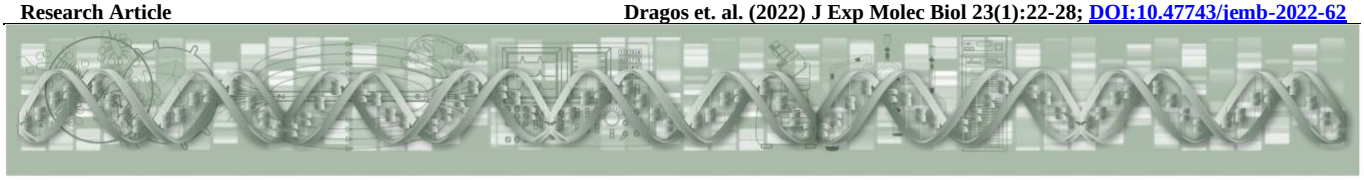

OPTIMIZATION OF PLASMA CELL ENRICHMENT FOR CNV DETECTION BY SNP ARRAY AND MLPA

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ABSTRACT

Multiple myeloma (MM) is characterized by the uncontrolled proliferation of an atypical plasma cell clone. The main factor preventing the characterization of plasma cells is their low rate in the analyzed samples. Through this study we tried to highlight the necessity of enrichment of MM samples to be analyzed by MLPA and SNParray. Samples from 5 patients diagnosed with MM were investigated and 2 of them were enriched by magnetic sorting with anti-CD138 antibodies and analyzed by SNParray and MLPA. Unsorted samples showed a lower incidence of abnormalities. By analyzing the data, we concluded that even a 30% malignant cell infiltration in the MM sample is not enough and the magnetic sorting is a mandatory for the enrichment of target cells from the sample.

Keywords: Plasma Cell enrichment, SNP ARRAY, MLPA, CNV.

INTRODUCTION

Plasma cell (PC) is the last stage of differentiation of B lymphocyte and is implicated in humoral immunity response. The accumulation of mutation in this cell type will determine an abnormal proliferation in the bone marrow which will create the environment necessary for the emerging of cancers. Multiple myeloma is a type of hematological malignancy characterized by the accumulation of at least 10 % abnormal PC in the bone marrow (Rajkumar 2019). Despite all advancements done until know in the understanding of the pathogenesis of PC disorders, these malignancies remain hard-to-treat (Morelli, Gullà et al. 2020) this is why for the diagnosis, risk stratification and approach to treatment the clear identification and characterization of plasma cells is crucial. Whole genome screening studies have shown that almost all MM patients harbor genetic abnormalities (Zang, Zou et al. 2015).

CD138 (syndecan-1, Sdc-1) is a member of the syndecan family and is implicated in cell-cell and cell-matrix interactions. In adult human tissues, CD138 is predominantly expressed in epithelial cells and plasmacytes (Palaiologou, Delladetsima et al. 2014). This fact makes CD138 the perfect target in identification and isolation of plasma cell by various technique.

The most frequent type of abnormalities in MM is the CNV's (copy number variations). The duplication of odd chromosome (3, 5, 7, 9, 11, 15, 19, 21) divides MM in hyperdiploid and non-hyperdiploid (Buedts, Smits et al. 2020) and is found in around 50% of MM and confer a good prognosis (Fonseca, Barlogie et al. 2004, Barilà, Bonaldi et al. 2020, Ankathil, Foong et al. 2021). The deletion of chr 1p has an incidence of approximatively 30% and give a poor prognosis especially if 1p32 is implicated (Qazilbash, Saliba et al. 2007, Ouyang, Gou et al. 2014). Chr 12p has been described as a negative prognostic marker (Li, Xu et al. 2015). Chr 13q (48-50% incidence) deletion has no prognostic by it's on but in association with other abnormalities it's a worsening prognostic factor (Binder, Rajkumar et al. 2017). TRAF3 gene (chr14q32) abnormalities, with an incidence of approximatively 15% modulate proteasome inhibitor response in MM (Wixted, Rothstein et al. 2012, Neja 2020). Chr16q deletion has an incidence of 20-35% is suggested to be associated with adverse clinical outcome in MM (Jenner, Leone et al. 2007, Walker, Leone et al. 2010). Del17p targeting gene TP53 is a well-known worse prognostic factor in cancers mutation of this gene are the most frequent somatic event(Bouaoun, Sonkin et al. 2016). In MM it confers a bad prognosis especially when is associated with another DNA lesion (double hit) (Corre, Perrot et al. 2021)

For many years the only way to identify chromosomal abnormalities was conventional cytogenetics (Karyotype and FISH). In MM these techniques have a 30-50% success due to low infiltrate of PC in the BM samples and/or PC low mitotic index because of their dependents of BM environment (Saxe, Seo et al. 2019).

SNParray and MLPA are two molecular techniques that can provide information about CNV. SNParray can also identify loss of heterozygosity, chromothripsis, chromoanasyntesis and complex copy number changes at a high resolution (<200 kb)(Marcozzi, Pellestor et al. 2018).

The aim of this study is to show the importance of increasing the plasma cell infiltration in the sample for the detection of CNV by SNParray and MLPA.

MATERIALS AND METHODS

The **study group** included 5 patients with various PC infiltrations which were diagnosed with MM in Regional Institute of Oncology, Iași Romania, in 2017-2018. This study was approved by the local ethics commission and the

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written consent to participate in studies involving human participants was signed by each patient. All the bone marrow (BM) samples were harvested on anticoagulant EDTA. The BM samples were first evaluated by flow cytometry to establish the PC infiltration.

PC's sorting was made using CD138 plasma cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacture protocol and to previous publications (Horst, Hunzelmann et al. 2002). Briefly, after the BM samples were centrifugated for 10 min at 300xg, the supernatant was discarded, the sample was resuspended in 1 ml RPMI 1640 and slowly put on top of 5 ml Ficoll Histopaque®-1077 (Sigma Aldrich) and centrifugated at 400xg for 40min.The mononucleate cells (BMMC) were recovered and washed 2 times with PBS by centrifugation for 10 min at 300xg. Then we incubated the BMMC with CD138 microbeads at a titre of 1:5. After 30 min of incubation at 4°C, cells were washed once with the recommended buffer (PBS with 5 mm EDTA and 0·5% BSA). After resuspending cells in 1 ml buffer, they were separated using MS-columns (Miltenyi Biotec).

Genomic DNA (gDNA) was **extracted** with Wizard® Genomic DNA Purification Kit – Promega according to the manufacture instruction. Briefly, 900µl lysis solution was applied to plasma cells suspension and incubated for 10 min at room temperature (TC). After the solution was centrifugated for 16.000xg, 20 sec, the supernatant was discarded, 300µl nuclei lysis solution was applied, mixed and 100µl precipitation solution was added. The whole mixture was vortexed, centrifugated and the supernatant was moved to a new tub with 300µl isopropanol. The pellet of DNA was obtained by centrifugation at 16.000xg for 1 min. Fallowed two washes with 70% ethanol and the rehydration in 100 µl nuclease free water.

The quality and quantity of isolated gDNA were measured by using Nano Drop 2000 (Thermo Fisher Scientific, USA). DNA samples were then stored at -20°C prior used.

For **Multiplex ligation-dependent probe amplification (MLPA) analysis,** according to the manufacture instruction, all DNA samples were diluted to 20ng/µl. 5µl of diluted DNA was denaturized for 5 min at 98^0C, mix with the probe and again denaturized for 1 min at 95^0C and incubated overnight for 16-20 h. The kit used in this analysis was SALSA MLPA P425-B1 MM probe mix (MRC-Holland, Amsterdam, Netherlands). This kit has 46 probe which target the following regions: 1p32.3 (FAF1, CDKN2C), 1p32.2 (PLPP3 and DAB1), 1p31.3 (LEPR), 1P31.2 (RPE65), 1p21.3 (DPYD), 1p21.1 (COL11A1), 1p12 (FAM46C), 1q21.3 (CKS1B), 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). After probe hybridization fallowed the ligation and PCR reaction. In the analysis for every six samples, we used 2 control samples from normal individuals. MLPA products were analyzed using ABI 3500 Genetic analyzer (Applied Biosystems, Foster City, CA, USA) and Coffalyser software (MRC Holland, Amsterdam, Netherlands) according to the manufacture instruction.

Interpretation of MLPA results: The median dosage quantity (DQ-ratio) represents the median of all ratios of the same type CNV.

The cut-off values for the DQ ratio of the probes, established by the manufacturer, were 0.8 and 1.2. Values grater then 0.8 and lower than 1.2 were considered normal. Values grated then 0.4 and lower than 0.6 were heterozygous deletion and grated then 1.75 were considered duplication. Value 0 was considered homozygous deletion.

SNP array was performed as previously described (Mikulasova, Wardell et al. 2017). Briefly, 2-3µg gDNA from patients and from control DNA were fragmented by enzymatic digestion with AluI and RsaI restriction enzymes and fluorescently labeled with Cy5 and Cy3. After purification of labeled DNA, patients and control DNA samples were combined with COT Human DNA (Hoffmann-La Roche) and hybridization mix (Oligo aCGH Hybridization Kit, Agilent Technologies), and co-hybridized to SurePrint G3 CGH+SNP, 4×180K (Agilent Technologies) arrays. After hybridization and washing, DNA microarrays were scanned using a Microarray Scanner (SureScan-Agilent Technologies) with 3 µm resolution. Feature Extraction Software 12.0.2.2 (Agilent Technologies) was used for data extraction and quality control evaluation. Genomic CytoGenomics software v2.0 was used for CNA calling by the ADM-2 algorithm with the following settings: ≥100 kb size, ≥0.2-fold change of log2 ratio, ≥5 consecutive probes. For the data interpretation as manufactures recommends we used the thresholds listed in the table below (Table 1). The median log. ratio is represented by the median of all ratios of the same type CNV. In the analysis we excluded chr X and chr Y and also CNV from 14q32.33 (which is an artifact based on duplication in the reference sample used).

Table 1. Logarithmic ratio (log. ratio) threshold of SNParray interpretation

aCGH data interpretation	
CNV	Log. ratio
LOSS/DELETION	<-0.2
NORMAL	-0.2<and <0.2
GAIN/AMP	0.2<

RESULTS AND DISCUSSIONS

In the first step we evaluated 3 unsorted samples using MLPA and SNParray: one patient with 77% PC infiltration (sample A), one with 43% PC infiltration (sample B) and one with 18% PC infiltration (sample C).

The aCGH analysis results show that **sample A** harbor deletion of chromosome (chr) 1p, 2, 4, 13, 18 and 22, duplication of chr 1q, 9, 19p and other small deletion and duplication. In total there were 80 DNA lesion. The median log. ratio for gain was 0.56 and for loss -0.68. The **sample B** harbor a hyperdiploid karyotype characterized by duplications of chr 1q, 3, 5, 7, 9, 15,18,19, 21 and deletion of 8p, 12q21.3-q23.3, 14q22.1-q31.1. In total there were 43 DNA lesions. The median log ratio for the gain was 0.37 and for loss -0.58. **Sample C presented** a normal karyotype (Figure1).

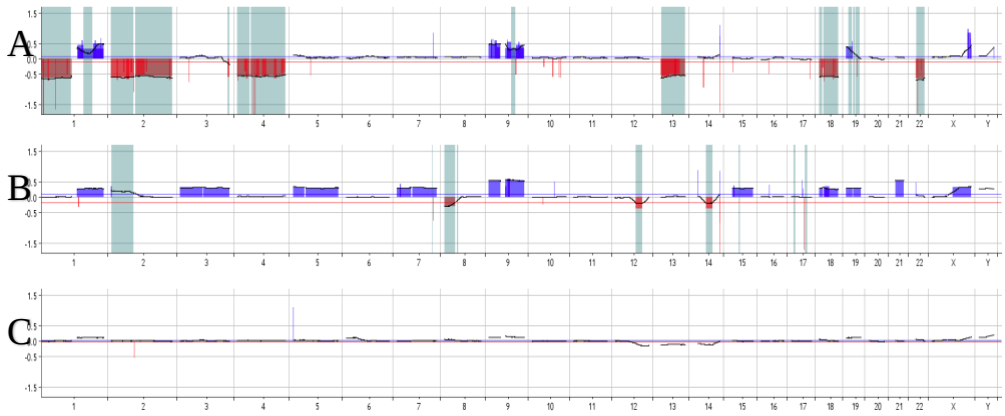


Figure 1. SNParray results of: A-sample with 77% plasma cell infiltration; B-Sample with 43% plasma cell infiltration; C-sample with 18% plasma cell infiltration.

Considering the median ratio, with the decrease of the percentage of PCs, the capacity to distinguish the anomalies presented in the clone of interest decreases, and at very small percentages (~20%), no copy number alterations can be identified at all (Table 2; Figure 1)

Table 2. Log ratio obtained by aCGH and MLPA in samples with different PCs percentage.

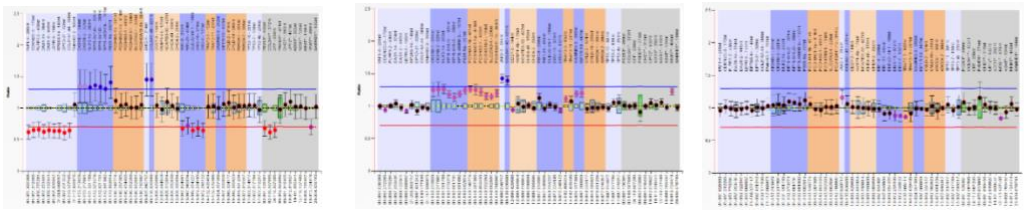
		log. ratio		
		A 77% PC	B 43% PC	C 18% PC
aCGH	GAIN	0.56	0.37	0
	LOSS	-0.68	-0.58	0
MLPA	GAIN	1.37	1.23	1
	LOSS	0.64	1	1

From the MLPA analysis results show that **sample A** presented deletion on chr1p and chr13 and duplication on chr9 median DQ ratio for gain was 1.37 and for loss 0.64. Additionally in the control chr reagion the signals of the probe on chr 2, 4 and 18 are lower suggesting the presence of deletion in this region.

Sample B harbor duplication of chr9 and with a lower signal also duplication of chr 1q, 5, 9 and 15. Median DQ ratio for gain was 1.23.

Sample C didn't present any CN (fig2)

Even if the MLPA couldn't presented a genomic view, all the abnormalities that was identified are in concordance with aCGH results for all three samples.



A B C

Figure 2. MLPA results of: A-sample with 77%plasma cell infiltration (1797); B-Sample with 43% plasma cell infiltration (1884); C-sample with 18% plasma cell infiltration (2434).

Based on these observations (aCGH and MLPA) and considering that there is no MM without cytogenetic abnormalities, the absence of alterations in case C was considered a normal false result due to the low percentage of PC.

Those, in the second step we investigated by SNParray 2 samples: **sample D** with 30% PC and **sample E** with 23% PC infiltration from which we separated and analydes: CD138+ cell fraction, CD138- cell fraction and the unsorted sample.

SNParray results from **sample D CD138+** show the presence of duplication of chr 1p13.3-p11.2, 3, 5p, 9, 11, 15, 16p, 19p, 21, deletion of chr 12q12-q15 and 16q11.2-q24.3 and other small CNV. Total number of DNA lesion was 106 with a median log. ratio for gain of 0.66 and for loss -0.76 (Table 3). **Sample D unsorted** harbour only a part of the CNV presented in CD138+ fraction, which are duplication chr 3p12.2-p11.1, 5p, 9p23-p22, 11p15-p11, 15,19p13-q13 and deletion of chr 12q12-q15 and chr 16q22.2-q24.3. The total nomber of DNA lesion was 48 with a median log. ratio for gain of 0.57 and for loss -0.37 (Table 3). The **sample D CD138-** sample didn't present any abnormalities. (Figure. 3).

Table 3. Log ratio of sorted versus unsorted samples using cCGH and MLPA techniques.

		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
MLPA	GAIN	1.69	1.22	1	1.35	1.2	1
	LOSS	0.64	0.79	1	0.68	0.68	1

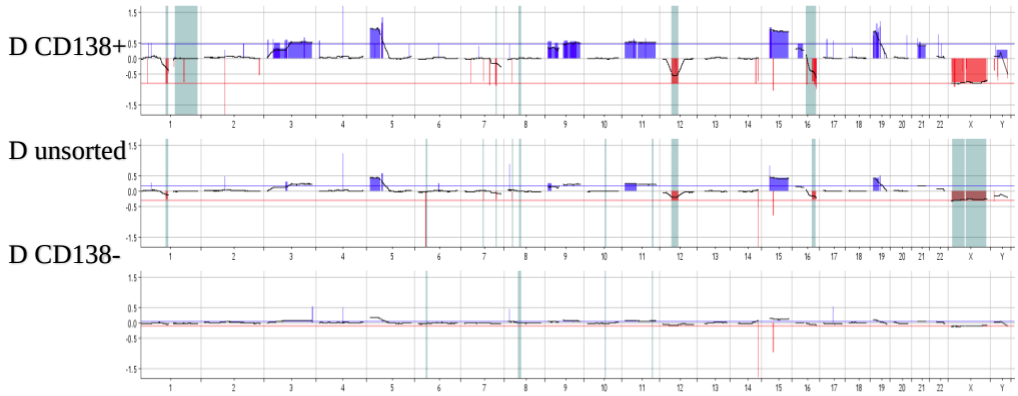


Figure 3. SNParray results sample D: D CD138+ is positively sorted fraction (only with PC); D unsorted is native sample; D CD138- is negatively sorted fraction (without PC).

Looking to ratio signal for each altered region separately, we concluded that only the alteration that exceed the +0.5 or -0.5 thresholds are those that could be identified in unsorted sample, even that the percentage of PC is representative (30%). The others alteration identified in CD138+ cells are missed because of the dilution with the other cell clones from the BM samples. **Sample E CD138+** presented duplication of 1q and chr9 and deletion of chr 4, 5, 6, 13, 14,16q. The total number of DNA lesion was 44 and the median log. ratio for gain was 0,52 and for loss -0,75 (Table 3).
Unsorted sample **E** presented only duplication of chr 1q with a log. ratio median of 0,25 (Table 3). The **sample E CD138-** sample didn't present any abnormalities. (fig.4). Likewise, in this sample E, only dup 1q which has an increased ratio than 0.5, is the one that is identified in the unsorted sample.

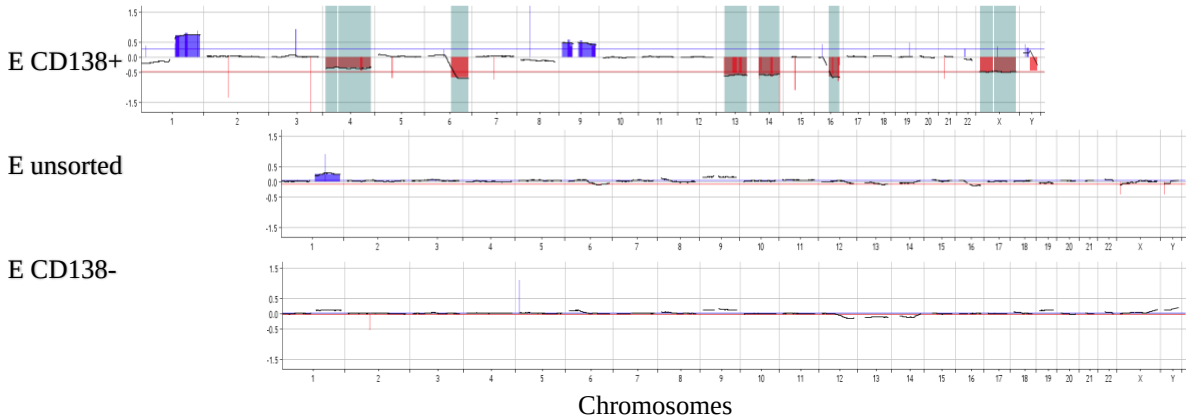


Figure 4. SNParray results sample D: D CD138+ is positively sorted fraction (only with PC); D unsorted is native sample; D CD138- is negatively sorted fraction (without PC).

We tested the samples D and E also using MLPA. The analysis show that sample **D CD138+** harbor duplication of chr9 and chr15 and deletion of chr1p12 (gene FAM46C) and 16q23 (gene WWOX). The median DQ. ratio for duplication was 1.69 and for losses was 0.64. All this information is consistent with that obtained through aCGH (Table 3).

Unsorted sample D presented all the CNV of **sample D CD138+** but at a lower intensity level, with a median DQ ratio for duplication was 1.22 and for losses 0.79.

Sample **D CD138-** didn't present any alterations in the targeted regions.

The situation was similar with **sample E CD138+** which presented deletion of chr 1p32,2 (PLPP3-gene), 1p21,1 (DPYD-gene), chr13 and chr16 and duplication of chr5, 9, 15 and 12p13,31 (CD27 and VAMP1 gene). The median DQ ratio for gain was 1,35 and for loss 0,68.

In **unsorted sample E** only the duplication of chr5 and 12 and deletion of chr 16 could be relatively distinguish with DQ median ratio of < 1,2 and >0,68 for gain and loss respectively (Figure 5).

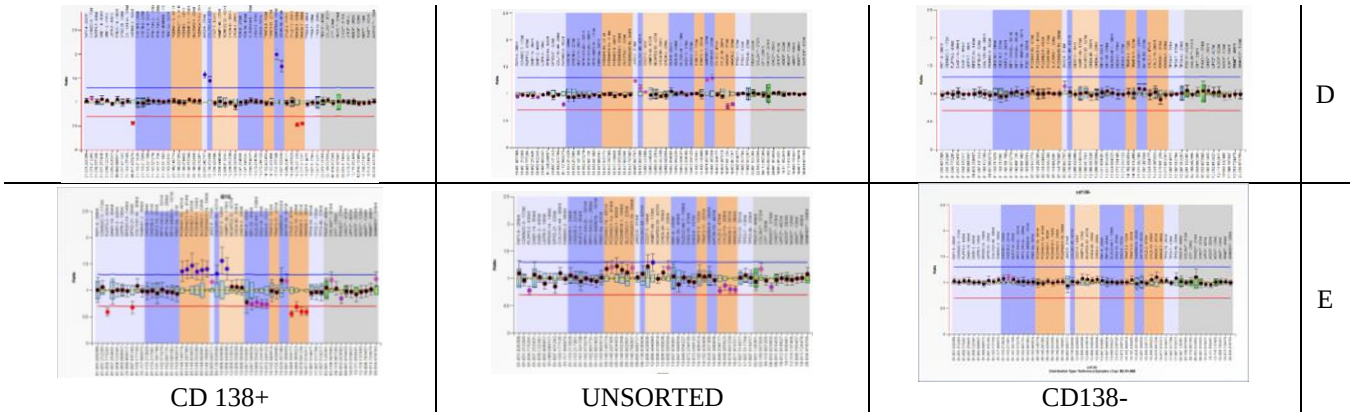


Figure 5. MLPA results of: D- sample of sorted fraction (CD138+); F- unsorted sample; E- sample of sorted fraction (CD138-)

Cancer is a disease which is characterized by genomic instability and MM is no exception. Classical cytogenetic by karyotyping theoretical should be able to provide all the information related with the chromosomal abnormalities but in cancers many factors make this impossible (Szuhai and Vermeer 2015) because of small infiltration of malignant cell in the sample, low mitotic index of this cells and sometimes low quantitate of sample. The developing of complementary techniques that can bring supplementary information became a real target.

MLPA is a technique that can detect in a single reaction up to 50 CNV, is relatively low time consuming and has a low cost but may have problems with mosaicism, tumor heterogeneity, or contamination with normal cells. (Stuppia, Antonucci et al. 2012).

From a genetic point of view, MM is a complex disease, with a high level of heterogeneity of which mechanisms of occurrence and evolution are not fully understood (Aksenova, Zhuk et al. 2021). This heterogeneity in same cases of MM is translated in the presence of clone and subclone. This may be the case of sample were all the abnormalities that were with a log ratio higher than 0.5/-0.5 in sample D CD138+ can also be seen in sample D unsorted. That suggests the presence of a major clone with duplication chr 3p12.2-p11.1, 5p, 9p23-p22, 11p15-p11, 15,19p13-q13 and deletion of chr 12q12-q15 and 16q22.2-q24.3 and 1 or more subclones that presented suplimentary anomalies.

Also, other causes that prevent a proper analysis of PC in MM is extramedullary disease, nonrepresentative in bone marrow (BM) aspirates either due to patchy tumor infiltration or hemodilution (Puig, Flores-Montero et al. 2021). Due to this causes the PC infiltration in BM sample can be as low as 0.01% cases in which without enrichment no anomalies can be detected.

CONCLUSION

By analyzing the data, we concluded that even a 30% malignant cell infiltration in the MM sample is not enough and the magnetic sorting is a mandatory for the enrichment of target cells from the sample.

Identification, in unsorted sample, only a few mutations presented in CD138+ PCs, suggested the existence of a major clone with those abnormalities and 1 or more subclones that presented supplementary genetic anomalies.

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