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BrdU incorporation in multiparameter flow cytometry : A new cell cycle assessment approach in multiple myeloma

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The authors have no conflict of interest to declare.

Abstract

Background

Mutiple myeloma (MM) is a neoplasia characterized by the accumulation of malignant plasma cells (PC) in the bone marrow. Although proliferation markers have been studied in MM, none of the current staging systems include them. Moreover, approaches used to analyze proliferation do not separate MM cells (MMCs) from normal PC.

Methods

In this study, we combined multiparameter flow cytometry and BrdU incorporation or Ki67 staining to analyze MM cell proliferation in 44 monoclonal gammopathy of undetermined significance (MGUS), 153 newly diagnosed MM patients and 69 MM patients at relapse. The prognostic value of proliferation assessment was analyzed in 60 newly diagnosed patients treated with high-dose chemotherapy supported by autologous hematopoietic stem cell transplantation.

Results

The median number of proliferating malignant PC significantly increases during MM disease progression. MM patients with a percentage of proliferating MMCs > 1.42% using BrdU/DAPI or > 1.1% using ki67/DAPI, are associated with a significantly shorter event free survival compared to patients with a lower percentage of proliferating MMCs.

Conclusions

Combination of flow cytometry with BrdU or ki67/DAPI staining could become a standard for the determination of MM cell proliferation. Furthermore, in the context of new effective myeloma treatment options, assessment of MM cell proliferation may be valuable, in clinical trials, to identify novel agents that could significantly affect the

small proliferative compartment of MM cells.

Introduction

Multiple myeloma (MM) is characterized by an accumulation of malignant plasma cells (PC) in the bone marrow (BM) leading to bone destruction, renal failure and organ dysfunction. Within the last decade, new treatment strategies have significantly improved the outcome of MM patients (1). However, despite these new treatments using drugs such as proteasome inhibitors or immunomodulatory agents (IMiDs), most patients will eventually relapse and die (2). A key biological question is the ability to perform an accurate appreciation of the tumor burden, in terms of infiltrating plasma cells, for the initial staging of patients and for the monitoring of the disease. Flow cytometry is widely used for the diagnosis and monitoring of hematological disorders including MM (3-5). Proliferating PC, i.e. the growing fraction of MM cells, have been evaluated by flow cytometry detection of newly synthesized DNA using techniques based on the uptake of tritiated thymidine or bromodeoxyuridine (BrdU) (6-8). The so-called plasma cell labeling index (PCLI) based on propidium iodure incorporation (9) has been shown to be a powerful and independent predictor of survival in MM (10,11) as the assessment of Ki-67 expressing cells in the malignant fraction (12). Although proliferation markers have been studied in MM, none of the current prognostic scores or staging systems, available to physicians nowadays, include these markers. Moreover, the approaches used to analyze proliferation in MM do not separate MMCs from their normal cell counterparts. Therefore, easy-to-perform markers of proliferation remain to be developed for routine practice. Novel

multiparameter flow cytometry (MFC) enables increased sensibility and depth of malignant PC detection (13). Furthermore, assessment of proliferating myeloma cells is of special interest, as proliferating MMCs can be targeted by available treatments and upcoming therapeutic treatment options (14). The study aimed to combine MFC and BrdU incorporation or Ki67 staining to analyze MM cell proliferation in samples from MGUS and MM patients. We also investigated the prognostic value of proliferation assessment in a cohort of newly diagnosed MM patients.

Materials and methods

Primary multiple myeloma cells

Bone marrow samples were collected after patients' written informed consent in accordance with the Declaration of Helsinki and institutional research board approval from Montpellier University hospital (DC-2008-417). In particular, bone marrow samples were collected from 44 MGUS patients, 153 MM patients at diagnosis and 69 at relapse. For 60 newly diagnosed patients treated with high dose Melphalan (HDM) and autologous stem cell transplantation (ASCT), MMCs were purified using anti-CD138 MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and their gene expression profile (GEP) obtained using Affymetrix U133 plus 2.0 microarrays.

Antibodies

Anti CD45 R Phycoerythrin-TexasRed (ECD) (clone-J33), anti CD138 allophycocyanin (APC) (clone-BB4), anti CD38 Pe-Cyanin 5.5 (PeCy5.5) (clone-LS198-4-3), CD20 allophycocyanin-AlexaFluor 750 (APC-AF 750) (clone-B9E9), CD19 R Phycoerythrin-Cyanine 7 (PC7) (J3-119), CD56 R Phycoerythrin-Cyanine 7 (PC7) (clone-N901), CD117 R Phycoerythrin-Cyanine 7 (PC7) (clone-104D2D1),

CD27 R Phycoerythrin-Cyanine 7 (PC7) (clone 1A4CD27) monoclonal antibodies (mAbs), were purchased from Beckman Coulter (Miami, FL, USA), CD200 Phycoerythrin-Cyanine 7 (clone-MRCOX-104), Kappa immunoglobin light chain phycoerythrin (PE) (clone-G20-193), Lambda immunoglobin light chain fluoroscein isothiocyanate (FITC) (clone-JDC-12), KI67 allophycocyanin (APC) (clone-B56) and anti BrdU-APC (BrdU Flow Kit from BD Biosciences) mAbs were purchased from Becton Dickinson Biosciences (San Jose, CA, USA).

Cell staining for immunophenotyping of MM

BM aspirates were harvested in heparin-containing syringes. Samples were filtered through a 100 µm filter to remove bone debris. Whole bone marrow was incubated with or without BrdU (10µM), (Flow Kit APC, Biosciences, San Jose, CA, USA) at 37°C during two hours. To lyse red blood cells, samples were incubated with a 4-fold volume excess of ammonium chloride for 20 minutes. Then, leukocytes were washed and incubated in phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, United Kingdom) containing 10% goat serum albumin (PAA Laboratories, Paris, France) for 10 minutes as blocking step. Erythrocytes-lysed bone marrow samples after saturation step using goat serum (PPA laboratories, Austria), were labeled with optimal concentrations of antibodies (Table 1). Cells were stained with anti CD19, anti CD20, anti CD27, anti CD38, anti CD56, anti CD200 and anti CD45 mAbs, during twenty minutes at 4°C, washed twice in PBS/10% goat serum, fixed and permeabilized (BrdU Flow Kit from BD Biosciences, BD Biosciences) and washed using PermWash buffer (BD Biosciences). Cells were incubated during one hour at 37°C in PBS containing DNAse I (300 µg/ml). Cells were then stained with anti-Kappa light chain, anti-Lambda light chain and anti BrdU-APC mAb (BrdU Flow Kit from BD Biosciences) during twenty minutes at 4°C (Table 1). For Ki67 staining, cells

were labeled as described in Table 1. After fixation and permeabilization, cells were stained with an APC conjugated anti-KI67 antibody or isotypic APC (BD Biosciences) and anti-Kappa light chain, anti-Lambda light chain mAb. Nuclei were stained in PermWash containing DAPI (2μg/ml) (4',6 Diamido-2-phenylindole, dilactate from Invitrogen, Life Technologies) during twenty minutes at 4°C. Cells were analyzed using Cyan cytometer (Beckman Coulter, FL, USA). Contaminating events were removed on both FCS and SSC plots. Singlets were plotted on FSC-A vs FSC-H and SSC-A vs SSC-H plots to remove debris and to select the total leukocyte population. PC and B cells were selected on CD45/CD38 and CD20/CD19 plots, respectively. Abnormal PC were selected based on the CD19, CD27, CD56, CD117 and CD200 signals (13). 5,000,000 events were acquired per tube and the minimum number of abnormal plasma cells needed was 20. The maximum sensitivity of the method is 0.0004% (13). The analyses have been done using FlowJo software (FlowJo, LLC).

Gene expression profiling and statistical analyses

Gene expression data were normalized with the MAS5 algorithm and analyses processed with GenomicScape (http://www.genomicscape.com) (15) the R.2.10.1 and bioconductor version 2.5 programs (16). Gene Set Expression Analysis (GSEA) was used to identify genes and pathways differentially expressed between populations (17). Univariate and multivariate analysis of genes prognostic for patients' survival was performed using the Cox proportional hazard model. Difference in event free survival between groups of patients was assayed using Maxstat algorithm (18) and survival curves plotted using the Kaplan-Meier method.

Results

MFC was used to delineate malignant and normal plasma cells (13) (Supplementary Figure S1A). PC were selected on CD45/CD38 plot. Specific malignant markers including CD117, CD56 and CD200 combined with CD19, CD27 normal PC markers and kappa or lambda light chains were used to identify malignant from normal plasma cells (Supplementary Figure1A) (Table 1). In order to assess proliferation of myeloma cells specifically, we combined MFC with BrdU incorporation or Ki67 staining (Table 1) (Supplementary Figure S1B).

MFC and BrdU incorporation was investigated in 44 MGUS, 153 newly diagnosed MM patients and 69 MM patients at relapse (Figure 1A). The median percentage of malignant PC in the S phase of the cell cycle in MGUS, newly diagnosed MM patients and relapsing patients was 0.2% (range: 0 to 1.23%), 0.4% (range: 0 to 17.3%) and 0.83% (range: 0 to 33.8%) respectively. The median number of proliferating malignant PC was 2-fold higher in newly diagnosed patients compared to MGUS (P = 3E-5) and 2-fold higher in MM at relapse compared to patients at diagnosis (P = 0.03) (Figure 1A). We also investigated the interest of Ki67 staining to assess proliferation of malignant plasma cells. MFC incorporating Ki67/DAPI staining was also investigated in 11 MGUS, 49 newly diagnosed MM patients and 18 MM patients at relapse (Figure 2A). The median percentage of $Ki67^{+}/DAPI^{+}$ (cells in S-G2-M phases) malignant PC in MGUS, newly diagnosed MM patients and relapsing patients was 0.38% (range: 0.07 to 1.75%), 0.59% (range: 0 to 18.8%) and 0.91% (range: 0 to 17.6%) respectively. The median number of $Ki67^{+}/DAPI^{+}$ malignant PC was significantly higher in newly diagnosed patients compared to MGUS and in MM at relapse compared to MGUS (P = 0.04). MMC proliferation assessment using BrdU incorporation or Ki67+/DAPI+ staining was highly correlated (r = 0.95, P < 0.001) (Supplementary Figure S2).

The prognostic value of proliferation assessment using BrdU incorporation was analyzed in 60 newly diagnosed patients treated with high dose chemotherapy supported by autologous hematopoietic stem cell transplantation (HSCT). Using R Maxstat algorithm(18), allowing to determine the optimal cutpoint for continuous variables (19-21), we identified that MM patients with a percentage of proliferating MMCs > 1.42 are associated with a significant shorter event free survival (20 months median EFS) (P = 0.0005) compared to patients with a percentage of proliferating MMCs ≤ 1.42 (not reached median EFS) (Figure 1B). Using the same methodology, MM patients with a percentage of ki67+/DAPI+ MM cells > 1.1% were significantly associated with an adverse EFS (19 months median EFS) compared to patients with a percentage of ki67+/DAPI+ MM cells ≤ 1.1% (not reached median EFS) (P = 3E-5) (Figure 2B). Therefore, proliferation staining using BrdU incorporation or ki67/DAPI was significantly higher in newly diagnosed patients classified in the proliferation subgroup of the myeloma molecular classification(22) (Supplementary Figure S3). MM cell proliferation using BrdU incorporation or KI67/DAPI was also significantly higher in patients with high growth proliferation gene index (GPI)(23), UAMS-high risk score (HRS)(24), high RS GEP-based score(25) and high IFM score(26) associated with an adverse outcome in MM (Supplementary Figure S4 and Supplementary Figure S5).

Gene Set Expression Analysis (GSEA) was performed comparing gene expression profiles of patients with high risk proliferation assessment (S phase > 1.42%) to patients with low risk proliferation (S phase < 1.42%). Accordingly, high risk patients are characterized by a significant enrichment of genes involved in cell cycle and proliferation (CHANG CYCLING GENES, MOLENAAR TARGETS OF CCND1 CDK4, ROSTY CERVICAL CANCER PROLIFERATION CLUSTER and CHIANG LIVER

CANCER SUBCLASS PROLIFERATION UP, $P < 0.005$) (Figure 1C). In contrast, MM patients with low risk proliferation assessment were characterized by a significant enrichment of genes related to mature bone marrow plasma cells (MOREAUX B LYMPHOCYTE MATURATION BY TACI UP, $P = 0.002$) (Figure 1D). Altogether, these data revealed that combination of MFC with BrdU incorporation or KI67/DAPI staining is a practical method for MM cell proliferation assessment. Furthermore, this methodology predicts EFS in newly diagnosed MM patients.

Discussion

During the last years, a significant expansion of MM therapeutic options occurred. This underlines the need of useful biological and prognostic informations to develop rational and targeted therapies. The plasma cell labeling index was shown to be one of the most powerful risk factor in MM (10,11). However, this approach did not separate malignant MM cells from normal PC. In the present study, we have described that combination of multiparameter flow cytometry with BrdU incorporation or ki67 staining allows specific MM cell proliferation assessment. We identified that MM patients with > 1.42% proliferating MM cells identified with BrdU/DAPI or > 1.1% using ki67/DAPI correlated with significant quicker relapse, in the transplantation settings. The methodology yields important information regarding myeloma prognosis at the time of diagnosis. In this study, as expected, results obtained with BrdU incorporation significantly correlated with ki67/DAPI analyses as described in other cancers (27,28). Both approaches assess myeloma-specific proliferation on routinely collected fresh bone marrow aspirates. Comparing the two methods, BrdU incorporation requires ex vivo labeling of viable PC, in real time, with equipment that could not be available in all clinical laboratories. The Ki67/DAPI methodology could be used in any laboratories with equipment for clinical multiparametric flow cytometry

analyses. BrdU incorporation allows detection of S-phase cells only. Ki67 is found in multiple phases of the cell cycle including S, G2, M (27,28) and could provide more information relative to quiescence (29).

Several groups have shown the value of minimal residual disease (MRD) analysis over previous response criteria in MM patients (3-5). Our methodology is also suitable for MRD with low population of tumor cells. Combining the high clinical sensitivity of MFC to the detection of a proliferative contingent of MM cells will enable the identification of patients with active disease during the follow-up of MRD.

Furthermore, in the context of new effective myeloma treatment options, assessment of MM cell proliferation may be valuable, in clinical trials, to identify novel agents that could significantly affect the small proliferative compartment of MM cells.

In conclusion, combination of MFC with BrdU incorporation or ki67/DAPI staining could become a standard for the determination of the fraction of proliferating malignant plasma cells.

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Figure legends:

Figure 1. Assessment of malignant plasma cell proliferation using BrdU incorporation

(A) The percentage of malignant proliferative MM cells was analyzed using BrdU incorporation and multiparameter flow cytometry in bone marrow samples from 44 patients with MGUS, 153 newly diagnosed patients and 69 patients at relapse.

(B) A percentage of proliferating MM cells > 1.42% could predict for shorter event free survival. Patients of a cohort of 60 newly diagnosed patients were ranked according to increasing proliferation defined by BrdU incorporation/DAPI and a maximum difference in event free survival (EFS) was obtained using the Maxstat R function.

(C) Top gene sets significantly associated with high risk proliferation assessment in MM.

GSEA enrichment plots with the absolute enrichment *P* value and the normalized enrichment score of the gene set.

(D) Top gene sets significantly associated with low risk proliferation assessment in MM.

GSEA enrichment plots with the absolute enrichment *P* value and the normalized enrichment score of the gene set.

Figure 2. Assessment of malignant plasma cell proliferation using ki67/DAPI

(A) The percentage of proliferating malignant plasma cells was analyzed by flow cytometry using ki67+/DAPI+ staining, in bone marrow samples from 11 patients with MGUS, 49 newly diagnosed patients and 18 patients at relapse.

(B) A percentage of proliferating MM cells defined by ki67+/DAPI+ staining > 1.1% could predict for shorter event free survival. Patients of a cohort of 31 newly diagnosed patients were ranked according to increasing proliferation defined by

ki67+/DAPI+ staining and a maximum difference in event free survival (EFS) was obtained using the Maxstat R algorithm.

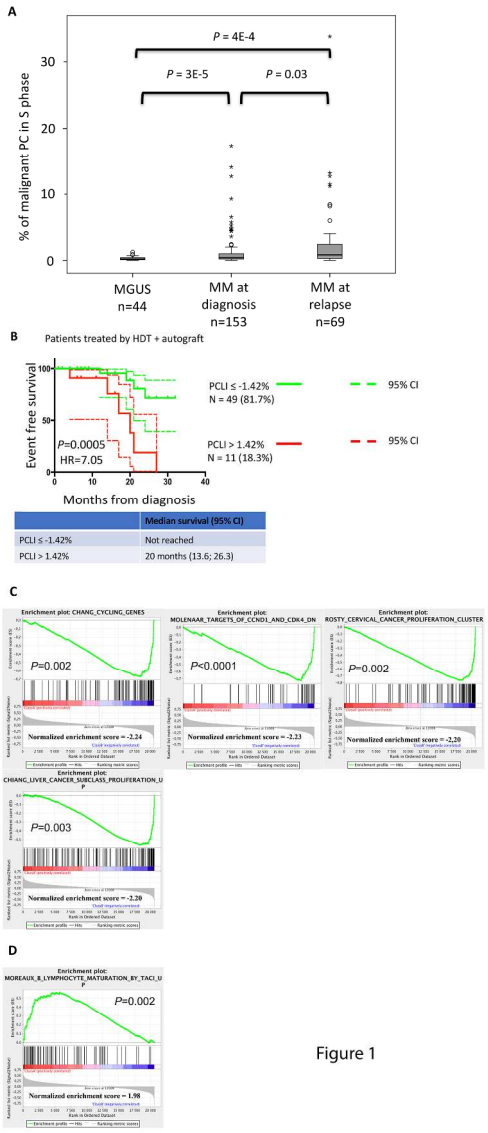
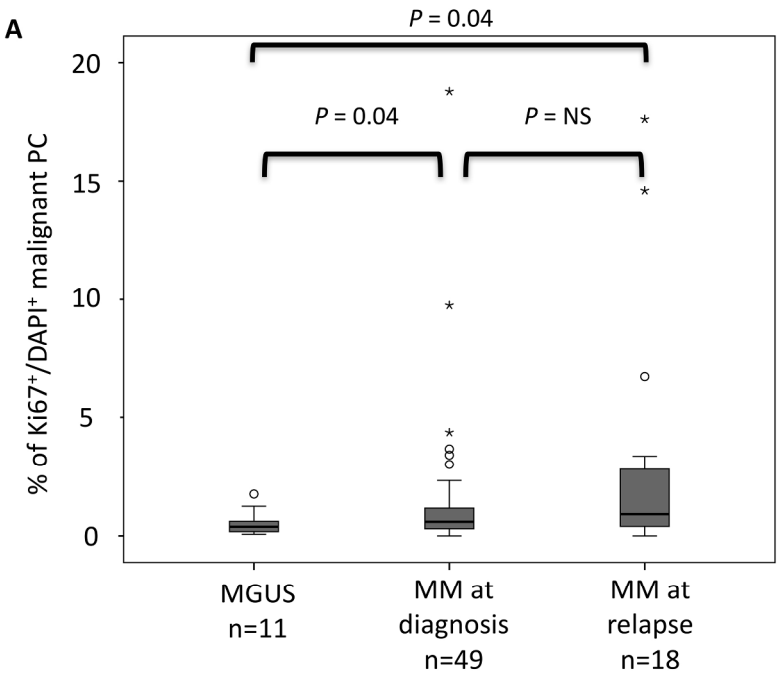
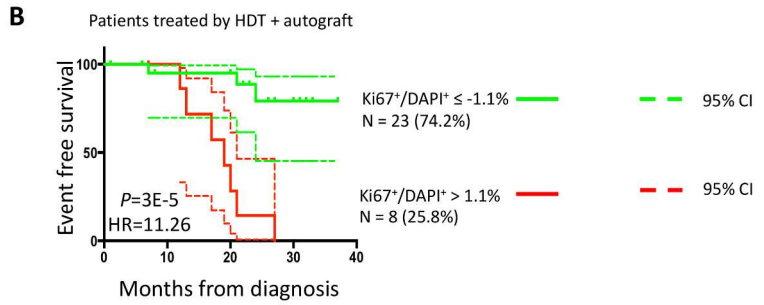


Figure 1

Figure 1

150x299mm (300 x 300 DPI)





	Median survival (95% CI)
Ki67+/DAPI+ ≤ -1.1%	Not reached
Ki67+/DAPI+ > 1.1%	19 months (13.8; 24.1)

Figure 2

Figure 2

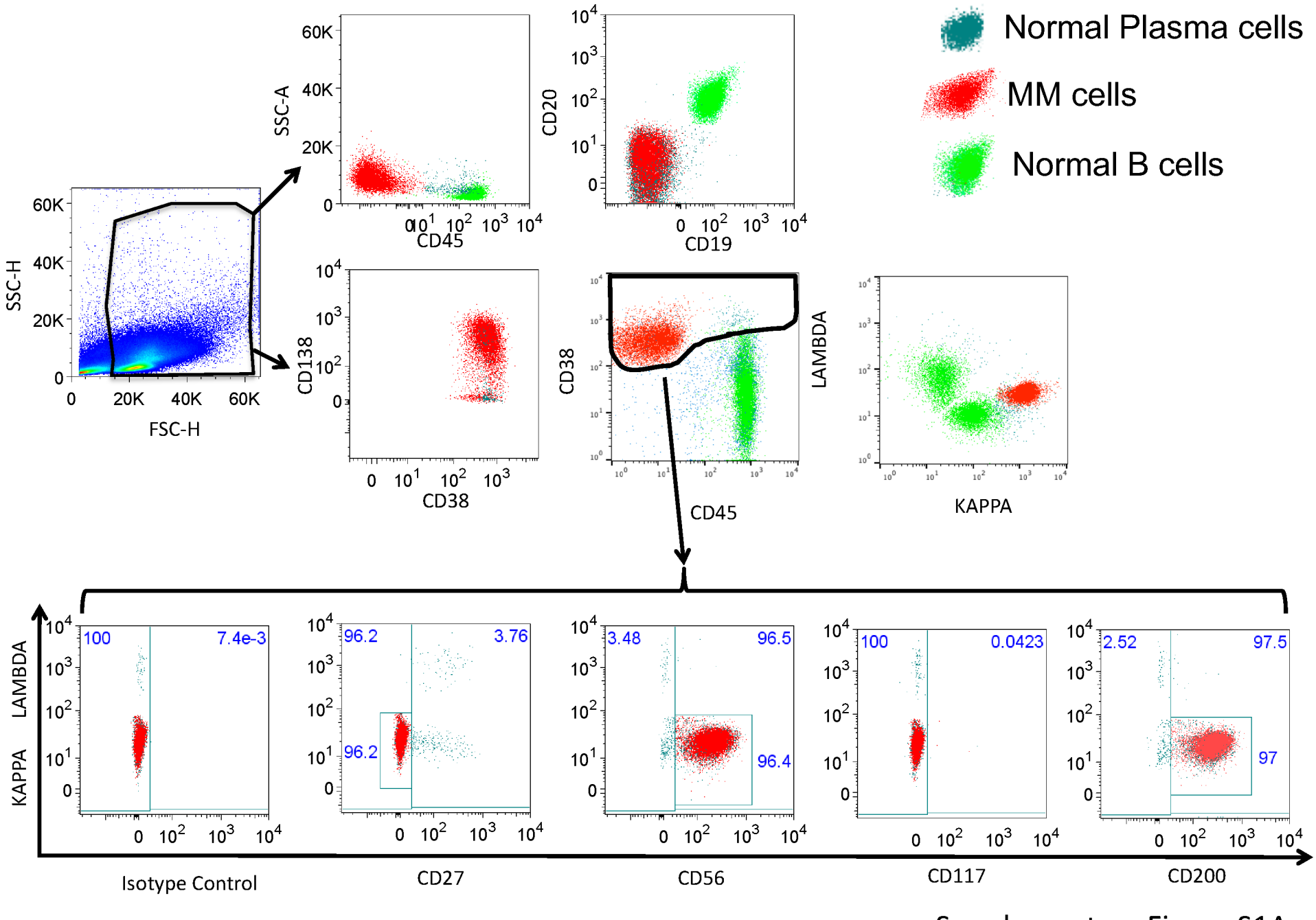
194x274mm (300 x 300 DPI)

Tubes	**DAPI**	**FITC**	**PE**	**ECD**	**PeCy5.5**	**PeCy7**	**APC**	**APC-AF750**
1 – Control	DAPI	Lambda	Kappa	CD45	CD38	CD19	Anti BrdU	CD20
2 - Negative Pool	DAPI	Lambda	Kappa	CD45	CD38	Negative Pool CD19/CD27	Anti BrdU	CD20
2 - Positive Pool	DAPI	Lambda	Kappa	CD45	CD38	Positive Pool CD56/CD117/CD200	Anti BrdU	CD20

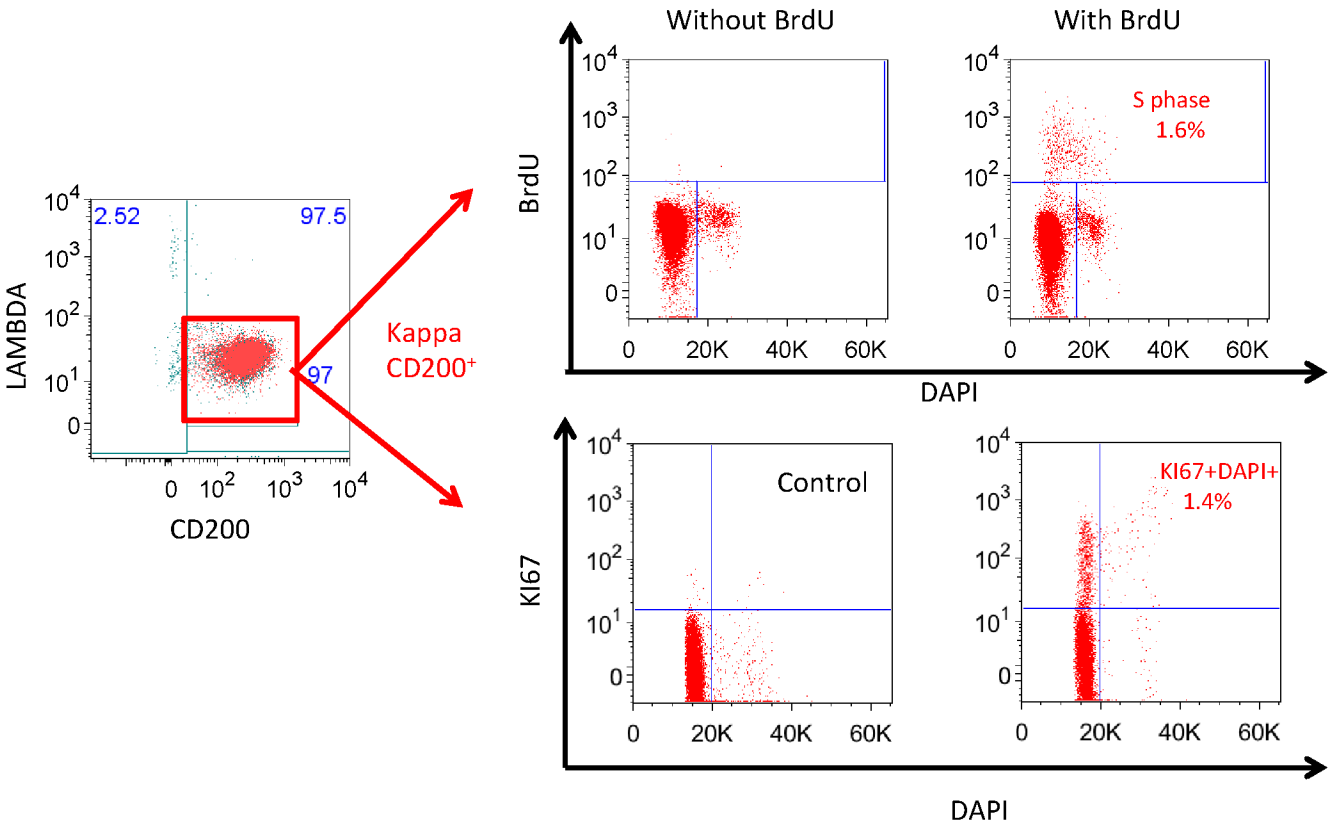
Table 1A: BrdU panel composition. The panel comprises 3 tubes that contain DAPI, anti-CD45, anti-CD20, anti-CD38, anti-BrdU, anti-kappa, anti-lambda antibodies, a positive antibody pool against markers that are overexpressed in malignant PC (CD56, CD117 and CD200) and a negative antibody pool against markers that are downregulated in malignant PC (CD19 and CD27) compared to normal PC.

Tubes	**DAPI**	**FITC**	**PE**	**ECD**	**PeCy5.5**	**PeCy7**	**APC**	**APC-AF750**
1 – Control	DAPI	Lambda	Kappa	CD45	CD38	CD19	Ki67	CD20
2 - Negative Pool	DAPI	Lambda	Kappa	CD45	CD38	Negative Pool CD19/CD27	Ki67	CD20
2 - Positive Pool	DAPI	Lambda	Kappa	CD45	CD38	Positive Pool CD56/CD117/CD200	Ki67	CD20

Table 1B: Ki67 panel composition. The panel comprises 3 tubes that contain DAPI, anti-CD45, anti-CD20, anti-CD38, anti-Ki67, anti-kappa, anti-lambda antibodies, a positive antibody pool against markers that are overexpressed in malignant PC (CD56, CD117 and CD200) and a negative antibody pool against markers that are downregulated in malignant PC (CD19 and CD27) compared to normal PC.



Supplementary Figure S1A



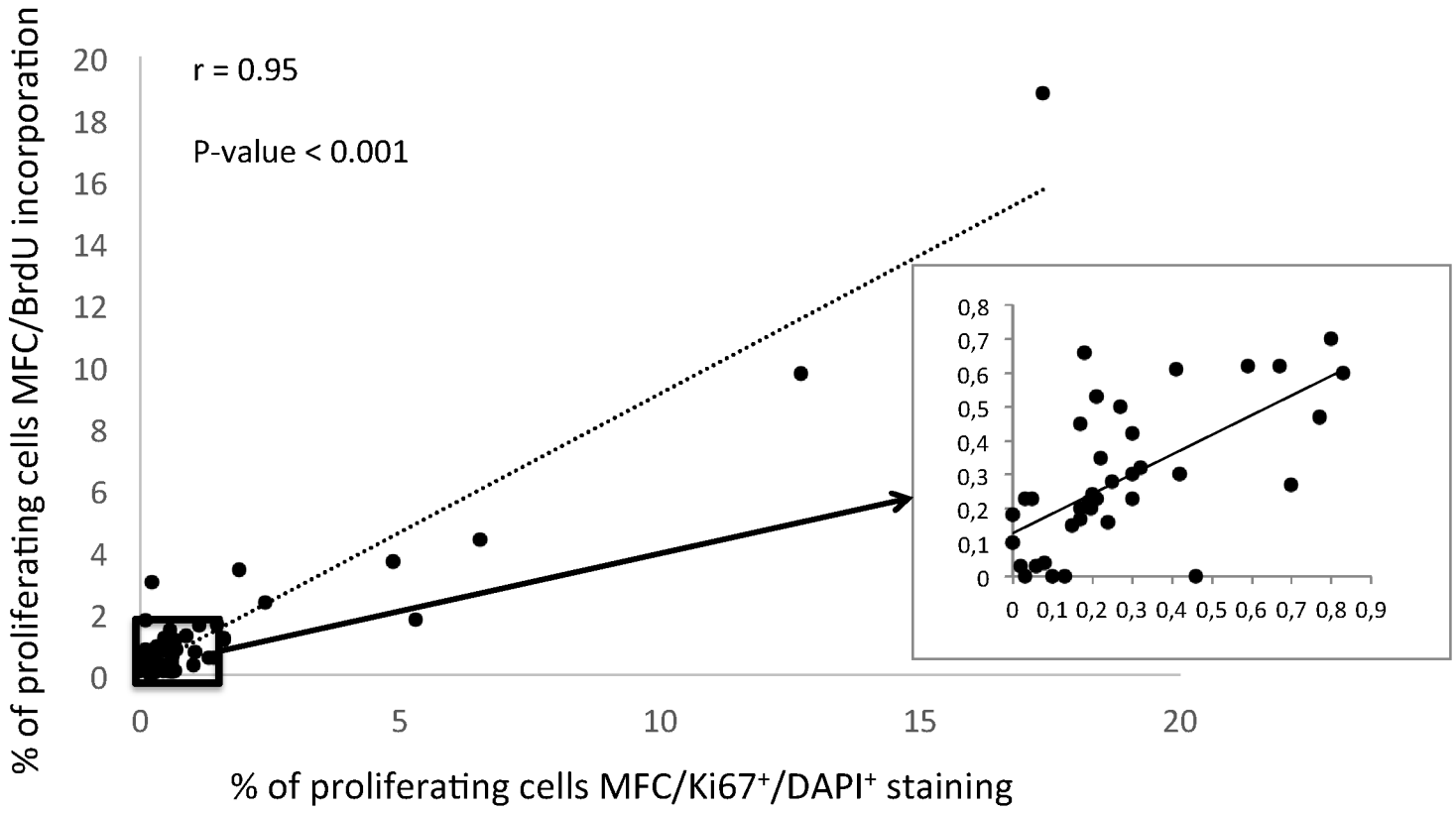
Supplementary Figure S1B

Supplementary Figure S1. Immunophenotyping of normal and malignant plasma cells.

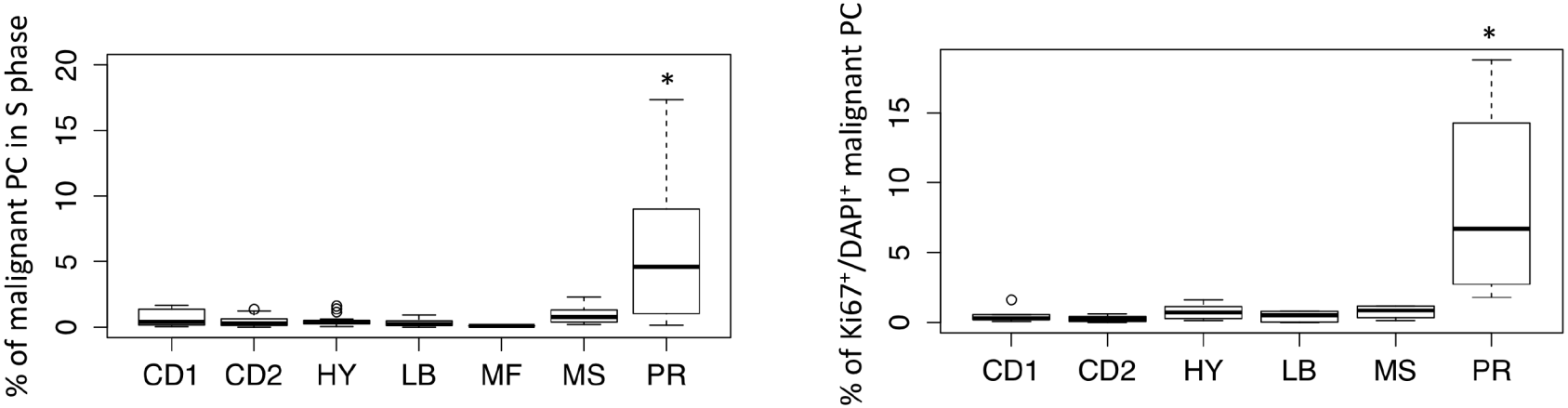
(A) Singlets are selected on FSC and SSC plots and total leukocytes on FSC/SSC plots. PC and B cells are selected on CD45/CD38 and CD20/CD19 plots. Events on the kappa/lambda diagonal are removed. Abnormal PC are selected on the CD27, CD56, CD117, and CD200 dimensions (Alaterre, et al. 2017).

(B) Assessment of proliferating MM cells using BrdU incorporation and ki67 immunostaining.

Alaterre E, Raimbault S, Garcia JM, Reme T, Requirand G, Klein B, Moreaux J. Automated and simplified identification of normal and abnormal plasma cells in Multiple Myeloma by flow cytometry. Cytometry B Clin Cytom 2017.

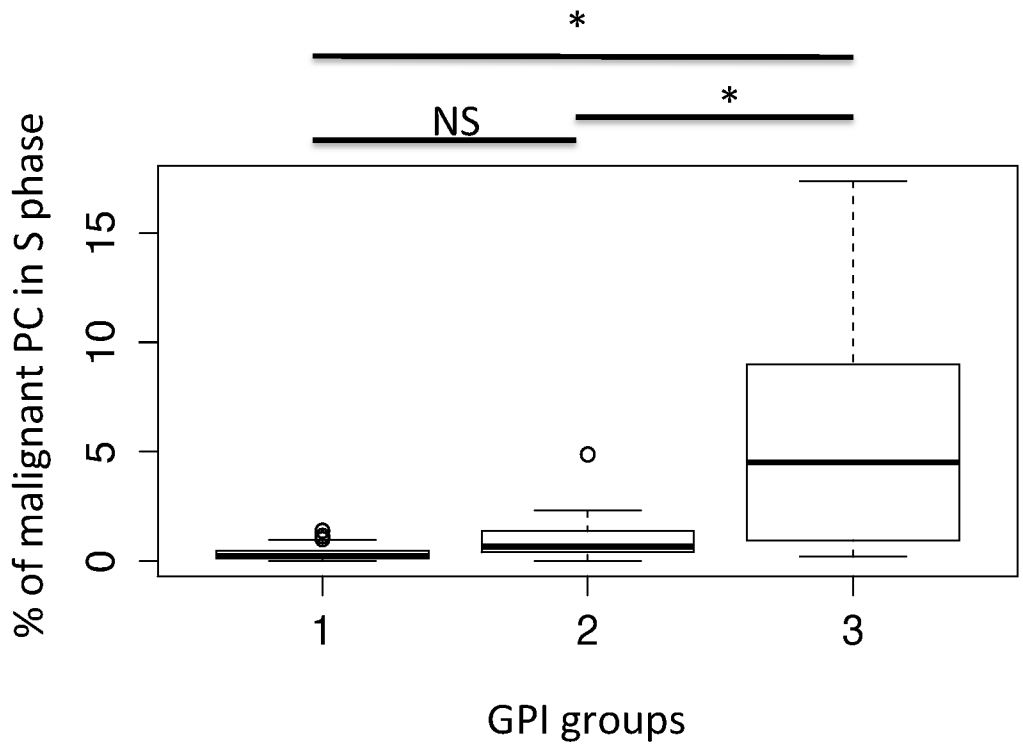


Supplementary Figure S2: Correlation between combined MFC/BrdU incorporation and MFC/Ki67$^+$/DAPI$^+$ staining for malignant plasma cell proliferation assessment. Proliferation has been investigated by flow cytometry in 11 MGUS and 49 MM patients at diagnosis.



Supplementary Figure S3: Proliferation staining combining (A) MFC and BrdU incorporation (n=81) or (B) MFC and Ki67 staining (n=30) in the 7 subgroups of the UAMS molecular classification of multiple myeloma. Proliferation staining was investigated in newly-diagnosed MM patients. PR: proliferation, LB: low bone disease, MS: MMSET, HY: hyperdiploid, CD1: Cyclin D1-Cyclin D3, CD2: Cyclin D1-Cyclin D3, MF: MAF, MY: myeloid. * Indicate that proliferation staining is significantly higher in the group compared to all the patients of the cohort ($P < 0.05$).

A



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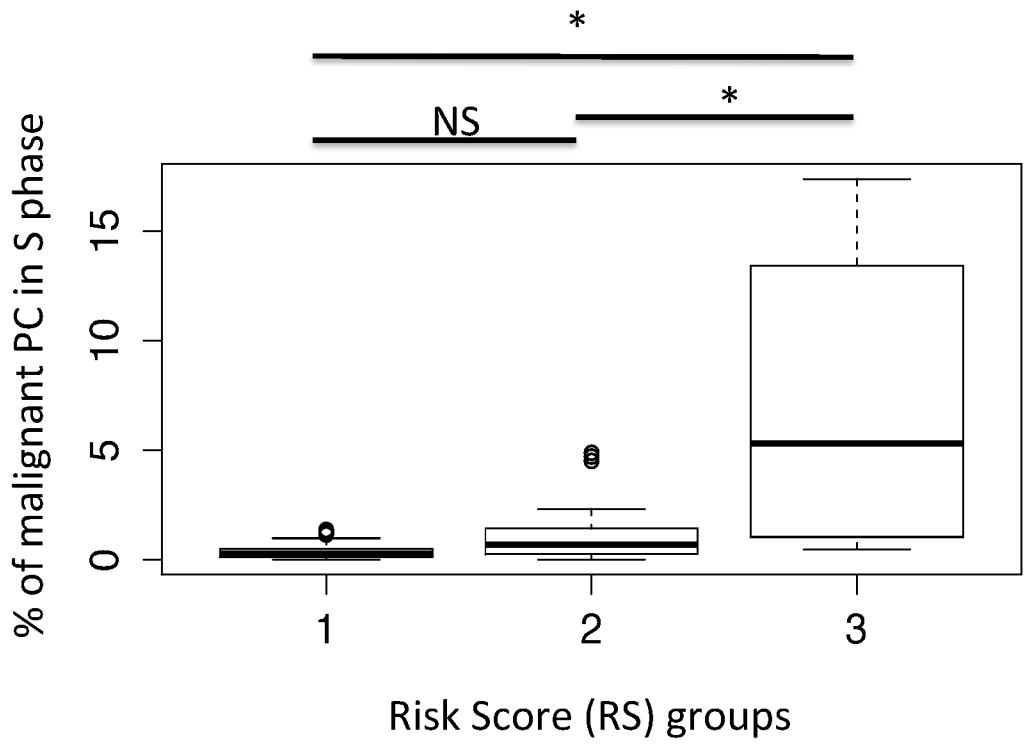
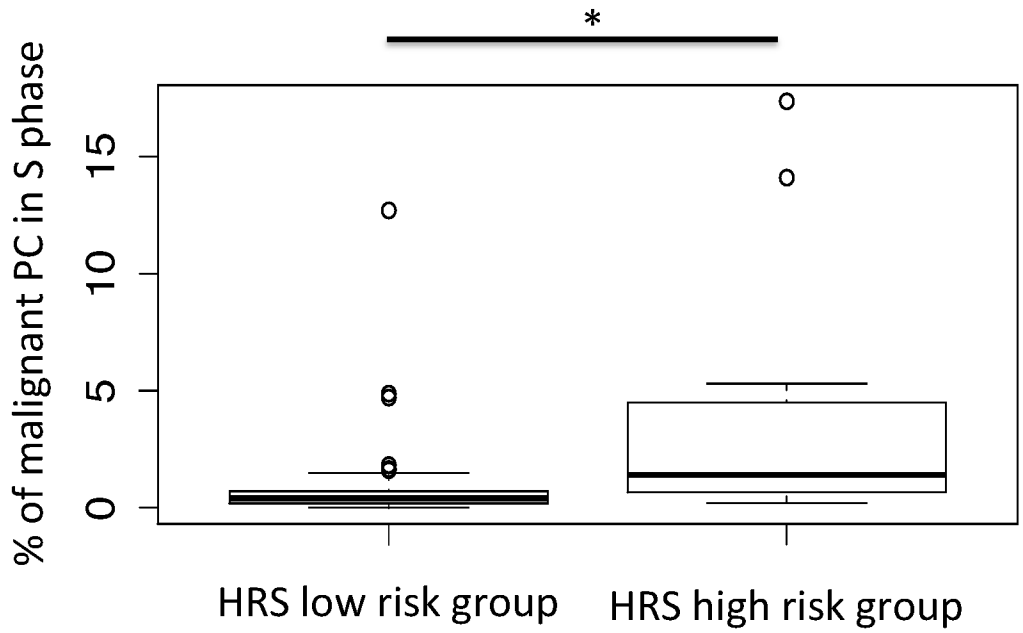
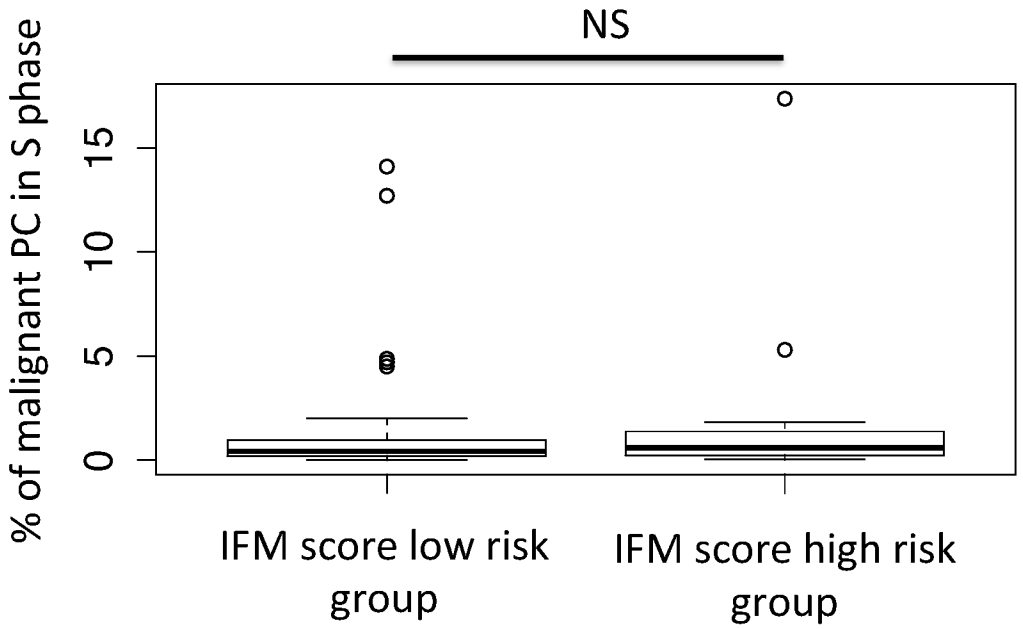


C


*
% of malignant PC in S phase
0
5
10
15
HRS low risk group
HRS high risk group

D



Supplementary Figure S4.

The percentage of proliferating MM cells using BrdU was assessed for patients classified according to GEP-based GPI index (Hose*, et al* 2011) (A), HRS (Shaughnessy*, et al* 2007) (B), RS (Reme*, et al* 2013) (C) and IFM (Decaux*, et al* 2008) (D) risk scores (n = 83). * indicates a P-value <0.05 using a Student t-test.

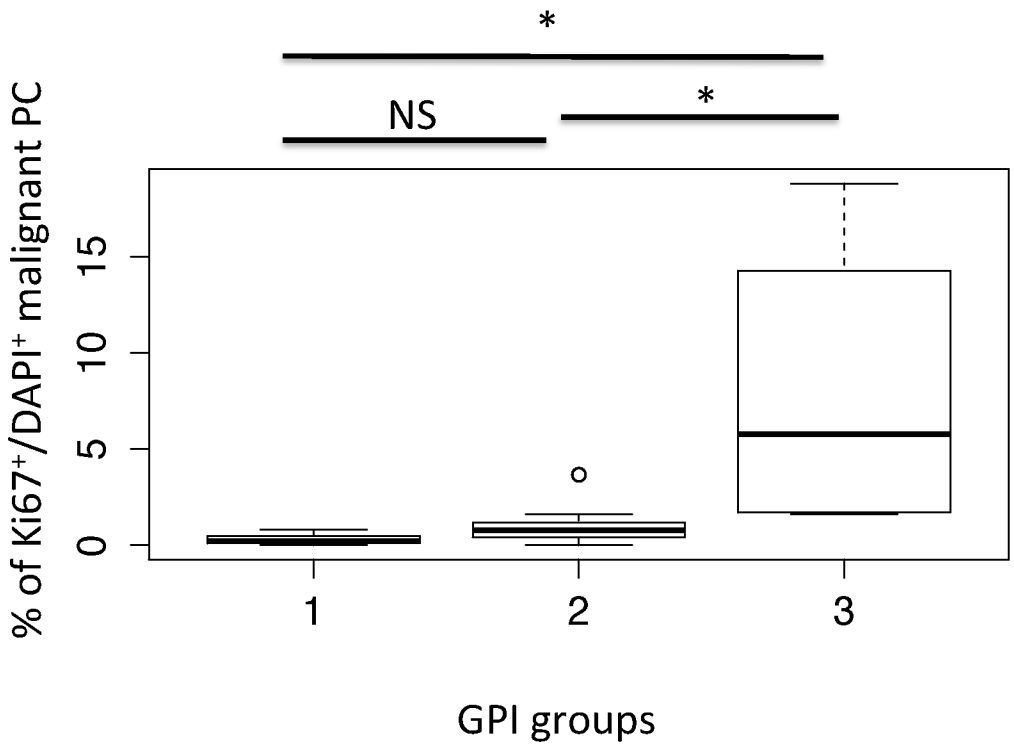
Hose, D., Reme, T., Hielscher, T., Moreaux, J., Messner, T., Seckinger, A., Benner, A., Shaughnessy, J.D., Jr., Barlogie, B., Zhou, Y., Hillengass, J., Bertsch, U., Neben, K., Mohler, T., Rossi, J.F., Jauch, A., Klein, B. & Goldschmidt, H. (2011) Proliferation is a central independent prognostic factor and target for personalized and risk-adapted treatment in multiple myeloma. *Haematologica,* **96,** 87-95.

Shaughnessy, J.D., Jr., Zhan, F., Burington, B.E., Huang, Y., Colla, S., Hanamura, I., Stewart, J.P., Kordsmeier, B., Randolph, C., Williams, D.R., Xiao, Y., Xu, H., Epstein, J., Anaissie, E., Krishna, S.G., Cottler-Fox, M., Hollmig, K., Mohiuddin, A., Pineda-Roman, M., Tricot, G., van Rhee, F., Sawyer, J., Alsayed, Y., Walker, R., Zangari, M., Crowley, J. & Barlogie, B. (2007) A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. *Blood,* **109,** 2276-2284.

Reme, T., Hose, D., Theillet, C. & Klein, B. (2013) Modeling risk stratification in human cancer. *Bioinformatics,* **29,** 1149-1157.

Decaux, O., Lode, L., Magrangeas, F., Charbonnel, C., Gouraud, W., Jezequel, P., Attal, M., Harousseau, J.L., Moreau, P., Bataille, R., Campion, L., Avet-Loiseau, H. & Minvielle, S. (2008) Prediction of survival in multiple myeloma based on gene expression profiles reveals cell cycle and chromosomal instability signatures in high-risk patients and hyperdiploid signatures in low-risk patients: a study of the Intergroupe Francophone du Myelome. *J Clin Oncol,* **26,** 4798-4805

A



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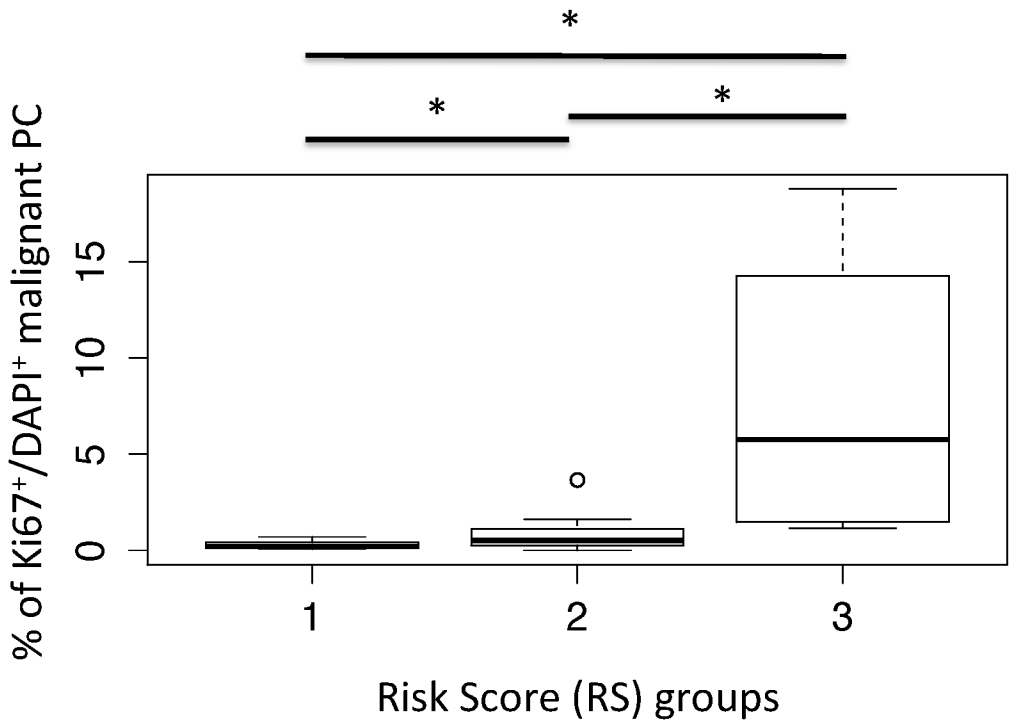
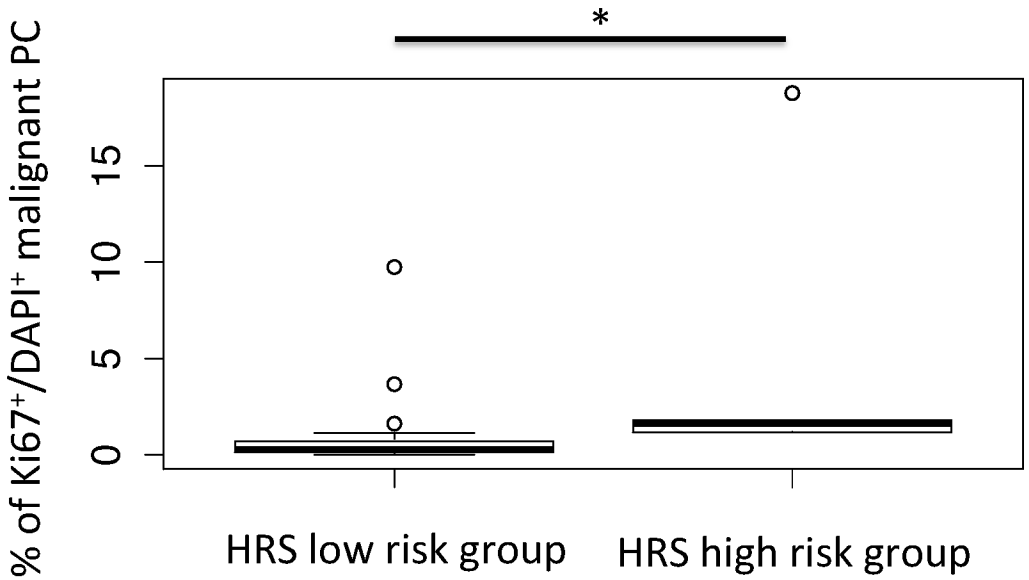
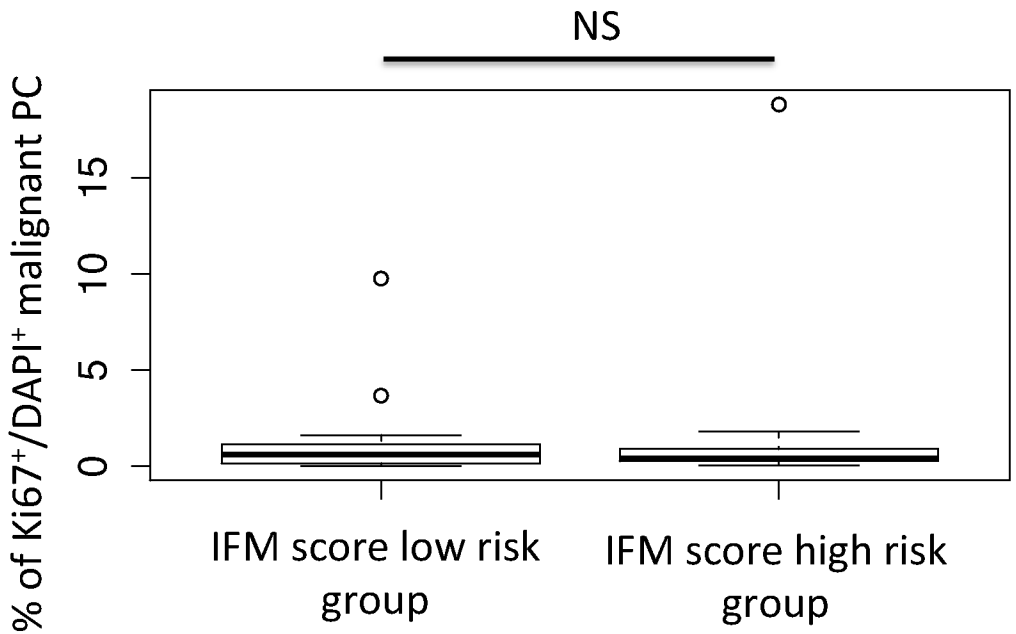


C


*
% of Ki67+/DAPI+ malignant PC
0
5
10
15
HRS low risk group
HRS high risk group

D



Supplementary Figure S5.

The percentage of proliferating MM cells was assessed for patients classified according to GEP-based GPI index (A), HRS (B), RS (C) and IFM (D) risk scores (n = 31). * indicates a P-value <0.05 using a Student t-test.