In-depth cytogenetic and immunohistochemical analysis in a real world cohort - reconsidering the role of primary and secondary aberrations in multiple myeloma

Multiple myeloma (MM) arises from terminally differentiated antibody-producing B cells and evolves from monoclonal gammopathy of undetermined significance (MGUS) to overt disease through a sequence of genetic changes. These can be diverse and subdivided into primary and secondary events based on their occurrence. It is postulated that oncogenesis in MM is driven by two different almost exclusive primary genetic events (hyperdiploidy and immunoglobulin [Ig] H-translocation) and that clonal evolution occurs with the acquisition of different secondary chromosomal aberrations, e.g., monosomies, deletions, duplications and mutations.[1] Via routine fluorescence *in situ* hybridization (FISH), hyperdiploidy is observed in ~50% of MM patients, whereas in the absence, MM is driven by class-switch recombination and somatic hypermutation during B-cell maturation in the loci encoding Ig on chromosome 14 resulting in chromosomal translocations.[2] Monosomy 13/del13q14 is discussed as a possibly third type of primary aberration and appears in ~45% of MM patients.[3] Some describe it as an independent MM-initiating event. Chesi *et al.*[4] classified del13q as an early event, appearing in germinal centers, in MGUS and intramedullary MM. This finding was updated based on a mouse model, indicating that the loss of a particular gene on chromosome 13, *Mir-15a/Mir-16-1*, regulates MM-progression in a copy number-dependent way, with a more aggressive tumor course in mice lacking both copies of the *Mir-15a/Mir-16-1* cluster.[4-8] A potential underlying mechanism may be the overexpression of cyclins D1-3 that are physiologically inhibited by the *Mir-15a/Mir-16-1* genes. Cyclin D upregulation has been discussed as a unifying MM-initiating event by mediating the cell cycle transition from G1- to S-phase.[9,10]

Due to the ambiguous description of primary and secondary aberrations occurring alone or combined, and the varying role of monosomy 13/del13q14, further analyses seemed warranted. Therefore, we performed this exploratory, single center study at our Comprehensive Cancer Center Freiburg (CCCF) from 2013 to 2020. Of 338 patients, 29 had to be excluded, who did not fulfill the inclusion criteria, namely i) bearing the diagnosis of MM and ii) FISH diagnostics being routinely performed at initial diagnosis/initial presentation (ID). Thus, FISH data of 309 patients were prospectively assessed, with fully documented clinical data being associated with FISH and cyclin D1-3 results. Patients were assigned to four subgroups (SG); SG1: with only hyperdiploidy/IgH-translocations (n=74, 24%), SG2: without hyperdiploidy/IgH-translocations, but other cytogenetic aberrations (oCA) (n=40, 13%), SG3: both primary and secondary aberrations (n=182, 59%) and SG4: no aberrations detectable (n=13, 4%; Table 1). In depth cytogenetic, immunohistochemical and statistical analyses were carried out with SG2.

Interphase FISH was routinely performed on CD138$^+$ bone marrow (BM) plasma cells (PC) from 'first pull' BM aspirates using an extended FISH panel from MetaSystem Probes, Germany as previously described.[11] An aberration was rated at a 10% cutoff-value.[12] Progression-free survival (PFS) was defined as the time from ID to MM recurrence or death and overall survival (OS) from ID to death from any cause. Observation times for patients alive/without disease progression at the time of the analysis were censored at last follow-up (01/2023). Survival probabilities were estimated using the Kaplan-Meier method and compared with the log-rank test. All data were analyzed with SAS 9.4 (SAS Institute, Inc. Cary, North Carolina) with a *P* value of <0.05 considered as statistically significant.

BM tissue sections (4 μm) were immunohistochemically analyzed as described.[13] Samples were incubated with cyclin D1-3 antibodies. The staining patterns were independently and blindly assessed (SW with pathologists SC and M-AC). The study was performed according to the guidelines of the Declaration of Helsinki and Good Clinical Practice. All patients gave their written informed consent for institutionally initiated research studies conforming to the Institutional Review Board guidelines. The trial protocol was approved by the ethics committee of the University of Freiburg (EV 22-1525-S1) and registered at https://frks.uniklinik-freiburg.de/FRKS004358.

Patients' characteristics of the entire cohort and SG1-4 are summarized in Table 1. Our entire cohort revealed a median age of 65 years, Karnofsky Performance Status (KPS) of 80% and MM paraprotein types with preponderance of IgGκ-MM, with advanced International Staging System (ISS), Revised-ISS (R-ISS), Revised-Myeloma Comorbidity Index (R-MCI) and proteasome inhibitor (PI)-based treatment in the majority of patients, reflecting a typical real-world cohort. SG2 showed light chain (LC)-MM frequencies of 35%, more λ-LC types (47%), increased ISS/R-ISS stage III (38%/25%), R-MCI frail patients (25%) and intense treatment: 75% received at least one autologous stem cell transplantation (ASCT) or even allogeneic SCT (allo-SCT). The best remission status during the disease course was rewarding: 42% achieved a complete remission.

Table 1. Patient characteristics of all patients and subgroups (SG) 1-4 with only hyperdiploidy/IgH-translocations (SG1) *versus* no hyperdiploidy/IgH-translocation, but other cytogenetic aberrations (SG2), both primary and secondary aberrations (SG3) *versus* no aberrations detectable (SG4).

Demographic data	All patients, N=309	Subgroup 1, N=74	Subgroup 2, N=40	Subgroup 3, N=182	Subgroup 4, N=13
Age in years at Initial diagnosis, median/mean (range)	65/64 (28-89)	68/66 (29-86)	62/63 (46-89)	66/65 (37-87)	58/55 (28-75)
Sex: male/female, N (%)	190 (61)/119 (39)	52 (70)/22 (30)	23 (58)/17 (42)	104 (57)/78 (43)	11 (85)/2 (15)
KPS %, median (range)	80 (20-100)	80 (30-100)	90 (20-100)	80 (30-100)	80 (60-100)
Type of MM, N (%)					
IgG/IgA/IgD LC only/asecretory / biclonal	173 (56)/48 (15)/1 (0.3) 82 (27)/3 (1)/2 (0.7)	55 (75)/5 (7)/0 12 (16)/1 (1)/1 (1)	17 (43)/9 (22)/0 14 (35)/0/0	93 (51)/33 (18)/1 (0.5) 52 (29)/2 (1)/1 (0.5)	8 (61)/1 (8)/0 4 (31)/0/0
κ:λ/both/asecretory LC type	189 (61):113 (37)/5 (1.5)/2 (0.5)	52 (70):20 (27)/2 (3)/0	21 (53):19 (47)/0/0	109 (60): 68 (37)/3 (2)/2 (1)	7 (54):6 (46)/0/0
Risk stratification, N (%)					
ISS: I/II/III	89 (29)/98 (32)/122 (39)	32 (43)/25 (34)/17 (23)	10 (24)/15 (38)/15 (38)	42 (23)/52 (29)/88 (48)	5 (39)/6 (46)/2 (15)
R-ISS: I/II/III	62 (20)/183 (59)/64 (21)	24 (32)/42 (57)/8 (11)	4 (10)/26 (65)/10 (25)	29 (16)/109 (60)/44 (24)	4 (31)/8 (62)/1 (7)
R-MCI: fit, 0-3/intermediate-fit, 4-6/frail, 7-9	84 (27)/160 (52)/65 (21)	29 (39)/35 (47)/10 (14)	11 (28)/19 (47)/10 (25)	35 (19)/103 (57)/44 (24)	9 (69)/3 (23)/1 (8)
Frequency monosomy13/del13q14	125 (41)	0	37 (93)	88 (48)	0
Therapy/outcome, N (%)					
x-line therapy: 0/1/2/≥3	6 (2)/152 (49)/62 (20)/89 (29)	3 (4)/17 (23)/38 (51)/16 (22)	1 (2)/17 (43)/9 (22)/13 (33)	2 (1)/91 (50)/32 (18)/57 (31)	0/6 (46)/4 (31)/3 (23)
First-line therapy by substance class: Proteasome-inhibitors/ alkylants/antibodies/IMiD/ Ø therapy	268 (87)/ 20 (6)/8 (3)/7 (2)/6 (2)	65 (88)/ 4 (6)/1 (1)/1 (1)/3 (4)	31 (78)/5 (12)/3 (8)/0/1 (2)	160 (88)/ 10 (6)/4 (2)/6 (3)/2 (1)	12 (92)/ 1 (8)/0/0/0
SCT: Yes/No	198 (64)/111 (36)	47 (64)/27 (36)	30 (75)/10 (25)	109 (60)/73 (40)	12 (92)/1 (8)
Best response: Ø therapy/ID/CR/ vgPR/PR/SD/PD	6 (2)/9 (3)/58 (18)/113 (37)/85 (28)/29 (9)/9 (3)	3 (4)/0/9 (12)/28 (38)/22 (30)/9 (12)/3 (4)	1 (2)/8 (20)/17 (42)/ (18)/2 (5)/3 (8)/2 (5)	2 (1)/7 (4)/37 (20)/65 (36)/53 (29)/15 (8)/3 (2)	0/0/4 (31)/3 (23)/3 (23)/3 (23) /0
Pts alive: Yes/No	214 (69)/ 95(31)	56 (76)/ 18(24)	27 (68)/13 (32)	123 (68)/59 (32)	8 (62)/5 (38)
PFS in months, median (range)	38 (0-288)	49 (1-194)	41 (0-288)	36 (0-149)	44 (7-72)
OS in months, median (range)	105 (0-431)	117 (1-431)	117 (0-288)	103 (0-387)	73 (14-88)

Interphase fluorescence *in situ* hybridization (FISH) was routinely performed on CD138$^+$ bone marrow (BM) plasma cells (PC) from 'first pull' BM aspirates using an extended FISH panel from MetaSystem Probes, Germany as described.[11] [XL t(4;14) FGFR3/IGH DF; XL t(6;14) CCND3/IGH DF; XL t(11;14) MYEOV/IGH DF; XL t(14;16) IGH/MAF DF; XL t(14;20) IGH/MAFB DF; XL 5p15/9q22/15q22 hyperdiploidy, XCE 3/7/17; XL DLEU/LAMP; XL 19p/19q del; XL RUNX 1; XL CDKN2C/CKS1B, XL MYC BA, XCE 8; XL IGH BA; XL TP53/NF1; XL DLEU/TP53]. ID: initial diagnosis; KPS: Karnofsky Performance Status; MM: multiple myeloma; LC: light chain: ISS: International Staging system; R-ISS: Revised ISS; R-MCI: Revised Myeloma Comorbidity Index; ImiD: immunomodulatory drugs; SCT: stem cell transplantation; CR: complete remission; vgPR: very good partial response; PR: partial remission; SD: stable disease; PD: progressive disease; pts: patients; PFS: progression-free survival; OS: overall survival.

Monosomy 13/del13q14 was present in our entire cohort in 41% and in SG1-4 only in SG2 and SG3 in 93% and 48%, respectively (Table 1). Detailed cytogenetic analyses were carried out in SG2 since these patients were of particular interest (Figure 1A): apart from monosomy 13/del13q14 in 37 of 40 patients (93%), gain1q21 was present in 21 of 40 (53%), del14p32 in 19 of 40 (48%), del17p13/monosomy 17 in ten of 40 (25%), monosomy 8 in eight of 40 (20%), monosomy 1/del1p32 in ten of 40 (25%), myc-A/-R in nine of 40 (22%), single trisomies in nine of 40 (22%) and other deletions in nine of 40 patients (22%).

PFS and OS were determined via Kaplan-Meier curves. Figure 1B depicts the PFS of SG1-4 with 49, 41, 36 and 44 months, respectively (P=0.4214). OS of SG1-4 is visualized in Figure 1C, showing no statistical significance (P=0.2985), albeit median OS in SG1 and 2 were 117 months, whilst in SG3 and SG4 only 103 and 73 months, respectively.

In order to determine, whether - based on their monosomy 13/del13q14 status – SG2 patients showed differences in demographics, MM-types, ISS/R-ISS, R-MCI and outcome, we compared three subgroups as outlined in the *Online Supplementary Table S1*. Patients with monosomy 13/del13q14 alone (i) were the youngest, all males, fit via KPS and R-MCI, had predominantly LC-only and κ-LC-types. All patients remain alive and showed median PFS of 43 months and OS of not reached. Patients with monosomy 13/del13q14 and ≥1 additional cytogenetic event (ii) were older, showed a median KPS of 80% and were frail in 26%. ISS and R-ISS II/III were predominant in 73% and 88%, respectively. Median PFS and OS in this group were 41 and 116 months, respectively. Patients with exclusively secondary aberrations (iii) were the oldest, frail in 33%, and had advanced ISS/R-ISS II/III. Whilst two of three patients remain alive, median PFS and OS were dismal with 13 and 21 months, respectively.

Due to the individual cytogenetic aberrations of SG2 and the varying role of monosomy 13/del13q14, SG2 was dissolved and regrouped in the remaining three SG1, SG3 and SG4, based on their monosomy 13/del13q14-status (*Online Supplementary Table S1*): patients with monosomy 13/del13q14 alone (i) were included in SG1 (IgH-translocation alone). Patients with monosomy 13/del13q14 with ≥1 additional cytogenetic event (ii) were regrouped in SG3 (both primary and secondary aberrations) and patients with exclusively secondary aberrations (iii) in SG4. This led to new SG1.1, SG3.1 and SG4.1 (n=77, 216, 16, respectively). The median PFS was 46, 37 and 38 months, respectively, which revealed a 9-month difference between SG1.1 and SG3.1, but did not reach significance (P=0.3628; Figure 1D). The median OS was 117, 105 and 65 months, respectively (Figure 1E), also failing to reach significance (P=0.0680). When PFS and OS in SG1.1 (n=77) *versus* merged SG3.1 and SG4.1 (n=232) were compared, *P values* of 0.1544 and 0.0262, respectively, were obtained (Figure 1F, G). Thus, patients with hyperdiploidy/IgH-translocation/monosomy13/del13q14-status alone (primary aberrations) *versus* those with primary and secondary aberrations (including patients with monosomy 13/del13q14 and ≥1 additional cytogenetic event and those with exclusively secondary aberrations) largely differed in OS (Figure 1G).

In order to investigate the role of cyclin D1, D2 and D3 (*Online Supplementary Table S2*), immunohistochemistry (IHC) was performed in SG2 and SG4 (control) patients. Thereof, 32 of 40 SG2 patients were analyzed and compared to ten of 13 SG4 patients (n=11 could not be assessed due to missing BM samples). Figure 2A depicts the underlying mechanism possibly leading to overexpression of cyclins D1-3 based on the loss of a specific gene on chromosome 13q14, *Mir-15a/Mir-16-1*, which physiologically inhibits cyclins D1-3 in the cell cycle and transition from G1- to S-phase. Figure 2B shows representative BM sections with intense nuclear cyclin D1 and D2 and cytoplasmic D3 expression. Cyclin D1-3 in the control (SG4) revealed an upregulation of cyclin D3 in five of ten patients (50%) alone (Figure 2C), and in SG2 patients in one of 32 (3%), three of 32 (9%) and eight of 32 (25%), respectively (Figure 2D). Figure 2E allied the upregulation of cyclins D1-3 in SG2 patients with their individual cytogenetic aberrations, indicating a heterogeneous distribution without a distinct cyclin D upregulation pattern. Monosomy 13/del13q14 alone occurred in three patients (patient #1-3), but in almost all others in conjunction with various additional aberrations. They revealed a cyclin D3 positivity in one patient as well as negativity for cyclins D1-3 in two others. Monosomy 13/del13q14 with one, two, three, four or five additional cytogenetic aberrations occurred in seven, 12, seven, six and two patients, respectively (#4-37; n=34). The heterogeneous distribution in cyclin D1-3 upregulation was confirmed in this subgroup. Of interest, three patients (#38-40) with exclusively secondary aberrations, i.e., gain1q21, myc-A/-R, del17p13/monosomy 17 or del14q32, showed cyclin D3 upregulation in one patient, but did not express cyclins D1-3 in both others. Consequently, no correlation between cytogenetics of patients without hyperdiploidy/IgH-translocations, but oCA (SG2) and cyclins D1-3 via IHC became apparent.

In summary, we initiated this study after observing symptomatic MM patients showing no hyperdiploidy/IgH-translocation, but oCA (termed SG2) over a 7-year period. Our interest was to address their frequency, MM-initiating events and outcome data. We found a remarkable fraction of patients without hyperdiploidy/IgH-translocations, but oCA (13%) who showed a predominance of monosomy 13/del13q14. Notable was that only few SG2 patients (3/309 i.e., 1%) did reveal an impaired PFS and OS, who showed no monosomy 13/del13q14, but secondary aberrations alone, suggesting them to belong to another genetic entity as compared to those with monosomy 13/del13q14 and secondary events (*Online Supplementary Table 1*). No distinct

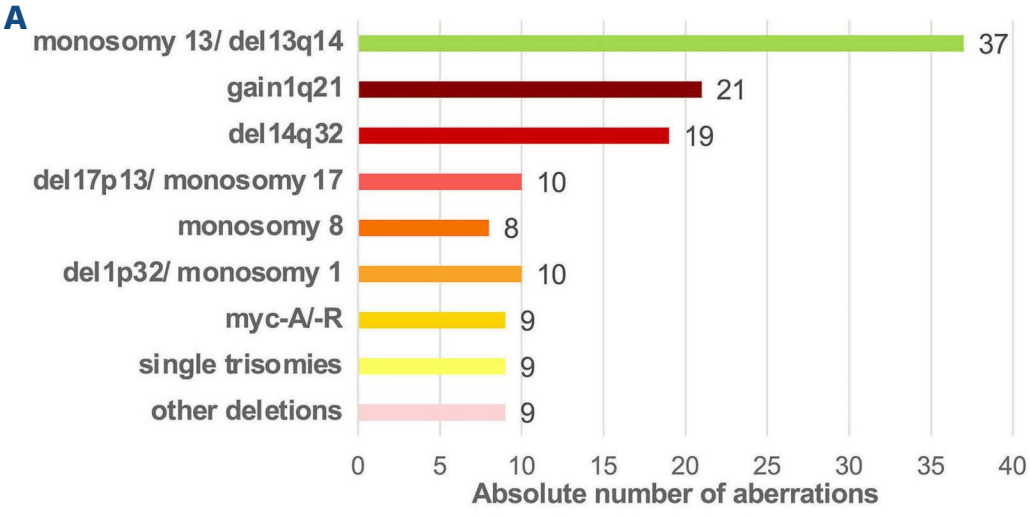
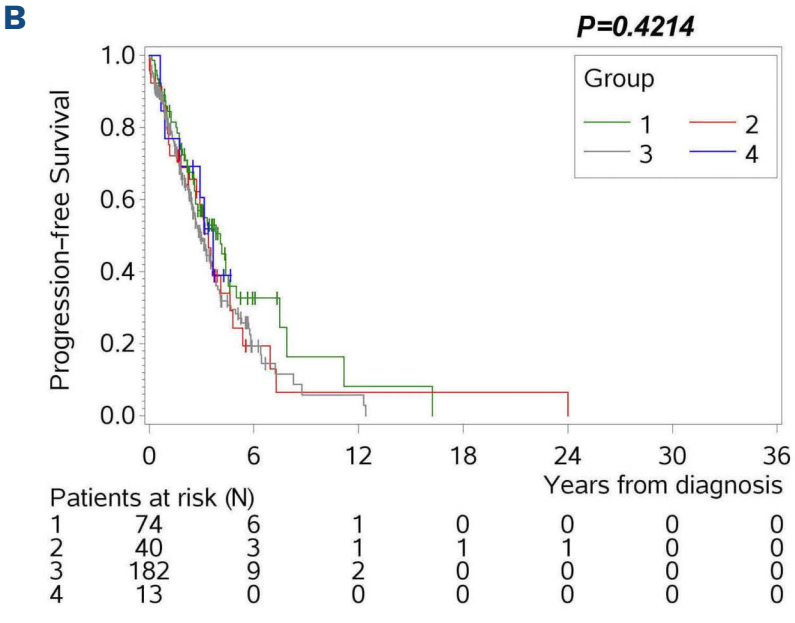
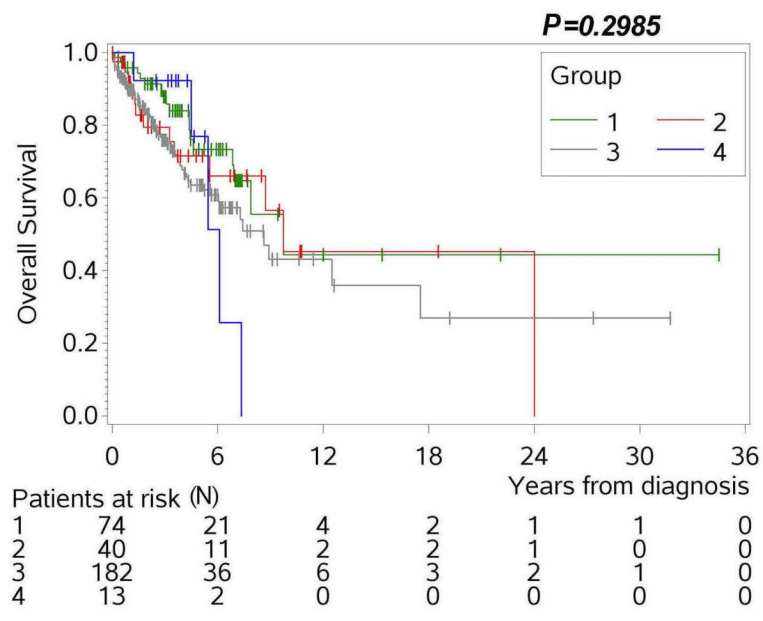
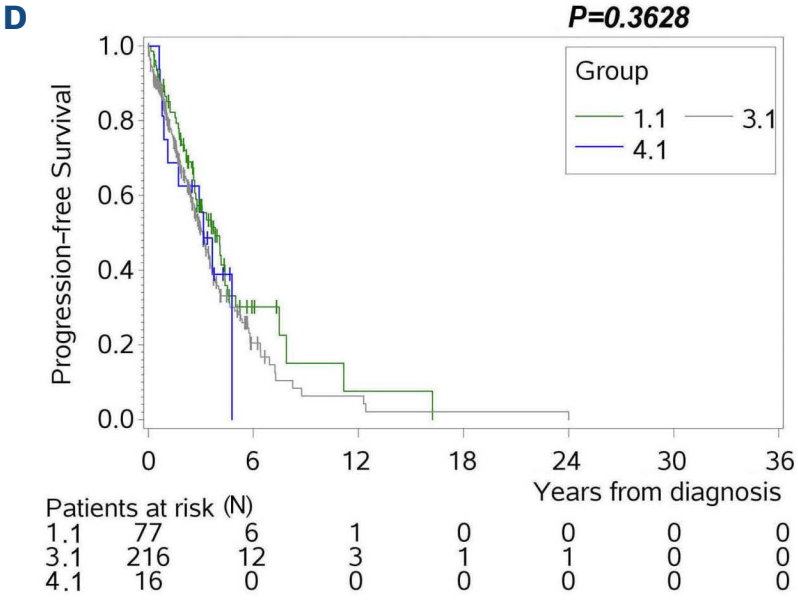
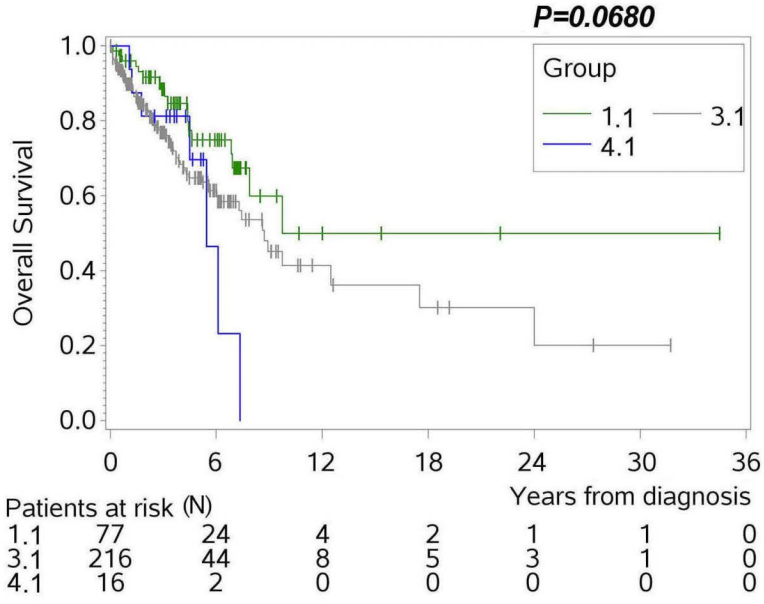


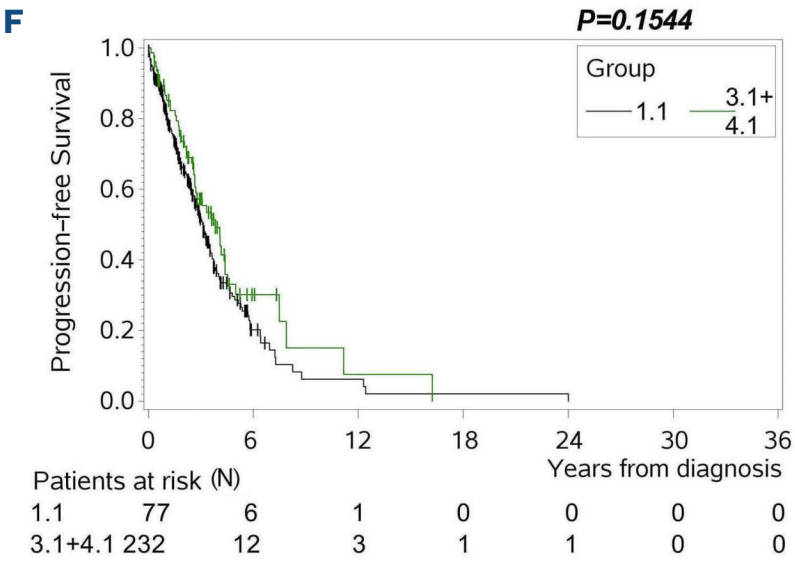
Figure 1. Comparison of progression-free survival and overall survival. (A) Detailed cytogenetic aberration numbers of respective interphase fluorescence *in situ* hybridization subset of patients without IgH-translocations, but other aberrations. (B) Progression-free survival (PFS) of subgroup (SG)1 *versus* SG2 *versus* SG3 *versus* SG4 and (C) overall survival (OS) of SG1 *versus* SG2 *versus* SG3 *versus* SG4. (D) PFS of SG1.1 *versus* SG3.1 *versus* SG4.1 and (E) OS of SG1.1 *versus* SG3.1 versus SG4.1. (F) PFS of SG1.1 *versus* merged SG3.1+4.1 and (G) OS of SG1.1 *versus* merged SG3.1+4.1.

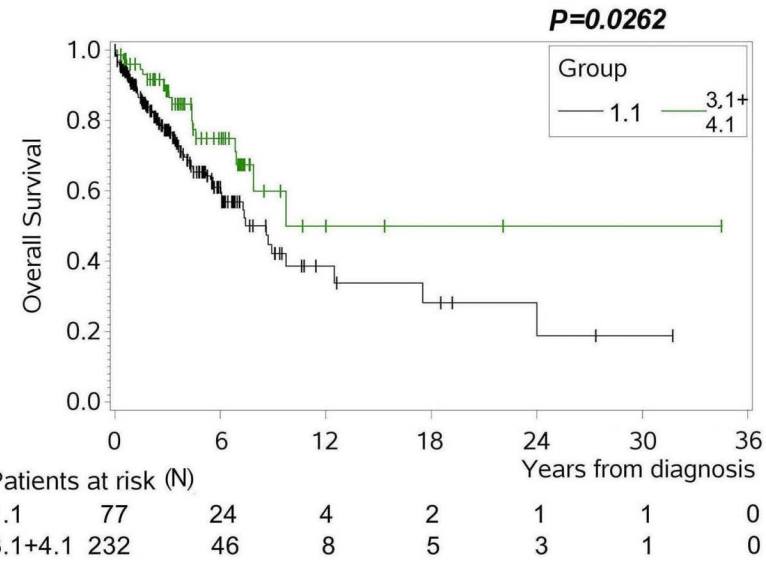


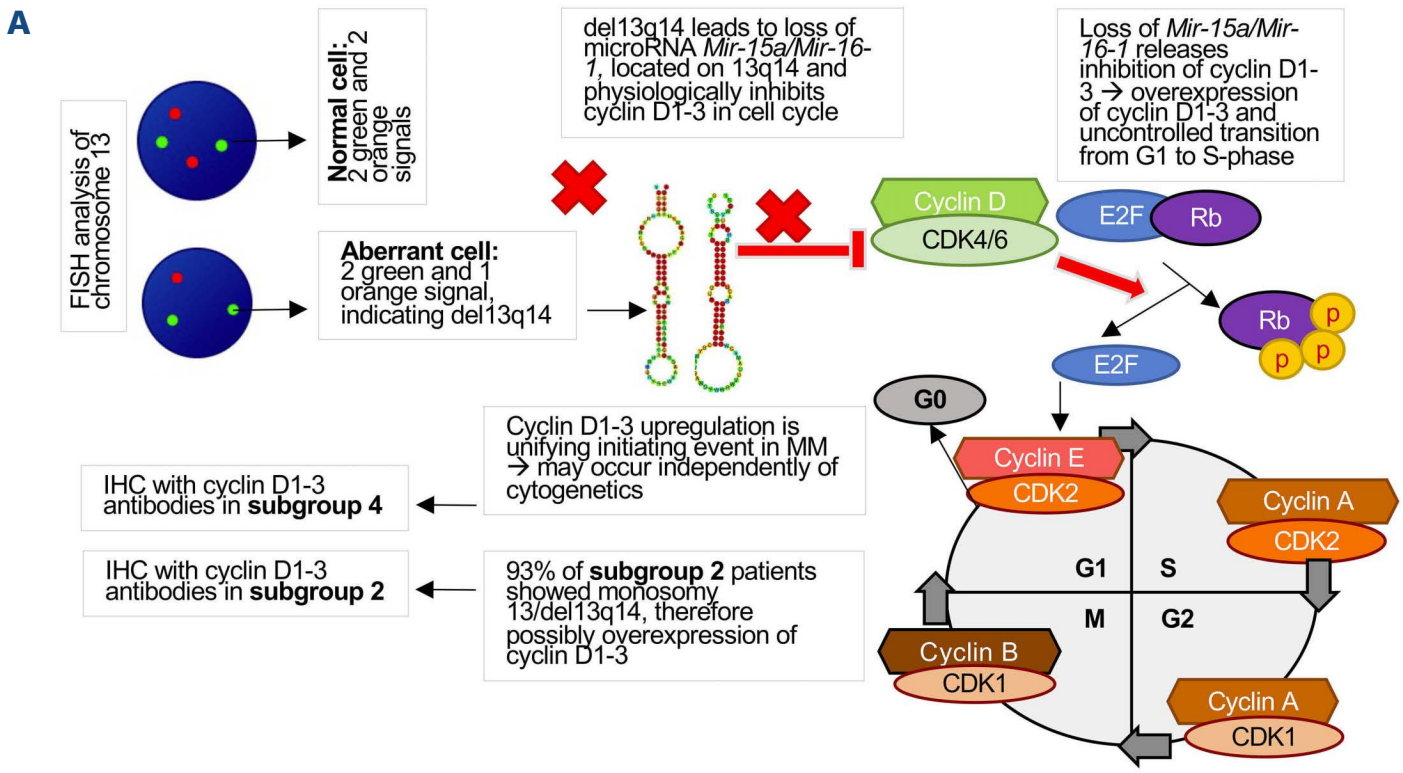


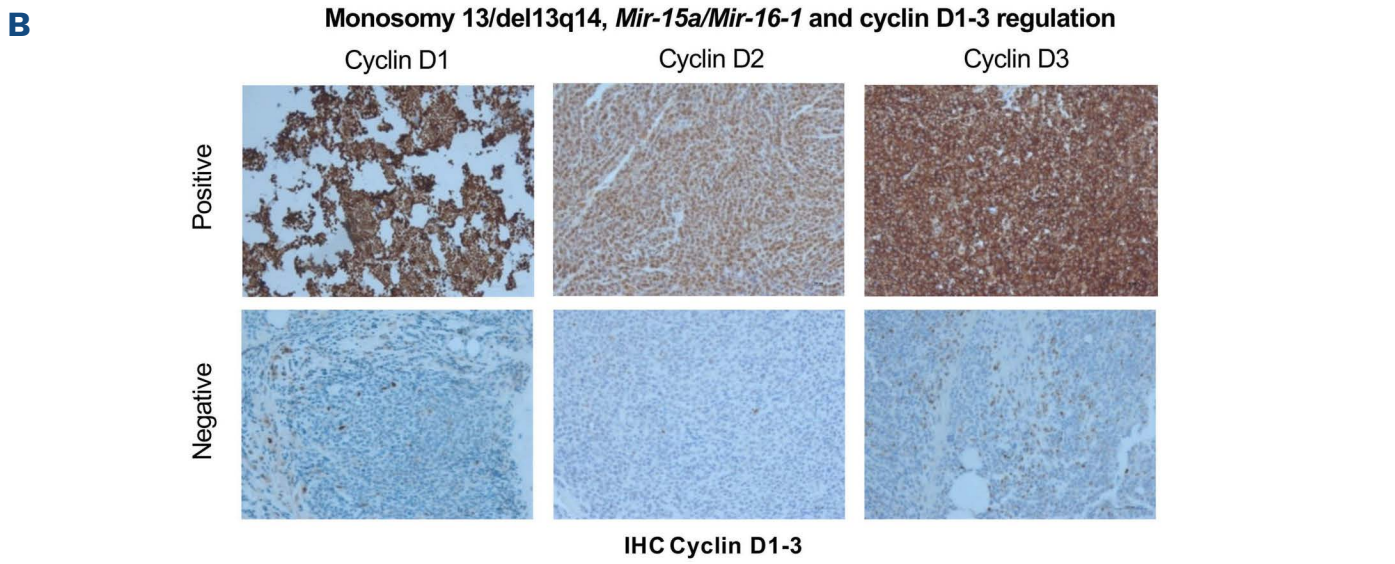


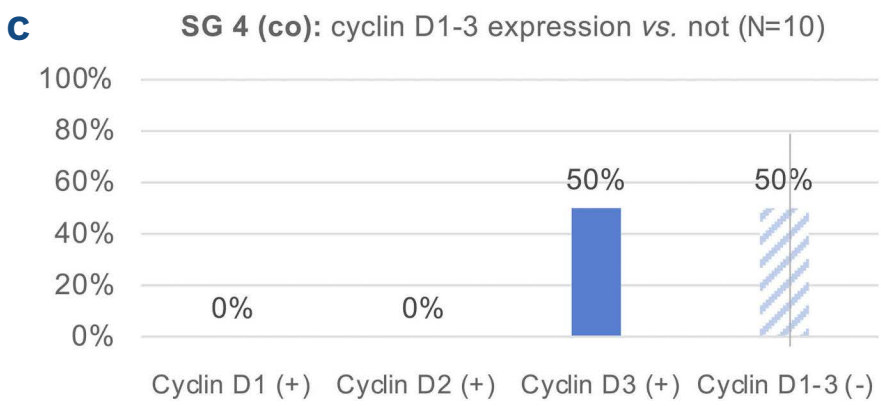


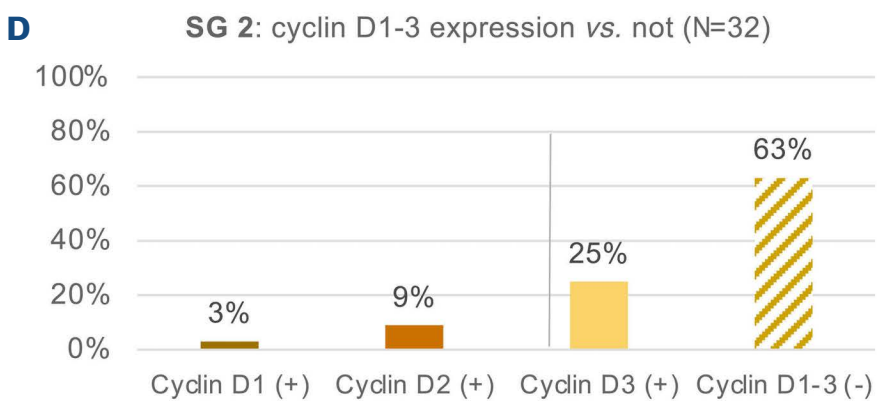












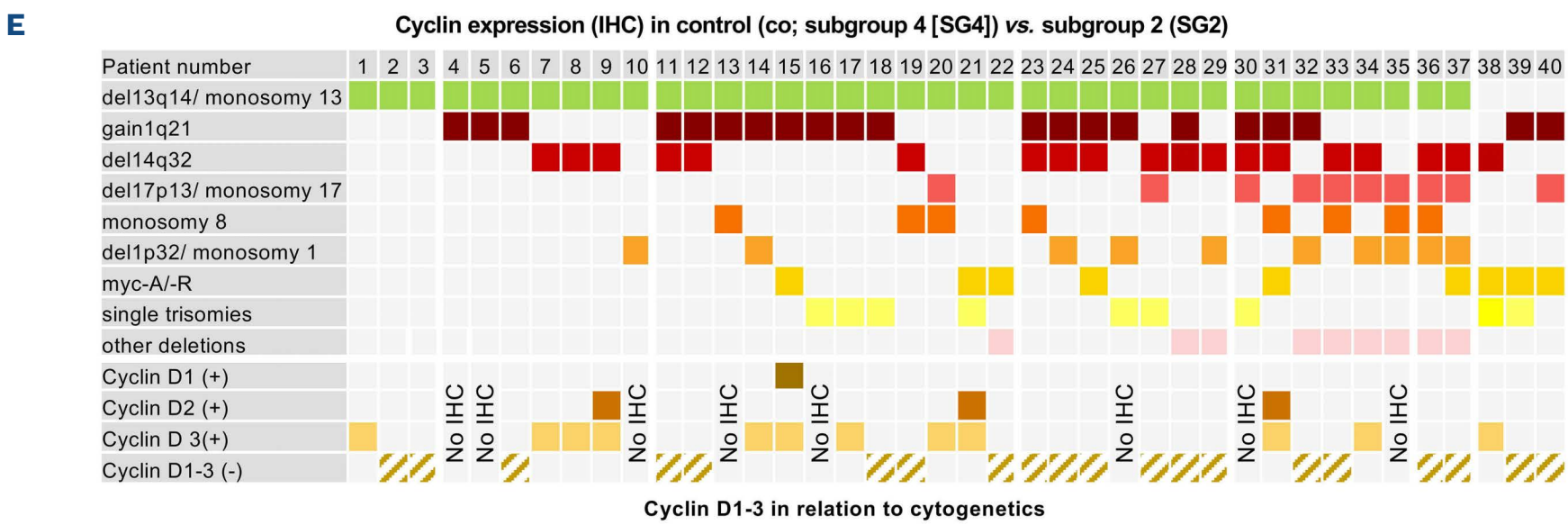


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Figure 2. Immunohistochemical analysis of subgroups 2 and 4 (control group). (A) Relation between monosomy 13/del13q14, *Mir-15a/Mir-16-1* and cyclin D1-3 upregulation. (B) Photomicrographs (x20) showing positive and negative immunohistochemical staining patterns for cyclin D1, D2 and D3. (C) Cyclin D1-3 overexpression via immunohistochemistry (IHC) in subgroups (SG)4 and SG2. (D) cyclin D1-3 expression in relation to the cytogenetics of SG2.

pattern in cyclin D1-3 upregulation could be observed and, therefore, no clear association of cyclin D1-3 upregulation via inhibited *Mir-15a/Mir-16-1* due to monosomy 13/del13q14 occurrence (Figure 2E).

Monosomy 13/del13q14 has been discussed as a third MM-initiating event, in addition to hyperdiploidy or IgH-translocations.[4,9,14-16] Del13 with loss of the microRNA *Mir-15a/Mir-16-1*, located on 13q14, was described as an early event in MM via a refined murine model.[15] Due to the loss of *Mir-15a/Mir-16-1*, proteins mediating the transition from G1- to S-phase in the cell cycle, like cyclin D1-3, were discussed to be overexpressed.[8] Deregulation of cyclin D1-3 had been proposed as a unifying oncogenic event.[9,17] We performed IHC in SG2 (and SG4 [control]) to determine cyclin D1-3, but observed upregulation of cyclins D1-3 in only 37% of SG2 and 50% in SG4 patients. Thus, deletion of 13q14 or the absence of the entire chromosome 13 with loss of *Mir-15a/Mir-16-1* did not mandatorily lead to overexpression of cyclins D1-3 and cyclin D upregulation was not the unifying MM-initiating event in our cohort. Additional analyses seem warranted to further elucidate the MM-initiating mechanisms in this subgroup, as chromosome 13 includes other driver genes, e.g., *Dis3* or *Rb1*.

Strengths of our analysis were the meticulous examination of newly diagnosed MM patients with clinical data and follow-up information. Our median observation period of 7 years was substantial, therefore, our genetic subgroup and outcome data robust. Cytogenetics were analyzed according to the S3-MM-guideline[18] with an extended FISH panel. Even more in-depth cytogenetic analyses can now be performed via whole genome sequencing (WGS) to verify our findings, thereby detecting additional driver genes. Nevertheless, the accumulation of monosomy 13/del13q14 in SG2 was remarkable and as WGS is not necessarily performed in clinical routine yet, but patients without hyperdiploidy/IgH-translocations, but oCA and predominance of monosomy 13/del13q14 occurred quite frequently, an accessible risk stratification and outcome analysis seemed of interest.

Limitations of our study were the single institution approach, yet due to strict inclusion criteria regarding patients' and therapy data, all patients included provided infinitely detailed information. Another criticism could be the heterogeneity of patients as typical for tertiary centers. Since our CCCF-treated patient population was relatively young and the majority received ASCT, we refrained from non- *versus* ASCT-based subgroup analyses, but considered all patients as one group. Besides, one could criticize the use of other than bortezomib-cyclophosphamide-dexamethasone (VCD)-induction in rare subgroups, however, we focused on cytogenetics in this study, therefore, we refrained from further subgroup analyses than those already extensively performed. Due to limited patients, it was not reasonable to further distinguish between monosomy 13 and del13q14, although those events can be associated with a different prognosis.[19] Certainly, SG2 with 13% of patients is a proportion that needs confirmation in even larger cohorts (i.e., within DSMM/GMMG, IFM, MRC or other study cohorts) before any definite assumptions are drawn.

In conclusion, patients without hyperdiploidy/IgH-translocations, but oCA detected via FISH occurred in >10% of our patients presenting with newly diagnosed MM. Most of SG2 patients showed a predominance of monosomy 13/del13q14, suggesting that monosomy 13/del13q14 may act as a MM-initiating event in the absence of other primary events. Expanded analyses, preferably in even larger cohorts should verify our data and further elucidate the impact of hyperdiploidy, IgH-translocations or monosomy 13/del13q14 *versus* secondary aberrations and their underlying mechanisms.

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Disclosures

No conflicts of interest to disclose.

Contributions

The results of this work are based on the results of the dissertation of SW. SW acquired und analyzed the data, and both SW and ME wrote the paper. MP provided the idea for this work and conducted the FISH analyses. BVK assisted in the immunohistochemical analysis. SW, SC and M-AC blindly assessed all bone marrow samples. GI performed the statistical analysis. All authors discussed the results and contributed to the final manuscript.

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Data-sharing statement

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

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