.003**
		no alteration	134 (86.5%)	542 (92.2%)	
		amp	10 (6.5%)	35 (6.0%)	
19q	743	del	21 (13.5%)	54 (9.2%)	0.25
		no alteration	121 (78.1%)	475 (80.8%)	
		amp	13 (8.4%)	59 (10.0%)	
20p	743	del	23 (14.8%)	75 (12.8%)	0.66
		no alteration	125 (80.6%)	492 (83.7%)	

		amp	7 (4.5%)	21 (3.6%)	
20q	743	del	4 (2.6%)	24 (4.1%)	0.121
		no alteration	134 (86.5%)	526 (89.5%)	
		amp	17 (11.0%)	38 (6.5%)	
21p	743	del	3 (1.9%)	16 (2.7%)	0.305
		no alteration	122 (78.7%)	427 (72.6%)	
		amp	30 (19.4%)	145 (24.7%)	
22q	743	del	18 (11.6%)	28 (4.8%)	**0.007**
		no alteration	135 (87.1%)	549 (93.4%)	
		amp	2 (1.3%)	11 (1.9%)	

[2] Pearson's Chi-squared
[1] n (%)

Table 10. *Distribution of CNAs in the 2 different sub-groups on study: ER and Not-ER patients; for each CNAs, deletion (del), amplification (amp) and absence of both (no alteration) are shown.*

Table 10 also highlights all differences observed in the distribution of each specific alteration between ER and Non-ER patients.

Notably, 6 CNAs were selected both from the reduced multivariate OO model and from the correlation analysis as significantly associated with patients who relapse early.

In details, the following differences should be noted:

- 1q amplification, as found in 39.4% (ER) vs 22.1% (not-ER) patients;
- 2p deletion, as found 5.2% (ER) vs 1.9% (not-ER) patients;
- 2p amplification, as found in 3.9% (ER) vs 1.9% (not-ER) patients;
- 12 p deletion, as found in 9.7% (ER) vs 1.5% (not-ER) patients;
- 17p deletion, as found in 7.7% (ER) vs 2.5% (not-ER) patients;
- 19p deletion, as found in 7.1% (ER) vs 1,9% (not-ER) patients;

Once selected these specific OO-defined CNAs most significantly recurrent in ER patients, we stratified patients according to the presence of at least one of them, to explore a novel approach to achieve a reliable MM risk assessment. To this aim, we stratified patients according to the presence of at least one of the ER-related CNAs into two subgroups, including 249 e 494 patients respectively, either carrying ("early_HR") or not ("not") these CNAs (**Figure 11a-b**).

In a univariate survival analysis, the "early_HR" sub-group of patients had a significantly lower PFS and OS (PFS, HR 2.15, CI 1.79-2.58 p<0.0001; OS, HR 2.37, CI 1.86-3.02 p<0.0001) as compared to those of the other sub-group of patients (**Figure 11a-b**).

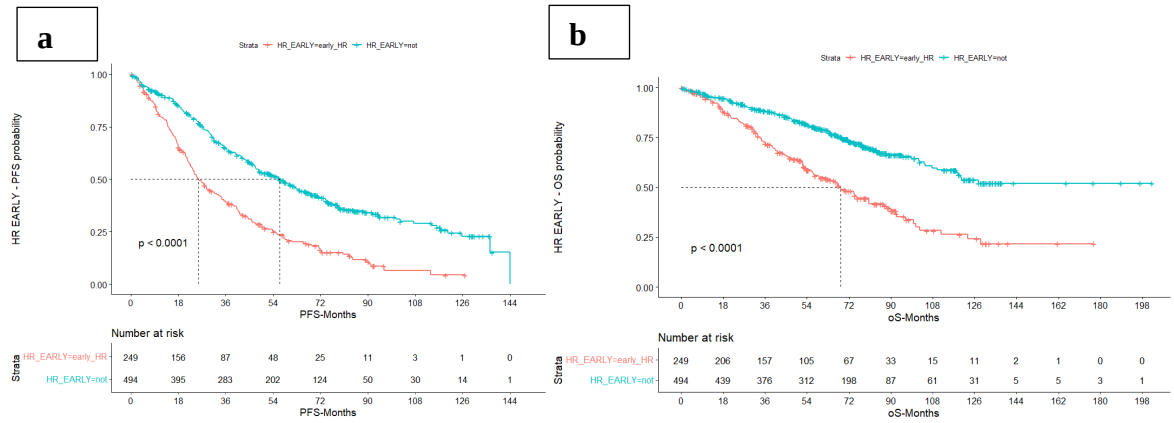


Figure 11. *Univariate analysis in PFS (**a**) and OS (**b**) of patients identified by the risk predictor, carriers of at least one CNAs-OO associated with ER.*

Notably, the bad prognostic impact was maintained also in absence of amp1q and/or del17p; in fact, even by excluding these CNAs, patients carrying at least one of the remaining ER-CNAs (del2p and/or amp2p and/or del12p and/or del19p, 38/249 patients, 15%) still have worse prognosis, as compared to patients without ER-CNAs.

Figure 12 displays the univariate survival analysis of these patients, showing a significantly higher HR, as compared to patients who did not present any of the ER-related CNAs detected by the model (494 patients), both in PFS (HR 2.57, CI 1.79-3.70 p <0.0001) and in OS (HR 2.49, CI 1.57-3.94 p=0.0001).

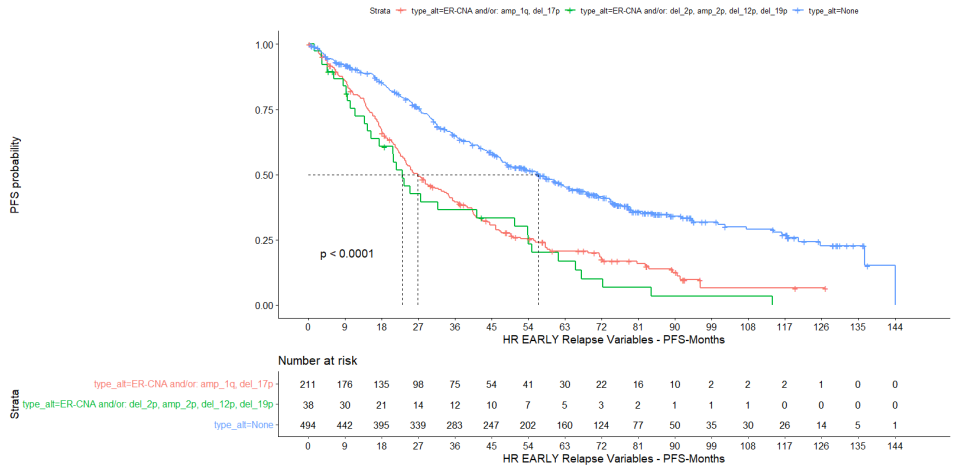


Figure 12. *Univariate analysis of PFS of patients stratified by ER-related CNA-OO combinations.*

Since the prognostic impact of ER-CNAs was explored by not taking into account their possible co-occurrence, we also evaluated this possibility, showing that, in a univariate analysis, the co-presence of two or more ER-CNAs progressively worsened the median survival of the patients, with progressively higher HR:

- presence of 1 ER-CNAs has a HR= 1.946, (C.I. 1.6-2.267)
- presence of 2 ER-CNAs has a HR= 3.586, (C.I. 2.536-5.072)
- presence of 3 ER-CNAs has a HR=11.147, (C.I. 3-531-35.193).

Notably, the co-occurrence of more than three ER-CNAs was never observed.

Univariate survival analysis is shown in **figure 13**.

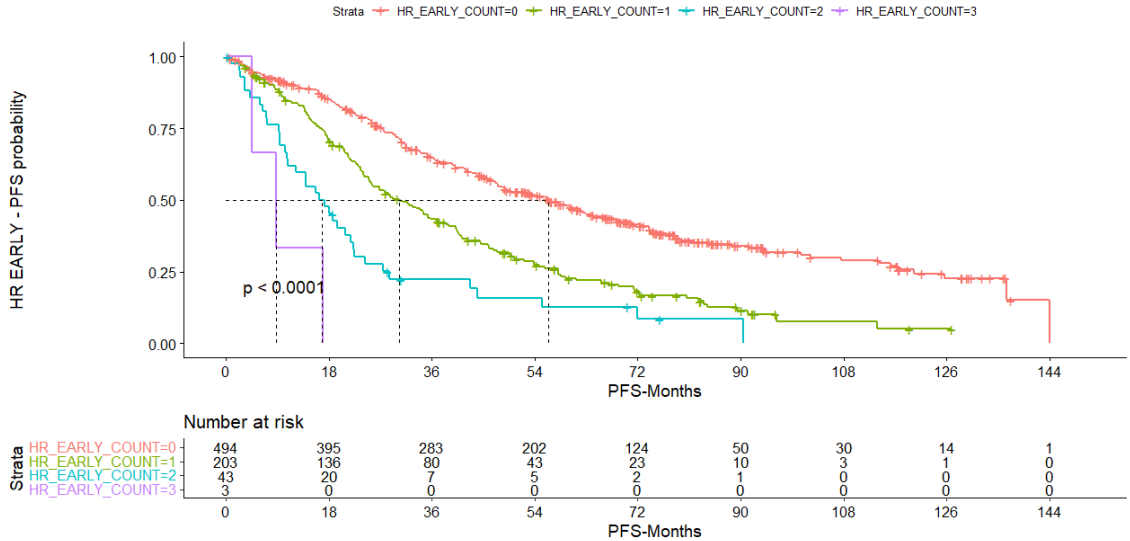


Figure 13. *Univariate analysis of PFS of patients stratified by ER-related CNA-OO.*

Finally, a Cox multivariate analysis was performed to assess the independency of ER-CNAs in predicting outcome. We showed that the risk of early relapse conferred by the presence of at least one of the previously mentioned ER-CNAs was independent both from that of being R-ISS III (R-ISS III HR=1.51 CI 0.17- 2.39-2.2; *p*=0.01), and from that of achieving low-quality responses (< stable disease, SD) to first-line therapy (HR=2.59 CI 0.33-2.84 *p*=0.004). Notably, multivariate analysis underscored the non-descriptive nature of the type of induction therapy provided to patients within this model. This implies that early relapse is primarily associated with the baseline genomic profile of patients, rather than the specific induction therapy provided (**Figure14**).

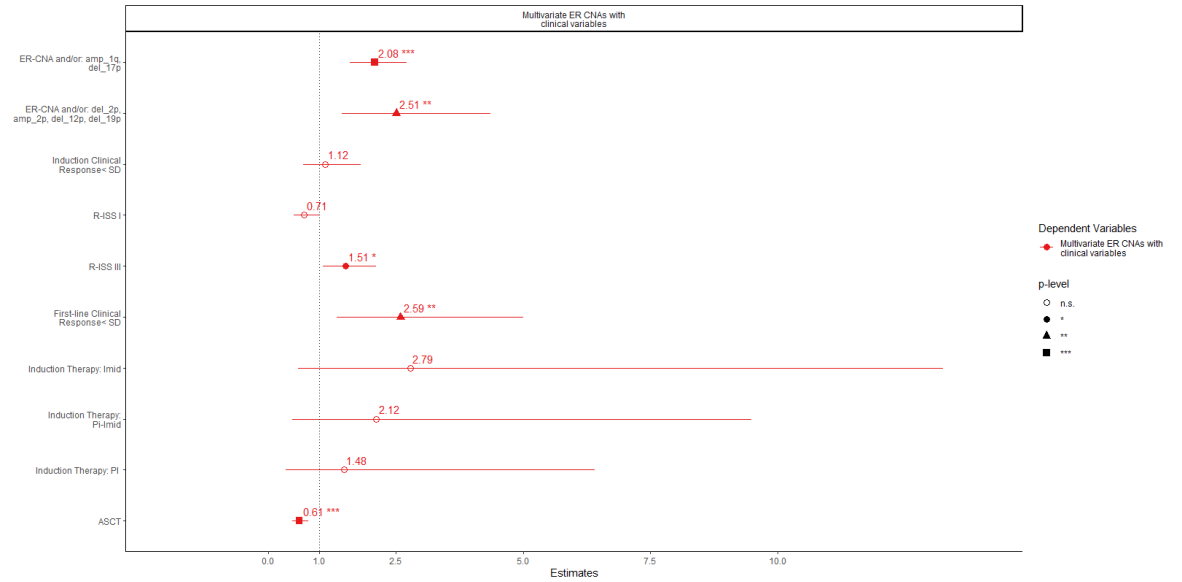


Figure 14. *Multivariate logistic regression model evaluating risk factors associated with ER-PFS in the patients at risk for the entire 18-month period. CI, confidence interval; ASCT, autologous stem-cell transplantation; IMiDs, immunomodulatory drugs; PI, proteasome inhibitor.*

5. Discussion

MM is a neoplastic disease characterized by the accumulation of B lymphocytes and plasma cells in the bone marrow. MM is a genetically complex disease, where the co-occurrence of multiple different genomic events leads to the development and progression of the tumour.

From a genomic point of view, CNAs are the most represented type of chromosomal aberrations in myeloma plasma cells, mostly contributing to the clinical and the molecular heterogeneity of this pathology. In fact, according to the different clonal architecture and the recurrence of certain CNAs, subgroups of patients with specific clinical outcomes can be identified.

CNAs are conventionally detected by cytogenetic technologies, particularly by FISH, that accurately identifies specific chromosomal abnormalities, such as deletions, translocations and amplifications, even though has a limited glance on MM genomic landscape, as can pick few alterations at a time. Recently, the introduction of high-throughput molecular technologies (e.g., SNPs array and NGS) has significantly improved the resources available to explore the genomic landscape of oncological diseases. Overall, it is undeniable that these techniques offer an exceptionally high resolution, enabling a thorough examination of the entire genome and providing a comprehensive overview of all potential CNAs. However, novel technical issues have emerged, linked to the use of these powerful techniques.

In the present study, we aimed at dealing with the critical issue of *cut-offs definition* for CNAs call from data derived from high-throughput technologies. We focused on the CNAs call from data deriving from SNPs array experiments, even though our approach might be extended to data deriving from NGS experiments, as well.

The starting point of our study emerged from a preliminary exploratory analysis of 743 newly diagnosed MM patients, whose plasma cells' clone(s) were previously characterized by SNPs array experiments, unveiling that a wide distribution of signals, allowing CNAs' call, should be managed, to provide a reliable CNAs call. This underscored the need of establishing precise thresholds for CNAs calls for each chromosome arm. In fact, these thresholds are crucial for distinguishing CNAs' signals from background noise and for ensuring precise calls of amplifications or deletions.

In the context of FISH analysis, the issue of cut-off definition has been solved by defining a consensus standard cut-off of 10% [37,40]. On the contrary, no consensus has been established so far for CNAs calling from molecular data, even though, by analogy with FISH, a conventional 10% cut-off is employed also in this context, meaning that all signals moving 10% around the 2N normality signal define amplifications and deletions.

A precise CNAs call allows the accurate genomic profiling of MM patients' plasma cells, thus supporting a reliable patients' stratification in sub-groups, that might also have different prognosis. However, the use of 10% standard cut-offs just considers the *presence* of any given chromosomal aberration but neglects the *dimension* of clone(s) carrying that specific aberration. This important aspect has been recently explored, showing that del(17p), as detected by FISH, impacts patients' outcome just when detected in 55% of plasma cells[64]. On the contrary, when present at diagnosis in small sub-clones (e.g., in 10% of plasma cells), del(17p) do not impact first relapse, even though the possibility that small sub-clone(s) carrying del(17p) might be selected and might expand during disease progression cannot be ruled out[4,31,64].

Similar considerations should be made for whole genome CNAs, called by molecular data; in fact, it can be easily anticipated that by shifting the CNAs molecular signals' cut-off up and down around the 2N signal, it would be possible to indirectly define the amount of plasma cells carrying that specific alteration, thus measuring the dimension of MM sub-clones. However, the issue here is far more complicated, as whole genome CNAs should be analyzed both individually (also considering that they might affect the whole chromosome, or just one of the two chromosomal arms) and in combination, to provide a clear interpretation of the patient's genomic landscape.

To address this issue, in the present study we developed a statistical pipeline aimed at the identification of the best cut-off to be employed for CNAs calls from molecular data. To this aim, we adopted an Outcome-Oriented (OO) approach, designed to select cut-offs for each CNAs according to predefined clinical endpoints. OO-defined cut-offs were selected for their ability to define the clone(s)' size most significantly correlated to the specified clinical endpoint. Therefore, not only did we accurately describe the type of CNAs characterizing the genomic landscape of patients included in the study, but also, we indirectly defined the dimension of clone(s) most significantly impacting patients' outcome.

Notably, the OO approach is highly versatile, as by modifying the clinical endpoint, the specific cut-offs for each CNAs call can be adjusted. This gives the possibility to identify new alterations that potentially allow a better stratification of patients on the aimed outcome, that may be different from early relapse.

The endpoint chosen for the present study is early relapse, i.e., relapse within 18 months from the start of therapy; early-relapsing patients are considered at high-risk, as these patients results frequently therapy-refractory and, even with the best treatments, do not perform well[58].

It is, therefore, critically important to catch early this high-risk sub-group of patients to provide them with intensified therapies, as soon as possible.

The use of the OO approach improved the accuracy of CNAs calls, as compared to the standard (i.e., CL) approach, and more importantly allowed the selection of the CNAs with the strongest prognostic impact in patients' outcome, supporting the early definition of patients at high risk of early relapse.

Notably, 36 out of 60 CNAs called by the OO approach had a higher prognostic impact than that of the same CNAs called by the CL method; among these, 1q amplification and 17p deletion should be considered paradigmatic, as, even though already known to be associated with high risk, their impact on prognosis resulted significantly higher when called by the OO approach, than when called with the conventional one.

Furthermore, the OO approach highlighted the role of CNAs that, until now, have never been associated with patients' prognosis, such as the chromosome 12p deletion, suggesting that risk stratification based on FISH data related to few chromosomal aberrations might be misleading, as it do not consider the role of aberrations not commonly explored in daily practice.

The OO approach was validated by comparing the effect of OO-defined CNAs with that of CNAs called with the standard approach; the comparison ended up with the definition of a PFS model with better fit. The comparison was performed by employing parameters describing the multivariate models (i.e., AIC values, as shown in **table 8**), for the selection a reduced number of variables by Backward stepwise selection, that kept 14 variables for the classical method and 25 for the OO method. Overall, the OO model even including a higher number of variables, provides a better goodness of fit, meaning a good trade-off between complexity and model fit, therefore it has a lower AIC value than the classical model. Consequently, alterations with specific cut-offs are included in the OO model includes and have an impact on survival.

Overall, 155 out of the 743 patients included in the study relapsed within 18 months from the start of therapy. We characterized this subgroup of patients using OO-cut-offs and performed a correlation analysis to define the changes that most correlated with early relapse. The analysis showed that patients who relapse early are enriched in amp(1q) and del(17p), as previously reported in literature[58]. Six CNAs (amp1q, del17p, del12p, amp2p, del2p, del19p), were selected both from the reduced multivariate OO model and from the correlation analysis as significantly associated with patients who relapse early and were called ER-CNAs. Therefore, according to these analyses, we have been able to stratify patients according to the presence of at least one of these ER-CNAs, into two different subgroups that included 294 and 494 patients,

respectively, with significantly different outcome and the presence of at least one ER-CNAs remained an independent unfavourable prognostic factor even in a multivariate Cox model, including the main known risk factors in myeloma, such as R-ISS III, and the obtaining of low-quality responses to first-line therapy.

Notably, among ER-CNAs, del2p, amp2p, del12p, del19p are alterations not broadly described, nor associated with a poor prognosis in MM. However, recently, del(12p) has been suggested as novel MM negative prognostic marker and has gained increasing attention[82], even though its prognostic impact remains inconsistent, due to few studies available considering this variable [83,84]. Nevertheless, our results are comparable with those reported by Avet-Loiseau et al.[83] and by Li et al.[82], booth demonstrating that patients with del(12p) have remarkably shorter PFS than those without del(12p). Therefore, the OO approach might be considered in support of a pivotal role of del(12p) in conditioning the early relapse in MM patients. It is worth of note that chromosome 12p13.31 carries *CD27*, which is commonly under-expressed in MM[85] and is linked to the transition from normal cells to MGUS to MM[86]. Recently, the role of CD27 has been explored by flow cytometry, showing that it is expressed by normal PCs, but is absent in PCs from patients with MM at diagnosis (36%), at relapse (47%) and in HMCLs (92%), and that CD27-negative patients have poor survival[87].

Our analysis showed that the presence of at least one of the ER-CNAs identified a subgroup of patients whose PFS and OS were significantly lower than those in the other subgroup. To exclude the possibility that the unfavourable prognosis could only be due to the presence of either amp(1q) or del(17p), already known to be high risk alterations, we compared the survival of patients carrying at least one of these two alterations, or at least one of the remaining ER-CNAs, (del2p, amp2p, del12p, del19p), confirming the unfavourable prognostic impact of all these ER-CNAs.

Finally, the presence of at least one ER-CNAs remains an independent unfavourable prognostic factor even in a multivariate Cox model, including the main known risk factors in myeloma, such as R-ISS III, and the obtaining of low-quality responses to first-line therapy.

In conclusion, the OO statistical approach used in this study allowed to define dynamic, CNA-specific clonality cut-offs, improving the accuracy of CNAs' calls to identify MM patients with the highest probability of early relapse.The use of the new cut-offs, associated with early relapse risk, showed a significant association between the clinical outcome of patients and the presence of particular CNAs, some already known in the literature (i.e., amp1q and del17p),

while the biological and prognostic role of previously undescribed CNAs other than del(12p), such as amp(2p), del(2p), del(19p), will have to be investigated in future studies.

The OO-approach employed here allowed to define CNAs-specific dynamic clonality cut-offs, improving the CNAs calls' accuracy to identify MM patients with the highest probability to early relapse. As being outcome-dependent, the OO-approach is dynamic and might be adjusted according to the pre-selected outcome of interest (e.g., MRD-negativity) thus providing outcome specific clonality cut-offs.

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