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**A longitudinal analysis of chromosomal abnormalities in disease progression from MGUS/SMM to newly diagnosed and relapsed multiple myeloma**

**This is a pre print version of the following article:**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1769647> since 2022-07-02T09:21:36Z

*Published version:*

DOI:10.1007/s00277-020-04384-w

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(Article begins on next page)

**This is the author's pre-print version of the contribution published as:**

Oliva S, De Paoli L, Ruggeri M, Caltagirone S, Troia R, Oddolo D, D'Agostino M, Gilestro M, Mina R, Saraci E, Margiotta Casaluci G, Genuardi E, Bringhen S, Boccadoro M, Omedé P. A longitudinal analysis of chromosomal abnormalities in disease progression from MGUS/SMM to newly diagnosed and relapsed multiple myeloma. *Ann Hematol.* 2021 Feb;100(2):437-443. doi: 10.1007/s00277-020-04384-w. Epub 2021 Jan 3. PMID: 33392702.

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**This is not the final published version. The publisher's version is available at:**

<https://link.springer.com/article/10.1007/s00277-020-04384-w>

<https://doi.org/10.1007/s00277-020-04384-w>

**When citing, please refer to the published version.**

**Link to this full text:**

<http://hdl.handle.net/2318/1769647>

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1 **Original article**

2  
3 **A Longitudinal Analysis of Chromosomal Abnormalities in Multiple Myeloma Disease**  
4 **Progression**

5  
6  
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20  
21 *Word count: 1885 words*

22 *References: 27*

23 *Tables: 3*

24 *Figures: 2*

25  
26 **Running title:** Chromosomal abnormalities in MM progression

27  
28  
29 **Declarations**

30  
31 **Funding**

32 No funding was provided for this contribution.

33  
34 **Conflicts of interest**

35 SO has received honoraria from Amgen, Celgene, and Janssen; has served on the advisory boards for Adaptive  
36 Biotechnologies, Janssen, Amgen, and Takeda.

37 MD has served on the advisory board for GSK.

38 RM has received honoraria from Amgen, Celgene, Takeda and Janssen; has served on the advisory boards for  
39 Janssen.

40 SB has received honoraria from Celgene, Amgen, Janssen, and Bristol-Myers Squibb; has served on the advisory  
41 boards for Celgene, Amgen, Janssen, and Karyopharm; has received consultancy fees from Janssen and Takeda.

42 MB has received honoraria from Sanofi, Celgene, Amgen, Janssen, Novartis, Bristol-Myers Squibb, and AbbVie; has  
43 served on the advisory boards for Janssen and GSK; has received research funding from Sanofi, Celgene, Amgen,  
44 Janssen, Novartis, Bristol-Myers Squibb, and Mundipharma.

45 PO has served on the advisory boards for Janssen.

46 The other authors declare no competing financial interests.

47  
48 **Ethics approval**

49 All procedures followed were in accordance with the ethical standards of the responsible committees on human  
50 experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Patients  
51 gave written informed consent before bone marrow aspiration and before starting therapy.

52  
53 **Availability of data and material**

54 After the publication of this article, data collected for this retrospective analysis and related documents will be  
55 made available to others upon reasonably justified request, which has to be written and addressed to the attention  
56 of the corresponding author Dr. Stefania Oliva at the following e-mail address: stefania.olivamolinet@gmail.com.  
57 The corresponding author Dr. Stefania Oliva is responsible to evaluate and eventually accept or refuse every  
58 request to disclose data and their related documents, in compliance with the ethical approval conditions, in  
59 compliance with applicable laws and regulations, and in conformance with the agreements in place with the

60 involved subjects, the participating institutions, and all the other parties directly or indirectly involved in the  
61 participation, conduct, development, management and evaluation of this analysis.

62

63 **Authors' contributions**

64 SO and PO conceived and designed the work that led to the submission.

65 All the authors acquired the data and interpreted the results.

66 SO drafted the first version of the manuscript.

67 All the authors revised the manuscript and approved the final version.

68 All the authors agreed to be accountable for all aspects of the work in ensuring that questions related to the  
69 accuracy or integrity of any part of the work are appropriately investigated and resolved.

70

71 **Acknowledgments**

72 We thank all the patients involved in this analysis, the nurses Manuela Grasso and Luca Merlone, the statistician  
73 Stefano Spada, the data manager Marco Poggiu, and Ugo Panzani from the Torino site.

74

75 **Abstract**

76 We analyzed variations in terms of chromosomal abnormalities (CA) by fluorescence in situ  
77 hybridization (FISH) analysis on purified bone marrow plasma cells throughout the  
78 progression from monoclonal gammopathy of undetermined significance/smoldering multiple  
79 myeloma (MGUS/SMM) to newly diagnosed MM/plasma cell leukemia (NDMM/PCL) at  
80 diagnosis and from diagnostic samples to progressive disease. High risk was defined by the  
81 presence of at least del(17p), t(4;14) and/or t(14;16). 1p/1q detection (in the standard FISH  
82 panel from 2012 onward) was not available for all patients. We analyzed 139 MM/PCL  
83 diagnostic samples from 144 patients, with a median follow-up of 71 months: high-risk CA at  
84 diagnosis (MGUS/SMM or NDMM) was present in 28% of samples, whereas 37-39% showed  
85 high-risk CA at relapse. In 115 patients with NDMM who evolved to relapsed/refractory MM,  
86 we identified 3 different populations: (1) 31/115 patients (27%) with gain of new CA (del13,  
87 del17p, t(4;14), t(14;16) or 1q CA when available); (2) 10/115 (9%) patients with loss of a  
88 previously identified CA; and (3) 74 patients with no changes. The CA-gain group showed a  
89 median overall survival of 66 months vs. 84 months in the third group (HR 0.56; 95% CI 0.34-  
90 0.92, p=0.023). Clonal evolution occurs as disease progresses after different chemotherapy  
91 lines. Patients who acquired high-risk CA had the poorest prognosis. Our findings highlight the  
92 importance of performing FISH analysis both at diagnosis and at relapse.

93  
94  
95

96 **Keywords:** multiple myeloma; cytogenetic abnormalities; fluorescence in situ hybridization;  
97 FISH; clonal evolution; disease progression;

## 99 **Introduction**

100 In multiple myeloma (MM), the heterogenous biology of plasma cells (PCs) confers an inter-  
101 and intra-patient complexity both at clonal and sub-clonal level [1–3]. Cytogenetic  
102 abnormalities (CA) may change throughout the progression from the premalignant stage of  
103 monoclonal gammopathy of undetermined significance (MGUS) to smoldering MM (SMM) to  
104 symptomatic MM, and the major subclone at the time of diagnosis can be different from the one  
105 at first or subsequent relapses [4, 5]. A Darwinian model has been proposed to explain a  
106 possible competition among the survival clones in the bone marrow (BM) niche [6–8]. In this  
107 view, clonal evolution may occur in different ways: stable evolution (same genomic profile at  
108 diagnosis and relapse), linear evolution (novel mutations at relapse, with the same mutational  
109 architecture), and branching evolution (‘disappearance’ of some mutations revealing evolution  
110 from a minor undetected subclone, or appearance of novel different subclones) [9, 10].  
111 Different characteristics could explain why clone selection and chemotherapy may play a major  
112 role, for instance in killing the most sensitive cells and selecting the more resistant ones.  
113 Prognosis is influenced by CA such as del(17p), t(4;14) and/or t(14;16) and amp1q [11–13];  
114 however, it remains unclear whether the CA changes during disease progression preserve the  
115 same impact showed at diagnosis. In this study, we investigated the CA changes in BM PCs from  
116 MGUS/SMM to MM/plasma cell leukemia (PCL) and from diagnostic samples to progressive  
117 disease.

118

## 119 **Patients and methods**

### 120 *Patients*

121 From 2002 to 2018, 144 patients were retrospectively analyzed by fluorescence in situ  
122 hybridization (FISH) during the MGUS/SMM phase, at MM/PCL diagnosis, and at MM relapse  
123 (RRMM). Patients were diagnosed in Turin and Novara (Italy); samples and data were stored

124 and analyzed in Turin. All procedures followed were in accordance with the ethical standards  
125 of the responsible committees on human experimentation (institutional and national) and with  
126 the Helsinki Declaration of 1975, as revised in 2008. Patients gave written informed consent  
127 before BM aspiration and before starting therapy. In conformance with the International  
128 Myeloma Working Group guidelines, MGUS/SMM patients were not considered for any therapy.  
129 Almost all patients with newly diagnosed MM (NDMM) or PCL received new drugs  
130 (immunomodulatory agents [IMiDs] or proteasome inhibitors [PIs]) at diagnosis in  
131 combination with conventional chemotherapy as upfront treatment or incorporated into pre-  
132 transplant induction or post-transplant maintenance strategies inside or outside clinical trials.  
133 Almost all patients evaluable at relapse received new drugs with or without transplantation  
134 both inside or outside clinical trials (**Table 1**). Baseline data included age, sex, isotype,  
135 laboratory values, International Staging System (ISS) stage, CA detected by iFISH, serum level  
136 of lactate dehydrogenase (LDH), and bone lytic lesions.

137

### 138 *FISH analysis*

139 FISH was performed on BM aspirates after red blood cell lysis. Bone marrow plasma cells  
140 (BMPCs) were enriched using anti-CD138-coated magnetic microbeads and AutoMACS Pro  
141 Separator (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) following manufacturer's  
142 instructions. Samples were then centrifuged at 1600 rpm for 5'; afterwards, the obtained dry  
143 pellets were fixed in Carnoy's solution. Purity was assessed by multiparameter flow cytometry  
144 (plasma-cell purity always exceeded 90%). Finally, the samples were stored at -80 °C without  
145 any technical difference in storage process over time.

146 DNA probes (Cytocell Lt, Cambridge, UK) were used to detect del13, del(17p), del1p32.3,  
147 t(11;14)(q13;q32), t(4;14)(p16;q32), t(14;16)(q32;q23) and gain1q21. The same panels for

148 DNA probes were used for the same patient at different stages of testing, from MGUS/SMM to  
149 NDMM/PCL to RRMM,  
150 Three hundred BMPC nuclei from each sample were scored. Cut-off levels for positive values  
151 represented the mean plus three standard deviations (SDs) of abnormal cells from 15 healthy  
152 donors' BMPC samples, and were adjusted at 15% for single fusion, 3% for 2 or more fusion  
153 signals in IgH translocations and 10% for deletions/gains. "Abnormal Chr1" category  
154 represented patients carrying at least del(1p) and/or gain(1q) and was implemented in the  
155 standard FISH panel from 2012 onward. As compared to standard risk, high risk was defined  
156 by the presence of at least del(17p), t(4;14) and/or t(14;16).

157

#### 158 *Statistical methods*

159 Differences in patient and disease characteristics for comparison between groups were  
160 investigated using Kruskal-Wallis and Fisher's exact tests, as appropriate, for continuous and  
161 categorical variables, respectively. Overall survival (OS) was calculated from the date of  
162 diagnosis to the date of death or the date the patient was last known to be alive. Time-to-event  
163 data were analyzed using the Kaplan–Meier method; groups were compared with the log-rank  
164 test. Adjusted hazard ratios (HRs), 95% confidence intervals (CIs) and two-sided p-values were  
165 estimated using Cox proportional hazards models. Data were analyzed as of January 2020 using  
166 R (v3.6.1).

167

## 168 **Results**

### 169 *Patients*

170 The median follow-up was 71 months (IQR 38-96); 139/144 patients had MM/PCL diagnostic  
171 sample, of which 29 had also MGUS/SMM evaluable FISH; 115/139 had relapse samples: 84

172 had the 1<sup>st</sup> relapse sample, whereas the other patients had  $\geq 2^{\text{nd}}$  relapse sample. Five out of 144  
173 total patients were evaluable at 2 subsequent relapses.

174 Patient characteristics of the 139 NDMM/PCL patients are illustrated in **Table 1**: median age  
175 was 64 years (IQR 57-72) and males were 70 (50%). The ISS scores were respectively 41%,  
176 39% and 17% for stages I, II and III.

177 Sixty-seven (48%) patients received front-line PI-based therapy; 66 (47%) lenalidomide-based  
178 therapy, 5 (4%) conventional chemotherapy/other; 1 patient did not start any therapy. Fifty  
179 patients (36%) received autologous stem-cell transplantation (ASCT).

180 A total of 293 samples were analyzed by FISH at different disease evolution stages (**Table 2**).  
181 Median time to progression (TTP) from MGUS/SMM to NDMM/PCL was 24 vs. 40 months for  
182 high-risk vs. standard-risk CA ( $p=0.04$ ). Median TTP from NDMM/PCL to 1<sup>st</sup> relapse was 25 vs.  
183 30 months for high-risk vs standard-risk CA ( $p=0.14$ ).

184

#### 185 *Clonal evolution*

186 Clonal evolution in terms of CA changes was evaluated in 29 patients evolving from MGUS/SMM  
187 to NDMM/PCL, 115 from NDMM/PCL to relapse or from 1<sup>st</sup> to  $\geq 2^{\text{nd}}$  relapse. One-third of  
188 patients showed CA changes during disease progression. Three patient populations were  
189 identified:

#### 190 A. 29 MGUS/SMM $\rightarrow$ NDMM/PCL:

191 - Group 1: 5/29 patients (17%) gained new CA [2 gained del13, 2 del(17p) and 1 t(14;  
192 16)].

193 - Group 2: 3/29 patients (10%) lost previously detected CA.

194 - Group 3: the other 21/29 (72%) patients showed no CA changes.

#### 195 B. 115 NDMM/PCL $\rightarrow$ RRMM

- 196 - Group 1: 31/115 patients (27%) gained new CA among del13, del(17p), t(4;14), t(14;16)  
197 and abnormal Chr1 [only 1 patient gained t(11;14)].  
198 - Group 2: 10/115 patients (9%) lost previously detected CA.  
199 - Group 3: the other 74/115 (64%) patients showed no CA changes.

200

201 The most frequent gains or losses were associated with del13 or del(17p) (**Figure 1**). A  
202 proportion of subclones emerged during disease progression: 40% of deletions/gains were  
203 detected in <10% of BMPC nuclei; 65% of single fusion abnormalities were detected in <15%  
204 of BMPC nuclei at the MGUS/SMM or NDMM/PCL phases.

205 Throughout the progression from NDMM to relapse, no baseline characteristics were  
206 significantly associated with CA gains vs. losses vs. no changes after progression (e.g.  
207 plasmacytoma 10% vs. 0% vs. 4% [p=0.73], ISS III 26% vs. 10% vs. 16% [p=0.57], LDH>ULN  
208 10% vs. 20% vs. 12% [p=0.36], respectively; **Table 3**).

209 No statistically significant differences among therapies in terms of CA gains vs. losses vs. no  
210 changes after progression were observed (IMiDs 61% vs. 60% vs. 49%; PIs 35% vs. 30% vs.  
211 49% [p=0.36]; ASCT 32% vs. 50% vs. 43% [p=0.50], respectively; **Table 3**).

212 Patients gaining new CA during disease evolution (group 1) showed the worst median OS with  
213 66 months (95% CI 58-82) vs. 84 months (95% CI 73-NR) in group 3 (group 1 vs. 3, HR 0.56;  
214 95% CI 0.34-0.92, p=0.023; **Figure 2**).

215

## 216 **Discussion**

217 Our results confirmed that genetic instability increases as disease progresses after different  
218 chemotherapies, with a group with acquired high-risk CA having the poorest prognosis. This  
219 suggests that some clones that might have existed at the diagnosis as a minor population could  
220 expand during treatment and drive relapse.

221 Although MGUS and SMM are genetically less complex than MM, clonal heterogeneity is already  
222 present at these early pre-malignant stages. Indeed, early reports detected fewer aneuploidies  
223 in MGUS than in MM [14–16], whereas next-generation sequencing (NGS) analysis of sample  
224 series showed variable degrees of spontaneous evolution of genes mutations, cytogenetic  
225 lesions and mutational signatures [17–24]. This suggests that MM evolves in discrete steps not  
226 just clinically but also from a molecular point of view, with the acquisition of subsequent  
227 genomic lesions underlying an increasingly aggressive clinical behavior. In this study, we  
228 observed a clonal evolution by FISH in a subgroup of patients progressing from MGUS/SMM to  
229 NDMM, although a larger series of patients and the analysis of mutational profiles could add  
230 biological significance to these findings.

231 Our study confirmed the importance of performing FISH analysis both at diagnosis and relapse,  
232 since the acquisition of structural genetic lesions has been associated with a dismal outcome,  
233 suggesting that clonal selection during the MM course is fundamental in the evolution of  
234 subsequent refractoriness and inferior survival.

235 This retrospective analysis has some limitations. The patient inclusion period was very long,  
236 from 2002 to 2018, with clearly different treatment approaches available over time. The  
237 heterogeneity of therapies could suggest that therapy is apparently not a significant factor  
238 influencing clonal evolution (e.g. ASCT vs. no ASCT, IMiDs, PIs) nor affecting the clone selection  
239 responsible for relapse. Indeed, this selection could be patient-dependent or related to internal  
240 factors, such as local BM and/or immune conditions. This was demonstrated by Corre et al.,  
241 who observed no significant changes in the mutational burden of MM patients undergoing the  
242 same treatment [25]. Future prospective clinical trials with homogeneously treated MM  
243 patients (e.g. young, transplant-eligible NDMM patients, or elderly NDMM patients treated with  
244 PIs or IMiDs) could include pre-planned BM aspirates with FISH at different time points in order

245 to perform a longitudinal comparison of changes in the genetic profiles of patients in remission  
246 (as control arm) and at relapse.

247 Corre et al. did not observe any differences between primary diagnosis and relapse in terms of  
248 defined IGH translocations, including t(4;14). These findings are in line with a model suggesting  
249 that trisomies of odd chromosomes and rearrangements involving the IGH gene are primary  
250 events occurring in the early disease phase, whereas other abnormalities (e.g. loss of  
251 chromosome 13, 17p deletions or MYC rearrangements) are considered secondary events.  
252 Accordingly, IGH translocations were observed in the large majority of PCs at diagnosis,  
253 whereas monosomy 13 or del(17p) were frequently detected only in subclones [26]. On the  
254 contrary, as also shown in 2013 by Hébraud et al. for t(4:14) [27], we observed a small fraction  
255 of patients gaining or losing translocations after progression, even if the gain or loss of  
256 chromosome 13 and the 17p deletions were the most frequent. One hypothesis is that small  
257 positive subclones involving IgH translocations at the time of diagnosis could exist, since most  
258 of our patients were considered negative at diagnosis with a translocation in a subclonal  
259 fraction (<15% of PCs) and were subsequently considered positive because these subclones  
260 appeared with higher frequency at relapse ( $\geq 15\%$  of PCs).

261 In conclusion, we retrospectively confirmed the occurrence of CA changes, which may reflect a  
262 clonal evolution during the disease course, and the importance of implementing FISH analysis  
263 at different phases of the disease. We need to understand whether different therapies may  
264 select resistant clones and favor the aggressiveness of MM at relapse and whether the evolution  
265 of FISH techniques will help tailoring clinical decision making.

266

267

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269

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338

339 **Tables**

340

341 **Table 1. Patient characteristics of NDMM patients**

	<b>N=139</b>
<b>Age, years</b>	
Median (IQR)	64 (57-72)
<b>Gender, N (%)</b>	
M	70 (50)
<b>ISS, N (%)</b>	
I	57 (41)
II	54 (39)
III	24 (17)
Missing	4 (3)
<b>Creatinine</b>	
Median, mg/dL (IQR)	0.96 (0.8-1.22)
Missing, N (%)	3 (2)
<b>LDH</b>	
Median, U/L (IQR)	330 (279-84)
>ULN, N (%)	14 (10)
Missing, N (%)	34 (24)
<b>Front-line therapies, N (%)*</b>	
PIs	67 (48)
IMiDs	66 (47)
Conventional chemotherapy/other	5 (4)
ASCT	50 (36)
None	1 (1)
<b>Therapy at 1<sup>st</sup> relapse, N (%)*</b>	
PIs	68 (49)
IMiDs	32 (23)
Conventional chemotherapy/other	5 (4)
ASCT	12 (9)
<b>Therapy at ≥2<sup>nd</sup> relapse, N (%)*</b>	
PIs	24 (17)
IMiDs	23 (17)
Conventional chemotherapy/other	5 (4)
ASCT	8 (6)

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343 *Percentages may not total 100 because of rounding.*

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345 *\*Percentages may not total 100 because patients may have received more than one type of treatment.*

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347 **Abbreviations.** ASCT, autologous stem-cell transplantation; IMiDs, immunomodulatory drugs; IQR, interquartile

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348 range; ISS, International Staging System; LDH, lactate dehydrogenase; M, male; N, number; NDMM, newly diagnosed multiple myeloma; U/L, units per liter; ULN, upper limit of normal; PIs, proteasome inhibitors.

349 **Table 2. FISH results at baseline (first sample) and at relapse**

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FISH risk* N (%)	MGUS/SMM N=29	NDMM N=139	1 <sup>st</sup> relapse N=84	≥2 <sup>nd</sup> relapse N=70
Standard	19 (66)	100 (72)	53 (63)	43 (61)
High	10 (34)	39 (28)	31 (37)	27 (39)
del(17p)	4 (14)	23 (17)	23 (27)	21 (30)
t(4;14)	7 (24)	20 (14)	10 (12)	11 (16)
t(14;16)	2 (7)	1 (1)	1 (1)	1 (1)
del(13p)	9 (31)	66 (47)	47 (56)	42 (60)
t(11;14)	6 (21)	32 (23)	19 (23)	20 (29)
1q/p CA**	6/8 (75)	20/27 (74)	7/16 (44)	24/38 (63)

352 \*FISH risk defined by the presence of at least one among del(17p), t(4;14)] and/or t(14;16).

353 \*\*Only in evaluable patients.

354 Percentages may not total 100 because of rounding.

355 **Abbreviations.** FISH, fluorescence in situ hybridization; CA, chromosomal abnormalities; MGUS, monoclonal  
 356 gammopathy of undetermined significance; MM, multiple myeloma; N, number; NDMM, newly diagnosed MM;  
 357 SMM, smoldering MM.  
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361 **Table 3. Baseline characteristics and therapies in the three groups in 115**

362 **NDMM/RRMM patients**

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	Group 1 [CA gain] N=31	Group 2 [CA loss] N=10	Group 3 [Unchanged] N=74	P-value
Median age, years (IQR)	67 (58-71)	63 (60-64)	62 (57-70)	0.32
Sex				0.28
Male	18 (58%)	3 (30%)	35 (47%)	
Female	13 (42%)	7 (70%)	39 (53%)	
Plasmacytoma	3 (10%)	0	3 (4%)	0.73
LDH>ULN	3 (10%)	2 (20%)	9 (12%)	0.36
ISS				0.57
I	13 (42%)	3 (30%)	32 (43%)	
II	10 (32%)	6 (60%)	27 (36%)	
III	8 (26%)	1 (10%)	12 (16%)	
Missing	-	-	3 (4%)	
Median Hb value	10.6	10.6	10.6	0.82
Median creatinine value	1.04	0.86	0.95	0.67
Bone lytic lesions ≥2	12 (39%)	4 (40%)	22 (30%)	0.85
IMiDs	19 (61%)	6 (60%)	36 (49%)	0.36
PIs	11 (35%)	3 (30%)	36 (49%)	
Conventional chemotherapy/other	1 (3%)	1 (10%)	2 (3%)	
ASCT	10 (32%)	5 (50%)	32 (43%)	0.50

365 *Group 1: patients who gained new CA among del13, del(17p), t(4;14), t(14;16) and abnormal Chr1 [only 1 patient*  
 366 *gained t(11;14)]; Group 2: patients who lost previously detected CA; Group 3: patients who showed no CA changes.*  
 367 *Percentages may not total 100 because of rounding.*

368 **Abbreviations.** ASCT, autologous stem-cell transplantation; CA, chromosomal abnormalities; Chr1, chromosome  
 369 1; Hb, hemoglobin; IQR, interquartile range; IMiDs, immunomodulatory drugs; ISS, International Staging System  
 370

371 stage; LDH, lactate dehydrogenase; NDMM, newly diagnosed multiple myeloma; PIs, proteasome inhibitors;  
372 RRMM, relapsed/refractory multiple myeloma; ULN, upper limit of normal.  
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381 **Figure titles and legends**

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386 **Figure 1. Frequency of FISH changes**

387 Panel A: Gain frequency (N=31)\*

388 Panel B. Loss frequency (N=10)\*\*

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392 *\*Chr1 abnormalities only in evaluable patients [N=3/10].*

393 *\*\*Chr1 abnormalities only in evaluable patients [N=0/4].*

394 *N.b. A single patient may present with more than 1 CA change.*

395 **Abbreviations.** FISH, fluorescence in situ hybridization; CA, chromosomal abnormalities; Chr1, chromosome 1.

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401 **Figure 2. Overall survival of 3 longitudinal FISH groups**

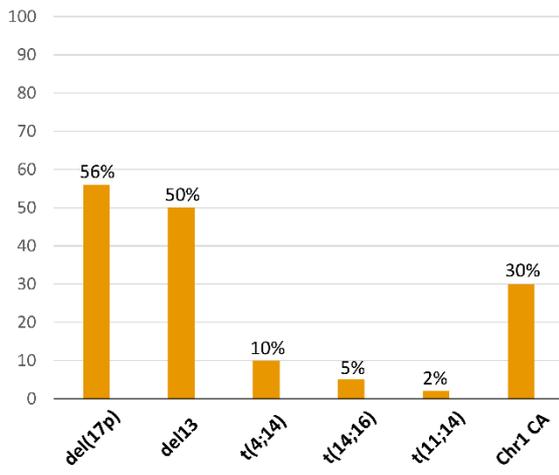
402  
403  
404

405 **Abbreviations.** FISH, fluorescence in situ hybridization; CI, confidence interval; HR, hazard ratio; p, p-value; unch.  
406 unchanged.

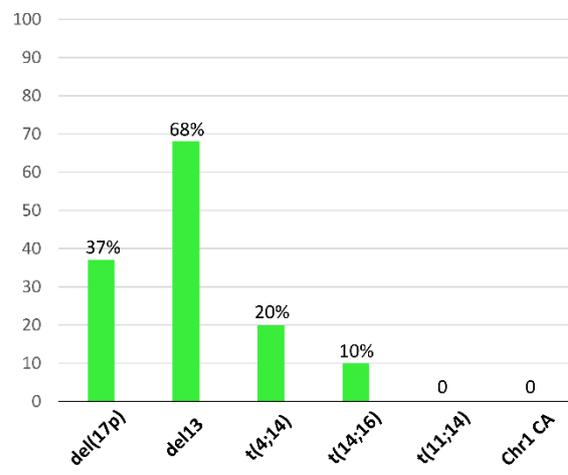
407

408 Figure 1  
409

**A. Gain frequency (N=31)\***

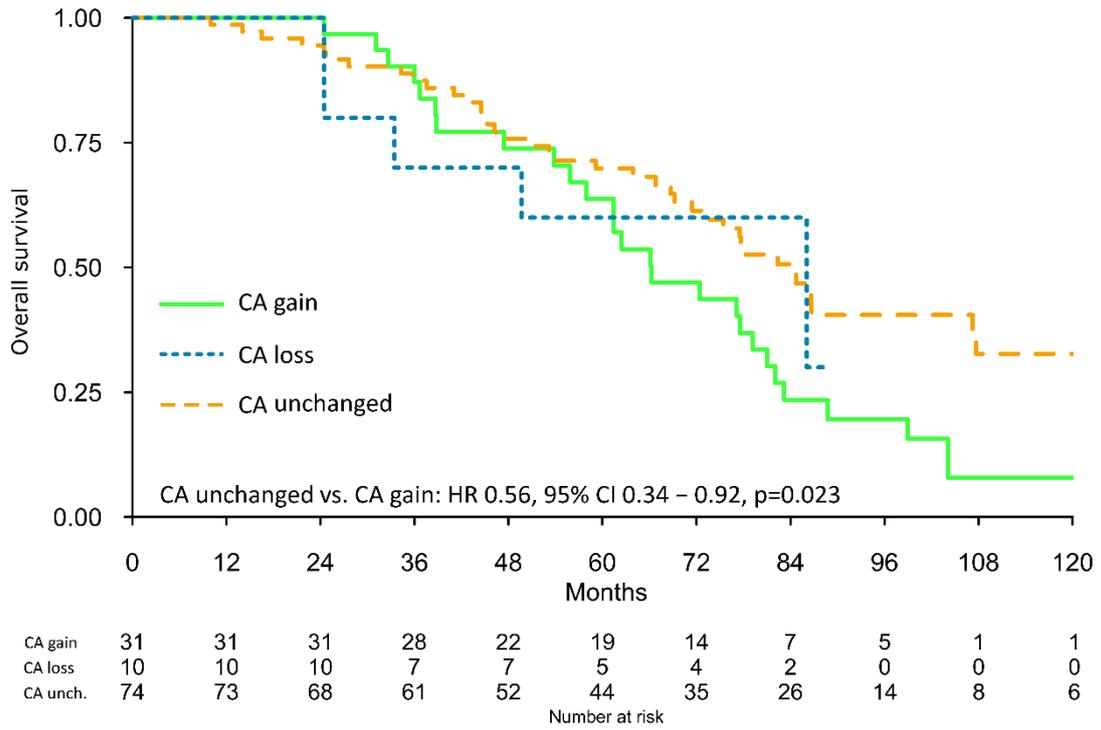


**B. Loss frequency (N=10)\*\***



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413 Figure 2  
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