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Stereological estimates of nuclear volume in squamous cell carcinoma of the uterine cervix and its precursors

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Summary. Using modern stereology, this study was carried out to obtain base-line data concerning three-dimensional, mean nuclear size in precancerous and invasive lesions of the uterine cervix. Unbiased estimates of the volume-weighted mean nuclear volume (nuclear $\bar{v}_V$) were obtained by point-sampling of nuclear intercepts in 51 pre-treatment biopsies from patients with invasive squamous cell carcinomas (SCC). Vertical sections from 27 specimens with cervical intraepithelial neoplasia (CIN) grades I through III were also investigated, along with 10 CIN III associated with microinvasion (CIN III+M). On average, nuclear $\bar{v}_V$ was larger in SCC than in CIN III and CIN III+M together ($2\ P = 8.9 \cdot 10^{-5}$). A conspicuous overlap of nuclear $\bar{v}_V$ existed between all investigated lesional groups. The reproducibility of estimates of nuclear $\bar{v}_V$ in biopsies with SCC was acceptable ($r = 0.85$ and $r = 0.84$ in intra- and inter-observer studies, respectively). The efficiency of the sampling scheme was high, with more than 60% and more than 80% of the total observed variance contributed by differences between individual lesions with CIN and SCC, respectively. Estimates of nuclear $\bar{v}_V$ based on sampling within the whole epithelial thickness and on sampling in the lower one-third in CIN I and the lower two-thirds in CIN II lesions were of the same magnitude. Approximate estimates of the absolute variation of nuclear $\bar{v}_V$ were directly proportional to individual estimates of nuclear $\bar{v}_V$, whereas the relative variation of nuclear $\bar{v}_V$ tended to decrease with increasing mean nuclear volume. Based on the rather small number of cases investigated, estimates of nuclear $\bar{v}_V$ are unable to distinguish between different grades of CIN. However, the estimation of nuclear $\bar{v}_V$ is well-suited for the purposes of objective grading of malignancy in SCC.

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Introduction

The development of invasive squamous cell carcinoma of the uterine cervix (SCC) is generally considered to take place during a multistep process involving precursor lesions of increasing severity in the mucosal epithelium. The malignant potential at the cellular level may be subdivided and subjectively assessed according to the WHO Classification (Poulsen and Taylor 1975). However, the developmental process from subtle premalignant, dysplastic changes, through carcinoma in situ, to invasive cancer may merely represent a progressive continuum (Campion et al. 1986; Koss 1978; McIndoe et al. 1984; Richart and Barron 1969). A simplified concept of grading precancerous lesions of the uterine cervix into three stages of cervical intraepithelial neoplasia (CIN) has therefore been proposed (Richart 1973; Buckley et al. 1982). Still, the cytological and histological diagnosis and grading of CIN relies on subjective and qualitative examination. This approach suffers from a disturbing and serious lack of reproducibility in histopathological interpretation (Bellina et al. 1982; Cocker et al. 1968; Holmquist et al. 1967; Ismail et al. 1990; Ringsted et al. 1978). In fact, pathologists agree best at morphological extremes and a reduced, two-tiered grading system of CIN may be the only way to improve diagnostic consistency (Ismail et al. 1990; Robertson et al. 1989). There seems to be an obvious need for supplementary techniques in the morphological evaluation of CIN. Uniform reporting of standardized criteria is necessary for the valid selection of optimal treatment and for comparative trials of treatment of CIN and SCC. These requirements

may be satisfied using objective and reproducible, quantitative methods.

Nuclear anaplasia is one of the main features of neoplastic dedifferentiation (Black and Speer 1957; Meyer-Arendt and Humphreys 1972), and quantitative estimation of nuclear size may provide valuable information of guidance in therapy and prognostic evaluation. Morphometrical estimates of nuclear mean profile areas represent the usual, technical approach in this regard. However, such two-dimensional estimates are critically dependent on nuclear shape, and even minor variability in section orientation may be of significance when dealing with CIN lesions. Modern stereology copes with this problem by the combination of two very convenient techniques that are easy to employ under routine laboratory conditions. First, unbiased estimates of the volume-weighted mean nuclear volume (nuclear $\bar{v}_V$) are independent of shape and provide realistic information concerning three-dimensional nuclear size (Gundersen and Jensen 1985). Secondly, when combined with sampling on so-called vertical sections, according to Baddeley et al. (1986), the problems of nuclear orientation are easily solved (Gundersen et al. 1988b; Sørensen 1991). The aim of the present study was to provide base-line data of this unbiased estimator of three-dimensional, nuclear mean volume in CIN lesions of various severity and in SCC. Technical aspects are presented, and the efficiency and reproducibility of the stereological method are considered with regard to the use in the prognostic evaluation of SCC.

Materials and methods

The files of the University Institute of Pathology were searched for histological biopsies showing CIN, taken from patients in the period 1982–1988. Diagnostic biopsies with CIN I and CIN II were retrieved from 7 and 10 patients, respectively, and cone biopsies showing CIN III and CIN III + microinvasion (CIN III + M) were included from 10 patients each. Pre-treatment biopsies from 51 patients, taken in the period 1977–1982, with invasive SCC, were supplied from the Department of Oncology, Aarhus. All specimens had been routinely processed in the same histological laboratory. In the cases of diagnostic and pre-treatment biopsies, only one histological specimen was available, and from cone biopsies three representative tissue blocks were selected for the study. One new, 4-μm-thick section was cut from each of the original paraffin-embedded cones, whereas three sections, 25 μm apart, were cut from all other biopsies. These procedures yielded sufficient amounts of tissue for the stereological investigation. Staining was carried out with haematoxylin and eosin (H & E). The histological diagnoses were re-evaluated and confirmed in all cases. Follow-up of patients with SCC was obtained for a preliminary prognostic investigation.

The stereological measurements were carried out using a monocular Olympus BHS microscope with projection attachment and equipped with an 100x oil immersion lens (numerical aperture of 1.4). Fields of vision were projected at a final magnification of about 1850× (calibrated regularly, using a micrometer graticule).

Unbiased estimates of the three-dimensional nuclear $\bar{v}_V$ were obtained according to the method developed by Gundersen and

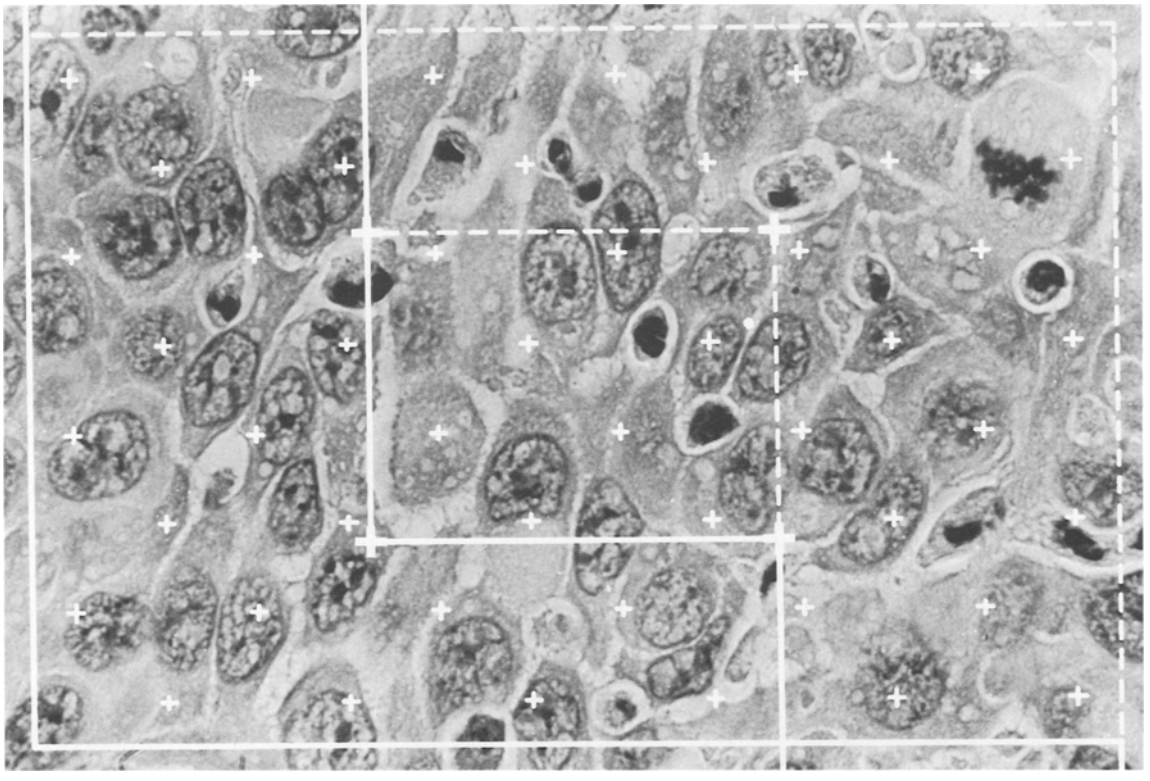

Fig. 1. Field of vision from a squamous cell carcinoma of the uterine cervix, projected onto a test system with points and unbiased sampling frames. The intercept through each point-sampled nuclei is measured from nuclear border to nuclear border in one arbitrary direction, assuming isotropy of the nuclei. The sampling frames are used to obtain two-dimensional estimates (Sørensen et al., to be published). H & E, ×1333

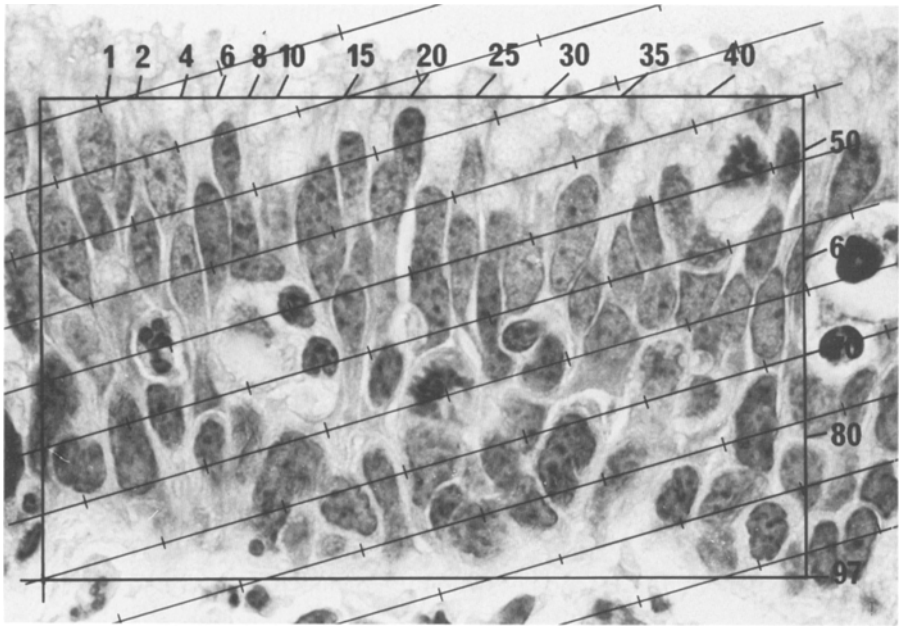

Fig. 2. Field of vision with cervical intraepithelial neoplasia (CIN) III of the uterine cervix, projected onto a test system suited for vertical design. The left-hand edge of the orientation frame is aligned with the vertical axis (i.e. perpendicular to the horizontal plane of reference = the macroscopical mucosal surface). A series of non-equidistant numbers has been marked off on the frame (see Fig. 16 in Gundersen et al. 1988a, and Fig. 2 in Sørensen 1991). A transparent test probe with points associated with orientation lines is superimposed onto the frame according to a random, starting orientation number (here 70). The intercepts are measured in point-sampled nuclei in the direction dictated by the lines in the test system. In the next fields of vision the directions are systematically selected by adding a constant to the starting number. H & E, ×958

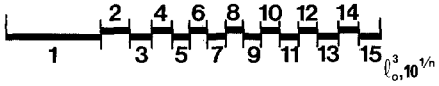

Fig. 3. Classified l_0^3-ruler used for measuring nuclear intercepts, shown magnified 3:5. The widths of the 1st and 15th classes have a ratio of 1:10 on a scale of (length)^3. The ruler is not an ordinary l_0^3-ruler, as described by Gundersen and Jensen (1985) and Nielsen et al. (1986). Using a log-transformed scale, the width of any class is approximately 17% larger than the preceding class (if it exists!). This modification eliminates the unnecessary bias of the ordinary l_0^3-ruler, as pointed out by Cruz-Orive and Hunziker (1986). For detailed explanation of the l_0^3-ruler, see Sørensen (1991)

Jensen (1985). This technique is based on point-sampling of nuclear test lines, and thus requires isotropy either of the nuclei to be measured or of the directions of sampled test lines. In the case of SCC, directional isotropy of nuclear orientation is assumed globally fulfilled. A simple test system with points was employed (Fig. 1), and intercepts through the point-sampled nuclei were measured in one arbitrary direction. In contrast, nuclei of CIN lesions show directional anisotropy, because the nuclei show a preferential pattern of orientation. However, by defining the surface of the epithelium as "the horizontal plane of reference" the biopsies with CIN lesions can be regarded as so-called vertical sections, cut perpendicular to the horizontal plane of reference (Baddeley et al. 1986; Gundersen et al. 1988a), even when the biopsies are studied in retrospect. By generating test lines of isotropical directions in three-dimensional space, nuclear intercepts can be measured along these test lines. This procedure is very conveniently accomplished by the test system shown in Fig. 2. The nuclear intercepts were measured from nuclear border to nuclear border, through the sampling point, by a modified l_0^3-ruler (Fig. 3). Multiplication of the averaged, cubed mean intercpet length, $\overline{l_0^3}$, by $\pi/3$, gives an estimate of nuclear $\bar{v}_V$.

Estimates of nuclear $\bar{v}_V$ are independent of nuclear shape, and larger nuclei are emphasized due to the point-sampling, because the chance that a point hits a nucleus is directly proportional to nuclear volume (Gundersen and Jensen 1983, 1985). Finally, nuclear $\bar{v}_V$ provides information of both nuclear three-dimensional size and variability of nuclear size, since $\bar{v}_V = \bar{v}_N \cdot (1 + CV_N^2(v))$, where N denotes the ordinary ("unweighted") number distribution of volume (Gundersen and Jensen 1985; Sørensen 1991; Sørensen and Erlandsen 1990). Estimates of nuclear $\bar{v}_V$ will thus increase as a function of the relative variability of nuclear volume, even for a fixed mean nuclear volume. Calculated examples of the estimation of nuclear $\bar{v}_V$ have been published elsewhere (Sørensen 1991).

Fifteen fields of vision (five from each of the three histological specimens of individual cases) were investigated in each lesion. In SCC the fields of vision were chosen systematically within the whole tumour, by adjusting the distance between different fields of vision approximately proportional to overall tumour sectional area. In CIN lesions the fields of vision were investigated after alignment of the test system to the vertical axis (Fig. 2), and the complete thickness of the epithelial layer was included for nuclear sampling. However, biopsies with CIN I and II were measured twice, the second measurements taking only the basal one-third and the basal two-thirds thickness of the epithelium into account, respectively. The microinvasive areas of CIN III + M lesions were not investigated. All specimens were examined by a laboratory technician (observer A), and the analysis of one case took about 10–15 min. In one-third of the investigated cases of SCC, the stereological measurements were carried out twice by observer A 4 months apart to study the intra-observer variability of nuclear $\bar{v}_V$. In addition, one of the authors (observer B) investigated the same set of 16 cases for the purpose of studying inter-observer variability.

The variation associated with estimates of nuclear $\bar{v}_V$ may arbitrarily be considered contributed by the three levels in the sampling hierarchy: (1) variation among nuclear intercepts and their classification; (2) variation among different fields of vision; and (3) variation among biopsies from different patients, that is, biological variation. The contribution by each source to the total observed variance was investigated by the method of nested analysis of variance, as illustrated in a stereological context by Gundersen and Østerby (1981). Variability within groups is reported either as absolute variances or as coefficients of variation, CV = SD/mean. Due to the skewed distribution of nuclear $\bar{v}_V$, comparisons of grouped means were carried out between geometric means after log-transformation, using unpaired Student's t-test, modified according to Welch (1949) in the case of inhomogeneity of variance. Least-square linear regression analysis was employed for analysing the relationship between the different sets of stereological measurements in SCC,

between estimates of nuclear $\bar{v}_V$ in CIN I and CIN II lesions based upon the whole and the restricted epithelial thickness, and finally, between estimates of nuclear $\bar{v}_V$ in SCC lesions and their associated variance and CV. Due to the log-scaled data, the location of the regression lines in the reproducibility studies were analysed using paired Student's *t*-tests, because intersections with fixed ordinates are nonsensical after this data transformation. Furthermore, the intra- and inter-observer reproducibility was analysed by comparing the residual variance (unexplained variation of the locations of points around the regression lines) with the variance to be expected due to the known precision of the estimates. For the latter purpose, the squared standard error of the mean ($SEM = SD/\sqrt{n}$) of individual estimates of nuclear $\bar{v}_V$ in the whole group of SCC lesions (based on the variation among fields of vision) was plotted against individual mean nuclear $\bar{v}_V$ (log-log plot), and a straight line was encountered (the slope = 2.13 μm^3 was not significantly different from 2, $2P = 0.46$, $r = 0.86$, $2P = 7.0 \cdot 10^{-16}$). Finally, a preliminary screening of the prognostic value of nuclear $\bar{v}_V$ in cases of SCC was carried out using Kendall's rank correlation. The limit of significance for acceptance was $2P < 0.05$.

Results

The absolute variation and the contribution from each sampling level to total observed variance of estimates of nuclear $\bar{v}_V$ are shown in Table 1. For all five groups of cervical lesions, the largest contribution to total variance is from the highest level of sampling, that is to say, it is attributable to differences in nuclear $\bar{v}_V$ among individual lesions. Indeed, in SCC the variation associated with differences among lesions is by far the largest contributor to overall variance, because in these tumours less than 10% of total variation can be attributed to each of the two lower levels of sampling.

In Fig. 4 estimates of nuclear $\bar{v}_V$ are shown grouped for each of the morphological categories investigated. A considerable overlap is seen between individual groups. This also applies to the interesting distinction between nuclear $\bar{v}_V$ in CIN III and CIN III + M. The geometric mean of nuclear $\bar{v}_V$ in SCC was significantly larger than that of CIN III and CIN III + M together ($2P = 8.9 \cdot 10^{-5}$). The overlap between these groups was also conspicuous.

The relationships between estimates of nuclear $\bar{v}_V$ based on the whole epithelial thickness and the lower one third and two-thirds in CIN I and CIN II lesions, respectively, is shown in Fig. 5. In both groups the slopes of the regression lines were not significantly different

Table 1. The contribution from each level of the sampling hierarchy to total observed variation associated with the estimation of nuclear $\bar{v}_V$ in precancerous lesions (CIN) and invasive squamous cell carcinomas (SCC) of the uterine cervix, calculated by the method of nested analysis of variance[a]

Lesional type	Nuclear intercepts and their measurement	Fields of vision	Among lesions
CIN I[b]			
Absolute variation	52810	15940	4550
(mean no. per lesion)	(71)	(15)	(7)
Relative contribution to total variance	16.3%	7.3%	76.4%
CIN II[b]			
Absolute variation	141300	67110	13460
(mean no. per lesion)	(98)	(15)	(10)
Relative contribution to total variance	10.7%	22.5%	66.8%
CIN III			
Absolute variation	60590	27370	5050
(mean no. per lesion)	(127)	(15)	(10)
Relative contribution to total variance	9.5%	26.7%	63.8%
CIN III + microinvasion			
Absolute variation	130200	44230	8120
(mean no. per lesion)	(135)	(15)	(10)
Relative contribution to total variance	11.9%	24.4%	63.7%
SCC			
Absolute variation	224800	53300	20850
(mean no. per lesion)	(145)	(15)	(51)
Relative contribution to total variance	7.5%	9.6%	82.9%

[a] The relative contribution to variance is estimated by regarding the observed variance at each level of the sampling hierarchy as the sum of the true variance at that level plus the variance of the mean, $(SEM)^2$, at the lower level of sampling. Large variances are thus diminished in their contribution to total observed variance according to the number of observations at that particular level of sampling (Gundersen and Østerby 1981)

[b] Nuclear intercepts obtained from the lower one-third and two-thirds of the epithelial height in CIN I and CIN II lesions, respectively

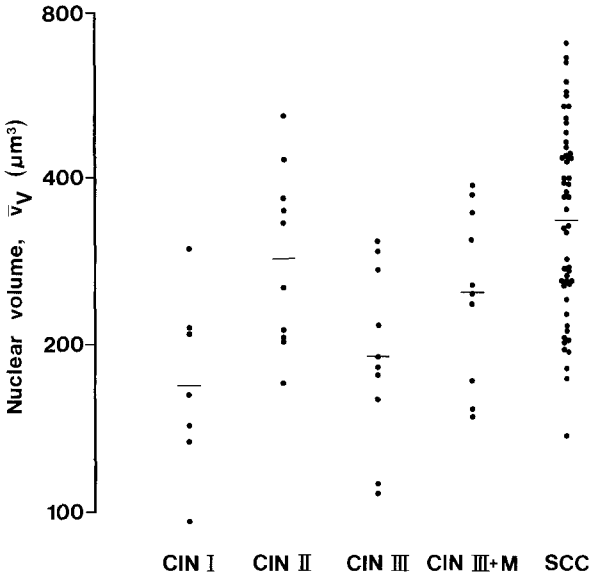

Fig. 4. Estimates of the volume-weighted mean nuclear volume, nulear $\bar{v}_V$ (on a logarithmic ordinate), of the investigated lesional categories of the uterine cervix: *CIN I-III*, cervical intraepithelial neoplasia, severity grades I, II, and III; *CIN III+M*, cervical intraepithelial neoplasia grade III, with associated focal microinvasion of less than 5 mm depth; *SCC*, squamous cell carcinoma

from unity ($2P>0.50$), and the correlation coefficients were $r=0.73$ and $r=0.90$ in CIN I and CIN II lesions, respectively.

The results from the observer variability studies carried out on SCC lesions are shown in Fig. 6. Both the intra- and the inter-observer reproducibility studies showed regression lines with slopes not significantly different from unity ($2P>0.16$). The intra-observer study showed a correlation coefficient of 0.85, whereas the inter-observer study yielded a correlation coefficient of 0.84. Using paired test design, no difference of the geometric means was found in the intra-observer study ($2P=0.33$). The regression line was not significantly different from the ideal one. The intra-observer correlation was acceptable, because the SEM from the correlation and the theoretical SEM were in the same order of magnitude (93 μm^3 and 62 μm^3, respectively). A significant difference was disclosed between the geometric means in the interobserver study ($2P=0.03$, paired design). On average, observer B showed an overall tendency to underestimate nuclear $\bar{v}_V$ by about 11%. However, the inter-observer reproducibility was still robust, showing an SEM of 114 μm^3 from the correlation, whereas the theoretical SEM was 60 μm^3. Thus the unexplained variation was about 35% larger than the theoretical one, when one observer estimates nuclear $\bar{v}_V$ in the same invasive tumours 4 months apart, and about 70% larger than the theoretical one, when two different observers measure the same SCC lesions. Using the theoretical values of SEM, the theoretically best obtainable coefficients of correlation were estimated at $r=0.93$ and $r=0.95$ in the intra- and interobserver reproducibility studies, respectively, compared with the observed values of 0.85 and 0.84, respectively.

The relationship between estimates of nuclear $\bar{v}_V$ in SCC lesions and approximate estimates of the variability of nuclear $\bar{v}_V$ is shown in Fig. 7a. Using the variation at the lowest level of sampling as an estimate of nuclear size variability, the variation was shown to be directly proportional to individual estimates of nuclear $\bar{v}_V$ ($r=0.88$, $2P=4.0\cdot10^{-17}$); the slope = 1.82 μm^3 was not significantly different from 2 ($2P=0.26$), as would be expected if the variation of nuclear $\bar{v}_V$ is only a scaling parameter of nuclear $\bar{v}_V$). Although only marginally significant, the relative variation of nuclear $\bar{v}_V$, expressed by the CV (Fig. 7b), interestingly showed an inverse relationship to nuclear $\bar{v}_V$ ($r=-0.26$, $2P=0.06$). Nuclear populations with larger estimates of nuclear $\bar{v}_V$ thus showed a tendency to diminished relative variability of nuclear volume. However, these estimates of the variability of nuclear $\bar{v}_V$ are only approximations, because estimation of the volume-weighted second moment of nuclear volume is necessary to assess the true variability associated with estimates of nuclear $\bar{v}_V$, as shown elsewhere (Gundersen and Jensen 1985; Sørensen 1991).

The screening for prognostic impact of nuclear $\bar{v}_V$ in SCC lesions is outlined in Fig. 8. No obvious correlation between estimates of nuclear $\bar{v}_V$ and survival time is noticed (Kendall's $\tau=0.11$, $2P=0.41$).

Discussion

Quantification of changes in nuclear size during malignant dedifferentiation of the mucosal lining of the uter-

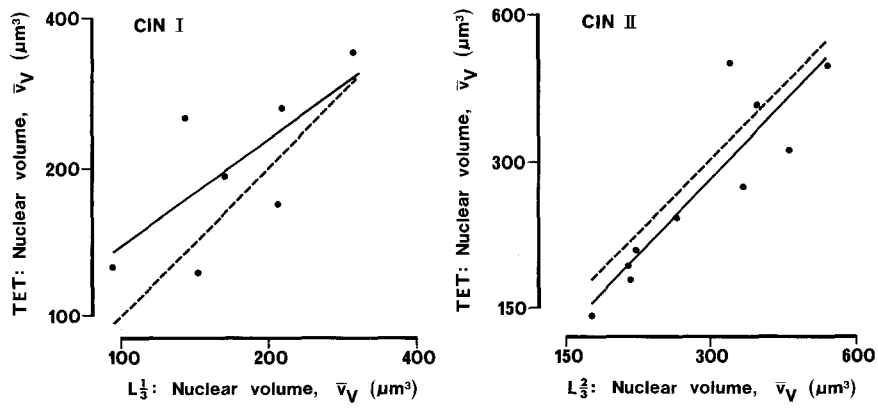

Fig. 5. Regression of estimates of nuclear $\bar{v}_V$ (log-log scaled), based on nuclear sampling in the whole epithelial thickness (*TET*), against the lower one-third (*L1/3*) and lower two-thirds (*L2/3*) in cervical intraepithelial neoplasia grade I and II, respectively. In CIN I, the correlation is poor ($r=0.73$, $2P=0.06$; the slope is not significantly different from unity, $2P=0.50$). A significant correlation exists in CIN II ($r=0.90$, $2P=4.6\cdot10^{-4}$; the slope is not significantly different from unity, $2P=0.91$)

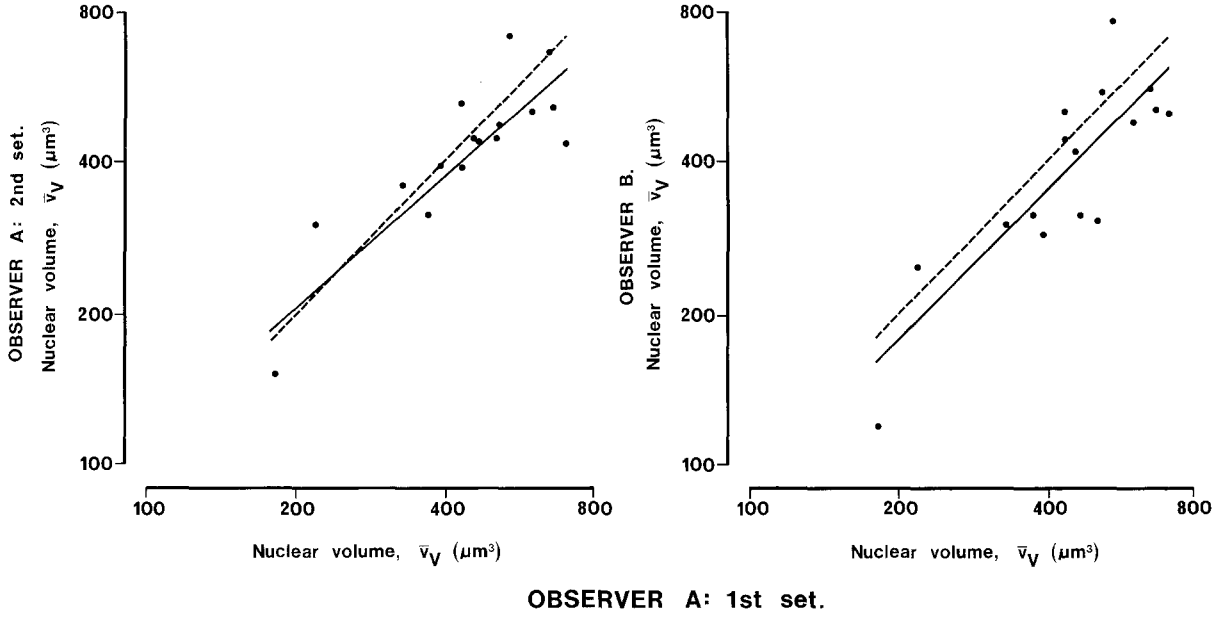

Fig. 6. Regression of estimates of nuclear $\bar{v}_V$ (log-log scaled) in biopsies with invasive squamous cell carcinoma of the uterine cervix, as obtained by observer A, against estimates of nuclear $\bar{v}_V$ obtained 4 months later by the same observer (*left*), and against estimates of nuclear $\bar{v}_V$ obtained by observer B (*right*) in the same series of biopsies. A significant correlation exists in both analyses with regression lines not significantly different from unity, $2P > 0.16$ (intra-observer study: $r = 0.85$, $2P = 3.4 \cdot 10^{-5}$; inter-observer study: $r = 0.84$, $2P = 4.0 \cdot 10^{-5}$)

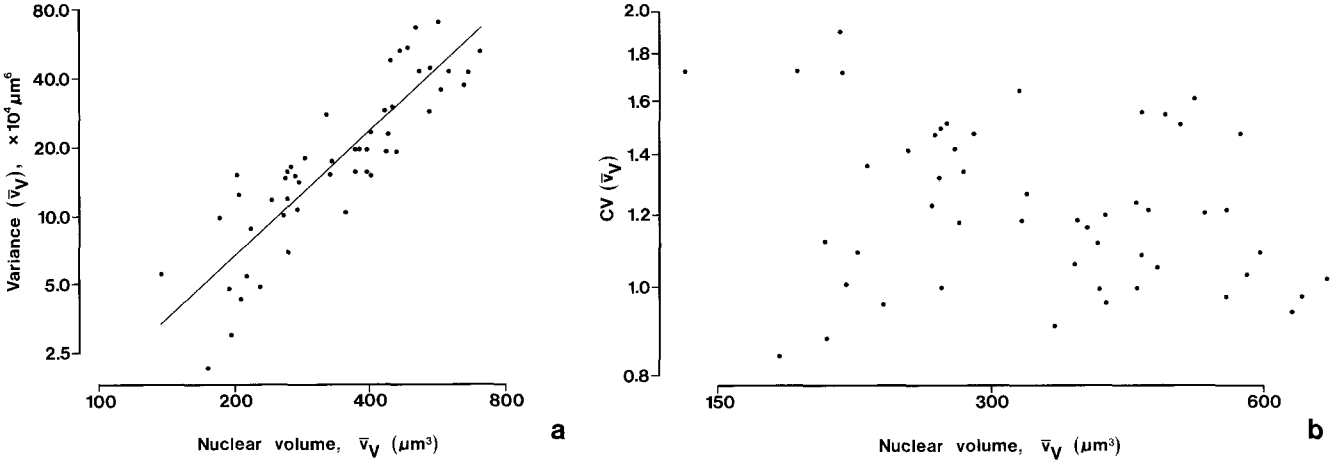

Fig. 7. a Regression (log-log-transformed) of the observed variation at the lowest level of sampling of estimates of nuclear $\bar{v}_V$ against estimates of mean nuclear $\bar{v}_V$ in 51 biopsies with squamous cell carcinomas from the uterine cervix ($r = 0.88$, $2P = 4.0 \cdot 10^{-17}$). **b** Scatterplot (log-log-transformed) of the relative variation of nuclear $\bar{v}_V$, as expressed by CV = SD/mean, at the lowest level of the sampling hierarchy, against estimates of nuclear $\bar{v}_V$ in the same biopsies ($r = 0.26$, $2P = 0.06$)

ine cervix is not a new discipline. Using estimates of the average nuclear diameter, Strodtbeck (1937) was unable to distinguish between SCC and normal cervical epithelium, but the distribution of nuclear sizes was wider and skewed to higher values in SCC. Cramer (1953) and Pedersen (1955), who made similar one-dimensional measurements, also found such distributions of nuclear sizes in cancerous lesions. Two-dimensional estimates of nuclear mean profile area, $\bar{a}_H$(nuc), were obtained by Foraker and Reagan (1956) in histological specimens with metaplasia, atypical hyperplasia, and carcinoma in situ (CIS). They demonstrated a great overlap between the lesional types. The nuclear size distributions, based on both one- and two-dimensional measurements, have also been demonstrated to be identical in epithelium diagnosed to be normal, metaplastic, dysplastic, and showing a CIS pattern (Reagan and Hamonic 1956). However, Wiernik et al. (1973) later showed differences in estimates of $\bar{a}_H$(nuc) and in the distribution of $\bar{a}_H$(nuc) in normal epithelium of the uterine cervix and in SCC. Detailed morphometrical investigations of cytological specimens have shown that estimates of projected nuclear areas were largest in dysplasia, followed by CIS and SCC (Reagan et al. 1957). In

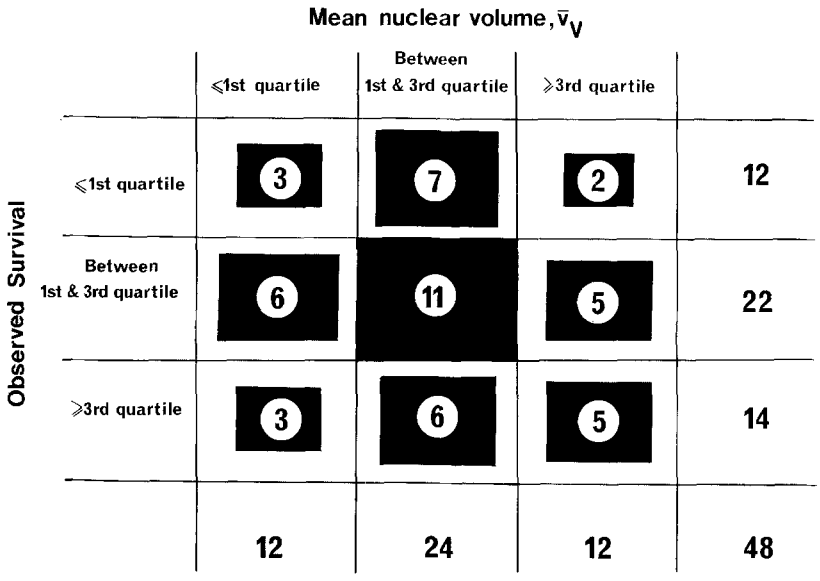

Fig. 8. The observed survival time (>5 years of follow-up) of 48 patients with squamous cell carcinomas of the uterine cervix is compared to the nuclear $\bar{v}_V$, obtained in pre-treatment biopsies (both variables are grouped on their quartiles). Three patients of the original group of 51 cases have been excluded due to deaths from other causes. No correlation exists between the two parameters (Kendall's $\tau = 0.11$, $2P = 0.41$)

the recent literature, one study has shown a progressive increase in estimates of $\bar{a}_H$(nuc) from normal cervical epithelium over dysplasia to CIS and CIS+M, with a slight fall in $\bar{a}_H$(nuc) in SCC (Mariuzzi et al. 1989). Consequently, there seems to be some confusion in the literature as to the magnitude of two-dimensional estimates of $\bar{a}_H$(nuc) in various lesions of the uterine mucosa. This also applies to studies of nuclear area in lesions classified according to the severity of CIN. Measurements in the same strata of the epithelial lining thus differ considerably in their magnitude (Boon and Kok 1985; Tosi et al. 1988). Moreover, the largest estimates of $\bar{a}_H$(nuc) are found in CIN III in one study (Tosi et al. 1988), and in CIN I in another (Boon and Kok 1985).

One of the explanations for such discrepancies is that two-dimensional estimates of $\bar{a}_H$(nuc) are intimately dependent on nuclear shape and orientation. Thus, for precursor lesions of the uterine cervix, even minor departures from any standardized sectioning introduce bias and hamper the comparability. This is just one of the problems associated with nuclear sampling by sectional planes. Another, maybe even more serious limitation is that the relationship between estimates of $\bar{a}_H$(nuc) and the real nuclear volume is always unknown. The recent advances in modern stereology provide, however, an elegant solution to these problems. Estimates of nuclear $\bar{v}_V$ are independent of shape and yield realistic information on three-dimensional nuclear size. The key to obtaining such unbiased estimates is to use point-sampling; that is, to sample nuclei in a manner directly proportional to nuclear volume (Gundersen and Jensen 1983, 1985). All dimensions are utilized by this sampling design, and problems concerning nuclear shape are eliminated. One only has to think of nuclear volume in a distribution of volume, instead of number (see Fig. 8 in Sørensen and Erlandsen 1990). Estimates of nuclear $\bar{a}_H$(nuc) are actually weighted proportionally to nuclear height perpendicular to the sectional plane. This is a rather awkward sampling probe, when one wants to obtain a three-dimensional impression of nuclear changes in tissue specimens.

After the introduction of the "vertical sections" design (Baddeley et al. 1986), problems of preferential nuclear orientation are easily dealth with. The vertical axis can be made to the wish of the individual investigator, and does not necessarily need an internal horizontal plane of reference. As long as the vertical axis is identifiable, the simple generation of test lines with isotropic direction of orientation ensures the unbiasedness of the estimator (Sørensen 1991). Using modern stereological techniques, the present investigation clearly demonstrates the large overlap between unbiased estimates of nuclear $\bar{v}_V$ among CIN lesions. Although based on a limited number of cases, estimates of nuclear $\bar{v}_V$ are of the same magnitude when based either on measurements in selected strata of the premalignant epithelium or on the whole epithelial thickness. No stepwise increase in nuclear size from CIN I to SCC is encountered by entering the third dimension of nuclear volume. This may be difficult to understand at first glance. The retrospective study design is, however, liable to a number of uncontrollable factors that may introduce bias, and the comparisons of individual lesional groups must be seen in this light. For instance, one needs to assume that the tissue processing has the same impact on the nuclear volume; that is that the volumetric changes occur in the same direction and to the same extent, irrespective the severity of the lesions. In addition, the assumption of directional isotropy of nuclear orientation in SCC has to be considered. A low level of anisotropy in SCC, inherited from the highly orientated CIN precursor lesions, cannot be ignored with certainty. However, in the case of studying SCC in retrospect, "true" vertical sections are hard to obtain.

The great overlap of nuclear volume between different CIN types does not correlate with DNA studies of such precursor lesions. Although opposed by some investigators (Hughes et al. 1987), most agree that the

DNA content, as expressed by the DNA index, and the incidence of aneuploidy, increases continuously with rising severity of CIN (Barres et al. 1985; Dudzinski et al. 1987; Jakobsen et al. 1983; Wilbanks et al. 1967). However, the correlation between three-dimensional estimates of nuclear $\bar{v}_V$ and DNA indices have been shown to be rather poor in other malignancies (Nielsen et al. 1989b; Sørensen et al. 1990, 1991), and exceedingly poor in invasive SCC (Sørensen et al., to be published). Nuclear volume may thus express a wide range of biological constituents and events in the nucleus, and is not simply a physical reflection of DNA content.

The failure of nuclear $\bar{v}_V$ to distinguish between CIN III and CIN III+M is rather unfortunate, because no guidance for the pathologist in diagnosing inconspicuous microinvasion is provided by the technique. It seems more appropriate to use immunohistological demonstration of basement membrane components like laminin for the diagnosis of microinvasion in puzzling cases (Richards and Furness 1990).

Estimates of nuclear $\bar{v}_V$ are obtained with high efficiency, using the presented, simple sampling scheme. More than 80% of the total variance associated with estimates of nuclear $\bar{v}_V$ is contributed by biological differences between individual SCC lesions. The intra- and inter-observer reproducibility of nuclear $\bar{v}_V$ in SCC are quite acceptable, when compared with the theoretically best obtainable repeatability. Moreover, there is no need to invest more effort in improving the reproducibility and the precision of individual estimates of nuclear $\bar{v}_V$. The great biological diversity among lesions and the wide range of prognostic outcome once again stress that this extra effort will be too laborious and expensive. Other studies agree on the high reproducibility and efficiency of nuclear $\bar{v}_V$ (Nielsen et al. 1989a; Sørensen and Ottosen 1991).

Like in bladder cancer (Nielsen et al. 1986, 1989c) and malignant melanomas (Sørensen 1989; Sørensen et al. 1991), preliminary findings in retrospective series of patients suggest an independent prognostic value of estimates of nuclear $\bar{v}_V$ in SCC (Sørensen et al., to be published). However, in this study the worst prognosis is carried by small estimates of nuclear $\bar{v}_V$, in contrast to the other malignancies mentioned above. The sensitivity of nuclear $\bar{v}_V$ as an indicator of prognosis may be due to the combined information of nuclear size and variability of nuclear size (Sørensen 1991). In the present study, the variation of estimates of nuclear volume is a linear function of nuclear $\bar{v}_V$. However, the relative variation of nuclear volume, as expressed approximately by the coefficient of variation, tends to diminish for larger estimates of nuclear $\bar{v}_V$. Thus, small nuclei may show larger relative size variation, which may indicate a poorer prognosis. However, the chosen estimates of nuclear size variability are only approximations (Sørensen 1991).

In conclusion, unbiased estimates of nuclear $\bar{v}_V$ show great overlap between lesions with different morphological severity of cervical intraepithelial neoplasia. Based on the limited number of cases investigated, the estimator cannot be used as a guide for the classification of CIN or as an indicator of occult microinvasion. However, estimates of nuclear $\bar{v}_V$ are reproducible and are obtained with high efficiency, and are thus appropriate for the use in objective, prognosis-related grading of malignancy in SCC. Indeed, estimates of nuclear $\bar{v}_V$ have already shown independent, prognostic value in a retrospective series of patients with SCC of the uterine cervix (Sørensen et al., to be published).

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