" ALEXANDRU IOAN CUZA" UNIVERSITY OF IAŞI
FACULTY OF BIOLOGY
DOCTORAL SCHOOL OF BIOLOGY

STUDY OF MOLECULAR AND CYTOGENETIC ABNORMALITIES IN MULTIPLE MYELOMA

PHD THESIS SUMMARY

Scientific advisor:

Prof. Dr. Habil. Dragoș Lucian GORGAN

PhD student:

Mihaiela Loredana HANGAN (Căs. DRAGOȘ)

IAŞI
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LIST OF ABBREVIATIONS

Abreviere	Semnificație
MM	Multiple myeloma
NGS	Next generation sequencing
SNParray	Single nucleotid polymorphism array
aCGH	Comparative genomic hybridization array
ISH	In situ hybridization
FISH	Fluorescence in situ hybridization
BM	Bone marrow
BMSC	Bone marrow-derived stem/stromal cells
ADN	Deoxyribonucleic acid
ARN	Ribonucleic acid
HSC	Hematopoietic Stem Cells
NK	Natural Killer Cell
SMM	Smoldering Multiple Myeloma
MGUS	Monoclonal gammopathy of undetermined significance
BAC	Bacterial artificial chromosome
B2-mg	Beta 2 Microglobulin- B2
CSR	Class-Switch Recombination
CRAB	C- hypercalcemia, R- renal failure, A- anemia, B- bone disease
EPO	Eritropoietina
g	grams
HRD	Hyperdiploid
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor I
IGH	Immunoglobulin heavy chain
IL-..	Interleukin …
IL-3	Interleukin 3
IL-5	Interleukin 5
Mg	Milligrams
TPO	Thrombopoietin

IMWG	International Myeloma Working Group
MLPA	Multiplex ligation-dependent probe amplification
PCL	Plasma cell leukemia
PCR	Polymerase chain reaction
RT	Reverse transcription
R-ISS	Revised International Staging System
TGFB-1	Transforming growth factor beta 1
TNFR	Tumor necrosis factor receptor
VEGFA	Vascular endothelial growth factor A
WHO	World Health Organization
FLC	Free light chain
CNV	Copy number variation
CNA	Copy number alteration
Chr	Chromosom

INTRODUCTION

The evolution of genetics and in particular cytogenetics has had a major impact on the understanding of hematological diseases. Starting with the discovery of plant chromosomes in 1875 and continuing with the discovery of human chromosomes in 1879 (Arsham et al., 2017) which opened the door to new horizons and up to state-of-the-art techniques such as NGS and SNP-array have led to the increasingly accurate delineation of the picture of cellular evolution and hematological disorders respectively.

Multiple myeloma (MM) has been known since antiquity, but it was not until the early 19th century that case reports of „molii ossium" appeared, highlighting the bone lesions characteristic of what later became known as MM. The best documented case is that of Thomas Alexander McBean, a highly respected London merchant (1845), in whom pathologist Henry Bence Jones and clinical consultant William Macintyre described a new form of proteinuria associated with bone fragments and pathological fractures. This proteinuria became known as Bence Jones proteinuria. Patients with „molii ossium" and Bence Jones proteinuria died within just a few years of diagnosis, often after futile treatments with nonspecific remedies such as quinine, rhubarb, orange peel, and iron salts (Kyle et al., 2011, Steensma et. al, 2018).

In 1939 Longsworth and co-workers introduced protein electrophoresis into the MM study and demonstrated that this could reveal excess monoclonal immunoglobulins in serum (Ribatti, 2018).

In recent decades molecular cytogenetics has made a great technological leap forward giving researchers the ability to simultaneously visualize the loss or gain of genetic material throughout the genome at unprecedented resolution. Thus, in 1990 CGH (comparative genomic hybridization) microarrays with non-polymorphic probes (aCGH) were implemented followed by SNP („single nucleotide polymorphism") microarrays with polymorphic probes (SNParray) (Sherry et al., 2001, Ylstra et al., 2006, Smetana et al., 2011, Lockwood et al., 2006).

AIM OF THE STUDY

The aim of the thesis was to identify molecular and cytogenetic markers useful in the early diagnosis of patients with MM and to optimize the monitoring of cases under treatment by selecting appropriate molecular and cytogenetic tests for different treatment stages.

Chapter 1. MULTIPLE MYELOMA

1.1. Definition, classification and prevalence

MM (Kahler's disease), a neoplasia of B lymphocytes resulting from accelerated proliferation of atypical plasma cells that accumulate in the bone marrow (Dănăilă et al., 2011, Firth, 2019). These atypical cells produce/secrete immunoglobulins (Ig) or a polypeptide subunit of Ig, of a single type that is subsequently determined as a monoclonal protein (M-protein) in serum or urine (Remily-Wood et al., 2014, Tathineni et al., 2020).

The main elements that make up the clinical picture of the MM patient are hypercalcaemia (C), renal impairment (R), anaemia (A), bone disease (B) - in short CRAB (Padala et al., 2021). Added to these are neurological impairment (Egan et al., 2020, Smith et al., 2019), recurrent and persistent bacterial infections and hypervascularity (Blimark et al., 2015).

The stages of MM progression are: gammopathy of unknown significance (MGUS), indolent/asymptomatic multiple myeloma (SMM), multiple myeloma (MM) proper, and the last and most severe phase is plasma cell leukemia (Maura et al., 2020).

MM ranks second in terms of the incidence of haematological malignancies (Kazandjian, 2016). According to data published by the NIH (https://seer.cancer.gov/statfacts/html/mulmy.html, 2022) MM accounts for 1.8% of all cancers (Padala et al., 2021). Approximately 34,470 new cases are diagnosed annually and the 5-year mortality is 57%. Incidence is higher in Western Europe, North America and Australia compared to Asia and sub-Saharan Africa most likely due to limited access to differential diagnosis in these areas (van de Donk et al., 2021).

The average age at diagnosis is 66-70 years (depending on the study) (Kyle et al., 2003). In terms of the difference between males and females studies are conflicting (Callander et al., 2022). The incidence of MM appears to be 2-3 times of black versus white race but is lower in Asian and Hispanics (Kumar et al., 2017).

According to Myeloma Euronet Romania 500 new cases were diagnosed in 2020.

1.2. Recurrent molecular and cytogenetic abnormalities

MM results from the accumulation of chromosomal alterations throughout the evolution of the B lymphocyte lineage (de Moraes Hungria et al., 2018).

Chattopadhyay et al. identified 16 interacting loci in 3999 patients that could play a role in the familial transmission of a predisposition to the condition (Chattopadhyay et al., 2019).

Translocations involving the IGH (immunoglobulin heavy chain) gene located at position 14q32 are considered initial events in the pathogenesis of MM (Duek et al., 2019). Other important abnormalities are odd chromosome trisomies, MYC gene rearrangements, 1p deletion, 1q amplification, partial deletion or monosomy of chromosome 13, deletions at chromosome 14, and 17p deletion (Gay et al., 2019).

First events in MM can be grouped into two subtypes: (i) hyperdiploid (HRD) and (ii) nonhyperdiploid. The first category is characterized by trisomies involving the odd chromosomes: 3, 5, 7, 9, 11, 15,17, 19 and/or 21. The second category has as a predominant trait the presence of translocations of the IGH gene mainly: t(4;14), t(6;14), t(11;14), t(14;16) and t(14;20) (Cowan et al., 2022).

There are numerous events required for tumor progression such as: the vast majority of DNA sequence copy number variations (CNV/CNA), translocations involving the MYC gene and somatic mutations in MAPK, NFkB and DNA repair pathways that are only found in the MM stage and not in premalignant stages being considered secondary events (Manier et al., 2016).

Hyperdiploid myelomas have been associated with a good prognosis unlike non-hyperdiploid myelomas (Avet-Loiseau et al., 2012). This could be due to the action of tumor suppressor genes or sensitivity to therapy (Kumar et al., 2012). Patients with hyperdiploid MM who also have structural abnormalities usually develop a more aggressive form of the

disease involving IGH translocations and/or duplication of the long arm of chromosome 1 (Carrasco et al., 2006, Chng et al., 2006).

Hypodiploid MMs (with less than 48 chromosomes) and near tetraploid MMs (with more than 75 chromosomes) are associated with a very low survival rate (Hoctor and Campbell, 2012, Smadja et al., 2001).

Chapter 2: MATERIAL AND METHODS

2.1 Study group

Between December 2017 and December 2021, 343 patients raised the suspicion of MM (and agreed to be included in this study) following clinical examination at the Regional Institute of Oncology, Iași.

Following clinical and laboratory tests for 45 patients the diagnosis of MGUS was confirmed, 13 solitary plasmacytoma, 27 plasma cell leukemias and 102 with other diagnoses. For 156 patients the diagnosis of Multiple Myeloma was confirmed.

All patients signed informed consent. The study was approved by the ethics committee of the Regional Institute of Oncology, Iași.

Factors that prevented the correct and complete evaluation of atypical plasma cells in the bone marrow aspirate were „patchy disease" (uneven distribution of plasma cells in the bone marrow), hemodilution, clonal diversity and extramedullary disease.

2.1.1 Study group – IMMUNOFENOPATHYPING

Of the 156 patients diagnosed with MM, only 143 could be diagnosed by immunophenotyping, 13 samples were clotted and therefore non-compliant. 72 were male and 71 females. The average age was 66 years ranging from 39 to 87 years.

2.1.2 Study group- MLPA

The following inclusion criteria were applied to perform this analysis: the PC infiltrate in the marrow, according to cytological analysis, should be greater than 10%; the PC infiltrate assessed by flow cytometry should be greater than 1.5% and all patients should be diagnosed with active MM. Also, all patients who died immediately after diagnosis without any treatment being implemented were excluded. Thus, 107 patients remained (Table 1).

Table 1- Characteristics of the study group

Title 1	n	
Patients with MM	n=107	
Median age at diagnosis	65	Range (39-87)
Age<60	29	27.1%
Age 60-70	44	41.1%
Age<70	34	31%
Sex		
Male	51	47.66%
Female	56	52.33%
ISS Stage		
1	21	19.6%
2	39	36.4%
3	47	43.9%
Anemia	82	76.6%
Bone lesions	98	91.5%
Hypercalcemia (>=11mg/dl)	30	28%
Renal lesion	53	49.5%
Treatment		
VCD	74	69.1%
Other therapies	33	30.8%
Autologus transplant	30	40.5%

2.1.3 Study group- SNParray

For 50 patients, SNParray analysis was performed to highlight DNA copy number anomalies (CNAs) additional to those detected by MLPA. 20 (40%) were male and 30 (60%) females. The average age of the analyzed group was 67 years.

2.1.4 Study group- KARYOTYPE and FISH

Between December 2017 and January 2022, 156 samples from patients diagnosed with MM were sent to the cytogenetics department of Regional Institute of Oncology, Iași in order to carry out KARYOTIP analysis. Of these, 46 had a mitotic index of 0, 85 were with 46XX/46XY, 3 presented unanalyzable numerical and structural abnormalities and for 22 metaphases presented chromosomal abnormalities.

To confirm various anomalies, the FISH technique was used in 13 cases.

2.2 Research methods

2.2.1 Flow cytometric immunophenotyping

Flow cytometry is a multiparametric analysis that allows the characterization of cells from the point of view of membrane proteins called clusters of differentiation (CD).

The method is successfully used in the immunophenotyping of samples from MM patients including normal and atypical plasma cells. Table 2 shows the entire panel of antibodies used. It is divided into two tubes. Table 3 shows the differences in antibody expression in normal versus abnormal plasma cells.

Table 2: Panelul utilizat în imunofenotiparea Mielomului Multiplu

Tube no.	FITC	PE	PC5	PC7	APC	AC7	PB	PO
1	CD38	CD56	Cy-beta2m(4)	CD19	Kappa (3)	Cy-lambda(3)	CD45	CD138
2	CD38	CD28(5)	CD27(3)	CD19	CD117	CD81	CD45	CD138

Table 3. The normal/abnormal expression profile of phenotypic markers

CD	NORMAL	ABNORMAL
CD19	+	-
CD56	-	+/-
CD27	+	-
CD28	-	+
CD81	+	-
CD117	-	+/-
CD45	+	-
CD38	+	+
CD138	+	+

The panel of antibodies used is consistent with EuroFlow EuroFlow recommendations (Roshal et al., 2017, Flores-Montero et al., 2016).

Samples were analyzed using Navios (Beckman Coulter) and FACS ARIA III (Becton Dickenson) cytometers - 3 lasers, 10 colors, from TRANSCENT laboratory, Regional Institute of Oncology, Iași. An average of 100,000 cells was acquired for each tube/sample analyzed. The cytometer was calibrated and monitored daily according to the manufacturer's recommendations and EuroFlow standards (Kalina et al., 2012, Glier et al., 2019). For data analysis, files (.fcs or .lmd) were processed using Infinicyt 1.8 software (Cytognos SL, Salamanca, Spain). Plasmocytes were highlighted by selecting CD38 and CD138 positive populations. A threshold (threshold) of 10% was applied for a CD to be considered a positive, exception was made for CD19 where the threshold was set to 50% (Paiva et al., 2017b).

2.2.3 Sorting of plasma cells with magnetic beads

Sample enrichment by flow cytometry raised various problems and thereforeit was chosen to sort plasma cells with magnetic beads labeled with anti-CD138 antibodies (ProtocolCD138MicroBeads, Shin et al., 2012, Bansal et al., 2021).

The first stage of this process consists in the separation of mononuclear cells - BMMC/PBMC (bone marrow mononuclear cells/peripheral blood mononuclear cells) by density gradient with Ficoll (Hystopaque). The cell pellet thus obtained is incubated with magnetic beads marking CD138+ cells. After labeling, the cell suspension was transferred to a separation column and exposed to a magnetic field. Finally the labeled cells are eluted from the column (ProtocolCD138MicroBeads),

The human CD138 MicroBeads kit from Miltenyi Biotec was used, and the separated plasma cells were used for DNA isolation.

After sorting, the percentage of isolated plasma cells was verified by labeling with CD138 and CD38. It was possible to raise the percentage of atypical plasma cells from values of 5-6% to 87%.

2.2.4 Extraction and quantification of nucleic acids

DNA extraction

It was performed using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). The basic principle of this kit consists in precipitation of genomic DNA with isopropanol (after the steps of cell lysis and removal of proteins and RNAs) (Rodríguez-Riveiro et al., 2022).

The QIAamp DNA Mini Kit-Qiagen kit, which isolates nucleic acids with the help of silicon membranes, was also used for genomic DNA extraction. Initially, the sample is lysed with proteinase K and a lysis buffer, after which a membrane attachment buffer is added (which gives the lysate a certain PH and a certain salinity, which will determine that by centrifugation the DNA remains fixed on the membrane and the proteins and the rest of the contaminants to be removed). Followed by two washing steps and elution with buffer solution (Handbook))

Quantification of nucleic acids

The first control step is spectrophotometric quantification of the isolated products, reading at 260nm and 280nm, using a NanoDrop 2000 spectrophotometer. This step is based on the absorbance for ultraviolet light of nucleic acids at a wavelength of 260nm and of proteins at 280 nm. RNA and single-stranded DNA have an extraction coefficient of 0.027 (μg/ml)-1 cm-1. To evaluate the purity of nucleic acids, the ratio between absorbance 260nm and 280nm is used. A pure DNA has this ratio equal to ~1.8, and pure RNA ~2.

2.2.5 MLPA technique - kit P0425-B2 for Multiple Myeloma

To identify cytogenetic abnormalities (variations in the number of DNA copies) in patients with MM, the MLPA P425-B1 kit (MRC-Holland, Amsterdam, Netherlands) was used, which can reveal deletions or duplications of the following chromosomal regions and target genes: 1p21.1 (COL11A1), 1p12 (FAM46C), 1p32.2 (PLPP3 and DAB1), 1p32.3 (FAF1, CDKN2C), 1q21.3 (CKS1B), 1p31.3 (LEPR), 1p21.3 (DPYD) , 1P31.2 (RPE65), 1q23.3 (NUF2, RP11, and PBX1), 5q31.3 (PCDHA1, PCDHAC1, PCDHB2, PCDHB10,

SLC25A2, and PCDHGA11), 9p24.1 (JAK2), 9q34.3 (COL5A1) , 12p13.31 (CD27, VAMP1, NCAPD2, CHD4), 13q14.2 (RB1 and DLEU2), 13q22.1 (DIS3), 14q32.32 (TRAF3), 15q12 (GABRB3), 15q26.3 (IGF1R), 16q12 .1 (CYLD), 16q23.1 (WWOX) and 17p13.1 (TP53). Each probe is between 129 and 499 nucleotides.

Sorted samples versus the fraction of CD138- cells and the unsorted sample were analyzed and the results obtained emphasized the fact that the enrichment of the sample is absolutely necessary in the case of this pathology and for the use of this technique (Figure 1, Table 4).4).
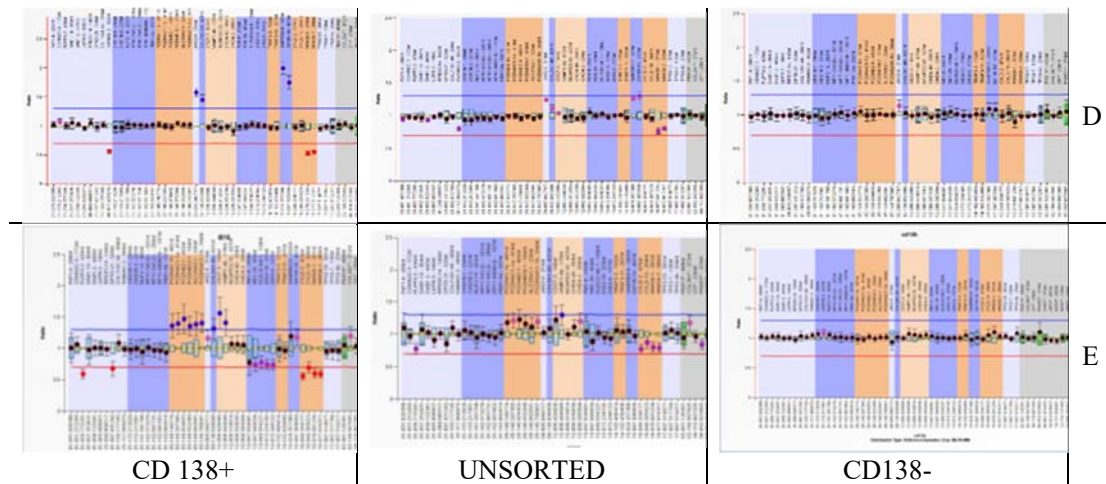

| | CD 138+ | UNSORTED | CD138- |

Figure 1. Graphs resulting from MLPA analysis

Table 4. Logarithmic value of CNAs in cells of two samples from which CD138+, CD138- and unsorted sample was tested

		D			E		
		CD138+	UNSORTED	CD138-	CD138+	UNSORTED	CD138-
MLPA	GAIN	1.69	1.22	1	1.35	1.2	1
	LOSS	0.64	0.79	1	0.68	0.68	1

2.2.6 SNP-array analysis in Multiple Myeloma

The SNParray technique is generally used to investigate constitutional genetic mutations, however, in the last decade its usefulness has also been highlighted for somatic changes. When affected cells are dependent on the microenvironment in the body this

technique can aid karyotyping and FISH techniques (Busse et al., 2017, Peterson et al., 2018, Rack et al., 2019, Munshi et al., 2011).

Array Comparative Genomic Hybridization (aCGH) with Single Nucleotide Polymorphism (SNP) probes was performed on a slide with 4x180,000 (180K) probes (110 112 CGH probes, 59 647 SNP probes, 3 000 replicate probes and 8 121 control probes) covering the entire human genome with a spatial resolution of ~25.3 kb DNA (Agilent). Scan files were interpreted using Agilent's CytoGenomics v2.0 software using standard interpretation parameters.

For the use of SNParray in patients with MM it is absolutely necessary to enrich the sample by sorting CD138 positive cells (Munshi and Avet-Loiseau, 2011) .(Figure 2, Table 5).
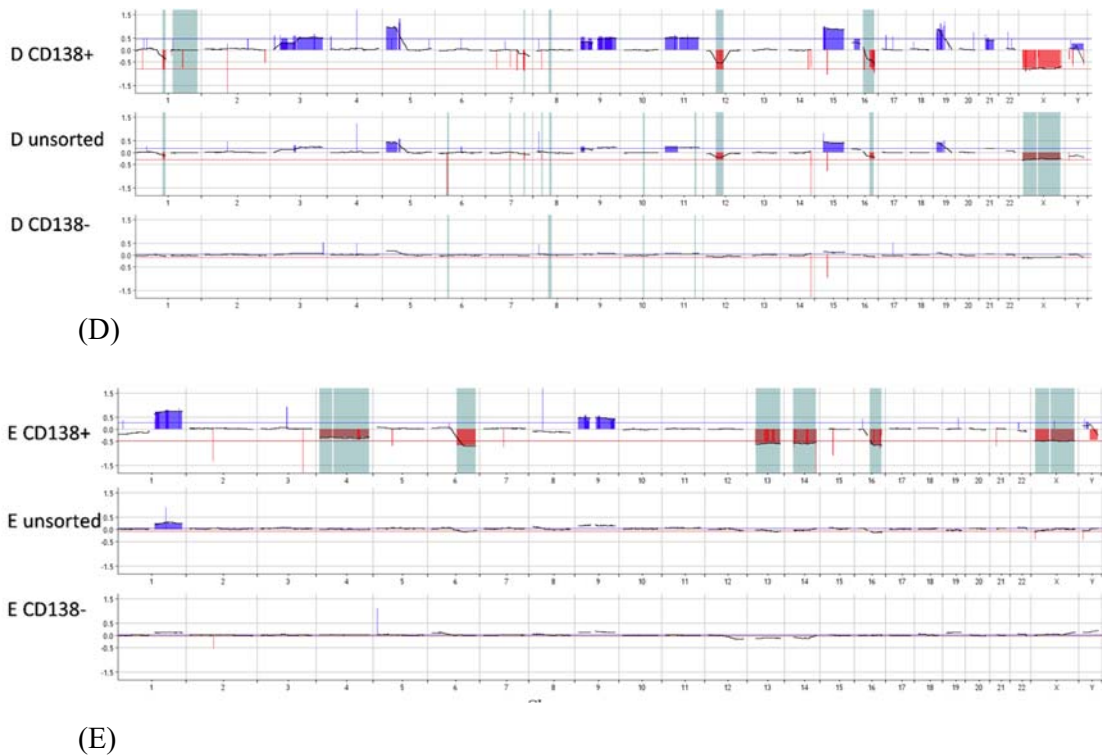

(D)

(E)

Figure 2. SNParray images from two patients diagnosed with MM.

Table 5. Logarithmic rate value for two patients analysed by SNParray

		D			E		
		CD138+	UNSORTED	CD138-	CD138+	UNSORTED	CD138-
aCGH	GAIN	0.66	0.57	0	0.52	0.25	0
	LOSS	-0.76	-0.37	0	-0.75	0	0

14

The analysis of the data concluded that a minimum plasma cell infiltrate of 30% in the sample analysed is necessary for the results obtained by this technique to be accurate.

Recurrent abnormalities that can be identified by array in MM are: hyperdiploidy, chr 13 deletion, 17p deletion, 1p deletion, 1q amplification/duplication, microdeletions, chromothripsis, chromanase, loss of heterozygosity.

2.2.7 Standard chromosome analysis Karyotype and FISH technique

Cytogenetic analyses are methods by which chromosomes are analysed. The karyotype is the standard arrangement of chromosomes in a cell, performed to detect numerical or structural, homogeneous or mosaic chromosomal abnormalities of autosomes or gonosomes.

The method involves obtaining dividing cells (unstimulated culture with unsynchronised mitogens, with cells from actively dividing tissues, which are then processed in several successive steps (metaphase division arrest, hypotonisation, fixation, slide staining and staining). The chromosomes are then photographed and arranged in homologous chromosome pairs according to standard criteria.

Clonal plasma cells are dependent on the medullary microenvironment and therefore their proliferative activity in culture is low. Because of this, only 30-50% of newly diagnosed cases of MM analysed by classical cytogenetics show an abnormal karyotype (Chin and Zakaria, 2014). Only numerical and structural abnormalities larger than 5Mb can be described by karyotyping (Riegel, 2014). Also, various prognostically important abnormalities such as t(4;14) are cryptic in classical cytogenetics (Stevens-Kroef et al., 2012). In contrast, with this technique, each cell is analysed individually and therefore we can observe clones and subclones of a particular cell line.

The introduction of FISH (fluorescence in situ hybridization) testing of interphase nuclei has improved the detection of genetic abnormalities in MM. This technique consists of hybridising fluorescent probes to the target DNA.

European guidelines published in 2019 recommend for routine in diagnosis karyotyping and a panel of FISH including: t(4;14), t(11;14), del17p, t(16;14), t(14;20) plus 1q amplification, 1p deletion (Rack et al., 2019). Amplification techniques (based on DNA extraction) are also suggested as an alternative if analyzable metaphases are not obtained.

2.2.8 Statistical analysis

Statistical analysis was performed using IBM® SPSS Statistics 21.0, R v4.0.3 and Office-Excel software. All parameters were analysed in relation to progression-free survival (PFS) and overall survival (OS). PFS was defined as the duration from the start of treatment to disease progression or patient death (regardless of cause of death). PFS analysis events included disease progression, relapse and patient death. Disease progression was diagnosed according to IMWG diagnostic criteria. OS was defined as the time from the date of diagnosis to the date of patient death. OS analysis events included death only. Patient loss to follow-up was treated as censored information. Kaplan and Meier method and log-rank test were used for survival analysis. Each graph contains the relevant statistical information: n - total number of patients and p-value (a $p < 0.05$ was considered statistically significant). Cox regression analysis was used to generate the relative hazard ratio (HR). HR is a mathematical value that calculates the probability that a person in the target group will experience events such as disease progression or death.

The development of the ,,heatmap" graphs was performed in R software using the Complex Heatmap package (Gu et al., 2016).

Chapter 3: RESULTS AND DISCUSSION

3.1 Rezultate și discuții – IMMUNOPHENOTYPING

Specific surface antigens (CDs) are commonly used in flow cytometry assays to discriminate malignant from normal plasma cells (Alaterre et al., 2017, Sato et al., 2021). Some of these surface antigens by association become prognostic factors and others are independent prognostic factors. Their initial cellular functions are not always understood, and their implications for diagnosis, prognosis, and risk stratification are variable and not uniformly agreed upon. There is a consensus on flow cytometry-assayed CDs that can identify the pathogenic clone and further characterize it (Paiva et al., 2010, Caers et al., 2018).

Regardless of the disease category, plasma cells have similar immunophenotypic characteristics, which are distinct from those of normal PC. Usually, CD38 and CD138 are the most important markers for discriminating PC from other cells in the BM. In addition, expression of CD45, CD19, CD56, CD117, CD28, CD27 and CD81 along with cytoplasmic immunoglobulin light chain allows clear discrimination between normal/reactive PC versus monoclonal PC. Together, these markers are used by the EuroFlow consortium to create a standardized panel, allowing the identification and immunophenotypic characterization of neoplastic PC (Flores-Montero et al., 2016).

CD19

A expression higher than 50% of this marker was found in only five cases (3.49%) with a median survival of 17 months for PFS. Eight patients showed an expression between 8% and 41% (Figure 3).
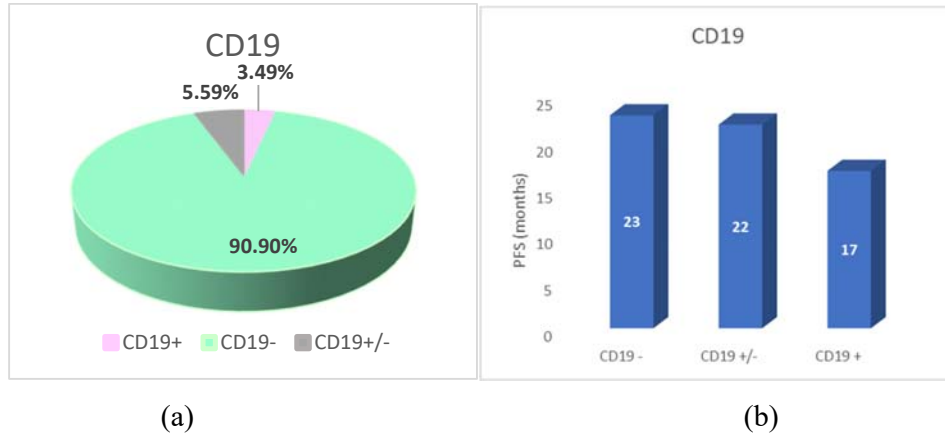
Figure 3. (a) CD19 presence in the analysed group; (b) Progression-free survival (median calculated using Kaplan Meier analysis) of patients grouped according to CD19 expression.

CD81

Analysis of this marker was performed for only 139 cases of which 62.5% (n=87) showed expression of this protein at the membrane level and 37.41 (n=52) lacked it. In the literature it is known as an independent negative prognostic factor especially for PFS (Paiva et al., 2012). In our group p was insignificant but HR indicates negative prognosis for both PFS (0.73, 95% CI 0.42-1.26) and OS (0.62 95% CI 0.31-1.24) (Figure 4).
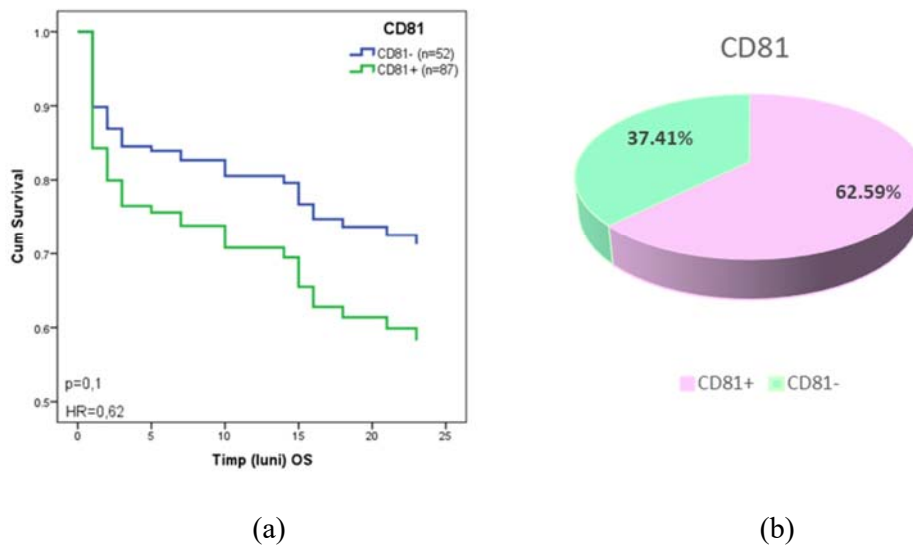

Figure 4. (a) Representation of overall survival patterns of CD81+/CD81- groups from COX regression analysis; (b) Presence of CD81 in the analysed group.

Paiva et al. ((Paiva et al., 2017a) divide plasma cells according to CD19 and CD81 expression into three differentiation groups. CD19(+)CD81(+) immature PCs („less-differentiated"), CD19(-)CD81(+) intermediate-differentiated PCs („intermediate-differentiated") and CD19(-)CD81(-) mature PCs („fully differentiated"). In our group five cases (3.49% similar to the mentioned study where the incidence was 3% for this clone) had immature PCs with a median PFS survival of only 17 months (HR 0.65, 95% CI 0.29-1.43). 58.34% (n=82) had second stage differentiated PCs with a median PFS of 22 months (HR 0.86 95% CI 0.20-3.55) and 37.4% (n=52) had mature PCs with a median PFS of 40 months. In terms of overall survival the median for the undifferentiated PC clone was also 7 months and for the other categories exceeded 48 months (Figures 5).
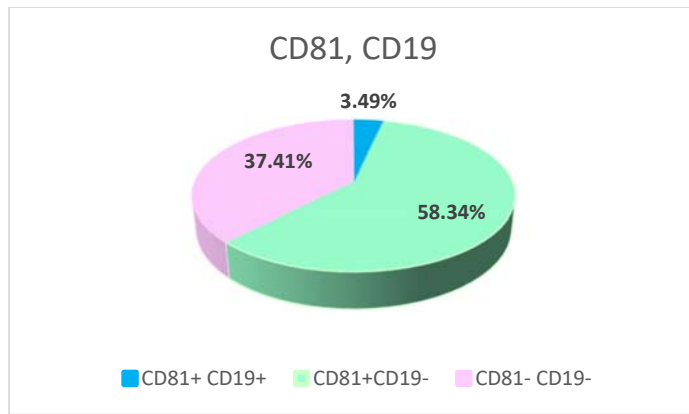
(a)
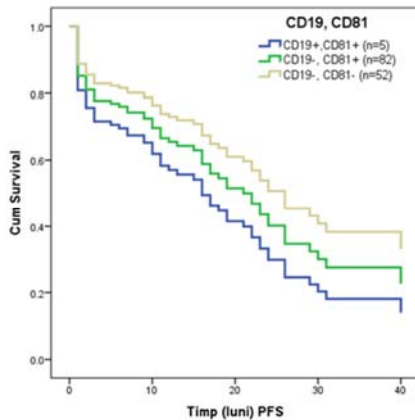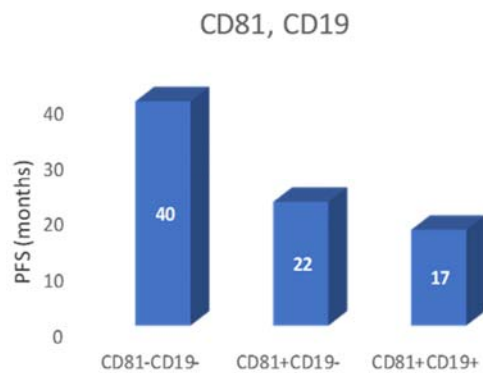
(b)   (c)

Figure 5. (a) Distribution of CD19, CD81 expression in the study group; (b) Progression-free survival assessed as a function of CD19, CD81 expression by COX regression; (c) Median PFS calculated by Kaplan Meier assessed as a function of CD19, CD81 expression.

CD117

      This CD was evaluated in 142 cases of which 66.42% (n=93) had CD117+ and 33.57% (n=49) had CD117-. The presence of this protein was a positive independent prognostic factor with a median PFS of 24 months (versus those with CD117- at 19 months), p=0.04 (HR=1.61, 95%CI 1.0-2.6) and a median OS greater than 48 months (versus cases with CD117- that had a median at 23 months), HR=1.63 (95%CI 0.90-2.92) (Figure 6).
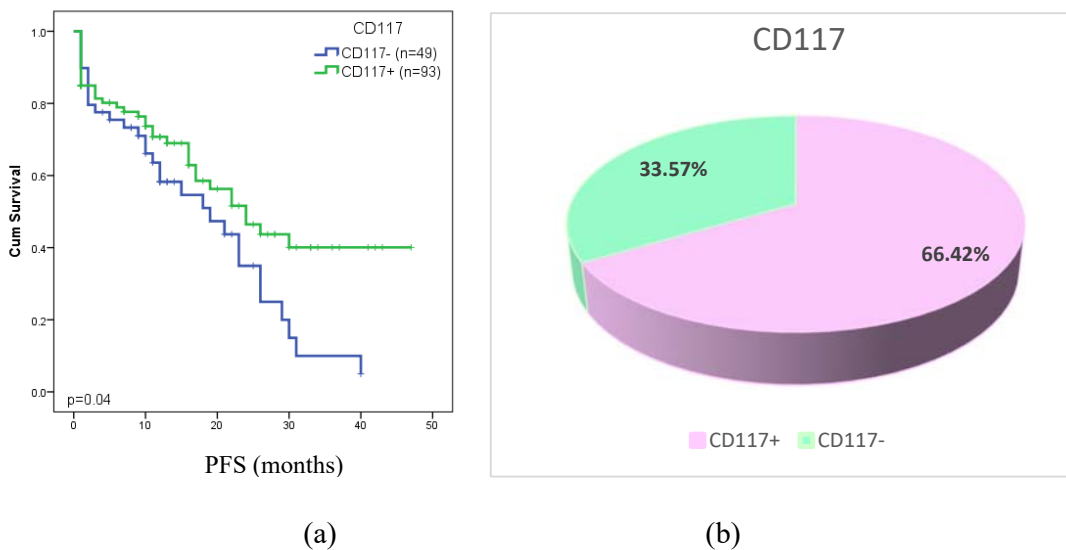

(a)   (b)

Figure 6. (a) CD117 incidence in the analyzed group; (b) Kaplan Maier survival curve for PFS as a function of CD117 expression.

3.2 Results and discussion - MLPA

      Genomic instability, involving complex numerical and structural abnormalities, is a hallmark of atypical CP in MM (Kim et al., 2015). FISH, karyotyping and cytology are known to be standard ("gold standard") methods for the diagnosis of MM (Dimopoulos et al., 2021).

      Xiaofei et al., 2021 (Ai et al., 2021), highlighted the correlation of iFISH and MLPA data obtaining a similarity percentage of 97.1% (1354 results from 1395 comparisons, were concordant), although, there were some discrepancies in resolution, point mutations and subclones caused by the probes used in both methods. In addition, according to previous studies iFISH analysis is only able to detect deletions or large duplications of 20-50 kb (He

et al., 2016), while MLPA can recognize sequences of 50-100 nt in length (Stuppia et al., 2012). Thus, the need for alternative methods arises, both in MM [49, 50] and in other hematological diseases.

Chr abnormalities identified with the MLPA P-425 B1 kit are generally known to have a negative prognosis, except for hyperdiploidy (HRD) which is known to have a positive prognosis (Chretien et al., 2015, Zhan et al., 2006, Zang et al., 2015), (Figure 7).
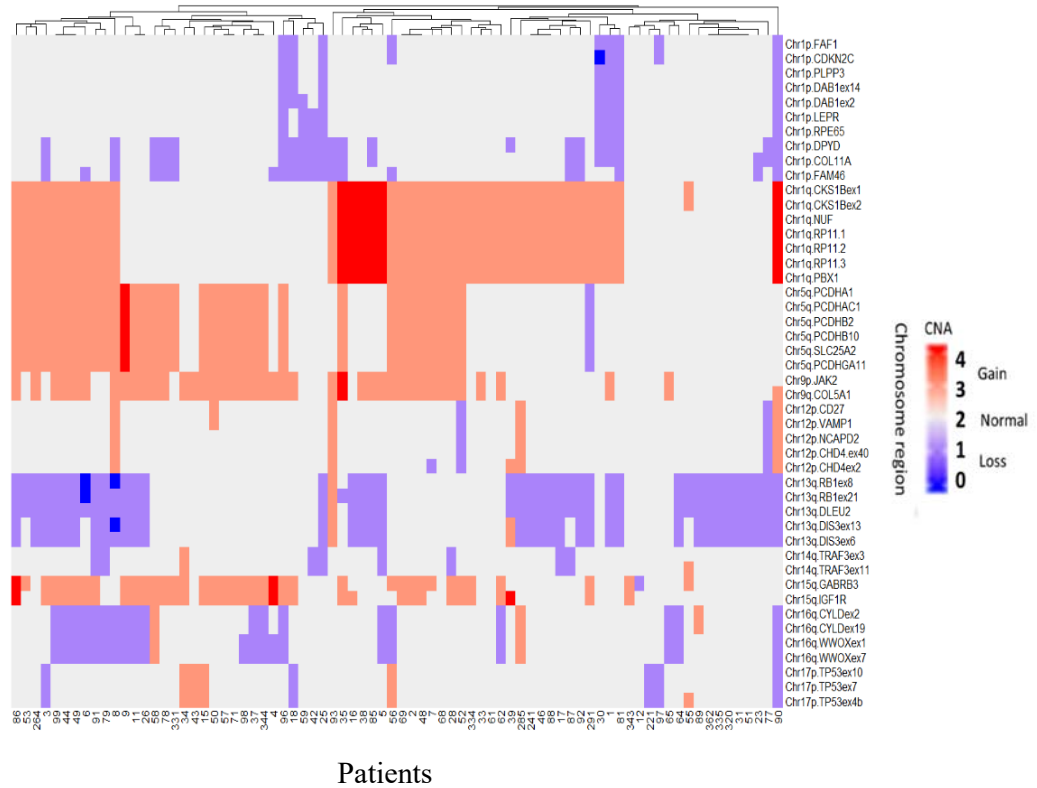

Figure 7. Overview of all CNAs present in the study group.

Previous studies have shown that chromosomal abnormalities, in different proportions and combinations, can affect the prognosis of patients with MM (Walker et al., 2015), which was also observed in the studied group, where the association of del1p with dup1q and/or del13q resulted in poorer survival than each alteration taken individually.

Shah et al. (Shah et al., 2018) divided MM based on the presence of cumulative adverse DNA lesions into three groups: with zero adverse lesions, with one adverse lesion, and with two adverse lesions. This concept of cumulative lesions, also used by other researchers (Baysal et al., 2020), made the diversity of cytogenetic abnormalities easier to

understand and apply in assessing prognosis and treatment response for clinical practice. Similarly, considering only adverse CNAs, the studied group was divided into four subgroups (no adverse CNAs, one adverse CNA, two adverse CNAs and at least three adverse CNAs). The worst survival was observed when at least three changes were associated resulting in a PFS, with a median of 16 months (n = 21), compared to patients without adverse CNAs (PFS greater than 48 months). OS was lower only in patients with at least three adverse changes (38 months), while the remaining patients exceeded the study time frame (Figures 8 and 9).
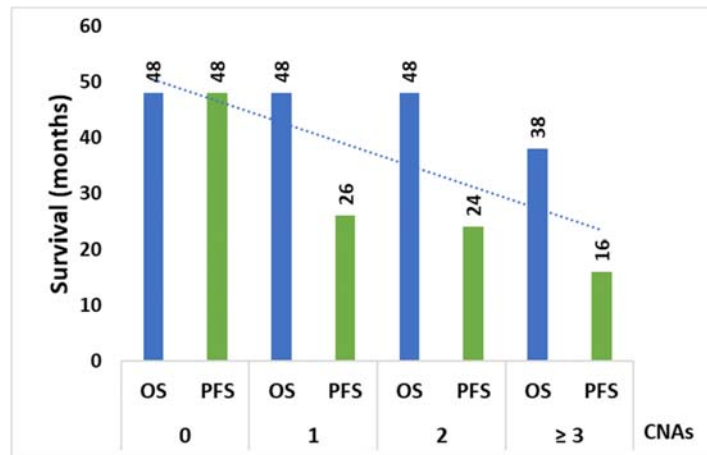
Figure 8. Overall survival (OS) and progression-free survival (PFS) correlated with adverse CNAs. The median PFS and OS were calculated using Kaplan-Mayer means and medians for survival time. 0- no adverse CNAs, 1-one adverse CNAs, 2- two adverse CNAs and ≥ - at least three adverse CNAs..

PFS (months)
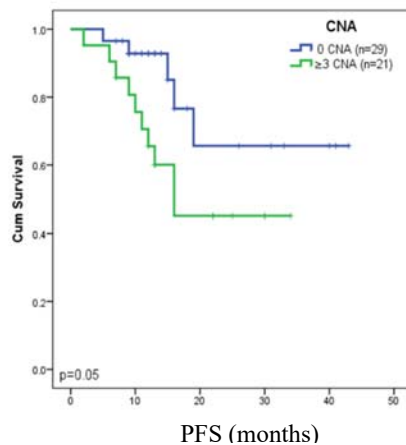
Figure 9. Progression-free survival (PFS) correlated with adverse ANC. using Kaplan-Meier analysis. 0- no adverse NACs, ≥ 3- at least three adverse CNAs..

3.3 Correlation of results obtained from the analyses: MLPA and immunophenotyping

Monoclonal PCs have been identified and phenotypically characterized by MFC. CD19 was predominantly negative except for four cases. Among the surface markers analysed, the following markers were prognostic indicators: CD81, CD19 and CD117, correlated with the identified CNAs.

The CD81 marker was expressed in 62.8% of patients, being an adverse marker with a median OS of 45 months (p=0.012). More than 30% associated with either del13q or dup1q. Association with the latter had a significant negative impact on OS (median survival of 38 months) (p=0.002) and PFS (median survival of 14 months) (p=0.001). In patients in whom CD81+ was associated with more than three adverse NACs (n=13), PFS was low, with a median of 13 months (p=0.045) and OS of 38 months (p=0.017) (Table 6).

Table 6. Correlation of CD81 expression with adverse CNA and clinical prognosis of MM patients estimated by COX and Kaplan-Meier regression analysis.

| Adverse CNAs | N | CD81+ Atypical PCs | | | | | |
| | | OS | | | PFS | | |
		Months	Hazard ratio (95%CI)	p	Months	Hazard ratio (95%CI)	p
del13q	23	38	0.39 (0.18-0.85)	0.013	19	0.62(0.33-1.1)	ns
dup1q	29	38	0.31 (0.14-0.68)	0.002	14	0.38 (0.21-0.7)	0.001
del1p	14	34	0.41 (0.17-0.99)	0.04	16	0.47(0.23-0.97)	0.035
≥3 CNA	13	38	0.36 (0.15-0.87)	0.017	13	0.46 (0.21-1.0)	0.045

Abbreviations: n- number of cases; ns - not statistically significant; p<0.05 was considered statistically significant, months-mean calculated by Kaplan Meier, Hazard ratio resulted from COX regression analysis.

Given the expression of CD19 and CD81, the maturation stages (Paiva et al., 2017a) of atypical PC were correlated with the identified CNAs (Table 20). Patients with CD19(+) CD81(+) (poorly differentiated or undifferentiated) PC (n=4) had a median PFS of 17 months and in 75% of cases were associated with HRD. Patients with CD19(-) CD81(+) (n=62) (intermediately differentiated PC) had a median PFS of 23 months and cases with CD19(-) CD81(-) (mature/differentiated PC) had a median PFS of 40 months. When CD19 and CD81 expression was compared with fewer or more than three adverse NACs, Kaplan-

Maier results revealed: CD19(+) CD81(+) (n= 1) had the lowest median PFS of 7 months; CD19(-) CD81(+) (n=11) had a median PFS of 12 months; CD19(-) CD81(-) (n=7) had a median PFS greater than 48 months (Table 7, Figures 10 and 11)≥.

Table 7. Association between PC and CNA maturation stages.

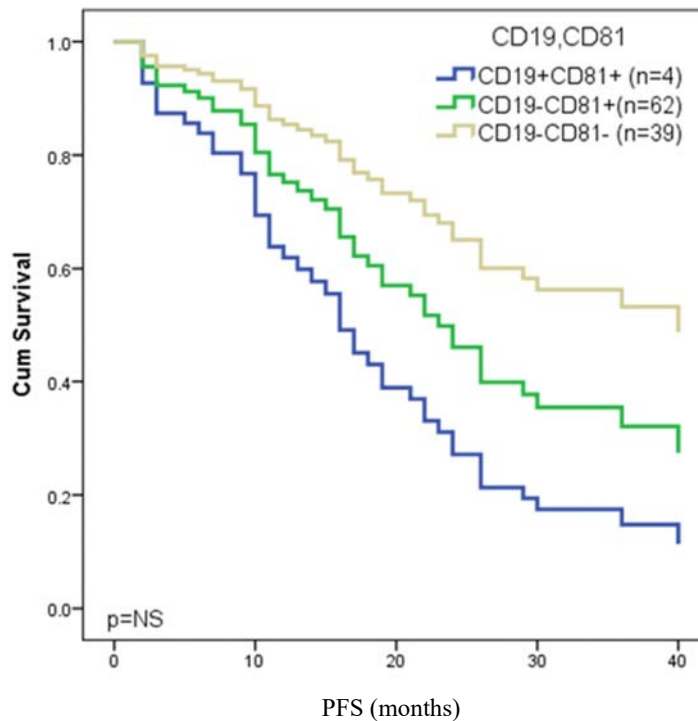| PCs maturation stages	N	Genomic alterations (CNAs)							
		del1p	dup1q	HRD	del12p	del13q	del14q	del16q	del17p
CD19 (+) CD81(+)	4	0%	25%	75%	0%	50%	0%	25%	0%
CD19(-) CD81(+)	62	22.6%	43.6%	46.8%	3.2%	33.9%	8.1%	22.6%	6.5%
CD19(-) CD81(-)	39	20.5%	33.3%	48.7%	0%	41%	7.7%	12.8%	0%

Figura 10. Analiza COX regression a supraviețuirii PFS a grupului de pacienți repartizați în funcție de expresia CD81, CD19.
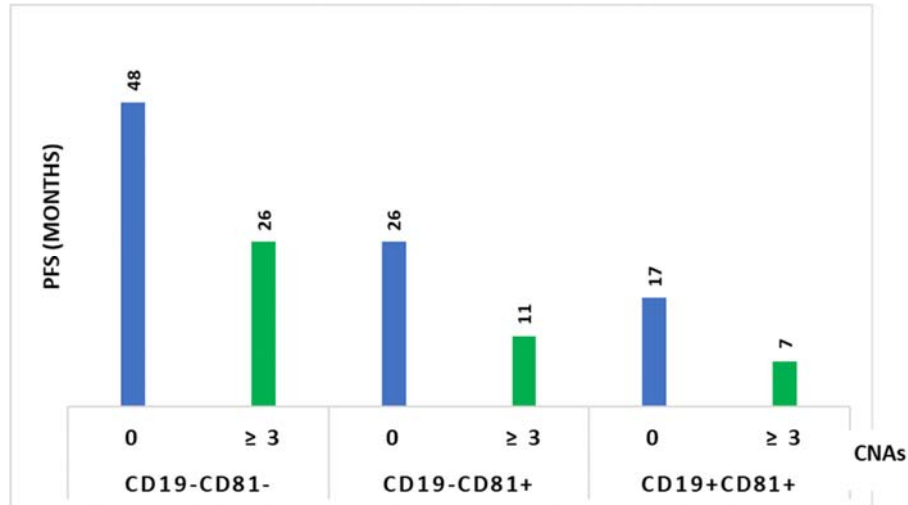
Figura 11. Survival analysis of association between CD19-CD81 and at least three adverse DNA damages. The median PFS and OS was calculated using Kaplan-Mayer means and medians for survival time.

CD117 marker expression was identified in 67 patients and had a favorable impact on PFS (p=0.008) and OS (p=0.019). This prognosis was affected by CD81 co-expression as follows: median PFS increased from 18 months to 36 months in CD117(+) CD81(+) vs CD117(-) CD81(+) (p=0.025) , HR 0.48 (95% CI 0.23-0.90) and median OS from 40 months to more than 48 months (p=0.046, HR 0.44 (95% CI 0.19 -0 .99). There was no difference in PFS or OS within the CD81(-) group with or without CD117 expression (p=0.82/p=0.84) (Figure 11 a and b).

An analysis of the association of CD117 with at least three adverse CNAs was also performed. CD117 expression had a positive impact in the group with less than 3 CNAs. Thus, in this group, CD117 expression increased PFS from 21 to 36 months (p=0.008) and OS from 45 to over 48 months (p=0.02). In the group with more than 3 CNAs, CD117 expression showed no statistical significance, perhaps due to the low number of patients in this group (Figure 11 c and d).

In addition, different stages of PC maturation were compared with CD117 expression and the presence or absence of three adverse CNAs. Thus, in patients with differentiated PC (CD19(-) CD81(-)) with CD117(-) and less than three adverse CNAs, PFS was 24 months compared to the group with at least three adverse CNAs, which had a lower PFS of 13 months. In the group of intermediate differentiated PC (CD19(-), CD81(+)) with CD117(-),

25

the median PFS was 19 months in the absence of three adverse CNAs and significantly decreased to 7 months, when the at least three opposing CNAs.
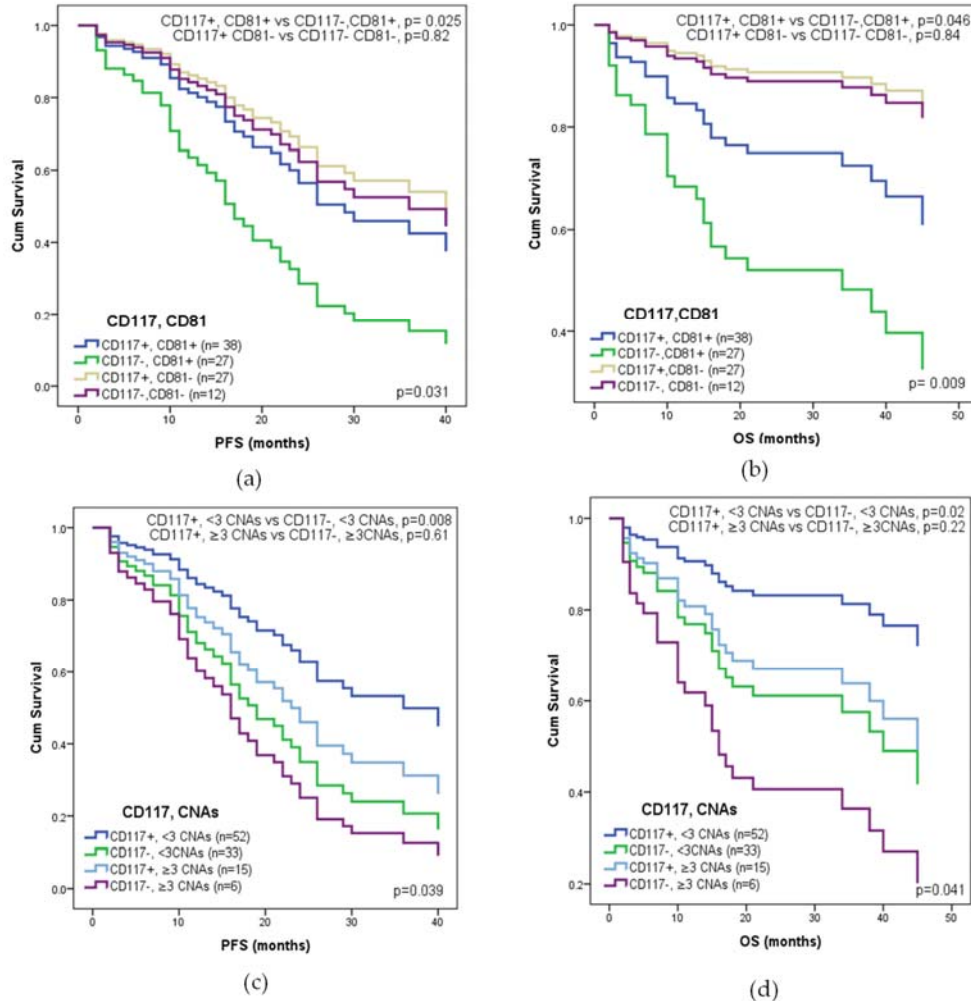
Figure 11. Survival models of MM patients taking into account the co-expression of CD81, CD117 (a)-PFS , (b)-OS and CD117, CNA (c)-PFS , (d)-OS calculated by COX regression analysis .

In the last group, less differentiated PC (CD19(+), CD81(+), only four patients were analyzed: for three of them CD117 was positive and less than three CNAs were identified, leading to a median PFS of 17 months; for the last patient in this group, who had three adverse CNAs and CD117(-), the median PFS was only 7 months. Thus, even though three adverse CNAs decrease PFS in all three stages of maturation, CD117 expression may improve outcome (Figure 12, Table 8).

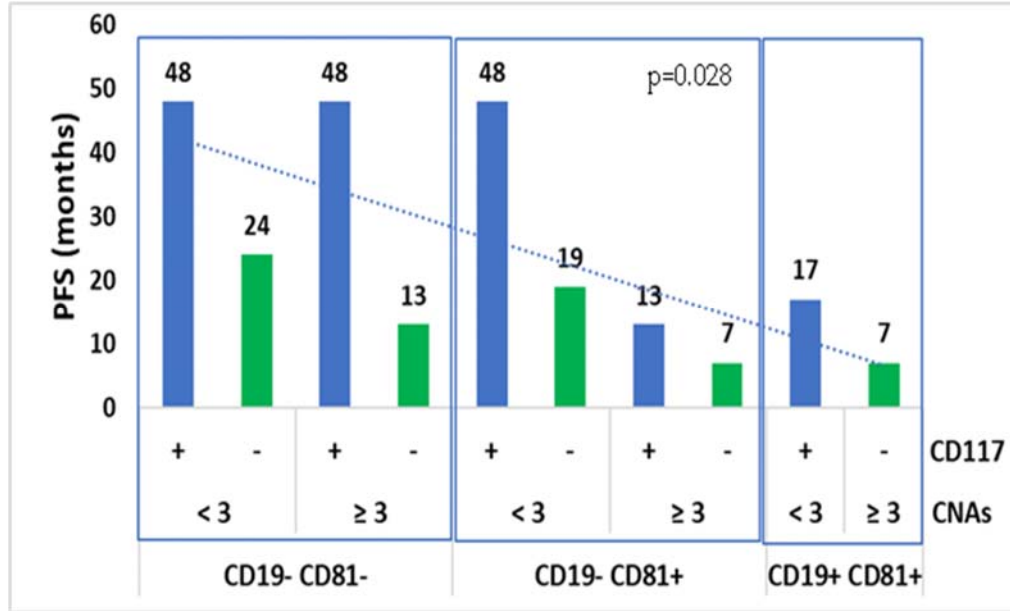Figure 12. Correlation of PCs maturation stages with both the expression of CD117 and adverse CNAs affects the progression-free survival of myeloma patients. The median PFS and OS were calculated using Kaplan-Mayer means and medians for survival time.

Table 8. Correlation between CD19, CD81, CD117 and CNAs

CD19, CD81 (PCs maturation stage)	CNAs	CD117 Positive or negative	Subgroups	N	Progression-free survival Months	Hazard ratio (Subgroups compared)	p value (For subgroups compared)
CD19- CD81- (differentiated)	<3	+	(a)	22	>48	1.88 (0.22-15.69)	-
		-	(b)	10	24	0.68 (0.06-7.70) (b vs a)	0.75
	≥3	+	(c)	5	>48	0.56 (0.06-4.48) (c vs a)	0.53
		-	(d)	2	13	x	x
CD19- CD81+ (intermediate)	<3	+	(e)	27	>48	3.021 (1.28-7.08)	-
		-	(f)	23	19	0.33 (0.14-0.77) (f vs e)	0.008
	≥3	+	(g)	9	13	0.35 (0.12-1.04) (g vs e)	0.049
		-	(h)	3	7	0.21 (0.042-1.05) (h vs e)	0.051

| CD19+ CD81+ (little or non-differentiated) | <3 | + | (i) | 3 | 17 | 0.28 (0.76-1.09) (i vs e) | 0.052 |
| | ≥3 | - | (j) | 1 | 7 | x | x |

Analysis of PC groups together with adverse CNAs and CD117 expression revealed relevant results only in the CD19-CD81+ group. Thus, CD117 expression had a positive impact when fewer than three adverse CNAs were identified.

In general, CD117 expression improved outcome in intermediate differentiated PCs, regardless of the number of DNA alterations. As a result, the favorable prognostic factor CD117 should be considered in the disease staging and follow-up algorithm. Consequently, this study shows that CD19 and CD81 divide MM patients into three distinct PC differentiation groups, which associated with adverse CNAs and CD117, allow the identification of new subgroups with different prognoses, which may represent additional targets for future studies and even and for specific therapies.

3.4 Results and discussion - SNParray

The standard techniques for identifying genetic abnormalities in MM are karyotyping and FISH (Moreau et al., 2021), but as our knowledge of genetic alterations has grown, so has the methodology to detect new genetic information. The cytogenetics of atypical PC is often patchy and difficult to characterize especially in the early stages of the disease (Neuse et al., 2020). FISH is limited to a small set of common abnormalities and karyotyping has a success rate according to previous studies of only 30% (Gole et al., 2014). Thus, SNParray comes to the aid of classic techniques by being able to detect small changes in the number of DNA copies, at a very high resolution (<200 kb) (López-Corral et al., 2012).

50 patients were investigated by this technique. Data were visualized using CytoGenomics v3.0 software. All analyzed patients showed chromosomal abnormalities (Figure 13). All the data obtained were compared with those obtained by MLPA, observing a 100% concordance.

After analyzing the slides, it was observed that 52% (n=26) of the patients presented trisomies of the odd chromosomes (more than 3 chromosomes involved) and 5.76% (n=3) presented small anomalies (Figure 13 and 14) .
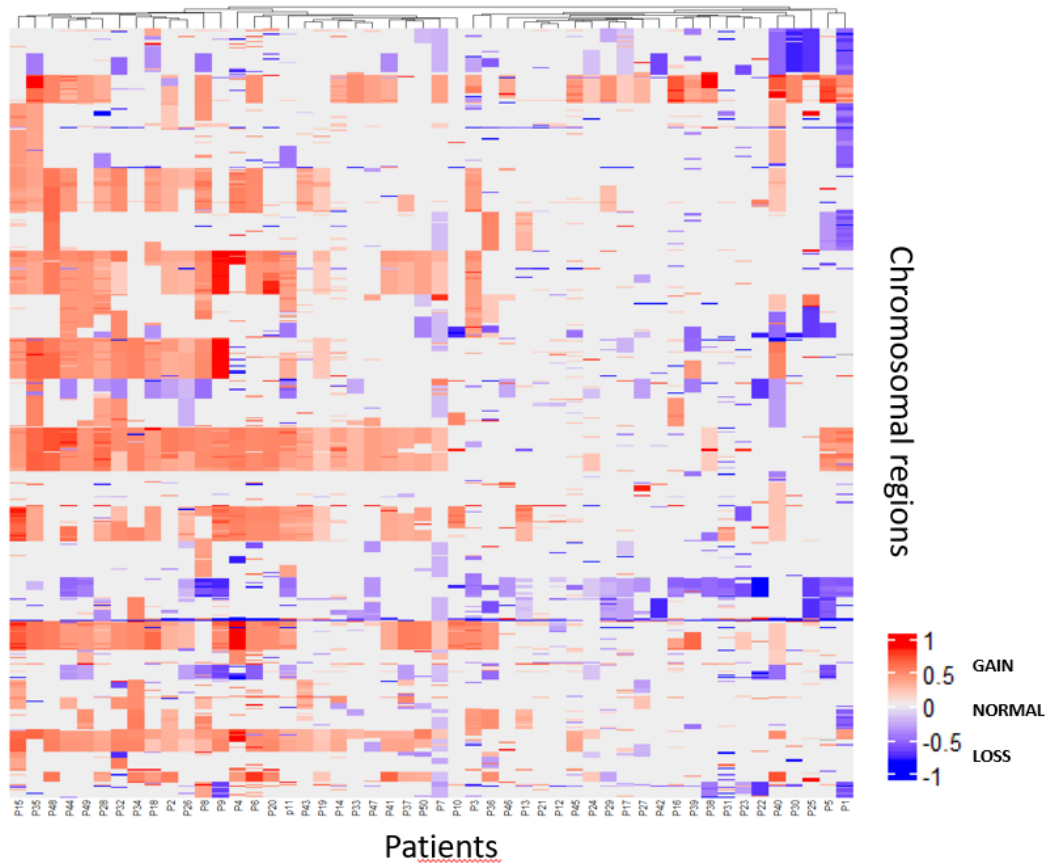
Figure 13. Genetic profile of patients investigated by SNP array

Odd chromosome trisomies are an important diagnostic diagnostic criterion (van de Donk et al., 2021). The trisomies of chromosomes 9 and 19 were most frequently encountered. According to the literature, duplications of chromosomes 3,5 and 21 are the most frequent (González et al., 2007) (Figure 15).
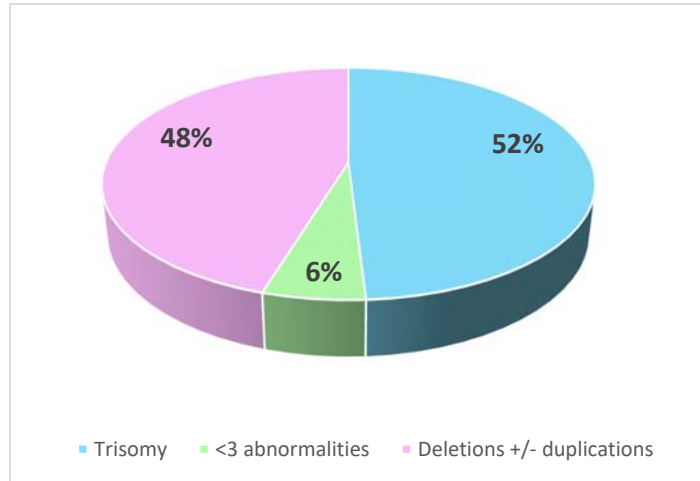
Figure 14. Distribution of the study group according to the anomalies presented
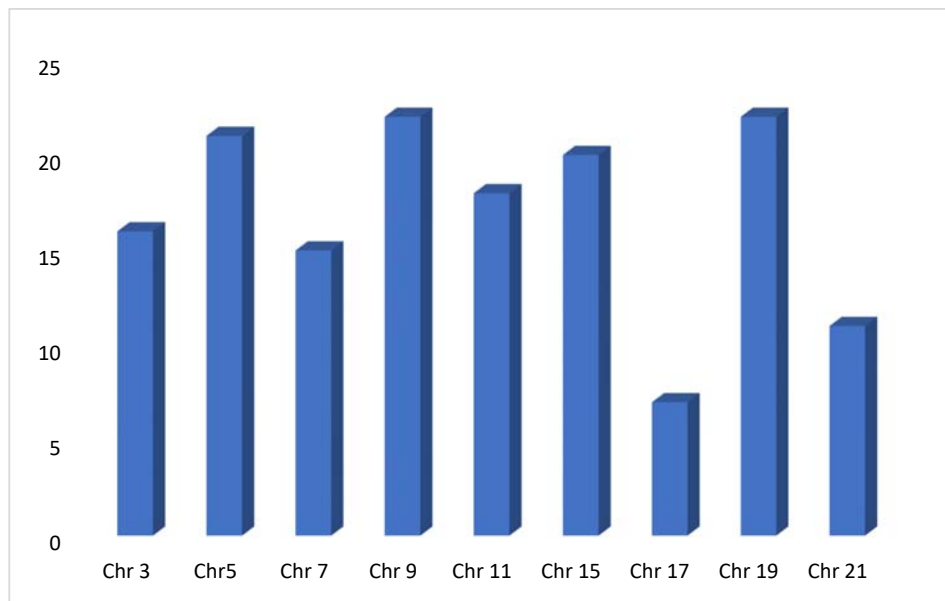
Figure 15. Frequency of odd chromosome duplications

Deletion of the p arm of chromosome 1 was found in 18 cases. Statistically del1p22.1 conferred a negative prognosis to patients (p=0.002, n=17) with median survival for PFS being 13 months. The most frequent deletions were 1p22 (17/18) and 1p13 (17/18). Overall progression-free survival of the group presenting this abnormality was only 16 months with an HR of 0.84 (95% CI 0.43-1.62).

Dup1q was present in 27 cases. Even though survival did not show statistical significance, the median calculated by the Kaplan Meier method was only 13 months for PFS and the HR was 0.66 (95%CI 0.34-1.27) (Figures 16 and 17).

Patients
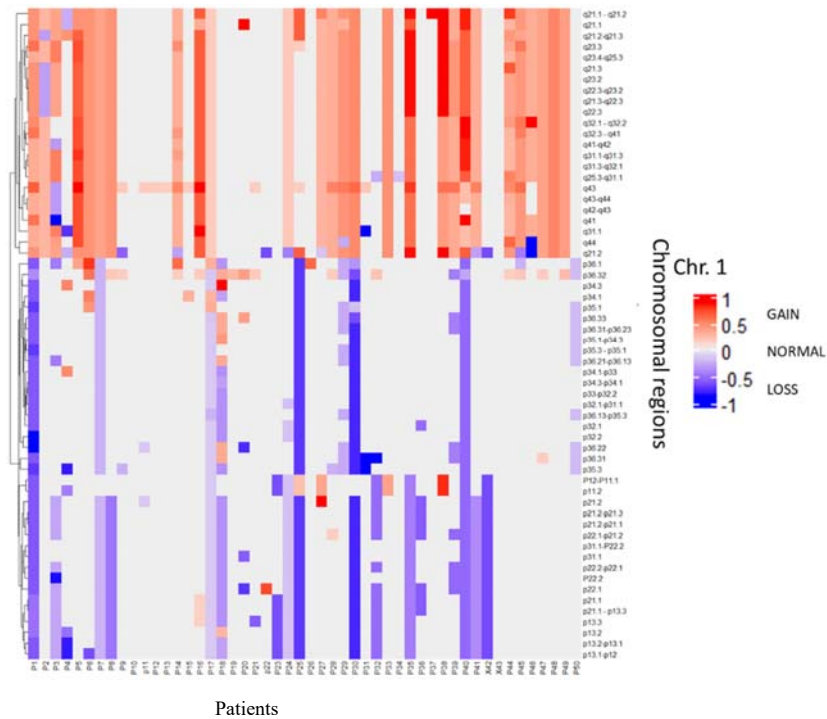
Figure 16. The genomic profile of chromosome 1 in the studied group.

(a)   (b)
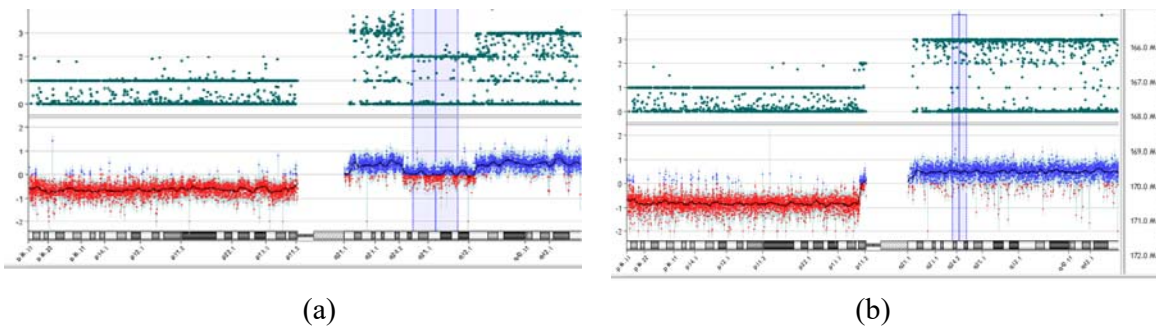
Figure 17. (a) Case#1 – Deletion of arm p of chromosome 1 and partial amplification of arm q (presence of multiple complex processes) is observed, (b) Case 30 - Deletion of arm p and loss of heterozygosity for arm q with amplification up to 3 copy.

On chromosome 2 in 3 cases (p15,p35 and p41) dup2q37 was observed suggesting the presence of der(2)t(1;2)(q12-21;q37) a rare abnormality described only in 8 cases with

MM (Busson Le Coniat et al., 2000, Sawyer et al., 2014) (Figure 96). In the case of the analysed group it seems that this abnormality conferred an adverse prognosis. In 6 cases large duplications of chr 2 were observed and in 3 deletions without statistical significance (Figure18).
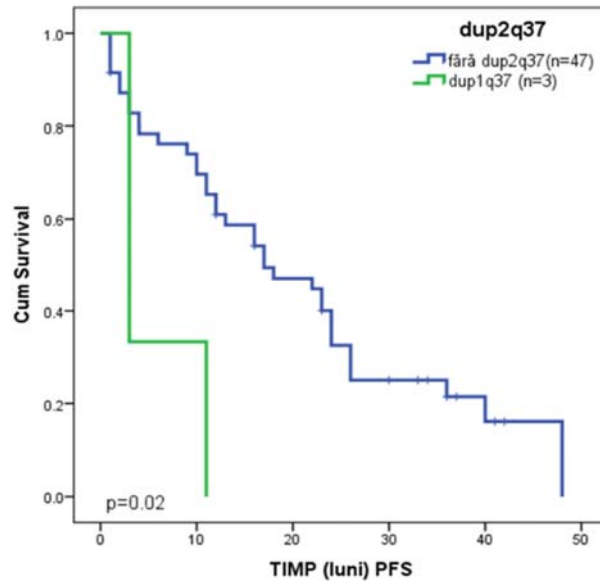
Figure 18. Survival (PFS) of studied patients distributed in groups according to the presence or absence of dup2q37.

Even though chromosome 3 trisomy was reported to be present in patients with hyperdiploidy and therefore with a positive prognosis in our group this abnormality had a negative impact being present in 17/50 of cases with a median PFS of only 11 months (p=0.046) and for OS of only 17 months (p=0.05) (Figure 19).

(a)   (b)
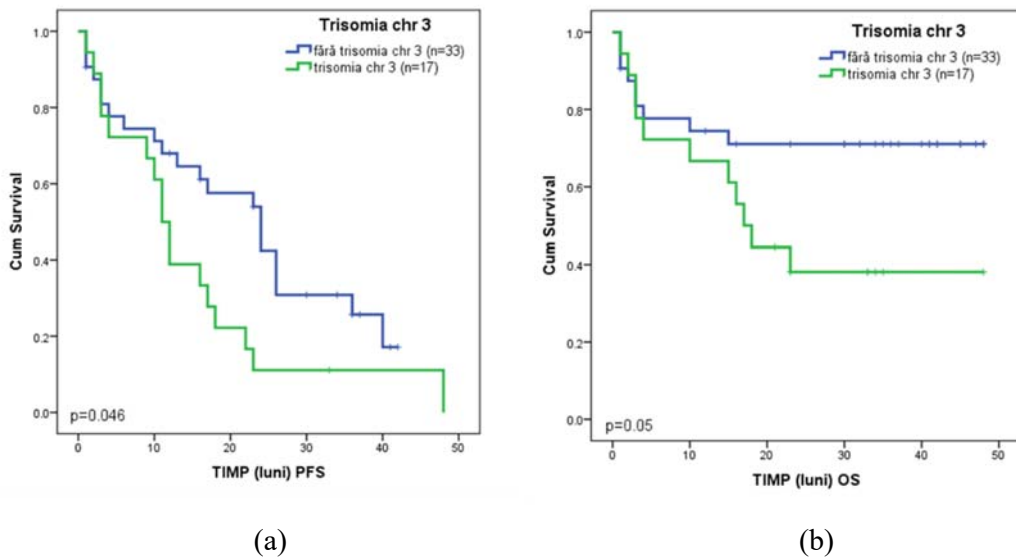
Figure 19. Overall survival (OS) (b) and progression-free survival (PFS) (a) of multiple myeloma (MM) patients correlated with the presence or absence of trisomy chr 3.

The 5q deletion was found only in one patient who had a survival of only 2 months. This deletion is mostly found in myelodysplastic syndromes and is always associated with a negative prognosis (Ortega et al., 2013).

The presence of 20 diverse processes (chromoplexi, chromotrypsis and chromanosynthesis) was also observed in 18 patients (Figure 20).

| Case 4 chr 16- complex event | Case 11 chr 6-complex event |
| Case 17 chr 1- complex events | Case 44 chr 1-Chromoanasynthesis |
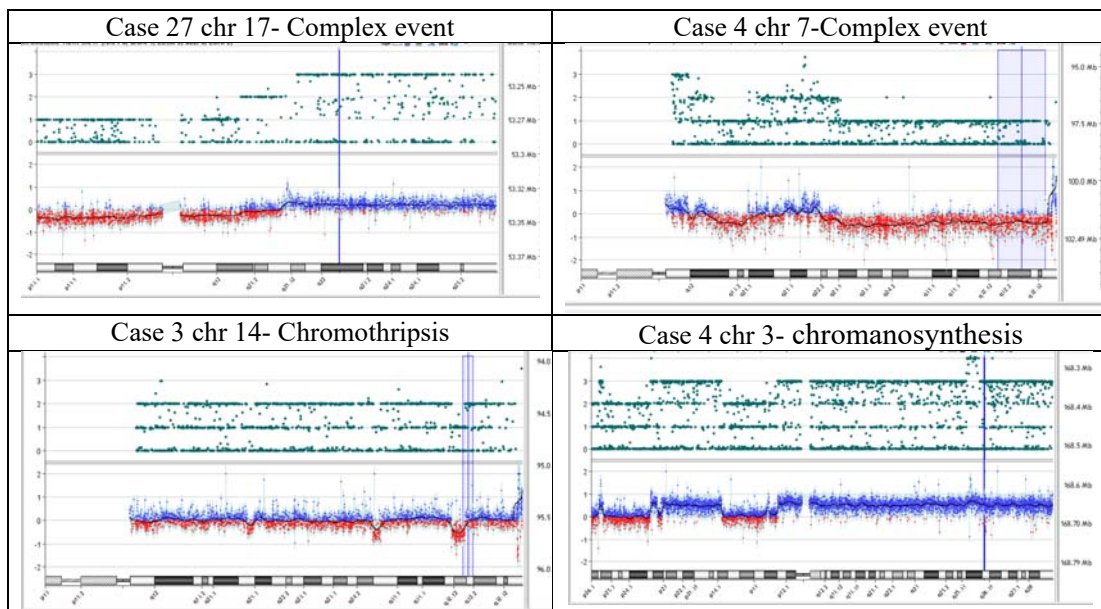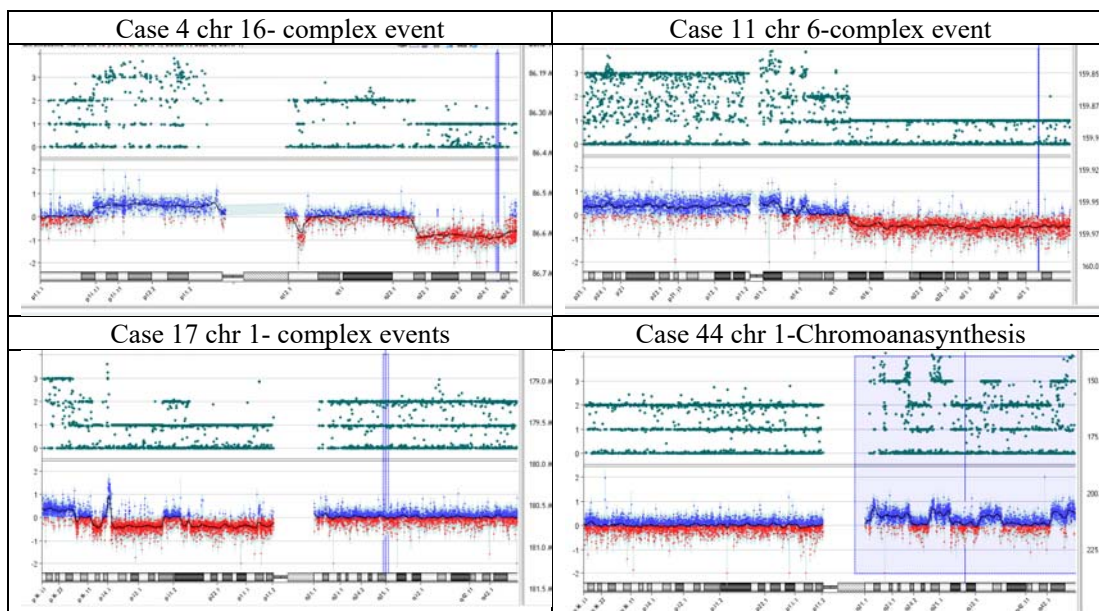
Figure 20. Examples of complex events/chromothripsis/chromoanasynthesis

Patients with these complex abnormalities had a more aggressive outcome with a PFS of only 17 months versus patients without these chromosomal alterations who had a PFS of 30 months. All samples whose PCs expressed CD19+, CD81+ at the membrane level showed such complex events.

The 13q deletion another important factor in the diagnosis of MM was found in 30% (n=7) of the group of samples presenting trisomy of odd chromosomes and in 61% (n=16) of cases presenting less than 3 amplified odd chromosomes. In the whole study group 44% (n=23) had del13q which is in agreement with previously reported studies (approx. 50%) (Königsberg et al., 2000).

Loss of heterozygosity is a sporadic common event in the study group with no apparent statistical significance in terms of disease progression and response to treatment (Figure 21).

A	B	C
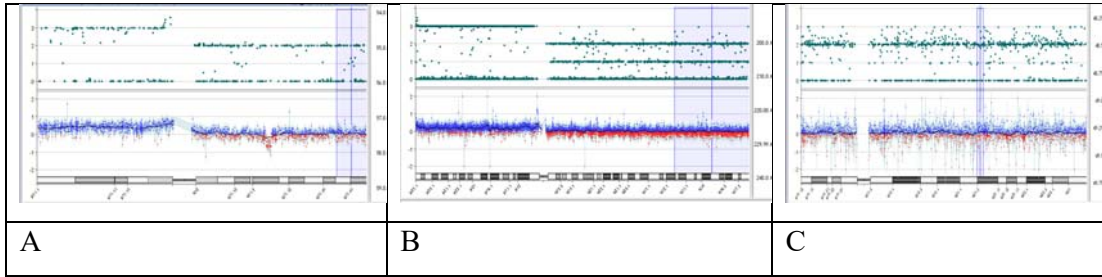
Figure 21. A- Chr 19, case 1- loss of heterozygosity of the entire chr with amplification of up to 3 copies of the p-arm is observed, B. Case 2, LOH with amplification of p arm chr 2, C. Case 25 chr 18 -loss of whole chromosome heterozygosity.

The results obtained by this technique illustrate, as in the case of MLPA, a high genomic instability. Unlike MLPA, the array is a more expensive technique but it provides an overview of the whole genome, which in the case of MM seems to be increasingly necessary for a correct diagnosis and for the patient to have access to targeted therapy.

3.5 Results and discussions - CARIOTIP and FISH

Of the total samples processed, only 22 provided analyzable metaphases (Figure 22).
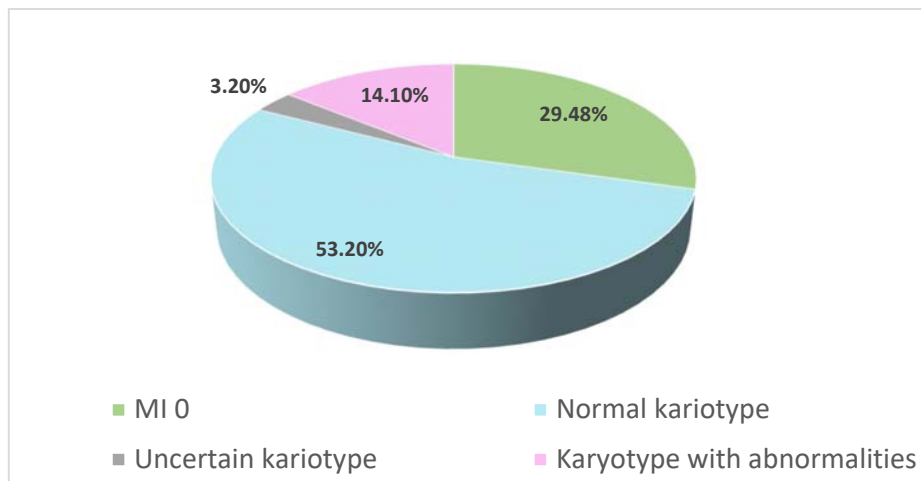
Figure 22. Distribution of results obtained from karyotype analysis

The success rate for this analysis was below the average reported in the literature (12.8 vs. 30% of cases) (Hamdaoui et al., 2020).

Hyperdiploidy was found in 11/22 cases (falling within previously reported limits (Robiou du Pont et al., 2017). Most frequently chromosome 5 and 19 duplications were present followed by chromosomes 9 and 15 (Figure 23) .
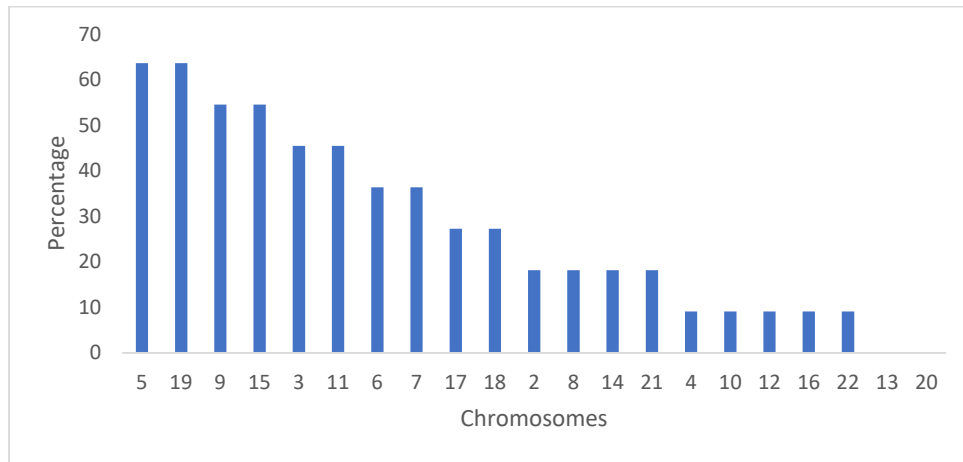
Figure 23- Frequency of chromosomes with trisomies identified by karyotype.

The most common deletion was chromosome 13 deletion followed by chromosome 8 deletion. Del13 was associated in 2/22 cases with t(11;14).

Translocations of chromosomes 4 and 14 (t(4;14)), 14 and 16 (t(14;16)) and deletion of chromosome 17p (del(17p)) are criteria for disease stages according to R-ISS (Greipp et al,2005). Ongoing research reveals a complex genomic landscape in MM characterized by major chromosomal instability. t(11;14) has been characterized as having an intermediate prognosis for MM patients (Paner et al., 2020). MM cells with t(11;14) have a unique biology, with relatively higher expression of the anti-apoptotic protein BCL2 and lower expression of MCL1, in contrast to MM cells without this translocation. The t(11;14) translocation emerged as the first predictive marker in MM, indicating susceptibility to BCL2 inhibitors (Kaufman et al., 2021). In our group a total of 4 patients showed this translocation (identified by FISH- Figure 24).
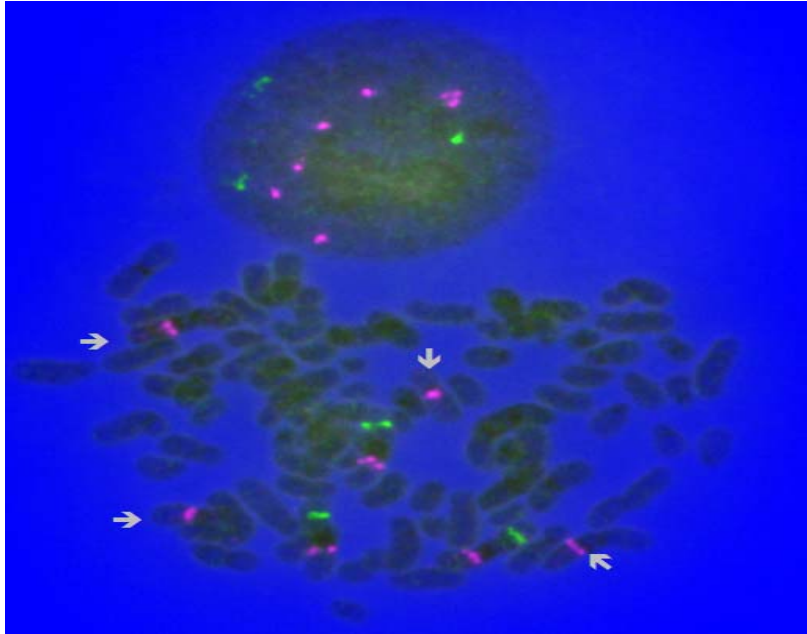
Figure 24. FISH image showing t(11;14).

The 17p deletion was present in 4 cases. This DNA copy number variation has negative implications in many cancers (Kaufman et al., 2021). In our group, patients with this abnormality who were investigated by classical cytogenetics had an average survival of only 10 months.

Cases showing multiple clones and tetraploidy were also detected by karyotyping. A statistical evaluation could not be performed due to the small number of samples with analysable metaphases.

In conclusion, FISH provides information on the presence of translocations with a prognostic role in patients with MM and karyotyping provides a genome-wide overview with characterisation of clones and subclones in the sample but at a low resolution and directly proportional of atypical PC efficiency of proliferation in the extramedullary environment.

GENERAL CONCLUSIONS

A better understanding of the genomic instability of clonal plasma cells in multiple myeloma (MM) patients contributes to the selection of optimal treatment strategies with long-term benefits.

The identification of new markers in the context of streamlining methodology is essential in the early diagnosis of MM.

A complete description of the mechanisms of transformation of plasma cells into atypical plasma cells and the mutations involved in this process will contribute to the understanding of the process of tumour progression in multiple myeloma and the identification of targeted solutions in therapy.

The results of this study led to the following conclusions:

➤ Enrichment of bone marrow samples (from patients diagnosed with MM) by sorting, gives better resolution when identifying genetic abnormalities by both classical cytogenetic techniques (iFISH) and molecular biology techniques (SNParray, MLPA, etc.).

➤ The results obtained by immunophenotyping analysis demonstrated that in the case of differentiation cluster 117 (CD117) it can be considered an independent positive prognostic factor, as the progression-free survival in the analysed group was statistically significantly better (p=0.04).

➤ CD81 is a negative prognostic factor in the analysed group, indicated by COX regression analysis, hazard ratio being 0.62.

➤ CD19 and CD81 co-expression divide PC into three differentiation stages (poorly differentiated PC: CD19+,CD81+; intermediate differentiated: CD19-, CD81+ and differentiated: CD19-, CD81-) with different treatment response and survival rate respectively.

➤ In multiple myeloma, karyotyping is not always a reliable method to identify abnormalities, as atypical plasma cells have a low proliferation rate in vitro and are dependent on the bone marrow microenvironment.

➤ High-performance diagnostic tests such as SNParray and MLPA allow both the identification and description of new genetic abnormalities with implications for the development of MM and the optimisation of diagnostic strategies and follow-up of disease progression under treatment.

- Correlation of MFC data revealed that in atypical plasma cell differentiation groups characterized by coexpression of CD19 and CD81 the presence of CD117 confers a positive prognosis (p=0.024).

- Within the CD19-, CD81+, CD117+ group the presence of at least three adverse copy number abnormality (CNA) areas resulted in decreased patient survival (p=0.008).

- Although only the CD19-, CD81+ group was statistically relevant regarding overall outcomes in all 3 stages of plasma cell differentiation, groups with a high risk of progression characterized by CD117- and ≥3 adverse CNAs emerged.

- Immunophenotyping provides details on the expression of various proteins at the membrane level, and the correlation of these data with chromosomal abnormalities, expression of molecular markers, treatment and survival rates will allow the identification of new treatment strategies.

- Compared to classical karyotyping and genotyping methods, the SNParray technique provides a higher resolution overview of genome-wide abnormalities.

- Processes involving complex events, chromotripsis and chromatinase, have negative effects on the onset and progression of different cancers. In the group analysed, these events reduced median PFS at 10 months (p=0.05) and OS at 15 months (p=0.049).

- All patients with CD19+,CD81+ plasma cells showed such complex processes.

- Karyotyping although has a low resolution, by cell-by-cell analysis provides important information about the clones present in the sample.

- The FISH analysis technique allows the detection of translocations that have a profound adverse prognosis.

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