

## THE PROGNOSTIC SIGNIFICANCE OF PLOIDY AND DNA-HETEROGENEITY IN THE PRIMARY DIAGNOSIS AND MONITORING OF PATIENTS WITH LOCALLY ADVANCED PROSTATIC CARCINOMA

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**Abstract.** Single-cell DNA cytophotometry was employed to analyze the tumors of 271 patients with locally advanced prostatic carcinoma as to DNA ploidy and heterogeneity and the distribution of the phases of the cell cycle before and during therapy, with the intention of establishing prognostic factors apart from those already known (stage, grade). Follow-up periods ranged from 1 to 9 years.

One hundred and ninetyeight (73%) of the 271 patients had carcinoma stage T3 N0 M0, and 73 (27%) of them had carcinoma stage T3/T4 N+ M1. The tumors were evaluated cytologically to establish the grades of malignancy. 11.8% were grade-I carcinoma, 64.3% were grade-II and 23.8% were grade-III carcinoma. Single-cell DNA cytophotometry demonstrated aneuploidy rates of up to 73% and diploidy rates of up to 23.8% for the higher grades of malignancy, whereas the diploidy rate established for grade I carcinoma was 71 % and the respective aneuploidy rate was 15.2%. These differences are significant ( $p < 0.001$ ).

There was a significant correlation between the results of DNA cytometry and the clinical course of the disease. Patients with diploid tumor-cell nuclei developed no metastases and no local tumor progression during the follow-up period of 9 years, whereas patients with aneuploid tumor-cell nuclei showed metastases and local tumor progression within 8-22 months, despite changes in therapy. These patients died of carcinoma after an average 18 months following primary diagnosis.

**Key words:** Nuclear DNA analysis, DNA heterogeneity, diagnosis of prostatic carcinoma, therapy control

The pathomorphological classification of malignant tumors of the prostate is often difficult, even today. Morphologically speaking, carcinoma of the prostate is characterized by a highly heterogeneous appearance with overlapping histological characteristics, variable biological behavior and clinical course.

Treatment could be improved by developing criteria of malignancy apart from the histological ones, and in addition to precise grading, determination of hormone receptors, histochemical and immunological analyses and analyses of molecular genetics. The DNA ploidy and the proliferative activity of the different tumors could be such criteria of differentiation (1-5,7,9,14,18,19,24).

If the DNA content of tumor-cell nuclei deviates

from that of normal tissue, it is an indication of a tumor cell population with aneuploid characteristics (12,17). The DNA heterogeneity of tumor-cell nuclei in malignant tumors of different organs has become very important clinically during the last few years because of its relation to tumor behavior before and during therapy (1-5,7,8,10,13,19,22,23,26,30,31,33,35).

In an experimental study - from 1980 to 1989 - the authors analyzed tumor cell material obtained by aspiration biopsy, with the intention of establishing prognostic factors in addition to those already known. The DNA ploidy and heterogeneity and the proliferative activity of prostatic carcinoma were analyzed, by means of absorption scanning cytophotometry, before and during therapy.

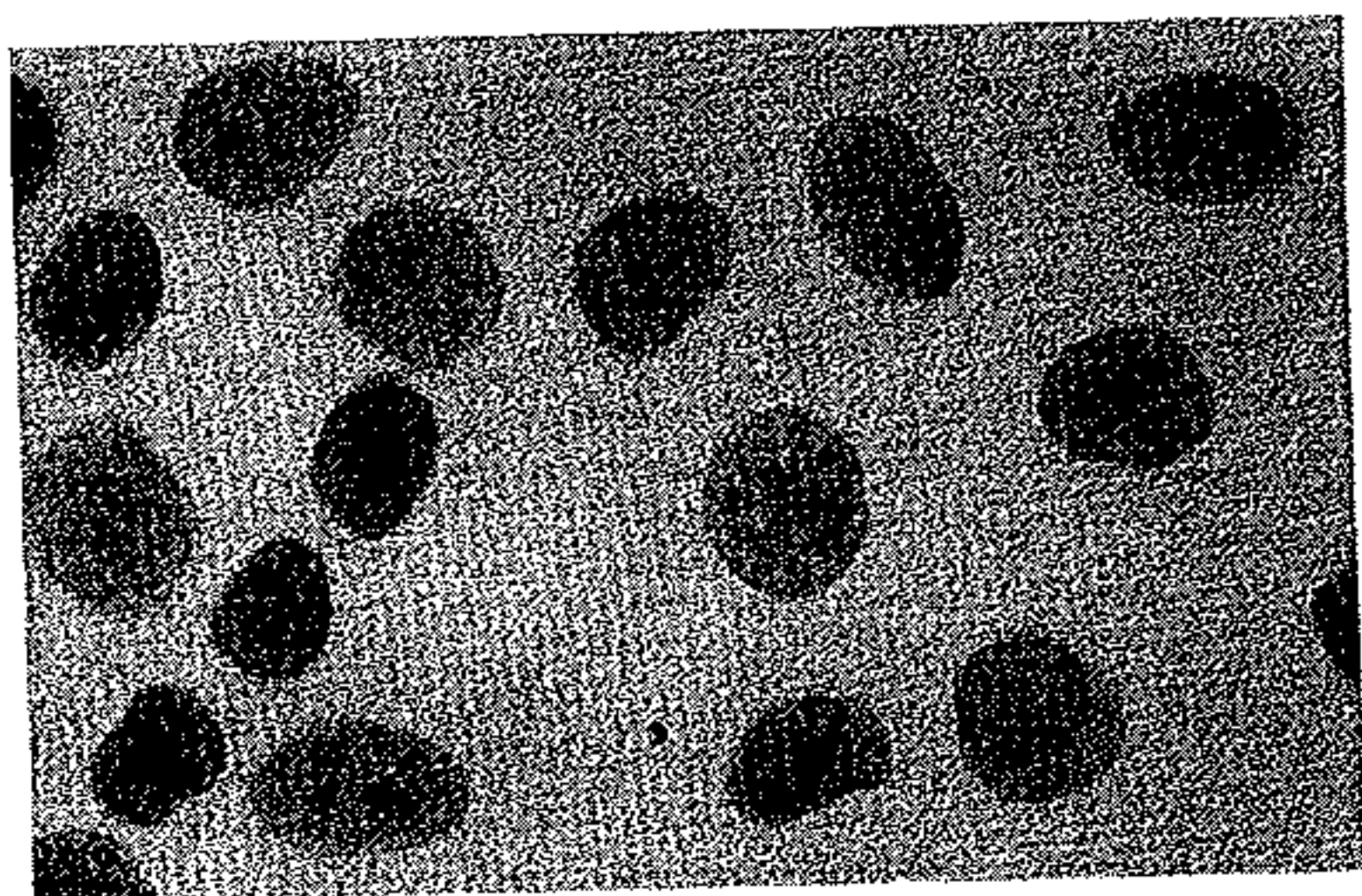


Fig. 1. Feulgen's reaction: isolated, clearly distinguishable cell nuclei without overlapping or impurities.  $\times 1000$ .

## PATIENTS AND METHODS

From January 1980 to December 1989, single-cell scanning absorption cytophotometry was employed to analyze the tumors of 271 patients with locally advanced prostatic carcinoma, and the clinical course of the disease in the respective patients was documented. Follow-up periods ranged from 1 to 9 years. The average age of the patients was 68.9 years (42–88 years).

The cellular material for DNA analysis was obtained by means of transrectal fine-needle aspiration biopsy (Franzén *et al.* 1960) (17). The aspirate was stained with Papanicolaou's stain. It was then assessed cytologically to establish the diagnosis and differentiation of the tumor, according to the recommendations of the Urological Study Group on Prostatic Carcinoma (20). Following the determination of the grade of malignancy, the aspirate was re-stained with Schiff's reagent (30-min hydrolysis in 5 N HCl at room temperature, followed by Feulgen's reaction) (16) (Fig. 1) and analyzed by means of cytophotometry. Approximately 100 tumor cell nuclei were measured, sometimes more. The DNA content of these nuclei was determined by means of single-cell scanning absorption photometry. Fine scanning with an MPV-2 cytophotometer was the method chosen for measuring. A process computer was employed to control the scanning stage, measuring procedure and

Table I. Frequency of stages in 271 patients with locally advanced prostatic carcinoma at the time of diagnosis.

Stage	n	%
T3 N0 M0	198	73
T3/T4 N+ M1	73	27

Table II. Frequency of grades of malignancy in 271 patients with prostatic carcinoma.

Grade of malignancy	n	%
I	32	11.8
II	174	64.3
III	65	23.9

registration of absorption according to the program, via an interface (pdp-8a computer). The respective total extinction values measured were printed out by Teletype, together with the mean value, standard deviation, variation and coefficient of variation.

Following Sprenger *et al.* (29) and Leistenschneider and Nagel (22), the authors have termed the DNA content of a cell nucleus either *euploid* or *polyploid* if it is within the range of the mean value of the diploid standard cell population ( $\pm 25\%$ ), according to the coefficient of variation of the standard cell population. All cell nuclei showing DNA levels outside of the euploid or polyploid ranges are termed *aneuploid*. The DNA content of about 100 cell nuclei determined at each measurement is summarized in a cytophotogram and expressed as an absolute value in arbitrary units (AU). The number of cell nuclei measured by cytophotometry is given as 'n'. A cytophotogram is produced by recording the values obtained on the abscissa and ordinate (Fig. 6–8). The homogeneity of the grades of ploidy, according to  $\chi^2$ , has been verified statistically for the three grades of malignancy.

Survival curves have been obtained by employing the Kaplan-Meier product limit method. They have been compared statistically by means of the Long Rank Test (15,21,25).

## RESULTS

### Tumor stages and grades of malignancy

The staging in this study follows the recommendations of the UICC applying the TNM classification (32). When first diagnosed, 198 (73%) of the patients had carcinoma stage T3 N0 M0, and 73 (27%) patients had carcinoma stage T3/T4 N+ M1 (Table I). The total number of cases studied shows the cytological grade of malignancy G II to prevail with 64.3%, followed by grade III with 23.9% and grade I with 11.8% (Table II).

### Cytological criteria of tumor response to therapy

The *case material* described by the authors is evidently different from the cases reported by other authors as *only 27% of the patients included in this study had metastases* whereas 73% had none although they had stage T3 carcinoma.

This is explained by the fact that the authors have been able to *monitor* the effect or failure of therapy in the *primary tumor* itself by means of *cytology* and

Table III. Frequency pattern of tumor-cell proliferation and grades of malignancy in 271 patients with prostatic carcinoma.

Grade	(n)	Aneuploidy (%)
I	(32)	15.2
II	(174)	51.0
III	(65)	73.0

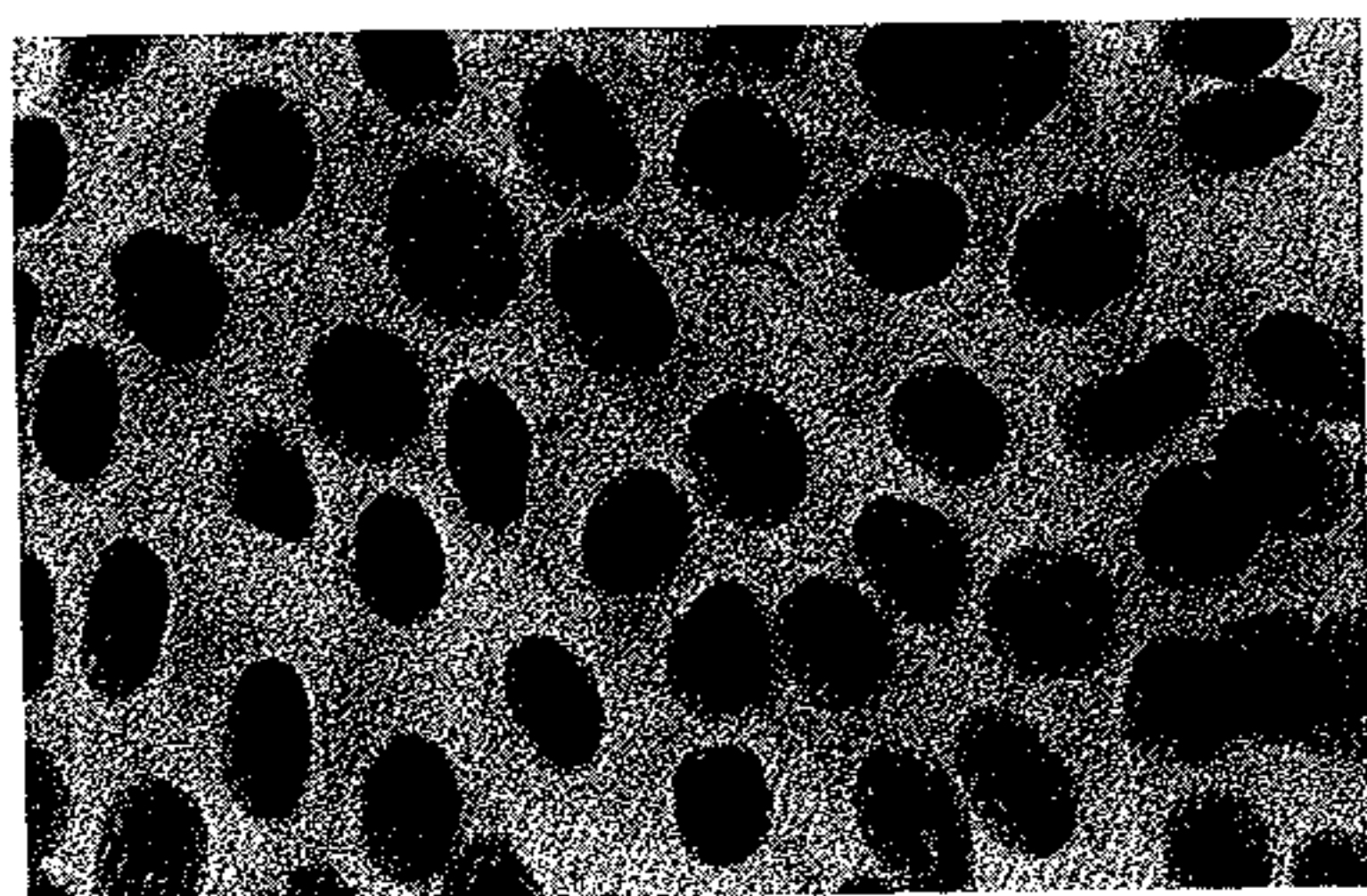


Fig. 2a. Case 1. K.H., 63 years, prostatic carcinoma stage T<sub>3</sub> NX M0: primary cytological diagnosis prior to BUSERELIN therapy. Cytological sample obtained by aspiration. Grade-I carcinoma. Stained with Papanicolaou's stain. ×1000. Prior to therapy: moderate nuclear polymorphism, pronounced nuclear hypochromatism, prominent nucleoli, Grade-I carcinoma (score 9).

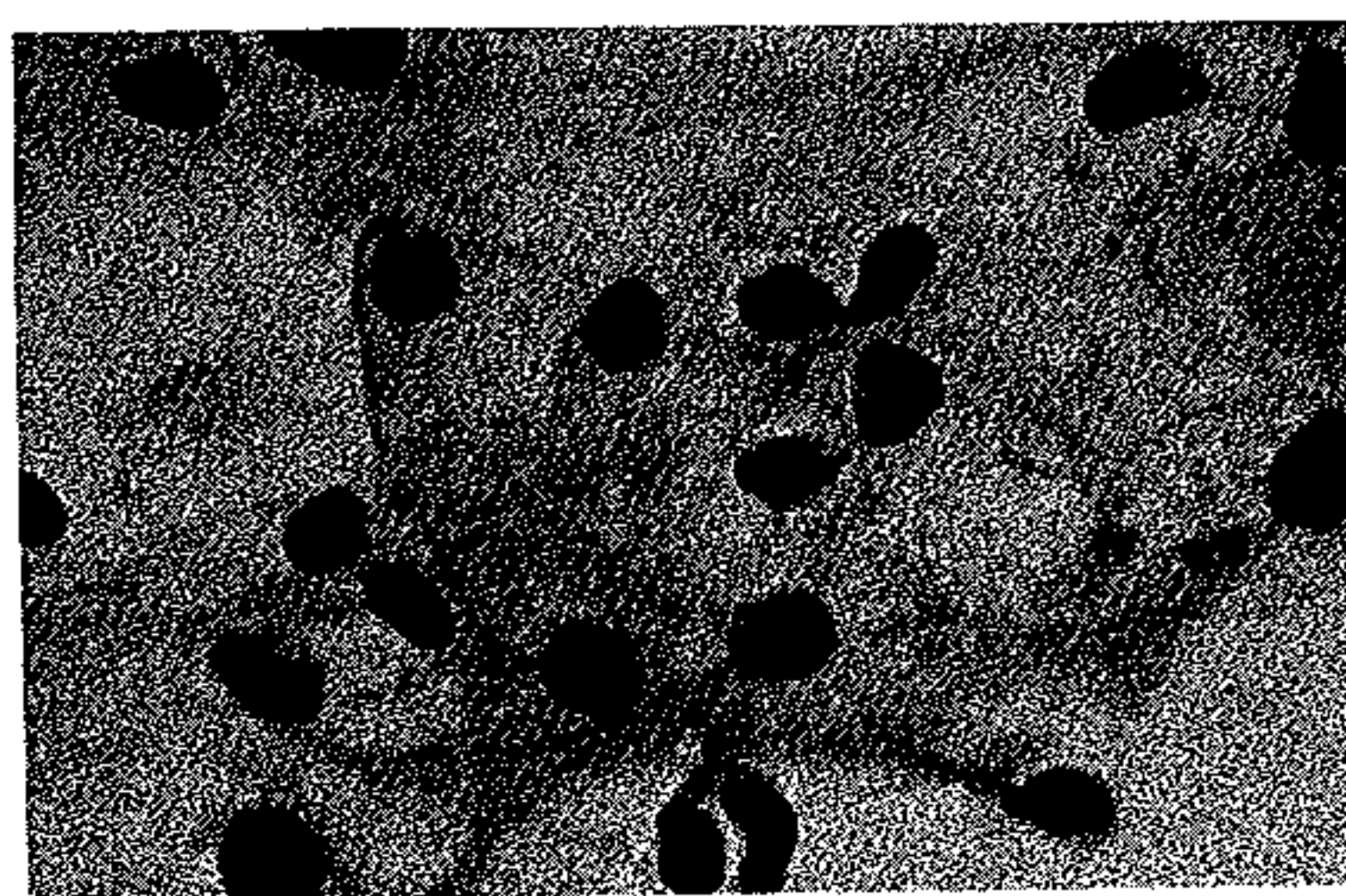


Fig. 2b. Same patient, after 2.5 years of BUSERELIN therapy. Regression grade II. Stained with Papanicolaou's stain. ×1000.

After 2.5 years of BUSERELIN therapy: reduction in nuclear size, nuclear rarefaction, diminution of nucleoli, pyknotic nuclei, fine vacuolation of cytoplasm and nuclear chromatin. Overall, good therapeutic effect, corresponding to regression grade II.

DNA cytophotometry, and performed more than 18.000 aspiration biopsies in the last 15 years.

At present, these two methods – and the morphological examination of material gained by punch biopsy – are the only objective parameters which permit an evaluation of the response of locally advanced, non-metastatic carcinoma to treatment.

The most important signs of tumor regression are

found in the cell nucleus (pyknosis, reduction in size) and in the nucleoli (reduction in size and number) (14,23).

The cytological criteria of regression correspond with those defined for the regression grading by histological methods (14,23). The cytological regression grading adopted by the Uropathological Group in Germany distinguishes six grades (Table IV).

Table IV. Regression grading according to the recommendations of the Uropathological Group in the FRG.

Grade of regression	Effect of therapy	Cytomorphological criteria
0	very good	no carcinoma, marked regressive changes
II	good	epithelial atypias, carcinoma?, marked regressive changes
IV	satisfactory	few and small carcinoma cell clusters ('residual carcinoma'), marked regressive changes
VI	sufficient	carcinoma with marked regressive changes
VIII	poor	carcinoma with poor regressive changes
X	none	carcinoma without regressive changes

Table V. Nuclear DNA ploidy pattern obtained by single-cell cytophotometry compared with clinical findings and bone metastases in 271 patients with prostatic carcinoma.

			M1		died		clinically stable	
	n	%	n	%	n	%	n	%
diploid	67	25	-	-	8*	12	59	88
polyploid/near polyploid	63	23	33	52	33	52	24	38
aneuploid	141	52	138	98	137	97	4	3
	271	171	63	170	63	87	32	
				14*	5			

\* Cardiocircular failure

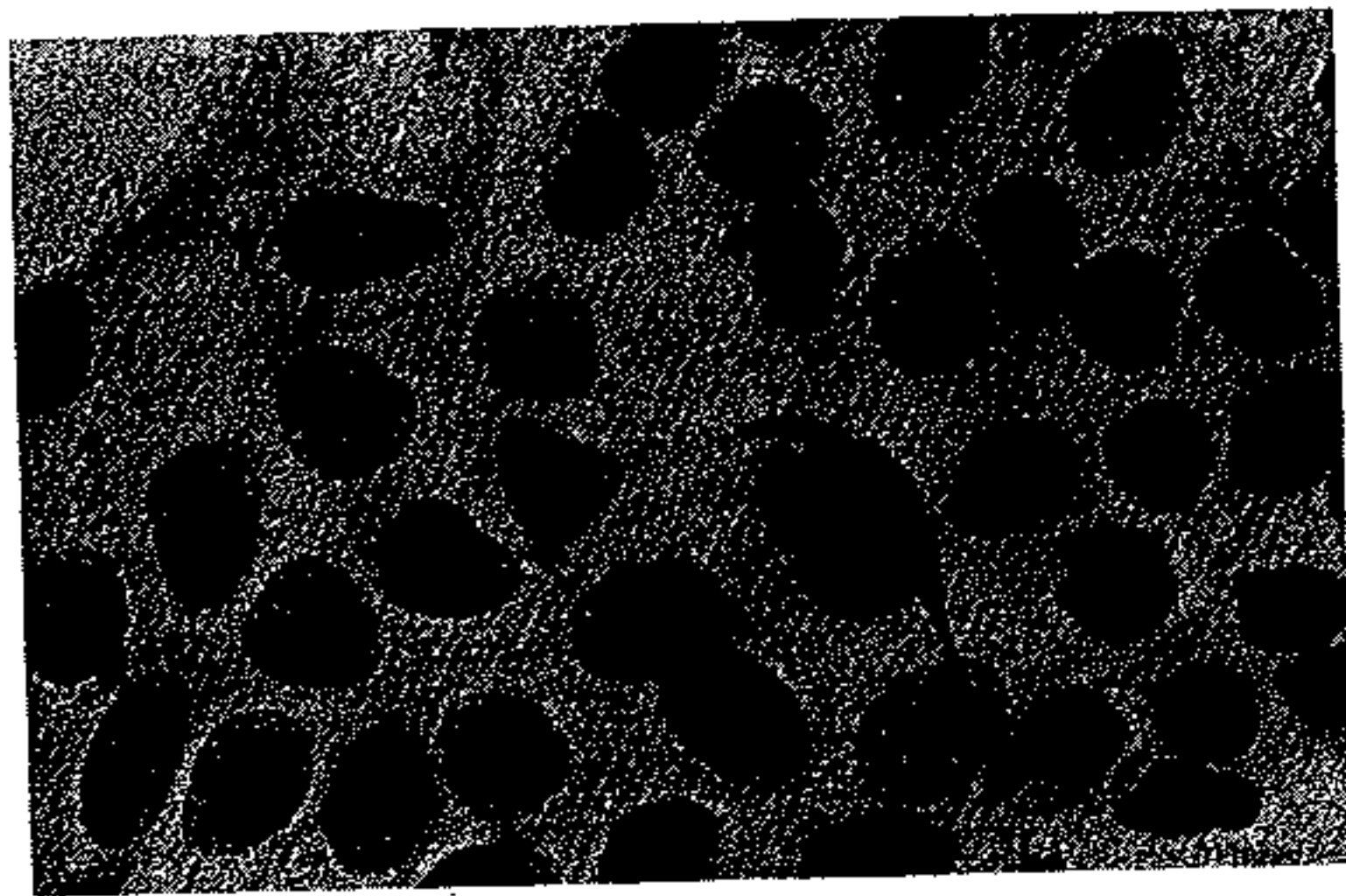


Fig. 3a. Case 2. W.K. 65 years, prostatic carcinoma stage T<sub>3</sub> NX M0: primary cytological diagnosis prior to BUSERELIN therapy. Cytological sample obtained by aspiration. Grade-II. Stained with Papanicolaou's stain.  $\times 1000$ .

Prior to therapy: marked nuclear polymorphism and hyperchromatism, disturbance of nuclear arrangement, prominent nucleoli displaying loss of circularity; some nuclei contain several nucleoli. Grade-II carcinoma (score 13).

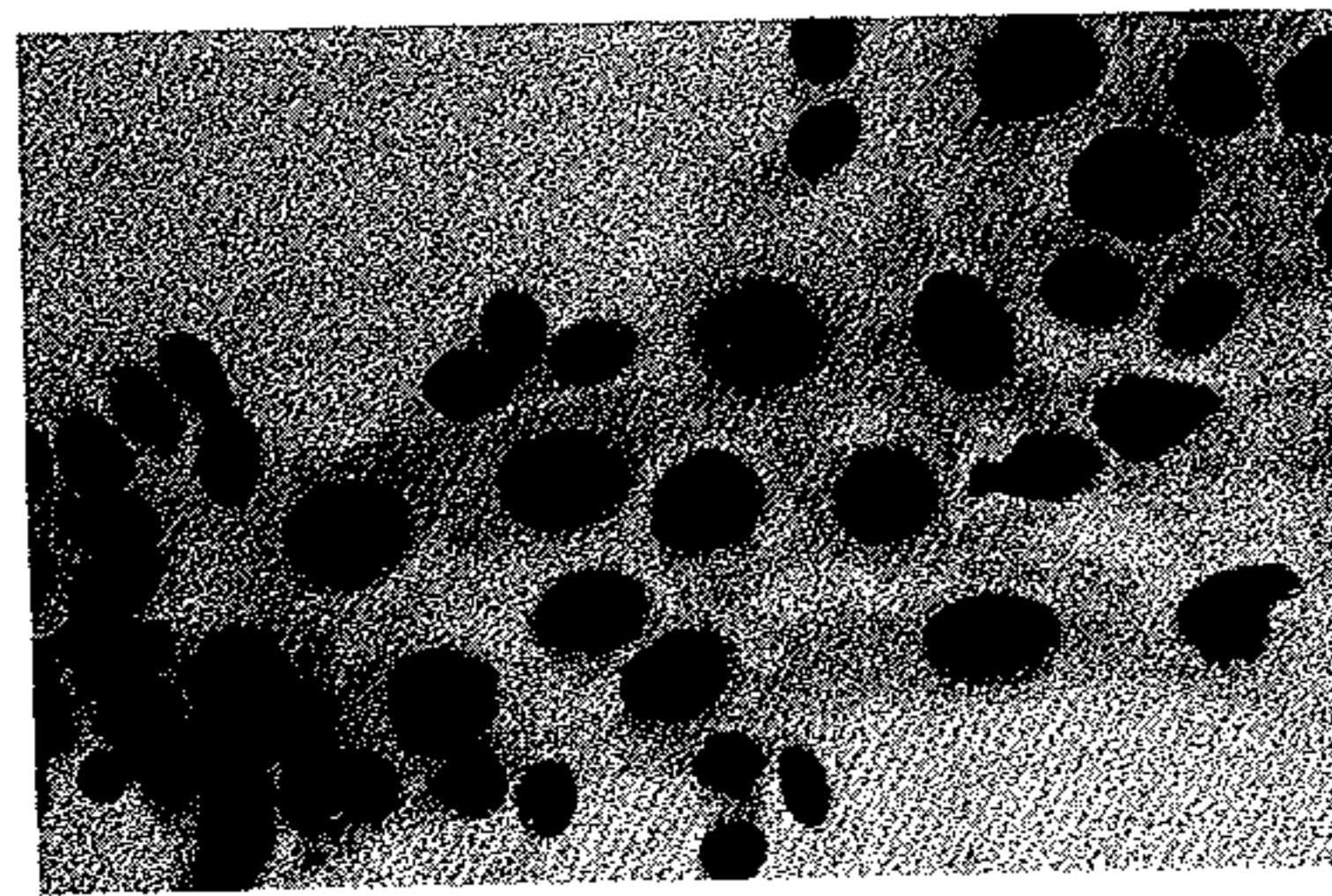


Fig. 3b. Same patient, after 2.5 years of BUSERELIN therapy. Regression grade IV. Stained with Papanicolaou's stain.  $\times 1000$

After 2.5 years of BUSERELIN therapy: reduction in nuclear size, localized pyknotic nuclei, scarcely distinguishable nucleoli. Regression grade IV.

The six grades are defined by characteristic *signs of regression*. The *regression grades II to VI* indicate good to sufficient therapy response of the tumor whereas the *regression grades VIII and X* signal poor or no response of the tumor to therapy.

The difference between good (grades II–VI) and poor regression (grade VIII or X) is *statistically significant* (14,23).

The regression signs occur irrespective of the form of therapy. This has been proven in an earlier study the authors undertook with 600 patients under six different forms of therapy. What has been established furthermore is *the point of time* when a poor or lacking therapeutic effect can be unmistakably demonstrated in the tumor itself for the different forms of therapy. When the tumor shows *poor or no regression* after *six months* of anti-androgen treatment such as *orchiectomy*, therapy with estrogens, *LH-RH analogues* or *anti-androgens*, it is unlikely to respond to continued therapy. The therapeutic effect of estramustine phosphate (ESTRACYT) or cyclophosphamide can be reliably evaluated after only *three months*

of treatment (23).

According to the authors' observations, the regression grades VIII and X signal that local tumor progression or metastases are very likely to occur within few months.

*The interindividual reproducibility of the cytological regression grading averaged 85%, which is a remarkably high percentage.*

The three cases described below show different responses to BUSERELIN therapy and the respective characteristic cytological changes.

#### Single-Cell Scanning Cytophotometry

While cytology yields morphological results and thus permits a *qualitative rating* of the effect of therapy, the analysis of the DNA content of tumor-cell nuclei by single-cell scanning cytophotometry permits a *quantitative grading* of regression in the primary tumor. Both methods, particularly DNA cytophotometry, can contribute greatly to a better understanding of biological tumor activity.

Table VI. Survival chances of 271 patients with prostatic carcinoma, grouped according to DNA ploidy.

Survival Probabilities	n = 271		
	3 years	5 years	9 years
diploid	99 %	97 %	88 %
polyploid/diploid	71 %	51 %	38 %
aneuploid	34 %	6 %	—
Acc. to Kaplan-Meyer	p = 0.001		



Fig. 4a. Case 3. F. Sch., 51 years, prostatic carcinoma stage T<sub>3</sub> NX M0: primary cytological diagnosis prior to BUSERELIN therapy. Cytological sample obtained by aspiration. Grade-III carcinoma. Stained with Papanicolaou's stain. ×1000.

Prior to therapy: markedly polymorphic and hyperchromatic nuclei, disturbance of nuclear arrangement, prominent nucleoli displaying loss of circularity, conspicuously dissociated nuclei. Grade-III carcinoma (score 17).

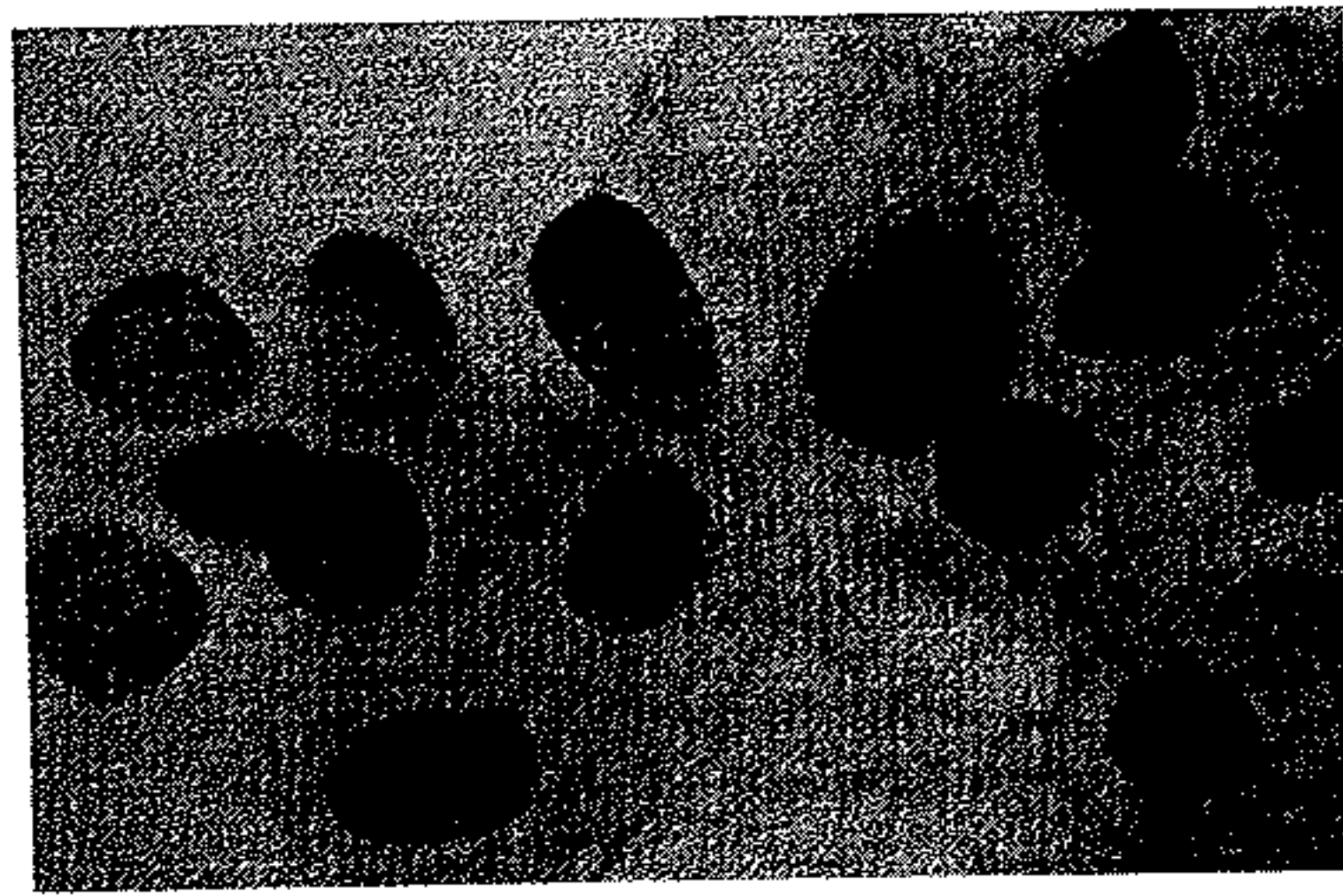


Fig. 4b. Same patient, after 6 months of BUSERELIN therapy. Regression grade X. ×1000.

After six months of BUSERELIN therapy: the nuclei are still hyperchromatic and polymorphic, with prominent nucleoli; rare and only localized regressive changes. Regression grade X, therefore cross-over to secondary therapy with ESTRACYT.

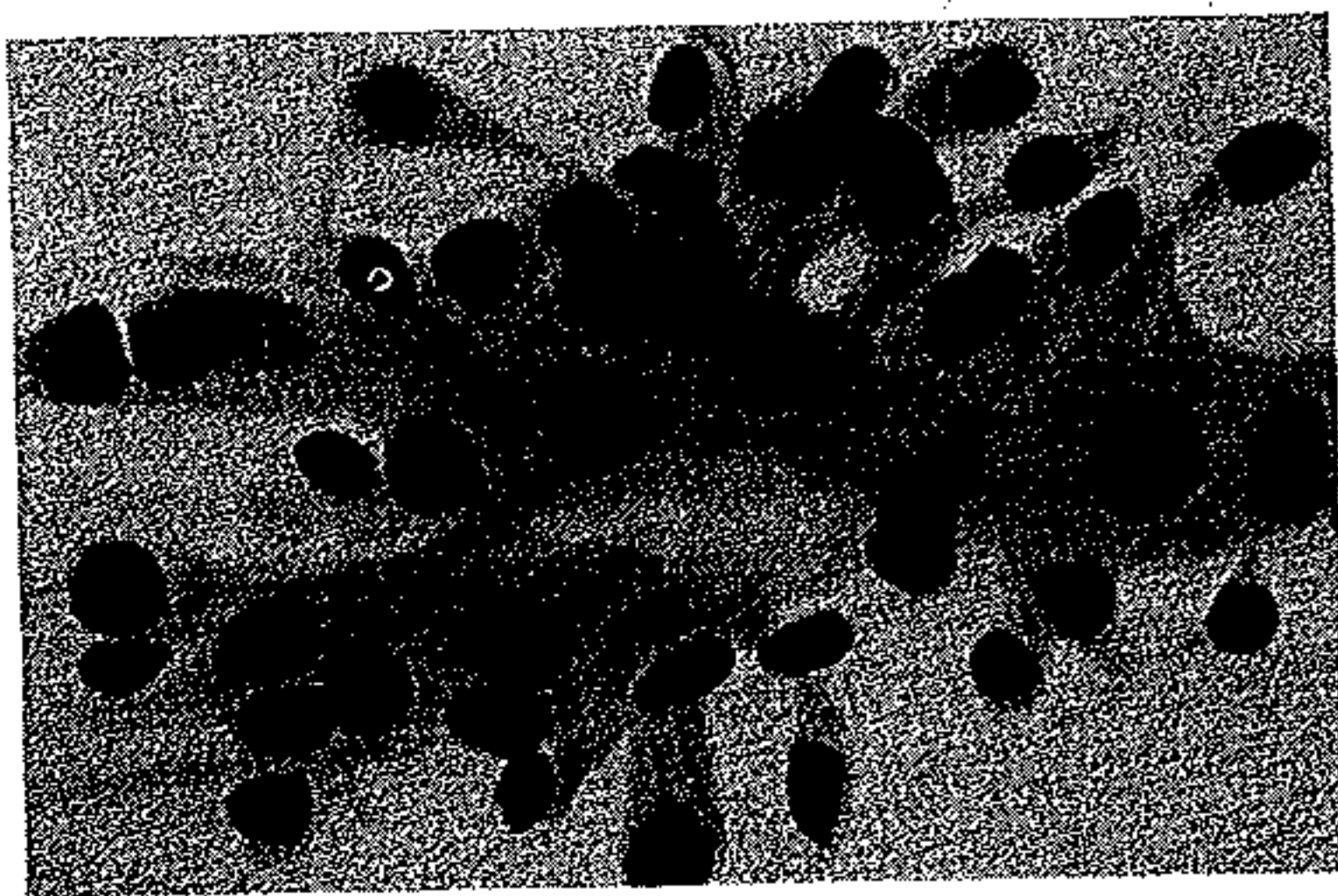


Fig. 4c. After 1.5 years of ESTRACYT therapy: nuclear rarefaction and reduction in size, localized cytoplasmic vacuolation, nucleoli reduced in size. Sufficient therapeutic effect. Regression grade VI.

Same patient, after 2.5 years of secondary ESTRACYT therapy. Regression grade VI. Stained with Papanicolaou's stain. ×1000

It has been known for years that tumor cell populations, corresponding to a change in the number of chromosomes towards aneuploidy, show higher DNA contents than normal tissues. This has been confirmed by investigations of different organs, particularly the mammas, lungs, and urinary bladder.

It also applies to prostatic carcinoma whose DNA content differs substantially from that of adenoma. This fact assigns DNA cytophotometry its place in the treatment and therapy control of prostatic cancer (24,28,35).

As far as is ascertainable, the first sequential studies assessing the response of nuclear DNA in prostatic carcinoma to various therapeutic measures were done at this clinic (14,22,28).

The authors have observed that a statistically significant fall of the grade of ploidy from aneuploid towards diploid occurs in prostatic carcinoma when therapy is successful. If an aneuploidy remains unchanged during therapy, a negative clinical course of the disease may be predicted. Furthermore, the authors have shown that therapy-resistant carcinomas of the prostate differ in their nuclear DNA content significantly from tumors with positive response to therapy.

#### Results of DNA cytophotometry

The prognostic value of DNA cytophotograms for BUSERELIN therapy is the same as for any other therapy; the results are statistically significant and can be reproduced in the sample at any time. The figures 9-11 show the cytophotograms of three patients with different responses to therapy.

#### DNA ploidy, heterogeneity of DNA distribution and grade of malignancy

The grade of ploidy in prostatic carcinoma shows DNA values ranging from 2c (diploid) to 19c. The ploidy values of DNA frequency peaks most frequently seen were in the 5c, 6c, 9c, 10c and 12c ranges.

Out of 32 patients with grade-I carcinoma, 68.3% exhibited DNA values in the diploid (2c) range, 10.9% showed DNA values in the polyploid (4c) range

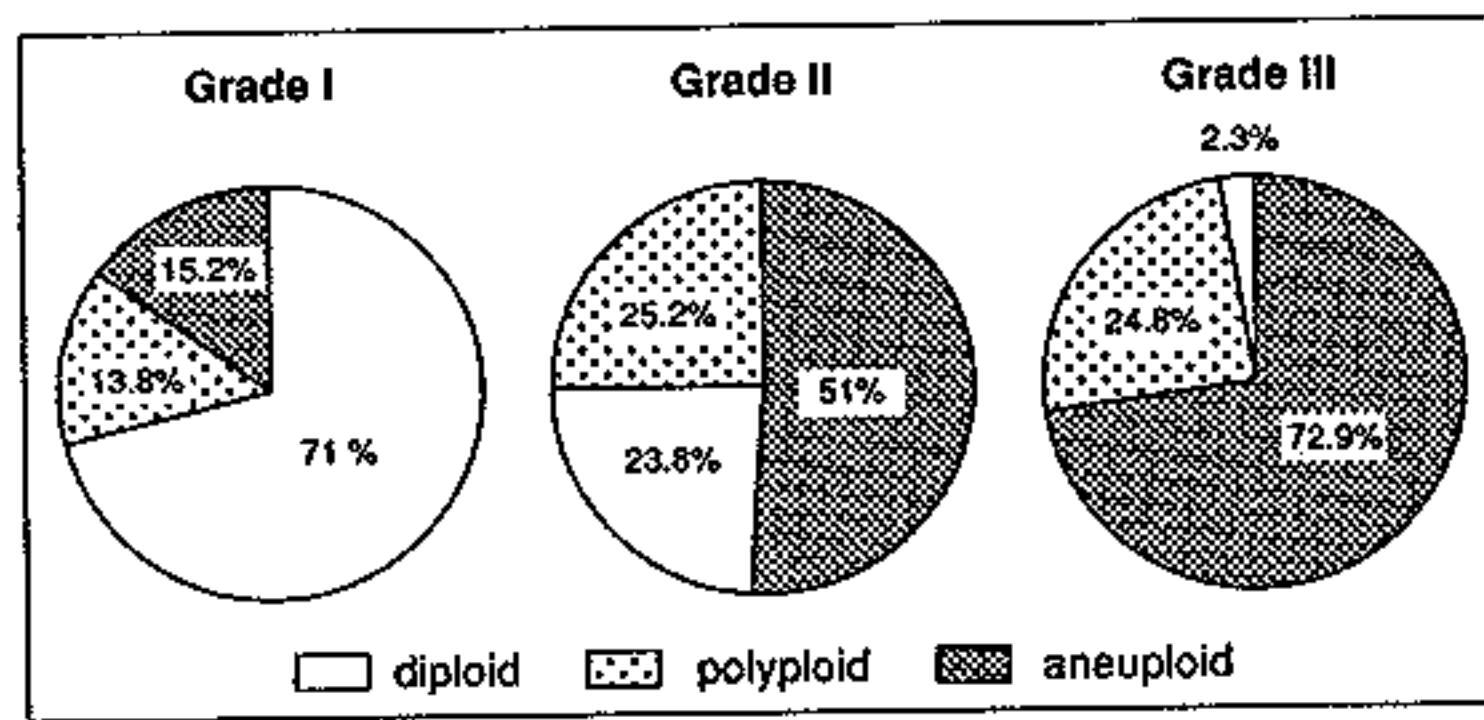


Fig. 5. Correlation between the grades of malignancy and the DNA contents of several tumor-cell nuclei or 271 patients with prostatic carcinoma.

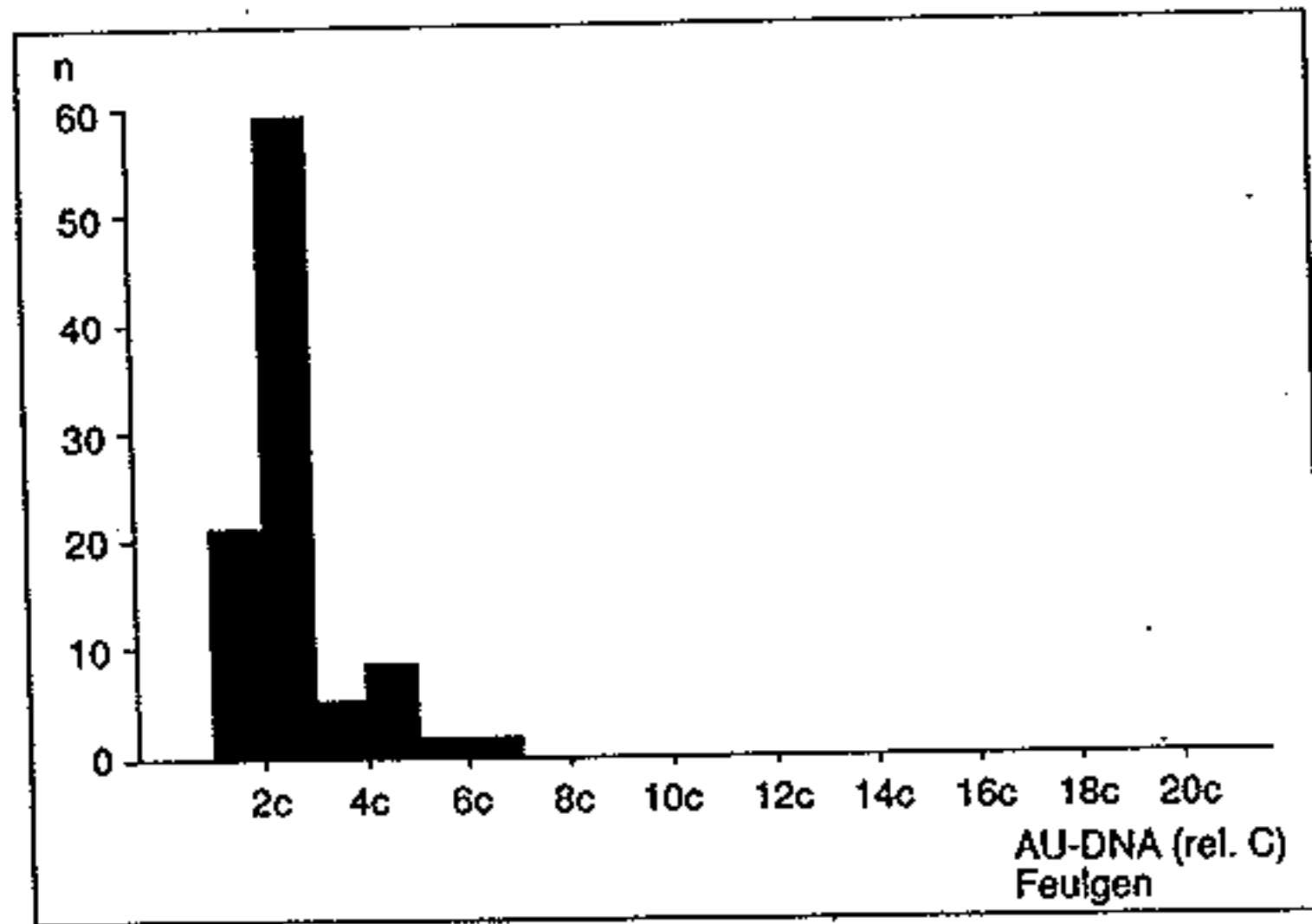


Fig. 6. Grade (of malignancy) I: DNA histogram with frequency peak in the diploid range with values scattering up to 7c.

and 15.2% had DNA values in the aneuploid (3c and 5c) range (Figs. 5 and 6).

The 174 patients with grade-II carcinoma showed highly varying DNA frequency patterns although the cyto-morphological differentiation of the tumors was uniform.

As can be seen from Table III, the DNA values measured were in the diploid (2c) range in 23.8% of the cases, in the polyploid (4c) range in 25.2% and in the aneuploid (3c, 5c) range in 51% of the cases.

Out of the 65 patients with grade-III carcinoma, only 4.2% exhibited diploid DNA distributions, whereas 24.8% had polyploid DNA values and 71% had aneuploid DNA values with more than two DNA frequency peaks and scattered DNA ploidy values up to 19c (Figs. 5, 7, 8).

*Grade of malignancy and distribution of the phases of the cell cycle*

Single-cell scanning cytophotometry affords a means for analyzing the cell cycle and establishing the

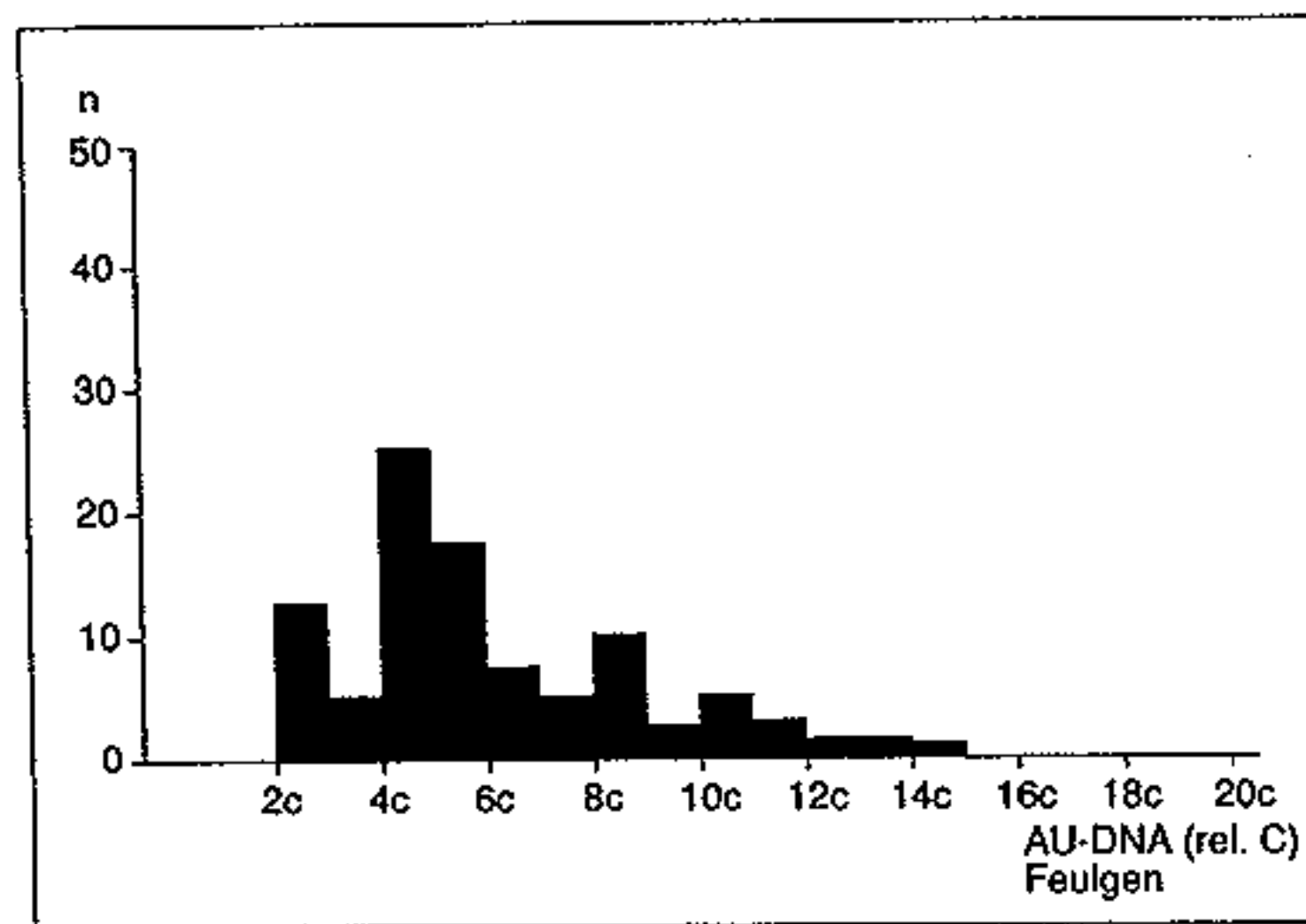


Fig. 7. Grade (of malignancy) II: DNA histogram with highly heterogeneous distribution pattern ranging from 2c to 15c

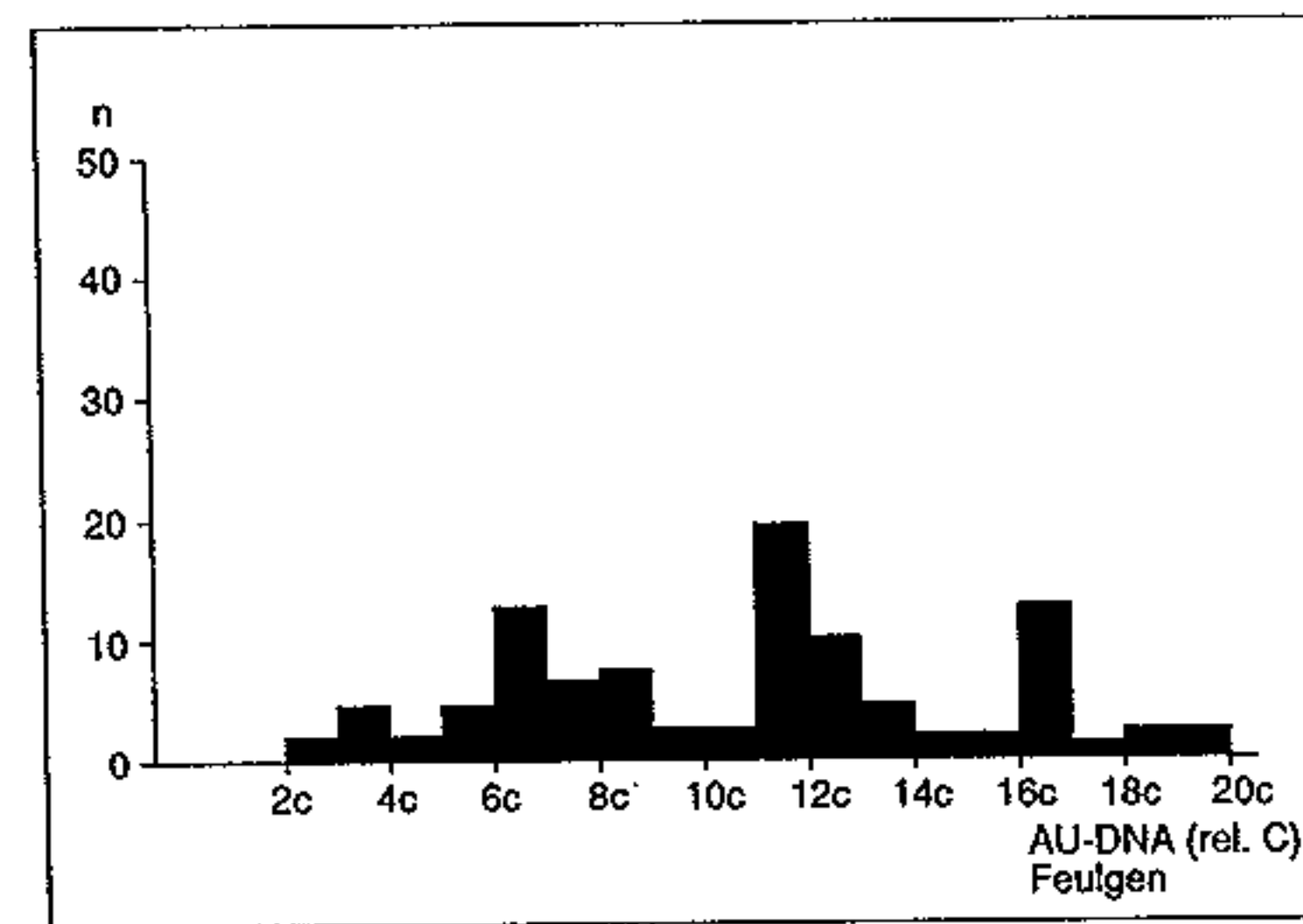


Fig. 8. Grade (of malignancy) III: Heterogeneous DNA histogram with frequency peaks in 6c, 8c, 12c and 16c and values scattering up to 19c.

number and rate of tumor cells in the G<sub>0</sub>/G<sub>1</sub> phase, in the S phase and in the G<sub>2</sub>/M phase.

Table III shows that grade-I carcinomas contain 15.2% of proliferating tumor cells whereas grade-II and grade-III carcinomas exhibit an increase in this rate to 51 and 71%, respectively. Patients with a high rate of aneuploidy suffered clinical progression earlier than patients with minor rates of aneuploidy (S-G<sub>2</sub>/M <15%).

*Prognostic relevance of DNA ploidy*

During follow-up periods of up to 9 years, 67 (25%) patients with a DNA frequency peak in the diploid (2c) range (Fig. 6) developed no metastases and showed no tumor progression, as can be seen in the Tables V and VI.

Sixty-five of the 141 patients (52%) with two or more DNA frequency peaks or with widely scattered aneuploid DNA distribution (Fig. 7 and 8) already

T<sub>3</sub> NxM0

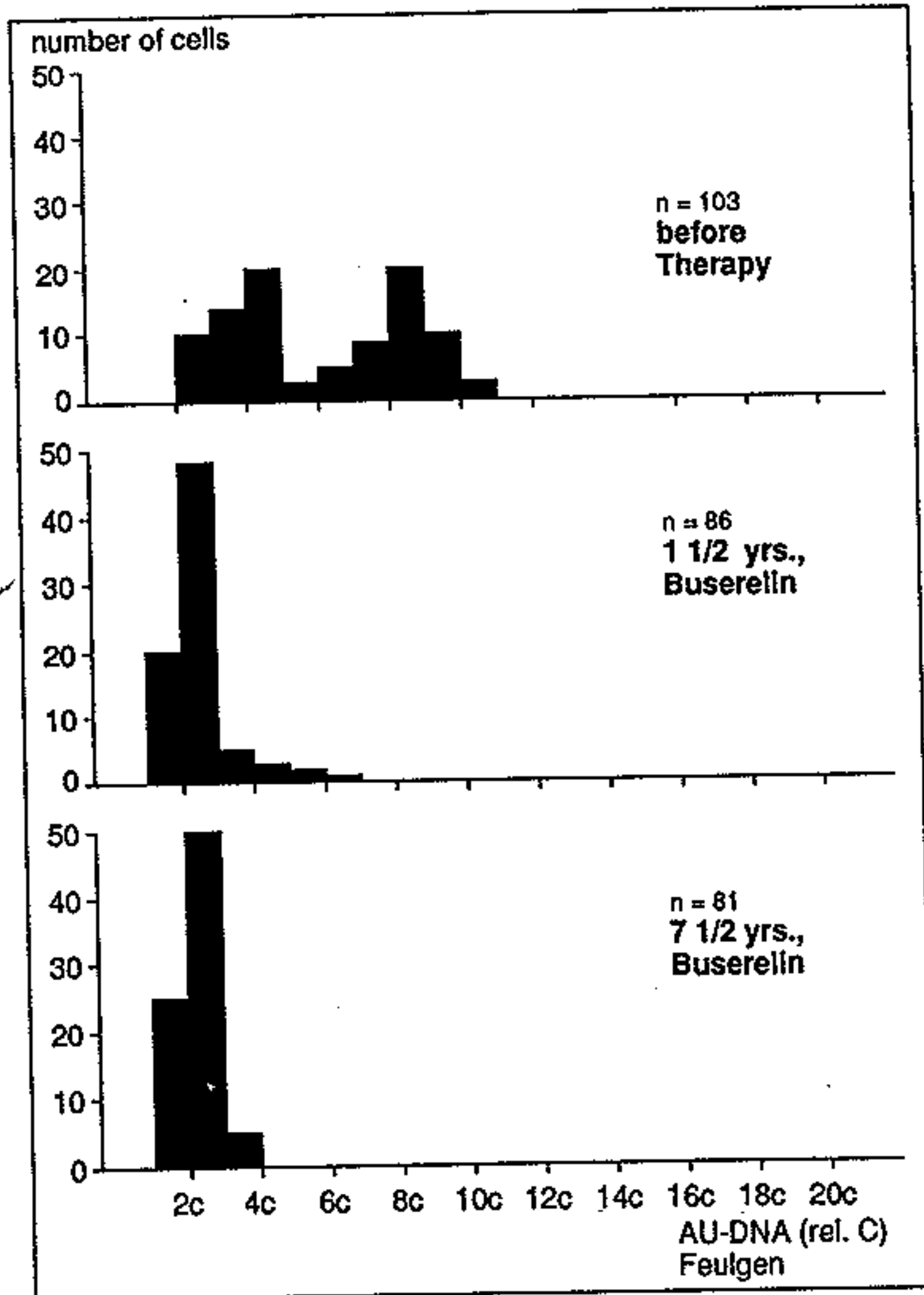


Fig. 9. DNA histograms for patient K. H., T<sub>3</sub> NX M0, grade-II carcinoma (see also Figs. 2a and 2b).

Top graph prior to therapy, middle graph after 1.5 years of BUSERELIN therapy, bottom graph after 7.5 years of BUSERELIN therapy. Good therapeutic effect.

Prior to therapy, there is a broad DNA frequency peak between 8c and 10c, with some values spreading to 12c. After 1.5 and 7.5 years of BUSERELIN therapy, there are slim peaks in the diploid (2c) range. The histograms correspond with the morphological findings and the clinically stable condition of the patient after 8 years of therapy.

had distant metastases when they were first diagnosed, and they died of carcinoma within the first 22 months. Seventythree of the remaining 76 patients in this group developed bone metastases after periods ranging from 9 to 36 months following diagnosis; 67 of them died of carcinoma within the follow-up period.

Thirty-three of the 63 patients with polyploid DNA distribution tending towards diploid had distant metastases and died of carcinoma despite a change in therapy; another 6 patients died of cardiocirculatory failure. Twenty-four of the patients in this group remained clinically stable.

T<sub>3</sub> NxM0

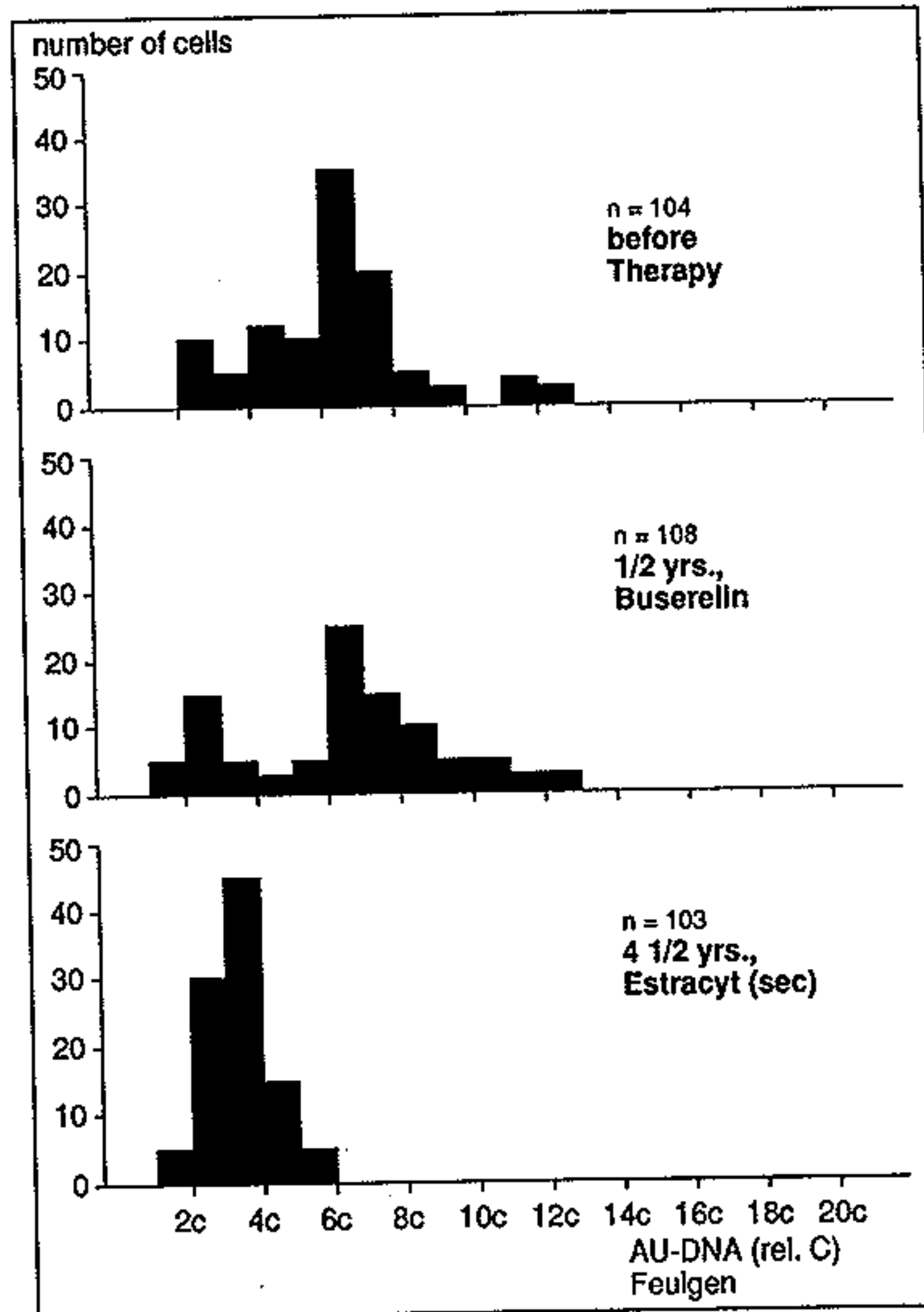


Fig. 10. DNA histograms for patient K. W., T<sub>3</sub> NX M0, grade-III carcinoma (see also Figs. 4a, 4b and 4c).

Top graph prior to therapy, middle graph after 6 months of BUSERELIN therapy; unsatisfactory therapeutic effect. Bottom graph after 1.5 years of secondary treatment with ESTRACYT; satisfactory therapeutic effect.

Prior to therapy, the DNA frequency peak reads between 4c and 8c, with some values spreading to 12c. After six months of BUSERELIN therapy, the DNA frequency peak is still between 6c and 8c. As no signs of regression were demonstrated by cytological examination, either (Fig. 4b), therapy was changed to estramustine phosphate. 1.5 years after the change in therapy, there is a statistically significant DNA frequency peak in the diploid range signalling good regression. This result corresponds with the morphological findings and the clinically stable condition of the patient, i.e. there is no evidence of metastatic development (Fig. 4c).

DISCUSSION

The results of the DNA analyses presented in this study are indicative of the variable biological behavior of prostatic carcinoma and thus of its heterogeneous nature. The few studies published to date on investigations of prostatic carcinoma by means of both sing-

T<sub>3</sub> NxM<sub>0</sub>

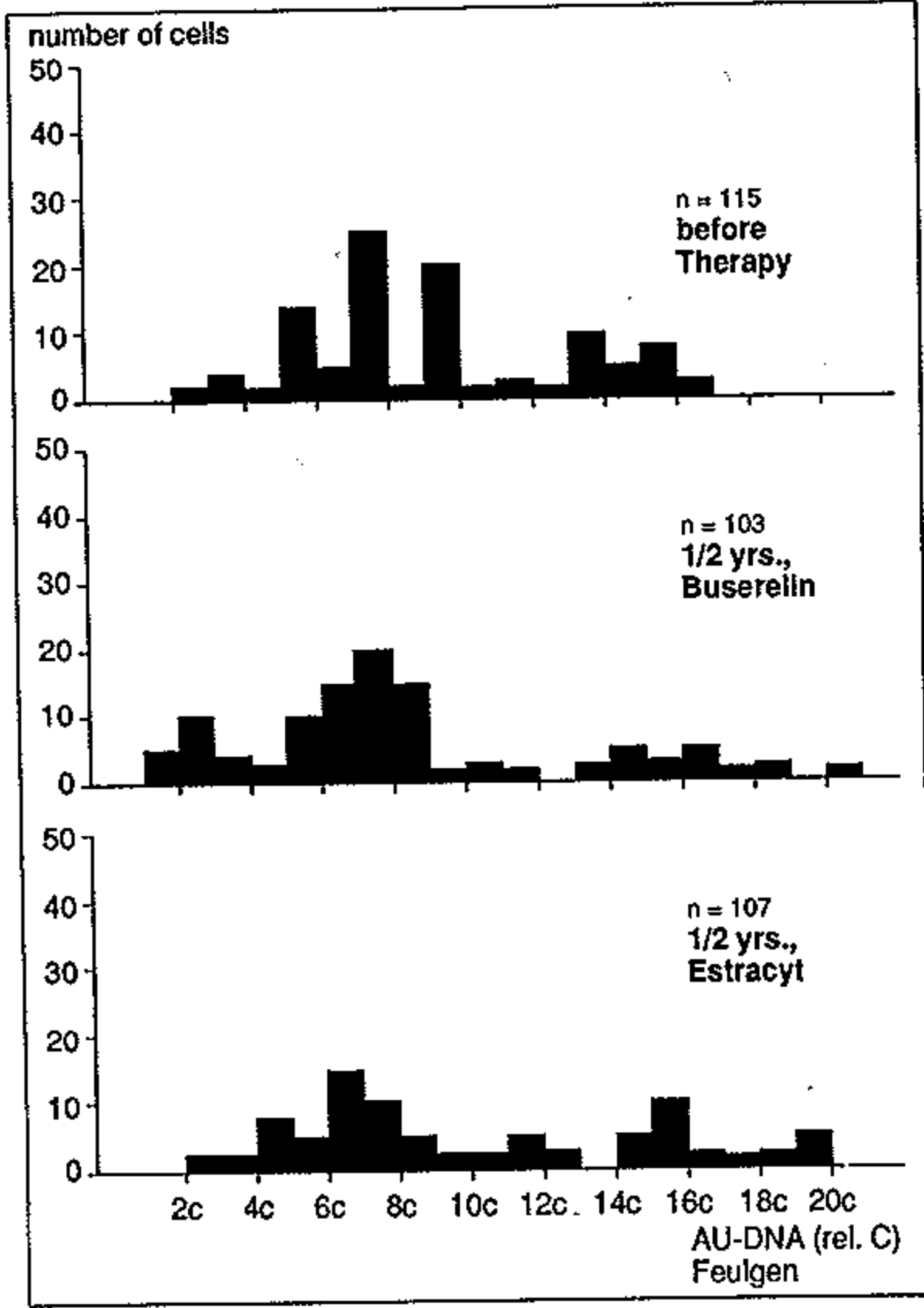


Fig. 11. DNA histograms for patient F. H., T<sub>3</sub> Nx M<sub>0</sub>, grade-III carcinoma (score 17). Top graph prior to therapy, middle graph after failure of BUSERELIN therapy. Bottom graph after 6 months of unsuccessful secondary treatment with ESTRACYT.

This is an example of *primary* resistance to BUSERELIN and resistance to *secondary* ESTRACYT therapy as well. Prior to BUSERELIN therapy, there are DNA frequency peaks near 5c and 7c, another near 9c, and some values reach as far as 16c.

After six months of BUSERELIN therapy, the DNA frequency peak is in the octaploid range, with values spreading to 19c. As such a DNA distribution pattern definitely signals a very poor response to treatment, therapy was changed to ESTRACYT.

After six months of *secondary* ESTRACYT therapy, the tumor proved to be resistant to ESTRACYT as well. It is demonstrated by the DNA distribution beyond the 19c range. The cytophotogram shows a pronounced aneuploidy.

This finding was confirmed *clinically* by a *breakthrough of the tumor* into the urinary bladder which was detected for the first time then, although no bone metastases were found yet at this point. Therefore therapy was changed to *cyclophosphamide*, the third form of treatment. Despite this change in therapy the patient died from wide-spread metastases after eight months of treatment with cyclophosphamide

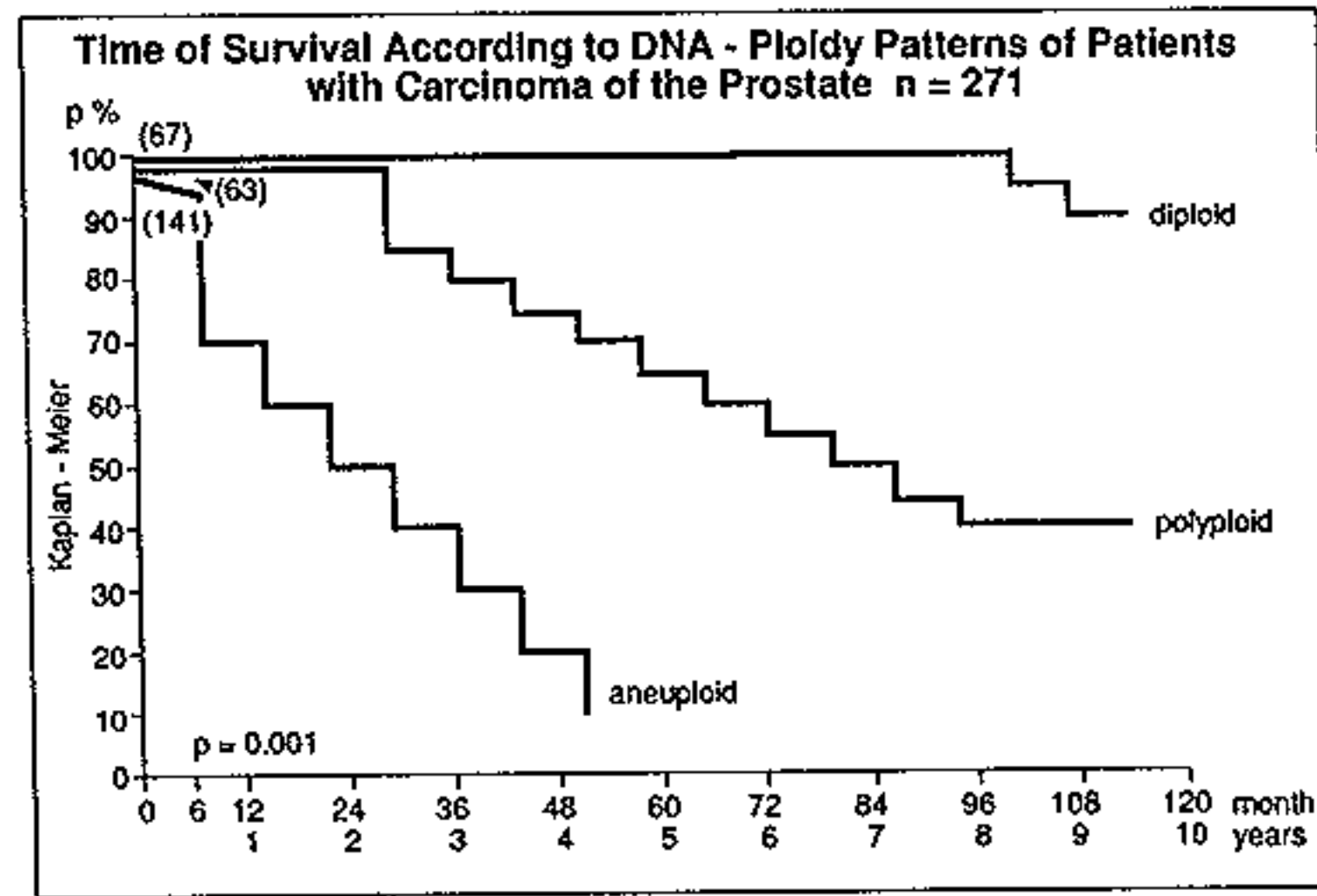


Fig. 12. Survival time of patients with carcinoma of the prostate, grouped according to DNA ploidy.

le-cell cytophotometry and flow-through cytophotometry show differing results and report high DNA values particularly for poorly differentiated tumors (1-3,6,11,22,23,28).

This investigation established the rate of DNA aneuploidy for highly differentiated tumors at 15% and for poorly differentiated tumors at 71%.

Single-cell cytophotometry is the only method permitting exact DNA analyses because it measures single tumor cells which have previously been identified as carcinomatous. Single-cell photometry is a time-consuming procedure because the technician has to place each cell in the measuring instrument. The time disadvantage is outweighed by the advantage afforded by the fact that it is possible to carry out qualitative cell morphology and quantitative measurement of nuclear DNA in the same material and to match the results. The cells can be fixed on the slide so that their position remains unaltered and the measurement can be repeated at any time (1-5,7,29).

In flow-through cytophotometry the DNA is measured in suspended cells, which are directed along the microscope's optical path. The continuous measuring of cells in the suspension requires rapid measuring. However, it is impossible to assign the DNA content measured to the morphology of the cell examined. During flow-through cytophotometry, all fluorescing particles present in the sample - including damaged nuclei, incompletely separated nuclei and



fluorescing impurities - are measured and recorded in the DNA distribution (1-5,7,29).

The results of the DNA analyses of cell nuclei reported in this study and the findings of other authors have shown that the measuring of the DNA content of cells forms a reliable basis for a quantitative evaluation of on the success or failure of the therapy in question.

A shift towards euploidy or an increase in the hypodiploid DNA distribution and a decrease in the aneuploid DNA values seen prior to therapy indicate a therapy-induced remission of the tumor. If the DNA distribution pattern remains aneuploid during therapy, it is a sign of tumor progression (11,14,22,23,28).

The prognosis tends to be more negative when cytophotometry shows widely scattered DNA values. The relation of diploid and aneuploid cells is of similar significance (1-5,7,11,14,22,34). This relation is especially relevant in respect of G-II (grade of malignancy) carcinomas which are morphologically homogeneous but have highly varying malignant potentials.

If the rate of diploid cells (G0/G1 phase) is >60%, the prognosis for the patient is more favorable than with a rate of < 60 %. In such a case, a high rate of cells in the G0/G1 phase can be taken to signal a low growth rate of the tumor.

The same differences in respect of survival periods can be seen in the groups with G-II and G-III carcinoma showing a decrease in diploid cells and an increase in proliferating aneuploid cells.

The single-cell cytophotometric analyses of 271 patients in this study enabled the authors to draw valid clinical and prognostic conclusions on the basis of the rates obtained for the ploidy, the DNA frequency peaks and the phases of the cell cycle.

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