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Research Article

Prediction of Non-Progression in Prostate Cancer Patients under Active Surveillance by DNA-Karyometry

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Abstract

The option of Active Surveillance for patients with localized prostate cancer depends on a low Gleason-Score (GS) of 6. Nevertheless about 30%have to face clinical progression within five years.

Our hypothesis is that automated measurements of DNA-content of prostate cancer cells yield a DNA-grade of malignancy [1-4] that is able to predict non-progression of prostate cancers at much higher accuracy than the subjective GS.

Nuclear DNA-measurements were performed from cancer tissue in residual biopsy-material of 80 patients from the HAROW-study. Local- and a reference pathologists GSs were available. Follow-up of mean 4,1 years included repeated PSA-values, number and GSs of positive biopsies, clinical stage, and in19cases results from prostatectomies.

Reproducibility of GSs was 55% without and 45% with differentiation between 6 and 7a. Progression occurred in 37.5% if upgrade of any inclusion criterion was used as evidence and in 18,8% if only PSA-DT <36 months or upstaging to pT3. Prevalence of DNA-grade 1 was 40%.Sensitivity, specificity and negative predictive value of local pathologists GS, reference pathologists GS and DNA-karyometry were: 0%, 95.0% and 74.0%, 20.0%, 86.7% and 76.5%, 85.0%, 51.0% and 90.6%, if upgrade of any criterion of inclusion was used as evidence of progression and 5.9%, 96.8% and 79.2%, 23.5%, 87.3% and 80.9%, 100%, 50.8% and 100% if only PSA-DT < 36 months and/or upstaging to pT3 were used.

Thus, objective automated DNA-karyometry can much more reliably exclude progression of prostate cancers under Active Surveillance within four years as compared with subjective Gleason-scoring.

Patient summary: The probability to exclude an objectively assessed progression of an untreated, localized prostate cancer under Active Surveillance was only 80.9% for the subjective microscopic Gleason-score but 100% for objective DNA-karyometry. Thus, patients with DNA-grade of malignancy 1 can much more safely rely on this conservative, non invasive therapeutic strategy.

Introduction

It is well established that prostate cancer reveals a wide range of malignancy. A significant proportion will neither become clinically significant nor kill its carrier, even if untreated [1]. The challenge is to individually identify those cancers that within about five years most likely will not become life threatening, even without treatment. Currently the mostly used prognostic index for cancers of the prostate is the subjective Gleason-score (GS, 2), despite the fact, that its inter observer reproducibility is low and its prognostic validity moderate [3]. If the former is about 45.7% [3], a significant number of clinical decisions, based on this score, may be wrong. If 30% of patients with GS 6 (82.8%) or 7 (17.2%) under Active Surveillance (AS) have to face progression of their prostate cancers within 6.8 years [4], the prognostic validity of this index is limited as it does not identify those, that will progress with sufficient security. The estimated average 5 and 10 years probabilities of discontinuing AS are about 33% and 55% respectively [5]. The most frequent triggers for intervention were progression on re-biopsy and PSA-kinetics. Only a limited number (1-8.7%) discontinued AS because of own preference [5]. Thus many urologists and patients continue refusing to accept the AS-strategy.

As all cancers of men are chromosomally aneuploid, this also holds for all prostate cancers that are listed in the Mitelman Database of Tumor Cytogenetics [6]. Yet, no specific chromosomal changes exists for these cancers. The degree of this malignancy-specific feature may increase with time, what is called cytogenetic tumor progression. The malignant potential of cancers increases with increasing degree of chromosomal aneuploidy (genetic instability) [7].

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The existence of significant chromosomal aneuploidy and its degree can easily be estimated by measuring the DNA-content of nuclei [8]. This method, called DNA-cytometry is known since 1966 [9]. A large number of studies has been published that prove the prognostic validity of DNA-ploidy measurements in cancers of the prostate, independent of the Gleason-score [10]. Yet, so far prospective studies on untreated patients under active surveillance are missing [11].

As especially prostate cancers are rather heterogeneous, the samples for prognostic DNA-cytometry must be representative and thousands of cancer cells have to be measured to yield prognostically valid results. Flow- and image-cytometry are offered as methods. While in flow-cytometry measured nuclei cannot be microscopically assessed after measurement and nuclear doublets or artifacts not be eliminated, most pathologists prefer image cytometry. As these measurements so far were too time consuming, they were hardly accepted in routine diagnostics. Yet, meanwhile cancer cell-nuclei can automatically be identified by digital classifiers, trained by cytopathologists [12] and differentiated from nuclear doublets, artifacts, lymphocytes, granulocytes and fibroblasts (as potential reference cells). This new technology is called DNA-karyometry [14]. German pathologists have recently agreed on the Frankfurt Consensus in which most technical details of prognostic DNA-karyometry for cancers of the prostate are stated, including details of diagnostic interpretation of DNA-histograms [12].

The objective of this prospective cohort study is to compare the prognostic validity of DNA-karyometry with that of Gleason-scoring in 80 patients with localized prostate cancers under active surveillance. Further, the inter observer reproducibility of Gleason-scoring should be compared with that of DNA-karyometry, based on published results.

Material (Patients) and Methods

Patient acquisition

HAROW (Hormones, Active Surveillance, Radiation, Operation, Watchful Waiting) represents the first comprehensive prospective observational study, comparing treatment options in localized prostate cancer [14]. Data on primary treatment decisions and follow-up in 3.169 patients with diagnosed, histologically confirmed localized (T1a-T2c/N0/M0) prostate cancer were collected for up to 5 years. Among them 417 (13.2%) patients decided for AS. All of these had been asked in July2008by the study-secretariat of the "Stiftung Männergesundheit" in Berlinif they would also participate in another study, dedicated to the evaluation of prognostic DNA-karyometry in comparison with Gleason-scoring, named DNA-ProKo-study (prospectiv, kontrolliert). 234 patients agreed. Their respective 28 different pathologists then have been asked to send paraffin blocks and stained sections of all cancer tissue bearing prostate biopsies and prostatectomy-specimens, including copies of histological reports, to the department of Cytology, Institute of Pathology, University of Düsseldorf. This was successful in 94 cases. Later these materials were transferred to the Institute of Pathology, City hospital of Düren for prognostic DNA-karyometry. September 30th, 2015 a followup-questionaire has been sent to the respective urologists. The following information has been asked for: all PSA-values, including their dates, number, dates and GS of follow-up-biopsies, eventual changes of therapy including their cause, histological results of eventual prostatectomy and clinical upstaging. 14 patients had to be excluded because of lacking sufficient residual tissue for DNA-karyometry. The STARD-diagram in figure 1 illustrates the evolution of the final 80 patients.

Gleason-scoring

Gleason-scoring by local pathologists and by the reference pathologist Prof. Dr. Rainer Engers was performed according to [1]. He was blinded against the GS of the former. Sores were given per core.

Definitions of tumor-progression

Two different definitions for evidence of a tumor-progression during the follow-up period have been applied:

1. All changes of inclusion criteria for Active Surveillance mentioned in the German S3-guidelines from 2014 (15) towards higher values: stage >cT1-2b, PSA-DT < 36 months, > 2 tumor-positive cores or upgrading of GS ("soft" criteria). Tumor-volume per score was not considered.
2. Only PSA-DTs <36 months or histologically proven pT3 stages on radical prostatectomies ("hard" criteria).

Cell separation and Feulgen-staining

Pathologists had been asked to provide all paraffin-embedded core-biopsiesand prostatectomy tissue blocks that contained cancer and the respective stained sections for each patient. In order to prepare pure nuclear suspensions from cancer cells from each block, an experienced pathologist (A.B.) marked all cancer-foci with a

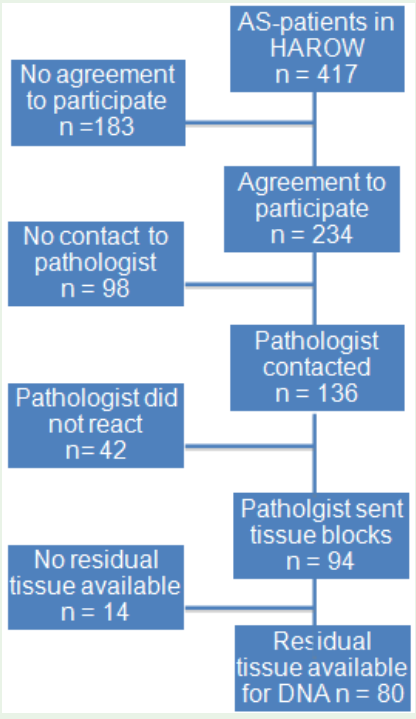

Figure 1: STARD-diagram to report flow of patients.

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Table 1: Predictive accuracy of Gleason-scores (GS) and DNA-karyometry for prostate cancer progress. Evidence of progression according to S3-guidelines.

	LocalPathologists Gleason-Scoring						Reference Pathologists Gleason-Scoring						DNA-Karyometry					
	cT1-2b			cT1-2a			cT1-2b			cT1-2a			cT1-2b			cT1-2a		
	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a
Number	80	77	3	78	78	0	80	68	12	78	66	12	80	68	12	78	66	12
Correct Negative	57	57	0	55	55	0	52	52	0	51	51	0	29	26	3	31	28	3
Correct Positive	0	0	0	1	0	1	4	0	4	4	0	4	17	14	3	18	15	3
False Negative	20	20	0	20	21	0	16	16	0	15	15	0	3	3	0	1	1	0
False Positive	3	0	3	2	0	2	8	0	8	8	0	8	31	25	6	28	22	6
Sensitivity	0%	0%	-	4.80%	0%	-	20.00%	0%	-	21.10%	0%	-	85.00%	82.40%	-	94.70%	93.80%	-
Specificity	95.00%	100%	-	96.50%	100%	-	86.70%	100%	-	86.40%	100%	-	48.30%	51.00%	-	52.50%	56.00%	-
Positive Predictive Value	0%	0%	-	33.30%	0%	-	33.30%	0%	-	33.30%	0%	-	35.10%	35.90%	-	39.10%	40.50%	-
Negative Predictive Value	74.00%	73.10%	-	73.30%	72.40%	-	76.50%	76.50%	-	77.30%	77.30%	-	90.60%	89.70%	-	96.90%	96.50%	-
Column #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

felt-tip pen on the slides and transferred these outlines to the block by cutting around its circumference with a pointed scalpel. After cutting three 50 µm sections per block, the cancer-bearing foci could be separated from the rest of the tissue. One 50 µm thick section from a formalin-fixed transurethral resection, containing only fibromuscular tissue without acini was added to provide fibroblasts for internal calibration. By enzymatic cell separation with pepsin according to Hedley [16] and cytocentrifugation on slides, nuclear monolayers were prepared. These were subjected to acid hydrolysis (5N HCl, 60 minutes, 27°C) and conventionally Feulgen-stained with pararosaniline (Merck, Darmstadt, Germany, Nr.1.09033.0500) and covered with coverslips.

DNA-karyometry

The instrument used was a MotiCyte-auto from Motic, Xiamen, PR China. It consists of a motorized BA600 microscope with automated change of objectives, autofocus and a motorized xy-stage. It was equipped with a Moticam Pro 2/3 CCD camera. DNA-measurements were performed with a 40x/0.65nA plan objective and 10x secondary magnification. The instrument was software controlled by a personal computer. The performance of the software has been described elsewhere [17]. Digital nuclear classifiers had been trained (by A.B.) to classify pararosaniline-stained objects of cell separation specimens from prostate cancer tissue into: artifacts, nuclei from cancer cells, lymphocytes, granulocytes and normal fibroblasts as internal reference cells. 6.51% of cancer cell nuclei are lost and 2.69% of artifacts were misclassified as cancer cells [18]. All nuclear images classified as derived from cancer cells or from fibroblasts were checked on an image gallery by an experienced cytopathologist, reclassified or eliminated if necessary, before DNA-histograms were presented. The coefficient of variation (CV) of reference cells was < 4% and their Coefficient of Correlation (CC) of nuclear IOD *vs* area <0.4 in all cases. The classification of DNA-histograms into: peridiploid (DNA-grade 1), peritetraploid (DNA-grade 2), x-ploid (DNA-grade 3) and multiploid (DNA-grade 4) was performed according to the consensus of the European Society for Analytical Cellular Pathology in, ESACP, on Diagnostic DNA-Image Cytometry [19] and the Frankfurt-Consensus on Objective DNA-Grading of Malignancy as Adjunct to the Histological GS [13].

Calculation of PSA-DTs

PSA-doubling times in months were calculated between the first and the last of the available values.

Results

The mean age of patients was 66.63 years, minimum 50.25, maximum 84.21 and standard deviation 7.15 years. Mean duration of follow-up was 4,1 years per patient, minimum 3, maximum 85 months.

At the beginning of the study 12 patients were in stage T1a, 1 in T1b, 48 in T1c, 12 in T2a, 2 in T2b and 1 in T2c. For 4 patients initial staging was not known. At the end of the study 8 patients were in stage T1a, 1 in T1b, 38 in T1c, 7 in T2a, 2 in T2b, 20 in T2c and 4 in pT3.

Mean number of PSA-values obtained during follow-up was 8.05, minimum 2, maximum17.

Mean number of taken biopsies was 10,4. Mean number of initially cancer-positive biopsies was 1,31.69 of the 28 local pathologists maximal Gleason-score was 4-6 (86.3%), 8x 7a (10%), 3x 7b (3.7%). 33 of the reference pathologists maximal Gleason-score was 4-6 (41.3%), 35x 7a (43.8%), 11x 7b (13.8%) and 1x 10 (1.2%).

44 maximal Gleason-scores were identical between local and reference pathologist, if full scores were considered (inter observer reproducibility: 55%), 36 when 6, 7a and 7b differentiated (45%). One case differed by two scores (6 to 10).Upgrading of reference pathologists GS in biopsies versus prostatectomies occurred in 7 cases.

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Table 2: Predictive accuracy of Gleason-scores (GS) and DNA karyometry for prostate cancer progress. Evidence of progression according to Böcking.

-	LocalPathologists Gleason-Scoring						Reference Pathologists Gleason-Scoring						DNA-Karyometry					
	cT1-2b			cT1-2a			cT1-2b			cT1-2a			cT1-2b			cT1-2a		
	GS 6 + 7ab	GS 6 +7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a	GS 6 + 7ab	GS 6 + 7a	GS>6 + 7a
Number	80	77	3	78	76	2	80	68	12	78	66	12	80	68	12	78	66	12
Correct Negative	61	61	0	60	59	1	55	55	0	53	53	0	32	29	0	32	29	3
Correct Positive	1	0	1	1	1	0	4	0	4	4	0	4	17	13	4	15	11	4
False Negative	16	16	0	15	14	1	13	13	0	13	13	0	0	0	0	0	0	0
False Positive	2	0	2	2	2	0	8	0	8	8	0	8	31	23	8	31	26	5
Sensitivity	5,9%	0%	-	6,3%	6,7%	-	23,5%	0%	-	22,2%	0%	-	100%	100%	-	100%	100%	-
Specificity	96,8%	100%	-	96,8%	96,7%	-	87,3%	100%	-	94,7%	100%	-	50,8%	58,2%	-	50,8%	51,8%	-
Positive Predictive Value	33,3%	0%	-	33,3%	33,3%	-	33,3%	0%	-	33,3%	0%	-	35,4%	36,1%	-	32,6%	30,0%	-
Negative Predictive Value	79,2%	79,2%	-	80,0%	80,8%	-	80,9%	80,9%	-	80,3%	80,3%	-	100%	100%	-	100%	100%	-
Column #	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36

Mean 1.78 biopsies per patients have been processed for DNA-measurements. Mean number of reference cells (fibroblasts) measured per biopsy by DNA-karyometry was 268.1 (minimum 2, maximum 4.615, standard deviation 554.3) and of cancer cells measured per biopsy was 4.669, 2 (minimum 83, z maximum 30.010, SD 6279.7). DNA-measurements yielded 32 maximal DNA-grades of 1(40.0%), 45-grades 2 (56.25) and 3 -grades 3 (3.75%) per patient (Figures 2 & 3).

The coincidence of Gleason-score 4-6 and DNA-grade 1 was 17/32x (53.1%). In 12/36 biopsies with Gleason-score 7a (33.3%) and

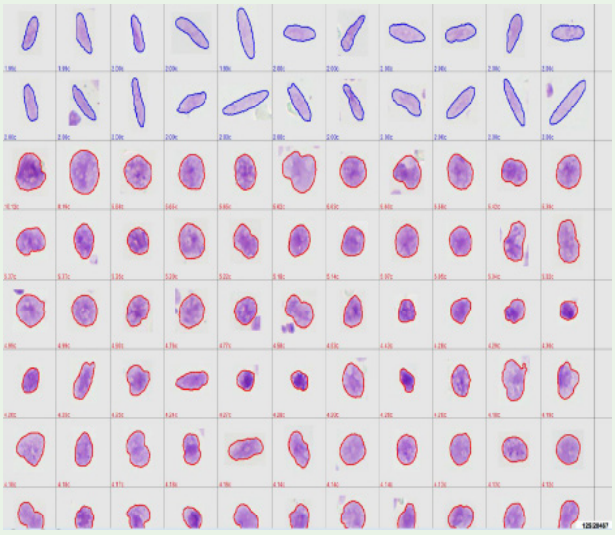

Figure 2: Screenshot from MotiCyte-auto representing automatically detected and classified nuclei from 22 Feulgen-stained fibroblasts and 66 prostate cancer cells.

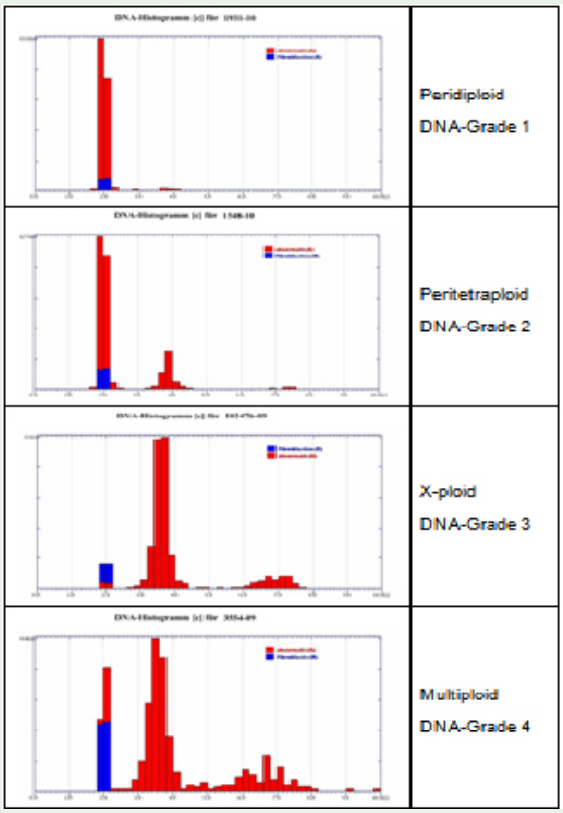

Figure 3: Representative nuclear DNA-histograms of four DNA-grades of malignancy.

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in 2/11 with 7b (reference pathologist) DNA-grade was 1 (18.2%), 44.2% of GS 7a & b.

Correlation of GSs of local pathologists, reference pathologist and DNA-karyometry with the occurrence of progression according to S3-citeria ("soft") where 0/20 (0%), 4/20 (20.0%) and 17/20 (85.0%). If "hard" criteria for the evidence of progression of cancers were applied, these figures were: 1/17 (5,9%), 4/17 (23,5%) and 17/17 (100%). Tables 1 and 2 present sensitivities, specificities, positive and negative predictive values for all combinations of GSs and stages as criteria of inclusion and different evidences of progression. These figures do not change significantly if only patients with stages cT1-2a were taken into consideration or with GSs 4-6.

1 patient changed therapy to radiation and 33 to radical prostatectomy (37.5%). 5 of these due to own decision, 28 due to their urologists recommendation (35.0%).

20 patients had suffered a progress of their prostate cancer during the period of observation due to change of one or more of the inclusion criteria: 1x stage >cT1-2b, 17x PSA-DT < 36 months, 7x > 2 tumor-positive cores or 2x upgrading of Gleason-score. In 4 cases 2 occurred together.17 patients had suffered a progress due to PSA-DT <36 months, 4 showed a stage pT3a on prostatectomy, 1 both.

Discussion

Active surveillance represents a non invasive strategy for clinically localized, low-grade cancers of the prostate that avoids overtreatment. Yet, uncertainty regarding individual patient outcomes remains a concern [20]. After 2,2 years 33% of patients underwent intervention in the Johns Hopkins experience, 24.3% because of reclassification on biopsy [21]. 24% of patients experienced adverse reclassification within 28 months in the Canary PASS cohort of which 12.7% were actively treated [20].

A main reason for this high rate of reclassifications and patients quitting this conservative strategy may be the low inter observer reproducibility and prognostic validity of subjective microscopical assessment of the grade of malignancy by the GS [1,2]. Additionally the known multifocal distribution of prostate cancers and their histological heterogeneity limit the reliability of prognostic markers, obtained on non sufficiently representative tissue specimens.

Different grades of cancer malignancy are tightly associated with different stages of cytogenetic tumor progression, characterized by different grades of chromosomal aneuploidy. As these are associated with different grades of DNA-aneuploidy [8], nuclear DNA-measurements can be used to objectively grade the malignancy of cancers. Tavares and coworkers were the first to publish respective encouraging results for cancers of the prostate 1973 [10]. Since then dozens of publications have proven the prognostic validity of DNA-cytometry for that tumor [11]. Yet, up to now, no prospective Oxford-level of evidence 1b cohort study on untreated prostate cancer patients under AS applying this method has been published.

Meanwhile the disadvantage of time-consuming manual selection of Feulgen-stained nuclei to be microphotometrically measured has been overcome by the development of digital nuclear classifiers that allow automated specific identification of nuclei from normal fibroblasts and cancer cells in enzymatic cell separation specimens of prostate cancer tissue biopsies [18]. Applying these in computer controlled, motorized microscopes as the MotiCyte-auto, allows the automated measurement of ten thousands of nuclei within a few minutes. In this study a mean number of 286nuclei from normal fibroblasts as internal reference cells and 4.615 from cancer cells have been measured per specimen. This may result in a higher representativity of the grading results obtained on few samples for the tumor as a whole. Standardized automated prognostic interpretation of DNA-distributions according to published algorithms [19] excludes effects of subjective DNA-histogram-analysis.

Comparing the Gleason-Scores of 28 different local pathologists with those assessed by a specialized urological Reference Pathologist (R.E) and finding an inter observer reproducibility of Gleason scores on initial biopsies of 55%, confirmed similar reports in the literature [3]. As the decision to recommend AS depends on the Gleason-score, this is dubious in nearly every second case. In comparison, the inter observer reproducibility of DNA-grading the malignancy of cancers of the prostate has been reported to be 93.0% and 90.2% [11].

Defining objective parameters for assuming a clinical progress of prostate cancer is controversial. Considering the fact that besides the number of cancer-tissue positive cores and their GS, also the assessment of the clinical stage cT is subjected to subjectivity, only the calculation of the PSA-doubling time seems to represent a reliable surrogate marker for the progression of an individual cancer. As the postoperative proof of tumor-spread over the organ capsule also represents an objective evidence of progress, we compared the above mentioned "soft" criteria according to S3-guidelines with those of two "hard" markers (Tables 1 and 2).

As the inclusion of 3 patients with GS 7b and those 2 with clinical stage cT2b may influence measures of diagnostic accuracy, we have separately calculated measures of diagnostic accuracy with and without these (Tables 1 and 2).

Our results show that, irrespective of excluding patients with GS 7b or stage cT2b, the sensitivity of the reference pathologists Gleason-scoring with 20% is much better than that of the local pathologists (0%) but it is outrun by that of DNA-karyometry with 85.0%.

This result is burdened by a lower specificity of 86.7% for reference pathologists grading and 48.3% for DNA-karyometry, while the local pathologists reach 95.0%. Yet, most likely specificity will increase if longer follow-up periods will be available as 4.1 years after assessing higher grades of malignancy, not yet all may have led to clinically manifest progresses.

The negative predictive value gives the rate at which a given patient can rely that within a given period (4.1 years) he will not suffer any progress of his cancer, if he presents GS 4-6 or DNA-grade 1. These figures were 74.0% for the locals and 76.5% for the reference pathologist but 90.6% for DNA-karyometry.

If only "hard" evidence for cancer progression was considered sensitivities were 5.9%, 23.5% and 100%, specificities 96.8%, 87.3% and 50.8% and negative predictive values 79.2%, 80.9% and 100%.

As we could only investigate 80 patients, we recommend further prospective level 1b-studies to confirm our results of a superior prognostic validity and reproducibility of DNA-karyometry for prostate cancer patients under active AS.

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Conclusions

Prognostic DNA-karyometry of prostate cancers under AS in order to exclude a progress during the next four years seems to be significantly more valid and reproducible as compared with the subjective Gleason-score.

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