in MM remains to be biologically heterogeneous with the median overall survival (OS) being 3–4 years.[3] Although several prognostic variables such as the International Staging System (ISS) that includes beta-2 microglobulin and albumin levels can be used to define the stage and predict the prognosis in the MM patients, yet, with the advancement in technology for laboratory testing and the emergence of new treatments, novel prognostic markers are being studied to predict disease outcomes.[4]

Flow cytometric DNA content evaluation is a widely used tool in the prognostication of many solid tumors as well as hematological malignancies.[3,5] DNA content is the most frequently measured entity of the cell and flow or laser scanning – cytometry is the method of choice for the analysis of cellular DNA content. The analysis of the DNA content reveals cell ploidy and provides information on the cell position in the cell cycle. DNA content measured by cytometry (DNA ploidy) is defined as DNA index (DI) and for normal (non-tumor, euploid) cells in G0-G1 phase of the cell cycle DI = 1.0. Cells in G2/M phase of the cell cycle have DI = 2.0 and the S phase normal cells are characterized by 1.0 <DI <2.0.[6] This method can also be used to estimate the frequency of apoptotic cells that are characterized by fractional DNA content with DI <1.0 due to extensive DNA fragmentation. They are defined as "sub-G1" or "sub-diploid" cell populations.[6,7]

In this single-institutional study, we have assessed the bone marrow DNA ploidy at baseline in newly diagnosed cases of MM and have studied its association with other prognostic markers and outcome.

MATERIAL AND METHODS

The study was approved by the Institute Ethics Committee (JIP/IEC/2017/0146). All new cases of MM diagnosed based on standard criteria undergoing routine diagnostic workup between July 2019 and July 2021 were included in the study. Data regarding baseline demographic, clinical characteristics and laboratory investigations were collected from the patient records. This included age, gender, clinical presentation, comorbidities, Eastern Cooperative Oncology Group Performance Status (ECOG-PS), routine biochemical tests, serum protein electrophoresis/immunofixation findings, hematological investigations, staging, therapeutic details (induction regimen), and outcome (post-induction and survival data). Fluorescence *in situ* hybridization (FISH) reports wherever available were collected. Response assessment was done using the International Myeloma Working Group (IMWG) response criteria.[8] Progression-free survival (PFS) was defined as the duration between the start of treatment to disease progression or death (regardless of the cause of death) whichever comes first. OS was defined as the duration between the start of treatment to death due to any cause.

Sample collection

Bone marrow aspirate was collected as part of a routine diagnostic workup taking all aseptic precautions and as per standard protocol. No additional invasive procedure was done solely for the study. A bone marrow sample (1–2 ml) was collected in the same syringe after the routine first pull of 0.2–0.5 ml sample for slide morphology to avoid dilution of morphology. Sample processing and data acquisition were done within 24 h of sample collection.

Cell preparation

The cell suspension was prepared by bulk erythrocyte lysis with ammonium chloride-based lysing reagent [0.15 M NH_4Cl (8.29 g NH_4Cl), 1.0 g $KHCO_3$, 37 mg EDTA, and 1 L distilled water]. A 500 µ1–1000 l of the bone marrow sample was added to 7–8 ml of RBC lysing reagent in a falcon tube. Incubation was done for 10 min at room temperature and cells were pelleted by centrifuging for 5 min at 540 g. The supernatant was discarded and cells were washed twice in a phosphate-buffered saline (PBS). After the final wash, the cells were resuspended with PBS. The cell count was performed in an automated cell counter and adjusted to get the final concentration of $1–2 \times 10^6$ cells in 50 l and stained for DNA ploidy analysis with propidium-iodide (PI) staining.

DNA ploidy staining

Step 1 (Fixation): Improper fixation of cells might cause an inadequate PI staining of cellular DNA mimicking apoptotic cells. In-house prepared 70% of ethanol was used to fix the cells. After adjusting the volume of the cells, the cells were fixed with ice-cold 70% of ethanol which was added dropwise to the cell suspension while vortexing (this should ensure the fixation of the cells and minimize clumping). The fixation time was a minimum of 30 min–2 h. In case of delay in further sample preparation, the fixed cells were stored in the refrigerator at 2–8°C.

Step 2 (Washing): After fixation, cells were washed twice with PBS, by centrifuging for 5 min at 850 g.

Step 3 (Staining): The DNA ploidy staining was done using PI kit (The BD Cycletest™ plus DNA Reagent Kit, Cat. No. 340242 BD Biosciences). The cells were treated with ribonuclease, by adding 50 µ1 of RNase. Thereafter, the cells were stained directly by adding 200 µ1 of PI and the sample was immediately acquired using the flow cytometer.

Quality control measures

The cytometer stability and sensitivity were checked daily; quality control measures were performed using Cytometer Setup and Tracking beads as per the manufacturer's recommendations. Using PI stained chicken erythrocyte

nuclei (CEN) and chick thymocyte nuclei (CTN), we established the linearity and resolution of PI labeling for DNA ploidy analysis (BD-Cat. No.349523). In CEN and CTN preparations, single nuclei, doublets, triplets, and certain bigger aggregates are all present. These aggregates produce numerous reference peaks in the fluorescence 2-area (FL2) histogram plots when labeled with PI [Figure 1].

Cell acquisition and analysis

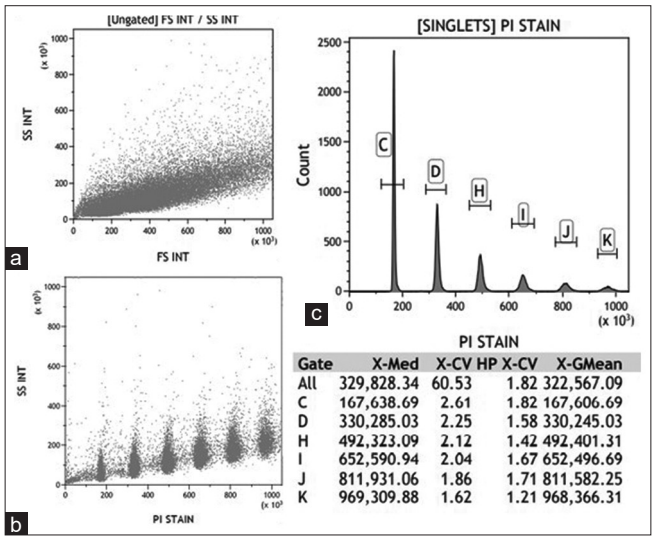
Approximately 10,000–30,000 events were acquired by flow cytometer (Navios-AY43297, Beckman Coulter, BC) to have a minimum of 100 tumor cell events. The cells for DNA ploidy analysis were acquired at a low rate (approximately 200 events/seconds). DNA ploidy was analyzed with Kaluza software (version 2.1) using Michael H. Fox's cell cycle algorithm.

Gating of myeloma cells

Based on the scatter properties (forward scatter [FS]/side scatter [SS]), and, in comparison with the stained immunophenotyping tube done simultaneously, normal lymphocytes (low FS/SS) and abnormal plasma cells (mostly medium FS/SS) were gated [Figure 2]. Events with high SS were not included in the plasma cell gate to avoid inclusion of normal myeloid cells in the bone marrow. For a few of the cases, we tried adding surface markers CD45 and CD38 in the DNA ploidy tube before the fixation step; however, the acquisition was not optimal.

Assessment of DNA ploidy

The frequency (%) of gated cells (plasma cells and control lymphocytes) in particular phases (G0-G1 vs. G2/M) of the cell cycle was noted. The DNA content of these cells was denoted by the mean fluorescence intensity (MFI) which was the peak value of fluorescence of the channel FL2. To assess the DNA index of the tumor sample, we took the ratio of DNA content (MFI) of the G0-G1 population of the myeloma cells with that of the normal control cells (lymphocytes) present in the same sample [Figure 3]). The categories of ploidy were diploidy, hypodiploidy, near hyperdiploidy, and hyperdiploidy based on DI >0.95–<1.06, ≤ 0.95, ≥1.06–<1.16, and ≥1.16, respectively.[5]



Gate	X-Med	X-CV HP	X-CV	X-GMean
All	329,828.34	60.53	1.82	322,567.09
C	167,638.69	2.61	1.82	167,606.69
D	330,285.03	2.25	1.58	330,245.03
H	492,323.09	2.12	1.42	492,401.31
I	652,590.94	2.04	1.67	652,496.69
J	811,931.06	1.86	1.71	811,582.25
K	969,309.88	1.62	1.21	968,366.31

Figure 1: QC for DNA ploidy using chick erythrocyte nuclei stained with propidium-iodide (PI) (a) FSC/SSC plot, (b) SSC/PI (FL2), and (c) count/PI in histogram plot showing CV of the peaks.

Statistical analysis

Continuous variables were summarized as the mean ± standard deviation or median (range) and categorical data were expressed as frequency and percentages. The data were corrected to the first decimal place or the nearest whole number, respectively, wherever possible. The normality of data was checked using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Parametric or non-parametric tests were chosen based on the distribution of data. The differences in the continuous variables between groups (lymphocytes and plasma cells) were evaluated using an independent Student's *t*-test or Mann–Whitney U-test depending on the distribution of data. The differences in the categorical variables between groups (DNA index

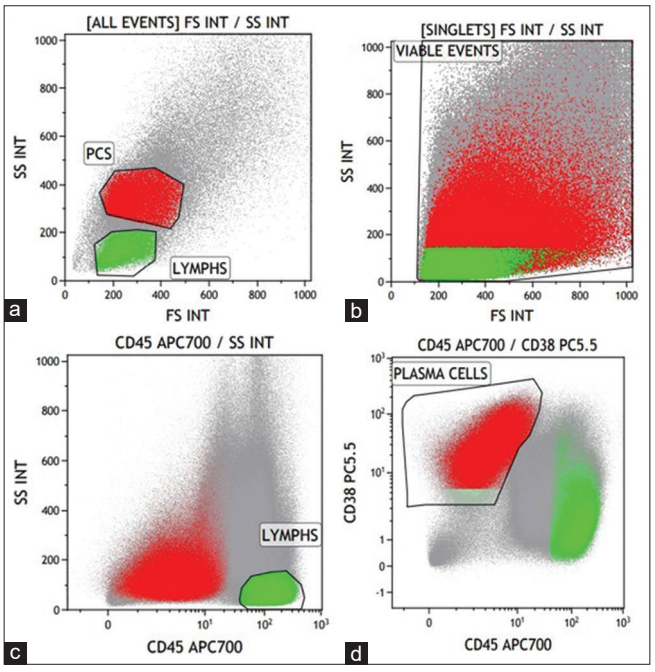


Figure 2: Gating strategy for lymphocytes and plasma cells for ploidy analysis in a case of multiple myeloma (a) FSC/SSC plot of DNA ploidy analysis with gated plasma cells and lymphocytes based on the scatter properties compared with immunophenotyping tube, (b) FSC/SSC plot from the corresponding immunophenotype tube of the same case, (c) CD45/SSC from immunophenotyping tube with gated lymphocytes, and (d) CD38/CD45 from immunophenotyping tube showing gated PC population.

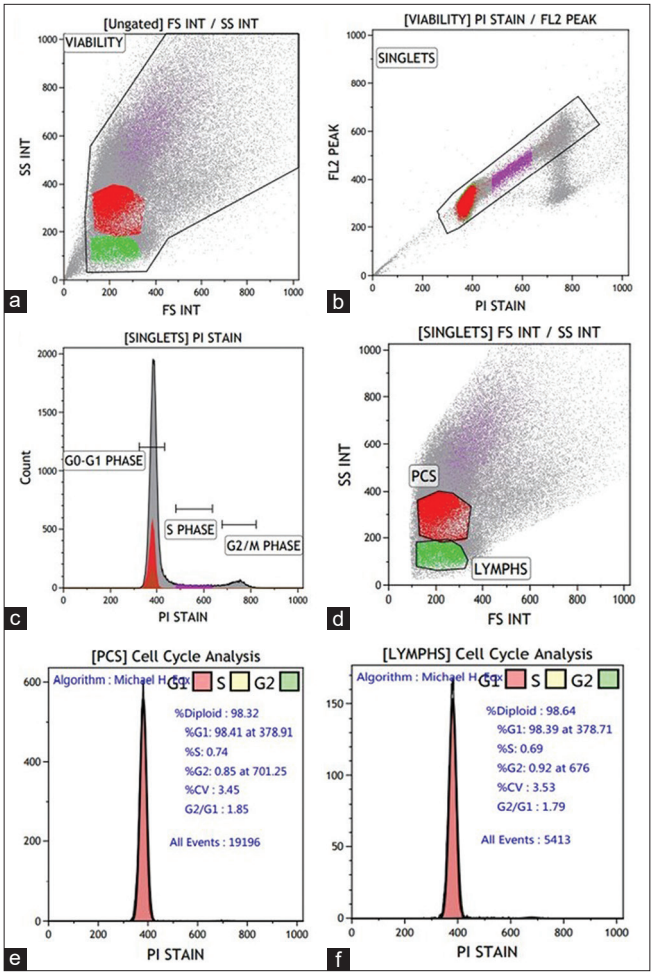


Figure 3: Ploidy analysis in a case of multiple myeloma showing (a) SSC/FSC plot with scatter gate of viable cells, (b) FL2 peak/FL2 integral with singlets gate, (c) count/PI showing various phases of cell cycle among singlets, (d) FSC/SSC plot with gated plasma cells and lymphocyte among singlets based on scatter properties, and (e and f) count/FL2 showing plasma cells and lymphocytes analyzed by cell cycle analysis (Algorithm; Michael H Fox).

vs. ECOG-PS, ISS staging, FISH, and post-induction remission status) were evaluated using the Chi-square test or Fisher's exact test depending on the distribution of data. Kaplan–Meier method using the log-rank test was used to compare the DNA index and survival outcomes (PFS and OS). All statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). All values were two sided and $P < 0.05$ was considered statistically significant.

RESULTS

Calculation of DNA index

A total of 32 patients diagnosed with MM were included in the study. The DNA ploidy analysis showing the percentage of cells and DNA content in various phases of the cell cycle among the lymphocytes and the plasma cells is given in [Table 1]. The percentage of cells in various phases of the cell cycle showed that plasma cells in the S phase were lower than lymphocytes which were not statistically significant. The DNA content of the lymphocytes and plasma cells in the G0-G1 and G2/M phases of the cell cycle denoted by MFI value showed higher DNA content in plasma cells compared to lymphocytes in both these phases which were not statistically significant. The median DNA index of the plasma cells of the patients was 1.02 (0.97–1.31). None were found to have hypodiploid DNA content, 3 (9%) patients had hyperdiploidy (median DI 1.24 [1.21–1.31]), 5 (16%) patients had near hyperdiploidy (median DI 1.09 [1.07–1.15]), and 24 (75%) patients had diploid DNA content (median DI 1.01 [0.97–1.04]). For the study of the association between DNA index and other parameters, hyperdiploidy and near hyperdiploidy have been considered as one category designated as hyperdiploid ($n = 8$) MM and compared with diploid MM ($n = 24$).

Baseline characteristics and their association with DNA index

Overall, 21 (66%) of the patients were male with a median age of 55 (49–60) years. The majority of the patients, 15 (58%) had ISS Stage III disease. Most of the patients, 20 (74%) had an ECOG PS greater than or equal to 2. At our institute, a three-drug combination is the preferred standard of care for MM induction therapy. The three classes of drugs are proteasome inhibitors, immunomodulatory agents, and steroids. The combination of the drugs is chosen based on comorbidities, level of renal dysfunction, and fitness status. The most common induction regimen used for the patients was VRd (Bortezomib + Lenalidomide+ Dexamethasone) in 13 (44.84%), VPD (Bortezomib + Pomalidomide + Dexamethasone) in 7 (24.15%), and VD (Bortezomib + Dexamethasone) in 6 (20.68%) of the cases. The post-induction response assessment was done after a median number of 5 (3–9) cycles. For the study of the association between DNA index and response outcome, very good partial response (VGPR), complete response, and stringent complete response have been considered good outcomes and partial response, stable disease, and progressive disease have been considered as poor outcomes. Post-induction outcome was available for 21 patients among whom 15 (71%) had VGPR and above. The patients were segregated into two categories as diploid MM and hyperdiploid MM as mentioned earlier. [Table 2] shows the baseline characteristics of the study patients overall and segregated into diploid and hyperdiploid categories. The median age of the patients with hyperdiploid DNA index was higher than those with diploid DNA index which was statistically not significant ($P = 0.5$). There was no significant association between DNA index and the following parameters: ISS staging ($P = 0.68$), ECOG PS ($P = 0.59$), and post-induction remission status ($P = 0.10$).

Table 1: DNA ploidy analysis showing percentage of cells and DNA content in various phases of cell cycle.

Parameters	Control (lymphocytes) (*n*=32)	Plasma cells (*n*=32)	*P*-value
Phases of cell cycle (%) median (range)			
G0-G1	92.1 (48.2–99.7)	92.8 (54.9–99.5)	0.8
S phase	5.3 (0.06–49.6)	3.9 (0.01–35.1)	0.49
G2/M	2.2 (0.06–18.5)	2 (0.08–12.5)	0.24
MFI values median (range)			
G0-G1	274.5 (73.8–513.6)	278.7 (96.9–504.4)	0.54
G2/M	554.8 (114–1023)	589.1 (245.8–1023)	0.32
Ratio of MFI values Median (range)			
G2/G1	1.8 (1.5–3)	1.9 (1.5–2.9)	0.12
CV (%) of peak median (range)			
G0–G1	4.4 (2.1–16.6)	6 (2.2–17.5)	0.17

MFI: Mean fluorescence intensity

Table 2: Baseline characteristics of the study patients and its association with DNA index.

Characteristics	Overall (*n*=32)	Diploid DNA index (*n*=24)	Hyperdiploid DNA index (*n*=8)	*P*-value
Age in years – median (range) (*n*=32)	55 (49–60)	53 (48–59)	60 (56–63)	0.5
Gender – *n* (%) (*n*=32)				
Male	21 (66%)	16 (67%)	5 (63%)	0.83
Female	11 (34%)	8 (33%)	3 (37%)	
ISS staging – *n* (%) (*n*=26)				
ISS-I	3 (12%)	3 (14%)	0	0.68
ISS-II	8 (30%)	7 (32%)	1 (25%)	
ISS-III	15 (58%)	12 (54%)	3 (75%)	
ECOG PS – *n* (%) (*n*=27)				
0–1	7 (26%)	5 (24%)	2 (33%)	0.59
≥2	20 (74%)	16 (76%)	4 (67%)	
Post-induction outcome – *n* (%) (*n*=21)				
VGPR	11 (52%)	6 (35%)	5 (100%)	0.10
CR	3 (14%)	3 (18%)	0	
sCR	1 (5%)	1 (6%)	0	
PR	3 (14%)	4 (23%)	0	
SD	2 (10%)	2 (12%)	0	
PD	1 (5%)	1 (6%)	0	

ECOG PS: Eastern Cooperative Oncology Group Performance Status, VGPR: Very good partial response, CR: Complete response, sCR: Stringent complete response, PR: Partial response, SD: Stable disease, PD: Progressive disease

FISH test results were available for nine patients; five of them had cytogenetic translocations/abnormality on FISH while the remaining four had no translocations. The FISH abnormalities detected were isolated del13q14.3 in three patients, del13q14.3 with t (4;14), del 13q14.3, and Trisomy 1 in one patient each. There was no significant association between DNA index and the presence or absence of any FISH abnormality ($P = 0.77$).

Survival outcome and its association with DNA index

The median OS in the study patients was 20 (CI 11.41–28.85) months and the median PFS was not reached. There was no difference in the OS among patients with diploid MM and hyperdiploid MM ($P = 0.84$) with the median OS in diploid patients being 20 (CI 10.80–29.45) months and the median OS in hyperdiploid patients was not reached. We could not compare the difference in PFS and DNA index between the two groups as there were no events in the hyperdiploid group.

DISCUSSION

For the diagnosis, staging, and evaluation of the response in MM, new systems have been developed. The IMWG, with some modifications and clarifications, served as the main inspiration for the diagnostic and response criteria that are suggested. Myeloma staging is currently done using the ISS and Revised-ISS, but other stratification models have also been developed.[9,10] A new genetic categorization of MM has identified a distinct subgroup of the disease that is associated with hyperdiploidy and a favorable prognosis.[11] Even though

almost all patients with MM respond to initial treatment, myeloma that does not respond to initial chemotherapy remains a serious concern.

Flow cytometry allows multiple attributes of individual cells to be measured at the same time. In the bone marrow, MM cells tend to be bigger than normal small lymphocytes and thus can be distinguished using cell size and light scatter properties as were done in our study for initial gating. A commonly used flow cytometry DNA ploidy analysis method is based on PI.[12] Morgan *et al*. used propidium-iodide without surface markers for DNA ploidy analysis. They have analyzed myeloma cells based on the light scatter (SSC vs. FSC) properties.[13] In our study, we used the same approach using scatter properties and PI fluorescence intensity to determine the DNA content of the two cell populations and to calculate the DNA ploidy of myeloma cells.

There are several flow cytometric DNA ploidy strategies used in various studies. Tembhare *et al*. investigated a multiparametric approach for concurrent evaluation of six color immunophenotyping and DNA content analysis utilizing a novel dye, FxCycle Violet, which is stimulated with a violet laser. With the benefit of concurrent 6–7 color immunophenotyping, which enables more precise identification of malignant cells based on light scatter characteristics and tumor-specific markers, this technique produced results that were comparable to those of other conventional DNA ploidy measures.[5] Sidana *et al*. studied the rapid assessment of hyperdiploidy using the plasma cell proliferative index and concurrently trisomies were found using interphase FISH on the bone marrow.[14] Almeida *et al*. proved the utility of a high-sensitivity DNA ploidy assay for determining minimal residual disease.[15]

DNA ploidy analysis includes the estimation of DI calculated by the ratio of mean fluorescence of G0-G1 peak of tumor cells to normal lymphocytes. The DI cutoff for determining hyperdiploidy has been given in various studies. Orfäo *et al*. found that in hyperdiploidy, the DI varied from 1.08 to 1.32 in MM.[16] Bezić *et al*. defined a near-hyperdiploid group with a DI of 1.06–1.3 and its importance in early breast cancer.[17] Sidana *et al*. considered values of 0.95–1.05 to be diploid, below 0.95 as hypodiploid, and between 1.06 and 1.50 as hyperdiploid. Near-tetraploid values ranged from 1.51 to 1.7, whereas tetraploid values ranged from >1.7 to 10% clonal G2/M cells with a visible 4n population.[14] Tembhare *et al*. categorized ploidy groups as hypodiploidy (<0.95), diploidy (0.95–1.05), near hyperdiploidy (1.06–1.15), and hyperdiploidy (>1.16), respectively.[5] In our study, we have also used a similar cutoff to define ploidy categories.

In our study on 32 patients with MM, we found no cases of hypodiploid MM, but we did find hyperdiploid in 25% (8 of 32) of the patients. In their study on 124 B-ALL and 50 MM patients, Tembhare *et al*. had 2% of hypodiploidy, 58% of hyperdiploidy, 8% of near hyperdiploidy, and the remaining 30% of diploidy among MM cases.[5] The prognostic value of DNA content of the plasma cells in MM has been explored in earlier studies with better prognosis reported in hyperdiploid MM.[3,18] Sidana *et al*. found hyperdiploidy in 53% of newly diagnosed MM patients, which was associated with better survival outcomes.[14] Similarly, there was a correlation between hyperdiploidy and having a better OS in other studies by Morgan *et al*.,[13] Avet-Loiseau *et al*.,[19] and in a large study on 349 patients by Greipp *et al*.,[20] whereas other studies have shown a worse prognosis for MM patients with hyperdiploidy.[21] Hypodiploid MM by DNA content flow cytometry is rarer, occurring in <2% of newly diagnosed MM patients; the previous studies have shown these patients to be unresponsive to therapy and to have short survival.[13,20] In recent studies, flow cytometric ploidy analysis is increasingly being used in the analysis of B-ALL with its correlation of cytogenetic-based ploidy stratification and risk assessment,[22] and other studies are being done to assess the potential role in the detection of measurable residual disease.[23]

In summary, this is the first study from our center on ploidy analysis using flow cytometry which helped us in standardizing the method. The phases of the cell cycle, and the DNA content of aberrant and normal cells, were comprehensively analyzed. Based on the calculated DNA index, DNA ploidy was categorized which was correlated with clinical indicators. The frequency of aneuploidy detected was 25% of hyperdiploidy. There are two limitations of our study. First, we were unable to get optimal results in doing DNA ploidy staining along with surface markers. Having simultaneous immunophenotypic markers in the same tube would lead to better delineation of the abnormal population. Second, we had a small sample size and so significant associations with clinical characteristics and outcomes could not be established. Studies have shown the flow cytometric DNA content of plasma cells from MM patients to be a prognostic factor independent of previously established variables. Patients with myeloma who have a high level of hyperdiploid DNA in their plasma cells may have a better prognosis than those who have a normal level of DNA. Better characterization of abnormal and normal cell populations and bigger sample size can be enrolled to correlate better with outcome measures.

CONCLUSION

Flow cytometry is a useful tool in DNA ploidy analysis and its role as a prognostic factor can be explored in larger studies in MM as well as other hematologic malignancies.

Declaration of patient consent

Institutional Review Board (IRB) permission obtained for the study.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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