DNA Ploidy as a Potential Adjunct Prognostic Marker of Low-Risk Prostate Cancer Progression after Radical Prostatectomy

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Abstract

Purpose

Post prostatectomy PSA kinetics and General Grade Groups (GGG) are the strongest prognostic markers of biochemical recurrence (BCR) and prostate cancer (PCa)-specific mortality after radical prostatectomy. Despite having low-risk PCa, some patients will experience BCR, for some, clinically significant BCR. There is a need for an objective prognostic marker at the time of prostatectomy to improve risk stratification within this population. In this study, we investigated the prognostic potential of DNA ploidy.

Materials and Methods

Prostatectomy samples from 97 patients with GGG1 and GGG2 with a low-risk CAPRA-S score were included in this study. PCa tissue with the worst Gleason pattern underwent tissue disaggregation, cell isolation and staining with a DNA stoichiometric stain. Using image cytometry, DNA ploidy was measured and a Ploidy Score (PS) was generated.

Results

Among the 97 patients, 79 had no BCR, 18 experienced BCR, of which 14 had a PSA doubling time (PSA-DT) >1 year (low-risk group) and 4 had a PSA-DT of <1 year (high-risk group). Using Logistic regression analysis, only pathological T stage (pT) and PS independently predicted BCR with PS being the most significant (p=0.001). The number of aneuploid cells was significantly higher in the high-risk group compared to the other groups ($p=1.7x10^{-11}$). PS combined with GGG diagnosis further stratified

risk groups of biochemical recurrence free survival within CAPRA-S low-risk cohort.

Conclusion
DNA ploidy is an independent prognostic marker of BCR in low-risk PCa after radical prostatectomy, which could early on identify potentially aggressive PCa recurrences and introduce a more personalized approach to salvage treatments.

Introduction

Prostate cancer (PCa) is one of the most common cancers diagnosed in men in the world[1]. More than 50% if PCa cases are low-risk of death at diagnosis[2], however, 5-10% of these low-risk cases will have a poor outcome even after radical treatment.[3] For postoperative cases, the Gleason score (GS) remains the strongest predictor of biochemical recurrence (BCR). However, GS's subjectivity results in significant inter-observer variability, especially among general pathologists.[4] GS is part of validated predictors of BCR and systemic progression, such as CAPRA-S score.[5,6] Recently, it has been shown that GS and post-prostatectomy prostate-specific antigen (PSA) kinetics are the strongest predictors of metastases and mortality. Despite having low CAPRA-S scores, some patients will experience BCR, and some of those will have adverse PSA kinetics (PSA doubling time [PSA-DT] < 1 year), which would classify them into the high-risk BCR group as per the European Association of Urology (EAU).[7,8] However, salvage radiotherapy (RT) is most effective when delivered before PSA levels reach 0.5 ng/mL, after which, the opportunity to avoid use of androgen deprivation therapy in conjunction with salvage radiation could be missed. There is need for a prognostic marker that could better predict BCR at time of prostatectomy and differentiate between low (PSA-DT > 1 year) and high-risk (PSA-DT < 1 year) BCR to aid in the decision making process regarding salvage treatments.

Recently, molecular panels like the Decipher® Prostate Cancer Test (GenomeDx Biosciences, San Diego, CA) are attempting to provide better prognostic information.[9] Unfortunately, these tests are expensive and the genetic diversity of tumors limits their use in clinical decision-making.[10,11] DNA ploidy analysis by detecting large-scale genomic alterations could be a promising alternative.[12] DNA ploidy is now accepted as an objective prognostic biomarker in epithelial cancers[12-14] such as PCa.[12, 14-17] DNA ploidy correlates with recurrence-free survival and has added prognostic information to GS,[12,14,18] especially in patients with GS < 7.[15-17,19,20] To our knowledge, this is the first study to

investigate the correlation of DNA ploidy with BCR and PSA kinetics after radical prostatectomy in patients with low-risk PCa.

Materials and Methods

1. Patient selection and study design

The study was reviewed and approved by the Slovenian National Medical Ethics Committee as well as the General Hospital of Celje (GH Celje). The study and work comply with the principles of the Declaration of Helsinki. A cohort of 99 low-risk patients (CAPRA-S scores of 0, 1 or 2, and GG1 or GG2) who have undergone radical retro-public prostatectomy (RP) without lymphadenectomy at GH Celje between 2003 and 2009 were included in this study. All patients had a negative resection margin.

2. Pathological evaluation

Radical prostatectomy GS, defined by a group of general pathologists from GH Celje, was labeled General Grade Group (GGG) for this study. Study samples were reviewed by an experienced genito-urinary pathologist (MV) at the Institute of Pathology in Ljubljana. The review was labeled as Expert Grade Group (EGG). All specimens were evaluated according to the 2014 modified Gleason scoring system and ISUP Grade Groups (1-5). EGG1 and EGG2 would therefore represent GS 6 and GS 3+4=7, respectively.

3. Follow up and endpoint

Patients were followed with PSA every four months during the first year, every six months during the second and third years, and then annually or until BCR. Van den Broeck *et al.* developed a BCR risk group definition: EGG 1 or 2 case with BCR and PSA-DT <1 year is considered high-risk and a case with BCR and PSA-DT >1 year is defined as low-risk BCR.[7] PSA-DT was calculated using the Memorial Sloan Kettering Cancer Centre calculator by entering the at least two measurements of PSA above 0.1 ng/mL spaced at least two months apart.[21] Patients with persistently elevated PSA (above 0.1 ng/mL 6-8 weeks after RP) were excluded. Local recurrence was identified by a choline or PSMA PET/CT scan or prostate bed biopsy. Distant metastases were diagnosed by imaging (choline or PSMA PET/CT scan or CT scan and bone scintigraphy). The primary endpoint of the study was BCR. The secondary endpoint was defining or stratifying the EAU BCR risk groups.

4. Tissue processing and staining

Using the H&E section from the formalin-fixed paraffin-embedded prostatectomy sample, MV delineated the cancerous tissue on the slide. The slide with the worst GS pattern was used for this analysis. Three sections were cut from the corresponding paraffin block: the first and third 5 μm thick sections were stained with H&E to confirm the presence of the cancerous tissue; the second 70 μm thick section underwent tissue disaggregation and cell isolation followed by staining with a modified Feulgen-thionin a stoichiometric DNA stain.[22] It was transferred in nylon gauze (pore size 50 μm), placed into plastic cassettes, underwent deparaffinzation in xylene and rehydration by immersion into decreasing ethanol concentrations. The rehydrated tissue was then digested with a pepsin mixture, incubated at 37 C and stopped with phosphate-buffered saline. Sample was then shaken until the cells diffused into solution. After centrifugation, the resulting supernatant was removed and the remaining 2 ng/mL of solution was equally divided to each of the chambers on the cytospin slide. This slide was then centrifuged to create the cell monolayer. After air-drying, slides were stained with thionin-eosin.[22]

5. DNA Image Cytometry

Feulgen-stained slides were scanned with the Health Canada-approved DNA Image Cytometry system (ICM) ClearCyte (Perceptionix Inc.). The system uses a Zeiss microscope with a 20x objective and a high-resolution monochrome CCD camera. An illumination wavelength of 600 nm was used, which corresponds to the absorption peak of the thionin stain. The effective pixel sampling was 0.37 μm. ClearCyte automatically scans the area of the stained cell deposition. Two spots per cytospin slide were used in order to maximize cell yield per sample. The two spots were scanned separately and the resultant files were subsequently merged.

Using proprietary algorithms combining morphometric features, the ClearCyte software automatically classified objects into epithelial cell nuclei, inflammatory cells, overlapping cells, and out of focus cells.[23] Cells were visually reviewed by a cytotechnician who manually removed misclassified objects. Only in focus epithelial cell nuclei were used for DNA ploidy analysis. The DNA content of each cell was determined by measuring their integrated optical density (IOD). The DNA Index (DI) is a normalized measure of DNA content. It is obtained from cell IOD divided by the average IOD of the reference cell population (defined by the cytotechnician). The mean IOD value of normal epithelial cells was used as an internal reference as advised by the manufacturer.

6. DNA Ploidy features

Cells were classified into 13 different groups or "bins" according to their DI values. Cells with a DI: lower than 0.9 were moved into bin 0; between 0.91 and 0.95 were moved into bin 1; between 0.96 and 1 into bin 2; between 1.01 and 1.10 into bin 3; between 1.11 and 1.25 into bin 4; between 1.26 and 1.60 into bin 5; between 1.61 and 1.85 into bin 6; between 1.86 and 1.95 into bin 7; between 1.96 and 2.05 into bin 8; between 2.06 and 2.15 into bin 9; between 2.16 and 2.25 into bin 10; between 2.26 and 2.5 into bin 11; and finally cells with a DI more than 2.5 into bin 12. Cells with a DI >2.5 are likely true aneuploid cells as their DI is too large to correspond to cycling diploid cells. All other cells could either be normal diploid epithelial cells at different phases of the cell cycle or aneuploid cells. Frequencies (#) and percentage (%) of cells in each of these bins were calculated as well as the frequency exceeding rate (E#) and the percentage exceeding rate of cells with a DI higher than the corresponding value of the bins (supplement tables S1-S4).

7. Statistical evaluation

The R statistical package (version 1.2.1335) was used for all statistical analyses including the non-parametric Wilcoxon rank sum test. Differences between more than two groups were assessed by one way ANOVA. Univariate and multiple linear regression analysis were used to measure the association between one or more variables and recurrence. Biochemical recurrence-free survival (BCRFS) was studied using the Kaplan-Meier method and the log-rank test.

Results

Among the 99 patients, two patients were excluded due to lack of tissue material. The final cohort consisted of 97 patients. Clinical characteristics of this cohort are outlined in Table 1. The mean observation period was 12.1 years (median = 11.24 years , IQR [9.50-12.95] The mean age of patients at RP was 61.0 years. The mean preoperative PSA level was 4.8 ng/mL. Three patients died, one from cancer, two from unrelated causes so the overall survival was 96%.

Of the 97 patients, 79 had no BCR (referred to as BCR0). Among the 18 patients who experienced BCR (BCR1), 14 had a PSA-DT >1 year (low-risk BCR) and 4 had a PSA-DT of <1 year (high-risk BCR). Mean PSA-DT of high-risk patients was 0.62 years versus low-risk patients of 2.0 years. Overall survival and cancer-specific survival was 95.9% and 99%, respectively. 55.6% of patients with recurrence

received salvage therapy, including both high-risk and low-risk BCR patients. No patients received adjuvant radiotherapy. Four patients were treated with salvage radiotherapy with or without androgen deprivation treatment (ADT), being delivered at PSA level above 0.5 ng/mL. Six patients were treated with ADT and one also underwent chemotherapy.

Comparisons of DNA ploidy between patients with and without BCR

On average, 2175 cells were analyzed per specimen (min 76, max 10799). Mean and standard deviation of all DNA ploidy features are given in Supplemental tables 1-4. Fourteen DNA ploidy features were statistically different between BCR0 and BCR1 (Wilcoxon test, $p < 0.001$). All these features showed the same trend: there were significantly more aneuploidy cells or cells with a higher DI in patients that experienced BCR than patients that did not.

Two DNA ploidy histograms of specimens from a patient with BCR and without BCR are depicted in Figure 1. Figure 2 shows nuclear images of nuclei with increasing Dis (from the specimen with BCR shown in Figure 1B)

DNA Ploidy Score

All features were entered into a stepwise forward linear discriminant analysis (LDA) to differentiate between BCR0 and BCR1. To avoid overtraining, the number of features entered was limited to two. The algorithm selected 2.5# and 2.15#. The canonical score (linear combination of these two features) was named Ploidy Score (PS). Using a threshold value of 0.16, patients with a PS value below 0.16 were classified as PS- and PS+ otherwise.

The sensitivity and specificity of this PS test to detect BCR patients with a sensitivity of 44.4% (8 of 18) with a 95% CI of 22.0% to 66.0% and a specificity of 92.4% (73 of 79) with a 95% CI of 86.0% to 97.0% (Table 2). We then assessed the performance of the PS in EGG2 subgroup of patients. The sensitivity and specificity were 50% (6 of 12) and 100.0% (25 of 25).

Gleason Score and BCR

Sensitivity and specificity of the EGG to predict BCR was 66.7% (12 of 18) (95.0% CI of 41.0 to 87.05) and 68.4% (54 of 79) (95% CI of 57.0% to 78.0%) respectively. Sensitivity of GGG to predict BCR was 33.3% (6 of 18) (95.0% CI of 13.0% to 59.0%) and 78.5% (62 of 79) (95.0% CI of 56.0% to 59.0%) respectively.

Univariate and multivariate regression analysis

To be consistent and allow a more logical comparison between EGG and GGG, which are intrinsically binary (grade 1 or 2, see Table 1), we transformed all other variables into binary variables. Patients younger than 60 years old were coded as 0 and equal or over 60 years old as 1. PSA values below 6 ng/mL were coded as 0 and values between 6.01 and 10 ng/mL were coded as 1. For pathology grade, pT2 was coded as 0 and pT3 as 1.

Univariate analyses showed that PS, pT and EGG were statistically significant predictors of BCR. Age, preoperative PSA and GG were not significant (Table 3). Multivariate regression analysis was used to access the prognostic value of PS adjusted by EGG, pT, preoperative PSA level and age. Only PS and pT were statistically significant (Table 3).

Correlation of DNA Ploidy Score with high-risk BCR

To investigate the potential of the PS to detect most aggressive BCR cases, we compared the distribution of PS between three groups classified as: group 0 (patients with no BCR); group 1 (patients with a PSA-DT of >1 year); and group 2 (patients with a PSA-DT of <1 year). There was a statistically significant difference in the PS and in the frequency of cells with a DI higher than 2.5 (likely truly aneuploidy cells) between these three groups (Figure 3).

Ploidy Score and time to biochemical recurrence

We examined the correlation between PS and EGG with BCR-free survival using the Kaplan-Meier analysis (Figure 4). Mean time to BCR was significantly shorter in PS+ patients compared to PS- patients: 44.7 months versus 85.2 months (log rank test, $p<0.001$ Figure 4A). The 10-year BCRFS rates for PS+ were 42% (95% CI 22-78%) and PS- were 90% (95% CI 84-97%). For EGG1 and EGG2, they were 93% (95% CI 87-99%) and 68% (95% CI 54-84%) respectively. Using a combination of PS and EGG, four risk groups were generated (Figure 5). Group A consists of patients with PS- and EGG1 diagnosis with a BCR proportion of 7.5% (4/53). Group B consists of patients with PS- and EGG2 diagnosis with a BCR proportion of 20.7% (6/29). Group C consists of patients with PS+ and EGG1 diagnosis with a BCR proportion of 28.6 (2/7). Group D consists of patients with PS+ and EGG2 diagnosis with a BCR proportion of 75% (6/8). All four high-risk BCR cases were classified into group D. The cumulative proportion of patients with BCRFS decreased from group A to D (Figure 5).

Discussion

Our study suggests that DNA ploidy obtained on radical prostatectomy specimens can stratify patients with low-risk PCa into low and high risks of subsequent BCR. DNA ploidy combined with Gleason groups further refines risk stratification of BCR. This implies that low-risk group based on traditional clinicopathological criteria is heterogeneous and DNA ploidy has potential to tackle this issue. Moreover, our data suggests that DNA ploidy could identify very early on patients at high-risk of aggressive BCR (PSA-DT <1year) and patients with a risk of non-significant BCR (PSA-DT >1 year) much sooner than actual PSA recurrence.

The negative predictive value of PS for BCR was 88%, suggesting a high likelihood of excluding BCR, while a lower positive predictive value indicates that some recurrences are not detected. However, as the number of aneuploidy cells (DI >2.5) is significantly higher in the high-risk patients (Figure 3), PS could detect aggressive cancers.

Regression analysis to predict BCR showed another interesting property of DNA ploidy and GGG assessment. Ploidy score and expert pathologist assessment were significant in predicting BCR in univariate analysis, while general pathologist assessment was not (Table 3). Despite differences in prognostic performance between expert and general pathologists (Table 2), multivariate analysis indicated that only pT and PS were independent variables to predict BCR, suggesting that a DNA ploidy assessment adds important prognostic information despite already having an expert pathologist assessment. Kaplan-Meier analysis of BCR clearly showed that adding PS to EGG improved risk stratification. Including DNA ploidy into pathology report from radical prostatectomy may refine clinical management. For example, for patients who are EGG1 and PS-, a follow-up with family practitioner could be sufficient. In contrast, PS+/EGG2 patients, at higher risk of early recurrence, would require early consultation with radiation oncology, consideration for adjuvant or early multimodal salvage treatment. On the other hand, a more frequent PSA based follow up my be sufficient for PS+/EGG1 and PS-/EGG2 patients.

Evidence for DNA ploidy-independent prognostic value in PCa was summarized in two recent reviews.[12,14] Most studies reported statistical significance of DNA ploidy in multivariate statistical models reflecting no added value of DNA ploidy to GGG, however some of these studies used flow

cytometry, which has poorer resolution compared to image cytometry.[24-27] In these studies, expert pathologists gave GGG assessment, which may not be available in all institutions; additionally experts could have discordant reports.[4] Efforts have been made to incorporate genomic prognostic markers into risk stratification tools, benefitting mostly high-risk patients.[9,28] Genomic classification increased the C-index for 10-year distant metastasis from 0.76 to 0.81 compared to the clinical model alone.[9] Only one contemporary study has assessed the prognostic value of DNA ploidy in favorable risk PCa (pre-treatment setting) showing its independent prediction of BCRFS.[15] In a post-RP setting, several studies have shown that abnormal DNA ploidy was a predictor of BCR in a cohort of patients with GS 7.[16,19,20] A study from Lau *et al.* determined multivariate prognostic significance of DNA ploidy GS 7 cohort but not in the GS 8-10 cohort.[24,29] Pretorious *et al.* reported that DNA ploidy was the only significant predictor of early recurrence in a GS 7 cohort subset.[20] Lennartz *et al.* indicated that DNA aneuploidy with deletion of PTEN and 6q15 have shown the strongest prognostic information in subgroup of patients with GG2.[16]

Study limitations

The low number of recurrences limits the strength of our findings and a larger cohort is definitively needed. Also two-thirds of recurrent patients received salvage treatments after RP, therefore evaluation of time to metastases as an endpoint was not possible. However, studies indicate that a PSA threshold of 0.4 ng/mL for BCR best predicts metastases.[30]

Conclusion

Abnormal DNA ploidy can be an independent prognostic marker of BCR in low-risk PCa and increases prognostic value when combined with Gleason assessment, allowing a more personalized approach to salvage treatments.

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Table 1. Clinical characteristics of the study cohort (97 patients).

Variable	mean (IQR) or Number (%)
Observation period (years)	12.1(3.7–16.2)
Age at RP (y-old)	61.0 (56.6-66.3)
Pre-operative PSA (ng/mL)	4.8 (1.0 - 10.0)
Pathological T stage	
pT2	88 (91%)
pT3a	9 (9%)
ISUP GG, general pathology (GGG)	
GGG1	74(76%)
GGG2	23 (24%)
ISUP GG, expert pathology (EGG)	
EGG1	60 (92%)
EGG2	37 (38%)
Biochemical Recurrence (BCR)	
None	79 (82%)
Low-risk	14 (14%)
High-risk	4(4%)
Salvage therapy when BCR	10 (56%)
Distant metastases	1 (1%)
Overall Survival	96 (96%)
Cancer-specific survival	99 (99%)

FIGURES:

Figure 1. DNA ploidy histograms of two study samples. (A) Patient without biochemical recurrence and (B) patient with biochemical recurrence.

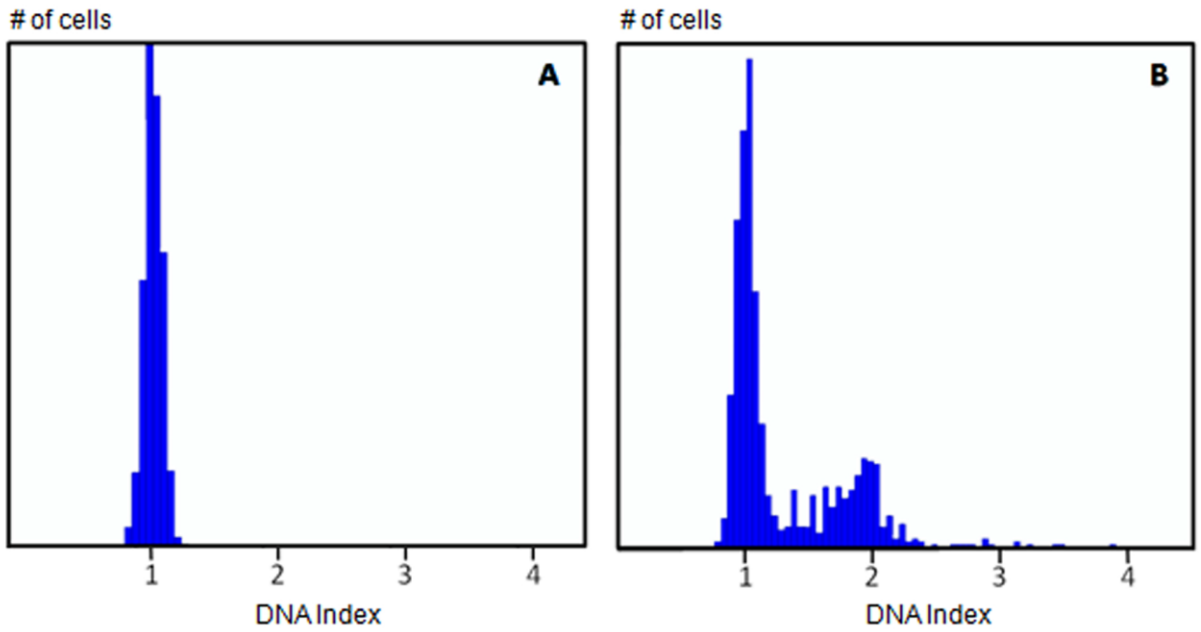


Figure 2. Nuclear images of cells. A) Diploid cells, B) cells with a DNA index around 1.5 and C) cells with a DNA index higher than 2.2 (highly aneuploid cells).

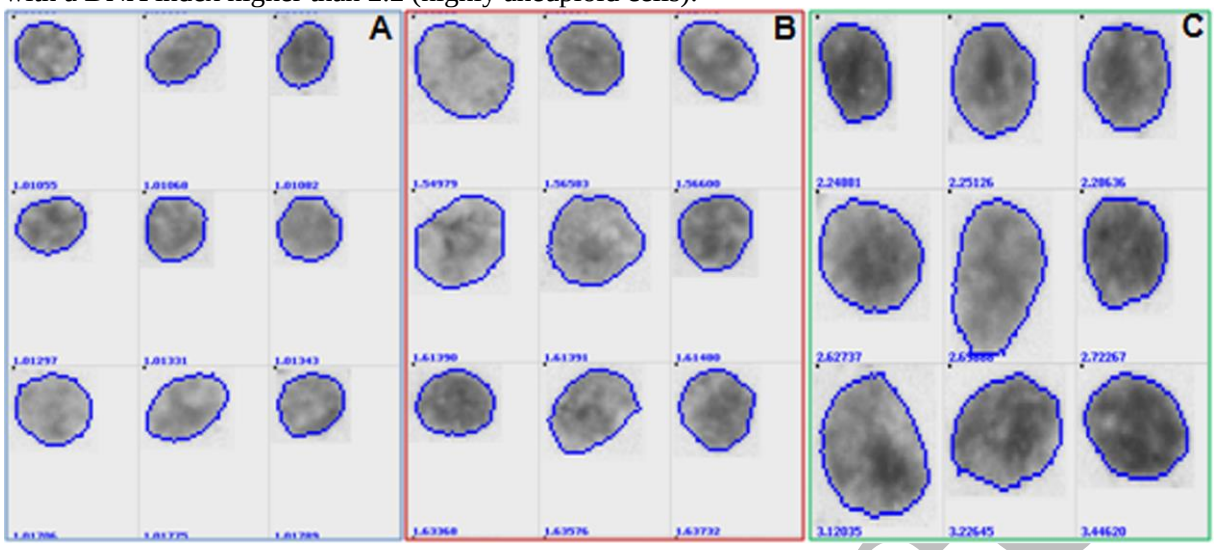


Figure 3. Correlation between DNA ploidy features and PSA doubling time. None: no recurrence (79 cases); low-risk: PSA doubling time >1 year (14 cases) and high-risk: PSA doubling time ≤1 year (4 cases). (A) Box plots of the Ploidy score (ANOVA, p=9.6x10-8). The bold line represents the median, the boxes represent the 50th percentile and error bars represent 5th and 95th percentile of cell frequencies. (B) Number of aneuploid cells (DI >2.5) (ANOVA, p=1.7x10-11).

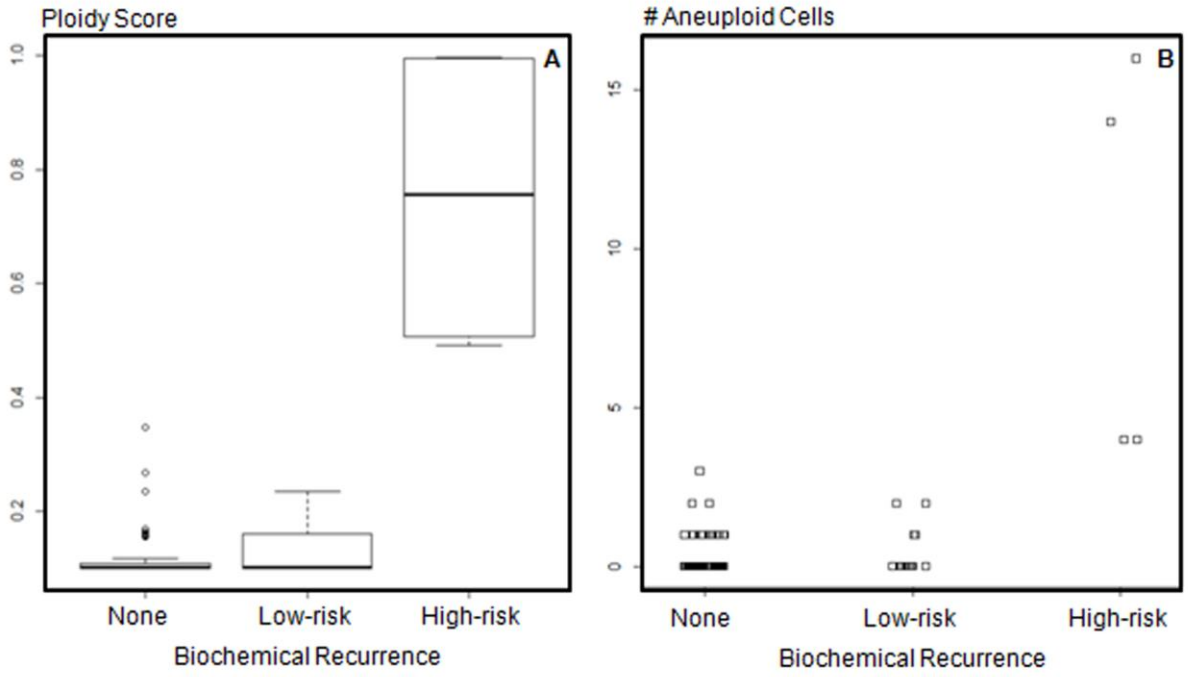


Figure 4. Cumulative proportion of BCR-free survival (BCRFS) according to the Ploidy Score (A) (Log rank test, p=4x10-7) and EGG (B) (p=0.004, respectively).

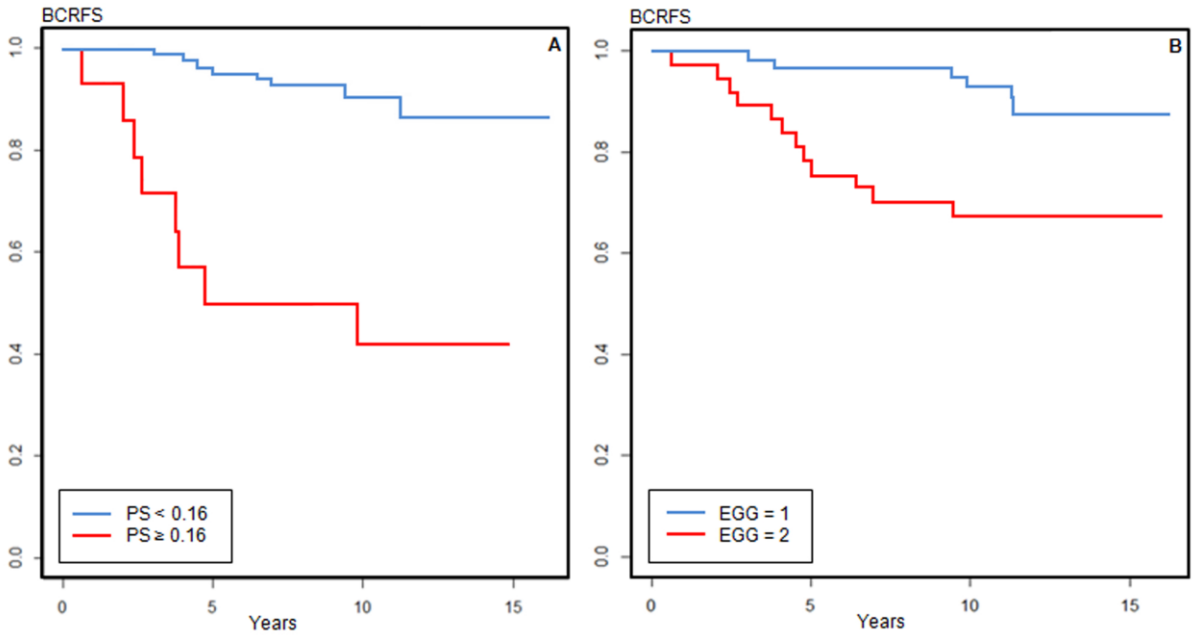
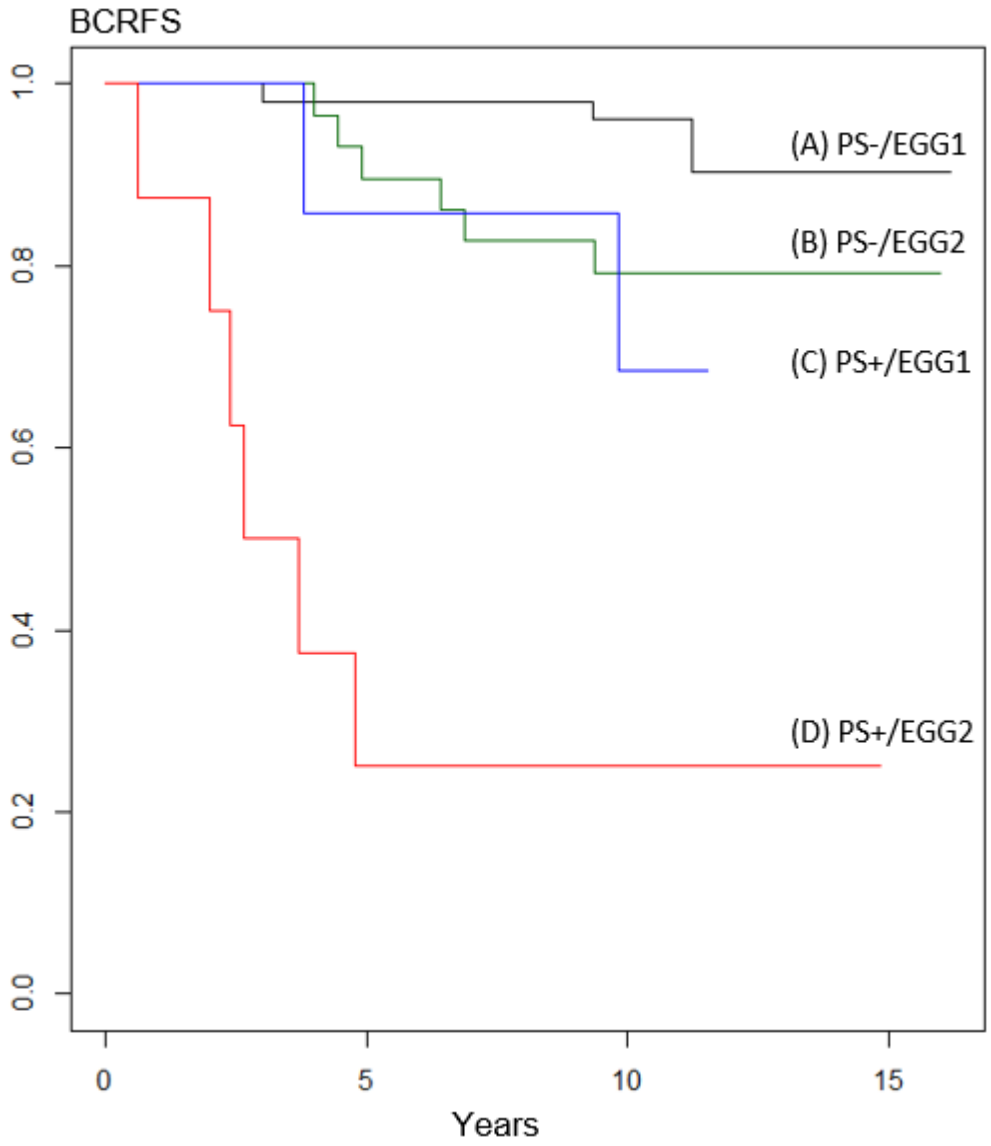


Figure 5. Kaplan-Meier plot of BCR-free survival for patients grouped according to EGG diagnosis and Ploidy score. (A) PS- / EGG1, (B) PS- / EGG2, (C) PS+ / EGG1 and (D) PS+ / EGG2. Using log-rank test, there was a significant difference between groups A and C (p = 0.04), between groups A and D (p=7x10-10) between groups B and D (p=4x10-5) and between groups B and C (p = 0.04).


BCRFS
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
Years
(A) PS-/EGG1
(B) PS-/EGG2
(C) PS+/EGG1
(D) PS+/EGG2