Aus dem Institut für Cytopathologie der Heinrich-Heine Universität Düsseldorf

Direktor: Universitätsprofessor Dr. med. A. Böcking

IDENTIFICATION OF PROGRESSIVE CERVICAL SQUAMOUS INTRAEPITHELIAL LESIONS USING DNA-IMAGE-CYTOMETRY

A STUDY ON DIAGNOSTIC VALIDITY AND RELIABILITY

Dissertation

zur Erlangung des Grades eines Doktors der Medizin

Der Medizinischen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Vu Quoc Huy NGUYEN

2003

Als Inauguraldissertation

gedruckt mit Genehmigung der Medizinischen Fakultät

der Heinrich-Heine-Universität Düsseldorf

gez.: **Univ.-Prof. Dr. med. Dr. phil. Alfons Labisch, M.A.**

Dekan

Referent: **Univ.-Prof. Dr. med. A. Böcking**

Korreferent: **Priv.-Doz. Dr. med. V. Küppers**

My wife, Phương Anh

My children, Phương Thảo and Quốc Bảo

TABLE OF CONTENTS

1. INTRODUCTION

1.1. Epidemiology of cervical cancer and its precursors

1.1.1. Incidence and prevalence of cervical cancer ad its precursors

Cervical cancer is one of the most frequent cancer with 470,000 new cases occurring among women worldwide each year, the vast majority of them in developing countries. Of the 230,000 women who die of cervical cancer annually, some 80 percent are from developing countries, where cervical cancer is the most common cause of cancer deaths among women. Cervical cancer screening is a cost-effective way to save lives. A 1993 World Bank study found that screening women every five years with standard follow-up for identified cases costs about $100 per disability-adjusted life year (DALY) gained, compared with about $2,600 per DALY for treatment of invasive cancer and palliative care [PATH 2000a].

Table 1. Incidence of cervical cancer worldwide. Source: GLOBOCAN 2000. Cancer Incidence, Mortality and Prevalence Worldwide, Version 1.0. IARC Cancer Base No. 5. Lyon, IARC Press, 2001.

Nr.	Regions	Cases	Deaths
1	World	470,606	233,372
2	More developed countries	91,451	39,250
3	Less developed countries	370,153	194,025
4	Africa	67,076	35,220
5	Caribbean	6,670	3,143
6	America	85,466	34,497
7	Asia	245,669	131,544
8	Europe	64,929	28,560
9	Australia and Oceans	2,110	989

Cervical cancer precursors are divided into different grades from mild, moderate to severe dysplasia and carcinoma in situ. Other terminology used for these precursors is Cervical Intraepithelial Neoplasias [Richart 1973] or more recently Squamous Intraepithelial Lesions [Kurman and Solomon 1994, Solomon et al. 2002]. They predominantly occur in women within their reproductive years, with large population impact and risk factors characteristics of a sexually transmitted disease. In United States, the most comprehensive survey was carried out by the College of American Pathologists, which compiles rates of cytological abnormalities diagnosis from more than 300 U.S. cytology laboratories. According to this survey, in 1997, 1.97% of all Pap smears were reported as LSIL and 0.5% as HSIL [Jones and Novis 2000]. Another similar survey performed in 1993 reported rates of 1.83% and 0.45% for cytological diagnoses of LSIL and HSIL, respectively [Jones et al. 1993]. The term Atypical Squamous Cell of Undetermined Significance was introduced by the Bethesda system to cover the broad spectrum separating morphologically normal or benign changes of epithelia from definite squamous intraepithelial lesions. Rate of ASCUS diagnoses has been estimated between 2.8-5.0% [Stoler 2000, Jones et al. 1987].

Prevalence of cytological abnormalities was found to be age-related. In a study carried out at U.S. Planned Parenthood clinics, the prevalence of cytologically diagnosed CIN I and II peaked at 2.6% in women 25-29 years of age and decreased to 0.9% in women over the age of 50 years. The peak prevalence of CIN III was 0.5%, which occurred in cytological smears from women 35-39 years old [Sadeghi et al. 1988].

1.1.2. Causal factors of cervical cancer and its precursors

Epidemiological studies have identified a number of potential risk factors for the development of both cervical cancer and its precursors lesions. The most important factor was infection with a variety of sexually transmitted diseases, especially Human Papillomavirus infection. Other risk factors included early age of the first sexual intercourse, age at first pregnancy, number of sexual partners, history of cigarette smoking, oral contraceptive use, low socioeconomic class and parity [Schiffman et al. 1995]. Since the late 1970s, zur Hausen suggested that there might be an association between HPV and cervical cancer [zur Hausen 1977]. A large number of epidemiological,

clinicopathological and molecular studies have subsequently linked the presence of specific types of HPV to the development of anogenital cancer and its precursors. A recent study estimates the worldwide HPV prevalence in cervical carcinomas at 99.7 percent (Walboomers et al., 1999) [Walboomers et al. 1999]. Nowadays it is widely accepted that HPVs play the critical role in the pathogenesis of most cervical cancers and their precursor lesions. More than 100 types of HPVs have been identified, high oncogenic risk types are 16, 18, 33, 45, 56 and 58 [Wright et al. 2002].

1.1.3. Natural history of cervical intraepithelial lesions

Human Papillomavirus can exist throughout most of the anogenital area (including areas not covered by male condoms) and can remain infectious for years [PATH 2000a]. HPV cannot be treated, but infection becomes undetectable in the majority of cases. In some women, however, HPV infection persists and leads to precancerous lesions. Immunocompromised women may be at particularly high risk of persistent infection [Temmerman et al. 1999]. Detectable HPV infection is most common in younger women. Although prevalence varies among regions, it generally reaches a peak of about 20 percent among women aged 20 to 24, with a subsequent decline to approximately 3 percent among women over age 30 [Meijer et al. 1998]. Many women with HPV infection likely will develop mild dysplasia, most of which regresses or does not progress, particularly among women under age 35. Progression to detectable, precancerous lesions can take as long as 10 years. One study estimates that the risk of progression from moderate to severe precancerous lesions is 32 per-cent within 10 years [Holowaty et al. 1999]. Clinical impressions of increasing cervical cancer rates among younger women may reflect a population's age structure or screening patterns rather than a shift in age-specific rates [PATH 2000b]. Some country data suggest, however, that age-specific rates for dysplasia and cervical cancer have shifted downward by about five years, possibly due to increasing sexually transmitted infection and HIV/AIDS rates [McIntosh et al. 2000].

Studies on the natural history of different grades of dysplasia provided a widely varying estimate of the rates of regression and progression. Some results from these studies are summarized in table 2.

Table 2. Progression and regression rates of SILs and CISs

Authors	No. of patients	Follow-up time	Regressed (%)	Persisted (%)	Progressed (%)
Low-grade SIL					
Campion et al. (1986)	100	-	7	67	26
Heinzl et al. (1982)	2417	-	46	44	10
Robertson et al. (1988)	1347	-	57	27	15
CIS					
Koss et al. (1963)	67	6 years	25	61	6
McIndoe et al. (1984)	131	1-28 years	8	69	22
Spriggs (1971)	37	>2 years	40	60	-

The theory that had been widely accepted is that high-grade SILs always develop from low-grad SILs. Richart assumed that high-grade SIL usually begins as a small focus within a low-grade SIL [Richart 1966]. This small focus then gradually expands and replaces the low-grade lesion. According to this theory, the transition from a low-grade SIL to a high-grade SIL represents a monoclonal event within a HPV-infected epithelum.

However, it should be pointed out that several evidences suggest that high-grade SILs may develop as an independent event without progressing from low-grade SILs. Koss (1992) suggests that high-grade SILs develop de novo from epithelium adjacent to low-grade SILs [Koss 1992]. Prospective follow-up studies also provide the evidence that at least some high-grade SILs can develop independently from low-grade lesions. In a study on women visiting a clinic for sexually transmitted diseases, Koutsky et al. (1992) found that most cases of high-grade SILS arose de novo in this population in the absence of a cytologically detectable low-grade SILs [Koutsky et al. 1992].

1.2. Diagnostic validity of methods used in the fight against cervical cancer and its precursors

Cervical exfoliative cytology or Papanicolaou smear is a highly effective screening and diagnostic test in pathology. Its effectiveness has been proved in detecting various pathologic conditions of the female genital tract, especially the preneoplastic lesion of the uterine cervix. A considerable reduction has been observed in incidence of cervical cancer in developed countries, from 18.5 per 100,000 women in 975 to 16.4 per 100,000 in 1980 [Parkin et al. 1998]. Since HPV infections were recognized as a causal factor of cervical dysplasias and invasive cancers [zur Hausen 1987, zur Hausen 1991], different methods have been developed to detect these viruses and to differentiate them into high-risk or low-risk types [Lörinz 1996, Manos et al. 1999].

1.2.1. Diagnostic validity of cytology and histology

Cervical dysplasia represents squamous cells or tissues which are microscopically suspicious for cancer, but without sufficient evidence for its definite assumption. Resulting from weakness of morphologic criteria to early and unequivocally identify malignant transformation in epithelial cells, dysplasias are not a disease entity. The assumption widely accepted is that the higher the grades of dysplasia the higher the probability of progression to cancer [Wright 2002]. However, as a result of insufficient morphological criteria, neither histological nor cytological evaluation can predict if a lesion will progress to cancer in an individual case [Böcking 1998].

These problems partially explain the wide variation of reported sensitivities of cytological screening for cervical cancer from 20% to 83% [Schneider et al. 2000, Renshaw 2002]. The positive predictive value of cytological screening varies from 22% for HSILs (of ASCUSs and LSILs) to 55.7% for invasive carcinoma (of ASCUS and SILs) [Schneider et al. 1996, Johnson and Wahehra 2001]. Within a sample of 2845 cases of mild or moderate dysplasias, the PPV for invasive carcinoma was only 13% [Soost and Baur 1990]. Most PPVs were published without mentioning the intervals of progression from test positivity to histologically proven cancer. As the development from CIN I to CIN III/carcinoma in situ

and invasive cancer may take several years, time must be taken into account if PPVs are given for a specific test.

As diagnoses of cervical dysplasias (or SILs) are not only poorly reproducible but also of limited biological meaning for the individual patient, the number of resulting control procedures is usually high. These range from repeated cytological smears and biopsies to unnecessary operations (conizations). Missed early diagnoses of cancers may also result from cytomorphological uncertainties. This also results in unnecessary costs and avoidable anxiety of the patients.

1.2.2. Diagnostic validity of adjuvant methods

Adjuvant diagnostic methods currently proposed to solve these problems are assays for detection of Human Papillomavirus (HPV) and HPV typing [Munoz et al. 1996, Cuzick et al. 1995] and DNA image cytometry [Böcking 1995b, Böcking and Motherby 1999, Wright et al. 2002]. Proposed applications of HPV testing in cervical cancer prevention programs include (1) where Pap smear screening is the norm, as a triage for women with Pap smear findings of atypical squamous cells of unknown significance; women who test positive for high-risk HPV types would be monitored closely or referred for colposcopy; (2) as a means of surveillance of women after treatment for high-grade lesions or microinvasive cancer; those who test positive for high-risk HPV types would be monitored more closely than those who test negative; and (3) as a primary screening method for high-grade lesions among women aged 30 to 35 or older; those who test positive for high-risk HPV would undergo diagnosis via colposcopy or another visualization technique [Cuzick 2000]. In general, however, proposed approaches such as administering HPV tests to women with mild dysplasia in order to determine whether treatment is necessary have had varying levels of effectiveness and are likely to be relatively costly [Bollen et al. 1997, Kaufman et al. 1997; Lytwyn et al. 2000]. HPV high-risk detection actually yielded a wide variation of PPVs from 13% to 33.5% [Clavel et al. 1999, Cuzick et al. 1999, Manos et al. 1999, ALTS Group 2000, Adam et al. 2000]. Using Hybrid-Capture II test, several study reported the specificity of high-risk HPV testing for detection of histological high-grade SIL from 58% to 85% [Cuzick 2000, Clavel et al. 1999, Wright et al. 2000]. Transient incident infection, especially in young women, is common

Adam et al. 2000. Specificity thus remains a concern with use of HPV testing for primary screening, and more research is needed to determine optimal approaches Cuzick 2000, Koss 2000. One study in South Africa suggests that specificity can be improved by adjusting the level of HPV DNA used to define a positive result Kuhn et al. 2000. In contrast, DNA image cytometry has so far provided more encouraging data on its diagnostic validity.

1.3. DNA image cytometry

1.3.1. Biological background

Chromosomal aneuploidy is associated with most malignant and some benign tumors Böcking 1998b. Structural and/or numeric chromosomal aberrations have been found in most cervical squamous carcinomas Murty et al. 1988, Norming et al. 1992, even in HSILs Fahmy et al. 2002. DNA-aneuploidy represents the quantitative cytometric equivalent of chromosomal aneuploidy and has been internationally accepted as a marker for neoplastic cell transformation Haroske et al. 2001.

Quantitation of nuclear DNA content by cytometry has come into practice for assistance in the diagnosis and grading of malignant tumors, including pre-invasive cervical lesions and cervical cancer. The DNA content cannot be measured directly by cytometry. After quantitative DNA staining according to Feulgen, the nuclear IOD (Integrated Optical Density) is the cytometric equivalence of its DNA content. The quantitation of nuclear DNA requires a rescaling of the IOD values by comparison with those from cells with known DNA content. Therefore the DNA content is expressed in a „c“ scale in which 1c is half the mean nuclear DNA content of cells from a normal (non-pathological) diploid population in G0/G1 cell cycle phase.

For practical reasons, a term being widely accepted and used throughout the literature is „DNA ploidy“. However, the meanings of „DNA ploidy“ and „chromosomal ploidy“ are not identical. Whereas „chromosomal ploidy“ is theoretically detectable by cytogenetic methods in each single cell, its DNA content cannot be equated with a

certain chromosomal outfit. The term „DNA ploidy“ should therefore preserved for the description of DNA stemlines, but not for single cells [Haroske et al. 1998].

Indeed, the quantity of nuclear DNA may be changed by the following mechanisms: replication, polyploidization, gain or deletion of chromatids. Each affects the size or the number of chromatids. Furthermore viral infections may change the nuclear DNA content detectable by flow and image cytometry. Among others, the unspecific effects of cytostatic or radiation therapy, vitamin B12 deficiency, apoptosis, autolysis and necrosis on nuclear DNA content play also a role. All these effects have to be taken into consideration when a diagnostic interpretation of DNA histograms is performed [Böhm and Sandritter 1975, Winkler et al. 1984, Sandberg 1990, Biesterfeld et al. 1994].

The basic aims of diagnostic DNA cytometry are to identify DNA stemlines outside the euploid regions as abnormal (or aneuploid) at a defined statistic level of significance. Furthermore DNA image cytometry should give information about:

- Number of abnormal DNA stemlines
- Polyploidizytion of euploid or aneuploid DNA stemlines
- Cell cycle fractions
- Occurrence of rare cells with an abnormally high DNA content [Haroske et al. 1998]

Increasing information on chromosomal aneuploidy not only as a highly specific marker of neoplastic cell transformation, but also on its role in tumor pathogenesis and progression support the biological basis of diagnostic DNA-image cytometry [Duesberg et al. 1998, Li et al. 2000].

1.3.2. Principles of the method

Because DNA image cytometry results in nuclear IOD values, equivalent but not identical with nuclear DNA content, the quantitation of nuclear DNA requires a rescaling of IOD values by comparison with those from cells with known DNA contents, so-called reference cells. By means of reference cells the arbitrary unit scale will be transformed

in a reference unit scale (2c, 4c, 8c, for example). In general, there are two types of reference cell systems: external and internal ones, respectively. Whereas the external reference cells are very easily to identify by the investigator, but often not to prepare in parallel with the clinical sample, the internal reference cells have the advantage of sharing all preparatory steps with the analysis cells in the clinical specimens. The nuclear IOD values of reference cells own the same methodological limitations in terms of precision of the measurements as the appropriate IOD values of the analysis cells.

The mean ratio between the modal IOD values of the non-pathologic cells of the tissue under study and the reference cells used is called corrective factor. This corrective factor must be applied to DNA measurements from the clinical sample before any DNA histogram interpretation. Due to the methodological variability, mentioned above, the corrective factor is not constant. The accuracy of each diagnostic DNA evaluation depends decisively on the standard deviation (SD) of the corrective factor used during the rescaling procedure [Haroske et al. 1997].

Because most of the interpretations of DNA measurements are population-based, the results are usually displayed as DNA histograms. The bin size of such histograms should be adapted to the precision of the actual measurements, i.e. the lower the variability in the reference cell peak, the smaller the bin size of histogram classes could be [Haroske et al. 1998].

1.3.3. Standardization

DNA image cytometry can be used as an objective adjuvant method to establish the diagnosis of (prospective) malignancy in different preneoplastic lesions and for grading of tumor malignancy of manifest cancers. Four international consensus reports of the European Society of Analytical Cellular Pathology on standardized diagnostic DNA-image cytometry (Böcking et al. 1995, Haroske et al. 1998, Giroud at al. 1998, Haroske et al. 2001) provided guidelines and performance standards for diagnostic DNA measurements, definitions of terms and algorithms for diagnostic data interpretation. International consensus has also been reached on the application of

DNA-ICM for the identification of high-grade intraepithelial lesions in cervical cytology, which need further clinical management (Hanselaar et al. 2001).

1.3.4. Diagnostic accuracy of DNA image cytometry in cervical and endometrial pathology

Various studies have demonstrated the value of DNA aneuploidy as a marker for histologically confirmed and/or prospectively neoplastic development in cervical dysplasia [Fu et al. 1982, Böcking et al. 1986; Chatelain et al. 1989a, Kashyap et al. 1990, Bollmann u Böcking 1996, Nenning et al. 1997, Bollmann et al. 2001]. Fortunately, DNA aneuploidy cannot be found in benign or reactive changes of cervical squamous epithelium and other non neoplastic cells and tissues [Sandritter and Fischer 1961, Shevchuck and Richard 1982, Winkler et al. 1984, Böcking et al. 1984]. As low-grade SILs are mostly DNA-euploid and high-grade SILs usually -aneuploid, Wright et al. (2002) and other authors proposed DNA ploidy as a distinguishing feature of both lesions [Wright et al. 2002, Bollmann et al. 2001]. The finding of DNA-aneuploidy qualifies an ASCUS or LSIL lesion as high-grade, obligatory precancerous or prospectively malignant, which should be removed. Böcking and Motherby published a positive predictive value of DNA-aneuploidy of 92% after two years of follow-up in ASCUS/LSIL lesions. Grote et al. (2001) have also reported on the significant prognostic impact of DNA-ICM in invasive cervical cancer [Grote et al. 2001]. Using multivariate analysis, both DNA stemline ploidy with a cut-off value of 2.20 and the 5cEE have been found in that study to be of pre- and post-surgical prognostic value.

During the last decade, the clinical application of DNA-image cytometry (DNA-ICM) as an adjuvant diagnostic and prognostic method has increased. The summary statement of the International Consensus Conference on the Fight Against Cervical Cancer task force #18 recommended DNA-ICM as a useful adjunctive method to separate low-grade from high-grade cervical intraepithelial lesions which need further clinical management [Haanselar et al. 2001].

Sudbø & coworkers have recently demonstrated that it may take up to 5 years until invasive squamous cancer; predicted by demonstration of DNA aneuploidy in oral

squamous dysplasias can be proven histologically. While the PPV for DNA aneuploid dysplasias for the development of histologically proven cancer was only about 10% after one year, it increased to 90% after five years [Sudbø et al. 2001].

1.4. Reproducibility of gynecological cancer diagnosis

The reliability of a diagnostic method depends on different variables, like validity and reproducibility. The reproducibility of a method includes two aspects: intra- and interobserver agreement. Features, which have an impact on reproducibility, are objectivity of diagnostic criteria, number of diagnostic categories, study population and experience of the test performers. Interobserver variability has important implications for diagnostic error, thus patient care and also medical litigation.

In cancer screening and diagnosis, cytological and histological investigations so far played the crucial role and have greatly contributed to the fight against cancer worldwide. The most successful cancer screening program ever carried out is the early detection of cervical cancer and its precursors using exfoliative cytology. Despite its great contribution to the increasing number of detected preinvasive cervical lesions and the decreasing number of invasive cancers, its reproducibility is still insufficient and causes clinical problems. Reproducibilities of cytological and histological diagnoses of precancerous lesions and cancers including typing and grading have been largely investigated.

1.4.1. Reproducibility of cytology and histology in diagnosis and grading of dysplasias and cancers

Grading of dysplasia or intraepithelial lesions is a daily task in diagnostic pathology and cytopathology. It is notoriously subjective and thus lacks sufficient intra- and interobserver reproducibility. This is partly due to the lack of validated morphological criteria, upon which pathologists and cytologists have reached consensus [Bosman 2001]. Variability among histopathologists was assessed in a study including 106 cervical biopsy specimens [de Vet et al. 1990]. Four experienced histopathologists assigned them to

one of five diagnostic categories: no dysplasia, mild, moderate, severe dysplasia and carcinoma in situ. Considerable disagreement among pathologists was observed: the unweighted kappa was only 0.28. All grades of dysplasia were equally difficult to distinguish from adjacent categories. Using modified Bethesda grading system for histological reporting of SILs, McCluggage and coworkers reported a weighted kappa of only 0.36 (95%CI 0.21-0.61) [McCluggage et al. 1998].

Tezuka et al. (1992) reported a study on 70 cytological specimens containing endometrial cells. Nineteen pathologists assigned the smears to one of three diagnostic categories: negative for, suspicious of and positive for malignancy. The agreement was better on negative and positive categories (kappa = 0.46 and 0.47, respectively) and poor in grading suspicious cells. The overall kappa for all smears was only 0.36 [Tezuka et al. 1992]. A study of Gynecologic Oncology Group comparing the three-level architectural grading system (AG) with the two-level nuclear grading system (NG) in 88 cases of stage I endometrial adenocarcinomas and demonstrated a moderate reproducibility (kappa = 0.49, AG; kappa = 0.57, NG) [Zaino et al. 1994]. Another study also dealing with endometrial adenocarcinomas showed acceptable results for interobserver reproducibility: 0.65 for overall diagnoses, 0.70 for AG and 0.55 for NG [Nielsen et al. 1991]. In an analysis of 244 ovarian immature (malignant) teratomas (IT), the reproducibility between pathologists of the traditional grading system for IT is only moderate if a three-tiered scale is used and limited by the results of the least skilled observer (kappa = 0.54). Interobserver variability is reduced if a two-tiered system is used (kappa = 0.66) [O'Connor and Norris 1994].

Histological typing of cancer also presents difficulties in diagnostic histopathology. Most publications on this concern also showed insufficient reproducibilities. In an assessment which involved 50 slides of breast cancer and 10 pathologists, the overall kappa of histological tumor typing was only 0.23 [Cserni 1999]. The kappa increased to 0.65 if only diagnoses from 6 specialized pathologists were taken into account.

1.4.2. Reproducibility of adjuvant methods in cancer diagnosis and prognosis

Assessment of proliferative activity plays an important role in prognosis of many cancers. The most common proliferation marker is Ki-67, detected by using immunohisto- or cytochemistry. Using multi-sample tissue microarray technique for evaluation of Ki-67 labeling on breast cancer tissue blocks, a recent study demonstrated a wide variation of weighted kappa of 0.39-0.84 [Mengel et al. 2002]. When the interobserver bias possibility was excluded, this study showed an interlaboratory agreement of 75.7%. The authors concluded that immunohistochemical determination of the Ki-67 labeling index needs to be standardized. Biesterfeld et al. (1996) reported a better interobserver agreement on the immunocytochemical receptor status of 89% for the estrogen receptor and 93% for the progesterone receptor [Biesterfeld et al. 1996]. Applying the Cell Analysis Systems (CAS) 200/486 image analyzer for quantitative immunohistochemical (IHC) analyses, Makkink-Nombrado et al. (1995) showed a low reproducibility with overall agreement of 56% for Ki-67, 70% for estrogen receptor and 70% for progesterone receptor [Makkink-Nombrado et al. 1995].

Recently, overexpression of $p16^{INK4a}$ induced by oncogenic high-risk HPV has been reported as a specific marker for CIN 2-3 or higher lesions [Sano 1998, Milde-Langosch et al. 2001, Klaes et al. 2001]. Klaes et al. (2002) have demonstrated that immunostaining of $p16^{INK4a}$ may contribute to improve the interobserver agreement in the diagnosis of cervical intraepithelial neoplasia [Klaes et al. 2002].

In a study on 56 cases of colon, breast and lung carcinoma, Böcking et al reported an interobserver reproducibility of the DNA grading system of 82.2%. In comparison, the histopathological grading of breast cancers according to Bloom and Richardson in that study yielded an interobserver reproducibility of 57%. By using Auer DNA-histogram classification for breast cancer, Böcking et al found an intraobserver and interobserver reproducibility of 70% and 45%, respectively [Böcking et al. 1989].

1.5. Objectives

In the past, most studies on DNA-ICM of cervical dysplasia were retrospectively designed and of descriptive nature, i.e. at the time of DNA-ICM the cytological or histological follow-up results were known [Böcking et al. 1984, Böcking et al. 1986, Göppinger et al. 1986, Chatelainet al. 1989]. While data on diagnostic accuracy of DNA-ICM in cervical cytology are encouraging, no data have been published so far on the interobserver reproducibility of this adjuvant method if applied to establish the qualitative diagnosis of DNA-aneuploidy as a marker of progressive behavior in cervical intraepithelial lesions. Therefore the objectives of this study are:

- To investigate the diagnostic validity of DNA-ICM for the identification of progressive cervical intraepithelial lesions in Pap smears.

- To investigate the interobserver reproducibility of DNA-ICM applied to the routine Pap smears classified as ASCUS or higher lesions.

2. MATERIALS AND METHODS

2.1. Materials

Two hundred and two women with Pap smears diagnosed as ASCUS or higher during the period from January 1996 to January 2002 have been included in this study. The samples derived from a previous study, which used the Munich Nomenclature II to classify cytological findings [Leick 2003]. The cytological samples were consecutively taken from routine input of the Institute for Cytopathology, University of Düsseldorf. Correspondent data on follow-up cytology and histology were retrospectively collected. Follow-up histology was classified according to the CIN system [WHO 1994]. The mean age of patients was 37.6 years (range 16 – 88 years).

2.2. Methods

2.2.1. Cytological investigation

Samples from the uterine cervix were taken using Ayres spatula or Cervex brush and fixed in 96% alcohol for at least 15 minutes. All slides were then stained according to Papanicolaou and routinely examined. The Bethesda nomenclature [Kurman and Solomon 1994, Solomon et al. 2002] was used for cytological classification in order to achieve internationally accepted diagnostic results.

The 2001 Bethesda System for reporting results of cervical cytology (adapted from Solomon et al., 2002)

GENERAL CATEGORIZATION (optional)
Negative for Intraepithelial Lesion or Malignancy
Epithelial Cell Abnormality: See Interpretation/Result

I**NTERPRETATION/RESULT**
NEGATIVE FOR INTRAEPITHELIAL LESION OR MALIGNANCY
ORGANISMS

- Trichomonas vaginalis
- Fungal organisms morphologically consistent with *Candida spp.*
- Shift in flora suggestive of bacterial vaginosis
- Bacteria morphologically consistent with *Actinomyces spp.*
- Cellular changes consistent with *Herpes simplex* virus

OTHER NON NEOPLASTIC FINDINGS

- Reactive cellular changes associated with
  - inflammation (includes typical repair)
  - radiation
  - intrauterine contraceptive device (IUD)
- Glandular cells status post hysterectomy
- Atrophy

OTHER

- Endometrial cells (in a woman > 40 years of age)

EPITHELIAL CELL ABNORMALITIES

- SQUAMOUS CELL
  - Atypical squamous cells
    - of undetermined significance (ASC-US)
    - cannot exclude HSIL (ASC-H)
  - Low grade squamous intraepithelial lesion (LSIL) encompassing: HPV/mild dysplasia/CIN 1
  - High grade squamous intraepithelial lesion (HSIL) encompassing: moderate and severe dysplasia, CIS/CIN 2 and CIN 3
    - with features suspicious for invasion (if invasion is suspected)
  - Squamous cell carcinoma
- GLANDULAR CELL
  - Atypical
    - endocervical cells (NOS or specify in comments)
    - endometrial cells (NOS or specify in comments)
    - glandular cells (NOS or specify in comments)
  - Atypical
    - endocervical cells, favor neoplastic
    - glandular cells, favor neoplastic
  - Endocervical adenocarcinoma in situ
  - Adenocarcinoma
    - endocervical
    - endometrial
    - extrauterine
    - not otherwise specified (NOS)

OTHER MALIGNANT NEOPLASMS

2.2.2. DNA cytometry

2.2.2.1. Smear processing

Directly after morphological investigation, the smears underwent destaining and restaining according to Feulgen [Feulgen 1924]. For that purpose the Papanicolaou prestained smears were immerged in xylene to remove coverglasses. Feulgen staining was performed automatically using a modified staining machine, Varistain 24-4 (Shandon, Pittsburgh, Pennsylvania, USA) as described by Chatelain et al. (1989) [Chatelain et al. 1989]. The hydrolysis is performed in a temperature-adjusted cuvette, which is made from acid-resistant material. It maintained a constant temperature of 27.0°C. After rehydratation in decreasing ethanol concentrations and re-fixation in buffered 10% formalin, 5N HCl for hydrolysis was applied at 27.0°C for 60 minutes, followed by staining in Schiff's reagent (Merck, Darmstadt, Germany, No. 1.09033.0500) for another 60 minutes in room temperature, then rinsing in SO_2-water and dehydratation at increasing ethanol concentrations. Sulfur bathes are prepared extemporaneously by adding 5g Na or K metabisulfite with 50ml HCl 1N to distilled water to form a volume of 1000 ml. The slides were then covered with Entellan (Merck, Darmstadt, Germany, No. 1.07961.0500). The complete protocol for Feulgen staining is listed below.

Protocol for Feulgen staining

Procedure	**Substance**	**Condition**	**Time and temperature**
HYDROLYSIS	HCl	5N	60 min. at 27.0°C
RINSING	H_2O (distill.)	4 bathes	1 min. each bath at room temperature
STAINING	Schiff's reagent		60min. at 25°C
	Sulfite solution	4 bathes,	1min. each bath at room temperature
RINSING	Tap water		10 min. at room temperature
DEHYDRATION	H_2O (distill.)	3 min.	
	Alcohol 70%	5 min.	

	Alcohol 95%	3 min.
	Alcohol 95%	5 min.
	Alcohol 100%	3 min.
	Alcohol 100%	10 min.
MOUNTING	Xylene	5 min.
	Xylene	5 min.
	Mounting medium	

The covered slides were then keep in darkness until measurement.

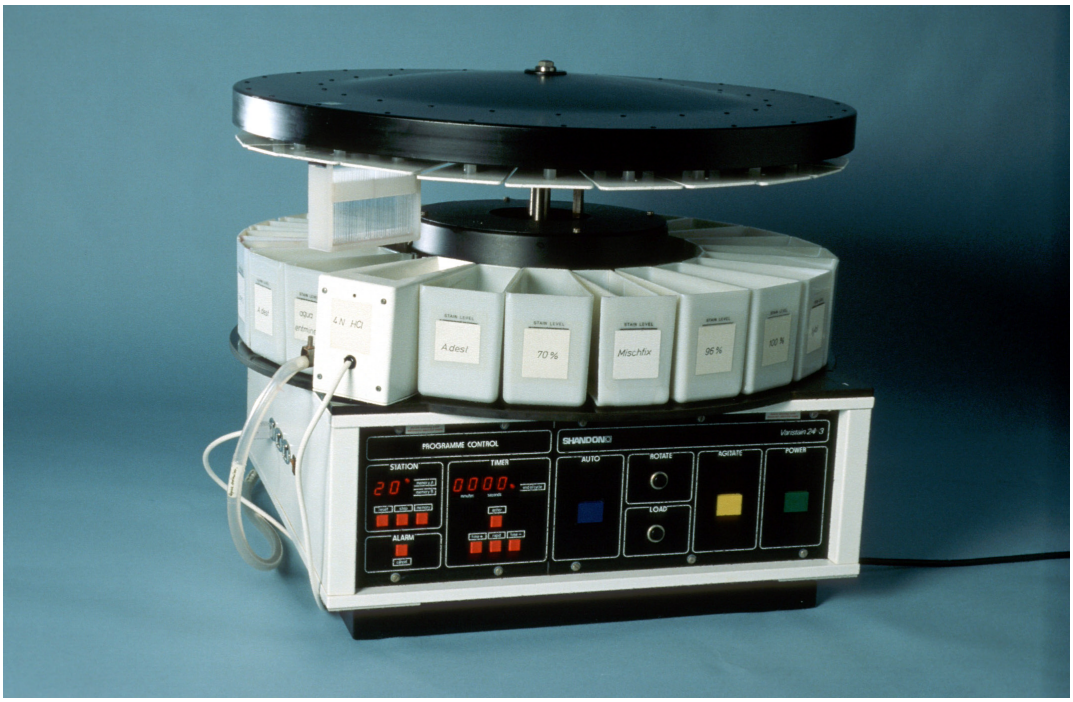

Figure 1. Automated staining machine Varistain 24-3 (Shandon, U.K.)

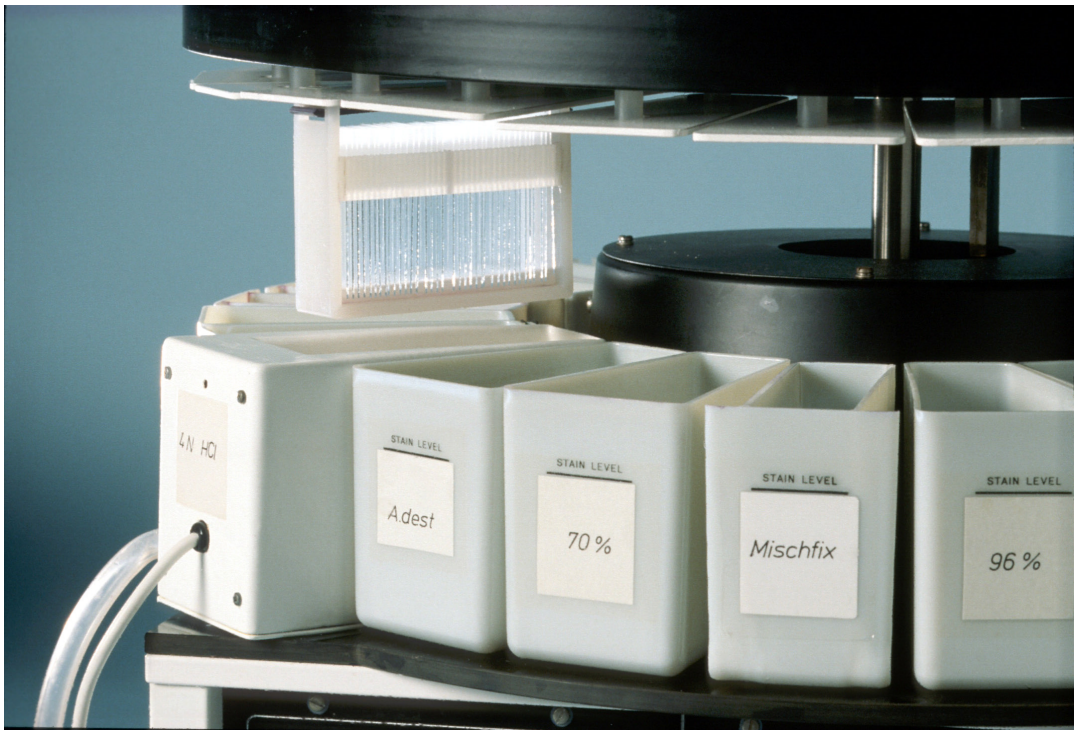

Figure 2. Temperature-controlled hydrolysis bath

2.2.2.2. DNA cytometry workstation

Measurements of nuclear DNA contents were performed using a computer-based image analysis system consisting of a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) with a 40x objective, NA 0.75; Köhler illumination was applied to reduce stray light. A CCD black and white video-camera with 572 lines resolution (VariCam, Model CCIR, PCO Computer Optics, Kehlheim, FRG) was adapted to the microscope and connected to an IBM PC compatible computer through a frame-grabber board (Matrox Meteor Board / Matrox Electronic Systems, Unterhaching, FRG). The software used in this study was the AutoCyte QUIC-DNA-Workstation (AutoCyte Inc., Burlington, N.C., USA), which provides shading-, glare- as well as local background per nucleus correction procedures. The latter was performed at a rate of 2.2%. Measurement results in form of DNA histograms, scattergrams and clinical reports were printed out using a color inkjet printer (Epson Stylus Color 640, EPSON Co., Japan). All technical instruments and the software used in the study met the standard requirements of the ESACP consensus reports [Böcking et al. 1995, Haroske et al. 1998, Giroud et al. 1998, Haroske et al. 2001].

Figure 3. DNA cytometry workstation

2.2.2.3. DNA measurements

Measurements were performed under the following rules: (1) nuclei to be measured were in focus; (2) no change of instrumentation adjustment during measurements under Köhler-illumination, light intensity, field diaphragm, analogue and digital adjustment; (3) visual control during measurements for artifact rejection, manual correction of inappropriate nuclear segmentation if necessary.

Nuclei to be measured were sampled in a systematic random manner. Only diagnostically relevant cells were measured, i.e. cells of a certain cytological entity, e. g. all tumor cells or all dysplastic cells, which could be identified by their morphology. A selective sampling for rare nuclei characterized by a high DNA content is allowed only if the occurrence per se is of diagnostic relevance.

To assure that relevant cells, i.e. dysplastic or tumor cells should be measured and to facilitate the relocalization of these cells on Feulgen-stained smears during DNA measurement session, their positions on the smear were imaged. The position of these cells was first marked by felt-tip pen on the Papanicolaou-stained smears. A photocopy of the whole smear with marked positions was made. After Feulgen staining and covered by coverglass, these marked positions were then re-transferred from the photocopy again onto the smear.

In each case at least 300 nuclei of abnormal squamous cells were randomly measured. At the beginning, the relevant cells from marked positions were measured. The whole smear was then rescreened to detect other cells of interest. At least 30 normal intermediate squamous cells were measured as internal reference cells. The mean IOD of these cells was defined as 2c. A correction factor of 1.00 was used to obtain the normal 2c value. The coefficient of variation of reference cells was always maintained below 5% [Haroske et al. 1998].

The first measurements were done as routine workup of ASCUS or higher cases. The second measurements were consecutively performed during this study and blinded to the results of the first measurements.

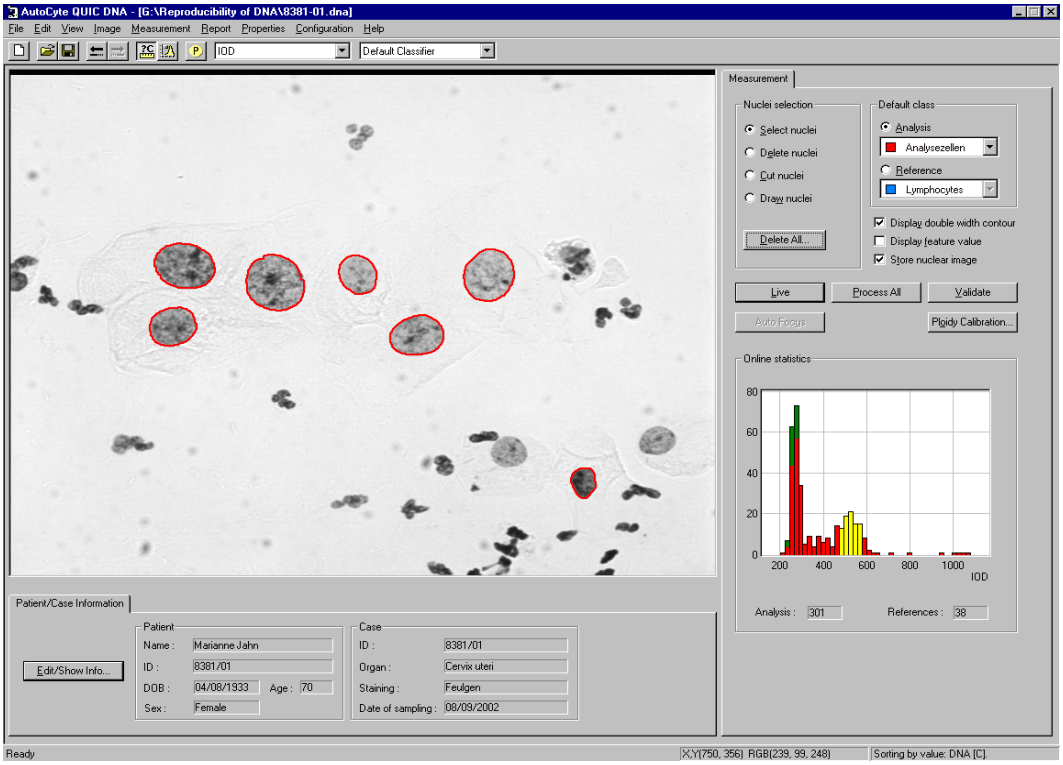

Figure 4. Screen for interactive nuclear DNA-measurements. Live image of Feulgen stained, segmented and measured nuclei left; online DNA-histogram right.

2.2.2.4. Definition of terms and algorithms for diagnostic interpretation of DNA cytometry

- DNA histogram: frequency distribution of IOD values obtained by quantitative DNA stains and rescaled by reference cells in “c” units. The class width should be twice the standard deviation of the IOD of the G0/1-phase-fraction of reference cells.

- Modal value of a histogram peak: the most frequent value, i.e. the mean value of the histogram class containing the highest number of nuclei.

- DNA stemline: the G0/G1 cell-phase fraction of a proliferating cell population (with a first peak and a second doubling one, or nuclei in the doubling region) Böcking et al. 1995, Haroske et al. 2001.

- DNA stemline ploidy: this was defined as the modal value of a DNA stemline in the unit c (c = content of DNA) [Böcking et al. 1995, Haroske et al. 1998].

- DNA-euploidy: those types of DNA distribution which cannot be differentiated from that of normal cell populations (resting, proliferating, or with polyploidization) [Haroske et al. 1998].

- Diploid euploidy: DNA-stemlines with a modal value between 1.8c and 2.2c [Haroske et al. 1998].

- Tetraploid euploidy: DNA-stemlines with a modal value between 3.6c and 4.4c [Haroske et al. 1998].

- DNA stemline aneuploidy: this was assumed, if the modal value of a stemline was <1.80c and >2.20c or <3.60c and >4.40c [Böcking et al. 1995, Haroske et al. 2001].

- Single cell aneuploidy: occurrence of at least one cell with a DNA content >9c (9cEE ≥ 1) per slide [Chatelain et al. 1989a].

2.2.2.5. Diagnostic assessment of abnormal DNA stemline patterns

According to Haroske et al (1998), a classification of the entire DNA-histogram based on the position of the DNA stemlines may be of prognostic value. The following terms with their respective definitions were used to classify the histogram for prognostic purpose:

- peridiploid: a single DNA-stemline has a modal DNA value between 1.8c and 2.2c.

- peritetraploid: a single DNA-stemline or a stemline additional to a peridiploid one with a modal DNA value between 3.6c and 4.4c.

- x-ploid: a single DNA-stemline or a DNA-stemline additional to a peridiploid or peritetraploid one has a modal DNA value outside the thresholds mentioned above. “x” will be substituted by the DNA ploidy value of this stemline (e.g. peritriploid, hyperdiploid etc.).

- multiploid: more than one abnormal DNA-stemline occurs (often called “Manhattan skyline”).

An experimental assessment of frequency distribution of these four stemline groups was performed by correlating the aneuploid stemline types with follow-up histological findings. In order to achieve an internationally accepted equivalence in diagnostic results, these histological diagnoses were reclassified into the modified Bethesda system for reporting results of cervical histopathology, which combines CIN II and CIN III into high-grade lesions.

2.2.3. Statistical analysis

Differences in proportions were evaluated using the chi-square test. Diagnostic validity of cytological diagnoses within the study sample was evaluated by the calculation of sensitivity and positive predictive value. Diagnostic validity of DNA-ICM encompassing sensitivity, specificity, positive and negative predictive values were calculated based on the second DNA cytometric measurements.

Kappa value was calculated for assessment of interobserver agreement and scored according to Landis and Koch guidelines [Landis and Koch 1977]:

- 0.0 – 0.2: slight agreement
- 0.2 – 0.4: fair agreement
- 0.4 – 0.6: moderate agreement
- 0.6 – 0.8: substantial agreement
- >0.8: almost perfect agreement

Two softwares, SPSS for Windows 10.0 (SPSS Inc., Chicago, USA) and Microsoft Excel 9.0 (Microsoft, Redmond, USA) were used for statistical analysis. Statistical significance was considered if $p < 0.05$.

3. RESULTS

3.1. Descriptive statistics of study sample

Of 202 patients entered in the study, more than one diagnostic cytology/DNA-ICM was performed in 19 patients. In these cases, each DNA-histogram has been regarded as an individual case. In all cases the time intervals between the initial cytological diagnosis/DNA-ICM and each cytological/histological follow-up diagnosis was assessed. The following data were obtained: 221 initial cytological diagnoses of ASCUS+ lesions and 221 DNA-ICM diagnoses, 139 cytological and 135 histological diagnoses of the follow-up. Initial cytological diagnoses were 25 ASCUSs, 72 LSILs, 118 HSILs and 6 invasive cancers.

The mean interval between the initial cytological/DNA-cytometric diagnoses and their cytological/histological verification was two months (range, 1 to 26 months).

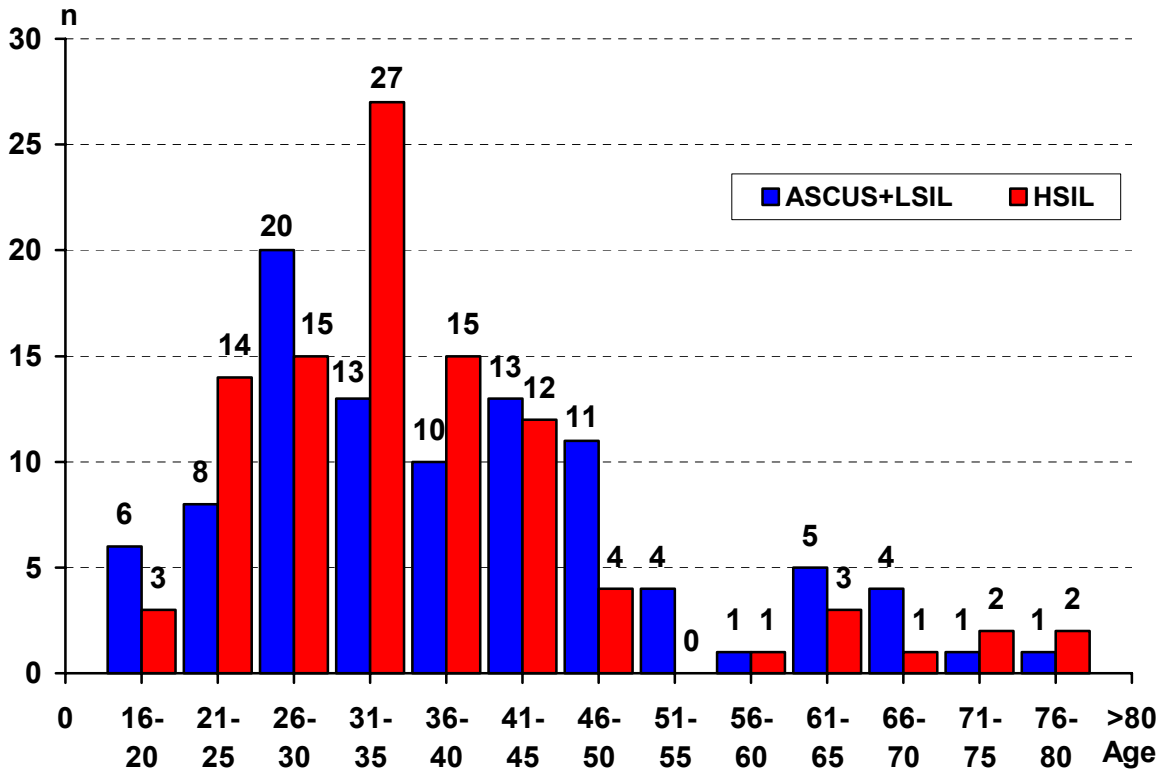

Figure 5. Frequency distribution of patients age

3.2. DNA measurements

Of 221 DNA measurements, 68 (30.8%) were found to be DNA-euploid. One hundred fifty three measurements were interpreted as DNA-aneuploid, including 17 stemline-, 49 single cell- and 87 stemline-and single cell-DNA-aneuploidies.

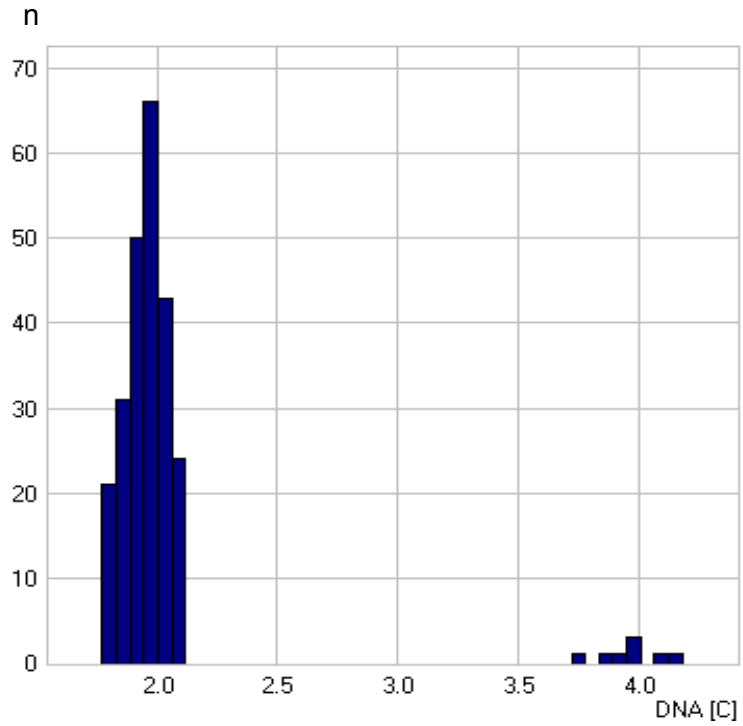

Figure 6. DNA-histogram from a Pap smear diagnosed as ASCUS: euploid (diploid) pattern. Stemline at 2.0c, no cells >9c

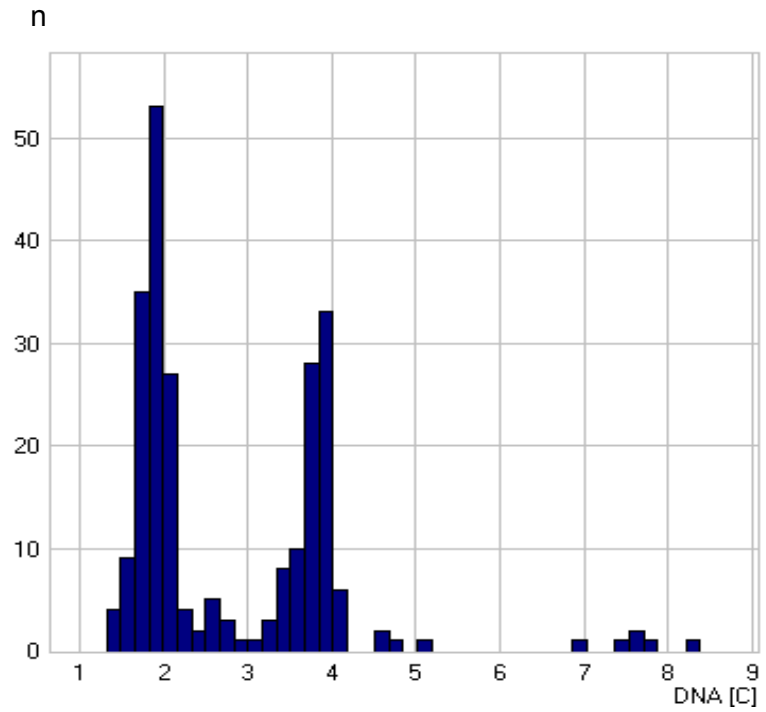

Figure 7. DNA-histogram from a Pap smear diagnosed as LSIL: euploid (polyploid) pattern. Stemlines at 2c and 4c, no cells >9c

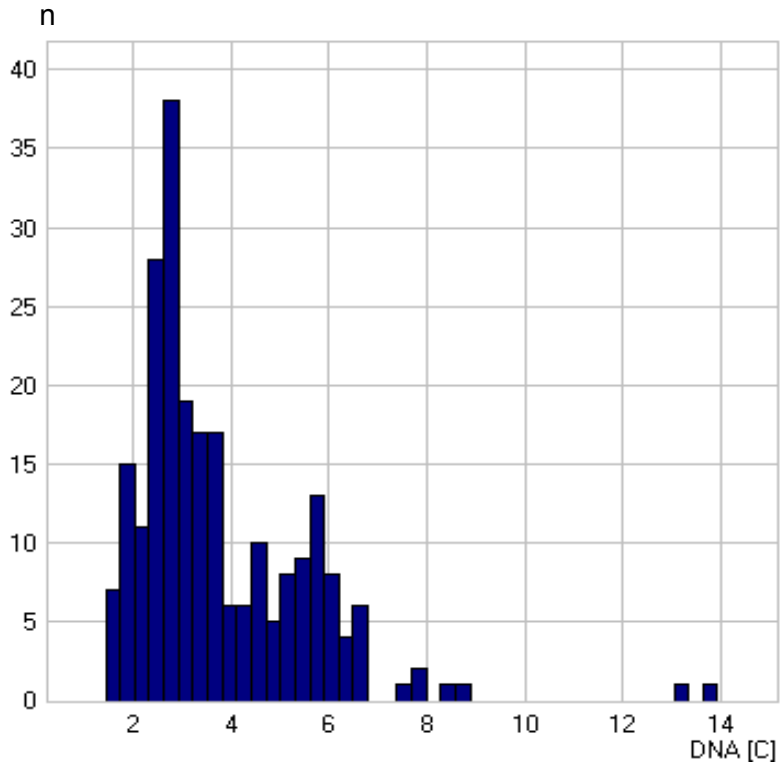

Figure 8. DNA-histogram from a Pap smear diagnosed as HSIL: aneuploid (multiploid) pattern. Stemlines at 3c and 6c, 2 cells >9c

3.3. Prevalence of DNA-aneuploidy

Prevalence of DNA-aneuploidy in different cytological subgroups is shown in table 3. The group of HSIL and higher lesions revealed an increased proportion of DNA-aneuploidy, reaching 100% in the invasive cancer cases. There was no significant difference in the percentage of DNA aneuploidy between ASCUS and LSIL cases (χ^2=0.11; p=0,74). Fourty four percent of ASCUS smears were classified DNA-aneuploid.

Table 3. DNA-aneuploidy in different cytological subgroups (percentage in brackets)

Histogram	Input cytology				
	ASCUS	LSIL	HSIL	Cancer	Total
n	25	72	118	6	221
Mean age	44.8	36.6	36.0	46.8	37.6
Euploid	14 (56)	43 (59.7)	11 (5.1)	0	68 (30.8)
Aneuploid	11 (44)	29 (40.3)	107 (90.7)	6 (100)	153 (69.2)
9cEE	2	15	32	0	49
STL	3	2	12	0	17
9cEE + STL	6	12	63	6	87

3.4. Cytological/histological follow-up

Histological follow-ups were available in 135 cases. The rate of concordance between initial cytology and follow-up histology in LSILs, HSILs and invasive cancer cases were 26.3% (10/38), 87.9% (73/83) and 33.3% (2/6), respectively. In 11 cases with cytological diagnosis of ASCUS, all types of histologically confirmed lesion ranging from WNL to CIN III were found. ASCUS/LSIL lesions turn out to be high-grade lesions or invasive carcinoma in 34.0% (33/97) of cases.

Table 4. Results of cytological/histological follow-up

Cytological diagnosis	Nr. of cases	Cytological Follow-up		Number of cases with histological follow-up	Histological Follow-up				
		WNL, BCC	HSIL (CINIII)		WNL	CIN I	CIN II	CIN III	Invasive Cancer
ASCUS									
Euploid	14	12	1	2	2	-	-	-	-
Aneuploid	11	1	-	9	1	1	1	6	-
Σ	25	13	1	11	3	1	1	6	-
LSIL									
Euploid	43	29	1	14	2	4	5	3	-
Aneuploid	29	4	1	24	1	5	5	12	1
Σ	72	33	2	38	3	9	10	15	1
HSIL									
Euploid	11	8	0	3	1	2	0	1	0
Aneuploid	107	14	5	80	2	5	20	52	1
Σ	118	22	5	83	3	7	20	53	1
Invasive cancer									
Euploid	0	-	-	-	-	-	-	-	-
Aneuploid	6	-	-	6	1	1	0	2	2
Σ	6	-	-	6	1	1	0	2	2

3.5. Correlation of DNA-aneuploidy with histological follow-up

Table 5 describes the correlation between histological diagnoses and DNA cytometric interpretations on preceding Pap smears. The prevalence of DNA-aneuploidy increased from 66.7% (12/18) in CIN I over 93.1% (67/72) in CIN III to 100% (4/4) in invasive cancer cases.

Table 5. DNA-ICM vs. follow-up histology

Aneuploid histogram	Histology					
	WNL	CIN I	CIN II	CIN III	Ca.	Total
n	10	18	31	72	4	135
9cEE	2	3	8	16	1	30
STL	1	2	5	18	0	26
9cEE + STL	3	7	13	33	3	59
Total (%)	6 (60)	12 (66.7)	26 (83)	67 (93.1)	4 (100)	115 (85.2%)

The percentages of peritetraploid DNA-aneuploidy tend to be decreased (from 40% to 25%) and of multiploid DNA-aneuploidy tend to be increased (from 0% to 75%) with the severity of lesions. These differences were not statistically significant (p=0.9 and 0.3, respectively).

Table 6. Distribution of different abnormal DNA histograms according to histology (modified Bethesda classification)

Aneuploid histogram	Histology				
	WNL	LSIL	HSIL	Ca.	Total
n	10	18	103	4	135
Peritetraploid	4 (40)	5 (27.7)	31 (30.1)	1 (25)	41
X-ploid	2 (20)	1 (5.6)	18 (17.5)	0	21
Multiploid	0	6 (33.3)	44 (42.7)	3 (75)	53

3.6. Diagnostic accuracy of cytology and DNA-ICM

Diagnostic accuracy of cytological and DNA-ICM diagnoses were calculated by comparing initial cytology/DNA-ICM and follow-up results of cytology/histology. In 78 cases the final diagnosis was only based on follow-up cytology. Table 7 summarizes the diagnostic accuracy of cytological and DNA-ICM diagnoses. Sensitivity of cytological diagnosis of ASCUS/LSIL and DNA aneuploidy to detect the CIN III or higher lesions was 25.9% and 60.6%, respectively. The positive predictive values were 25.9% and 50.0%, respectively. DNA-ICM yielded a negative predictive value of 94.7%. Since the initial cytological diagnosis of all smears included in the study was at least ASCUS, it was impossible to calculate specificity and negative predictive values of cytological diagnoses.

Table 7. Diagnostic accuracy of ASCUS/LSIL diagnoses and DNA-ICM

	Cytology n = 221	DNA-ICM n = 221
Sensitivity	25.9% (22/85)	60.6% (20/33)
Specificity	-	71.2% (54/71)
Positive predictive value	25.8% (25/97)	50.0% (20/40)
Negative predictive value	-	94.7% (54/57)

3.7. Diagnostic accuracy of combination of DNA-ICM with cytology

When lesions graded higher then LSIL were included in initial cytological input criteria, sensitivity of DNA-aneuploidy increased from 60.6% in ASCUS/LSIL lesions to 92.9% in ASCUS or higher lesions. The PPV of DNA-aneuploidy slightly increased from 50.0% in ASCUS/LSIL to 51.1% in ASCUS/SILs lesions. Table 8 presents these values of diagnostic validity of DNA-ICM and the combination of DNA-ICM and cytology in our study using different diagnostic input criteria.

Table 8. Diagnostic validity of DNA-ICM and combination of DNA-ICM with cytology for different cytological diagnostic categories. Output criteria: histological CIN III or higher.

Initial cytological diagnoses	Method	Sensitivity	PPV	NPV
ASCUS + LSIL	DNA-ICM	60.6% 20/33	50.0% 20/40	94.7% 54/57
	DNA-ICM + Cytology		55.7% 54/97	
ASCUS + LSIL + HSIL	DNA-ICM	91.5% 75/82	51.1% 75/147	96.5% 65/68
	DNA-ICM + Cytology		71.6% 156/218	
ASCUS + LSIL + HSIL + Invasive Cancer	DNA-ICM	92.9% 79/85	51.6% 79/153	96.5% 65/68
	DNA-ICM + Cytology		72.5% 166/229	
LSIL + HSIL	DNA-ICM	93.5% 72/77	52.9% 72/136	94.4% 51/54
	DNA-ICM + Cytology		71.6% 139/194	

3.8. Reproducibility of DNA-ICM measurements

Prevalence of DNA-aneuploidy in two measurements from 202 cases is shown in Table 9. The group of HSIL and higher lesions revealed an increased proportion of DNA-aneuploidy, reaching 100% in the invasive cancer cases. The rates of DNA-aneuploid lesions are also increased from the group of histologically confirmed CIN I to invasive cancer as shown in Table 10.

Table 9. Prevalence of DNA aneuploidy in different cytodiagnostic categories

Cytological diagnosis	N	1. Measurement n (%)	2. Measurement n (%)
ASCUS	25	9 (36)	10 (40)
LSIL	72	26 (36.1)	28 (38.8)
HSIL	99	85 (85.8)	93 (93.9)
Invasive Carcinoma	6	6 (100)	6 (100)

Table 10. Prevalence of DNA-aneuploidy in correlation to histological follow-up

Histological diagnosis	N	1. Measurement n (%)	2. Measurement n (%)
WNL	10	6 (60)	6 (60)
CIN I	18	11 (61.1)	12 (66.7)
CIN II	31	24 (77.4)	26 (83)
CIN III	72	65 (90.3)	67 (93.1)
Invasive Carcinoma	4	4 (100)	4 (100)

Table 11 demonstrates results from detailed DNA-histogram interpretations. From two independent measurements DNA-euploidy was divided into two categories (diploid and polyploid), DNA-aneuploidy into three categories (9cEE only, aneuploid stemline only or both 9cEE and aneuploid stemline). None of the cases with DNA-aneuploidy in the first measurements had been interpreted as DNA-euploidy in the second measurements. Yet, there were 12 cases with a discrepancy between the first and second measurements. Nine out of these cases were classified as aneuploidy by detection of only one or a few cells with 9cEE. Correlation of two-category diagnostic classification is shown in table 12. The overall proportion of observed agreement was 94.1%, $\kappa = 0.87$, 95%CI 0.74-0.99.

Table 11. Comparison of detailed results from diagnostic DNA-image cytometry of cervical smears - the first and second measurement

1. Measurement / 2. Measurement		Euploidy		Aneuploidy		
		Diploid	Polyploid	9cEE only	Stemline only	9cEE + Stemline
Euploidy	Diploid	5	2	0	0	0
	Polyploid	4	51	0	0	0
Aneuploidy	9cEE only	1	4	29	2	11
	Stl. only	0	3	0	9	1
	9cEE + Stl.	0	4	19	14	43

Table 12. Comparison of two-category classification of diagnostic DNA-image cytometry of cervical smears

1. Measurement 2. Measurement	Euploidy	Aneuploidy	Σ
Euploidy	62	0	62
Aneuploidy	12	128	140
Σ	74	128	202

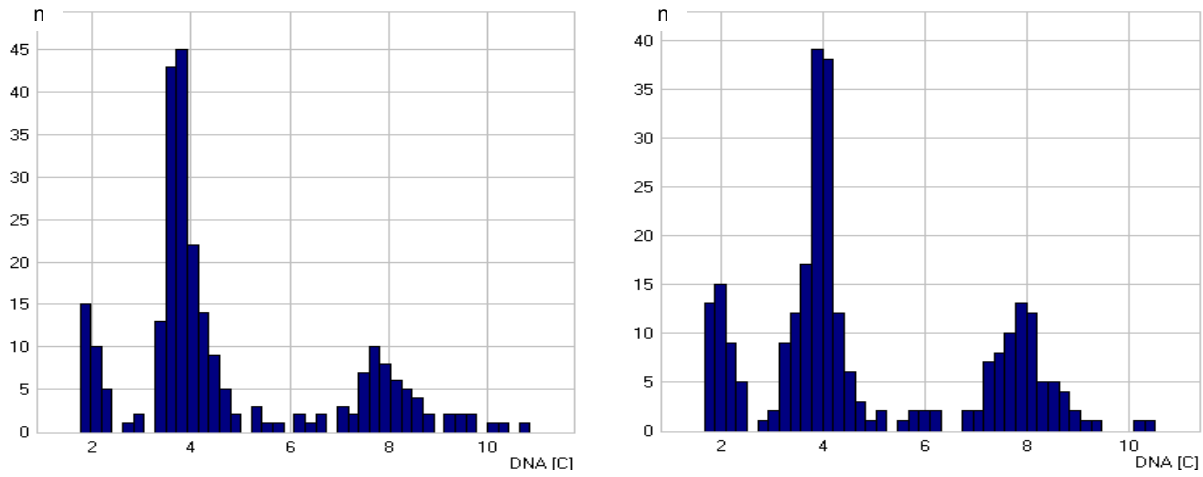

Figure 9. Case Nr. 6614/00. Minor differences of DNA histograms with identical diagnostic interpretation: Cytology: LSIL; DNA-aneuploidy (peridiploid, peritetraploid and perioctoploid stemlines, 9cEE = 7 resp. 4)

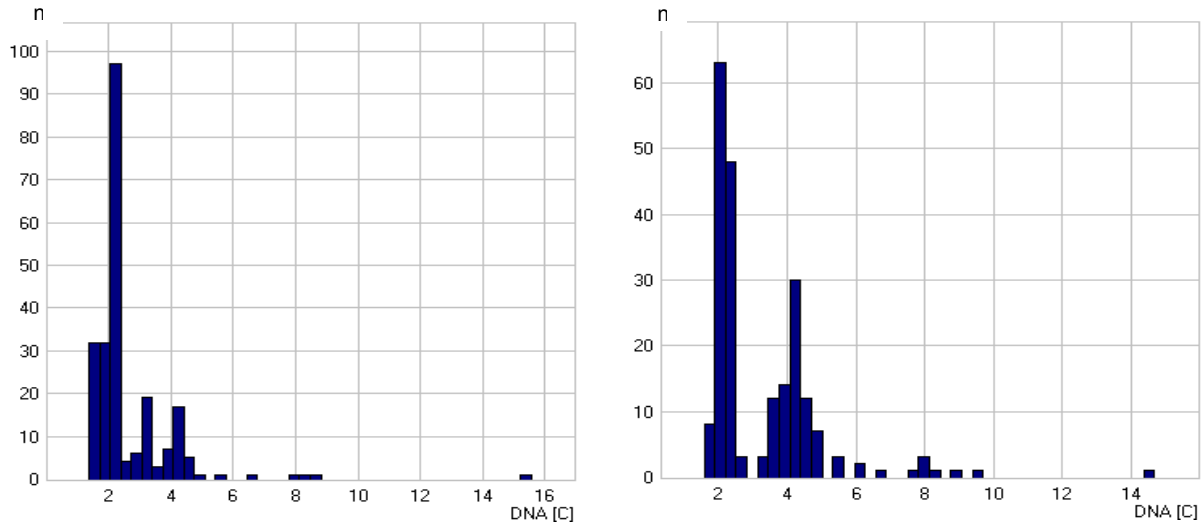

Figure 10. Case Nr. 144/01. Minor differences of DNA histograms with identical diagnostic interpretation: Cytology: ASCUS; DNA-aneuploidy (peridiploid and peritetraploid stemlines, 9cEE = 1 resp. 2)

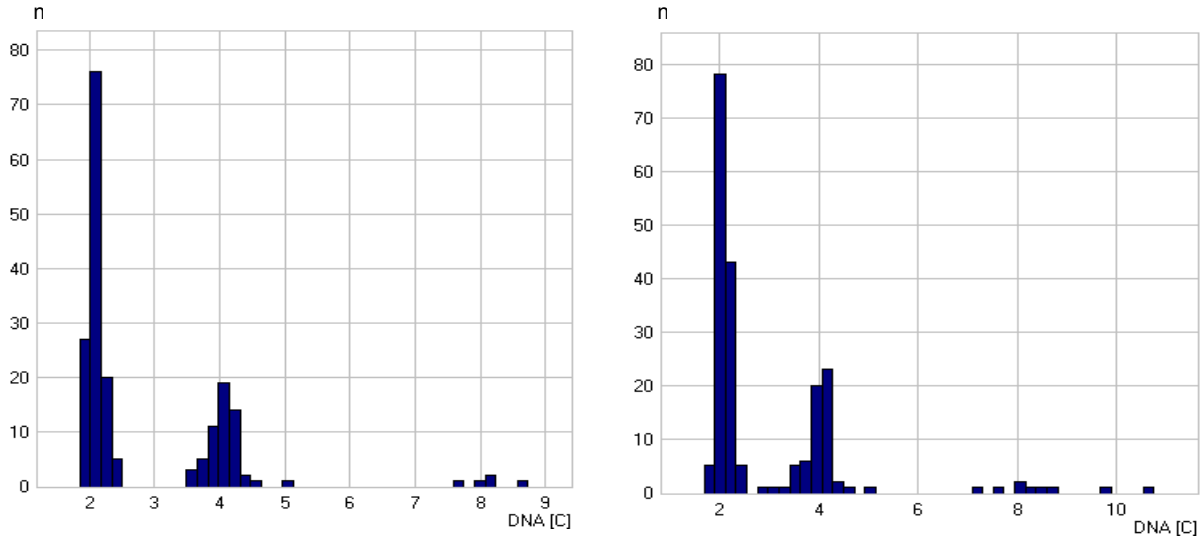

Figure 11. Case Nr. 14193/98. Minor differences of DNA histograms resulting in different diagnostic interpretations: Cytology: HSIL; DNA-euploidy left (peridiploid and peritetraploid stemlines, 9cEE=0), -aneuploidy right (9cEE=2).

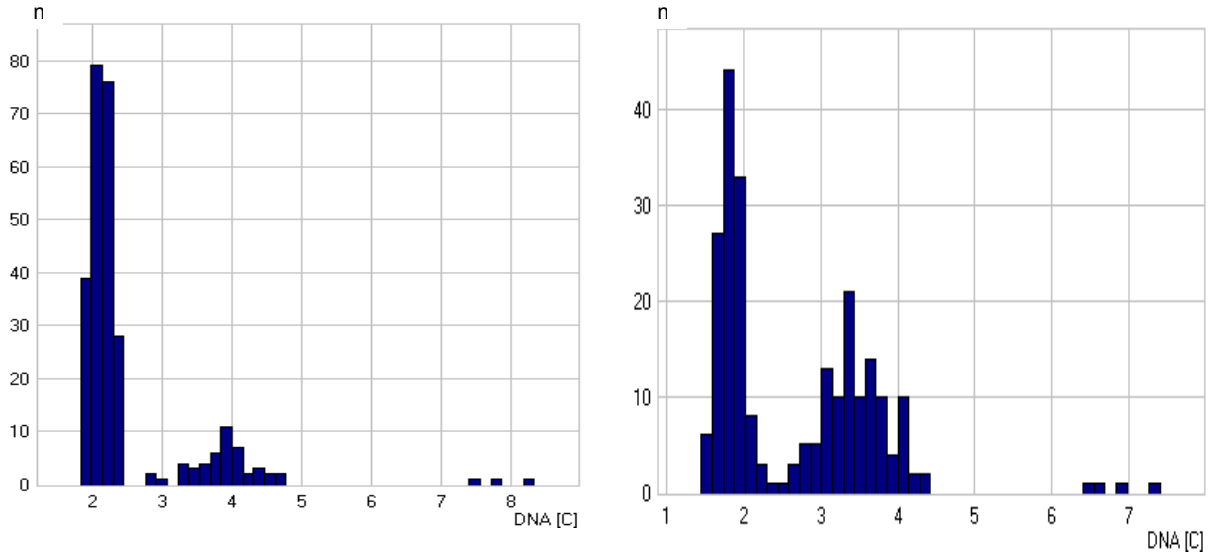

Figure 12. Case Nr. 1113/00. Small differences of DNA histograms resulting in different diagnostic interpretations: Cytology: ASCUS; DNA-euploidy left (peridiploid and peritetraploid stemlines, 9cEE=0), -aneuploidy right (peridiploid, peritetraploid stemlines and stemline at 3.3c, 9cEE=0).

4. DISCUSSION

4.1. Subjectivity and reliability of cytological/histological diagnoses

In cancer screening and diagnosis, cytological and histological investigations have greatly contributed to the fight against cancer worldwide. Despite its successfulness in screening and early detection of cervical precancerous lesions, diagnostic accuracy of histology and cytology in pathology of the uterine cervix still have their limits. Histomorphological and cytomorphological criteria for the diagnosis of different grades of precancerous intraepithelial squamous lesions (dysplasias) and even of in situ carcinoma of the uterine cervix are neither objectively defined nor internationally agreed upon [Vooijs 1991, Koss 1994, Kurman and Solomon 1994]. These facts explain the poor intra- and interobserver reproducibilities of histological and cytological diagnoses in pathology of the uterine cervix as reported by many authors. In the College of American Pathologists Interlaboratory Comparison Program in Cervicovaginal Cytology Study, Woodhouse and coworkers examined interobserver variability in the classification of SILs [Woodhouse et al. 1999]. The concordance rates of LSIL and HSIL diagnoses were 77.4% and 65.9%, respectively. Intra- and interobserver reproducibilities of conventional cytological diagnoses as reported in the literature are summarized in Table 13.

Table 13. Reported reproducibilities of conventional cervical cytology

Author	Number of cases	Participants	Intraobserver reproducibility	Interobserver reproducibility
Kern and Zivolic (1977)	112	2 cytopathologists, 13 cytotechnologists	75% - 100%	62.5% - 90.4%
Young et al. (1994)	20	5 cytopathologists	-	65%
Woodhouse (1999)	7228	2089 laboratories	-	65.9% - 77.4%
Smith et al. (2000)	10	49 cytopathologists	-	77.5%
Stoler et al. (2001)	4948	7 clinical centers and 4 quality control pathologists	-	43.0% - 47.1%

Results from a recent large-scale multicenter randomized prospective clinical trial involving well-trained pathologists, demonstrated a concordant cytological interpretation in only 43.0% of Atypical Squamous Cell of Undetermined Significance (ASCUS), in 42.6% of Low-grade Squamous Intraepithelial Lesion (LSIL) and in 47.1% of High-grade Squamous Intraepithelial Lesion (HSIL) cases [Stoler and Schiffman 2001]. In this study, diagnostic reproducibility for cervical biopsies and LEEP specimens with CIN1 were equivalent to cytological reproducibility. Forty-one percent of the biopsy specimens originally diagnosed as CIN1 were interpreted as negative by the QC group. The findings were similar for LEEP specimens. These findings suggest that the histological category of CIN1 is just a mixed entity as the cytological category of ASCUS. Using the CIN system and the modified Bethesda system for reporting of cervical colposcopic biopsy specimens, McCluggage et al. (1996) achieved an unweighted kappa of 0.20 and 0.30, respectively. The weighted kappa for both classifications was 0.36 [McCluggage et al. 1996]. Sherman and Paull (1993) reported the reproducibility of cytological and histological diagnoses of vaginal intraepithelial neoplasia in a series of 124 vaginal smears and 70 corresponding biopsies [Sherman and Paull 1993]. Consensus in cytopathological diagnoses was reached in 46% and in histopathological diagnoses in 55% of cases. In a European collaborative study on interobserver variation of histopathological grading of vulvar intraepithelial neoplasia, agreement was observed with overall weighted kappa of 0.65 [Pretti et al. 2000]. Exact agreement between two pathologists was observed in only 63.6% of paired readings. Only 5.0% of vulvar intraepithelial neoplasia grade 1 diagnosis was concordant in paired analysis. Using different morphological criteria, van Aspert van Erp et al. (1996) reported an interobserver agreement on endocervical columnar cell intraepithelial neoplasia of different grades from 74% to 94% [van Aspert van Erp et al. 1996]. A correct cytological diagnosis might be followed by an incorrect histological diagnosis and vice versa. Thus the reproducibility of diagnosis and grading dysplasias is still insufficient and needs to be improved.

4.2. Diagnostic accuracy of cytology, rates of progression/regression and histology/cytology correlation

Diagnostic accuracy of cervical cytology has often been studied. Reports showed a wide variation of sensitivity of Pap smears for the histological identification of at least severe dysplasias from 20% (Schneider et al 2000) to 67.9% (Wright et al 2002), 71% (Koss 1993) to 80% (Gay et al. 1985) [Gay et al. 1985, Koss 1993, Schneider et al. 2000, Wright et al. 2002]. Specificities of cervical cytology for correct identification of non-dysplastic or non-neoplastic lesions vary between 99.1% (Yobs et al 1985) and 87% (Wright et al 2002) [Yobs et al. 1985, Wright et al. 2002]. Positive predictive values largely depend on the definition of diagnostic input and output criteria. Commonly used input criteria are cytological diagnoses of ASCUS+ or LSIL+; output criteria are histological diagnoses of CINII+, CINIII+ or HSIL. Using ASCUS/LSIL as cytological input diagnosis and CIN III or higher as histological output diagnosis, our study yielded a PPV of 25.8%. Comparable values from the literature range from 13.0% (Soost and Baur, 1993) to 16.7% (Solomon et al, 2001). Many authors reported very high negative predictive values of cytology which were over 90% (Schneider et al. 2000: 97.54%, Ratnam et al. 2001: 98.1%) [Schneider et al. 2000, Ratnam et al. 2001]. In a recent study, Tuon et al. (2002) achieved an NPV of cytology of only 45% [Tuon et al. 2002].

In our study, the relatively low sensitivity of cytology alone is explained by the calculation based on the detection of high-grade CIN III or higher lesions in the follow-up (mean time: 2 months) and not on the detection of an abnormal finding. Using ASCUS/LSIL as input criteria, we obtained a higher PPV in comparison with those from previous studies, in which the input cytology was ASCUS+ (Manos et al. 1999, Solomon et al. 2001) or Munich group III/IIID. As discussed above, there is neither precise consensus on diagnostic criteria of different grades of cervical dysplasias resp. of squamous intraepithelial lesions. The application of different diagnostic criteria for identical diagnoses in cervical cytology might explain such differences.

Table 14 summarizes different published PPVs of cervical cytology and their respective definitions.

Table 14. Reported positive predictive values of atypical cervical cytological findings

Author	Criteria for calculation of PPV		PPVs of cytology (%)
	Input cytology	Output histology	
Soost and Baur (1990)	B	1	13.0
Manos et al. (1999)	A	2	12.9
Solomon et al. (2001)	A	2	16.7
This study	C	1	25.8
This study	A	1	32.7
This study	D	1	43.5

A: ≥ ASCUS

B: Groups III/III D (Munich Nomenclature II)

C: ASCUS/LSIL

D: ≥ HSIL

1: CIN III or invasive squamous carcinoma

2: CIN II/III or invasive squamous carcinoma

Poor interobserver reproducibility of cytological and histological diagnoses of cervical dysplasias also results in poor correlation of cytological and subsequent histological diagnoses on identical lesions. Cioc et al. (2002) demonstrated a surprisingly high concordance rate between diagnoses on Pap smears and cervical biopsies of 86.9%. The remaining discrepancy included cytology sampling errors (5.1%), biopsy sampling errors (6.8%) and cytology interpretation errors (1.2%) [Cioc et al . 2002]. Results from other studies yielded a cytology/histology correlation of only 69% in 576 LSIL cases (Dvorak et al. 1999) or 50% (Tuon et al. 2002) [Dvorak et al. 1999, Tuon et al. 2002]. Sampling errors still remain the principal source of discrepancies between cytology and histology.

Due to the definition of epithelial dysplasia as lesions with an increased probability to later develop into cancer, its diagnosis in an individual patient does not allow to predict progression or regression with sufficient accuracy. NPVs of only 45-55% for LSILs do not allow to return to a regular screening interval of one year as this would result in too many missed early cancers. PPVs of about 15% for LSILs do not allow to perform conizations on all these patients as this would result in overtreatment too often. Missed early diagnoses may result in patients who lack compliance. Finally conizations may often be performed without sufficient evidence for a progressive behavior of an individual lesion.

Reported rates of progression of low-grade to high-grade SILs or invasive carcinomas are also quite different. A major limitation of current cohort studies in developed countries is that treatment following the diagnosis of cervical cancer precursors interrupts the natural history and makes it impossible to determine whether the lesions would have progressed or regressed if left untreated. In a follow-up series of 2845 Pap smears with mild or moderate dysplasia, 64% were regressive, 20% persistent and 13% progressive to squamous cell carcinoma [Soost and Baur 1990]. In another study on 5894 women with mild cervical dysplasia, the regression, persistence and progression rates were 47%, 37% and 16%, respectively [Wright et al. 2002]. In an historical cohort including more than 17,000 women with diagnoses of different grades of dysplasia, Holowaty et al. (1999) conclude that “both mild and moderate dysplasias were more likely to regress than to progress”. The risk of progression from mild to severe dysplasia or worse in that study was only 1% per year, but the risk of progression from moderate to marked dysplasia was 16% within 2 years and 25% within 5 years [Holowaty et al. 1999]. In our study, 34.0% (33/97) of ASCUS/LSIL cases were histologically diagnosed as HSILs/invasive carcinomas after the mean time of two months. This comparably high rate may also be explained by the application of cytodiagnostic criteria rather specific for the prospective behavior of the lesions.

Table 15. Rate of progression ad regression of cervical dysplasias

Change of state	Author(s)	Means of detection at start / end	Follow-up duration	Nr. of patients	% Transition
Progression					
Mild to severe or worse	Jones et al. (1992)	Cytology/histology	Approx. 2 years	214	23
	Campion et al. (1987)	Cytology/histology	20 months	100	25
	Nasiell et al. (1986)	Cytology/histology	48 months	555	16
	Flannelly et al. (1994)	Cytology/cytology	0–24 months	621	25
	Carmichael and Maskens (1989)	Histology/histology	24 months	235	7
Moderate to severe or worse	Nasiell et al. (1983)	Cytology/histology	78 months	894	30
	Flannelly et al. (1994)	Cytology/histology	0–24 months	281	53
	Flannelly et al. (1994)	Cytology/cytology	0–24 months	281	47
	Galvin et al. (1955)	Histology/histology	Up to 4 years	63	24
	Syrjanen et al. (1992)	Histology/histology	72 months	68	21
Regression					
Mild to normal	Flannelly et al. (1994)	Cytology/histology	24 months	621	30
	Flannelly et al. (1994)	Cytology/cytology	24 months	621	30
	Campion et al. (1987)	Cytology/cytology	20 months	100	25
Moderate to normal	Flannelly et al. (1994)	Cytology/histology	24 months	281	21
	Flannelly et al. (1994)	Cytology/cytology	24 months	281	17
	Nasiell et al. (1983)	Cytology/cytology	78 months	894	54

4.3. Latency period

As it may take an interval period from months up to ten years for a mild or moderate squamous dysplasia cytologically diagnosed on a Pap smear to develop into histologically proven cancer, simultaneously obtained histological diagnoses may not be

adequate to decide correctly on the positive predictive value of cytological diagnoses or adjuvant methods. In a long-term follow-up study on women with ASCUS diagnoses, Emerson et al. (2002) have shown that the mean time until detection between ASCUS and SIL res. HSIL was 13.5 res. 19.9 months, respectively [Emerson et al. 2002]. As Sudbø et al. (2001) have shown, 10% of all histologically diagnosed oral squamous dysplasias developed into invasive carcinomas after one year. This rate increased to 25% after five years. Applying DNA-ICM, the progression rate based on the detection of DNA-aneuploidy was also 10% after one year but increased significantly to 90% after five years [Sudbø et al. 2001]. In our study, the peak frequency of ASCUS/LSIL diagnoses has been observed in women 26-30 years old, while that of HSIL cases has been observed in women 31-35 years old. Despite the number of cases was not high, these data again may indirectly provide information about the duration of progression from low-grade to high-grade SIL.

4.4. Diagnostic “golden standard”

Therefore the question arises if one should generally accept simultaneously obtained and insufficiently reproducible routine histology as a “gold standard” for cytological diagnoses? In an editorial article recently published in Journal of Pathology, Bosman stated that “ … the gold standard for dysplasia classification could be the correlation of molecular parameters with clinical outcome, regardless of histopathological classification” [Bosman 2001]. To assure the accuracy, only quality-assured histological diagnosis obtained after an adequate follow-up period should be accepted as a “gold standard” for cytological diagnoses including adjuvant methods.

4.5. Adjuvant methods

The question arises if there are adjuvant tests with a sufficiently high negative predictive value to allow the avoidance of unnecessary surgical interventions on the one hand and with a sufficiently high positive predictive value to early identify unequivocally progressive lesions, which should be removed ?

Currently, different adjuvant methods are applied to increase diagnostic accuracy in cervical cancer screening, especially in cases of ASCUS or LSIL, like colposcopy, HPV-DNA-typing and DNA-image cytometry. HPV DNA detection and typing in cytological smears from the uterine cervix using Polymerase Chain Reaction or Hybrid Capture test yielded high sensitivities but low specificities. In the study of Zielinski et al. (2001), the sensitivity of a positive high-risk HPV test for CIN 2/3 was 96.3%, but specificity only 60.2%. The positive and negative predictive values were 20.6% and 99.3%, respectively [Denise Zielinski et al. 2001]. Schneider et al. (2001) found a PPV of high-risk HPV testing of 29.1% and a NPV of 99.9% [Schneider et al. 2000]. Most calculations on diagnostic validity of HPV testing are based on the detection of cytological and/or histological high-grade lesions. The reported PPVs of high-risk HPV detection for high-grade lesions are mostly below 30%. This may also lead to uncertainty on both patients and physicians side concerning the prospective behavior of a given cervical/vaginal lesions. Recently, the initial results of the ASCUS/LSIL Triage Study (ALTS) have become available. These results confirm that HPV DNA testing is not useful for determining which women with LSIL diagnoses should be referred to colposcopy because 83% of the women diagnosed as having LSIL were also positive with high-risk HPV DNA using Hybrid Capture II testing [Anonym 2000]. In another study, among women diagnosed with ASCUS, HPV DNA testing identified more women with biopsy-confirmed high-grade SIL than did a repeat liquid-based cytology, but both triage methods would have referred equal numbers of women to colposcopy [Solomon 2001].

Most human neoplasms reveal light microscopically detectable chromosomal aberrations [Heppner and Miller 1998]. Chromosomal aneuploidy is defined as numerical and/or structural aberrations and may be used as a marker for neoplasia if it differs from the rest of cells in an individual patient [Böcking 1995]. Chromosomal aneuploidy has been found in most cervical squamous carcinomas [Murty et al. 1988, Norming et al. 1992], even in HSILs [Fahmy et al. 2002]. A recent study using in situ hybridization to analyze cervical intraepithelial neoplasias has provided sufficient evidence that 1, 7 and X aneusomies in squamous intraepithelial lesions are associated with progression towards cervical carcinoma [Bulten et al. 1998]. Aberrations of chromosome 1 have been only found in those SILs, which progressed to invasive cancer [Murty et al. 1988].

DNA-aneuploidy is the cytometric equivalent of chromosomal aneuploidy [Böcking 1995]. In a series of 276 Pap smears, 73.2% of DNA-aneuploid mild or moderate cervical dysplasias developed into carcinoma in situ or higher lesions, the histological diagnosis remained dysplastic in 17% [Böcking et al. 1986]. Mean time interval between diagnostic DNA-ICM and histological follow-up was up to three years. For the detection of invasive lesions, DNA-aneuploidy had a sensitivity of 98.6, and a PPV of 86.4%.

DNA-image cytometry has been repeatedly proposed as an adjunctive diagnostic and prognostic method for cervical intraepithelial lesions and invasive cervical cancer [Fu 1981, Böcking et al. 1984, Böcking et al. 1986, Chatelain et al. 1989, Kashyap et al. 1990, Hering et al. 2000, Bollmann et al. 2001, Grote et al. 2001]. Many authors reported high positive predictive values for the development of in situ or invasive cancer out of mild/moderate cervical dysplasias with proven DNA-aneuploidy; varying from 84% to 100% [Böcking et al. 1986, Chatelain et al. 1989, Kashyap et al. 1990, Bollmann et al. 1996, Nenning et al. 1997]. Yet all of these studies were retrospectively designed. Intervals between detection of DNA-aneuploidy and histological follow-up were one to three years [Böcking 1986]. According to the statement of the International Consensus Conference on the Fight Against Cervical Cancer task force #18, indication of DNA-ICM is “the identification of prospectively malignant cells in SILs and ASCUS” [Hanselaar et al. 2001].

4.6. Biological background of the relationship between aneuploidy and cancer pathogenesis

The scientific debate on different models of carcinogenesis is still going on. Chromosomal and thus DNA-aneuploidy seem to play a crucial role in cancer development. The hypothesis that aneuploidy itself may be a cause of cancer was first proposed by Bovery at the beginning of the 20th century [Bovery 1914]. During the last decades, this hypothesis has been ignored since most efforts have been centred on the hypothesis of somatic gene mutations. Increasing number of recently published scientific papers on the relationship between aneuploidy and cancer pathogenesis helped to understand more about this concern. Using a molecular cytogenetic approach termed Comparative Genomic Hybridization, Heselmeyer et al. (1996) found that the

gain of chromosome 3q that occurs in HPV 16-infected, aneuploid cells was a pivotal genetic aberration at the transition from severe dysplasia/CIS to invasive cervical carcinoma [Heselmeyer et al. 1996]. A codiscoverer of viral oncogenes in the 1970s, Peter Duesberg, recently stated that “the correlation between aneuploidy and cancer was very solid”, “it is present in nearly all solid cancers”, “never in normal/reactive cells”, “it explains the growing list of nonmutagenic carcinogens and why human oncogenes cannot turn human cells into cancer cells” [Webb 2001]. Duensing and coworkers in a series of experimentation on genomic instability have found that high-risk HPV may cause aneusomy through two mechanisms [Duensing et al. 2001a, Duensing et al. 2001b]. One is through the ability of viral protein E7 to uncouple the duplication of the centrosome from the cell division cycle, probably by targeting the pRb pathway. The second mechanism would be the disturbing effect of E6 on the checkpoint function of the cell cycle by degrading p53. Recently, Bollmann and coworkers have found that ASCUS cases with DNA rare events >9cEE were found exclusively in combination with high-risk HPV infection [Bollman et al. 2003]. These findings supports the concept (Böcking et al. 1984, 1986, 1995) that DNA-aneuploidy represents an objective, very early and highly specific marker of (prospective) neoplasia and that its detection in epithelial dysplasias identifies those lesions that most likely will progress to histologically manifest cancer. The finding of a relatively high PPV of 50.0% and the NPV of 94.7% for DNA aneuploidy/euploidy in cervical intraepithelial lesions after two months of follow-up is in accordance with this concept.

4.7. Standardization of diagnostic DNA-ICM

Up to 1995 DNA-image cytometry lacked international standardization of measurement performance and diagnostic interpretation of data. This has changed since the European Society of Analytical Cellular Pathology ESACP has published its “Consensus Reports for Standardized Diagnostic DNA-Image Cytometry” [Böcking et al. 1995, Haroske et al. 1997, Giroud et al. 1997, Haroske et al. 2001]. These papers did not only define the meaning of frequently used terms like DNA-euploidy, -polyploidy, -aneuploidy, they also provided standardized algorithms for the calculation of diagnostically or prognostically relevant indices of DNA-distribution. They further defined minimum performance standards for

measurements and also algorithms for diagnostic data interpretation. Our measurements were in strict accordance with these standards: e.g. more than 30 reference cells were measured, coefficients of variation of reference cell population always were below 5% (mean ± SD: 2.94 ± 0.71), coefficients of correlations between nuclear areas and integrated optical densities of reference cells always were below r=0.4. This was mainly achieved applying a software correction of glare- (at 2%) and diffraction errors. Internal reference cells were only measured within the same slides and nearby the abnormal epithelial cells under analysis. DNA aneuploidy was only assumed if cells with a DNA-content >9c and/or DNA-stemlines with modal values (<1.8c or >2.2c) or (<3.6c or >4.4c) were observed. Occurrence of an additional DNA-stemline at 4c with single values up to 8c was defined as DNA-polyploidy.

Laser scanning DNA cytometry is a relatively new method for the analysis of cytological samples and still under investigation. The method performed the analysis of large cell population of more than 10,000 per smear and was able to detect rare events Bollman et al. 2003. Since the method itself is based on the automated scanning principle, i.e. normal cells were also measured and the number of cells to be scanned is too high, the detection of DNA-aneuploid stemlines becomes more difficult or even impossible. In contrast, DNA-ICM with a controlled visual sampling principle focused on morphologically relevant cells may provide the detection of rare evens as well as the identification of aneuploid stemline(s).

4.8. Geographic error

Koss (1994) pointed out that low-grade intraepithelial lesions might occur peripheral to high-grade lesions, carcinoma in situ or adjacent to invasive cancer. Yet the latter lesions may not be represented in a given smear, which only contains cells of the former (geographic error) Koss 1994. Biopsies of small, discrete lesions can only be correctly performed under colposcopic visualization. This procedure might not have been generally performed in the patients investigated in this study. Geographic errors of cell sampling may also explain for the discrepancies between cytology, DNA-ICM and histology in our series.

Another source of discrepancies may be processing errors of histological workup of tissues obtained by cone- or LEEP biopsy. Conization specimens might not have been worked up completely in the routine. Geographic errors may also explain the high rate of seemingly false positive cytological diagnoses, which at least in part may represent false negative diagnoses of histology. The unexpectedly and unbelievably high rate of DNA aneuploidy in histological WNL cases may be interpreted as false negative histological diagnoses as a consequence of missed identification of CIN III, due to geographic errors or due to incomplete tissue workup. Another explanation of this diagnostic discrepancy would be too flat conizations, not comprising tumor cells as identified by DNA-cytometry/cytology.

4.9. Diagnostic accuracy of DNA-ICM and its improvements

As shown in table 3, the rate of DNA-aneuploidy in our series increased significantly with the increasing severity of cytological lesions, from 40.3% in LSILs over 90.7% in HSILs to 100% in invasive cancers (χ^2= 59.26, p=0). Yet, there was no difference in the observed frequency of DNA-aneuploidy between ASCUS and LSIL smears (40% and 40.3%, respectively). Since ASCUS smears may contain a wide spectrum of histologically defined lesions from benign reactive changes to invasive cancer [Crabtree et al. 2002], DNA-ICM may find such a high rate of aneuploidy. Related to histological follow-ups of 135 of our cases, the rate of DNA-aneuploidy also increased significantly from CIN I (66.7%), CIN II (83%), CIN III (93.1%) to invasive cancers (100%) (χ^2=9.91, p=0.02). DNA image cytometry in this study increased the sensitivity of suspicious Pap smears to unequivocally identify histologically proven in situ or invasive cervical cancer by 34.7% (from 25.9% to 60.6%) and the PPV by 24.2% (from 25.8% to 50.0%). Since mild to moderate dysplasias or LSILs may need years to progress to invasive carcinomas, prospective studies with longer follow-ups are needed to further confirm the high predictive value of DNA-ICM.

As presented in table 8, the sensitivity of DNA-ICM increased significantly from 60.6% in ASCUS/LSIL group to 92.9% for all ASCUS or higher lesions (χ^2= 24.42, p=0); whereas their NPVs were still unchanged at a very high level. The PPVs of DNA-

aneuploidy in different groups were stable, even if HSILs and invasive cancers were included in the calculation.

The classification of DNA histograms into prognostic classes like peridiploid, peritetraploid, X-ploid or multiploid has been seldom applied for clinical purposes. Their prognostic relevance varies from one type of solid tumor to the other [Haroske et al .1998]. In our study, these classes were correlated with the follow-up histology according to the modified Bethesda system. An increasing tendancy of multiploid stemlines and a descreasing tendency of peritetraploid stemlines with the increasing severity of the lesions were observed. Unfortunately, no statistical significance was found, maybe due to the small number of cases.

The mean interval between cytological/DNA-ICM diagnosis and histological follow-up in our study was only two months (range from 1 to 26 months). Yet, that in our previous study was at least one year [Böcking et al. 1986]. This difference could explain the comparably low PPV found in our study for the prediction of CIN III lesions or invasive carcinomas by the detection of DNA-aneuploidy in ASCUS or LSILs. After a longer interval of at least one year the PPV could also be more than 90% as reported in other studies and by Sudbø et al. (2001) for oral dysplasias [Böcking and Motherby 1999, Sudbø et al. 2001].

Due to its objectivity and diagnostic validity, DNA-ICM has also been applied in the diagnosis of other gynecological malignancies. It has been found to be useful in the differentiation between malignant and nonmalignant endocervical epithelium as well as in the differentiation between endometrial hyperplasias and adenocarcinoma [Biesterfeld et al. 2001a, Biesterfeld et al. 2001b]. Gschwendtner et al. (1998) demonstrated the contribution of DNA-ICM in the differentiation between partial and complete molar pregnancies, which is difficult in cases of early molar pregnancy [Gschwendtner et al. 1998]. DNA-ICM has also been proposed as a means of quality control in cervical cytology, especially in the case of discrepancies between cytological and histological diagnoses. In a series of 170 seemingly false positive routine cervical smears, DNA-aneuploidy was found in 47 cases without histological explanation [Nenning et al. 1995]. Using DNA-aneuploidy as a solid

marker of malignancy in uterine epithelia, the authors classified these cases as histologically false negative and not as false positive cytological diagnoses.

Beside its diagnostic application, DNA image cytometry has been used as a prognostic tool in cervical cancers. Results from a recent study of Grote et al. (2001) on stage IB/II cervical carcinomas confirmed the high prognostic value of standardized DNA image cytometry and showed that a DNA stemline ploidy above 2.2c is correlated with an unfavorable prognosis [Grote et al. 2001].

DNA-ICM may also contribute to avoid costs resulting from unnecessary and repeated cytological or clinical controls as well as of surgical procedures in DNA-euploid ASCUS/LSIL cases. If DNA-aneuploidy was detected, the indication for a surgical intervention (conization, LEEP-conization…) should be assumed, also in order to avoid the costs occurring if advanced cancers have to be treated. DNA-ICM is paid by German health insurance companies in cytological doubtful lesions (about 50€ per measurement).

4.10. Reproducibility of DNA-ICM measurements

In histological cancer diagnosis, typing and grading of malignancy, interobserver agreement was also repeatedly found to be insufficient. van Aspert van Erp et al. reported an overall agreement in diagnosis of endocervical adenocarcinomas of 76% [van Aspert van Erp et al. 1996]. A study of the Gynecologic Oncology Group investigating a histological grading system in stage I endometrial adenocarcinomas and demonstrated a moderate interobserver reproducibility of kappa = 0.49 [Zaino et al. 1994]. Another study also on endometrial adenocarcinomas showed a kappa value of 0.65 for overall diagnoses [Nielsen et al. 1991]. Harris et al. (2003) compared histological typing on core biopsies with that on therapeutic excisions in 500 invasive breast carcinoma patients [Harris et al. 2003]. The correlation of the four most frequent tumor types (classical lobular, tubular, tubular mixed, ductal carcinomas/no special type) between both types of tissue specimens was 74%. Fenger et al. (2000) reported an intra- and interobserver kappa of 0.61 and 0.47, respectively, for the histological classification of anal squamous carcinoma into six

WHO-subtypes [Fenger et al. 2000]. Thus histological diagnoses were not highly reproducible, even in the cancer diagnosis and grading of malignancy.

Since DNA-ICM had an impact on diagnosis and prospective behavior of precancerous lesions and invasive cervical cancers, its application can help to improve the reproducibility of grading cervical dysplasias and invasive cancers. DNA-ICM is somewhat time-consuming and needs cytological expertise. Its advantages are that it may be performed retrospectively, irrespective of the type of preceding fixation and staining and even on archived slides.

The comparably high value of interobserver agreement of 94.1% achieved in this study is at least 20% higher than the rates reported in the literature for subjective histological or cytological diagnoses of cervical dysplasias. Explanations for this high value are on the one hand the high standardization of DNA measurements and diagnostic data interpretation and the subjectivity of the method on the other hand. In contrast to the subjective assessment of conventional diagnostic methods based on morphology, DNA-histogram interpretations are based on well-defined algorithms, which already proved its diagnostic validity in cervical pathology.

The second measurement did not yield false positive findings of DNA-aneuploidy. It rather reveals missed 9cEE in 9 cases. As these rare events can sometime only be detected by thorough screening of the slides, they may be missed if these are not screened carefully enough in a routine setting. To avoid seldom-false negative results, slides to be measured should be carefully screened for dark and large nuclei.

In few cases, sampling of 300 abnormal cells may not be representative enough to detect aneuploid stemlines. Measuring more than 300 cells may increase the ability of the method to detect diagnostically relevant DNA-aneuploidy. Since the sampling of reference cells played the crucial role in scaling procedure, they should be measured nearby the respective analysis cells to avoid the scaling errors due to staining inhomogeneity.

Through the data from our study, DNA-ICM thus proved its diagnostic validity in identification of progressive cervical intraepithelial lesions as well as its reliability. In order to achieve more accurate data, larger investigations with longer follow-up intervals and standardized cytological/histological workup are needed.

5. CONCLUSIONS

On the basis of the results presented in this study, we conclude that:

- DNA aneuploidy has a PPV of 50.0% for CIN III or higher lesion after two months in ASCUS and LSIL cases. This may allow such aneuploid lesions to be removed by conization or LEEP.

- DNA euploidy has a high NPV of 94.7% in cervical smears with ASCUS and LSIL diagnoses. This allows the respective patients to be returned to normal screening intervals.

- DNA-ICM contributed to increase the sensitivity of conventional cytological ASCUS and LSIL diagnoses for detection of CIN III or higher findings by 34.7%. Combined with the high interobserver reproducibility of 94.1%, this qualifies DNA-ICM as a useful and reliable tool to raise diagnostic accuracy of cervical cytology for early identification of cervical cancer as well as a suited tool for quality control in tumor cell doubtful and positive cytological diagnoses.

TABLES AND FIGURES

ABBREVIATIONS

9cEE	9c Exceeding Events
ASCUS	Atypical Squamous Cell of Undetermined Significance
BCC	Benign Cellular Changes
c	content
CIN	Cervical Intraepithelial Neoplasia
CIS	Carcinoma in situ
DNA	Desoxyribonucleotid acid
DNA-ICM	DNA image cytometry
ESACP	European Society of Analytical Cellular Pathology
HPV	Human Papillomavirus
HSIL	High-grade Squamous Intraepithelial Lesion
IOD	Integrated Optical Density
LSIL	Low-grade Squamous Intraepithelial Lesion
NPV	Negative Predictive Value
PPV	Positive Predictive Value
SIL	Squamous Intraepithelial Lesion
STL	Stemline
WNL	Within normal limits

REFERENCES

1. Adam E, Berkova Z, Daxnerova Z, Icenogle J, Reeves WC, Kaufman RH (2000). Papillomavirus detection: Demographic and behavioral characteristics influencing the identification of cervical disease. Am J Obstet Gynecol, 182(2):257-264.

2. ALTS Group (2000). Human papillomavirus testing for triage of women with cytologic evidence of low-grade squamous intraepithelial lesions: Baseline data from a randomised trial. J Nat Cancer Inst, 92:397-402.

3. Anonym (2000). Human papillomavirus testing for triage of women with cytologic evidence of low-grade squamous intraepithelial lesions: baseline data from a randomized trial. The Atypical Squamous Cell of Undetermined Significance/ Low-grade Squamous Intraepithelial Lesions Triage Study (ALTS) Group. J Nat Cancer Inst, 92:397-402.

4. Biesterfeld S, Gerres K, Fischer-Wein G, Böcking A (1994). Polyploidy in non-neoplastic tissues. J Clin Pathol, 47:38-42.

5. Biesterfeld S, Veuskens U, Schmitz FJ, Amo-Takyi B, Böcking A (1996). Interobserver reproducibility of immunocytochemical estrogen- and progesterone receptor status assessment in breast cancer. Anticancer Res, 16(5A):2497-2500.

6. Biesterfeld S, Leitloff M, Rath W, Schröder W (2001a). DNA image cytometry in the differential diagnosis of endometrial hyperplasia and adenocarcinoma. Anal Quant Cytol Hist, 23(2):123-128.

7. Biesterfeld S, Reus K, Bayer-Pietsch E, Mihalcea AM, Böcking A (2001b). DNA image cytometry in the differential diagnosis of endocervical adenocarcinoma. Cancer Cytopathology, 93(2):160-164.

8. Böcking A, Adler CP, Common HD, Hilgarth M, Granzen B, Auffermann W (1984). Algorithm for DNA cytophotometric diagnosis and grading of malignancy. Anal Quant Cytol Hist, 6:1-7.

9. Böcking A, Hilgarth M, Auffermann W, Hack-Werdier C, Fischer-Becker D, Kalkreuth von G (1986). DNA-cytometric diagnosis of prospective malignancy in borderline lesions of the uterine cervix. Acta Cytol, 30:608-615.

10. Böcking A, Chatelain R, Biesterfeld S, Noll E, Biesterfeld D, Wohltmann D, Goecke C (1989). DNA grading of malignancy in breast cancer. Prognostic validity, reproducibility and comparison with other classifications. Anal Quant Cytol Histol, 11(2):73-80.

11. Böcking A (1995a). DNA Measurements. When and Why? In: Compendium on quality assurance, proficiency testing, and workload limitations. Wied GL, Keebler CM, Rosenthal DL, Schenk U, Somrak TM, Vooijs GP (Eds). Tutorials of Cytology, Chicago, Illinois, USA, 170-188.

12. Böcking A, Giroud F, Reith A (1995b). Consensus report of the ESACP task force on standardization of diagnostic DNA image cytometry. Anal Cell Pathol, 8:67-74.

13. Böcking A (1998a). Abklärung plattenepithelialer Dysplasien mittels DNA-Bildzytometrie. Deutsches Ärzteblatt, 95:A658-663.

14. Böcking A (1998b). Biologische Grundlagen und Anforderungen an die Methodik der statischen DNA-Zytometrie. In: Fortschritt und Fortbildung in der Medizin, Bd. 21, Deutscher-Ärzte-Verlag, 21-40.

15. Böcking A, Motherby H (1999). Abklärung zervikaler Dysplasien mittels DNA-Bild-Zytometrie. Pathologe, 20:25-33.

16. Böhm N, Sandritter W (1975). DNA in human tumors: a cytophotometric study. Springer-Verlag, Berlin, Heidelberg, New York.

17. Bollen LJ, Tjong-A-Hung SP, van der Velden J, Brouwer K, Mol BW, ten Kate FJ, ter Schegget J (1997). Human papillomavirus deoxyribonucleic acid detection in mildly or moderately dysplastic smears: a possible method for selecting patients for colposcopy. Am J Obstet Gynecol, 77(3):548-553.

18. Bollmann R, Böcking A (1996). Prognostische Validität der DNA-Bild-Zytomtrie bei Dysplasien in Gebärmutterhalsabstrichen. Verh Dtsch Ges Path, 80:577.

19. Bollmann R, Bollmann M, Henson DE, Bodo M (2001). DNA cytometry confirms the utility of The Bethesda System for classification of Papanicolaou smears. Cancer Cytopathology, 93(3):222- 228.

20. Bollmann R, Méhes G, Torka R, Speich N, Schmitt C, Bollmann M (2003). Determination of features indicating progression in atypical squamous cells with undetermined significance. Cancer Cytopathology, EarlyView. http:// www3.interscience.wiley.com/cgibin/fulltext/101525802/. Accessed 15th Feb. 2003.

21. Bosman FT (2001). Dysplasia classification: pathology in disgrace? J Pathol, 194:143–144.

22. Boveri T (1914). Zur Frage der Entstehung maligner Tumoren. Verlag von Gustav Fischer, Jena.

23. Bulten J, Poddighe PJ, Robben JC, Gemmink JH, deWilde PC, Hanselaar GAGJM (1998). Interphase cytogenetic analysis of cervical intraepithelial neoplasia. Am J Pathol, 152:495-503.

24. Campion MJ, McCance DJ, Cuzick J, Singer A (1986). Progressive potential of mild cervical atypia: prospective cytological, colposcopic and virologic study. Lancet ii:237-240.

25. Campion MJ, Singer A, Mitchell HS (1987). Complacency in diagnosis of cervical cancer. BMJ, 294:1337-1339.

26. Carmichael JA, Maskens PD (1989). Cervical dysplasia and human papillomavirus. Am J Obstet Gynecol ,160:916-918.

27. Chatelain R, Schmunck T, Schindler EM, Schindler AE, Böcking A (1989a). Diagnosis of prospective malignancy in koilocytic dysplasia of the cervix with DNA cytometry. J Reprod Med, 34:505-510.

28. Chatelain R, Willms A, Bisterfeld S, Auffermann W, Böcking A (1989b). Automated Feulgen staining with a temperature controlled staining machine. Analyt Quant Cytol, 11:211-217.

29. Cioc AM, Julius CJ, Proca DM, Tranovich VL, Keyhani-Rofagha S (2002). Cervical biopsy/cytology correlation data can be collected prospectively and shared clinically. Diagn Cytopathol, 26(1):49-52.

30. Clavel C, Masure M, Bory JP, Putaud I, Mangeonjean C, Lorenzato M, Gabriel R, Quereux C, Birembaut P (1999). Hybrid capture II-based human papillomavirus detection, a sensitive test to detect in routine high-grade cervical lesions: a preliminary study on 1518 women. Br J Cancer, 80:1306-1311.

31. Crabtree D, Unkraut A, Cozens D, Smith T, Lucas C, Pennington D, Chaney L, Nestok B, Robinson-Smith T, Yassin R (2002). Role of HPV testing in ASCUS: a cytologic-histologic correlation. Diagn Cytopathol, 27(6):382-386.

32. Cserni G (1999). Reproducibility of a diagnosis of invasive lobular carcinoma. J Surg Oncol, 70(4):217-221.

33. Cuzik J, Szarewski A, Terry G, Ho L, Hanby A, Maddox P, Anderson M, Kocjan G, Steele ST, Guillebaud J (1995). Human papillomavirus testing in primary cervical screening. Lancet, 45:1533-1536.

34. Cuzick J, Beverley E, Ho L, Terry G, Sapper H, Mielzynska I, Lorincz A, Chan WK, Krausz T, Soutter P (1999). HPV testing in primary screening of older women. Br J Cancer, 81:554-558.

35. Cuzick J (2000). Human papillomavirus testing for primary cervical cancer screening [editorial]. JAMA, 283(1);108-109.

36. de Vet HC, Knipschild PG, Schouten HJ, Koudstaal J, Kwee WS, Willebrand D, Sturmans F, Arends JW (1990). Interobserver variation in histopathological grading of cervical dysplasia. J Clin Epidemiol, 43(12):1395-1398.

37. Denise Zielinski G, Snijders PJ, Rozendaal L, Voorhorst FJ, Runsink AP, de Schipper FA, Meijer CJ (2001). High-risk HPV testing in women with borderline and mild dyskaryosis: long-term follow-up data and clinical relevance. J Pathol, 195(3):300-306.

38. Duensing S, Duensing A, Crum CP, Muenger K (2001a). Human Papillomavirus type 16 E7 oncoprotein-induced abnormal centrosome synthesis is an early event in the evolving malignant phenotype. Cancer Research, 61:2356–2360.

39. Duensing S, Duensing A, Flores ER, Do A, Lambert PF, Munger K (2001b). Centrosome abnormalities and genomic instability by episomal expression of human papillomavirus type 16 in raft cultures of human keratinocytes. J Virol, 75(16):7712-7716.

40. Duesberg P, Rausch C, Rasnick D, Hehlmann R (1998). Genetic instability of cancer cells is proportional to their degree of aneuploidy. Proc. Natl. Acad. Sci. USA, 95:13692–13697.

41. Dvorak KA, Finnemore M, Maksem JA (1999). Histology correlation with atypical squamous cells of undetermined significance (ASCUS) and low-grade squamous intraepithelial lesion (LSIL) cytology diagnoses. Diagn Cytopathol, 21(4):292-295.

42. Emerson RE, Puzanov A, Brunnemer C, Younger C, Cramer H (2002). Long-term follow-up of women with atypical squamous cells of undetermined significance (ASCUS). Diagn Cytopathol, 27(3):153-157.

43. Fahmy M, Skacel M, Brainard J, Biscotti C, Waterman J, Dolar S, Procop G, Yen-Lieberman B, Tubbs RR (2002). Chromosomal alterations in Low- and High-Grade Squamous Intraepithelial Lesions (LSIL and HSIL) of the uterine cervix detected by multicolor fluorescence in situ hybridization (FISH). Acta Cytol, 46(5):928.

44. Fenger C, Frisch M, Jass JJ, Williams GT, Hilden J (2000). Anal cancer subtype reproducibility study. Virchows Arch, 436:229–233.

45. Feulgen R, Rosssenbeck H (1924). Mikroskopisch-chemischer Nachweis einer Nucleinsäure vom Typus der Thymonucleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. Hoppe Seylers Z Physiol Chem, 135:203-248.

46. Flannelly G, Anderson D, Kitchener HC, Mann EM, Campbell M, Walker F, et al. (1994). Management of women with mild and moderate cervical dyskariosis. BMJ, 308:1399-1403.

47. Fu YW, Reagen JW, Fu AS, Janiga KE (1982). Adenocarcinoma and mixed carcinoma of the uterine cervix, 2: prognostic value of nuclear DNA analysis. Cancer, 49:2572-2577.

48. Galvin GA, Jones HW, Te Linde RW (1955). The significance of basal-cell hyperactivity in cervical biopsies. Am J Obstet Gynecol, 70:808-821.

49. Gay JD, Donaldson LD, Goellner JR (1985). False-negative results in cervical cytologic studies. Acta Cytol, 29:1043-1046.

50. Giroud F, Haroske G, Reith A, Böcking A (1998). 1997 ESACP consensus report on diagnostic DNA image cytometry. Part II: Specific recommendations for quality assurance. Anal Cell Pathol, 17:201-207.

51. GLOBOCAN 2000 (2001). Cancer Incidence, Mortality and Prevalence Worldwide, Version 1.0. IARC Cancer Base No. 5. IARC Press, Lyon.

52. Göppinger A, Freudenberg N, Ross A, Hillemans HG, Hilgarth M (1986). The prognostic significance of the DNA distribution in squamous-cell carcinomas of the uterine cervix. Anal Quant Cytol Histol, 8(2):148-151.

53. Grote HJ, Friedrichs N, Pomjanski N, Guhde HF, Reich O, Böcking A (2001). Prognostic significance of DNA cytometry in carcinoma of the uterine cervix FIGO stage IB and II. Anal Cell Pathol, 23:97-105.

54. Gschwendtner A, Neher A, Kreczy A, Muller-Holzner E, Volgger B, Mairinger T (1998). DNA ploidy determination of early molar pregnancies by image analysis: comparison to histologic classification. Arch Pathol Lab Med, 122(11):1000-1004.

55. Hanselaar AG, Böcking A, Gundlach H, Palcic B, Markovic N, Patterson B, Ueda M (2001). Summary statements of Task Force No. 8: Quantitative cytochemistry (DNA and molecular biology). Acta Cytol, 4:499-501.

56. Haroske G, Dimmer V, Meyer W, Kunze KD (1997). DNA histogram interpretation based on statistical approaches. Anal Cell Pathol, 15(3):157-173.

57. Haroske G, Giroud F, Reith A, Böcking A (1998). 1997 ESACP consensus report on diagnostic DNA image cytometry. Part I: Basis considerations and recommendations for preparation, measurement and interpretation. Anal Cell Pathol, 17:189-200.

58. Haroske G, Baak JP, Danielsen H, Giroud F, Gschwendtner A, Oberholzer M, Reith A, Spieler P, Böcking A (2001). Fourth updated ESACP consensus report on diagnostic DNA image cytometry. Analyt Cell Pathol, 23:89-95.

59. Harris GC, Denley HE, Pinder SE, Lee AH, Ellis IO, Elston CW, Evans A (2003). Correlation of histologic prognostic factors in core biopsies and therapeutic excisions of invasive breast carcinoma. Am J Surg Pathol, 27(1):11-15.

60. Heinzl S, Szalmay G, Jochum L, Roemer V (1982). Observations on the development of dysplasia. Acta Cytol 26:453-456.

61. Heppner GH, Miller FR (1998). The cellular basis of tumor progression. Int Rev Cytol, 177:1-56.

62. Hering B, Horn LC, Nenning H, Kuhndel K (2000). Predictive value of DNA cytometry in CIN 1 and 2. Image analysis of 193 cases. Anal Quant Cytol Histol, 22(4):333-337.

63. Heselmeyer K , Schroeck E, du Manoir S, Blegen H, Shah K, Steinbeck R, Auer G, Ried T (1996). Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. Proc Natl Acad Sci USA, 93:479–484.

64. Holowaty P, Miller AB, Rohan T, To T (1999). Natural history of dysplasia of the uterine cervix. J Natl Cancer Inst, 91(3):252-258.

65. Johnson SJ, Wadehra V (2001). How predictive is a cervical smear suggesting invasive squamous cell ? Cytopathology, 12(3):144-150.

66. Jones BA, Novis DA (2000). Follow-up of abnormal gynecologic cytology: a College of American Pathologists Q-probes study of 16132 cases from 306 laboratories. Arch Pathl Lab Med, 124:665-671.

67. Jones DED, Creasman WT, Dombroski RA, Lentz SS, Waeltz JL (1987). Evaluation of the atypical Pap smear. Am J Obstet Gynecol, 157:544-549.

68. Jones MH, Jenkins D, Cuzick J, Wolfendale MR, Jones JJ, Balogun-Lynch C (1992). Mild cervical dyskaryosis: safety of cytological surveillance. Lancet, 339:1440–3.

69. Kashyap V, Das DK, Luthra UK (1990). Microphotometric nuclear DNA analysis in cervical dysplasia of the uterine cervix: its relation to the progression to malignancy and regression to normalcy. Neoplasma, 37: 487-500.

70. Kaufman RH, Adam E, Icenogle J, Lawson H, Lee N, Reeves KO, Irwin J, Simon T, Press M, Uhler R, Entman C, Reeves WC (1997). Relevance of human papillomavirus screening in management of cervical intraepithelial neoplasia. Am J Obstet Gynecol, 176(1):87-92.

71. Kern WH, Zivolich MR (1977). The accuracy and consistency of the cytologic classification of squamous lesions of the uterine cervix. Acta Cytol, 21:519-523.

72. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, von Knebel Doeberitz M (2001). Overexpression of the $p16^{INK4a}$ as specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer, 92:276-284.

73. Klaes R, Benner A, Friedrich T, Ridder R, Herrinton S, Jenkins D, Kurman RJ, Schmidt D, Stoler M, von Knebel Doeberitz M (2002). $p16^{INK4a}$ immuno-histochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. Am J Surg Pathol, 92:276-284.

74. Koss LG, Stewart FW, Foote FW et al. (1963). Some histological aspects of behavior of epidermoid carcinoma in situ and related lesions of the uterine cervix. Cancer 16:1160-1211.

75. Koss LG (1993). Cervical (Pap) smear: New directions. Cancer (suppl), 71:1406-1412.

76. Koss LG (1994). Diagnostic cytopathology and its histopathologic bases, 4th edition. Lippincott, Philadelphia, 388-398.

77. Koss L (2000). Human papillomavirus testing as a screening tool for cervical cancer. JAMA, 283(19):2525-2526.

78. Koutsky LA, Holmes KK, Critchlow CW, Stevens CE, Paavonen J, Beckmann AM, DeRouen TA, Galloway DA, Vernon D, Kiviat NB (1992). A cohort study of the risk of cervical intraepithelial neoplasia grade 2 or 3 in relation to papillomavirus infection. N Engl J Med, 327:1272-1278.

79. Kuhn L, Denny L, Pollack A, Lorincz A, Richart RM, Wright TC (2000). Human papillomavirus DNA testing for cervical cancer screening in low-resource settings. J Natl Cancer Inst, 92(10):818-825.

80. Kurman RJ, Solomon D (1994). The Bethesda system for reporting cervical/vaginal cytologic diagnoses. Springer, New York.

81. Landis JR, Koch GG (1977). The measurement of observer agreement for categorical data. Biometrics, 33:159–174.

82. Leick A (2003). Identifizierung prospektiv maligner Dysplasien der Zervix uteri mittels DNA-Bildzytometrie. Promotionsschrift, Universität Düsseldorf.

83. Li R, Sonik A, Stindl R, Rasnick D, Duesberg P (2000). Aneuploidy vs. gene mutation hypothesis of cancer: Recent study claims mutation but is found to support aneuploidy. Proc. Natl. Acad. Sci. USA, 97(7):3236–3241.

84. Lorincz AT (1996). Molecular methods for the detection of human papillomavirus infection. Obstet Gynecol Clin North Am, 23(3):707-730.

85. Lytwyn A, Sellors JW, Mahony JB, Daya D, Chapman W, Ellis N, Roth P, Lorincz AT, Gafni A (2000). Comparison of human papillomavirus DNA testing and repeat Papanicolaou test in women with low-grade cervical cytologic abnormalities: a randomized trial. Canadian Medical Association Journal 163(6):701-707.

86. Makkink-Nombrado SV, Baak JP, Schuurmans L, Theeuwes JW, van der Aa T (1995). Quantitative immunohistochemistry using the CAS 200/486 image analysis system in invasive breast carcinoma: a reproducibility study. Anal Cell Pathol, 8(3):227-245.

87. Manos MM, Kinney WK, Hurley LB, Sherman ME, Shieh-Ngai J, Kurman RJ, Ransley JE, Fetterman BJ, Hartinger JS, McIntosh KM, Pawlick GF, Hiatt RA (1999). Identifying women with cervical neoplasia using human papillomavirus DNA testing for equivocal Papanicolaou results. JAMA, 281:1605-1610.

88. McCluggage WG, Bharucha H, Caughley LM, Date A, Hamilton PW, Thornton CM, Walsh MY (1996). Interobserver variation in the reporting of cervical colposcopic biopsy specimens: comparison of grading systems. J Clin Pathol, 49(10):833-835.

89. McCluggage WG, Walsh MY, Thornton CM, Hamilton PW, Date A, Caughley LM, Bharucha H (1998). Inter- and intraobserver variation in the histopathological reporting of cervical squamous intraepithelial lesions using a modified Bethesda grading system. Br J Obstet Gynaecol, 105(2):206-210.

90. McIndoe WA, McLean MR, Jones RW, Mullins PR (1984). The invasive potential of carcinoma in situ of the cervix. Obstet Gynecol 64:451-458.

91. McIntosh N, Blumenthal P, Blouse A (2000). Cervical cancer prevention guidelines for low-resource settings. JHPIEGO Corporation, Baltimore.

92. Meijer CJ, Helmerhorst TJ, Rozendaal L, van der Linden JC, Voorhorst FJ, Walboomers JM (1998). HPV typing and testing in gynaecological pathology: has the time come? Histopathology, 33(1):83-86.

93. Mengel M, von Wasielewski R, Wiese B, Rudiger T, Muller-Hermelink HK, Kreipe H (2002). Inter-laboratory and inter-observer reproducibility of immunohistochemical assessment of the Ki-67 labelling index in a large multi-centre trial. J Pathol, 198(3):292-299.

94. Milde-Langosch K, Riethdorf S, Kraus-Poppinghaus A, Riethdorf L, Loning T (2001). Expression of cyclin-dependent kinase inhibitors p16MTS1, p21WAF1, and p27KIP1 in HPV-positive and HPV-negative cervical adenocarcinomas. Virchows Arch, 439(1):55-61.

95. Munoz N, Bosch FX (1996). The causal link between HPV and cervical cancer and its implications for prevention of cervical cancer. Bulletin Pan-American Health Organization, 30:362-377.

96. Murty UV, Mitra AB, Das BC, Murty NS, Luthra UK (1988). Chromosomal phenotypes in patients with precancerous lesions of the uterine cervix progressed to cancer during follow up. Oncology, 45:384-388.

97. Nasiell K, Nasiell M, Vaclavinkova V (1983). Behavior of moderate cervical dysplasia during long-term follow-up. Obstet Gynecol, 61:609–614.

98. Nasiell K, Roger V, Nasiell M (1986). Behavior of mild cervical dysplasia during long-term follow-up. Obstet Gynecol, 67:665–669.

99. Nenning H, Horn LC, Kuhndel K, Bilek K (1995). False positive cervical smears: a cytometric and histological study. Anal Cell Pathol, 9(1):61-68.

100. Nenning H, Hering B, Horn LC, Kuhndel K (1997). Prognostischer Wert der DNA-Zytometrie bei Zervixzytologien PAP IIID. Verh Dtsch Ges Path, 81:651.

101. Nielsen AL, Thomsen HK, Nyholm HC (1991). Evaluation of the reproducibility of the revised 1988 International Federation of Gynecology and Obstetrics grading system of endometrial cancers with special emphasis on nuclear grading. Cancer, 68(10):2303-2309.

102. Norming U, Tribukait T, Gustafson H, Nyman CP, Wang N, Wijkstrom H (1992). Desoxyribonucleic acid profile and tumor progression in primary carcinoma in situ of the bladder: A study of 3 patients with grade 3 lesions. J Urol, 147:11-15.

103. O'Connor DM, Norris HJ (1994). The influence of grade on the outcome of stage I ovarian immature (malignant) teratomas and the reproducibility of grading. Int J Gynecol Pathol, 13(4):283-289.

104. Parkin DM, Läära E, Muir CS (1998). Estimates of the world-wide frequency of sixteen major cancer in 1980. Int J Cancer, 41:184-197.

105. PATH (2000a). Planning appropriate cervical cancer prevention programs. 2nd ed. PATH, Seattle.

106. PATH (2000b). Preventing cervical cancer in low-resource settings. Outlook, 18(1).

107. Preti M, Mezzetti M, Robertson C, Sideri M (2000). Inter-observer variation in histopathological diagnosis and grading of vulvar intraepithelial neoplasia: results of an European collaborative study. Br J Obstet Gynaecol, 107(5):594-599.

108. Ratnam S, Franco EL, Ferenczy A (2001). Human papillomavirus testing for primary screening of cervical cancer precursors. Cancer Epidemiol Biomarkers Prev, 9:945-951.

109. Renshaw AA (2002). Measuring sensitivity in gynecologic cytology. Cancer Cytopathology, 96(4):210-217.

110. Richart RM (1966). Colpomicroscopic studies of cervical intraepithelial neoplasia. Cancer, 19:395-405.

111. Richart RM (1973). Cervical intraepithelial neoplasia: a review. In: Sommers SC (ed), Pathology annual. Appleton-Century-Crofts, East Norwalk, 301-328.

112. Robertson JH, Woodend BE, Crozier EH, Hutchinson J (1988). Risk of cervical cancer associated with mild dyskaryosis. Br Med J, 297:18-21.

113. Sadeghi SB, Sadeghi A, Robboy SJ (1988). Prevalence of dysplasias and cancer of the cervix in a nationwide Planned Parenthood population. Cancer, 61:2359-2361.

114. Sandberg AA (1990). The chromosomes in human cancer and leukemia. Second Edition. Elsevier, New York, Amsterdam, Oxford.

115. Sandritter W, Fischer R (1961). Der DNS-Gehalt des normalen Plattenepithels und des invasiven Carcinoms der Portio. In: Proceedings of the First International Congress of Exfoliative Cytology. Lippincott, Philadelphia, 189-195.

116. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T (1998). Immunohistochemical overexpression of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia. Pathol Int, 48(8):580-585.

117. Schiffman M, Brinton LA (1995). The epidemiology of cervical carcinogenesis. Cancer, 76:1888-1901.

118. Schneider A, Zahm DM, Kirchmayr R, Schneider VL (1996). Screening for cervical intraepithelial neoplasia grade 2/3: validity of cytologic study, cervicography and human papillomavirus detection. Am J Obstet Gynecol, 174: 1534-41.

119. Schneider A, Hoyer H, Lotz B, Leistritza S, Kuhne-Heid R, Nindl I, Muller B, Haerting J, Durst M (2000). Screening for high grade cervical intraepithelial neoplasia and cancer by testing for high risk HPV, routine cytology or colposcopy. Intl J Cancer, 89:529-534.

120. Sevchuk MM, Richard RM (1982). DNA-content of condyloma accuminatum. Cancer, 49:489-492.

121. Sherman ME, Paull G (1993). Vaginal intraepithelial neoplasia. Reproducibility of pathologic diagnosis and correlation of smears and biopsies. Acta Cytol, 37(5):699-704.

122. Smith AE, Sherman ME, Scott DR (2000). Review of the Bethesda System atlas does not improve reproducibility or accuracy in the classification of atypical squamous cells of undetermined significance smears. Cancer, 90(4):201-206.

123. Solomon D, Schiffman M, Tarone R (2001). Comparison of three management strategies for patients with atypical squamous cells of undetermined significance: Baseline results from a randomized trial. J Natl Cancer Inst, 93:293-299.

124. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Raab S, Sherman M, Wilbur D, Wright T Jr, Young N (2002). The 2001 Bethesda system - Terminology for reporting results of cervical cytology. JAMA, 287(16):2114-2119.

125. Soost HG, Baur S (1990). Gynäkologische Zytodiagnostik. Lehrbuch und Atlas. Thieme, Stuttgart, New York.

126. Spriggs AI (1971). Follow-up of untreated carcinoma-in-situ of the cervix uteri. Lancet, 2:599-600.

127. Stoler MH (2000). Advances in cervical screening technology. Mod Pathol, 13(3):275–284.

128. Stoler MH, Shiffman M (2001). Interobserver reproducibility of cervical cytologic and histologic interpretations. Realistic estimates from the ASCUS-LSIL triage study. JAMA, 285(11):1-10.

129. Sudbø J, Kildal W, Risberg B, Koppang HS, Danielsen HE, Reith A (2001). DNA content as a prognostic marker in patients with oral leukoplakia. N Engl J Med, 344:1270-1278.

130. Syrjanen K, Kataja V, Yliskoski M, Chang F, Syrjanen S, Saarikoski S (1992). Natural history of cervical human papillomavirus lesions does not substantiate

the biologic relevance of the Bethesda system. Obstet Gynecol, 79(5 Pt1):675–682.

131. Temmerman M, Tyndall MW, Kidula N, Claeys P, Muchiri L, Quint W (1999). Risk factors for human papillomavirus and cervical precancerous lesions, and the role of concurrent HIV-1 infection. Int J Gynaecol Obstet, 65 (2):171-181.

132. Tezuka F, Namiki T, Higashiiwai H (1992). Observer variability in endometrial cytology using kappa statistics. J Clin Pathol, 45(4):292-294.

133. Tuon FF, Bittencourt MS, Panichi MA, Pinto AP (2002). Sensibility and specificity of cytology and colposcopy exams with the histological evaluation of cervical intraepithelial lesions. Rev Assoc Med Bras, 48(2):140-144.

134. van Aspert van Erp AJ, van't Hof Grootenboer AE, Brugal G, Vooijs GP (1996). Endocervical columnar cell intraepithelial neoplasia (ECCIN). 3. Interobserver variability in feature use. Anal Cell Pathol, 10(2):115-135.

135. Vooijs GP (1991). Benign proliferative reactions, intraepithelial neoplasia and invasive cancer of the uterine cervix. In: Bibbo M (Ed). Comprehensive Cytopathology, 2nd edition. WB Saunders, Philadelphia, 153-230.

136. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol, 189(1):12-19.

137. Webb T (2001). When theories collide: Experts develop different models for carcinogenesis. J Natl Cancer Inst, 93(2): 92-94.

138. WHO (1994). Histological typing of female genital tract tumours, 2nd edition. Springer, Berlin, 40-41.

139. Winkler B, Crum C, Fujii T, Ferenczy A, Boon M, Braun L, Lancaster WD, Richart RM (1984). Koilocytic lesions in the cervix: the relationship of mitotic abnormalities to the presence of papillomavirus antigens and nuclear DNA-content. Cancer, 53:1081-1087.

140. Woodhouse SL, Stastny JF, Styer PE, Kennedy M, Preastgaard AH, Davey DD (1999). Interobserver variability in subclassification of squamous intraepithelial lesions. Arch Pathol Lab Med, 123:1079-1084.

141. Wright TC Jr, Denny L, Kuhn L, Pollack A, Lorincz A (2000). HPV DNA testing of self-collected vaginal samples compared with cytologic screening to detect cervical cancer. JAMA, 283:81-86.

142. Wright TC, Kurman RJ, Ferenczy A (2002). Precancerous lesions of the cervix. In: Kurman RJ(ed.). Blaustein's Pathology of the female genital tract. 5th edition. Springer, New York, 253-324.

143. Yobs AR, Swanson RA, Lamotte LC Jr (1985). Laboratory reliability of the Pap smear. Obstet Gynecol, 65:235-244.

144. Young NA, Naryshkin S, Atkinson BF, Ehya H, Gupta PK, Kline TS, Luff RD (1994). Interobserver variability of cervical smears with squamous-cell abnormalities: a Philadelphia study. Diagn Cytopathol, 11(4):352-357.

145. Zaino RJ, Silverberg SG, Norris HJ, Bundy BN, Morrow CP, Okagaki T (1994). The prognostic value of nuclear versus architectural grading in endometrial adenocarcinoma: a Gynecologic Oncology Group study. Int J Gynecol Pathol, 13(1):29-36.

146. zur Hausen H (1977). Human papillomavirus and their possible role in squamous cell carcinomas. Curr Top Microbiol Immunol, 78:1-30.

147. zur Hausen H (1987). Papillomaviruses in human cancer. Cancer, 59:1692-1696.

148. zur Hausen H (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. Virology, 184:9-13.

SUMMARY

Cervical Intraepithelial Neoplasias (CIN) are lesions with an increased probability to later develop into cervical cancer. Its diagnosis in an individual patient unfortunately does not allow predicting progression or regression with sufficient accuracy. Cytomorphological criteria for the diagnosis of these lesions are neither objectively defined nor internationally agreed upon. These problems partially explain the wide variation of reported sensitivities of cytological screening for cervical cancer from 20% to 83% (Schneider et al. 2000, Renshaw 2002). The positive predictive value of cytological screening varies from 22% of Atypical Squamous Cell of Undetermined Significance (ASCUS) and Low-grade Squamous Intraepithelial Lesion (LSIL) for histological High-grade Squamous Intraepithelial Lesions (HSIL) to 55.7% of ASCUS and SILs for invasive carcinoma (Schneider et al. 1996, Johnson and Wadehra 2001). As diagnoses of cervical dysplasias are not only poorly reproducible but also of limited biological meaning for the individual patient, the number of resulting control procedures is usually high. These range from repeated cytological smears and biopsies to unnecessary operations (conizations). Missed early diagnoses of cancers may also result from cytomorphological uncertainties.

DNA-aneuploidy represents the quantitative cytometric equivalent of chromosomal aneuploidy and has been internationally accepted as a marker for neoplastic cell transformation (Haroske et al. 2001). Various studies have demonstrated the value of DNA aneuploidy as a marker for histologically confirmed and/or prospectively neoplastic development in cervical dysplasia (Fu 1982, Böcking et al. 1986, Chatelain et al. 1989, Kashyap 1990, Bollmann and Böcking 1996, Nenning et al. 1997, Bollmann et al. 2001).

The objectives of this study are to investigate:

- the diagnostic validity of DNA-ICM for the identification of progressive cervical intraepithelial lesions in Pap smears.
- the interobserver reproducibility of DNA-ICM applied to the routine Pap smears classified as ASCUS or higher lesions.

Cervical smears diagnosed as ASCUS or higher lesions from 202 patients were investigated. The smears first underwent Papanicolaou staining and were classified according to The Bethesda System 2001 for reporting results of cervical cytology. They were then destained and restained according to Feulgen. The first measurements were done as routine workup of ASCUS or higher cases. The second measurements were consecutively performed during this study and blinded to the results of the first measurements. The hard- and software used in the study as well as terms and algorithms for diagnostic interpretation of data were in strict accordance with the standard requirements of the European Society of Analytical Cellular Pathology consensus reports. Correspondent data on follow-up cytology and histology were retrospectively collected. The mean follow-up interval was two months.

Diagnostic validity of cytological diagnoses within the study sample was evaluated by the calculation of sensitivity and positive predictive value. Diagnostic validity of DNA-ICM encompassing sensitivity, specificity, positive and negative predictive values were calculated based on the second DNA cytometric measurement.

Initial cytological diagnoses were 25 ASCUSs, 72 LSILs, 118 HSILs and 6 invasive cancers. In 19 cases more than one diagnostic cytology/DNA-ICM was performed and each has been regarded as an individual case. The following data were obtained: 221 initial cytological diagnoses of ASCUS+ lesions and 221 DNA-ICM diagnoses, 139 cytological and 135 histological diagnoses of the follow-up.

The rate of DNA-aneuploidy in this study increased significantly with the increasing severity of cytological lesions, from 40.3% in LSILs over 90.7% in HSILs to 100% in invasive cancers ($\chi 2 = 59.26$, $p=0$). The sensitivity of cervical smears diagnosed as ASCUS / LSIL and DNA-ICM for the detection of CIN III or higher lesions were 25.9% and 60.6%, respectively. In these cases, DNA aneuploidy had a positive predictive value (PPV) of 50.0% and DNA euploidy had a negative predictive value (NPV) of 94.7%. The sensitivity of DNA-ICM increased significantly from 60.6% in ASCUS/LSIL group to 92.9% for all ASCUS or higher lesions; whereas their NPVs were still unchanged at a high level (94.7% for ASCUS & LSIL, 96.5% for ASCUS & LSIL & HSIL

and 96.5% for ASCUS and higher). The PPVs of DNA-aneuploidy in different groups were stable, even if HSILs and invasive cancers were included in the calculation (50.0% for ASCUS & LSIL, 51.1% for ASCUS & LSIL & HSIL and 51.6% for ASCUS and higher).

DNA-ICM thus contributed to increase the sensitivity of conventional cytological ASCUS and LSIL diagnoses for detection of CIN III or higher findings by 34.7%. Combined with the high interobserver reproducibility of 94.1%, this qualifies DNA-ICM as a useful and reliable tool to raise diagnostic accuracy of cervical cytology for early identification of cervical cancer as well as a suited tool for quality control in tumor cell doubtful and positive cytological diagnoses.

ZUSAMMENFASSUNG

Zervikale intraepitheliale Neoplasien (CIN) sind Läsionen der Schleimhaut des äußeren Muttermundes, die eine erhöhte Wahrscheinlichkeit aufweisen, sich zu einem Zervixkarzinom weiter zu entwickeln. Ihre Diagnose läßt aber im Einzelfall keine genügend sichere Vorhersage über Progression oder Regression der Läsion zu. Zytomorphologische Kriterien für eine reproduzierbare Diagnose dieser Veränderungen sind weder hinreichend objektiv definiert noch international standardisiert. Dies erklärt auch die Unterschiede der berichteten Sensitivitäten der gynäkologischen Zytologie zur Früherkennung des Zervixkrebses von 20% bis 83% (Schneider et al. 2000, Renshaw 2002). Der positive Vorhersagewert der gynäkologischen Zytologie schwankt von 22% für Atypical Squamous Cells of Undetermined Significance (ASCUS) und Low-grade Squamous Intraepithelial Lesions (LSIL) zur Erkennung von histologischen High-grade Squamous Intraepithelial Lesions (HSIL) bis 55,7% zur Erkennung des invasiven Zervixkarzinoms (Schneider et al. 1996, Johnson und Wadehra 2001). Da zytologische Diagnosen zervikaler Dysplasien von begrenzter prognostischer Bedeutung für den einzelnen Patienten sind und zudem schlecht reproduzierbar, ist die Zahl klinischer Kontrollen meist hoch. Diese reichen von wiederholten Abstrichen und Biopsien bis zu unnötigen Konisationen. Aus den zytodiagnostischen Ungewißheiten resultieren aber auch übersehene Frühdiagnosen des Zervixkarzinoms.

DNA-Aneuploidie stellt das zytometrische Äquivalent chromosomaler Aneuploidie dar und ist international als Marker für neoplastische Zelltransformation akzeptiert (Haroske et al. 2001). Die Validität der DNA-Aneuploidie als Marker für histologisch belegte und/oder prospektive maligne Transformation zervikaler Dysplasien wurde durch mehrere Studien nachgewiesen (Fu 1982, Böcking et al. 1986, Chatelain et al. 1989, Kashyap 1990, Bollmann und Böcking 1996, Nenning et al. 1997, Bollmann et al. 2001).

Die Zielsetzungen dieser Studie war:

- die diagnostische Validität der DNA-Bildzytometrie (DNA-ICM) für die Identifizierung progressiver zervikaler intraepithelialer Läsionen in Zervixabstrichen zu untersuchen
- die interindividuelle Reproduzierbarkeit der DNA-ICM an Routine-Abstrichen von der Zervix uteri, die als ASCUS oder höher-gradige Läsionen eingestuft worden waren, zu untersuchen.

Das Untersuchungsgut umfasste Abstriche von 202 Patientinnen, die als ASCUS oder höhere Läsionen diagnostiziert worden waren. Diese wurden zuerst nach Papanicolaou gefärbt und entsprechend dem aktuellen Bethesda System 2001 zur Befundung gynäkologischer Zytologie eingestuft. Danach folgte eine Umfärbung der Präparate nach Feulgen. Die ersten Messungen erfolgten im Rahmen der routinemäßigen zytologischen Bearbeitung. Die jeweils zweiten Messungen wurden in Unkenntnis der Ergebnisse der ersten während dieser Studie durchgeführt. Verwendete Geräte samt Software sowie Definitionen und Algorithmen für DNA-Messungen und diagnostische Bewertungen der Daten befanden sich in Übereinstimmung mit den Anforderungen der Europäischen Gesellschaft für Analytische Zelluläre Pathologie (ESACP). Daten bezüglich des zytologischen und histologischen Follow-ups wurden retrospektiv gesammelt. Das mittlere Followup-Intervall betrug 2 Monate.

Die diagnotische Validität der zytologischen und DNA-zytometischen Diagnosen wurde durch die Berechnung von Sensitivität und positivem Vorhersagewert ermittelt. Die Maßzahlen der diagnosischen Treffsicherheit der DNA-ICM bezogen sich auf die zweite DNA-Messung.

Die zytologischen Erstdiagnosen waren 25 ASCUSs, 72 LSILs, 118 HSILs und 6 invasive Karzinome. In 19 Fällen wurde mehr als eine zytologische Befundung/DNA-ICM durchgeführt. Jede wurde als einzelner Fall angesehen. Folgende Diagnosen wurden ausgewertet: 221 zytologische ASCUS+ Läsionen, 221 DNA-Bildzytometrien, 139 zytologische und 135 histologische Follow-up Diagnosen.

Der Prozentsatz von DNA-Aneuploidie stieg erheblich mit der zunehmenden Schwere der zytologischen Läsionen, von 40,3% in LSILs über 90,7% in HSILs bis zu 100% in invasiven Karzinomen ($\chi 2=59,26$; $p=0$). Die Sensitivität der Diagnosen ASCUS oder LSIL bzw. der DNA-Aneuploidie für die Identifizierung von CIN III oder höheren Läsionen betrug 25,9% bzw. 60,6%. In diesen Fällen hatte der Nachweis von DNA-Aneuploidie einen positiven Vorhersagewert (PPW) von 50,0%, DNA-Euploidie hatte einen negativen Vorhersagewert (NPW) von 94,7%. Die Sensitivität der DNA-ICM erhöhte sich erheblich von 60,6% in der ASCUS/LSIL Gruppe auf 92,9% für ASCUS oder höhere Diagnosen, während ihre NPWs auf einem hohen Niveau unverändert blieben (94.7% für ASCUS & LSIL, 96.5% für ASCUS & LSIL & HSIL und 96.5% für ASCUS und höhere Läsionen). Die PPWs für DNA-Aneuploidie in den unterschiedlichen zytodiagnostischen Gruppen waren auch konstant, selbst wenn HSILs und Karzinome in die Berechnung einbezogen wurden (50.0% für ASCUS & LSIL, 51.1% für ASCUS & LSIL & HSIL und 51.6% für ASCUS und höhere Läsionen).

Die DNA-ICM hat daher dazu beigetragen, die Sensitivität der zytologischen Diagnosen ASCUS und LSIL für die Identifizierung von CIN III oder höheren Befunden um 34,7% zu erhöhen. Mit einer hohen interindividuellen Reproduzierbarkeit von 94,1% qualifiziert sich die DNA-ICM als nützliche und zuverlässige Methode, um die diagnostische Validität der gynäkologischen Zytologie zur Früherkennung des Zervixkarzinomes und seiner Vorstufen zu verbessern. Weiter kann die Methode auch zur Qualitätskontrolle in zweifelhaften und Tumorzell-positiven zytologischen Diagnosen beitragen.

ACKNOWLEDGMENTS

First I would like to express my deepest thanks and appreciation to my Mentor, Prof. Dr. med. A. Böcking. He not only accepts me as his doctoral candidate and made it possible for me to work in his Institute but also took a large effort and a lot of time to train me in cytomorphological diagnosis, in scientific publication and especially in DNA-image cytometry. It is a great opportunity for me to work under his direction and I hope that a fruitful cooperation will be continued in the future.

To all of my colleagues and friends from the Institute of Cytopathology, especially, Dr. N. Pomjanki and Dr. H.J. Grote, whether or not mentioned by name, I express my thanks for their kindness and their cooperation.

I express my sincere thanks to the Düsseldorf Entrepreneurs Foundation and Gesellschaft von Freunden und Förderern der Heinrich-Heine Universität Düsseldorf e.V., without their generous support this dissertation could not have been carried out and completed.

In particular, I say my appreciation to my parents and my wife, without their encouragements and supports I could not be able to work toward the completion of this thesis.

Please let me say thanks again to all the persons who helped me during my study.

CURRICULUM VITAE

Family name:	NGUYEN
Given name:	Vu Quoc Huy
Date of birth:	November 22nd, 1969
Nationality:	Vietnamese
Father:	NGUYEN Van Tuan, 1936, retired.
Mother:	NGUYEN Thi Dung, 1943, retired.
Familial status:	Married with Mrs. TON NU Phuong Anh, MD, two children.

ELEMENTARY EDUCATION

1975 - 1980: Phu Hiep Elementary School, Hue, Vietnam.

HIGH SCHOOL EDUCATION

1980 - 1987: Quoc Hoc National High School, Hue, Vietnam. High School Education Diploma: 1987; Grade: Very Good.

UNIVERSITY EDUCATION

10.1987 - 08.1993: Medical student, Hue University School of Medicine, Hue, Vietnam. Graduation and Medical licensure: 1993; Grade: Good.

POSTGRADUATE MEDICAL TRAINING

03.1994 - 03.1997: Residency training in Obstetrics and Gynecology, Hue University School of Medicine / Hue Central Hospital.

10.1997 - 09.1998: Full-time training in Gynecological Cytopathology and Colposcopy at Cytology Laboratory and Cervical Dysplasia Unit, University Women's Hospital of Freiburg, Germany.

10.1999 - 10.2001: Master's Degree student in Obstetrics, Genecology and Reproductive Health, Hue University School of Medicine. Graduation: November 2001. Grade: Very Good.

12.2000 - 02.2001: Training and clinical research on gynecologic liquid-based cytology and Human Papillomavirus DNA detection, Institut Jules Bordet, Brussels, Belgium.

FELLOWSHIP

04.1997 - 09.1998:	DAAD Longterm-Fellowship
12.2000 - 02.2001:	UICC ICRETT Fellowship
07.2002 - 06.2003:	Düsseldorf Entrepreneurs Foundation Fellowship

AWARD

05.1996: Young Scientist Award of Vietnamese Medical and Pharmacy Universities Conference for the scientific report titled “The role of ultrasonography in diagnosis and treatment of in utero fetal death”.

WORKING POSITION

Since 01.1997: University Lecturer and Physician, Department of Obstetrics and Gynecology, Hue University School of Medicine / Hue Central Hospital (lifetime position).

Düsseldorf, February 25th 2003

(NGUYEN, Vu Quoc Huy)

ABSTRACT

IDENTIFIZIERUNG PROGRESSIVER ZERVIKALER INTRAEPITHELIALER LÄSIONEN MITTELS DNA-BILDZYTOMETRIE. DIAGNOSTISCHE VALIDITÄT UND REPRODUZIERBARKEIT

Vu Quoc Huy NGUYEN

Zervikale intraepitheliale Neoplasien (CIN) sind Läsionen der Schleimhaut des äußeren Muttermundes, die eine erhöhte Wahrscheinlichkeit aufweisen, sich zu einem Zervixkarzinom weiter zu entwickeln. Ihre zytologische oder histologische Diagnose läßt aber im Einzelfall keine genügend sichere Vorhersage über Progression oder Regression der Läsion zu. Zytomorphologische Kriterien für eine reproduzierbare Diagnose dieser Veränderungen sind weder hinreichend objektiv definiert noch international standardisiert.

Die Zielsetzungen dieser Studie war:

- die diagnostische Validität der DNA-Bildzytometrie (DNA-ICM) für die Identifizierung progressiver zervikaler intraepithelialer Läsionen in Zervixabstrichen zu untersuchen
- die interindividuelle Reproduzierbarkeit der DNA-ICM an Routine-Abstrichen von der Zervix uteri, die als Atypical Squamous Cells of Undetermined Significance (ASCUS) oder höher-gradige Läsionen eingestuft worden waren, zu untersuchen.

Das Untersuchungsgut umfasste Abstriche von 202 Patientinnen, die als ASCUS oder höhere Läsionen diagnostiziert worden waren. Diese wurden zuerst nach Papanicolaou gefärbt und entsprechend dem aktuellen Bethesda System 2001 zur Befundung gynäkologischer Zytologie eingestuft. Danach folgte eine Umfärbung der Präparate nach Feulgen. Die ersten Messungen erfolgten im Rahmen der zytologischen Routine-Diagnostik. Die zweiten Messungen wurden ohne Kenntnis der vorangehenden Diagnosen während dieser Studie durchgeführt. Verwendete Geräte samt Software sowie Definitionen und Algorithmen für DNA-Messungen und diagnostische Bewertungen der Daten befanden sich in Übereinstimmung mit den Anforderungen der Europäischen Gesellschaft für Analytische Zelluläre Pathologie (ESACP). Daten bezüglich des zytologischen und histologischen Follow-ups wurden retrospektiv gesammelt. Das mittlere Followup-Intervall betrug 2 Monate.

In 19 Fällen wurde mehr als eine zytologische Befundung/DNA-ICM durchgeführt, jede wurde als einzelner Fall angesehen. Folgende Diagnosen wurden ausgewertet: 221 zytologische ASCUS+ Läsionen, 221 DNA-Bildzytometrien, 139 zytologische und 135 histologische Follow-up Diagnosen.

Die Sensitivität der Diagnosen ASCUS oder Low-grade Squamous Intraepithelial Lesion (LSIL) bzw. der DNA-Aneuploidie für die Identifizierung von CIN III oder höheren Läsionen betrug 25,9% bzw. 60,6%. In diesen Fällen hatte der Nachweis von DNA-Aneuploidie einen positiven Vorhersagewert (PPW) von 50,0%, DNA-Euploidie hatte einen negativen Vorhersagewert (NPW) von 94,7%. Die Sensitivität der DNA-ICM erhöhte sich von 60,6% in der ASCUS/LSIL Gruppe auf 92,9% für ASCUS & LSIL & High-grade Squamous Intraepithelial Lesions (HSIL) & invasive Karzinome, während ihre NPWs auf einem hohen Niveau unverändert blieben (94.7% für ASCUS & LSIL, 96.5% für ASCUS & LSIL & HSIL und 96.5% für ASCUS und höhere Läsionen). Die PPWs von DNA-Aneuploidie in den unterschiedlichen zytodiagnostischen Gruppen waren auch konstant, selbst wenn HSILs und Karzinome in die Berechnung einbezogen wurden (50.0% für ASCUS & LSIL, 51.1% für ASCUS & LSIL & HSIL und 51.6% für ASCUS und höhere Läsionen). Die DNA-ICM hat damit dazu beigetragen, die Sensitivität der zytologischen Diagnosen von ASCUS und LSIL für die Identifizierung von CIN III oder höheren Befunden um 34,7% zu erhöhen. Mit einer hohen interindividuellen Reproduzierbarkeit von 94,1% qualifiziert sich die DNA-ICM als nützliche und zuverlässige Methode, um die diagnostische Validität der gynäkologischen Zytologie zur Früherkennung des Zervixkarzinomes und seiner Vorstufen zu verbessern. Weiter kann die Methode auch zur Qualitätskontrolle in zweifelhaften und Tumorzell-positiven zytologischen Diagnosen beitragen.

Universitätsprofessor Dr. med A. Böcking

A Böcking
27.02.03