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Leiterin: Prof. Dr. Irene Esposito

-Funktionsbereich Cythopathologie-

# Multicolour fluorescence in situ hybridization in cervical smears: detection of amplification of hTERC, MYC, and EGFR for the diagnosis of intraepithelial neoplasia

Dissertation

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Bruno Eduardo Silva de Araujo

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Dekan: Univ.-Prof. Dr. med. Nikolaj Klöcker

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Zweitgutachter: Prof. Dr. med. Monika Hampl

Dedicated to my family

## Summary

**Introduction**: The introduction of cytological cervical cancer screening has reduced the number of cervical cancer deaths worldwide. The inexpensive and easy-to-use cytopathological examination of cervical smears enables the detection of precancerous lesions, which in many cases can prevent progression to invasive carcinoma. Cytology is highly specific, but sensitivity is in need of improvement, which has to be considered when choosing the precautionary interval. The target lesion of the screening program in Germany is cervical intraepithelial neoplasia grade 3 (CIN 3), which corresponds to obligatory precancerous disease. These lesions can be resected using conisation procedures. Earlier precursor lesions (CIN 1 or CIN 2) have a much higher tendency for regression and are therefore preferably monitored by regular cytological examinations. This process can, however, lead to a psychological strain on the patients, who, depending on the algorithm, have to wait up to 6 months, repeated if necessary, until they are checked again in order to obtain answers to the development of the lesion. Thus, alternative methods complementary to cytology are required to predict the biological behavior of an intraepithelial cervical lesion earlier and more accurately. Many of these methods, however, require a high number of suspicious cells or do not allow examination of already microscopically examined abnormal cells. Fluorescence in situ hybridization (FISH) is a suitable alternative because it can detect a malignant lesion with high accuracy even with a small number of required analysis cells. It is also possible to perform cytology and FISH sequentially on the same slide and thus to combine morphological and genetic information of a lesion. The aim of the study is to analyze the potential of FISH as a confirmatory test for malignant transformation in dysplastic cell changes of the uterine cervix.

**Methods:** For our retrospective case control study, we used cervical smears from 132 women treated at the Department of Obstetrics and Gynecology of Heinrich Heine University, Düsseldorf, from January 2014 to August 2015, mostly in the dysplasia consultation ambulance. The cases were selected due to their cytological diagnosis according to groups I, IIp, IIID1, IIID2, IVa-p, or V, respectively, of the Munich Nomenclature III. Resulting from a sample size estimation, 25 patients were initially determined for each group. The interphase FISH was performed with three locus-specific probes: LSI MYC SpectrumAqua Probe (8q24.21), LSI TERC SpectrumGold Probe (3q26) and LSI EGFR SpectrumGreen Probe (7p11.2-p12). The FISH results and the cytological diagnoses were compared with the corresponding histological diagnoses (biopsies (n=126), conisations (n=28), hysterectomy specimens (n=4)), whereby the time of biopsy had to be within 3 months and the time of conisation (or hysterectomy) within 6 months after the cytological diagnosis. In addition, the clinical course and, if performed, the result of an HPV test were taken from the patient's records within 18 months of the cytological smear. The following statistical variables were used to evaluate the diagnostic accuracy of FISH: Sensitivity, specificity, positive and negative predictive value and area under the ROC curve (AUC).

**Results:** The definition of the cutoff required to distinguish a positive from a negative FISH result was initially based exclusively on statistical analysis (Youden index). Since the cutoff values of these analyses were too high (sometimes more than 30 required genetically modified cells) or the specificity was below 90%, additional analyses were performed to find better suited cutoff values for clinical application. Although the active modification of the cutoffs showed better results, they still remained lower than the requirements of clinical application. In many cases (n=94), the histological biopsy was used as a reference standard, as is usual in the literature. However, biopsies will not always be representative for the lesion as a whole. Thus, the FISH examinations with histological diagnoses after conisation were evaluated as a more

suitable reference standard as a partial analysis. As expected, this type of analysis provided the best results. A positive FISH result using a pre-defined cutoff of ≥10 genetically abnormal cells correlated with the histological diagnosis of conization ≥CIN2 achieved a sensitivity of 78.6% and a specificity of 92%. However, these results, which were encouraging for the overall collective, could not be confirmed in subgroup analyses in the diagnostic groups IIp, IIID1 or IIID2 which are also interesting for FISH application.

**Perspective:** The study provides valuable information on the basic applicability of FISH probes to cervical smears. It has been shown that the detection of genetic alterations using FISH as a method for estimating an actual at least CIN2 lesion has good diagnostic accuracy when examining a mixed cytological collective of diagnostic groups. For an application on defined cytologically unclear diagnostic groups, e. g. IIID1, the clinical applicability with the above mentioned probe combination is not yet proved. Nevertheless, factors were identified that might negatively influence the results, such as the low quality of the smears (often suboptimal alcoholic fixation) and the problem of the representative reference standard in the case of a histological biopsy. The introduction of liquid-based cytology and the choice of a longer clinical follow-up period could improve the significance of the FISH results. The addition of a fourth FISH probe and its combined evaluation, as already published for other organ systems (lung, biliary tract, urinary cytology) as applicable for routine cytodiagnostics, could also improve the accuracy of the method and should be tested in future work.

# Zusammenfassung

**Einleitung:** Die Einführung der zytologischen Krebsvorsorge hat weltweit zu einem Rückgang der Zahl der Todesfälle durch das Zervixkarzinom geführt. Die kostengünstige und einfach durchzuführende zytologische Untersuchung von Zervixabstrichen ermöglicht bereits die Erkennung von präkanzerösen Läsionen, wodurch in vielen Fällen ein Fortschreiten zu einem invasiven Karzinom vermieden werden kann. Die Zytologie ist sehr spezifisch bei allerdings verbesserungsbedürftiger Sensitivität, was bei der Wahl des Vorsorgeintervalls zu beachten ist. Die Zielläsion des Vorsorgeprogramms in Deutschland ist die zervikale intraepitheliale Neoplasie Grad 3 (CIN3), welche einer obligaten Präkanzerose entspricht. Diese kann kolposkopisch mittels Konisationsverfahren reseziert werden. Frühere Vorläuferläsionen (CIN1 oder CIN2) haben eine wesentlich höhere Regressionsneigung und werden daher zunächst durch regelmäßige zytologische Untersuchungen überwacht. Dieser Prozess kann jedoch zu einer psychischen Belastung der Patientinnen führen, die je nach Algorithmus bis zu 6 Monate, ggf. wiederholt, bis zur erneuten Kontrolle warten müssen, um Antworten auf die Entwicklung der Läsion zu erhalten. Entsprechend wären alternative, die Zytologie ergänzende Methoden erforderlich, die früher und genauer das biologische Verhalten einer intraepithelialen zervikalen Läsion vorhersagen können. Viele dieser Methoden benötigen aber eine hohe Anzahl von verdächtigen Zellen oder erlauben keine Untersuchung an bereits mikroskopisch untersuchten auffälligen Zellen. Die Fluoreszenz in situ Hybridisierung (FISH) bietet sich als geeignete Alternative an, da sie bereits bei einer geringen Anzahl erforderlicher Analysezellen maligne Läsionen mit hoher Genauigkeit detektieren kann. Darüber hinaus besteht die Möglichkeit, Zytologie und FISH nacheinander auf demselben Objektträger durchzuführen und auf diese Weise morphologische und genetische Informationen einer Läsion zusammenzuführen. Ziel der Studie ist die Analyse des Potenzials der FISH als Bestätigungstest für eine maligne Transformation in dysplastischen Zellveränderungen der Cervix uteri.

**Methodik:** Für unsere retrospektive Fall-Kontroll-Studie verwendeten wir Zervixabstriche von 132 Frauen, die von Januar 2014 bis August 2015 an der Klinik für Frauenheilkunde und Geburtshilfe der Heinrich-Heine-Universität Düsseldorf, meist in der Dysplasieprechstunde, behandelt wurden. Die Fälle wurden nach ihrer zytologischen Diagnose entsprechend der Gruppen I, IIp, IIID1, IIID2, IVa-p und V der Münchner Nomenklatur III ausgewählt. Gemäß einer Stichprobenschätzung wurden initial für jede Gruppe 25 Patienteninnen vorgegeben. Die Interphase FISH wurde mit drei genortspezifischen Sonden durchgeführt: LSI *MYC* SpectrumAqua Probe (8q24.21), LSI *TERC* SpectrumGold Probe (3q26) und LSI *EGFR* SpectrumGreen Probe (7p11.2-p12). Die FISH-Ergebnisse und die zytologischen Diagnosen wurden mit den korrespondierenden histologischen Diagnosen (Biopsien (n=126), Konisationen (n=28), Hysterektomiepräparate (n=4)) verglichen, wobei der Zeitpunkt der Biopsie innerhalb von 3 Monaten und der Konisation (oder Hysterektomie) innerhalb von 6 Monaten nach der zytologischen Diagnose liegen musste. Außerdem wurden der klinische Verlauf und, soweit durchgeführt, das Ergebnis eines HPV-Tests (innerhalb von 6 Monaten nach dem zytologischen Abstrich) den Krankenakten entnommen. Die folgenden statistischen Variablen wurden für die Bewertung der diagnostischen Treffsicherheit der FISH bestimmt: Sensitivität, Spezifität, positiver und negativer prädiktiver Wert sowie die Fläche unter der ROC-Kurve (AUC).

**Ergebnisse:** Die Definition des zur Abgrenzung eines positiven von einem negativen FISH-Resultat erforderlichen Cutoffs erfolgte zunächst ausschließlich auf Basis der statistischen Analyse (Youden-Index). Da die Cutoffs dieser Analysen zu hoch waren (zum Teil mehr als 30

erforderliche genetisch veränderte Zellen) oder die Spezifität unter 90% lag, wurden zusätzliche Analysen zur Findung geeigneter Cutoff-Werte für die klinische Anwendung durchgeführt. Die Veränderung der Cutoffs zeigte zwar bessere Ergebnisse, die aber immer noch unter den Anforderungen einer klinischen Anwendung lagen. Hierbei zeigte sich als Problem, dass bei vielen Fällen (n=94), wie auch in der Literatur üblich, die histologische Biopsie als Referenzstandard verwendet wurde. Diese muss nicht unbedingt für die Gesamtläsion repräsentative Veränderungen aufweisen. Deswegen wurden ergänzend als Teilanalyse die FISH Untersuchungen in Bezug auf die histologischen Diagnosen nach Konisation als besser geeignetem Referenzstandard ausgewertet. Diese Art der Analyse lieferte erwartungsgemäß die besten Ergebnisse. Ein positives FISH-Ergebnis unter Verwendung eines vordefinierten Cutoffs von ≥10 genetisch auffälligen Zellen erreichte nach Korrelation mit der histologischen Diagnose der Konisation ≥CIN2 eine Sensitivität von 78,6% und eine Spezifität von 92%. Diese für das Gesamtkollektiv ermutigenden Ergebnisse konnten in Subgruppenanalysen in den für eine FISH-Anwendung ebenfalls interessantesten Diagnosegruppen IIp, IIID1 oder IIID2 allerdings nicht bestätigt werden.

**Perspektive**: Die Studie lieferte nützliche Informationen bezüglich der grundsätzlichen Anwendbarkeit von FISH-Sonden an Zervixabstrichen. Es wurde gezeigt, dass der Nachweis von genetischen Veränderungen mittels FISH als Methode zur Abschätzung einer tatsächlich vorliegenden mindestens CIN2 Läsion eine gute diagnostische Treffsicherheit aufweist, wenn ein bezüglich der Diagnosegruppen gemischtes zytologisches Kollektiv untersucht wird. Für eine Anwendung an definierten zytologisch unklaren Diagnosegruppen, z.B. IIID1, ist die klinische Anwendbarkeit mit der oben genannten Sondenkombination noch nicht gegeben. Dennoch wurden Faktoren herausgearbeitet, die die Ergebnisse möglicherweise negativ beeinflussen, wie etwa die geringe Qualität der Abstriche (oft suboptimale alkoholische Fixation) und die Problematik des repräsentativen Referenzstandards im Falle einer histologischen Biopsie. Die Einführung der flüssigkeitsbasierten Zytologie und die Wahl eines längeren klinischen *follow-up*-Zeitraumes könnten hier Verbesserungen in der Aussagekraft der FISH-Resultate bringen. Die Hinzunahme einer vierten FISH-Sonde und deren kombinierte Auswertung, wie bereits für andere Organsysteme (Lunge, Gallenwege, Urinzytologie) publiziert und in der klinischen zytologischen Diagnostik anwendbar, könnte die Treffsicherheit der Methode ebenfalls verbessern und sollte in zukünftigen Arbeiten erprobt werden.

**List of abbreviations**

AJCC - American Joint Committee on Cancer

ALK - Anaplastic lymphoma kinase

ASC-H - Atypical squamous cells of undetermined significance cannot exclude HSIL

ASR - age-standardized rates

CIN1 - Cervical intraepithelial neoplasia-grade I

CIN3 - Cervical intraepithelial neoplasia grade 3

DAPI - 4',6-diamidino-2-phenylindole

DIC - Differential interference contrast

DNA - Deoxyribonucleic Acid

DNA-ICM - DNA image cytometry

EGFR - Epidermal growth factor receptor

FDA - US Food and Drug Administration

FIGO - International Federation of Gynecology and Obstetrics

HC2 - The Digene Hybrid Capture 2

HCl - Hydrochloric acid

HIV - Human immunodeficiency virus

HPV - Human Papillomavirus

Hr-HPV – High-risk Human Papillomavirus

HSIL - High-grade intraepithelial lesion

hTERC - human telomerase RNA component

hTERT - Human telomerase reverse transcriptase

IAC - The International Consensus Conference on the Fight Against Cervical Cancer International Academy of Cytology

IARC - International Agency for Researcher on Cancer

IHC - Immunohistochemistry

KCL - Potassium chloride

KH2PO4 - Monopotassium phosphate

LAST - US-based Lower Anogenital Squamous Terminology

LBC - Liquid Based Cytology

LSD - Least Significant Difference

LSIL - Low-grade intraepithelial lesion

Na2HPO4 - Disodium phosphate

NaCl – Sodium chloride

NaOH - Sodium hydroxide

NILM - Negative for Intraepithelial Lesion or Malignancy

NISH- Inonisotopic in situ hybridization

NP-40 - Tergitol-type NP-40

NPV - Negative predictive value

NSCLC - non-small cell lung cancer

p53 - Tumor protein p53

PBS - Phosphate-buffered saline

PCR - Polymerase chain reaction

pH - Potential of hydrogen

PPV - Positive predictive value

pRB - Progesterone receptor B

RTK - Receptor tyrosine kinase

RT-PCR – real-time polymerase chain reaction

SCC - Squamous Cell Carcinoma

SNP - Common single nucleotide polymorphisms

SOPs - Standard operating procedures

SSC - Saline-sodium citrate buffer

TBS - The Bethesda System for reporting cervical cytology

TGFα - Transforming growth factor-α

TIS - ThinPrep Imaging System

VIA - Visual Inspection of the cervix with Acetic acid

WHO - World Health Organization

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# 1 Introduction

## 1.1 Epidemiology of Cervical Cancer and Precursor Lesions

Cervical cancer is the fourth most common cancer in women and the seventh overall with an estimated 528,000 new cases and 266,000 deaths in 2012 accounting for 7.5% of all female cancer deaths in 2012 (Ferlay et al., 2013a). A large majority (around 85%) of the global incidences and deaths occur in the less developed regions, where it totals almost 12% of all female cancers. High-risk regions with estimated age-standardized rates (ASR) over 30 per 100,000 include Eastern Africa (42.7), Melanesia (33.3), Southern (31.5) and Middle Africa (30.6). Rates are lowest in Australia/New Zealand (5.5) and Western Asia (4.4) (Ferlay et al., 2015). The reason for such a large geographic variation in cervical cancer rates is related to the difference of the availability of screening programs that allow for the detection and removal of precancerous lesions and human papillomavirus (HPV) infections (Vaccarella et al., 2013, Bruni et al., 2010, Forman et al., 2012).

In 2013 4,610 women were diagnosed with cervical cancer in Germany. The 5-year prevalence of invasive cervical cancer is 17,780. The median age of the disease is 55.3 years, 20 years more than that of the median age of women with premalignant lesions. 1,550 women in Germany die of cervical cancer every year. 30 years ago the number was more than twice as high. The relative 5-year survival rate after diagnosis of an invasive cervical tumor is 69 % (Barnes and Kraywinkel, 2017).

In Germany, cervical cancer screening results are currently reported according to the *Münchner Nomenklatur III*, that was introduced in 2014 (for description refer to chapter 1.4.2). That differs from the procedure in other countries which use The Bethesda System for reporting cervical cytology (TBS). 16,237,698 women participated in the German cervical cancer screening program in 2013 and had the following cytological diagnoses according to the *Münchner Nomenklatur II*, that was the nomenclature then in force: 0.27% had a PAP III result, 1.16% a PAP IIID, 0.17% to PAP IVa-p and 0.02% a PAP V result (Marquardt et al., 2015). The rates of premalignant lesions of the uterine cervix reported according to the Bethesda System for reporting cervical cytology vary in different regions of the world from 0.2% to 0.8% for atypical squamous cells of undetermined significance not excluding HSIL (ASC-H), 0.8% to 6.2% for low-grade squamous intraepithelial lesions (LSIL), 0,4% to 2,2% for high-grade squamous intraepithelial lesions (HSIL) and 0,001% to 1,4% for squamous cell carcinoma

(SCC) (Zheng et al., 2015, Sankaranarayanan et al., 2004, Bal et al., 2012, Altaf and Mufti, 2012, Bukhari et al., 2012, Verma et al., 2014).

## 1.2 Natural History of Cervical Cancer and HPV infection

The evidence accumulated from virological, molecular, clinical and epidemiological studies allows the statement that cervical cancer is, in fact, a consequence of a long-term unresolved infection by certain genotypes of the HPV (Bosch et al., 2002).

The main steps in cervical carcinogenesis include the infection of the metaplastic epithelium in the cervical transformation zone with one or more carcinogenic types of HPV, viral persistence rather than clearance, the clonal progression of the persistently infected epithelium and invasion (Moscicki et al., 2012).

The HPVs are DNA double-strand viruses and of small size (approximately 8000 basepairs) that have cohabited with the human species over dozens of millennia suffering relatively few changes in their genetic composition. These more than 200 different types of papillomaviruses thus far identified express a characteristic tropism (McGhee et al., 2017). Some types are cutaneotropic (for example HPVs 1, 4, 5, 8, 41, 48, 60, 63 and 65) and they are frequently isolated in cutaneous and plantar warts, in cutaneous lesions in the patients with verruciform epidermodysplasia, in cutaneous lesions in immuno-depressed patients after a transplant and in some epithelial tumors. Another group of HPVs are mucosotropic (for example HPVs 6, 11, 13, 44, 55, 16, 31, 33, 35, 52, 58, 67, 18, 39, 45, 59, 68, 70, 26, 51, 69, 30, 53, 56, 66, 32, 42, 34, 64, 73, 54) and they are seen in benign and (pre-)malignant lesions of the anogenital tract in both sexes. Occasionally, these viral types are isolated in tissues and lesions in the oral cavity, oropharynx, larynx, and esophagus. Finally, another group of HPVs is isolated indifferently in cutaneous or mucous tissues and lesions (for example HPVs 2, 3, 7, 10, 27, 28, 29, 40, 43, 57, 61, 62 and 72) and their association with malignant lesions is less established (Castellsague, 2008).

HPV16 and HPV18 are the two most carcinogenic HPV types and are responsible for 70% of cervical cancers and about 50% of cervical intraepithelial neoplasia grade 3 (CIN3). In contrast, HPV6 and HPV11 are responsible for about 90% of genital warts (Smith et al., 2007).

The HPV genome codes for only eight genes (Doorbar, 2006). E6 and E7 are the primary HPV oncoproteins. Each has many cellular targets but p53 and retinoblastoma tumor

suppression proteins are the most important (Doorbar, 2006, MuÈnger et al., 2001, Mantovani and Banks, 2001). E6 inhibition of p53 blocks apoptosis, whereas E7 inhibition of pRB abrogates cell-cycle arrest. E7 is the primary transforming protein. Both proteins are expressed at low levels during the infectious process. At some still undefined point in progression to precancer, E6 and E7 expression is deregulated by either genetic or epigenetic changes, leading to their over-expression in the full-thickness epithelial lesion (Schiffman et al., 2005)

The anogenital transmission of HPV occurs mainly by skin-to-skin or mucosa-to-mucosa contact (Burchell et al., 2006, Roberts et al., 2007)**.** The probability of infection per sexual act is high, with no known difference between HPV types (Burchell et al., 2006). As HPV types have the same transmission route they are usually transmitted together resulting in a proportion of 20–30% of concurrent infections (Mendez et al., 2005, Plummer et al., 2007, Forman et al., 2012). Independent of type, infecting viral particles reach the germinal cells in the basal layer presumably via tiny tears in the mucosa (Munoz et al., 2006).

The most common HPV type (HPV 16) is also the most persistent (Schiffman et al., 2005, Nielsen et al., 2010). However, the average persistence of some non-carcinogenic types (eg, HPV61) can also be long (Stanley, 2006). The risk for the diagnosis of a premalignant lesion increases with longer HPV persistence since the probability of subsequent clearance decreases (Plummer et al., 2007).

Prevalent infections persist longer in women older than 30 years, probably because they are more likely to represent infections that are already of long duration and the immune response is not as good as that of a younger age (Herrero et al., 2005). The median time to clearance of HPV infections is 6–18 months (Plummer et al., 2007).

Due to current ethical issues the precise magnitude, the timing of risk of invasion and the point of non-return in untreated premalignant lesions will remain unknown (Peto et al., 2004)**.** However, crude estimates from early studies suggested a 20–30% risk of invasion over a 5–10-year time frame (Chang, 1990, Kinlen and Spriggs, 1978).

Apart from age, risk factors for invasion are the viral subtype and the integration of the HPV genome into the host genome (Peitsaro et al., 2002). Notably, integration might not be necessary to cause invasion because not all women with invasive cancers have measurable integration (Pirami et al., 1997, Arias-Pulido et al., 2006). HPV16, 18 and 45 are found in a higher fraction of invasive cancers than in premalignant lesions when compared with other HPV types (Smith et al., 2007).

## 1.3 Etiological Co-factors for Cervical Cancer

The best established etiologic co-factors for cervical cancer in addition to HPV-infections are smoking, long-term hormonal contraceptive use, multiparity and human immunodeficiency virus (HIV) infection (Moscicki et al., 2012). Furthermore, immunosuppression and coinfection with herpes simplex virus type-2 or Chlamydia trachomatis are also probable cofactors. On the other hand, a balanced diet with high fruit and vegetable contents can help to prevent the carcinogenesis (Munoz et al., 2006).

High parity has been found to be associated with cervical high-grade lesions in some studies (Chelimo et al., 2013, Liao et al., 2012). Furthermore, most of the studies restricting the analysis to HPV-positive women report an increased risk for HSIL or cervical cancer with increasing number of (early) pregnancies (Louie et al., 2009, Al-Halal et al., 2013). In a large International Agency for Researcher on Cancer (IARC) study, women with seven or more full-term pregnancies had a four-fold increase in the risk of developing cervical cancer as compared with nulliparous women (OR=3.82, 95% CI: 2.66–5.48) (Munoz et al., 2002).

A possible explanation for the high risk of cervical cancer with high parity is the maintenance of the transformation zone on the ectocervix for many years, facilitating the direct exposure to HPV (Autier et al., 1996). Hormonal changes induced by pregnancy may also decrease the immune response, thus allowing the persistence of HPV and influencing the risk of progression (Plummer et al., 2012).

Long time use of oral contraceptives has been associated with an increased risk of developing cervical cancer (Cogliano et al., 2005, Humans et al., 2007). Women positive for HPV DNA and users of oral contraceptives for five years or longer are more likely to have cervical cancer than those who have never used contraceptives (Smith et al., 2003). Furthermore, this association seems to be stronger for invasive carcinoma than carcinoma in situ (Moreno et al., 2002).

Various epidemiological studies demonstrated that smoking tobacco may influence the risk of progression to cervical cancer (González et al., 2006, Castellsague et al., 2002, De Gonzalez and Green, 2007). The risk of progression is also associated with some smoking habits such as intensity and duration (González et al., 2006). Some data show that women who had stopped smoking for more than 20 years had a two-fold decreased risk of progression in comparison with current smokers and recent quitters (Roura et al., 2014).

Smoking could increase the risk of cervical neoplasia by local immunosuppressive effect and DNA damage in squamous epithelial cells (Harris et al., 2004, Poppe et al., 1995, Szarewski et al., 2001). Tobacco metabolites that have been found in the cervical mucus of female smokers could produce a detrimental effect on the ability of the host to develop an effective immune response against viral infections, increasing the risk of persistent infections in the cervix (Kapeu et al., 2009, Prokopczyk et al., 1997).

## 1.4 Squamous Cervical Lesions

### *1.4.1 WHO Classification*

The cervical squamous cell precursor lesions have been formerly classified histologically as a three-tier system by the World Health Organization (WHO): Mild dysplasia (CIN1, cervical intraepithelial neoplasia-grade I): The extension of the atypical epithelium is limited to the lower third of the epithelium thickness; Moderate dysplasia (CIN2): It affects the lower two-thirds of the epithelium thickness; Severe dysplasia (CIN3): The upper third is also involved by the atypical epithelium.

The current 4th edition of the WHOs Classification of Tumors of the female reproductive organs uses a two-tier grading system: Low-grade squamous intraepithelial lesions encompass the former CIN1, whereas high-grade squamous intraepithelial lesions include the former CIN2, CIN3 and squamous carcinoma in situ (Kurman et al., 2014). Regarding the latter, there are no mature cells migrating towards the surface. The biological significance of these alterations does not differ from severe dysplasia and therefore may be grouped according to the concept on the lesion (High-grade squamous intraepithelial lesion, CIN3) (Kumar et al., 2007).

Certain features of high-grade CIN increase the likelihood of a coexisting early invasion. These include the extensive CIN3, widespread, expansion and deep extension into endocervical crypts. The first sign of invasion is referred to as early stromal invasion; this is an immeasurable lesion less than 1mm in depth that can be managed in the same way as high-grade CIN. Early stromal invasion is encompassed in the term microinvasive carcinoma (Tavassoli and Devilee, 2003)

Invasive squamous cell carcinomas of the cervix vary in the pattern of growth, cell type and degree of differentiation. However, most carcinomas exhibit sheet-like growth and infiltrate as networks of anastomosing bands or single cells with an intervening desmoplastic or

inflammatory stroma. Superficial stroma invasion may be associated with stroma loosening, desmoplasia and/or increased epithelial cell cytoplasmatic eosinophilia (Kurman et al., 2014).

### *1.4.2 TNM Classification*

The present seventh edition of TNM Classification contains rules of classification and staging that correspond with those appearing in the seventh edition of the AJCC Cancer Staging Manual (2009) and have the approval of all national TNM committees.(Edge et al., 2009).

The TNM system for describing the anatomical extent of disease is based on the assessment of three components:

T – The extent of the primary tumor

N – The absence or presence and extent of regional lymph node metastasis

M – The absence or presence of distant metastasis

The addition of numbers to these three components indicates the extent of the malignant disease, thus: T0, T1, T2, T3, T4 N0, N1, N2, N3 M0, M1 (Sobin et al., 2011).

The American Joint Committee on Cancer (AJCC) TNM classification and the International Federation of Gynecology and Obstetrics (FIGO) staging system for cervical cancer are provided below (Pecorelli, 2009):

Stage I - The carcinoma is strictly confined to the cervix (extension to the corpus would be disregarded)

IA - Invasive carcinoma which can be diagnosed only by microscopy, with the deepest invasion ≤ 5.0 mm and largest extension ≤ 7.0 mm

IA1 - Measured stromal invasion of ≤ 3.0 mm in depth and horizontal extension of ≤ 7.0 mm

IA2 - Measured stromal invasion of > 3.0 mm and not > 5.0 mm with an extension of not > 7.0 mm

IB - Clinically visible lesions limited to the cervix uteri or pre-clinical cancers greater than stage IA∗

IB1 -Clinically visible lesion ≤ 4.0 cm in greatest dimension

IB2 -Clinically visible lesion > 4.0 cm in greatest dimension

Stage II - Cervical cancer invades beyond the uterus, but not to the pelvic wall or to the lower third of the vagina

IIA - Without the parametrial invasion

IIAl - Clinically visible lesion ≤ 4.0 cm in greatest dimension

IIA2 - Clinically visible lesion > 4.0 cm in greatest dimension

IIB - With obvious parametrial invasion

Stage III - The tumor extends to the pelvic wall and/or involves a lower third of the vagina and/or causes hydronephrosis or non-functioning kidney∗∗

IIIA - Tumor involves a lower third of the vagina, with no extension to the pelvic wall

IIIB - Extension to the pelvic wall and/or hydronephrosis or non-functioning kidney

Stage IV - The carcinoma has extended beyond the true pelvis or has invaded (biopsy proved) the mucosa of the bladder or rectum. Bullous edema, as such, does not permit a case to be allotted to Stage IV

IVA - Spread of the growth to adjacent organs

IVB - Spread to distant organs

### *1.4.3 Cytological Classification*

Between 1990 and 2014 the *Münchner Nomenklatur II* was used in Germany as the reporting scheme for cytological smears of the uterine cervix (Wagner, 1990). But in view of the development and new challenges involved in the cytological diagnosis, specialists in agreement with the professional associations have proposed an overhaul of the Munich Nomenclature. Thus, from July 2014 the *Münchner Nomenklatur III* was adopted (Griesser et al., 2013). This nomenclature includes diagnostic groups for normal cytology, inconclusive cytology, squamous and glandular precancerous lesions and invasive carcinomas of the uterine cervix and other (adjacent) organs:

- **Group 0:** unsatisfactory for evaluation.
- **Group I and II-a:** normal smears (II-a with conspicuous history).

- **Group II:** with restricted predictive value. II-p, II-g, II-e with abnormal squamous, glandular or with endometrial cells at age >40, respectively.
- **Group IIID:** Dysplasia with the tendency of regression. IIID1 and IIID2, representing mild and moderate squamous dyskeratoses, respectively.
- **Group III:** unclear and equivocal results. III-p, III-g, III-e, III-x, smears are suspicious for high-grade lesions of squamous, endocervical glandular, endometrial or uncertain glandular origin, respectively.
- **Group IV:** Direct precursors of cervical carcinoma. IVa–p, IVa-g represent severe squamous dyskaryoses/ squamous carcinoma in situ or adenocarcinoma in situ, respectively. IVb-p and IVb-g are categorized if an invasive carcinoma cannot be excluded.
- **Group V:** Invasive carcinomas of squamous (V-p), endocervical (V-g), endometrial (V-e) or uncertain origin (V-x).

This nomenclature is mandatory in Germany according to the national guidelines for early cancer detection (Qualitätssicherungsvereinbarung, 2015).

In large parts of the world, the Bethesda System for reporting cervical cytology (TBS) is used as the standard reporting system for cervical cytological specimens. It was developed in the late 1980s at the United States National Cancer Institute and revised in 1991, 2001 and 2014.

In the Bethesda System, in brief, nuclear enlargement more than three times the area of normal intermediate nuclei, contour of nuclear membranes ranging from smooth to very irregular with notches are classified as LSIL (low-grade intraepithelial lesion), whereas less mature or immature squamous dyskaryotic cells with nuclear to cytoplasmatic ratio higher than LSIL are classified as HSIL (high-grade intraepithelial lesion), the latter includes cells of squamous carcinoma in situ (Nayar and Wilbur, 2015). Like the *Münchner Nomenklatur III*, diagnostic categories for normal cytology, inconclusive or atypical cytology, squamous and glandular precancerous lesions, invasive carcinomas of the uterine cervix and other (adjacent) organs are also included. This classification is recommended by the WHO for cytological reports (Health et al., 2006).

For a short overview of precancerous lesions, the above mentioned cytological reporting systems are shown in excerpts in comparison with the WHO-classification (Table 1).

| WHO, 2003 | WHO, 2014 | MN III | TBS 2014 | Description |
|---|---|---|---|---|
| | | Group II-p | ASC-US | Abnormal cells, koilocytosis without dyskaryosis |
| **CIN1** | LSIL | Group IIID1 | LSIL | Mild squamous dyskaryosis/dysplasia |
| **CIN2I** | HSIL | Group IIID2 | HSIL | Moderate squamous dyskaryosis/dysplasia |
| **CIN3, CIS** | | Group IVa-p | | Severe squamous dyskaryosis / dysplasia, squamous carcinoma in situ |
| | | Group III-p | ASC-H | Atypical squamous cells, a high-grade lesion cannot be excluded |
| **Adenocarcinoma in situ** | Adenocarcinoma in situ | Group IVa-g | AIS | Adenocarcinoma in situ |

**Table 1: Resume of the stages of intra-epithelial lesions according to the diverse reporting systems. WHO: World Health Organization; MN: *Münchner Nomenklatur III*; CIN: Cervical intra-epithelial neoplasia; TBS: The Bethesda System for Reporting Cervical Cytology.**

The main difference between MN III and the Bethesda System is the division of the HSIL group. This was subclassified by MN III in groups IIID2 and IVa-p, standing for moderate dysplasia and severe dysplasia, respectively.

## 1.5 Detection of Cervical Lesions

### *1.5.1 Cervical Cancer Screening, an overview*

In several Western countries, where screening programs have long been established, cervical cancer rates have decreased by as much as 65% over the past 40 years (Torre et al., 2015). For example, in Norway cervical cancer incidence rates decreased from 18.7 per 100,000 in 1970 to 9.6 per 100,000 in 2011 (Engholm et al., 2014). Rates have also decreased in some high-incidence areas including Colombia, the Philippines, and India. This is likely due to increased awareness and improved socioeconomic conditions (Forman et al., 2012).

The main options for cervical screening currently are cytology, HPV testing, and cytology plus HPV co-testing (Felix et al., 2016, Wentzensen and Schiffman, 2014).

Because the cytology and HPV testing require trained cytopathologists and technicians, sometimes it is not viable in poor countries to perform these methods, particularly in remote regions. Therefore, many of the patients are no longer attendant during the diagnostic tests and are subsequently lost to follow-up. Thus, an alternate screening procedure, in that case, may be

Visual Inspection of the cervix with Acetic acid (VIA). VIA is a highly sensitive screening test, simple to perform and does not require a high number of specialized professionals. However, the main disadvantage is its low specificity leading to high false positive screens (Gami et al., 2016).

### *1.5.2 Cytological Methods*

#### *1.5.2.1 Conventional smears*

Since George N. Papanicolaou along with Herbert F. Traut published "The diagnostic value of vaginal smears in carcinoma of the uterus'' in 1941 the mortality rate from uterine cancer declined by more than 80% (Siegel et al., 2016).

For the preparation of a smear, a sample of the ectocervix is taken using a spatula. The notched end of the spatula that corresponds to the contour of the cervix is rotated 360º around the circumference of the cervical orifice, retaining the sample on the upper surface of the spatula. A sampling of the endocervix requires insertion of the endocervical brush into the endocervical canal in the same rotation 45-90º and then removed. At this time, the samples on the spatula and endocervical brush are spread evenly and thinly lengthwise down a slide. The entire slide is then rapidly fixed by immersion or spray and the collection devices are discarded. Subsequently, the slides are stained according to Papanicolaou (refer to chapter 2.3.1, table 2).

Cytology of the uterine cervix has succeeded as a screening test because it has all the qualities of a diagnostic tool: it is cheap, simple to use and minimally invasive. Some studies show that the cytology´s accuracy varies between 64.2% and 78.4% (Dillner et al., 2008, Herrero et al., 2005, Sorbye et al., 2011, Katki et al., 2011, Szarewski et al., 2008). Thus, it has survived virtually unchanged for 50 years. However, during the last 15 years some technological advances, such as liquid-based cytology and automation, have emerged as possibly better alternatives (Alves et al., 2004).

#### *1.5.2.2 Liquid-based Cytology (LBC)*

Due to the sometimes poor quality of sampling or preparation like obscuration by blood or inflammation, bad fixation or inhomogeneous distribution of the cells, the conventional smears lead to false-negative and rarely false-positive test results (Siebers et al., 2009). Liquid-based cytology can surpass this problem because the cervical cells are rinsed into a pot with a

preservation solution. Just a representative portion of the sample is used and the cells are centrifuged on a glass-slide. Thus, the slide has a more homogenously spread cell preparation without clumping and obscuring by inflammatory cells (Kitchener et al., 2006). Therefore, the residual material in the pot may be used later for adjuvant methods such as human papillomavirus (HPV) testing, cytometry, FISH and other molecular tests (Arbyn et al., 2004, Hutchinson et al., 1994, Arbyn et al., 2007). Particularly, the standardized preparation and good fixation provide a good basis for the above-mentioned adjuvant methods.

However, some studies did not show increased accuracy with liquid-based cytology in comparison to the conventional smear. According to a randomized controlled trial performed by Siebers et al., the adjusted detection rate ratios for CIN1+ was 1.01, for CIN2+, 1.00, for CIN3+, 1.05 and for carcinoma in situ, 1.69 (Siebers et al., 2009). The only advantage described in the literature is the lower number of necessary samples to the final diagnosis (Bergeron et al., 2001, Ferenczy et al., 1996, Hessling et al., 2001, Coste et al., 2003). Furthermore, the costs of manually screened ThinPrep LBC and automated LBC are relatively high compared to the costs of conventional cytology. The difference is caused mainly by higher material and logistic costs. Thus, in some countries like Germany, LBC is not adopted as a standard screening method, by it's not reaching the required cost-effectiveness (Lee et al., 2006, Deshou et al., 2009, Kavatkar et al., 2008).

#### *1.5.2.3 Automated screening*

The second technological development of greater significance in cervical cytology is automation, in which computer technology using algorithms of cell recognition can identify the most abnormal areas of an entire slide and present them for the purpose of reading (Anttila et al., 2011). There are only two computer-assisted devices approved by the US Food and Drug Administration (FDA) available for primary screening of cervical specimens: ThinPrep Imaging System (TIS; Hologic Inc., Boxborough, MA) and FocalPoint Slide Profiler (BD Diagnostic Inc., Burlington, NC) (Levi et al., 2012). Likewise, LBC`s high cost of this method does not allow its introduction as a screening method. Kitchener et al. concluded in 2011 that the reduced sensitivity of automation-assisted reading compared to manual did not warrant the investment needed to introduce automated primary screening to the program (Kitchener et al., 2011).

### *1.5.3 HPV-Test*

The understanding that HPV is necessary for the cause of cervical cancer has led to major advances in primary and secondary prevention of cervical cancer. Initially, HPV DNA tests were approved for the triage of women with equivocal cytologically results (i.e. ASC-US according to TBS). Later, the regulatory approval was extended to HPV plus cytology co-testing and HPV DNA tests for primary cervical cancer screening (Iftner et al., 2015).

More than 190 commercial HPV tests are available, which target only all but two alpha-HPV types. The classification of alpha-HPV types into different cervical cancer-promoting risk categories is extremely challenging, especially for weakly carcinogenic and rare HPV types (Poljak et al., 2016).

HPV tests can be divided into groups and subgroups regarding the kind of approach:

- **High risk-HPV DNA screening tests**
- **High risk-HPV DNA screening tests with concurrent or reflex partial genotyping for the main hr-HPV types**
- **HPV DNA full genotyping tests**
- **HPV DNA type- or group-specific genotyping tests**
- **High risk-HPV E6/E7 mRNA tests**
- **In situ hybridization DNA-based HPV tests**
- **In situ hybridization mRNA-based HPV tests**
- **HPV DNA tests targeting miscellaneous HPV types**

Only a few HPV tests are approved by the FDA for cervical cancer screening. Four of the five approved hr-HPV tests, Cobas HPV test (Roche, Pleasanton, CA), Hybrid Capture 2 (Qiagen, Gaithersburg, MD), Cervista HPV HR (Bedford, MA) and Cervista HPV16/18 qualitatively detect viral DNA sequences. The fifth FDA-approved hr-HPV test, Aptima HPV assay (AHPV, Hologic), is a qualitative test for detecting mRNA expressed from viral E6/E7 oncogenes (Castle et al., 2015).

The Digene Hybrid Capture 2 (HC2) HPV DNA Test (Qiagen, Gaithersburg, MD) has been the most widely used molecular HPV assay in most clinical trials and has been extensively reviewed (Cuzick et al., 2008). This assay is FDA approved for triage in cases of equivocal cytology results in the presence of atypical squamous cells of undetermined significance (ASC-US) to determine which patients should be referred for a colposcopy and as a screening test for use in addition to cytology in women ≥30 years (Poljak et al., 2016).

Cytological screening which is still the most adopted has lower sensitivity compared to both, HPV and co-testing, and needs to be repeated at shorter intervals to achieve a good sensitivity. Notwithstanding, it is not plausible to send all HPV-positive women to colposcopy since HPV testing has a low specificity and would substantially increase the number of false positive women compared to cytology (Wentzensen et al., 2016).

### *1.5.4 Cervical Cancer Screening in some selected Countries*

#### *1.5.4.1 England:*

In England, the LBC+ is the adopted primary screening test. Women between 25 and 49 years are invited to the program consecutively every three years if they have had a negative screening-result. The screening-interval is longer for women between 50 and 64 years (5 years). Patients with a borderline or low-grade cytological result are referred to colposcopy and the hr-HPV Test. High-grade dyskaryosis or worse results are directly referred to a histological evaluation (Public Health England, 2015).

#### *1.5.4.2 Sweden:*

In Sweden, the primary screening test for women at age 23 to 29 is conventional cytology. An hr-HPV test is performed in women over the age of 30. The interval between negative screens is 3 years for women between ages 30-50 and 7 years for women between ages 51-64. In the case of hr-HPV or cytological positive results, a cytological or hr-HPV test control has to be performed, respectively. A co-test is required for women older than 41 years. Women younger than 28 years with an ASCUS or LSIL result are not referred to colposcopy, but to repeated cytology. Patients with a positive reflex test, HSIL results or with two positive hr-HPV test results within 3 years are referred to a colposcopy (Elfstrom et al., 2016).

*1.5.4.3 USA:*

The American Cancer Society recommends that all women should begin cervical cancer testing (screening) at age 21. Women aged 21 to 29, should have a cytological smear test every 3 years. Hr-HPV testing should not be used for screening in this age group, although it may be used as a part of follow-up for an abnormal cytological diagnosis. Beginning at age 30, the preferred way to screen is with a Pap test combined with an hr-HPV test every 5 years. This is called co-testing and should continue until age 65. Another reasonable option for women between ages 30 and 65 is to get tested every 3 years with just the cytological smear test. Women older than 65 years who have had a regular screening in the previous 10 years should stop cervical cancer screening as long as they haven't had any serious pre-cancerous lesions (like CIN2 or CIN3) in their last 20 years (Saslow et al., 2012).

### *1.5.5 Cervical cancer screening in Germany*

In Germany, the cervical cancer screening starts at age 20 with yearly intervals. The overall participation rate in the German population has increased in the last years. The annual screening rates were 2.8% higher in 2011 when compared with 2006 (Schneider, 2012). The cumulative participation rates obtained for 3-year intervals (2006/2008-2009/2011) were 63.4–66.5% (Geyer et al., 2015). Although the comparison of participation rates among countries is rather difficult due to differences in the approach (for example organized or opportunistic screening), it is noted that there is a significantly higher overall rate in the United Kingdom of 83.5% (Bang et al., 2012).

In Germany, the latest version of the Cancer Prevention Guidelines was published in June 2009 and revised in April 2016. The Joint Federal Committee (Gemeinsamer Bundesausschuss) decided not to use LBC and primary hr-HPV screening as an early detection method for cervical cancer. The main reason was that there was insufficient evidence for the improved clinical efficacy of LBC compared to conventional cytology. In other words, the significantly higher costs of LBC were not justified by better clinical efficacy. (Bundesausschuss, 2009).

Currently, the German Joint Federal Committee plans to change the algorithm for cervical cancer screening. Women aged between 20 and 60 years will be informed by their health insurance once every five years about cervical cancer screening. Yearly Cytology is offered to woman aged 20-34. Cytology and hr-HPV co-testing are offered to all women aged

35 and older once every three years. The hr-HPV test is not offered to the younger age group because of higher HPV-prevalence and risk of a false positive and none clinically relevant test results. They intend to review this strategy after six years (actual discussion of German cytopathologists and the Joint Federal Committee, personal communication Dr. Schramm).

### *1.5.6 Recommendations for Proceeding Conspicuous Screening Results in Germany*

In Germany, new different recommendations for further evaluation by the physicians are published with the advent of the *Münchner Nomenklatur III* (Griesser et al., 2015).

- **Group 0:** In this case, when the patient has cytological unsuspected preexisting findings and clinically inconspicuous findings, a cytological control within 6 months is recommended.
- **Group I:** Even in the absence of cells of the transformation zone, the smear will be repeated in the usual screening interval.
- **Group II-a:** The most frequent reason for the definition of a sample to be Group II-a is a previous finding of group IIID or group III. In this case, the cytopathologist indicates an increased risk for the patient despite a currently unsuspicious cell image. The smear should be repeated in a normal screening interval.
- **Group II-p and II-g:** If group II-p or II-g occurs for the first time, a control in 12 months is sufficient. If a group II-p or group II-g is given again in the control examination, a cytological control is indicated in 6 months for women up to 35 years of age; an additional HPV test should be considered for women over 35 years of age. If high-risk types are detected, the procedure is the same as for group IIID1.
- **Group II-e:** requires assessment by the gynecologist. In the case of anamnestic or clinical abnormalities, he will decide whether further diagnostic procedures would be required.
- **Group III-p and III-g:** If the presence of carcinoma is considered, a colposcopy is indicated. In the case of consideration of a CIN2, a CIN3 or

adenocarcinoma in situ, a cytological control is initially acceptable (optionally after inflammatory treatment and/ or local hormonal treatment, possibly in combination with additive methods, e.g. hr-HPV test or molecular/immunochemical indicators of (pre-)malignant transformation). Colposcopy should be carried out at the latest when there is another doubtful cytological result.

- **Group III-e:** further clinical diagnosis (sonography, hysteroscopy, and curettage, as long as the extra cervical examination has not produced any results: colposcopy) is to be considered.
- **Group III-x:** a further clinical evaluation is needed (colposcopy, sonography, hysteroscopy, fractional abrasion).
- **Group IIID1:** a cytological control is recommended at 6-month intervals (also in pregnant women). If the lesion persists longer than 12 months, colposcopy should be performed. After 2 years of persistence, a histological examination should be attempted by biopsy.
- **Group IIID2:** The interval between cytological controls should be 3 months. If the results are persistent for more than 6 months, a colposcopy is recommended. In pregnancy, a colposcopy is desirable as early as possible. A cytological colposcopy should be performed once per trimester. In the case of a discrepant finding between cytology and colposcopy, histological examination is recommended by biopsy.
- **Group IV:** Colposcopy and therapy. In pregnancy, the first presentation should occur as early as possible. Additional cytologic-colposcopic controls are recommended every 8-12 weeks.
- **Group V:** Further diagnostic and therapeutic measures.

### *1.5.7 Selected Adjuvant Methods in Diagnostic Cervical Cytopathology*

With the aim of improving the quality of the cytological diagnosis, some adjuvant methods were developed to use in conjunction with conventional cytology: In the Department of Cytopathology Düsseldorf, DNA image cytometry and immunocytochemistry are currently

applied in the diagnostic routine. Fluorescence in situ hybridization, which is being adopted by some cytopathology laboratories, is intended for application in cervical cytology in Düsseldorf with the aid of this study.

DNA image cytometry (DNA-ICM) is a method used to measure DNA aneuploidy, which in turn represents the quantitative cytometric equivalent of chromosomal aneuploidy and has been accepted internationally as a well standardized marker of neoplastic cell transformation (Haroske et al., 1998, Giroud et al., 1998, Bocking et al., 1995). Since then DNA aneuploidy, according to various studies, has been demonstrated to indicate either invasive carcinoma or prospectively neoplastic development in cervical dysplasia, The International Consensus Conference on the Fight Against Cervical Cancer International Academy of Cytology (IAC) Task Force 8 recommended DNA-ICM as a useful adjunctive method for identifying cervical intraepithelial lesions, which require further clinical management (Bocking et al., 1986, Hering et al., 2000, Bollmann et al., 2001, Webb, 2001, Hanselaar et al., 2001). This method is still in use at our and some other cytopathological departments for equivocal cervical cytology like group IIID1, IIID2, III-p or III-g.

Among several markers that have been presented as possible candidates to optimize the accuracy of cytology-based screening of the underlying cause of ASC-US or LSIL, the tumor suppressor protein p16INK4a (p16) and the cell proliferation marker Ki-67 have been considered important for the routine cytological evaluation. The co-expression of these two proteins in individual squamous cells, analyzed with immunocytochemistry, is associated with a persistent HPV-infection and a pre-(malignant) transformation of the cell (Brown et al., 2012, Schmidt et al., 2011, Possati-Resende et al., 2015, Tjalma, 2017).

Florescence in situ hybridization (FISH) is a technique that uses cDNA probes, labeled with fluorescent dyes, to detect genetic and chromosomal alterations in the cell. Since tumors emerge from genetic and epigenetic alterations that activate oncogenes and inactivate tumor suppressor genes, FISH could be able to detect structural and numerical chromosomal alterations of possibly premalignant and malignant cells independently from morphological alterations. The use of this method in tumor pathology that includes some research on its application in cervical cytology is described in the following chapter.

## 1.6 Fluorescence in situ Hybridization

In situ hybridization, the detection of a nucleic acid sequence "in its original place," was first described by Gall and Pardue (Gall and Pardue, 1969). This early work involved detection of target DNA sequences with radiolabelled complementary sequences (probes). The application of nonisotopic in situ hybridization (NISH) to cytogenetic research was first described by Manning et al. (Manning et al., 1975).

FISH, like Northern and Southern blotting, relies on base pairing between two polynucleotides with complementary sequences. For FISH, however, the target sequences are left within the tissue and there is no need to isolate nucleic acids prior to the probing as there is with Northern and Southern blot techniques. The FISH probe, whether directly or indirectly labeled, is allowed to hybridize with complementary target sequences within a cell, which usually is fixed onto a microscope slide.

Properly performed in situ hybridization is a sensitive and versatile tool that has many applications. These uses include analyzing nuclear organization throughout the cell cycle, studying DNA repair dynamics, providing very precise information on the location of specific DNA sequences (mapping), identifying rearrangements of DNA sequences within both interphase and metaphase (or prometaphase) cells, identifying the gain or loss of specific sequences, and studying the presence or expression of cell- or tissue-specific gene sequences.

Recent advances in FISH-based cytogenetic tests support or aid the physician in diagnosing cancer. It can be used to predict progression, for therapy planning (i.e. ALK-translocation) and also to monitor the regression of a tumour. The high sensitivity and specificity of FISH and the speed with which the assay can be performed have made FISH a powerful technique with numerous applications, and it is extensively used as a clinical laboratory tool. Therefore it has gained great acceptance such as other techniques like RT-PCR, PCR and immunohistochemistry (IHC) for cancer diagnosis and treatment (Futreal et al., 2004, Belaud-Rotureau et al., 2002).

Patient care with non-invasive or minimally invasive methods is appealing to the patient. A few of the applications where FISH can be utilized for the non-invasive detection of exfoliated tumour cells are for detection of bladder cancer in urine or for lung cancer in bronchial brushings/washings during bronchoscopy. Selective markers for dysplasia in Barrett's oesophagus can be identified to improve the differentiation between low-grade dysplasia (LGD) and high-grade dysplasia (HGD). FISH can also be used clinically to detect tumour cells of

biliary tract malignancy in endoscopic retrograde cholangiopancreatography- obtained from biliary brushing specimens (Voss et al., 2010).

Unlike conventional urinary cytology and cystoscopy, which depend on visible microscopic or macroscopic changes, FISH allows identification of chromosomal abnormalities associated with malignant development before phenotypic expression of those alterations. FISH of interphase nuclei for chromosomes 3, 7, 9, and 17 centromeric and gene loci has been optimized for detection of urothelial carcinoma and increases the sensitivity of tumour findings. The FDA approved UroVysion Kit is commercially available (Halling et al., 2000, Sokolova et al., 2007). It can also reduce the number of cystoscopies in the follow-up of bladder cancer patients. These findings suggest that the use of morphologic cellular changes by conventional cytology in combination with FISH-based molecular diagnostics increases the sensitivity of tumor findings and can efficiently detect cancer and predict its recurrence.

Disadvantages of FISH in relation to cytology include its expense. However, the greater ability compared to cytology of FISH to detect early and peripheral disease can have an impact on overall survival of the patient (Halling et al., 2000, Sokolova et al., 2007). In conclusion, FISH can detect cells that have chromosomal abnormalities consistent with neoplasia in exfoliative and aspiration cytology specimens.

There are several types of FISH probes, for example, chromosome enumeration probes (CEPs) and locus-specific indicator (LSI) probes. CEPs are used to detect aneusomy, whereas LSI probes are generally used to detect deletions, duplications, amplifications or translocations of specific genes (Halling and Kipp, 2007).

FISH has been proposed as an additional noninvasive test on cervical smears to detect chromosomal abnormalities (markers of chromosomal damage) and manufacturers are starting to promote the use of FISH testing to triage women for colposcopy on the basis of their cytological, hr-HPV test and FISH test findings (Uhlig et al., 2013). However, FISH is not currently used for screening cervical lesions.

Sokolova et al. assessed biopsy specimens showing high-grade dysplasia and cancer with FISH probes to 35 unique loci and identified 2 loci, the 3q26.3 region (comprising the hTERC gene) and the 8q24 region (comprising the c-MYC gene), which showed the highest frequency of copy number gains in high-grade dysplasia and cancer (Sokolova et al., 2007).

Technical details on the method are described in chapter 2.

## 1.7 Role of EGFR, MYC, and hTERC in Cancer Development

### *1.7.1 EGFR gene*

The epidermal growth factor receptor (EGFR) gene is located on chromosome 7p12-13 and codes for a 170kDa receptor tyrosine kinase. EGFR overexpression was observed in a variety of human tumors, mainly in the lung (Cheng et al., 2017, Savage et al., 2017).

The receptor tyrosine kinase (RTK) super-family of cell surface receptors serve as mediators of cell signaling by extra-cellular growth factors (Krause and Van Etten, 2005). Members of the ErbB family of RTKs, such as EGFR (also known as ERBB1 or HER1), ERBB2 (also known as HER2), ERBB3 (also known as HER3) and ERBB4 (also known as HER4) have received much attention, given their strong association with malignant proliferation (Sharma et al., 2007).

Ligand-induced activation of the EGFR can instigate a wide range of cellular responses such as growth, differentiation, migration, and survival through various signaling pathways (Yarden and Sliwkowski, 2001). Accordingly, it has been shown that persistent activation of the EGFR enables cancer cells to engage in autonomous proliferation, which is the first and debatably the most critical hallmark of cancer (Hanahan and Weinberg, 2000). Moreover, EGFR expression has long been recognized as a prognostic marker of advanced tumor stage, resistance to standard therapeutic approaches and reduced patient survival (Arteaga, 2002).

The dependence of certain cancer cells on the EGFR for growth and survival combined with the above-mentioned factors has directed much attention to the EGFR, which is currently a central target for cancer therapy (Mendelsohn and Baselga, 2000).

EGFR overexpression has been reported in 62% of non-small cell lung cancer (NSCLC) cases (Nicholson et al., 2001, Hirsch et al., 2003). In some cases, genomic analyses documented the amplification of chromosomal region 7p12, where the EGFR gene is located (Testa and Siegfried, 1992). In addition to EGFR overexpression, its cognate ligands, epidermal growth factor (EGF) and transforming growth factor-α (TGFα) are also frequently expressed in NSCLCs and can establish autocrine loops that lead to receptor hyperactivity (Putnam et al., 1992). The disruption of these autocrine loops is the primary rationale for antibody-based EGFR-targeted therapeutics (Mendelsohn, 1992).

Most of lung-cancer–specific EGFR mutations comprise a leucine-to arginine substitution at position 858 (L858R) and deletion mutants in exon 19 that affect the conserved sequence LREA (delE746-A750), causing a constitutive activation of the tyrosine kinase of the EGFR by destabilizing its autoinhibited conformation, which is normally maintained in the absence of ligand stimulation (Lynch et al., 2004, Kosaka et al., 2004, Yun et al., 2007, Rosell et al., 2009). The activating mutations confer hypersensitivity to the tyrosine kinase inhibitors gefitinib and erlotinib (Lynch et al., 2004, Pao et al., 2004, Rosell et al., 2009).

Gefitinib is a selective EGFR (ErbB1) tyrosine kinase inhibitor approved for the treatment of patients with non-small cell lung cancer after failure of both platinum-based or docetaxel chemotherapies (Arora and Scholar, 2005). The possible specific mechanism of antitumor activity is the up-regulation of the cyclin-dependent kinase inhibitor p27 via EGFR kinase inhibition leading to inhibited cyclin-dependent kinase activity and arrest in the G1 cell cycle phase (Arteaga and Johnson, 2001).

Erlotinib hydrochloride is a potent, reversible, and selective inhibitor of the EGFR (ErbB1) tyrosine kinase (Ranson, 2004). In a placebo-controlled trial, Erlotinib improved symptoms and increased survival rates of patients with advanced stage III or IV NSCLC and who had progressive disease after standard chemotherapies (Perez-Soler, 2004).

Since in HPV-infected patients, the majority of HPV-associated lesions regress spontaneously, it is possible to infer that additional genomic alterations are involved in the transformation of cervical epithelial cells and progression of cervical cancer. There is evidence showing that the HPV E6 protein, the Hippo pathway and the EGFR signaling pathway interact with each other to regulate cervical cancer progression (He et al., 2015).

### *1.7.2 MYC gene*

The MYC gene has long been known to be altered by chromosomal translocations and gene amplification in many human cancers. In addition, common single nucleotide polymorphisms (SNP) on human chromosome 8q24, which predispose to cancer, have been implicated in deregulated MYC expression (Wokołorczyk et al., 2008). This proto-oncogene contributes to the genesis of many human cancers, encodes a transcription factor c-Myc, which links altered cellular metabolism to tumorigenesis. c-Myc regulates genes involved in the

biogenesis of ribosomes and mitochondria, and regulation of glucose and glutamine metabolism (Dang et al., 2009).

Cervical tumours, especially in HPV-infected cases, have low-levels of Myc oncogene amplification, with 3–7 copies of the c-myc gene. This finding indicates that HPV-associated cervical carcinomas bear frequent alterations in this gene, which may have a critical biologic impact on the development and progression of carcinomas of the uterine cervix (Zhang et al., 2002). In addition, the integration of HPV sequences close to the c-Myc locus has been shown in some cervical cell lines and genital tumours, suggesting a synergistic role for HPV and this proto-oncogene in the development of cervical carcinoma (Nair et al., 1997)

### *1.7.3 hTERC gene*

Among the various components of the human telomerase, only human telomerase RNA component (hTERC), localized on chromosome 3q26 and human telomerase reverse transcriptase (hTERT) are essential for the reconstitution of telomerase activity (Ishikawa, 1997, Weinrich et al., 1997). It is proposed that telomere dysfunction may first promote chromosomal instability that drives early carcinogenesis, and telomerase activation can later restore genomic stability to a level permissive for tumour progression (Liu et al., 2004). In addition, it is assumed, that telomere-length shortening is prone to chromosomal aneuploidy. The shortened telomeres are more "sticky", and lead to chromosomal fusions and as a possible result of chromosomal breakage during the mitosis (Stindl, 2008).

About 90% of cervical carcinoma cases are characterized by an amplification of the human telomerase RNA gene (hTERC) (Yang et al., 2001). This specific genetic abnormality was also found in premalignant CIN II/CIN III and is considered as a genetic aberration occurring in the early stages of tumor development with greater value to predict malignant transformation and progression of the disease (Hopman et al., 2006a, Heselmeyer-Haddad et al., 2003, Alameda et al., 2009).

## 1.8 Aims of the study

The present work deals with an attempt to develop a FISH protocol, to detect an underlying malignant transformation in cervical lesions, sampled for cytology. In the case of a

mild dysplasia of the squamous epithelium (group IIID1 according to the *Münchner Nomenklatur III*), a cytological control has to be done in 6 months, and again after 1 year. In the case of persistence, referral to colposcopy is recommended. A diagnosis of moderate dysplastic changes of the squamous epithelium demands a cytological control in 3 months, and if necessary again after 6 months. Persisting lesions should be further evaluated by differential colposcopy. This kind of control brings up some personal issues, such as the psychological suffering that the patient endures during the waiting period between the diagnoses. An adjuvant method, applied to the cytological specimen, with the potential to confirm the first suspicious cytological result and to predict the potential of progression to cancer would be ideal. With this result, repeated controls could potentially be avoided. Since the carcinogenesis is a process involving genetic and chromosomal alterations from the beginning, FISH emerges as a possible adjuvant method in diagnostic cytopathology, since it has the capacity to detect this type of alterations with high sensitivity and specificity. In the current work, archived smears of women referred to the colposcopy unit at the University Hospital of Düsseldorf (UKD) between 2014 and 2015, are further analyzed subsequent to routine cytology with three FISH probes (hTERC, MYC, EGFR). All premalignant and malignant stages of squamous cervical lesions are included, as well as negative smears. The results of cytology and FISH are compared to a histological (biopsy, conization) and clinical follow-up. It is analyzed, whether our FISH protocol for adjuvant cytological investigation could provide enough information about the risk of progression of a given lesion at the time of examination and therefore possibly prevent repeated cytological examinations. An ethics vote was obtained from the Ethics Committee of the Medical Faculty of the Heinrich Heine University Düsseldorf (study number: 4923, Date: December 22, 2015).

# 2 Material and Method

## 2.1 Patients and Follow-up

For our retrospective case-control study, cervical smears of 132 women, who were treated in the period from January 2014 until August 2015 at the Universitätsklinik Düsseldorf (UKD), *Klinik für Frauenheilkunde und Geburtshilfe* (Department of Obstetrics and Gynecology), were included.

From a list with all "positive" smears with squamous lesions (classified as PAP IIp, IIID1, IIID2, IVa-p and V), 101 were selected for the study according to the number of suspicious cells available and a correspondent histological outcome.

The smears were chosen according to the cytological diagnosis: Pap I, Pap IIp, Pap IIID1, Pap IID2, Pap IVa-p and Pap V according to the *Münchner Nomenklatur III*. 25 smears were calculated as a representative sample for each diagnostic group on the basis of statistical analysis (calculated by Isabela Caroline de Santana Almeida Araujo, Department of Cytopathology). In group V, only 11 smears were available in the above-mentioned period. 25 specimens from the group I were included as negative controls resulting in a total number of 132 smears.

The negative smears were chosen consecutively in the above-mentioned period. As it is not common in the clinical routine to do a histological examination in cytologically negative lesions, this was not adopted as an inclusion criterion for group Pap I.

The patients were gathered with the support of the data bank system DC Pathos (dc-system Informatik GmbH, Heiligenhaus, DE), which includes data from the routine diagnostic cytopathology and histopathology. Only patients with near-term biopsy or conization for histological diagnosis in addition to cytology were eligible. The analysis was made taking into consideration the result of the histological diagnosis in a period of 3 months for a biopsy and 6 months for conization after the first cytological diagnosis. The clinical course of the patients was followed as long as available in order to obtain more information about the progression of the disease in comparison with the FISH outcome. Only cytological samples with enough suspicious cells (±50) were included.

## 2.2 Materials

### *2.2.1 Reagents, Probes, and Devices*

The following chemicals and items were used to prepare the solutions which were necessary to the FISH process or were directly applied to the process (Table 1).

| Solutions / Material | Order-Nr. | Company | Quantity |
|---|---|---|---|
| **Xylol** | 1.08685.2500 | Merck | 2,5l |
| **Ethanol series 70%,80% ,96% and 99.9%** | 3413128, 3400843, 3413131, 3417696 and 3413132 | VWR | 1l |

| | | | |
|---|---|---|---|
| **25% HCL** | 321-324416 | Merk | 1l |
| **Purified Water** | 3404939 | Otto Fischar | 10l |
| **Paraformaldehyde** | 8.18715.0100 | Merck | 100g |
| **Pepsin (essentially salt-free)** | P-6887 | Sigma | 1g |
| **DAPI II counterstain** | 6J5001 / 32-804831 | Abbott / Vysis | 2 x 500 µl |
| **Coverslips 15x15mm** | 631-0710 | VWR | 5x200 |
| **Coverslips 18x18mm** | 3217228 | VWR | 1x100 |
| **Coverslips Ø round 10mm** | 631-1340 | VWR | 10 x 100 |
| **Coverslips Ø round 12mm** | P 231.2 / 631-0713 | VWR | 1x200 |
| **Slide boxes (plastic) with closure** | 2-2438 (blau), 2-2436 (rot), 2-2435(grün) | NeoLab | For 100 Slides |
| **for PBS Buffer** | **Order-Nr.** | **Company** | **Quantity** |
| **Disodium phosphate $Na_2HPO_4$** | 106586 | Merck | 500g |
| **Monopotassium phosphate $KH_2PO_4$** | 104873 | Merck | 250g |
| **Sodium chloride NaCl** | 106404 | Merck | 1kg |
| **Potassium chloride KCL** | 104936 | Merck | 250g |
| **FISH probes** | **Order-Nr.** | **Company** | **Quantity** |
| **Vysis LSI TERC SpectrumGold** | 02N11-030 | Abbott | 3x 20 µl |
| **Vysis LSI MYC SpectrumAqua** | 02N22-020 | Abbott | 3x 20 µl |
| **Vysis LSI MYC SpectrumGreen** | 07N98-020 | Abbott | 3x 20 µl |
| **Additional FISH solutions** | **Order-Nr.** | **Company** | **Quantity** |
| **LSI/WCP- Hybridization Buffer** | 6J6701 /32-804826 | Abbott | 2 x 150µl |
| **NP 40** | 7J0501 | Abbott | 2x1ml |
| **20xSSC** | 2J1032/ | Abbott | 500g |
| **Vectashield with DAPI (Vector)** | 3410545 / H-1200 | Biozol | 10ml |
| **Fixogum** | 290117 | Marabu | 125g |
| **518F fluorescence free oil** | 444960 | Zeiss | 20ml |

**Table 1: List of the materials and solutions used in the study, describing the manufacturer and the quantity available by order. Order-Nr: order code.**

The following devices were necessary to the FISH staining process and evaluation (Table 2).

| Items (Staining) | Company | Model |
|---|---|---|
| **Analytical balance** | Satorius AG, Göttingen | Basic Plus 201 |
| **Precision balance** | Kern & Sohn | EW |
| **Precision balance** | Harry Gestig GMBH | PZ – 28 – 1T |
| **Centrifuge** | Qualitron INC | DW -41 -230 |
| **Minishaker** | JKA & Co. KG, Arnstein | MS 1 |
| **Waterbath** | Memmert GmbH | WB/0B7-45 WBU 45 |
| **Incubator** | Heraeus Holding GmbH | B 6060 |

| Refrigerator | Liebherr S.A. | ES |
|---|---|---|
| **Freezer** | Liebherr S.A. | Comfort |
| **Digital Thermometer** | VWR International | |
| **Items (Evaluation)** | **Company** | **Model or Exc/Sp/Em** |
| **Fluorescence Microscope** | Zeiss AG | AxioImager M1 |
| **Sp. Green Filter** | AHF Analysentechnik AG | 500 / 515 / 535 |
| **Sp. Red Filter** | AHF Analysentechnik AG | 575 / 593 / 624 |
| **Sp. Gold Filter** | AHF Analysentechnik AG | 546 / 555 / 575 |
| **Sp. Aqua Filter** | AHF Analysentechnik AG | 436 / 455 / 480 |
| **Sp. Orange Filter** | AHF Analysentechnik AG | 546 / 560 / 585 |
| **Axiovision Evaluation Software** | Zeiss AG | 4.6 |
| **Illuminator** | Zeiss AG | HBO 100 |
| **Power Supply** | Zeiss AG | 231 |
| **Microscope Camera** | Zeiss AG | AxioCam MRm |

**Table 2: List of devices and fluorescent filters (wavelengths in nm for excitation, splitting, and emission).**

### *2.2.2 FISH Probes*

The three probes used in this work were commercially available and purchased from Abbott (Abbott-Vysis, Downers Grove, IL, USA): Vysis LSI MYC SpectrumAqua Probe (8q24.21), Vysis LSI TERC SpectrumGold Probe (3q26) and Vysis LSI EGFR SpectrumGreen Probe (7p11.2–p12) (figure 1).

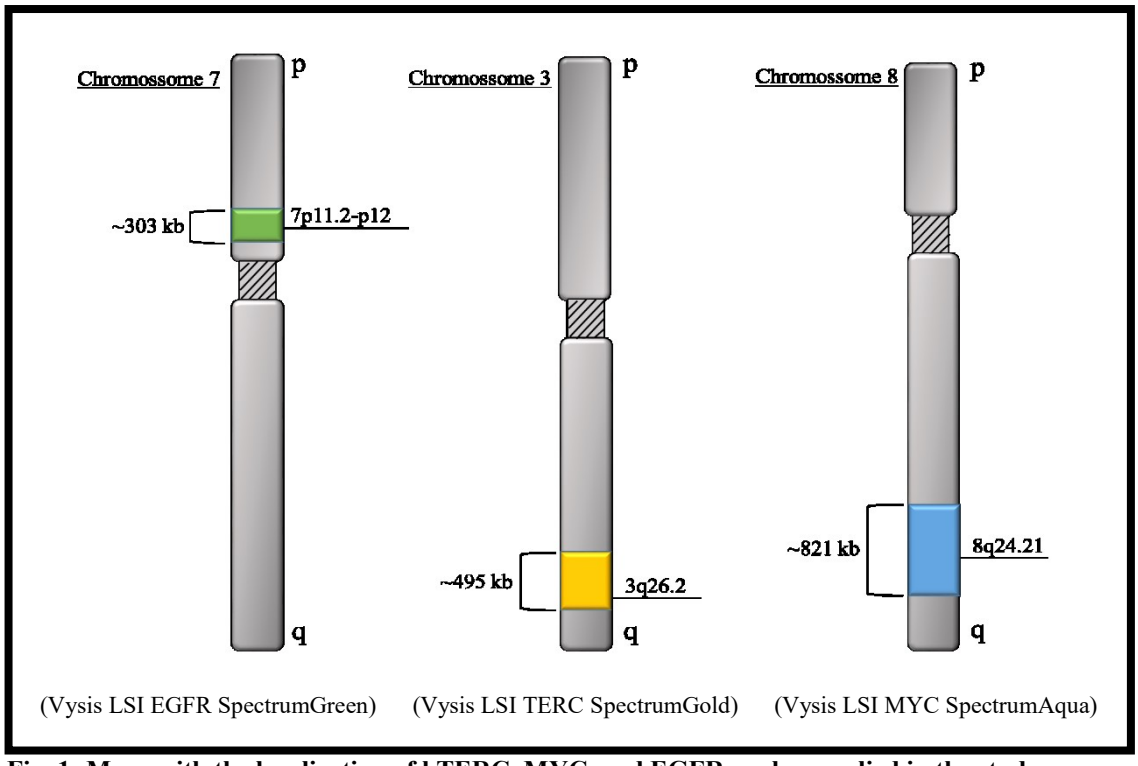


**Fig. 1: Maps with the localization of hTERC, MYC, and EGFR probes applied in the study.**

### *2.2.3 Preparation of the Solutions for FISH*

20x SSC Stock solution:

| | |
|---|---|
| 132 g | 20x SSC in |
| 400 ml | distilled water, adjust to pH 5.3 with HCl, add distilled water to |
| 500 ml | total. |

Filtrate with 0.45µm pore size filter.

2x SSC working solution:

| | |
|---|---|
| 100 ml | 20x SSC (pH 5.3), add |
| 850 ml | distilled water, adjust to pH 7 with NaOH, add dist. water to |
| 1000 ml | total. |

Discard working solution every day

0.4 x SSC + 0.1% NP-40 Working solution:

| | |
|---|---|
| 20ml | 20x SSC stock solution (pH 5.3), add |
| 950ml | distilled water |
| 1 ml | NP-40, adjust to pH 7-7.5 with NaOH, add dist. water to. |
| 1000ml | total. |

Discard working solution every day.

PBS stock solution:

| | |
|---|---|
| 1.46 g | Na2HPO4 x2H2O - disodiumhydrogen phosphate |
| 0.24 g | KH2PO4 - potassiumdihydrogen phosphate |
| 0.2g | KCL - potassium chloride |
| 8.0 g | NaCl - sodium chloride, add |
| 900ml | distilled water, dissolve with constant stirring |

adjust to pH 7.3 with 1N HCl, add

| | |
|---|---|
| 100ml | distilled water to |
| 1000ml | total. |

PBS working solution:

| | |
|---|---|
| 50ml | stock solution (1:20), add |
| 950ml | destilled water to |
| 1000ml | total. |

0.5% HCl / 70% Ethanol solution:

| | |
|---|---|
| 8ml | 25% HCl |
| 392ml | 70% Ethanol |
| 400 ml | total. |

Pepsin stock solution (10%, 100mg / ml):

| | |
|---|---|
| 100mg (0.1g) | pepsin, dissolve in |
| 1ml | distilled water |

Pipette 100 µl aliquots in Eppendorf tubes

Pepsin for use:

| | |
|---|---|
| 10µl | 10% pepsin, add |
| 4.9ml | distilled water, add |
| 100µl | 1M HCl to |
| 5ml | total. |

1% Formalin /PBS

| | |
|---|---|
| 1 g | Paraformaldehyde in |
| 100 ml | PBS buffer (at 50ºC on hot plate with a magnetic stirrer) |

## 2.3 Methods

### *2.3.1 Cytological Diagnoses*

The cytological smears of the uterine cervix were prepared by the Department of Obstetrics and Gynecology of the Düsseldorf University Hospital. The alcohol-fixed smears were then sent to our laboratory (Department of Cytopathology) and stained according to Papanicolaou in an automated slide stainer (Tissue-Tek Prisma; Sakura Finetek, Torrance, CA, USA). For detail of the staining protocol, refer to Table 3. The coverslip was placed with the support of an automated coverslipper (Tissue-Tek Glas; Sakura Finetek, Torrance, CA, USA).

This was followed by a routine cervical cytological diagnosis. Significant cellular changes were observed according to Standard operating procedures (SOPs). One cytotechnician and one cytopathologist made the cytological diagnoses. More complex cases were discussed by two experienced cytopathologists.

Before the *Münchner Nomenklatur III* was just adopted in July 2014, some older cases were classified using the *Münchner Nomenklatur II*. However, these cases were reclassified to *Münchner Nomenklatur III* within the scope of this study. This was possible due to the detailed description of cellular changes in the cytopathological reports.

| Step | Solution | Time | Process |
|---|---|---|---|
| **1** | 96% Ethanol | 2 min | Hydration of slides |
| **2** | 96% Ethanol | 1 min | |
| **3** | 70% Ethanol | 1 min | |
| **4** | 50% Ethanol | 1 min | |
| **5** | Aqua dest. | 1 min | |
| **6** | Hematoxylin (Merck®) (Aqueous solution) | 2 min | Nuclear stain |
| **7** | Tap Water | 3 min | Removal of dye excess |
| **8** | Tap Water | 3 min | |
| **9** | $NH_2OH$ | 1 min | Removal of traces of dye from the cytoplasm |
| **10** | 70% Ethanol | 1 min | Dehydration of slides |

| | | | |
|---|---|---|---|
| **11** | 70% Ethanol | 1 min | |
| **12** | 80% Ethanol | 1 min | |
| **13** | 96% Ethanol | 1 min | |
| **14** | Orange II 2b (Alcoholic solution) | 2 min | Cytoplasmatic stain |
| **15** | 96% Ethanol | 1 min | Removal of dye excess |
| **16** | 96% Ethanol | 1 min | |
| **17** | Polychrome 3b (Alcoholic solution) | 2 min | Cytoplasmatic stain |
| **18** | 96% Ethanol | 1 min | Removal of dye excess |
| **19** | 96% Ethanol | 1 min | |
| **20** | 99.5% Ethanol | 2 min | |
| **21** | Xylol | 1 min | Clarification |
| **22** | Xylol | 1 min | |
| **23** | Tissue Clear (Tissue-Tek®) | 1 min | Removal of xylol and further clarification |
| **24** | Tissue Clear (Tissue-Tek®) | 1 min | |

**Table 3: Description of the steps in the Papanicolaou Stain (Pap Stain) protocol.**

### *2.3.2 Histopathological diagnoses*

The formalin-fixed biopsy specimens and conization-specimens (loop electrical excision procedure (LEEP)-specimens in most cases) were handled in the Institute of Pathology, Düsseldorf University Hospital according to SOPs as part of the routine patient care. This included the brief preparation of paraffin-embedded tissue blocks and subsequent hematoxylin-eosin-stained slides. Histopathological diagnosis was made by an assistant physician and a consultant pathologist.

### *2.3.3 Preparation of Samples for FISH*

The cervical smears were routinely prestained with the Papanicolaou stain for cytological diagnosis. Due to the alteration of the morphological features of the cells during the FISH process, it was necessary to take representative microphotos from all slides in advance, since in Germany there is a legal obligation to maintain cytological samples in the archive for 10 years. The photos comprised suspicious cells and other features representative for the

cytological diagnosis as for example dysplastic or metaplastic cells, inflammation, and microorganisms. At least three photos were taken in each case.

The samples were analyzed in the optical microscope to define the section to be used in the FISH analysis. A round mark was made on the coverslip with a felt-tip pen to be used as a reference later. The section was selected according to the number of suspicious cells and the proximity to the center of the circle. The selected section was then engraved on the back side of the slides with an electronic engraving pen. In most cases, it is not possible to analyze the whole smear because of the high costs for the FISH probes. We chose a section with 12 mm diameter for hybridization.

### *2.3.4 Preliminary Tests*

Initial tests have shown that the three FISH probes did not work when applied together in one mix. In these cases, just the probe for hTERC demonstrated an appropriate fluorescence signal.

The first attempts to solve this problem involved the modification of the FISH protocol. Initially, possible interference with the mounting medium in the efficacy of the probe's signal was suspected. It was decided to increase the time of incubation in xylol, from the conventional 2 hours to 24, 48 and 72 hours after the removal of the coverslip.

A longer incubation-period of other solutions used in the FISH protocol was also attempted. These were 0.5% HCl /70% Ethanol and 1% formaldehyde solution.

Based on the experience of our technical assistants (Marietta Kazimirek and Birgit Buckstegge), it was decided to increase the temperature of the water bath to 82 °C prior to the pepsin-incubation, as in some cases they did make the experience of enhanced signals by this method in the laboratory routine.

All those tests were performed with a positive control. Fresh Cytospins of bronchial lavage specimens or oral brush samples were prepared just before the analysis to verify if the problem was linked to our protocol or to the archived samples. The positive controls demonstrated a better signal pattern but were still not sufficient for analysis.

Based on information provided by Abbotts FISH experts we also investigated a possible interference with the three probes together in the same solution. We suspected that the

Urovysion Hybridization Buffer was not the ideal medium to mix the three probes and this could possibly be the cause of the weak signals (Abbott Molecular Inc., Des Plaines, IL). We tested various combinations of probe-sets and the best solution was to use EGFR and hTERC probes together in a mix and the MYC probe alone in another area for hybridization, whenever possible. Thus, in 50 cases just hTERC and EGFR were tested. However, at some point in the study, we managed to overcome this problem by using the new developed Vysis IntelliFISH Hybridization Buffer (Abbott Molecular Inc., Des Plaines, IL). The components are not public and under patent protection.

### *2.3.5 FISH: Method and staining procedure*

In the fluorescent in situ hybridization (FISH) staining process, fluorescent molecules are deposited in chromatin at the sites of specific DNA sequences. Before any hybridization can occur, both the target and the probe sequences must be denatured with heat or chemicals. This denaturation step is necessary in order for new hydrogen bonds to form between the target and the probe during the subsequent hybridization step (O'connor, 2008). Complementary sequences in the probe and target are then allowed to reanneal. After washing and incubation in fluorescently labeled affinity reagents, a fluorescent signal is visible at the site of probe hybridization (Trask, 1991).

The process of FISH staining is resumed on table 4. To keep the conditions comparable in different runs of the FISH-procedure, usually, 4 slides were incubated in the cuvettes each time. The slides were incubated in xylol for 2 to 3 days in order to remove the coverslip. After removal of the coverslips, the slides were washed twice in xylene for 5 minutes each, to remove all traces of mounting medium. The samples were then rehydrated with two washes of 5 minutes in 99.9% ethanol, two in 96% ethanol and two in 80% ethanol and destained in a 0.5% HCl/70% ethanol solution for 15 minutes.

After 5 minutes in running tap water, the slides were soaked in a 2x sodium chloride and sodium citrate (SCC; pH ± 7.0) solution at 80 ± 1 °C (water bath) for 5 minutes. The slides were covered with a protease solution in a humidified box at 37 °C for 15 minutes

| Step | Duration | Process |
|---|---|---|
| Xylol | 1-3 days | Removal of the cover glass |
| Heat the 20x SSC buffer in the water bath at 80 °C | 1 hour | |

| | | |
|---|---|---|
| Incubation in xylol without coverslips | 1-4 hour | Removal of residual mounting medium |
| 2x incubation in 100% ethanol | 10 min. each | Hydration of the slides and destaining |
| 2x incubation in 96% ethanol | 5 min. each | |
| 2x incubation in 80% ethanol | 5 min. each | |
| 0.5% HCl / 70% ethanol solution | 15 min. | |
| Running tap water | 5 min. | |
| 20x SSC, 80°C (+-1°C) | 5 min. | |
| Slides covered with 1% pepsin and placed in a 37°C incubator | 15 min. | Enzymatic digestion |
| PBS solution | 5 min. | Removal of residual pepsin |
| 1% formalin / PBS solution | 5 min. | Fixation of cells |
| PBS solution | 5 min. | Removal of residual formalin |
| 70%, 85%, 100% ethanol solutions | 1 min. | Dehydration of slides |
| Incubation at room temperature | 1 min. | Drying |
| Prepare the probe mix (0.6µl of Probe, 0.3µl of distilled water and 2.1µl of FISH Buffer) | - | Preparation and application of the FISH probes |
| Apply 3 µl of the probe mix on the area covered by a round coverslip (12mm) | - | |
| Place the coverslip over the predefined region of the slide | - | |
| Seal the coverslip with Fixogum | - | |
| Place it in a 37°C incubator | 5 min. | |
| 73 °C heating plate (in the dark) | 10 min. | Denaturation |
| Incubate at 37°C humid chamber | 14-16 hours | Hybridization |
| **Second Day** | **Duration** | **Process** |
| 0.4% x SSC + 0.1% NP40 buffer solution (in the dark) | 1-5 min. | Removal of the coverslip |
| 0.4% x SSC + 0.1% NP40 Buffer (in the dark) | 2 min. | Removal of the probe excess |
| Rinse in distilled water | 1-3 seconds | Removal of residual SSC |
| Preparations complete dry | 2 min. | |
| Put 1 drop of DAPI on the cover glass (or sample) and cover the slide (24x60mm) | - | Nuclear counterstain |
| Seal with Fixogum and allow to dry | 30 min. | Avoid evaporation |
| Store in a refrigerator at 4°C until evaluation. Store permanently at -20°C | - | |

**Table 4: Protocol for the application of the FISH probes for hTERC, EGFR and MYC genes**

In the meantime, the hybridization mix was prepared. The probes were mixed with hybridization buffer (Vysis IntelliFISH Hybridization Buffer or Urovysion Hybridization Buffer - Abbott Molecular Inc., Des Plaines, IL) and distilled water. A 20% solution was adopted in this work after previous tests. Thus, the 3µl hybridization mix, enough for a 12mm in diameter-sized coverslip, contained 0.6 µl of probes, 0.3 µl distilled water and 2.1 µl of the buffer. In the cases with two regions (1- hTERC and EGFR; 2- MYC), the area with 2 probes

had 0.3 µl (concentration: 10%) of each probe and the area hybridized with the MYC-probe contained 0.6 µl (concentration: 20%). In the cases with just one area, i.e. three probes in the same mix, 0.2 µl of each probe was used.

Now, the slides were washed in phosphate-buffered saline (PBS) solution for 5 minutes, fixated on a 1% formaldehyde solution for 5 minutes and washed once more in PBS solution for 5 minutes. After dehydration with 70%, 85% and 99% ethanol (30 seconds each), residual alcohol was removed from the edges of a slide. It is important here not to let the specimens dry-up. The vessels with the probe mix were centrifuged for 1-3 seconds, vortexed for 10 seconds and recentrifuged in the meantime.

The hybridization mix, containing the FISH probes was applied to the slide, which was covered with a 12mm cover glass, sealed with rubber cement, placed in a humidified box and incubated for 5 minutes at 37 °C in a hybridization oven to dry the rubber cement. Then, every slice was placed on a heating block at 73 ± 1 °C for 10 minutes to denature the cellular double-stranded DNA. After replacing the slides back in the humidified box, they were incubated overnight in a hybridization oven at 37 °C.

The following steps were carried out in a darkened place in the lab. The next day, the rubber cement was removed with a tweezer and the slides were immersed in a washing solution (0.4x SSC / 0.3% NP-40) at room temperature for 2 minutes.

The coverslips were removed and the slides again immersed in the washing solution at 73 ± 1˚C for 2 minutes, washed once more for 2 minutes in the washing solution at room temperature and rinsed in distilled water. After 2 minutes drying at room temperature, the slides were mounted in Vecta-shield antifade medium (Vector Laboratories, Burlingame, CA, USA) with DAPI (4'.6-diamidino-2-phenylindole) as a counterstain. The slides were placed in a folder to protect against light and stocked in a refrigerator at 4˚C.

### *2.3.6 FISH Analysis*

After storing the samples at least one day in the refrigerator at 4°C, what in our experience allows the fluorescent signals to be more intense, the samples were analyzed using a microscope Axio Imager A1 (Carl Zeiss, Jena, Germany) with 63x/1.40 Oil DIC (WD=0.19mm) 1.4 NA and 100x/1.46 Oil DIC (WD=0.10mm) objective lenses. The microscope is fitted with a mercury lamp and single-bandpass filters for DAPI, SpectrumGreen

(EGFR), SpectrumGold (hTERC) and SpectrumAqua (MYC). The microscope is equipped with a charge-coupled device black and white video camera with 1.4 Megapixels (AxioCam MRm, München-Hallbergmoos, Germany). The computerized coloring of the black and white signals, the acquisition of cellular images in different focal plains and merging to a single image is provided by the AxioVysion and QuantiFISH software (Zeiss, Hallbergmoos, Germany).

Up to 60 suspicious nuclei were analyzed and the number of signals registered in a form. The nuclei of inflammatory cells (e.g. neutrophils) were adopted as an internal reference for the quality of hybridization. Just the cases which show two fluorescent signals for each probe in the nuclei in most of the inflammatory cells and with the satisfactory intensity of the fluorescence were accepted. Two signals close to each other were only counted as two signals when the distance between the two was bigger than the diameter of one common signal.

After analysis, the samples were stored in a freezer at -20˚C in order to preserve the fluorescence of the probes, making the repetition of the analysis possible later on.

### *2.3.7 Statistical Analysis*

All statistical analyses were calculated by Isabela Caroline de Santana Almeida Araujo (Bachelor of statistics, Department of Cytopathology). In order to check the diagnostic significance of FISH for the cervical lesions, the following statistical techniques were used: sensitivity, specificity, positive and negative predictive value, and area under the ROC curve (AUC).

| Test Result | Gold standard | | Gesamt |
|---|---|---|---|
| | Positive | Negative | |
| **Positive** | True Positive (TP) | False Positive (FP) | TP + FP |
| **Negative** | False Negative (FN) | True Negative (TN) | FN + TN |
| **Total** | TP + FN | FP + TN | TP + FP + FN + TN |

**Table 5: Contingency table: Ratios between test results and correct diagnosis.**

The sensitivity (Se) of a diagnostic test is the proportion of positives that are correctly identified by the test.

$$Se = \frac{True\ Positive}{True\ Positive + False\ Negative}$$

The specificity (Sp) of a diagnostic test is the proportion of negatives that are correctly identified by the test.

$$Sp = \frac{True\ Negative}{True\ Negative + False\ Negative}$$

The positive predictive value (PPV) is the proportion of patients with a positive test result that is correctly diagnosed.

$$PPV = \frac{Sensitivity\ *\ Prevalence}{Sensitivity\ *\ Prevalence + (1 - Specificity) * (1 - Prevalence)}$$

The negative predictive value (NPV) is the proportion of patients with a negative test result that is properly diagnosed.

$$NPV = \frac{Specificity * (1 - Prevalence)}{(1 - Sensitivity) * Prevalence + Specificity * (1 - Prevalence)}$$

The ROC curve is a graphical representation of the relationship between the probability of the correct result (sensitivity) and the false result (1-specificity).

First, a descriptive statistical analysis was done: the mean value, the standard deviation, and the concordance between cytology and histological/clinical follow-up were determined. After the descriptive statistics, the Kruskal-Wallis ANOVA (Analysis of Variance) was calculated. The Kruskal-Wallis variance analysis was used to compare the distribution of the number of positive cells between the diagnostic groups. The analysis was done with IBM SPSS Statistics (Released 2015. Version 23.0. Armonk, NY: IBM Corp.).

The hypotheses for the evaluation were:

$H_0$: The distribution of the number of positive cells from each specimen was equal in each cytological or histological diagnostic group (i.e. CINIII);

$H_1$: The distribution of the number of positive cells from each specimen was different at least in one cytological or histological diagnostic group.

If the p-value was a ≤ level of significance (α), the null hypothesis could be rejected. The applied level of significance was α = 0.05. If the null hypothesis was rejected, the multiple comparisons of the mean value for independent samples with LSD (Least Significant Difference) from Fishers' method must be applied. This method was used to identify the

different mean values of fluorescence signals for each of the FISH probes in each diagnostic group (i.e. group I).

After the multiple comparisons of the mean value, the cut-off was calculated for every single probe, two probes (EGFR and hTERC) and three probes. To calculate the cut-off, the youden index was applied.

$$Youden\ Index = Sensitivity + Specificity - 1$$

Since tetrasomy can be observed in a normal cell at the end of S-phase or the M-phase of the cell cycle, an additional analysis was made excluding the cells with four signals of each probe (deviation of ±1 signal in one probe was accepted). In cases with just EGFR and hTERC, only the 4-4 pattern was accepted (without allowing deviation of ±1). Tetrasomy at a significant level is observed for example in the following non-malignant conditions: inflammation, regeneration or viral infections.

# 3 Results

## 3.1 Patients

The flowchart of the study with the correlation of the smears to the cytological diagnoses and the histological/follow-up reference standard is shown in figure 2. A total of 132 patients and smears were added to the study (figure 2). In a further analysis, 6 patients were finally withdrawn due to problems with the histological diagnosis ((n=3), different site of analysis) and inadequate reaction with the FISH probes (n=3).

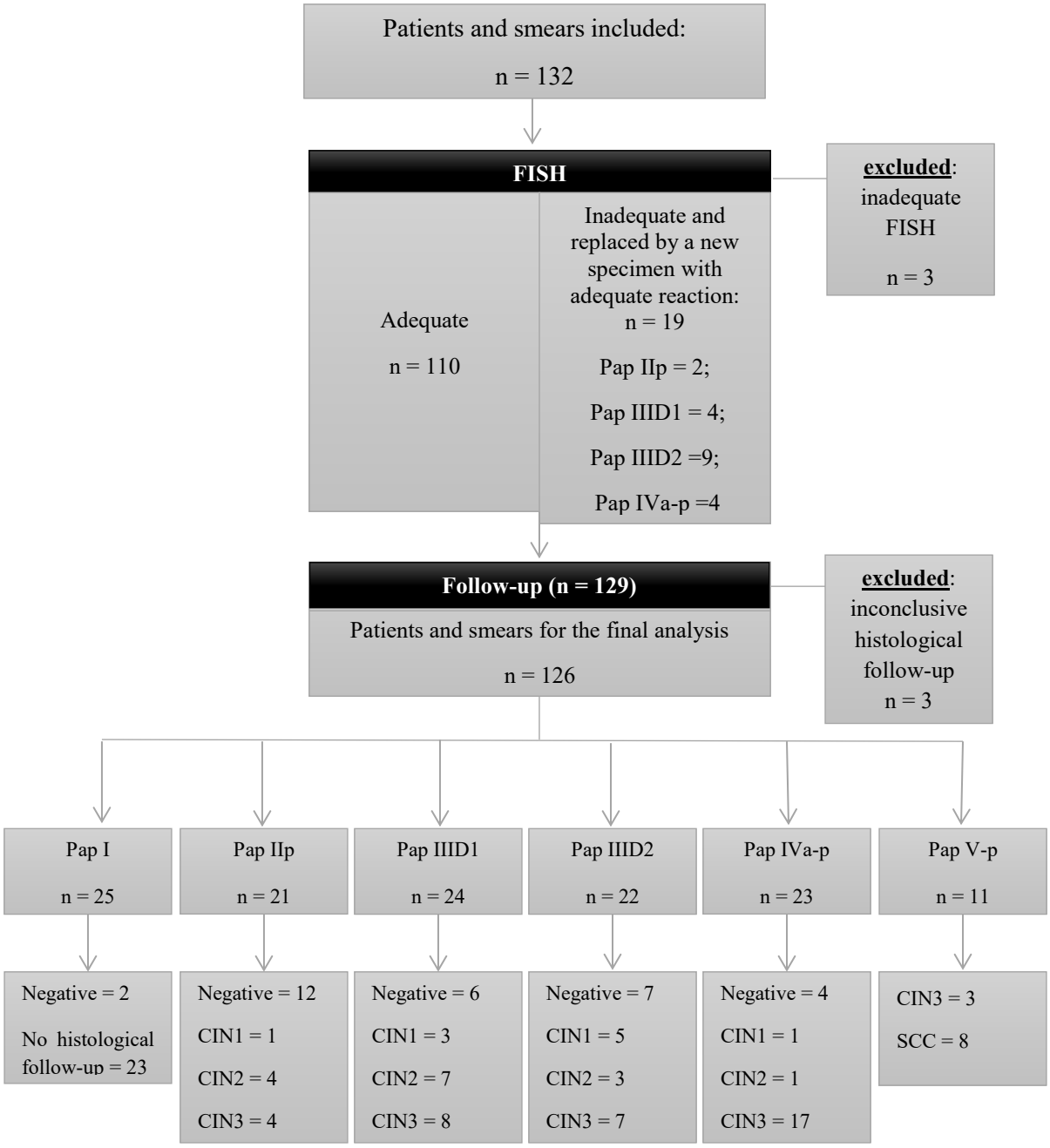


**Fig.2: Flow chart of the study. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma. FISH: Fluorescent in situ hybridization.**

The specimens with an insufficient FISH procedure (n=22) were in part substituted by others (n=19). However, the patients with an inappropriate site of the histological specimen were not substituted due to schedule problems. Finally, 126 patients were evaluated.

### *3.1.1 Age at cytological/histological diagnosis*

The 126 patients were between 18 and 79 years of age at the time of the first cytological diagnosis. The mean age was 38.1 years and the median age was 35.0 years. The standard deviation was 13.1 years. Most patients were between 25 and 38 years of age. The highest peak occurred at 25 to 30 years (figure 3).

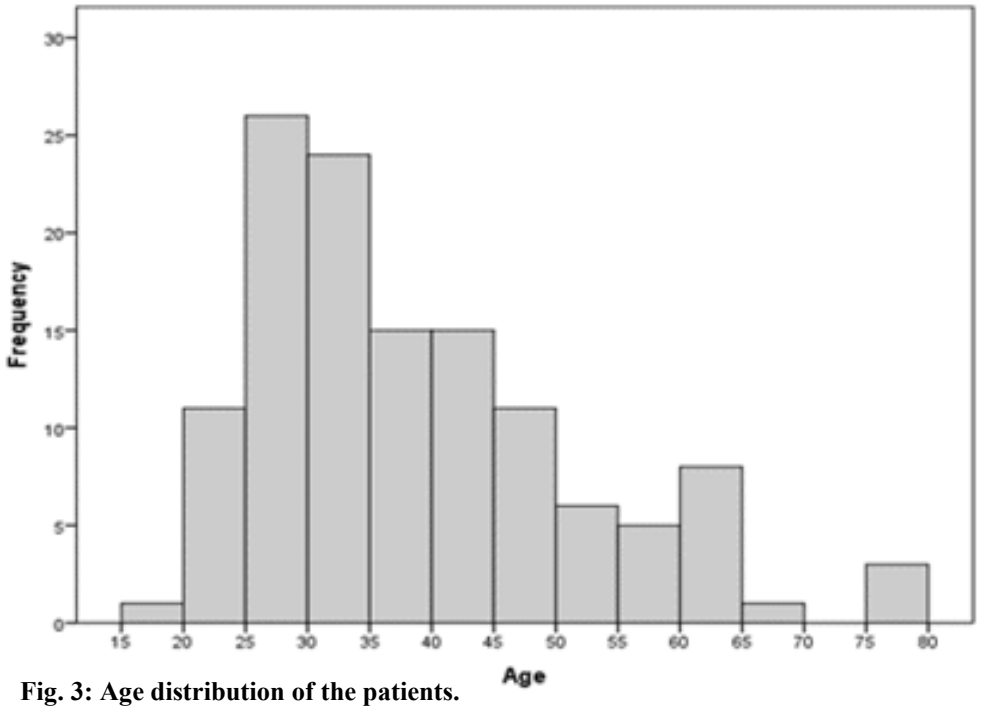


**Fig. 3: Age distribution of the patients.**

Considering the cytological results, the youngest patient (18 years old) belonged to group I. In the groups with conspicuous cytology (IIp - V), the youngest patients being (20 years old), some were diagnosed in cytology with IIp and IIID1. The oldest patient (79 years old) belonged to the negative group I, also. Considering groups IIp - V, the oldest patient belonged to group V (75 years old). The lowest mean age (31.27 ± 8.213) was found in group IIID2 and the highest (47.08 ± 15.623) in group I followed closely by group V (46.45 ± 13.545) (table 6, figure 4).

| | I | | IIp | | IIID1 | | IIID2 | | IVa-p | | V-p | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Age | 47.08 | 15.623 | 31.57 | 7.711 | 36.54 | 13.201 | 31.27 | 8.213 | 38.35 | 10.598 | 46.45 | 13.545 |

**Table 6: Descriptive statistic of the age of patients in different cytological groups. SD: standard deviation.**

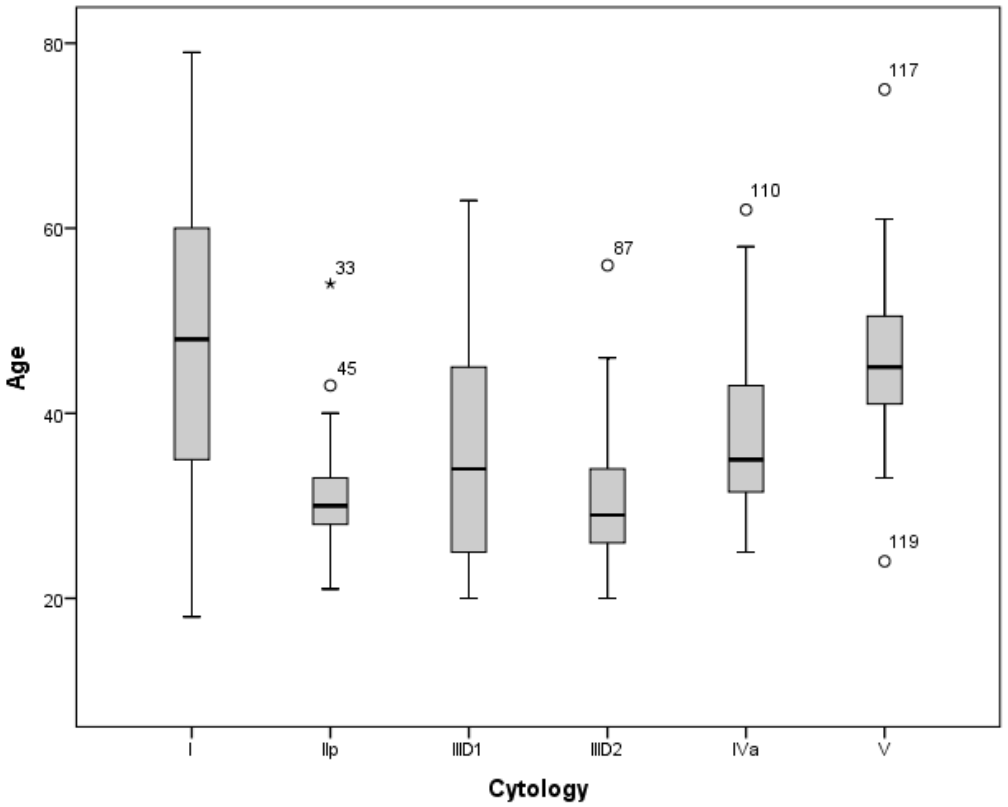


**Fig. 4: Box-plot representation of the age of patients related to the cytological result.**

Among the histological results, the youngest patient (18 years old) was found in the negative group, followed by the CIN2 results (20 years old). The oldest patient also belonged to the negative group (79 years old). The lowest mean age occurred in the group of the CIN2 results (34.20 ± 14.324) and highest in the SCC group (41.63 ± 9.273) (Table 7, figure 5).

| | Negative | | CIN1 | | CIN2 | | CIN3 | | SCC | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Age | 39.24 | 14.49 | 37.00 | 12.835 | 34.20 | 14.324 | 37.5 | 11.545 | 41.63 | 9.273 |

**Table 7 Descriptive statistic of the age of patients related to the histological outcome. CIN: Cervical intraepithelial neoplasia. SD: Standard deviation.**

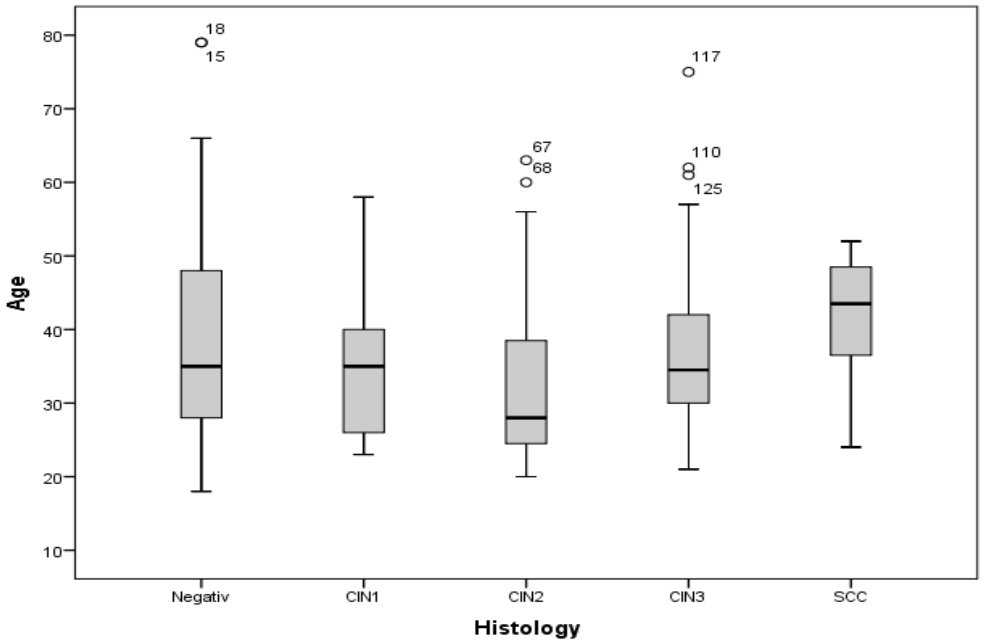


**Fig.5: Box-plot representation of the age of patients related to the histological result.**

### *3.1.2 HPV Prevalence*

The HPV tests were accomplished by a staff of the Department of Gynecology and Obstetrics. The corresponding test results of HPV-testing were gathered from the patient's charts on the digital hospital patient management system (KIS, Cerner Medico, Cerner Germany). Results of the Hybrid Capture 2 HPV DNA Test (hc2) (Digene Corporation, Gaithersburg, MD), performed at the same time or within 6 months of the cytological smear, were available in 77 (61.14%) cases, 56 (72.7%) cases were hr-HPV positive and 21 (27.3%) hr-HPV negative.

In group IIp 18 hr-HPV test results were available, corresponding to 23.4% of all samples and 85.7% of cases in group IIp. 19 HPV results in the group IIID1 were reported, representing 24.7% of all and 79.2% of IIID1 cases. 16 samples of group IIID2 group were tested, representing 20.8% of all HPV test cases and 72.3% of IIID2 cases. 14 hr-HPV were available in group IVa-p, corresponding to 18.2% of all cases and 60.9% of group IVa-p. Just 2 cases of group V had an hr-HPV test, 2.6% of all samples and 18.2% of the group.

The rate of positive hr-HPV tests increased proportionally to the grade of the lesions: 25% in group I to 100% in group V (Table 8). Taking into consideration that the hr-HPV test was performed in just 2 cases of group V, the group with a justifiable quantity of tests which had the higher rate of positive cases was the group IVa-p (92.9%).

The number of hr-HPV test results in women who also had a histological specimen was lower compared to cytology because of missing biopsies in women with a cytological group I test result. When compared with the histological outcomes, the hr-HPV test showed similar behavior, i.e., the proportion of positive hr-HPV test results raises with the severity of the lesion, except for CIN2 (table 9). No SCC case was tested for hr-HPV.

| Groups | hr-HPV Test (%) | |
|---|---|---|
| | Positive | Negative |
| **I** | 2 (25) | 6 (75) |
| **IIp** | 12 (66.7) | 6 (33.3) |
| **IIID1** | 14 (73.7) | 5 (26.3) |
| **IIID2** | 13 (81.3) | 3 (18.7) |
| **IVa-p** | 13 (92.9) | 1 (7.1) |
| **V** | 2 (100) | 0 (0) |
| **Total** | 56 (75) | 21 (25) |

**Table 8: Absolute number and percentile rates of hr-HPV positive test results in the diverse cytological groups. hr-HPV: High-risk human papilloma virus.**

| Groups | hr-HPV Test (%) | |
|---|---|---|
| | Positive | Negative |
| **Negative** | 17 (70.8) | 7 (29.2) |
| **CIN1** | 7 (87.5) | 1 (12.5) |
| **CIN2** | 7 (58.3) | 5 (41.7) |
| **CIN3** | 23 (92) | 2 (8) |
| **SCC** | 0 (0) | 0 (0) |
| **Total** | 54 (79.4) | 15 (20.6) |

**Table 9: Absolute number and percentile rates of positive cases for high-risk HPV in the diverse histological groups. hr-HPV: High-risk human papilloma virus. CIN: Cervical intraepithelial neoplasia**

In our study, the cervical specimens of 77 patients were tested for hr-HPV, 29 women were younger than 30 years at the time of the first cytological diagnosis, and 48 were 30 years old or older. The prevalence of high-risk HPV was lower in 30-year-old women or older (70.8%) than in those younger than 30 years (75.9%) (Table 10).

| Age | hr-HPV Test (%) | |
|---|---|---|
| | Positive | Negative |
| **< 30** | 22 (75.9) | 7 (24.1) |
| **≥ 30** | 34 (70.8) | 14 (29.2) |
| **Total** | 56 (72.7) | 21 (27.3) |

**Table 10: Absolute number and percentage rates of positive hr- HPV test results among women older and younger than 30 years. hr-HPV: High-risk human papilloma virus.**

## 3.2 Correlation of cytology with histology

### *3.2.1 Histological reference standard*

From the 126 cases of our work, 101 had a valid histological follow-up within the predefined periods (refer to chapter 2.1.). From these 31 30.7% were histologically classified as negative for intraepithelial neoplasia, 10 (9.9%) as CIN1, 14 (13.9%) as CIN2, 38 (37.6%) as CIN3 and 8 (7.9%) as squamous cell carcinoma, using the WHO histological classification of tumors of the uterine cervix in the year 2003. The old WHO classification is mentioned here because the yearly statistics in Germany according to the national guidelines for early cancer detection (Qualitätssicherungsvereinbarung Zervix-Zytologie, 2015) demands the comparison with the CIN1-CIN3 three-tier histological outcome.

Based on the WHO histological classification from 2014, 31 (30.7%) specimens were histologically classified as negative for intraepithelial neoplasia, 10 (9.9%) as low-grade intraepithelial neoplasia, 52 (51.5%) as high-grade intraepithelial neoplasia and 8 (7.9%) as squamous cell carcinoma.

All the patients were submitted to a biopsy with a suspicious lesion, but 9 (8.9%) of these had conization with a higher grade, which was taken into account as the valid histological outcome. In 62 cases, just one biopsy was performed. A second biopsy was performed in 39 cases and a third in 10 cases. In sum, 152 biopsies were performed.

A total of 32 conizations were performed. Hysterectomies occurred in 4 cases, 3 in the cytological group V-p, and one in the group IVa-p. The three cases that occurred in the group V-p were included as clinical follow-up results only due to the fact that they occurred more than 6 months after the first cytology. The hysterectomy case diagnosed as carcinoma in-situ (Pap IVa-p) in the cytology was evaluated together with the conizations since both provide a gold standard reference. Thus, for practical and statistical matters, it was considered that 32 women had conization, 31 of them underwent a loop electrosurgical excision procedure (LEEP) one underwent surgical removal of the uterus (Hysterectomy).

The histological reference standard defined as negative in this work was comprised of the samples diagnosed as a non-neoplastic or non-dysplastic lesion. These lesions comprise squamous metaplasia (6), condyloma acuminatum (1), inflammation (14), endocervical polyp (2), atrophy (1) and squamous hyperplasia (3). In four specimens, a lesion was not found.

### *3.2.2 Cytological/histological correlation with different reporting systems*

In order to gather more information about the quality of the cytological diagnosis, we have made a correlation analysis between the cytological and histological diagnoses. As explained above in the chapter material and methods, histological data up to three months after the first cytological diagnosis were accepted in the case of a biopsy and six months in the case of conization or hysterectomy. Further analyses with distinct reporting systems were performed as well. For cytology the Bethesda System and *Münchner Nomenklatur III,* and for the nosologic classification of the histological specimens, the 2003 and 2014 WHO classifications of tumors were adopted. The cytological samples, dated before July 2014 were classified for patient care according to the *Münchner Nomenklatur II*, the subsequent ones (from July 2014 to August 2015) according to the *Münchner Nomenklatur III*. The histological diagnoses for patient care used the 2003 WHO classification of tumors. In this study, the diagnoses according to the *Münchner Nomenklatur II* were reclassified according to the *Münchner Nomenklatur III*. For further comparison with the international literature, the diagnoses were again reclassified according to The Bethesda System and 2014 WHO classification.

The following tables show the absolute agreement between the cytological diagnosis and the histological outcome:

| | Negative | CIN1 | CIN2 | CIN3 | SCC | Total |
|---|---|---|---|---|---|---|
| IIp | 12 | 1 | 4 | 4 | 0 | 21 |
| IIID1 | 7 | **4** | 6 | 7 | 0 | 24 |
| IIID2 | 8 | 4 | **3** | **7** | 0 | 22 |
| IVa-p | 4 | 1 | 1 | **17** | 0 | 23 |
| V-p | 0 | 0 | 0 | 3 | **8** | 11 |
| Total | 31 | 10 | 14 | 38 | 8 | **101** |

**Table 11: Absolute agreement between biopsy or conization and cytology using 2003 WHO classification and *Münchner Nomenklatur III.* CIN: Cervical intraepithelial neoplasia. SCC: Squamous cell carcinoma.**

Comparing the 2003 WHO classification with the *Münchner Nomenklatur III* (Table 11), most cases (60 cases – 59.4%) were classified histologically as ≥CIN2. From these cases, 13.3% were classified cytologically as group IIp, 21.7% as IIID1 (low-grade dysplasia), 16.6% as IIID2 (moderate dysplasia), 30% as IVa-p (severe dysplasia or carcinoma in situ), and 18.7% as Pap V (invasive carcinoma). About 45.6% of the specimens were diagnosed as ≥CIN3. The main cytological corresponding result was group IVa-p (36.2%), followed by group V (23.4%)

and IIID1 (17%). 60.9% (28 out of 46) of ≥CIN3 lesions were preceded by a IVa-p or higher cytology. 82% (28 out of 34) of all IVa-p or higher diagnoses resulted in a ≥CIN3 lesion.

| | Negative | Low-Grade IEN | High-Grade IEN | SCC | Total |
|---|---|---|---|---|---|
| IIp | 12 | 1 | 8 | 0 | 21 |
| IIID1 | 7 | **4** | 13 | 0 | 24 |
| IIID2 | 8 | 4 | **10** | 0 | 22 |
| IVa-p | 4 | 1 | **18** | 0 | 23 |
| V-p | 0 | 0 | 3 | **8** | 11 |
| Total | 56 | 10 | 52 | 8 | 101 |

**Table 12: Absolute agreement between biopsy or conization and cytology using 2014 WHO classification and *Münchner Nomenklatur III*. IEN: Intraepithelial Neoplasia. SCC: Squamous cell carcinoma.**

Comparing 2014 WHO classification with the *Münchner Nomenklatur III* (Table 12), it was observed that most of the patients who had a high-grade intraepithelial lesion in the histological analysis were cytologically diagnosed as ≥ IIID2 (65%). High-grade lesions corresponded to 78.3% of the group IVa-p cases and high-grade lesions or invasive SCC to 100% of group V diagnoses, while in the groups IIID2, IIID1, and IIp, a high-grade lesion was found in 45.5%, 62.5% and 38.1% of the cases respectively. 65% (39 out of 60) of ≥ high-grade lesions were preceded by a IIID2 or higher cytology. 69.6% (39 out of 56) of all IIID2 or higher diagnoses resulted in a ≥ high-grade lesion.

| | Negative | CIN1 | CIN2 | CIN3 | SCC | Total |
|---|---|---|---|---|---|---|
| ASC-US | 12 | 1 | 4 | 4 | 0 | 21 |
| LSIL | 7 | **4** | 6 | 7 | 0 | 24 |
| HSIL | 12 | 5 | **4** | **24** | 0 | 45 |
| SCC | 0 | 0 | 0 | 3 | **8** | 11 |
| Total | 31 | 10 | 14 | 38 | 8 | 101 |

**Table 13: Absolute agreement between biopsy or conization and cytology using 2003 WHO classification and The Bethesda System. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma. ASC-US: Atypical squamous cells of undetermined significance. LSIL: Low-grade squamous intraepithelial lesion. HSIL: High-grade squamous intraepithelial lesion.**

Comparing diagnoses according to The Bethesda System and 2003 WHO classification (Table 13), it was found that HSIL and SCC comprise 73.9% of the ≥CIN3 diagnoses,

respectively. In comparison, the groups ≤ LSIL corresponded to just 23.9% of ≥CIN3 diagnoses. 62.5% (35 out of 56) of all HSIL or higher diagnoses resulted in a ≥CIN3 lesion.

| | Negative | Low-Grade IEN | High-Grade IEN | SCC | Total |
|---|---|---|---|---|---|
| ASC-US | 12 | 1 | 8 | 0 | 21 |
| LSIL | 7 | **4** | 13 | 0 | 24 |
| HSIL | 12 | 5 | **28** | 0 | 45 |
| SCC | 0 | 0 | 3 | **8** | 11 |
| Total | 31 | 10 | 52 | 8 | **101** |

**Table 14: Absolute agreement between biopsy or conization and cytology using 2014 WHO classification and The Bethesda System. IEN: Intraepithelial Neoplasia. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma. ASC-US: Atypical squamous cells of undetermined significance. LSIL: Low-grade squamous intraepithelial lesion. HSIL: High-grade squamous intraepithelial lesion.**

Classified according to 2014 WHO classification, 62.2% of the HSIL diagnoses correspond to a high-grade IEN (Table 14). LSIL diagnoses showed a higher agreement with diagnosed high-grade intraepithelial neoplasia (54.2%) than with low-grade intraepithelial neoplasia or negative diagnoses (45.8%), 65% (39 out of 60) of ≥high-grade lesions were preceded by a HSIL or higher cytology. 69.6% (39 out of 56) of all HSIL or higher diagnoses resulted in a ≥high-grade lesion.

### *3.2.3 Correlation of cytology and conization/hysterectomy specimens and overall agreement*

Conization specimens are obtained from the therapy of a suspected high-grade cervical lesion and have a much higher diagnostic accuracy compared to small tissue biopsies (Herzog et al., 1995). With respect to this, an agreement analysis between the cytological diagnoses and conization specimens was accomplished. It was assumed, that cytological results correspond better to the diagnoses of the conization specimens than to biopsies, because of sampling errors of the small biopsies (Massad et al., 1996, Boonlikit et al., 2006).

The following tables (15-18) show the absolute agreement between the cytological diagnoses and conization specimens:

| | Negative | CIN1 | CIN2 | CIN3 | SCC | Total |
|---|---|---|---|---|---|---|
| IIp | 0 | 0 | 0 | 2 | 0 | 2 |
| IIID1 | 1 | **1** | 1 | 6 | 0 | 9 |
| IIID2 | 0 | 2 | **1** | 4 | 0 | 7 |
| IVa-p | 0 | 0 | 0 | **12** | 0 | 12 |
| V-p | 0 | 0 | 0 | 1 | **1** | 2 |
| Total | 1 | 3 | 2 | 25 | 1 | 32 |

**Table 15: Absolute agreement between conization specimens and cytological diagnoses using 2003 WHO classification and *Münchner Nomenklatur III*. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma.**

| | Negative | Low Grade IEN | High-Grade IEN | SCC | Total |
|---|---|---|---|---|---|
| IIp | 0 | 0 | 2 | 0 | 2 |
| IIID1 | 1 | **1** | 7 | 0 | 9 |
| IIID2 | 0 | 2 | **5** | 0 | 7 |
| IVa-p | 0 | 0 | **12** | 0 | 12 |
| V-p | 0 | 0 | 1 | **1** | 2 |
| Total | 1 | 3 | 27 | 1 | **32** |

**Table 16: Absolute agreement between conization specimens and cytological diagnoses using 2014 WHO classification and *Münchner Nomenklatur III*. IEN: Intraepithelial Neoplasia. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma.**

| | Negative | CIN1 | CIN2 | CIN3 | SCC | Total |
|---|---|---|---|---|---|---|
| ASC-US | 0 | 0 | 0 | 2 | 0 | 2 |
| LSIL | 1 | **1** | 1 | 6 | 0 | 9 |
| HSIL | 0 | 2 | **1** | **16** | 0 | 19 |
| SCC | 0 | 0 | 0 | 1 | **1** | 2 |
| Total | 1 | 3 | 2 | 25 | 1 | **32** |

**Table 17: Absolute agreement between conization specimens and cytological diagnoses using 2003 WHO classification and The Bethesda System. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma. ASC-US: Atypical squamous cells of undetermined significance. LSIL: Low-grade squamous intraepithelial lesion. HSIL: High-grade squamous intraepithelial lesion.**

| | Negative | Low Grade IEN | High-Grade IEN | SCC | Total |
|---|---|---|---|---|---|
| ASC-US | 0 | 0 | 2 | 0 | 2 |
| LSIL | 1 | **1** | 7 | 0 | 9 |
| HSIL | 0 | 2 | **17** | 0 | 19 |
| SCC | 0 | 0 | 1 | **1** | 2 |
| Total | 1 | 3 | 27 | 1 | **32** |

**Table 18: Absolute agreement between conization specimens and cytological diagnoses using 2014 WHO classification and The Bethesda System. IEN: Intraepithelial Neoplasia. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma. ASC-US: Atypical squamous cells of undetermined significance. LSIL: Low-grade squamous intraepithelial lesion. HSIL: High-grade squamous intraepithelial lesion.**

Regarding the right medical indication for conization or hysterectomy therapy of a ≥CIN3 (WHO2003) or a high-grade CIN (WHO 2014) lesion, the following remarks could be made for the respective analyses:

- Table 15: 53.8% (14/26) of cytological group IVa-p and higher corresponded to a ≥CIN3 lesion. 15.3% (4/26) and 23.1% (6/26) of the ≥CIN3 lesions were cytologically diagnosed as group IIID2 and ≤ group IIID1, respectively.
- Table 16: 67.8% (19/28) of cytological group IIID2 and higher corresponded to a high-grade IEN and SCC. 32.1% (9/28) of those lesions were diagnosed as ≤ group IIID1.
- Table 17: 69.3% (18/26) of cytological diagnoses HSIL and SCC corresponded to a ≥CIN3 lesion. 30.8% (10/28) were diagnosed as ASC-US or LSIL.
- Table 18: 67.8% (19/28) of cytological diagnoses HSIL and SCC corresponded to high-grade IEN or SCC. 32.1% (9/28) were diagnosed as ASC-US or LSIL.

The agreement between the cytological and histological diagnosis in the following analysis (Table 19) refers to the cases which had an exact correspondent diagnosis on both methods: LSIL and IIID1 = CIN1 and low Grade IEN, HSIL and IIID2 = CIN2 and high-Grade IEN, HSIL and IVa-p = CIN3 and high-Grade IEN, V-p = SCC. The cytological group IIp has no exact correspondent and group I was not included due to the limit of groups that could be added to this statistical analysis.

| Reporting System for Cervical Cytology | WHO Classification | | | |
|---|---|---|---|---|
| | 2003 | | 2014 | |
| | Bio. and Conis. | Coniz. | Bio. and Conis. | Coniz.. |
| **The Bethesda System** | 51.56 | 63.3% | 51.6% | 63.3% |
| **MN III** | 45.2% | 50% | 51.6% | 63.3% |

**Table 19: Overall agreement between cytology and histology, given in percentages. MN III – *Münchner Nomenklatur III*; Bio. – Biopsy; Conis. – Conization. WHO: World Health Organisation.**

The Bethesda System showed a 6.4%-13-3% better agreement with the histological diagnoses according to 2003 WHO classification than the *Münchner Nomenklatur III* (Table 19). There was no difference between The Bethesda System and the *Münchner Nomenklatur III* if the histological specimens were diagnosed using 2014 WHO classification. The separate analysis of patients who were submitted to conization demonstrated a better agreement, regardless of the cytological reporting system. The Bethesda System had the same rate of agreement with both WHO systems. The *Münchner Nomenklatur III* had a better agreement with 2014 WHO classification.

## 3.3 Evaluation of FISH

As explained above, not all samples could be tested with the 3 probes together on the same nuclei as would have been desired because of a better assessment of reactive euploid polyploidization. From the 126 samples, 76 (60.3%) were hybridized with a mix of the three FISH probes and in 50 (39.7%) EGFR and hTERC were applied on one region and MYC separately on another. From these 50 samples, 25 (50%) were cytologically classified as group I, 11 (22%) as group IIp, 3 (6%) as group IIID1 and 11 (22%) as group IVa-p according to the *Münchner Nomenklatur III*. In 10 of the 126 smears (7.9% of all cases) the hybridization with the MYC probe could not be evaluated or there were not enough relevant cells on the slide for a separate analysis with MYC in addition to the EGFR/ hTERC probe mix. Of these 10 cases, 4 had a conization specimen for follow-up reference standard; of these 10 smears, 4 cases were cytologically classified as group IIp, 2 as group IVa-p and 4 as group I. In summary, 116 smears were finally evaluated, 76 of them with the 3-probe mix.

The number of hybridized FISH probes in a nucleus was analyzed for the individual probes each and all possible combinations of the probes, and the results subsequently compared to the follow-up, in order to formulate the best protocol for evaluation of suspicious cervical smears in the future. Atypical cells (nuclear enlargement, patchy DAPI staining, irregular shape) were detected, using the DAPI filter. The signals of the gene-specific FISH probes were recorded from these cells. Visualization of more than 2 signals of a probe in a nucleus was defined as a gain.

Moreover, an extra analysis was performed excluding the cells with tetrasomy, which may be observed under reactive (i.e. inflammatory) conditions in addition to (pre)neoplastic changes (Biesterfeld et al., 1994). In the cases analyzed with a 3 probe-mix, tetrasomy was defined as the presence of four copies of the genes in a nucleus. A deviation of +/-1 copy of one of the genes was accepted to consider diagnostic errors. Thus, the following patterns of gene copies were accepted as tetrasomy, regardless of the order: 4-4-4, 5-4-4, 3-4-4. In the 50 cases with a separate analysis of the MYC gene, these patterns could not be used, as the fluorescent signals of the three probes did not come from the same nuclei. So in these cases, tetrasomy was just considered for EGFR and hTERC and a deviation +/-1 of the copy number of one gene was not accepted. The following combination was adopted as tetrasomy for these cases: 4 (EGFR) - 4 (hTERC) – MYC did not influence.

### 3.4 FISH: Descriptive Analysis of individual genes and combinations

The descriptive analysis of the FISH results is resumed on the tables 20 and 21. The results were expressed as a mean number of cells with more than 2 gene copies per sample. The different patterns of analysis were, besides the individual probes and the combinations of two:

- **(+++): Gain of 3 probes in the same nucleus**
- **(+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus**
- **[(+)(+)(+)]: Gain of 3 probes, not necessarily in the same nucleus**
- **(+++) or (++-): 3 probes applied to the same nucleus, at least 2 with a gain**
- **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus.**

- **[(+)(+)(+)] or [(+)(+)(-)]: 3 probes not necessarily in the same nucleus, at least two of them with gain, at least 2 in the same nucleus.**

The + or - symbol inside the squared but outside the round brackets indicate the MYC gene analysis. Since this gene probe was not applied together with the probes for EGFR and hTERC in all the samples it was decided to let the signals + (more than 2 signals) or - (2 signals) outside the round brackets when the result was obtained from another group of cells.

### *3.4.1 Number of cells with gain of EGFR, hTERC, and MYC in relation to the cytological diagnostic groups*

| | I | | IIp | | IIID1 | | IIID2 | | IVa-p | | V-p | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| **EGFR 7p12** | 1.96 | 2.208 | 9.90 | 10.104 | 17.54 | 11.636 | 32.36 | 11.32 | 28.26 | 16.584 | 43.55 | 8.275 |
| **hTERC 3q26** | 1.64 | 1.440 | 16.62 | 17.313 | 28.54 | 16.003 | 37.64 | 9.82 | 37.78 | 13.389 | 46.73 | 6.084 |
| **MYC 8q14** | 0.86 | 0.854 | 18.71 | 18.834 | 16.42 | 11.367 | 30.41 | 10.87 | 34.29 | 13.922 | 43.64 | 7.827 |
| **EGFR and hTERC** | 0.04 | 0.200 | 7.52 | 9.988 | 15.08 | 12.406 | 30.73 | 12.11 | 27.13 | 17.831 | 42.00 | 8.955 |
| **EGFR and MYC** | - | - | 13.30 | 8.603 | 14.20 | 11.162 | 27.36 | 12.65 | 36.77 | 12.768 | 41.09 | 9.628 |
| **hTERC and MYC** | - | - | 17.40 | 9.721 | 17.20 | 11.884 | 29.32 | 11.77 | 37.85 | 12.422 | 42.64 | 7.646 |
| **(+++)** | - | - | 13.00 | 9.006 | 13.55 | 11.052 | 27.41 | 12.86 | 35.69 | 12.828 | 40.64 | 10.491 |
| **(+++) or [(++)+]** | 0 | 0 | 7.65 | 9.440 | 11.33 | 11.251 | 27.41 | 12.86 | 26.57 | 18.462 | 40,.64 | 10.491 |
| **(+++) or (++-)** | - | - | 20.40 | 11.177 | 21.60 | 12.479 | 33.36 | 10.79 | 40.23 | 10.963 | 45.18 | 6.570 |
| **(+++) or [(++)+] or [(++)-] or [(+-)+]** | 0.33 | 0.658 | 12.65 | 12.737 | 18.54 | 13.332 | 33.36 | 10.79 | 35.1 | 13.542 | 45.18 | 6.570 |

**Table 20: Mean number of cells with gain of the respective genes in the cytological groups obtained with the diverse analyses; "-" not examined; (+++): Gain of 3 probes in the same nucleus; (+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus; (+++) or (++-): 3 probes applied on the same nuclei, at least 2 with gain; (+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus.**

#### *3.4.1.1 Individual genes*

If compared to the grade of the cytological lesions, the three probes demonstrated an increase in the mean number of the analyzed cells with gains, with the exception of EGFR in group IIID2 and MYC in group IIp. The difference between the groups IVa-p and IIID2 in the hTERC analysis was just 0.14 (Figure 6, Table 20).

Except for group IIp, hTERC demonstrated a higher mean number of cells with a chromosomal gain in comparison with EGFR and MYC. The highest difference occurred in group IIID1, with an average of 11 more cells in comparison with EGFR and 12 more cells in comparison with MYC (Table 20). hTERC and EGFR had the highest and lowest mean number of cells with gains in group V and group IIp, respectively, regarding just the "positive" groups. For the analysis of MYC, the highest and the lowest mean numbers were observed in group V and group IIID1.

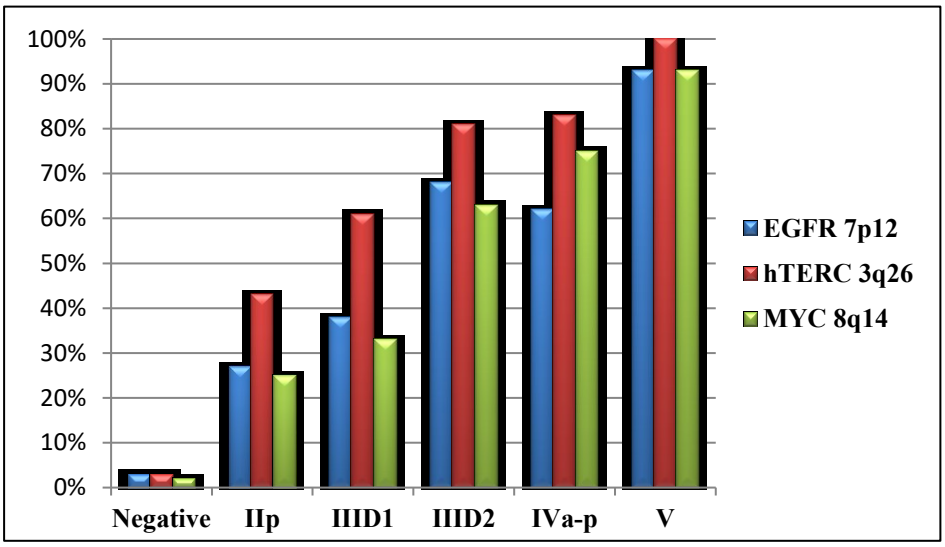


**Fig. 6: Graphic with the percentage of cells with gain of the individual probes in all cytological groups.**

### *3.4.1.2 Gain of two genes*

Analyzing the individual gain of two genes, it was possible to note, that only EGFR and MYC showed an increase in the number of cells in relation to the grade of the lesion. The highest increase occurred from group IIID2 to group IVa-p (9.38) (Table 20). hTERC and MYC demonstrated an early concurrent gain with a regular increase in respect to the cytological groups. The combinations with MYC did not provide results for the group Pap I as none of the samples in this group were tested with MYC and another probe together. The same situation occurred in the analysis of the concurrent gain of three genes. The analyses of gains in the same nuclei also showed increases in the number of cells in relation to the cytological groups.

### *3.4.1.3 Gain of three genes*

The analysis of the hybridized fluorescent signals of the three probes in the same nucleus demonstrated a proportional increase in the number of cells displaying gains of the genes. The

highest rise occurred from group IIID1 to group IIID2. The analysis of the gain of 3 probes, at least 2 in the same nucleus, included more cases (n=116) and showed an increase in relation to the grade of cytological lesions, with the exception of group IIID2 to group IVa-p. However, the difference between group IIID2 and group IVa-p was just 1.74 on average (Table 20).

#### *3.4.1.4 Analysis of tetrasomy*

Excluding the cells with 4 copies (possibly caused by tetrasomy) of the respective genes from the analysis led to a significant decrease in the number of cells with gains. In total, 721, 721 and 800 cells with 4 copies of EGFR, hTERC, and MYC, respectively, were considered as tetrasomic. The highest difference in the analysis including tetrasomy occurred in group IIID2. The mean of cells with a gain in this group (for hTERC, MYC, and EGFR) was 33.47 (including gain with 4 copies) and 19.38 without the same (Table 21, annex). All the individual genes, the combination of EGFR and hTERC and 2 of the analyses of all 3 genes (((+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]) and ((+++) or [(++)+])) had a regular increase in the number of cells with gains in relation to the cytological groups. All other combinations interestingly showed a lower number of cells with gains in cytological groups IIID1 and/or IIID2 compared to group IIp or IVa-p (Table 21). That means that in groups IIID1 and IIID2 of the respective analyses, the fraction of tetrasomic cells are higher than in groups IIp or IVa-p.

| **Excluding Tetrasomy** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | | IIp | | IIID1 | | IIID2 | | IVa-p | | V-p | |
| | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| EGFR 7p12 | 1.96 | 2.208 | 7.90 | 8.336 | 11.71 | 6.663 | 18.27 | 8.160 | 19.96 | 14.140 | 40.09 | 8.642 |
| hTERC 3q26 | 1.64 | 1.440 | 14.62 | 15.860 | 22.71 | 14.094 | 23.55 | 9.262 | 29.48 | 13.048 | 43.27 | 7.281 |
| MYC 8q14 | 0.86 | 0.854 | 9.41 | 9.314 | 10.58 | 7.424 | 16.32 | 6.564 | 26.95 | 14.572 | 40.18 | 8.436 |
| EGFR and hTERC | 0.28 | 0.614 | 5.52 | 8.220 | 9.25 | 7.225 | 16.64 | 8.438 | 18.83 | 15.084 | 38.73 | 8.968 |
| EGFR and MYC | - | - | 9.10 | 8.239 | 7.40 | 6.099 | 13.23 | 7.855 | 26.23 | 13.718 | 37.82 | 9.683 |
| hTERC and MYC | - | - | 13.30 | 9.546 | 10.35 | 7.569 | 12.18 | 7.102 | 27.31 | 13.444 | 39.36 | 7.762 |
| (+++) | - | - | 8.8 | 8.324 | 6.70 | 6.105 | 13.32 | 7.955 | 25.15 | 13.886 | 37.36 | 10.452 |
| (+++) or [(++)+] | 0 | 0 | 5.18 | 7.675 | 5.63 | 6.071 | 13.32 | 7.955 | 18.14 | 15.796 | 37.36 | 10.452 |
| (+++) or (++-) | - | - | 16.20 | 11.013 | 14.75 | 8.058 | 19.23 | 8.006 | 29.69 | 11.933 | 41.00 | 6.826 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (+++) or [(++)+] or [(++)-] or (+-)+] | 0.33 | 0.658 | 10.18 | 11.137 | 12.71 | 8.69 | 19.23 | 8.006 | 26.38 | 12.114 | 41.00 | 6.826 |

**Table 21: Mean number of cells with gain of the respective genes in the cytological groups obtained with the diverse analyses excluding cells with a tetrasomic pattern. (+++): Gain of 3 probes in the same nucleus; (+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus; (+++) or (++-): 3 probes applied on the same nuclei, at least 2 with gain; (+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus.**

### *3.4.2 Number of cells with gain of EGFR, hTERC, and MYC in relation to the histological follow-up*

| | Negative | | CIN1 | | CIN2 | | CIN3 | | SCC | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| **EGFR 7p12** | 9.98 | 12.474 | 25.33 | 14.883 | 16.47 | 13.814 | 29.25 | 15.829 | 41.88 | 9.094 |
| **hTERC 3q26** | 14.46 | 17.473 | 34.00 | 11.554 | 21.67 | 18.078 | 37.63 | 13.543 | 46.13 | 6.999 |
| **MYC 8q14** | 12.41 | 14.988 | 27.78 | 14.114 | 15.43 | 12.786 | 31.38 | 15.907 | 42.25 | 8.763 |
| **EGFR and hTERC** | 7.94 | 12.519 | 23.33 | 15.898 | 14.47 | 15.842 | 27.70 | 16.655 | 39.88 | 9.628 |
| **EGFR and MYC** | 18.21 | 13.402 | 22.63 | 16.097 | 21.00 | 13.901 | 28.81 | 15.382 | 38.88 | 10.398 |
| **hTERC and MYC** | 20.89 | 12.507 | 24.75 | 14.955 | 23.22 | 12.726 | 31.03 | 14.998 | 40.88 | 8.236 |
| **(+++)** | 17.63 | 13.512 | 22.13 | 16.057 | 20.22 | 13.33 | 28.59 | 15.475 | 38.25 | 11.386 |
| **(+++) or [(++)+]** | 7.54 | 12.149 | 20.00 | 16.317 | 13.00 | 14.507 | 25.51 | 17.195 | 38.25 | 11.386 |
| **(+++) or (++-)** | 24.63 | 12.316 | 28.75 | 14.270 | 27.44 | 12.856 | 34.16 | 14.242 | 44.00 | 7.309 |
| **(+++) or [(++)+] or [(++)-] or [(+-)+]** | 11.54 | 14.394 | 27.78 | 13.664 | 18.14 | 16.422 | 32.00 | 15.573 | 44.00 | 7.309 |

**Table 22: Mean number of cells with gain with the respective genes in the histological groups obtained with the diverse analyses. (+++): Gain of 3 probes in the same nucleus; (+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus; (+++) or (++-): 3 probes applied on the same nuclei, at least 2 with gain; (+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus. CIN: Cervical intraepithelial neoplasia.**

Relating the FISH copy number analysis of the respective genes to the histological outcomes revealed an irregular increase in the number of cells with gains. The mean number of these cells was higher in CIN1 than in CIN2 in all kinds of analyses. A high mean number of cells with gains was noted in the negative group, sometimes being similar to the CIN2 group (Table 22).

| | Excluding Tetrassomy | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Negative | | CIN1 | | CIN2 | | CIN3 | | SCC | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **EGFR 7p12** | 7,06 | 7,875 | 13,56 | 70,002 | 9,67 | 6,894 | 21,13 | 14,119 | 38,13 | 9,372 |
| **hTERC 3q26** | 11,54 | 14,402 | 22,22 | 9,244 | 14,87 | 13,938 | 29,5 | 12,836 | 42,38 | 8,400 |
| **MYC 8q14** | 7,39 | 8,352 | 13,78 | 4,324 | 8,14 | 6,298 | 23,41 | 15,229 | 38,5 | 9,396 |
| **EGFR and hTERC** | 5,13 | 7,724 | 11,56 | 7,178 | 7,67 | 8,457 | 36,38 | 9,471 | 36,38 | 9,471 |
| **EGFR and MYC** | 10,21 | 7,836 | 10,13 | 6,556 | 9,78 | 7,612 | 20,03 | 15,167 | 35,38 | 10,322 |
| **hTERC and MYC** | 12,89 | 7,571 | 12,25 | 5,800 | 11,89 | 7,574 | 22,28 | 14,552 | 37,38 | 8,245 |
| **(+++)** | 9,68 | 7,973 | 9,63 | 6,632 | 8,89 | 7,114 | 19,81 | 15,064 | 34,75 | 11,184 |
| **(+++) or [(++)+]** | 4,11 | 6,926 | 8,56 | 6,984 | 5,71 | 7,119 | 17,54 | 15,352 | 34,75 | 11,184 |
| **(+++) or (++-)** | 16,63 | 8,375 | 16,25 | 6,409 | 16,11 | 7,705 | 25,38 | 13,779 | 39,25 | 7,206 |
| **(+++) or [(++)+] or [(++)-] or [(+-)+]** | 8,09 | 9,899 | 16 | 6,042 | 10,86 | 9,502 | 23,87 | 14,146 | 39,25 | 7,206 |

**Table 23: Mean number of cells with gain in the histological groups obtained with the diverse analyses excluding tetraploid cells. (+++) 3 Probes on the same cell; (+++) or [(++)+] 3 Probes, three positive, at least two in the same nuclei; (+++) or [(++)+] or [(++)-] 3 Probes, at least two positive in the same nuclei; (+++) or [(++)+] or [(++)-] or [(+-)+] 3 Probes, at least two positive, at least two in the same nuclei. CIN: Cervical intraepithelial neoplasia.**

By excluding the cells with a tetrasomic pattern, it was possible to observe the same behaviour as in the previous analysis. That is, the mean number of cells with gains was higher in the CIN1 group than in the CIN2 group in all kinds of analyses (Table 23). In two kinds of analyses ((+++) and ((+++) or (++-))), the mean number of cells with gain (excluding gain with 4 copies) was even higher in the negative group than in the CIN1 or CIN2 groups.

## 3.5 FISH: ANOVA analyses

The tables 24 and 25 show the results of the ANOVA analyses. These analyses were made in order to verify if the FISH procedure shows significant differences in the number of cells with gains of the respective genes in specimens of the different cytological groups. The same was calculated between the histological follow-up diagnoses.

| | | EGFR | hTERC | MYC | EGFR and hTERC | EGFR and MYC | hTERC and MYC | (+++) | (+++) or [(++)+] | (+++) or (++-) | (+++) or [(++)+] or [(++)-] or [(+-)+] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cytology** | | | | | | | | | | | |
| **Negative** | **IIp** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | 0.001* | - | 0.001* |
| | **IIID1** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | <0.001* | - | <0.001* |
| | **IIID2** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | <0.001* | - | <0.001* |
| | **IVa-p** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | <0.001* | - | <0.001* |
| | **V-p** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | <0.001* | - | <0.001* |
| **IIp** | **IIID1** | 0.004* | 0.008* | 0.765 | 0.003* | 0.848 | 0.906 | 0.894 | 0.151 | 0.794 | 0.074 |
| | **IIID2** | <0.001* | <0.001* | 0.002* | <0.001* | 0.001* | 0.007* | 0.001* | <0.001* | 0.003* | <0.001* |
| | **IVa-p** | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| | **V-p** | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| **IIID1** | **IIID2** | <0.001* | 0.045* | <0.001* | <0.001* | <0.001* | 0.001* | <0.001* | <0.001* | 0.001* | <0.001* |
| | **IVa-p** | 0.005* | 0.016* | <0.001* | 0.005* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| | **V-p** | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| **IIID2** | **IVa-p** | 0.176 | 0.709 | 0.230 | 0.174 | 0.029* | 0.033* | 0.054 | 0.515 | 0.058 | 0.554 |
| | **V-p** | 0.026* | 0.010* | 0.003* | 0.028* | 0.003* | 0.001* | 0.003* | 0.014* | 0.002* | 0.003* |
| **IVa-p** | **V-p** | 0.001* | 0.022* | 0.041* | 0.001* | 0.393 | 0.265 | 0.272 | 0.003* | 0.237 | 0.011* |
| **Histology** | | | | | | | | | | | |
| **Negative** | **CIN1** | 0.001* | 0.002* | 0.004* | <0.001* | 0.558 | 0.559 | 0.489 | 0.005* | 0.488 | 0.002* |
| | **CIN2** | 0.046* | 0.109 | 0.261 | 0.066 | 0.662 | 0.726 | 0.654 | 0.142 | 0.731 | 0.088 |
| | **CIN3** | <0.001* | <0.001* | <0.001* | <0.001* | 0.012* | 0.009* | 0.009* | <0.001* | 0.011* | <0.001* |
| | **SCC** | <0.001* | <0.001* | <0.001* | <0.001* | 0.001* | 0.001* | 0.001* | <0.001* | 0.001* | <0.001* |
| **CIN1** | **CIN2** | 0.116 | 0.112 | 0.09 | 0.076 | 0.885 | 0.829 | 0.819 | 0.165 | 0.752 | 0.136 |
| | **CIN3** | 0.540 | 0.364 | 0.426 | 0.551 | 0.212 | 0.187 | 0.224 | 0.509 | 0.250 | 0.365 |
| | **SCC** | 0.044* | 0.054 | 0.038* | 0.053 | 0.020* | 0.012* | 0.018* | 0.029* | 0.017* | 0.023* |
| **CIN2** | **CIN3** | 0.004* | 0.001* | 0.001* | 0.002* | 0.137 | 0.098 | 0.119 | 0.008* | 0.109 | 0.002* |
| | **SCC** | <0.001* | <0.0001* | <0.001* | <0.001* | 0.012* | 0.006* | 0.008* | <0.001* | 0.006* | <0.001* |
| **CIN3** | **SCC** | 0.051 | 0.118 | 0.064 | 0.063 | 0.086 | 0.059 | 0.072 | 0.035* | 0.059 | 0.046* |

**Table 24: Results of the ANOVA analyses aiming to define the significance of the mean number of cells with gain of the respective genes among the diverse cytological and histological groups. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma.**

Some analyses were not possible in the cytological group I due to the fact, that there were no negative samples analyzed with the three probes in a mixing. The following remarks can be made on the ANOVA results with a focus on the analyses that did not reach the significance level (Table 24):

There was a significant difference between the cytological group I and all positive groups. The histological follow-up negative group, however, did not show a significant difference to CIN1 when analyzed with the following combinations: EGFR-MYC, hTERC-MYC, (+++) and ((+++) or (++-)). Besides that, the histological negative group did not demonstrate a significant difference to CIN2 in all individual and combined analyses of the genes, except for EGFR.

Except for the analyses with EGFR, hTERC, and EGFR-hTERC, the group IIp did not demonstrate a significant difference to the group IIID1. Group IIID1 showed a significant difference in relation to groups IIID2, IVa-p and V in all kinds of analyses. The histological follow-up CIN1 group, however, did not demonstrate a significant difference to the groups CIN2 and CIN3 in any kind of analysis. But compared to SCC, all analyses showed a significant difference with the exception of the analyses with hTERC and hTERC-EGFR. Between groups IIID2 and IVa-p (Table 24), a significant difference was only observed in the EGFR-MYC and hTERC-MYC analyses. In relation to the histological follow-up, the FISH results of the CIN2 group demonstrated significant differences to CIN3 in all kinds of analyses, except for EGFR-MYC, (+++) and ((+++) or (++-)). The group IIID2 demonstrated a significant difference in all kinds of analyses in relation to the group V. The FISH results in group CIN3 were significantly different to SCC in two analyses: ((+++) or [(++)+]) and the investigation of 3 probes, at least two of them with gain, at least 2 in the same nucleus.

| | | EGFR | hTerc | MYC | EGFR and hTERC | EGFR and MYC | hTERC and MYC | (+++) | (+++) or [(++)+] | (+++) or (++-) | (+++) or [(++)+] or [(++)-] or [(+-)+] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Negative | **IIp** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | <0.001* | - | <0.001* |
| | **IIID1** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | <0.001* | - | <0.001* |
| | **IIID2** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | <0.001* | - | <0.001* |
| | **IVa-p** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | <0.001* | - | <0.001* |
| | **V-p** | <0.001* | <0.001* | <0.001* | <0.001* | - | - | - | <0.001* | - | <0.001* |
| **IIp** | **IIID1** | 0.009* | 0.013* | 0.295 | 0.005* | 0.565 | 0.400 | 0.456 | 0.350 | 0.741 | 0.171 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **IIID2** | <0.001* | 0.005* | <0.001* | <0.001* | 0.082 | 0.409 | 0.077 | <0.001* | 0.32 | <0.001* |
| | **IVa-p** | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | 0.002* | <0.001* | <0.001* | 0.002* | <0.001* |
| | **V-p** | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| **IIID1** | **IIID2** | 0.007* | 0.677 | 0.005* | 0.005* | 0.005* | 0.041* | 0.002* | <0.001* | 0.103 | 0.010* |
| | **IVa-p** | 0.023* | 0.036* | <0.001* | 0.015* | <0.001* | <0.001* | <0.001* | 0.001* | <0.001* | <0.001* |
| | **V-p** | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| **IIID2** | **IVa-p** | 0.628 | 0.098 | 0.018* | 0.669 | 0.002* | 0.003* | 0.006* | 0.924 | 0.005* | 0.032* |
| | **V-p** | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| **IVa-p** | **V-p** | <0.001* | 0.003* | 0.009* | <0.001* | 0.041* | 0.022* | 0.035* | <0.001* | 0.022* | 0.001* |
| **Negative** | **CIN1** | 0.005* | 0.007* | 0.013* | 0.004* | 0.894 | 0.845 | 0.898 | 0.024* | 0.791 | 0.015 |
| | **CIN2** | 0.097 | 0.218 | 0.380 | 0.187 | 0.932 | 0.693 | 0.904 | 0.27 | 0.762 | 0.216 |
| | **CIN3** | <0.001* | <0.001* | <0.001* | <0.001* | 0.017* | 0.018* | 0.010* | <0.001* | 0.019* | <0.001* |
| | **SCC** | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| **CIN1** | **CIN2** | 0.21 | 0.136 | 0.128 | 0.110 | 0.852 | 0.873 | 0.833 | 0.246 | 0.982 | 0.227 |
| | **CIN3** | 0.282 | 0.201 | 0.149 | 0.289 | 0.104 | 0.050 | 0.076 | 0.215 | 0.045* | 0.125 |
| | **SCC** | 0.003* | 0.009* | 0.003* | 0.004* | 0.001* | <0.001* | 0.001* | 0.002* | <0.001* | 0.001* |
| **CIN2** | **CIN3** | 0.003* | <0.001* | <0.001* | 0.001* | 0.053 | 0.025* | 0.034* | 0.003* | 0.033* | 0.001* |
| | **SCC** | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| **CIN3** | **SCC** | 0.007* | 0.038* | 0.017* | 0.008* | 0.005* | 0.004* | 0.007* | 0.006* | 0.005* | 0.008* |

**Table 25: Results of the ANOVA analyses aiming to define the significance of the mean number of cells with gain of the respective genes excluding cell with a tetrasomic pattern among the diverse cytological and histological groups. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma.**

An additional analysis was performed excluding the cells with a tetrasomic pattern (Table 25). Except for the analyses of EGFR, hTERC, and EGFR-hTERC, the group IIp did not demonstrate significant difference to the group IIID1. In four combined analyses of at least two of the genes, this difference was too not noticed in group IIID2. Group IIID1 demonstrated significant differences to the group IIID2 in the analyses of individual genes with the exception of the hTERC analysis. The analysis of the application of (+++) or (++-), did not reach the statistical significance level too, but the same analysis in group CIN1 compared to CIN3 did. The CIN1 group, however, did not show a significant difference compared to groups CIN2 and CIN3 in the other analyses.

The analyses of EGFR, hTERC, EGFR-hTERC, and (+++) or [(++)+]) were not significantly different between groups IIID2 and IVa-p. The analysis with group CIN2 showed

significant differences to group CIN3 in all but one analysis: the combination of the probes for EGFR and MYC.

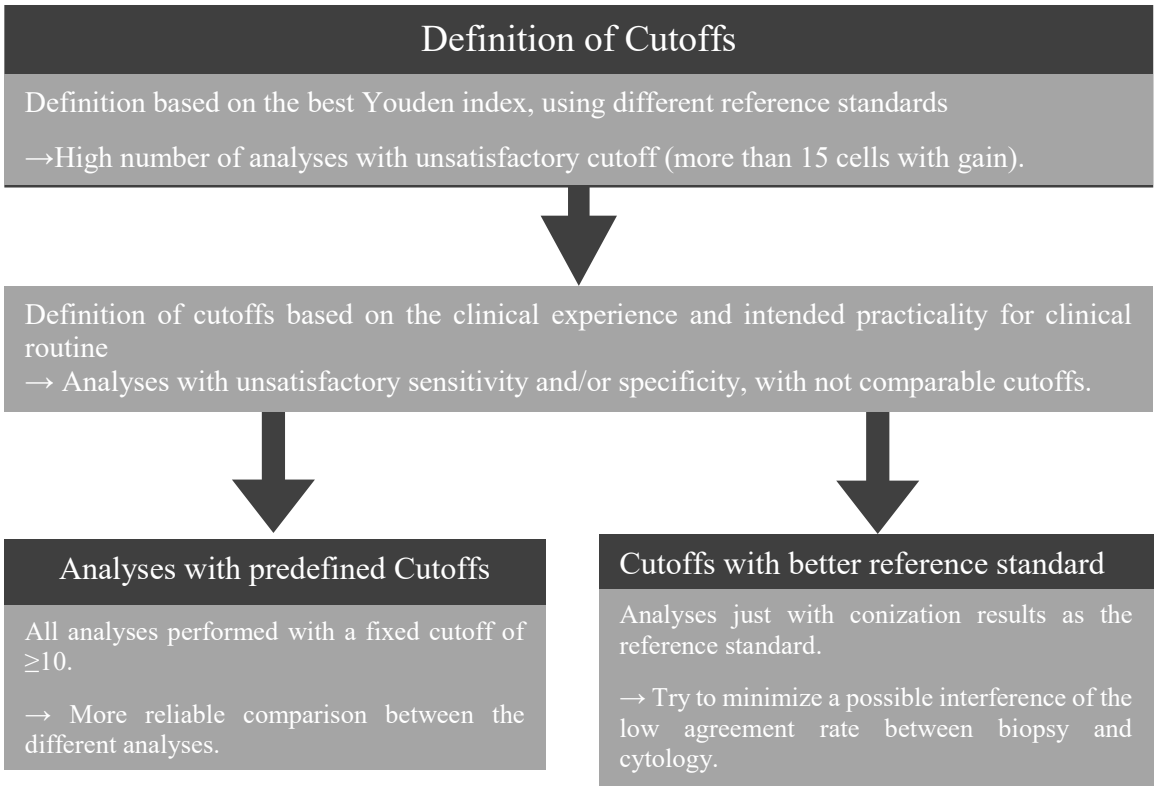


**Fig. 7: Scheme graphic with the description of the stepped process of cutoff development and a resume of the reasons for or the results of each step.**

### 3.6 FISH: Definition of Cutoffs

For the clinical routine, cutoff values for the separation of positive and negative FISH results should be established. Different analyses were performed in order to define the best cutoff for each individual gene probe or combination of probes. During the analysis process, the first results were objectively generated using the best Youden indices. Additional analyses were adopted to overcome problems due to the sometimes low practicality of this approach and bad comparability of different analyses. The steps of evaluation are described below (Figure 7).

For the cutoffs, based on the best Youden indices (tables 26-31, annex), several "reference standards" (≥CIN1, ≥CIN2, ≥CIN3) were tested. In many of these analyses, the cutoffs were higher than 15 (sometimes 30) cells, and in general, the specificity values are too low for a diagnostic test. A particular reanalysis was made in order to decide if a lower cutoff

would be more appropriate for the laboratory routine. The required number of cells with gain, the sensitivity and specificity were the parameters adopted to define the more suitable cutoffs.

The different kinds of analyzing the hybridized probes individually and the patterns of combinations are the same as described in chapter 3.4.

### *3.6.1 ≥CIN1 histology as a "reference standard"*

It is important to stress that the analysis with ≥CIN1 histology as a reference standard is just useful for research and comparisons purposes. The goal of the development of a new protocol or tool for the diagnosis of cancer precursors is to define the accuracy for that purpose. Since CIN1 represents an initial stage of the dysplastic process, in other words, represents a possible cancer precursor with a very high tendency of regression, there is no sense in using CIN1 as the lesion of interest in a (pre)cancer test.

The analyses of single genes using ≥CIN1 histology as the gold standard demonstrated the best result, with a high sensitivity of the three probes >85% and with a low number of cells with gain as the cutoff. Nevertheless, the specificities are lower than 65%. MYC had the lowest cutoff (>2) and the highest sensitivity (94.30%). However, MYC showed the lowest specificity (52.2%) (Table 26, 27).

| Probes | Cutoff | Sensitivity | Specificity | Youden Index | AUC | AUC (IC) | AUC (p) |
|---|---|---|---|---|---|---|---|
| **EGFR n=126** | >6 | 87.50% | 61.11% | 0.4861 | 0.815 | 0.739-0.890 | <0.001* |
| **hTERC n=126** | >10 | 87.5% | 64.80% | 0.523 | 0.805 | 0.724-0.886 | <0.001* |
| **MYC n=116** | >2 | 94.30% | 52.20% | 0.465 | 0.789 | 0.704-0.874 | <0.001* |
| **EGFR and hTERC n=126** | >2 | 88.90% | 61.10% | 0.5000 | 0.814 | 0.738-0.889 | <0.001* |
| **EGFR and MYC n=76** | >25 | 54.40% | 78.90% | 0.3333 | 0.687 | 0.553-0.820 | 0.015* |
| **hTERC and MYC n=76** | >32 | 50.90% | 89.50% | 0.4040 | 0.690 | 0.559-0.822 | 0.013* |
| **(+++) n=76** | >24 | 56.10% | 78.90% | 0.351 | 0.693 | 0.560-0.826 | 0.012* |
| **(+++) or [(++)+] n=116** | >10 | 71.40% | 76.10% | 0.475 | 0.783 | 0.699-0.868 | <0.001* |
| **[(+)(+)(+)] n=116** | >10 | 78.60% | 63.80% | 0.424† | 0.712 | 0.614-0.810 | <0.001* |
| **(+++) or (++-) n=76** | >36 | 52.60% | 89.50% | 0.421 | 0.687 | 0.555-0.820 | 0.015* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=116** | >2 | 92.90% | 54.30% | 0.472 | 0.798 | 0.712-0.884 | <0.001* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | >10 | 81.70% | 59.60% | 0.467† | 0.734 | 0.636-0.831 | <0.001* |

**Table 26: Definition of the cutoffs based on the best Youden Indices using CIN1 as the gold standard. The data obtained from this cutoff were reported: sensitivity, specificity and ROC curve value ($p < 0.05$). † indicates, that the chosen Youden Index was not the best but the more practical. AUC (IC): AUC 95% confidence interval. (+++): Gain of 3 probes in the same nucleus; (+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus; [(+)(+)(+)]:Gain of 3 probes, not necessarily in the same nucleus; (+++) or (++-): 3 probes applied on the same nuclei, at least 2 with gain; (+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus; [(+)(+)(+)] or [(+)(+)(-)]: 3 probes, at least two of them with gain, at least 2 in the same nucleus, not necessarily in the same nucleus.**

| Probes | Cutoff | Sensitivity | Specificity | Youden Index | AUC | AUC (IC) | AUC (p) |
|---|---|---|---|---|---|---|---|
| **EGFR n=126** | >5 | 84.70% | 63.00% | 0.477 | 0.800 | 0.723-0.877 | <0.001* |
| **hTERC n=126** | >10 | 87.5% | 68.50% | 0.560 | 0.795 | 0.712-0.878 | <0.001* |
| **MYC n=116** | >2 | 92.9% | 56.50% | 0.494 | 0.790 | 0.707-0.874 | <0.001* |
| **EGFR and hTERC n=126** | >3 | 79.20% | 68.50% | 0.477 | 0.797 | 0.720-0.875 | <0.001* |
| **EGFR and MYC n=76** | >17 | 45.60% | 84.20% | 0.298 | 0.669 | 0.542-0.796 | 0.028* |
| **hTERC and MYC n=76** | >17 | 52.6% | 78.9% | 0.3160 | 0.657 | 0.532-0.783 | 0.041* |
| **(+++) n=76** | >23 | 35.10% | 94.70% | 0.298 | 0.676 | 0.549-0.804 | 0.022* |
| **(+++) or [(++)+] n=116** | >3 | 75.70% | 69.60% | 0.453 | 0.773 | 0.688-0.858 | <0.001* |
| **[(+)(+)(+)] n=116** | >10 | 61.40% | 73.90% | 0.353† | 0.677 | 0.577-0.777 | 0.001* |
| **(+++) or (++-) n=76** | >24 | 47.40% | 84.20% | 0.316 | 0.656 | 0.530-0.782 | 0.043* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=116** | >3 | 90.00% | 56.50% | 0.465 | 0.787 | 0.701-0.873 | <0.001* |
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | >10 | 75.70% | 63.00% | 0.388† | 0.694 | 0.593-0.794 | <0.001* |

**Table 27: Definition of the cutoffs based on the best Youden Indices using CIN1 as the gold standard, excluding cells with a tetrasomic pattern. The data obtained from this cutoff (sensitivity, specificity, and ROC curve value) were reported ($p < 0.05$). † indicates, that the chosen Youden Index was not the best but the more practical; AUC (IC): AUC 95% confidence interval. (+++): Gain of 3 probes in the same nucleus; (+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus; [(+)(+)(+)]:Gain of 3 probes, not necessarily in the same nucleus; (+++) or (++-): 3 probes applied on the same nuclei, at least 2 with gain; (+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus; [(+)(+)(+)] or [(+)(+)(-)]: 3 probes, at least two of them with gain, at least 2 in the same nucleus, not necessarily in the same nucleus.**

### *3.6.2 ≥CIN2 histology as a reference standard*

| Probes | Cutoff | Sensitivity | Specificity | Youden Index | AUC | AUC (IC) | AUC (p) |
|---|---|---|---|---|---|---|---|
| **EGFR n=126** | >14 | 76.20% (48/63) | 69.80% (44/63) | 0.4600 | 0.776 | 0.695-0.857 | <0.001* |
| **hTERC n=126** | >15 | 84.10% | 58.70% | 0.429 | 0.772 | 0.690-0.853 | <0.001* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | (53/63) | (37/63) | | | | |
| **MYC n=116** | >15 | 75.40% (46/61) | 61.80% (34/55) | 0.372 | 0.752 | 0.664-0.839 | <0.001* |
| **EGFR and hTERC n=126** | >11 | 77.80% (49/63) | 68.30% (43/63) | 0.460 | 0.774 | 0.692-0.855 | <0.001* |
| **EGFR and MYC n=76** | >27 | 55.10% (27/49) | 74.10% (20/27) | 0.292 | 0.686 | 0.563-0.809 | 0.007* |
| **hTERC and MYC n=76** | >32 | 55.10% (27/49) | 85.20% (23/27) | 0.403 | 0.691 | 0.569-0.814 | 0.006* |
| **(+++) n=76** | >31 | 44.90% (22/49) | 85.20% (23/27) | 0.301 | 0.688 | 0.565-0.812 | 0.007* |
| **(+++) or [(++)+] n=116** | >10 | 74.10% (43/58) | 69.00% (40/58) | 0.431 | 0.716 | 0.620-0.811 | <0.001* |
| **[(+)(+)(+)] n=116** | >10 | 77.60% (45/58) | 53.40% (31/58) | 0.310 | 0.655 | 0.555-0.755 | 0.004* |
| **(+++) or (++-) n=76** | >36 | 57.10% (28/49) | 85.20% (23/27) | 0.423 | 0.682 | 0.557-0.807 | 0.009* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=116** | >36 | 50.80% (31/61) | 90.90% (50/55) | 0.417 | 0.763 | 0.675-0.850 | <0.001* |
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | >10 | 82.80% (48/58) | 44.80% (26/58) | 0.276 | 0.638 | 0.537-0.739 | 0.01* |

**Table 28: Definition of the cutoffs based on the best Youden Indices using CIN2 as the gold standard. The data obtained from this cutoff were reported: sensitivity, specificity and ROC curve value ($p < 0.05$). † indicates, that the chosen Youden Index was not the best but the more practical. AUC (IC): AUC 95% confidence interval. (+++): Gain of 3 probes in the same nucleus; (+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus; [(+)(+)(+)]:Gain of 3 probes, not necessarily in the same nucleus; (+++) or (++-): 3 probes applied on the same nuclei, at least 2 with gain; (+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus; [(+)(+)(+)] or [(+)(+)(-)]: 3 probes, at least two of them with gain, at least 2 in the same nucleus, not necessarily in the same nucleus. Between parentheses number of true positives (Sensitivity) and true negatives (Specificity).**

The tables 28 and 29 show the results of the cutoff analyses considering ≥CIN2 histological results as the reference standard, with and without cells with the tetrasomic pattern, respectively.

In a general way, the analysis using ≥CIN2 as reference standard showed no clinically applicable results. Either the cutoff was too high or the specificity and sensitivity were lower than acceptable for clinical routine (i.e. specificity approx. 90% or higher with good sensitivity approx. 70% or higher and an applicable cutoff of <15 cells with gain).

| Probes | Cutoff | Sensitivity | Specificity | Youden Index | AUC | AUC (IC) | AUC (p) |
|---|---|---|---|---|---|---|---|
| **EGFR n=126** | >8 | 76.20% (48/63) | 63.50% (40/63) | 0.387 | 0.775 | 0.686-0.854 | <0.001* |
| **hTERC n=126** | >10 | 85.70% (54/63) | 57.81% (37/63) | 0.444 | 0.771 | 0.688-0.841 | <0.001* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **MYC n=116** | >2 | 91.80% (56/61) | 47.30% (26/55) | 0.391 | 0.767 | 0.683-0.851 | <0.001* |
| **EGFR and hTERC n=126** | >3 | 77.80% (49/63) | 60.30% (38/63) | 0.381 | 0.770 | 0.690-0.851 | <0.001* |
| **EGFR and MYC n=76** | >20 | 42.80% (21/49) | 92.60% (25/27) | 0.354 | 0.690 | 0.574-0.807 | 0.006* |
| **hTERC and MYC n=76** | >21 | 46.90% (23/49) | 92.60% (25/27) | 0.395 | 0.693 | 0.577-0.808 | 0.006* |
| **(+++) n=76** | >23 | 40.80% (20/49) | 96.30% (26/27) | 0.371 | 0.699 | 0.583-0.815 | 0.004* |
| **(+++) or [(++)+] n=116** | >6 | 65.60% (40/58) | 74.50% (41/58) | 0.401 | 0.753 | 0.665-0.840 | <0.001* |
| **[(+)(+)(+)] n=116** | >10 | 62.10% (36/58) | 67.20% (39/58) | 0.293 | 0.647 | 0.546-0.747 | 0.006* |
| **(+++) or (++-) n=76** | >24 | 53.10% (26/49) | 85.20% (23/27) | 0.382 | 0.695 | 0.579-0.810 | 0.005* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=116** | >21 | 52.50% (32/61) | 85.50% (47/55) | 0.379 | 0.768 | 0.684-0.852 | <0.001* |
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | >10 | 75.90% (44/58) | 53.40% (31/58) | 0.293 | 0.647 | 0.546-0.747 | 0.006* |

**Table 29: Definition of the cutoffs based on the best Youden Indices using CIN2 as the gold standard excluding cells with a tetrasomic pattern. The data obtained from this cutoff (sensitivity, specificity, and ROC curve value) were reported (p < 0.05). † indicates, that the chosen Youden Index was not the best but the more practical. AUC (IC): AUC 95% confidence interval .(+++): Gain of 3 probes in the same nucleus; (+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus; [(+)(+)(+)]:Gain of 3 probes, not necessarily in the same nucleus; (+++) or (++-): 3 probes applied on the same nuclei, at least 2 with gain; (+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus; [(+)(+)(+)] or [(+)(+)(-)]: 3 probes, at least two of them with gain, at least 2 in the same nucleus, not necessarily in the same nucleus. Between parentheses number of true positives (Sensitivity) and true negatives (Specificity).**

Excluding the cells with a tetrasomic pattern from the analyses considering ≥CIN2 as a reference standard reduced the cutoff values of most of the kinds of approaches but did not, as well, show a clinically applicable result (Table 29). The best results were provided by the combined evaluation with EGFR and MYC showing a sensitivity of 42.8% and a specificity of 92.6%, and hTERC and MYC with a sensitivity of 46.9% and specificity 92.6%. The cutoffs were >20 and >21, respectively (Table 29).

### *3.6.3 ≥CIN3 as a reference standard*

The tables 30 and 31 show the results of the cutoff analyses considering ≥CIN3 histological results as the reference standard, with and without cells with the tetrasomic pattern, respectively.

| Probes | Cutoff | Sensitivity | Specificity | Youden Index | AUC | AUC (IC) | AUC (p) |
|---|---|---|---|---|---|---|---|
| **EGFR n=126** | >19 | 77.10% (37/48) | 74.40% (58/78) | 0.5140 | 0.815 | 0.740-0.889 | <0.001* |
| **hTERC n=126** | >25 | 83.33% (40/48) | 66.70% (52/78) | 0.500 | 0.820 | 0.748-0.892 | <0.001* |
| **MYC n=116** | >23 | 72.30% (34/47) | 76.80% (53/69) | 0.492 | 0.807 | 0.729-0.885 | <0.001* |
| **EGFR and hTERC n=126** | >17 | 77.10% (37/48) | 74.40% (58/78) | 0.514 | 0.818 | 0.744-0.892 | <0.001* |
| **EGFR and MYC n=76** | >27 | 62.50% (25/40) | 75.70% (27/36) | 0.375 | 0.716 | 0.600-0.832 | 0.001* |
| **hTERC and MYC n=76** | >32 | 62.50% (25/40) | 83.30% (30/36) | 0.458 | 0.726 | 0.611-0.842 | 0.001* |
| **(+++) n=76** | >27 | 62.50% (25/40) | 75.00% (27/36) | 0.375 | 0.718 | 0.602-0.834 | 0.001* |
| **(+++) or [(++)+] n=116** | >16 | 74.50% (35/47) | 75.40% (52/69) | 0.498 | 0.788 | 0.701-0.874 | <0.001* |
| **[(+)(+)(+)] n=116** | >10 | 82.2% (37/47) | 50.7% (36/69) | 0.329 | 0.665 | 0.565-0.764 | 0.003* |
| **(+++) or (++-) n=76** | >36 | 65.00% (26/40) | 83.30% (30/36) | 0.483 | 0.716 | 0.598-0.834 | 0.001* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=116** | >36 | 61.70% (29/47) | 89.90% (63/69) | 0.516 | 0.807 | 0.729-0.886 | <0.001* |
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | >10 | 88.9% (40/47) | 43.7% (31/69) | 0.326 | 0.663 | 0.564-0.761 | 0.003* |

**Table 30: Definition of the cutoffs based on the best Youden Indices using CIN3 as the gold standard. The data obtained from this cutoff were reported: sensitivity, specificity and ROC curve value ($p < 0.05$). † indicates, that the chosen Youden Index was not the best but the more practical. AUC (IC): AUC 95% confidence interval. (+++): Gain of 3 probes in the same nucleus; (+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus; [(+)(+)(+)]:Gain of 3 probes, not necessarily in the same nucleus; (+++) or (++-): 3 probes applied on the same nuclei, at least 2 with gain; (+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus; [(+)(+)(+)] or [(+)(+)(-)]: 3 probes, at least two of them with gain, at least 2 in the same nucleus, not necessarily in the same nucleus. Between parentheses number of true positives (Sensitivity) and true negatives (Specificity).**

As for the analysis ≥CIN2, the analysis using ≥CIN3 as the reference standard too showed no clinically applicable results in most of the analyses. The combined evaluation of 3 probes, at least two of them with gain, not necessarily on the same nucleus ([(+)(+)(+)] or [(+)(+)(-)]) showed a sensitivity of 88.9% and a specificity of 43.7%, with a cutoff of >10 (table 30). This kind of approach could be adopted as a screening test since it has demonstrated a high sensitivity. However, as described in chapter 4, FISH, due to the high costs, would hardly be included in a screening strategy.

Excluding the cells with four copies of genes from the analyses of cutoffs considering ≥CIN3 histology as a reference standard, it is possible to observe that, in a general way, the cutoffs were lower or equal to the cutoffs found in the analysis including the cells with a tetrasomic pattern. The evaluation of ((+++) or [(++)+]) presented a sensitivity of 61.7% and a

specificity of 87.0%, with a cutoff of >13 cells. The isolated evaluation of MYC gene probe showed a sensitivity of 59.6% and specificity of 91.3%. However, the cutoff (>19 cells with gain) was too high.

| Probes | Cutoff | Sensitivity | Specificity | Youden Index | AUC | AUC (IC) | AUC (p) |
|---|---|---|---|---|---|---|---|
| **EGFR n=126** | >14 | 68.80% (33/48) | 80.80% (63/78) | 0.495 | 0.821 | 0.746-0.895 | <0.001* |
| **hTERC n=126** | >13 | 91.70% (44/48) | 61.5% (48/78) | 0.532 | 0.831 | 0.761-0.900 | <0.001* |
| **MYC n=116** | >19 | 59.60% (28/47) | 91.30% (63/69) | 0.509 | 0.833 | 0.759-0.907 | <0.001* |
| **EGFR and hTERC n=126** | >13 | 66.70% (32/48) | 82.10% (64/78) | 0.487 | 0.828 | 0.755-0.900 | <0.001* |
| **EGFR and MYC n=76** | >15 | 65.00% (26/40) | 77.80% (28/36) | 0.428 | 0.740 | 0.626-0.853 | <0.001* |
| **hTERC and MYC n=76** | >21 | 55.00% (22/40) | 91.70% (33/36) | 0.467 | 0.752 | 0.641-0.864 | <0.001* |
| **(+++) n=76** | >15 | 62.50% (25/40) | 80.60% (29/36) | 0.431 | 0.751 | 0.641-0.862 | <0.001* |
| **(+++) or [(++)+] n=116** | >13 | 61.70% (29/47) | 87.00% (60/69) | 0.487 | 0.805 | 0.721-0.888 | <0.001* |
| **[(+)(+)(+)] n=116** | >10 | 71.1% (32/47) | 67.6% (48/69) | 0.387 | 0.694 | 0.594-0.793 | <0.001* |
| **(+++) or (++-) n=76** | >21 | 67.50% (27/40) | 77.80% (28/36) | 0.453 | 0.749 | 0.637-0.862 | <0.001* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=116** | >21 | 63.80% (30/47) | 85.50% (59/69) | 0.493 | 0.825 | 0.750-0.899 | <0.001* |
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | >10 | 82.2% (37/47) | 52.1% (37/69) | 0.343 | 0.672 | 0.573-0.771 | 0.002* |

**Table 31: Definition of the cutoffs based on the best Youden Indices using CIN3 as the gold standard, excluding cells with a tetrasomic pattern. The data obtained from this cutoff (sensitivity, specificity, and ROC curve value) were reported ($p < 0.05$). † indicates, that the chosen Youden Index was not the best but the more practical. AUC (IC): AUC 95% confidence interval. (+++): Gain of 3 probes in the same nucleus; (+++) or [(++)+]: Gain of 3 probes, at least 2 in the same nucleus; [(+)(+)(+)]:Gain of 3 probes, not necessarily in the same nucleus; (+++) or (++-): 3 probes applied on the same nuclei, at least 2 with gain; (+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]: 3 probes, at least two of them with gain, at least 2 in the same nucleus; [(+)(+)(+)] or [(+)(+)(-)]: 3 probes, at least two of them with gain, at least 2 in the same nucleus, not necessarily in the same nucleus. Between parentheses number of true positives (Sensitivity) and true negatives (Specificity).**

In the individual gene analyses, EGFR and MYC had a lower sensitivity and a higher specificity with consideration of tetrasomy than without. hTERC however, showed a higher sensitivity and a lower specificity.

## 3.7 FISH: Definition of Cutoffs, using only the histological result of conization specimens as the reference standard

In addition to the previous analyses, we determined the cutoffs using only the results of conization specimens as the reference standard, i.e., without the biopsy results (table 32). Based on the literature data (Boonlikit et al., 2006, Massad et al., 1996) it was presumed, that a conization specimen has higher accuracy and better concordance with cytology in relation to the targeted biopsies. Thus, this better agreement could provide a more reliable result.

It is important to notice that this kind of analysis used a lower number of samples, due to the reduced number of patients who underwent conization or hysterectomy (n=32).

| Probes | Cutoff | Sensitivity | Specificity | Youden Index | AUC | AUC (IC) | AUC (p) |
|---|---|---|---|---|---|---|---|
| **CIN2** | | | | | | | |
| **(+++) or [(++)+] n=53** | >1 | 89.30% | 84.00% | 0.733 | 0.888 | 0.795-981 | <0.001* |
| **[(+)(+)(+)] n=53** | >10 | 78.60% | 84.00% | 0.626 | 0.813 | 0.691-0.935 | <0.001* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=53** | >2 | 100.00% | 84.00% | 0.8400 | 0.942 | 0.880-1.00 | <0.001* |
| **[(+)(+)(+)] or [(+)(+)(-)] n=53** | >10 | 85.70% | 84.00% | 0.697 | 0.849 | 0.736-0.961 | <0.001* |
| **CIN2, excluding cells with a tetrasomic pattern** | | | | | | | |
| **(+++) or [(++)+] n=53** | >1 | 89.30% | 84.00% | 0.733 | 0.896 | 0.806-0.985 | <0.001* |
| **[(+)(+)(+)] n=53** | >10 | 71.40% | 92.00% | 0.634 | 0.817 | 0.697-0.937 | <0.001* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=53** | >2 | 100.00% | 84.00% | 0.8400 | 0.955 | 0.905-1.00 | <0.001* |
| **[(+)(+)(+)] or [(+)(+)(-)] n=53** | >10 | 78.60% | 92.00% | 0.706 | 0.853 | 0.743-0.963 | <0.001* |
| **CIN3** | | | | | | | |
| **(+++) or [(++)+] n=53** | >1 | 88.50% | 77.80% | 0.662 | 0.854 | 0.748-0.960 | <0.001* |
| **[(+)(+)(+)] n=53** | >10 | 76.90% | 77.80% | 0.547 | 0.774 | 0.642-0.905 | 0.001* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=53** | >2 | 100.00% | 77.80% | 0.7780 | 0.906 | 0.824-0.988 | <0.001* |
| **[(+)(+)(+)] or [(+)(+)(-)] n=53** | >10 | 84.60% | 77.80% | 0.624 | 0.812 | 0.689-0.934 | 0.001* |
| **CIN3, excluding cells with a tetrasomic pattern** | | | | | | | |
| **(+++) or [(++)+] n=53** | >1 | 88.50% | 77.80% | 0.662 | 0.873 | 0.775-0.970 | <0.001* |
| **[(+)(+)(+)] n=53** | >10 | 69.20% | 85.20% | 0.544 | 0.772 | 0.640-0.904 | 0.001* |
| **(+++) or (++-) or [(++)+] or [(++)-] or [(+-)+] n=53** | >2 | 100.00% | 77.80% | 0.7780 | 0.926 | 0.859-0.993 | <0.001* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **[(+)(+)(+)] or [(+)(+)(-)] n=53** | >10 | 76.90% | 85.20% | 0.621 | 0.811 | 0.687-0.934 | <0.001* |

**Table 32: Definition of the cutoffs based on the best Youden Indices using CIN2 and CIN3 conizations as the gold standard, with and without cells with the tetrasomic pattern. CIN: Cervical intraepithelial neoplasia.**

### *3.7.1 ≥CIN2 histology as a reference standard*

Many groups presented a clinically applicable aspect, but two of them deserve to be pointed out:

The groups ((+++) or (++-) or [(++)+] or [(++)-] or [(+-)+]), showed a sensitivity of 100.0% and a specificity of 84.0% at a cutoff of >2 cells with gain (table 32).

The groups ([(+)(+)(+)] or [(+)(+)(-)]), showed a sensitivity of 78.60% and specificity of 92.00% at a cutoff of >10 cells with gain. In this case, the cells with the tetrasomic pattern were excluded (table 32).

### *3.7.2 ≥CIN3 as a reference standard*

The groups ((+++) (++-) or [(++)+] or [(++)-] or [(+-)+]), showed a cutoff >2, sensitivity of 100.0% and specificity of 77.80% (table 32).

The exclusion of the cells with tetrasomic pattern did not show a considerable improvement.

The analyses with just the conizations results as the gold standard, in a general way, showed better results (i.e., lower cutoffs with higher sensitivity and specificity) than the analyses considering all histological results. This statement can only be made with limitation because of the very different cutoffs of the diverse analyses. To overcome this problem, further evaluation with a standardized cutoff was done (refer to chapter 3.8).

For clinical application, the FISH analysis should provide high specificity and a reasonable sensitivity. The best results presented by the analyses with just conizations results was related to the evaluation using ≥CIN2 as the reference standard and excluding the tetrasomic cells ([(+)(+)(+)] and [(+)(+)(+)] / [(+)(+)(-)] evaluations, table 32). The specificity of 92% allows this kind of evaluations to be adopted as a confirmation test.

Furthermore, applying the histological reference standard with conization or hysterectomy specimens only puts a bias towards higher grade intraepithelial lesions to the study population. Conization and hysterectomy are not diagnostic but therapeutic procedures. Because of this, non-dysplastic, negative lesions are less often treated in that way and a negative control is missing. Because of this limitation and the low number of samples included in this study, the comparison with all of the histological outcomes including biopsies is preferred.

## 3.8 FISH: Analyses with a defined cutoff

Based on the previous results, on the practical experience of our laboratory and on the necessity of comparing the results from different analyses, we decided to define a cutoff (>10) and analyze its application in different combinations of the FISH probes (tables 33-38).

| Probes | Sensitivity | Specificity | Youden Index | (Excluding Tetrasomy) | | |
|---|---|---|---|---|---|---|
| | | | | Sensitivity | Specificity | Youden Index |
| **(+++) or [(++)+] n=116** | 73.80% | 70.90% | 0.447 | 54.10% | 81.80% | 0.359 |
| **[(+)(+)(+)] n=116** | 78.70% | 56.40% | 0.351 | 62.30% | 69.10% | 0.314 |
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | 85.20% | 49.10% | 0.343 | 75.40% | 56.40% | 0.318 |

**Table 33: Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN2 histological results (biopsy and conization) as the reference standard.**

The analyses were made with either ≥CIN2 or ≥CIN3 histological results as the positive reference standard. Additional analyses were made considering just the conization and hysterectomy results as a reference standard or excluding the tetrasomic cells in the same way as in the last chapter. Furthermore, an analysis was made using all available histological, cytological and clinical information (clinical follow-up) up to 1.5 years after the index cytology as the reference standard.

| Probes | Sensitivity | Specificity | Youden Index | (Excluding Tetrasomy) | | |
|---|---|---|---|---|---|---|
| | | | | Sensitivity | Specificity | Youden Index |
| **(+++) or [(++)+] n=53** | 75.00% | 88.00% | 0.630 | 64.30% | 96.00% | 0.603 |
| **[(+)(+)(+)] n=53** | 78.60% | 84.00% | 0.626 | 71,40% | 92,00% | 0,634 |
| **[(+)(+)(+)] or [(+)(+)(-)] n=53** | 85.70% | 84.00% | 0.697 | 78,60% | 92,00% | 0,706 |

**Table 34: Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN2 histological results (conization only) as the reference standard.**

| Probes | Sensitivity | Specificity | Youden Index | (Excluding Tetrasomy) | | |
|---|---|---|---|---|---|---|
| | | | | Sensitivity | Specificity | Youden Index |
| **(+++) or [(++)+] n=116** | 69.70% | 70.00% | 0.397 | 50.00% | 80.00% | 0.300 |
| **[(+)(+)(+)] n=116** | 80.30% | 62.00% | 0.423 | 62.10% | 72.00% | 0.341 |
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | 89.40% | 58.00% | 0.474 | 80.30% | 66.00% | 0.463 |

**Table 35: Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN2 clinical follow-up with consideration of the available near-term histological results as the reference standard.**

In relation to the specificity, the best result was found in the analysis considering ≥CIN2 corresponding histological conization results as the reference standard and excluding cells with the tetrasomic pattern. The evaluation of ((+++) or [(++)+]) showed a specificity of 96% and a sensitivity of 64.3% (Table 34).

| Probes | Sensitivity | Specificity | Youden Index | (Excluding Tetrasomy) | | |
|---|---|---|---|---|---|---|
| | | | | Sensitivity | Specificity | Youden Index |
| **(+++) or [(++)+], n=116** | 78.70% | 65.20% | 0.439 | 66.00% | 82.60% | 0.486 |
| **[(+)(+)(+)] n=116** | 83.00% | 52.20% | 0.352 | 72.30% | 69.60% | 0.419 |
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | 91.50% | 46.40% | 0.379 | 83.00% | 55.10% | 0.381 |

**Table 36: Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN3 histological results (biopsy and conization) as the reference standard.**

| Probes | Sensitivity | Specificity | Youden Index | (Excluding Tetrasomy) | | |
|---|---|---|---|---|---|---|
| | | | | Sensitivity | Specificity | Youden Index |
| **(+++) or [(++)+], n=53** | 73.10% | 81.15% | 0.546 | 65.40% | 92.60% | 0.580 |
| **[(+)(+)(+)] n=53** | 76.90% | 77.80% | 0.547 | 69,20% | 85,20% | 0.544 |
| **[(+)(+)(+)] or [(+)(+)(-)], n=53** | 84,60% | 77,80% | 0,624 | 76,90% | 85,20% | 0.607 |

**Table 37: Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN3 histological results (conization only) as the reference standard.**

| Probes | Sensitivity | Specificity | Youden Index | (Excluding Tetrasomy) | | |
|---|---|---|---|---|---|---|
| | | | | Sensitivity | Specificity | Youden Index |
| **(+++) or [(++)+] n=116** | 72.50% | 63.10% | 0.356 | 60.80% | 81.50% | 0.423 |
| **[(+)(+)(+)] n=116** | 82.40% | 53.80% | 0.362 | 70.60% | 70.80% | 0.414 |
| **[(+)(+)(+)] or [(+)(+)(-)] n=116** | 92.20% | 49.20% | 0.414 | 84.30% | 58.50% | 0.428 |

**Table 38: Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN3 clinical follow-up with consideration of the available near-term histological results as the reference standard.**

In relation to the sensitivity, the best result was found in the analysis considering ≥CIN3 clinical follow-up with consideration of near-term histological results as the reference standard. The evaluation of ((+++) or [(++)-]) showed a sensitivity of 92.2% (Table 38).

The comparison of the FISH results (tetrasomy excluded) to the histological follow-up (biopsies and conizations) showed an increase in the sensitivity values but a retained specificity if ≥CIN2 or ≥CIN3 diagnoses were considered (Tables 33 and 36). If tetrasomy was not excluded, the specificity dropped. In the same analyses accepting only the conization results as the reference, the sensitivity and specificity were lower when comparing ≥CIN3 diagnoses than ≥CIN2 in all but one (Tables 34 and 37).

Using ≥CIN3 as reference standard led to an increase in the sensitivity in all kinds of evaluations in relation to the analysis taking into consideration ≥CIN2 as the reference standard. The highest sensitivity obtained increased from 89.4% using ≥CIN2 as the reference standard (Table 35) to 92.2% using ≥CIN3 as the reference standard (Table 38). The specificity, however, was lower in all kinds of evaluation.

The analyses with results obtained from available clinical follow-up data in addition to the histological reports did not show any improvement in the sensitivity or specificity that may lead to a possible clinical application. The probable reasons for this are discussed in chapter 4.

| | | | | | (Excluding Tetraploidy) | | |
|---|---|---|---|---|---|---|---|
| | **Probes** | **Sensitivity** | **Specificity** | **Youden Index** | **Sensitivity** | **Specificity** | **Youden Index** |
| **IIp** | (+++) or [(++)+] | 28.60% (2/7) | 70.00% (7/10) | -0.014 | 14.30% (1/7) | 80.00% (8/10) | -0.057 |
| | [(+)(+)(+)] | 28.60% (2/7) | 40.00% (4/10) | -0.214 | 14.30% (1/7) | 60.00% (6/10) | -0.257 |
| | [(+)(+)(+)] or [(+)(+)(-)] | 28.60% (2/7) | 40.00% (4/10) | -0.314 | 14.30% (1/7) | 50.00% (5/10) | -0.357 |
| **IIID1** | (+++) or [(++)+] | 46.20% (6/13) | 63.60% (7/11) | 0.098 | 23.10% (3/13) | 90.90% (10/11) | 0.140 |
| | [(+)(+)(+)] | 69.23% (9/13) | 9.10% (1/11) | -0.021 | 46.20% (6/13) | 72.70% (8/11) | 0.189 |
| | [(+)(+)(+)] or [(+)(+)(-)] | 61.54% (8/13) | 36.36% (4/11) | -0.217 | 61.50% (8/13) | 45.50% (5/11) | 0.070 |
| **IIID2** | (+++) or [(++)+] | 100.00% (10/10) | 16.70% (2/12) | 0.167 | 50.00% (5/10) | 41.70% (5/12) | -0.083 |
| | [(+)(+)(+)] | ---- | ---- | ---- | 60.00% (6/10) | 16.70% (2/12) | -0.233 |
| | [(+)(+)(+)] or [(+)(+)(-)] | 100.00% (10/10) | 8.30% (1/12) | 0.083 | ---- | ---- | -0.200 |
| **IVa** | (+++) or [(++)+] | 82.35% (14/17) | 75.00% (3/4) | 0.574 | 70.60% (12/17) | 75.00% (3/4) | 0.456 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | [(+)(+)(+)] | 94.10% (16/17) | 0.00% (0/4) | -0.176 | 70.60% (12/17) | 50.00% (2/4) | 0.206 |
| | [(+)(+)(+)] or [(+)(+)(-)] | 82.35% (14/17) | 0.00% (0/4) | -0.059 | 94.10% (16/17) | 0.00% (0/4) | -0.059 |

**Table 39: Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN2 histological results (biopsy and conization) as the reference standard. Between parentheses number of true positives (Sensitivity) and true negatives (Specificity).**

The analyses with the cytological groups IIp, IIID1, IIID2, and IVa-p, in general, showed either low sensitivity and or low specificity (tables 39 and 40). The exceptions were the analysis lacking the cells without tetrassomy at IIID1, which showed the sensitivity to be 42.90% and specificity to be 94.10% for CIN3+ diagnosis (table 40).

| | | | | | (Excluding Tetraploidy) | | |
|---|---|---|---|---|---|---|---|
| | **Probes** | **Sensitivity** | **Specificity** | **Youden Index** | **Sensitivity** | **Specificity** | **Youden Index** |
| **IIp** | (+++) or [(++)+] | 50.00% (2/4) | 76.92% (10/13) | 0.269 | 25.00% (1/4) | 84.60% (11/13) | 0.096 |
| | [(+)(+)(+)] | 50.00% (2/4) | 53.80% (7/13) | 0.115 | 25.00% (1/4) | 69.20% (9/13) | -0.058 |
| | [(+)(+)(+)] or [(+)(+)(-)] | 50,00% (2/4) | 61.50% (8/13) | 0,038 | 25,00% (1/4) | 61,50% (8/13) | -0,135 |
| **IIID1** | (+++) or [(++)+] | 42.90% (3/7) | 58.80% (10/17) | 0.017 | 42.90% (4/7) | 94.10% (16/17) | 0.370 |
| | [(+)(+)(+)] | 71.40% (5/7) | 17.56% (3/17) | -0.076 | 57.10% (4/7) | 70.60% (12/17) | 0.277 |
| | [(+)(+)(+)] or [(+)(+)(-)] | 57.14% (4/7) | 35.29% (6/17) | -0.11 | 57.10% (4/7) | 41.20% (7/17) | -0.017 |
| **IIID2** | (+++) or [(++)+] | 100.00% (7/7) | 13.30% (2/15) | 0.133 | 57.10% (4/7) | 46.70% (7/15) | 0.038 |
| | [(+)(+)(+)] | ---- | ---- | ---- | 71.40% (5/7) | 26.70% (4/15) | -0.019 |
| | [(+)(+)(+)] or [(+)(+)(-)] | 100.00% (7/7) | 6.67% (1/15) | 0.067 | 85.70% (6/7) | 6.67% (1/15) | -0.076 |
| **IVa** | (+++) or [(++)+] | 81.30% (13/16) | 60.00% (3/5) | 0.413 | 68.80% (11/16) | 60.00% (3/5) | 0.288 |
| | [(+)(+)(+)] | 93.80% (15/16) | 0.00% (0/5) | -0.187 | 68.80% (11/16) | 40.00% (2/5) | 0.088 |
| | [(+)(+)(+)] or [(+)(+)(-)] | 81.25% (13/16) | 0.00% (0/5) | -0.062 | 93.80% (15/16) | 0.00% (0/5) | -0.062 |

**Table 40: Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN3 histological results (biopsy and conization) as the reference standard. Between parentheses number of true positives (Sensitivity) and true negatives (Specificity).**

## 3.9 FISH: Analysis with high Gain

An additional analysis of high gain of the respective genes was performed with a minimum copy number of four required (Table 41 up to Table 46). This kind of analysis prevents the interference with tetrasomy that is found in euploid polyploidization. In addition, a gain of the hTERC gene is observed in early stages of dysplastic and atypical changes (i.e. CIN1, Figure 6). Thus, the adoption of 5 signals as the minimum necessary to define a cell with gain was expected to enhance the specificity of the analyses, mainly with three probes.

Most of the groups showed a specificity of 100%, the lowest being 96.9% (Table 46). The sensitivity, however, decreased dramatically. In most of the analyses, only the analysis with two of the three genes displaying gain ((+)(+)(+) or (+)(+)(-)) reached a sensitivity higher than 30%. But the maximum was 40.4% only if the results of conizations were used as the reference standard (Table 44). So, although the higher specificity in comparison to other kinds of evaluations, the high gain analyses did not provide a clinically suitable result.

| Proben | Sensitivity | Specificity | Youden Index |
|---|---|---|---|
| **(+++) or [(++)+], n=116** | 11.50% | 100.00% | 0.115 |
| **[(+)(+)(+)] n=116** | 21.70% | 100.00% | 0.217 |
| **[(+)(+)(+)] or [(+)(+)(-)], n=116** | 32.80% | 98.20% | 0.310 |

**Table 41: Analysis of high gain of the respective genes (>4 copies): Results using the predefined cutoff of >10 cells for FISH-positive and ≥CIN2 histological results (biopsy and conization) as the reference standard.**

| Proben | Sensitivity | Specificity | Youden Index |
|---|---|---|---|
| **(+++) or [(++)+], n=53** | 12.90% | 100.00% | 0.129 |
| **[(+)(+)(+)] n=53** | 19.40% | 100.00% | 0.194 |
| **[(+)(+)(+)] or [(+)(+)(-)], n=53** | 25.80% | 100.00% | 0.258 |

**Table 42: Analysis of high gain of the respective genes (>4 copies): Results using the predefined cutoff of >10 cells for FISH-positive and ≥CIN2 histological results (conization only) as the reference standard.**

| Probes | Sensitivity | Specificity | Youden Index |
|---|---|---|---|
| **(+++) or [(++)+], n=116** | 10.60% | 100.00% | 0.106 |
| **[(+)(+)(+)] n=116** | 20.00% | 100.00% | 0.200 |
| **[(+)(+)(+)] or [(+)(+)(-)], n=116** | 30.30% | 98.00% | 0.283 |

**Table 43: Analysis of high gain of the respective genes (>4 copies): Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN2 clinical follow-up with consideration of the available near-term histological results as the reference standard.**

| Probes | Sensitivity | Specificity | Youden Index |
|---|---|---|---|
| **(+++) or [(++)+], n=116** | 14.90% | 100.00% | 0.149 |
| **[(+)(+)(+)] n=116** | 25.50% | 100.00% | 0.241 |
| **[(+)(+)(+)] or [(+)(+)(-)], n=116** | 40.40% | 97.10% | 0.375 |

**Table 44: Analysis of high gain of the respective genes (>4 copies): Results using the predefined cutoff of >10 cells for FISH-positive and ≥CIN3 histological results (biopsy and conization) as the reference standard.**

| Probes | Sensitivity | Specificity | Youden Index |
|---|---|---|---|
| **(+++) or [(++)+], n=53** | 14.30% | 100.00% | 0.143 |
| **[(+)(+)(+)] n=53** | 21.40% | 100.00% | 0.214 |
| **[(+)(+)(+)] or [(+)(+)(-)], n=53** | 28.60% | 100.00% | 0.286 |

**Table 45: Analysis of high gain of the respective genes (>4 copies): Results using the predefined cutoff of >10 cells for FISH-positive and ≥CIN3 histological results (conization only) as the reference standard.**

| Probes | Sensitivity | Specificity | Youden Index |
|---|---|---|---|
| **(+++) or [(++)+], n=116** | 13.70% | 100.00% | 0.137 |
| **[(+)(+)(+)] n=116** | 23.50% | 98.40% | 0.220 |
| **[(+)(+)(+)] or [(+)(+)(-)], n=116** | 37.30% | 96.90% | 0.342 |

**Table 46: Analysis of high gain of the respective genes (>4 copies): Results of the analysis using the predefined cutoff of >10 for FISH-positive and ≥CIN3 clinical follow-up with consideration of the available near-term histological results as the reference standard.**

## 3.10 Results of the hr-HPV test

The hr-HPV tests were performed by the Department of Obstetrics and Gynecology at the University Hospital Dusseldorf (UKD). The HPV status of the patients was obtained from the digital hospital patient management system (KIS, Cerner Medico, Cerner Germany).

At first, a period of six months after the first cytological diagnosis was defined as a limit to consider the result. However, additional analysis with hr-HPV test results obtained up to 18 months after the first cytological diagnosis was done in order to observe if a late HPV diagnostic would be related to the dysplastic alteration of the cervix epithelium.

| HPV Tests | | | |
|---|---|---|---|
| **6 Months Follow-up; ≥CIN2** | | | |
| **Group** | **Sensitivity** | **Specificity** | **Youden Index** |
| **All Cytological Groups, n=77** | 83.33% | 36.59% | 0.199 |
| **II-p, n=18** | 57.14% | 27.27% | 0.156 |
| **IIID1, n=19** | 87.5% | 36.36% | 0.239 |
| **18 Months Follow-up; ≥CIN2** | | | |
| **All Cytological Groups, n=88** | 88.24% | 35.14% | 0.234 |
| **II-p, n=18** | 75% | 40% | 0.150 |
| **IIID1, n=23** | 87.50% | 14.29% | 0.018 |
| **6 Months Follow-up; ≥CIN3** | | | |
| **All Cytological Groups, n=77** | 92% | 36.54% | 0.285 |
| **II-p, n=18** | 66.67% | 33.33% | 0.000 |
| **IIID1, n=19** | 100% | 35.91% | 0.357 |
| **18 Months Follow-up; ≥CIN3** | | | |
| **All Cytological Groups, n=88** | 91.67% | 30.77% | 0.224 |
| **II-p, n=18** | 75% | 35.71% | 0.107 |
| **IIID1, n=23** | 100% | 20% | 0.200 |

**Table 47: HPV tests with Digene Hybrid Capture 2 performed at 6 months and 18 months after the index cytology. The HPV test results were compared to a histological follow-up with ≥CIN2 or ≥CIN3 as the reference standard. CIN: Cervical intraepithelial neoplasia. HPV: Human Papilloma Virus.**

The HPV-Test results were correlated with the histological follow-up at 6 or 18 months, using ≥CIN2 or ≥CIN3 as the reference standard. 11 cases of the hr-HPV test diagnosed after six months and up to eighteen months were added to the analyses and correlated with the 18 months follow up results.

The available hr-HPV test results were reported for all cytological groups or separately for group II-p and group IIID1 (table 47).

The following remarks can be made:

- The validity of this analysis is limited due to the low number of patients in group IIp (n=18, refer to table 8) and group IIID1 (n=19),
- The sensitivity of the hr-HPV test is better in women with underlying ≥CIN3 lesions than ≥CIN2 lesions, with the exception of group IIp (18-month period).
- The longer follow-up did only improve the sensitivity of the hr-HPV test in women with underlying ≥CIN2 lesions and group IIp cytology but not in the other analyses.
- The sensitivity of the hr-HPV test for a high-grade lesion is higher in women with group IIID1 cytology than with group II-p cytology.

# 4 Discussion

## 4.1 Status Quo of gynecologic cytology in the prevention of cervical cancer in Germany

When analyzing the past, the introduction of gynecological smear cytology for cervical cancer screening in Germany in the early 1970s, caused a great impact. Gynecological screening cytology, which is currently participated by more than 16 million women per year, had a decisive role in reducing the incidence of cervical carcinoma, which was > 35 cases per 100,000 women and year in the 1960s (Gustafsson et al., 1997). Today, the incidence is just below 10, i.e. being now just approximately a quarter. However, the declining trend of the incidence ceased since the 2000s in such a way that the numbers remain on a plateau-like level (Kaatsch et al., 2015). This is believed to be the result of constant participation of an estimated 80% women at least once in a three-year period. The enclosement of the remaining 20% is seen skeptically (Schneider, 2012).

This relative success is opposed to the fact that Germany still does not stand particularly well in comparison to central and southern European countries. In the current EUCAN comparison of 20 countries from this region (Ferlay et al., 2013b), we still have the sixth highest incidence despite all diagnostic progress. Similarly, the mortality rates are much lower in some neighboring countries, such as Switzerland, Italy or the Netherlands. In Germany, based on the numbers of the Robert Koch Institute for 2012, about 4,500 women a year are newly diagnosed

with cervical cancer, about 1,600 are dying from it, and the relative age-standardized 5 or 10-year survival rates are 68% and 65%, respectively (Kaatsch et al., 2015).

Thus, although the number of invasive cervical carcinomas in Germany has been significantly reduced, it has to be stated from an epidemiological point of view that the prognosis of those who manifestly suffer from this tumour entity is only on the average: In relation to all malignant tumours, the relative 5- or 10-year survival rates for female cancer patients in general, are 67% and 62%, respectively, and therefore are virtually identical to those of cervical carcinoma (Kaatsch et al., 2015).

The positive effect of cervical cytology in reducing the incidence of cervical cancer is the result of the identification of precursor lesions whose removal is possible before the lesions become an invasive tumour - but with the disadvantage of a surgical procedure with all its, although rare, but quite possible, intraoperative and postoperative risks. Accordingly, the diagnosis of precursor lesions must be as accurate as possible and must have a high degree of sensitivity, specificity, and predictive values.

For cervical cytology, the sensitivity per examination procedure is not particularly high, since sampling errors (crucial cells are not present in the smear), but also screening errors (crucial cells overlooked during microscopy or misinterpreted) have to be considered. The sensitivity of the method, in fact, is determined cumulatively by the repeated participation of the patients. The specificity, on the other hand, is accepted to be high with only a low rate of false positives. It should be considered that in the case of discrepant findings between positive cytology and negative histology, a false negative histological result may be responsible. Therefore, histology cannot be regarded unasked as the gold standard, but must instead remain as a complementary method beside cytology.

The most important data from the first Germany-wide annual statistics from 2012, that is still using the Munich nomenclature II, show that there is a good correlation between cytology and histology (Ziemke et al., 2014): From the histologically followed cases of Group V, 83.4% were invasive carcinomas (and 12.8% CIN3 as the immediately lower group). In group IVa, 86.3% corresponded to a CIN3 or higher (and 8.2% to CIN2). And even for Group IIID, where the correlation with histology is known to be lower, 69.1% of the histologically followed cases were CIN2 and higher (and 25.1% CIN1). However, due to the fact that only 9.4% of the cases of group IIID were clarified, the numbers are less significant than those of groups IVa and V with clarification rates of 81.9% and 78.8%.

The main problem of cytology-based cervical cancer screening remains the handling of cases of group IIID (MN II) or groups IIID1 and IIID2 (MN III). These diagnoses do not represent a primary indication for more invasive diagnostic procedures or therapeutic actions (biopsy, conization). This is the case because regression or persistence of the lesion may be expected (much) more frequently than progression to a higher-grade lesion. Accordingly, overtreatment should be avoided. Numbers originating from the 90s are showing about 10% risk of progression for a CIN1 lesion and about 20% for a CIN2 lesion over a period of several years (Ostor, 1993). So, physicians often just wait and observe, whether there is an increase in the morphological changes through cytological controls.

However, a recent study from Mecklenburg-Vorpommern, that reports the follow-up of more than 3300 patients with conspicuous cytology, shows that the cumulative risk of developing a CIN2+ or a CIN3+ lesion within three years is significantly different between the groups IIID1 and IIID2 (Marquardt and Ziemke, 2018). The risk of developing CIN3+ in this period increases from 2.1% to 17.1% with a IIID1 diagnosis, and from 25.0% to 62.4% with a IIID2 diagnosis at baseline. Obviously, at first line, women are affected, that had repeatedly conspicuous cervical smears. Regarding this, convincing data are presented in the study, which will not be discussed here.

The persistence or progression of conspicuous cytological or histological changes of the cervix is depending on the specific course of the HPV infection, which is etiologically underlying. Although HPV infection cannot be demonstrated in 100% of cervical carcinoma or CIN3 lesions, it is believed that almost all cases of these lesions are linked to persisting and transforming HPV infections. Accordingly, it often makes sense to determine the HPV status of the respective lesions in cytological or histological specimens. There are several methods available for this. However, a positive HPV status solely does not provide sufficient diagnostic impact, as in approximately 80% of cases a HPV infection disappears without symptoms within a period of three years (Schiffman et al., 2007, Grainge et al., 2005). As a complementary diagnostic procedure that is an indispensable part of cervical cancer prevention, the investigation of the HPV status is usually performed in relation to the high-risk HPV subtypes. The currently valid "*Einheitliche Bewertungsmaßstab*" (EBM), in which those health services are documented, that are accepted by the public health insurance, considers any cytological finding of Group III (IIID1, IIID2, III-p, III-g) according to MN III to be a prerequisite. The consequence of a positive high-risk HPV test results in the triage of abnormal cytology is the clarification of changes by colposcopy and if required biopsy.

## 4.2 Reorganization of gynecological cancer screening in Germany

There has been an ongoing discussion for many years about a reorganization of cervical gynecological cancer screening in Germany. The main purpose was to decide which diagnostic actions should be included in the screening procedure in the future and how they should be implemented. Therefore, models from other countries were related to the German system. In addition, the WHO recommendation of a preferably HPV-based and not cytology-based screening system has to be integrated into an acceptable German solution.

It was considered, among other things:

- whether cervical cancer screening should be switched to an invitation-based system ("organized screening") or not ("opportunistic screening"),
- for which patients cytology should be retained as the primary screening method, which kind of cytological preparation should be used for cytology: conventional smear or liquid-based cytology,
- for which patients cytology should be replaced by a high-risk HPV test as the primary screening method,
- which high-risk HPV test should be used and
- how a system, that combines cytology and HPV testing in a meaningful way, may look like.

The course of the discussion of these questions cannot and should not be traced in the context of this work. This particularly makes no sense, because, in addition to scientifically substantiated aspects, economic interests of the moderators and industry, as well as interests of the society as a whole, were quoted. This is evidenced by many passionate discussions at specialists' conferences and certainly also non-public in the decision-making bodies. At the end of these multi-year discussions emerged the approach outlined below, which is expected to be introduced in 2019 and is already described in the new S3 guideline "Prävention des Zervixkarzinoms" (Leitlinienprogramm Onkologie (Deutsche Krebsgesellschaft, 2017):

- For a transitional period of at least 6 years, all women from 20 years to 35 years of age retain the right of an annual cytological examination.

- For women older than 35 years, a combination of an HPV test and a cytological examination in a three-year period is provided instead.
- According to Table 7.1 of the above-mentioned S3 guideline, there are currently five HPV tests available that would meet the quality requirements for an appropriate assessment, namely the Hybrid Capture 2 test, the Cobas test, the Cervista / Genfind test, the Aptima test, and the Abbott RT test.

After the end of the transitional period, the aim is to further approximate the German approach to international recommendations in a kind of congruence model. This concerns both, the interval and the method of screening. This could mean, for example, to increase the interval for cytological screening from one to two years, to introduce a maximum age for participation in cervical cancer screening or to raise the minimum age for participation from 20 years to 25 years.

## 4.3 Biomarkers in the evaluation abnormal findings of the cervix uteri

So-called biomarkers, which also include DNA probes or DNA probe combinations examined by FISH, have been scientifically studied on various occasions, but have so far only a minor impact on collecting or triaging conspicuous findings of the cervix uteri. A review of the literature was conducted by the S3 guideline commission on the topic of biomarkers to determine whether these could be included in the screening process or for triaging.

There were only 10 studies, which met the PICO quality criteria. In six of the studies reported in detail in the S3 guidelines, studies on the E6 / E7 mRNA of various HPV types were available, two studies on p16 and combination of p16/Ki-67, one study on the immunocytochemical markers TOP2A and MCM2 and one immunochromatographic study on the E6 protein of HPV types 16, 18 and 45. Summarizing the statements 9.1 to 9.4 of the S3 guidelines, no benefits compared to the high-risk HPV DNA assays were reported in cross-sectional studies, but conventional cytology was exceeded regarding the sensitivity. Regarding specificity, the superiority of p16/Ki-67 double staining over cytology was observed.

Accordingly, it can be concluded that biomarkers currently have no significance in the primary diagnosis of cervical lesions or in the triage of conspicuous findings. This is also stated in the new S3 guideline (Leitlinienprogramm Onkologie (Deutsche Krebsgesellschaft, 2017), that there is currently not sufficient evidence for the use of biomarkers in cervical cancer

screening. The word "currently" [in German: "derzeit"], however, implicates, that in the case of new data, the application of biomarkers in the future would be quite possible. This in principle would also apply to FISH-based tests, for which there are seemingly no studies available that provide an epidemiologically acceptable conception.

## 4.4 Principles of FISH applications in cervical lesions

In principle, two different approaches should be considered for a FISH application in cervical cancer prevention. The first approach is a screening application with the idea to potentially replace cytology (or high-risk HPV-test). Up to date, no studies are available to answer this question. Some studies have used a "screening-like" approach. But nevertheless, despite a prospective design to investigate the natural course, only suspicious cytological diagnoses or CIN-lesions and not all of the normal cervical samples were further investigated with FISH. This might be caused by the high costs of the FISH probes and personal expenditure. In a study of 2499 cytologically screened women, the natural course of 74 histologically confirmed CIN1 lesions was followed for two years after determining hTERC gain and HPV-status at baseline. hTERC gain indicated a 3.24 fold higher risk of progression to CIN2/3, independent form baseline-HPV-status (Li et al., 2014a).

The utmost number of cervical FISH-studies are triaging suspicious cervical findings detected by other methods, in most instances by cytology. Most studies -some of them are mentioned later- compare the results of FISH with a histological reference to determine an underlying CIN2+/3+ lesion. But some studies, like the abovementioned one of Li and coworkers, conduct a longitudinal approach.

Nowadays, FISH is still an experimental method regarding triaging cervical cytology. For that application, several FISH probe kits are commercially available (summarized in Houldsworth (2014): FHAT, Cancer genetics Inc.; Cervical Cancer TERC, Quest Diagnostics; DTex, Neodiagnostix; oncoFISH, Ikonisys). Alike other new diagnostic methods as dual-stain with p16-Ki67, there is no consensus about how to implement these methods in current guidelines for cervical cancer screening or triaging.

### *4.4.1 Identifying interesting genomic regions for FISH*

If developing a new FISH assay, there is always the question of the genomic sites for hybridization. This could be aided by results of other methods, as for instance comparative genomic hybridization (CGH).

Since chromosomal segments with gains or losses, that are often associated with lesions on the cervix, were primarily detected using CGH, targets for FISH probes are usually defined based on these studies. A resume of the most commonly detected genomic aberrations by CGH of cervical cancer and separately for precursors is given on table 48 (Kirchhoff et al., 1999, Harris et al., 2003, Wilting et al., 2009, Sopov et al., 2004, Martin et al., 2006, Fitzpatrick et al., 2006, Zhai et al., 2007).

Genomic gains are more frequent than losses in cervical cancer (Huang et al., 2005). Gain of chromosome 3, where the human telomerase RNA gene (hTERC - 3q26) is located, is the most frequent genomic aberration found on this kind of tumour (Heselmeyer-Haddad et al., 2003, Kirchhoff et al., 1999). In addition, gain of 3q26 has been frequently found in low- and high-grade intraepithelial lesions and is therefore linked to neoplastic progression (Hopman et al., 2006b, Heselmeyer-Haddad et al., 2005, Heselmeyer-Haddad et al., 2003). Thus, most studies in the literature that tried to develop new diagnostic protocols based on FISH, adopted probes hybridizing at the hTERC gene locus (3q26) (Wang et al., 2014, Li et al., 2014c, Yin et al., 2012, Liu et al., 2012, Guo et al., 2012). The hTERC FISH was most often tested in cervical preneoplastic lesions and cancers on residual cytological specimens.

| Cytogenetic band | |
|---|---|
| **CIN2/CIN3** | |
| **Gain** | 1q22-q43; 3q13.33-q29; 7q31.1-q31.2; 20q12 |
| **Loss** | 6p25.2-p12.3; 12p13.31-p12.2; 17q25.3; 19p13.11-q12 |
| **SCC** | |
| **Gain** | 1p36.33–1p36.32; 3q13.11-q27.3; 7p11; 8q23 - 24.3; 9p21.3; 10q21; 11q13; 12q13.13; 16p13.3; 20q11.2; 20q13 |
| **Loss** | 2q12.1; 3p14.2; 4q21.1-q34.2; 18q |

**Table 48: Resume of the most commonly detected genomic aberrations in premalignant and malignant cervical lesions. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma.**

Policht and co-workers (Policht et al., 2010) used a literature-based approach to select FISH probes for a possible diagnostic application in cervical cytology. They tested 35 different

probes on formalin-fixed paraffin-embedded histological specimens of cervical cancer, CIN1-3 lesions, and normal cervix. They observed that only one probe, 3p14, exhibited significant losses, which were mainly detected in SCC. The others, especially probes for 3q26, 8q24, 5p13.2 and 5p15 genes, showed a higher rate of gains in CIN2+ lesions, and only low rates of genomic losses in all lesions. 3q26 and 8q24 probes were subsequently tested on residual cervical cytological specimens. Gain of one or the combination of the chromosomal regions correlated with HSIL or cancer with 92.3% sensitivity and 81% specificity.

### *4.4.2 Different ways of evaluating a FISH assay*

There are two important types of FISH probes: chromosome enumeration probes (CEPs) which are used to detect aneusomy of a given chromosome, and locus-specific indicator (LSI) probes that are generally used to detect deletion, duplication, or amplification of specific genes (Halling and Kipp, 2007).

Genomic or genetic amplification or deletion is detectable with the combination of a CEP probe and a LSI probe of the specific site, like HER2/neu gene for example, and is usually reported as a ratio of the number of the LSI signals divided by the number of the CEP signals. If only a CEP probe or a LSI probe are utilized in a FISH assay, the right term is gain for more than two signals or loss for less than two signals of the probe. To investigate the amplification of a gene is reasonable for oncogenes like MYC or EGFR. A deletion might be detectable for tumour suppressor genes.

Conesa-Zamora and co-workers investigated the amplification of HER2/neu (17q12) and EGFR (7p12) genes on 144 formalin-fixed and paraffin-embedded cervical specimens with normal findings, low-grade intraepithelial neoplasia, high-grade intraepithelial neoplasia, and squamous cell carcinoma. They observed no amplification of the two genes but polyploidy of chromosomes 7 and 17, that increases with the grade of the lesions (Conesa-Zamora et al., 2013). Li and co-workers reported increasing rates of EGFR gene amplification with a grade of a cervical lesion in cytological liquid-based cervical specimens and different amplification rates in LSIL cytology with positive (any lesion present) or negative (normal cytology) follow-up (Li et al., 2014b).

For the most often investigated FISH probe, hTERC at chromosome 3q26, most studies report gains (Heselmeyer-Haddad et al., 2005, Scotto et al., 2008, Sui et al., 2009). Even if

centromeric probes were adopted, some studies define the cutoffs for dichotomization of results based on the rate of cells with an abnormal number of the locus-specific probe (3q26), irrespective of the copy number of the centromeric probe. The results are usually displayed as a percentage of cases with positive cells above the cutoff value and were correlated to the grade of a cervical lesion. Some studies use different definitions for amplification from the abovementioned. Fan and co-workers showed the association of hTERC amplification and cytological or histological grade of cervical lesions (Fan et al., 2010). Using a probe for the hTERC gene and a centromeric probe for chromosome three (CEP3) it was defined that amplification of hTERC occurred if more than two hTERC signals and not less than two CEP3 signals were detected. Chen and co-workers suggest a hTERC-CEP3 ratio of >1 as isochromosome formation in a cell, whilst a ratio =1 represents a diploid cell (Chen et al., 2012).

The investigation of genetic losses in cervical specimens with FISH is rarely reported in the literature. Wang and co-workers investigated the 3p14.2 HPV integration locus including the FHIT gene with a corresponding FISH probe and reported decreasing 3p14.2 copy numbers when CIN transitioned to cervical cancer (Wang et al., 2017).

It is obvious that many of the studies adopt different kinds of cutoffs for dichotomization of positive or negative results, indicating an underlying high-grade CIN or progression of a given cytological lesion. Some of them report a certain percentage of the total cell count, others a minimum number of abnormal cells as a threshold.

The cutoffs and study designs for cervical FISH assays reported in the literature are very different. The great diversity of protocols and procedures established for acquisition and evaluation of the data makes the comparison and metaanalysis difficult. In a systematic review including studies regarding TERC, MYC and HPV in situ hybridization, Earley and colleagues consistently stated that the evidence of cervical FISH-testing is limited (Earley et al., 2014). Thus, up to now, there is not enough confidence provided for applying this technique in clinical routine.

From a scientific point of view, defining cutoffs with a percentage of the total cell counts can be considered a more precise cutoff alternative, since it could be reproduced in any situation independently of the number of evaluated cells. In most instances, the minimum number for that approach is approximately 100 cells. However, the clinical routine demands more simple and quick methods. It is not expected that the diagnostic centers adopt a method which needs a calculation of a minimum number of cells for every sample. It would be tedious, time-consuming and more expensive.

### *4.4.3 FISH assays with multiple probes*

Many FISH studies on cervical cancer and precursors utilize probes for different chromosomes or genetic loci in a mix, especially in order to improve the diagnostic accuracy of a FISH protocol implicating a probe for the hTERC gene solely. This allows even more different ways of defining a positive test result. The hybridization results (most often gains) can be evaluated individually for each probe and gain of only one of the utilized probes or different combinations could render a specimen as positive. But the combined evaluation of the probes, for example in a FISH protocol with four different probes, enables the detection of chromosomal aneuploidy to define a malignant transformation of the investigated cell population. This latter aspect is discussed in the next chapter. In the following, some FISH studies with several probes are scarcely reported:

Marzano and colleagues and Mian and colleagues, for example, tested centromeric probes for chromosomes 3, 7, and the X chromosome (Mian et al., 1999, Marzano et al., 2004). The chromosomes were analyzed individually in both studies. Mian et al found that just trisomy of X chromosome was significantly correlated with CIN2+ lesions ($P < 0.01767$). Statistical analysis gave highly significant correlations between cytology (HSIL), trisomy of chromosome 7 ($P < 0.00001$) and trisomy of X chromosome ($P < 0.00062$).

Marzano et al. found that polysomy of chromosomes 3 and X defined the transition from high-grade squamous intraepithelial lesions (HSIL) to cervical carcinoma ($p < 0.0001$). In addition, they examined the amplification of the gene located at 7p12 (EGFR). The CEP 7 probe was used to determine the amplification of EGFR. However, no samples showed gene amplification.

Kudela and co-workers focused on the gains of regions encoding the components of telomerase enzymes (3q26, 5p15) in cytological specimens (Kudela et al., 2018). A cutoff value of >11 cells with gain of >2 signals was defined as positive for the hTERC probe and >3 cells with gain of >2 signals for 5p15. Sensitivity and specificity were calculated for both probes, individually or combined. For hTERC, the sensitivity was 75.36% and the specificity 90.32%. MYC showed a sensitivity and specificity of 63.77% and 91.94%, respectively. For the combination of both probes, these values were 69.57% and 90.32%.

Some years ago, the same group worked with probes for hTERC, TERT and the centromere of chromosome 7 (Kudela et al., 2014). The chromosome 7 probe was used just for the detection of tetrasomic cells. A cell with a normal copy number of 2:2:2 (TERC-TERT-

Cen7) was determined as a healthy diploid cell, while tetraploid cells with four copies of each investigated region (4:4:4) were excluded from the evaluation process. In two different evaluation procedures, a cell was considered to be chromosomally abnormal if either the TERC or the TERT probe showed >2 or >3 signals per cell. The absolute count of cells with gain was determined for each specimen by the number of abnormal cells (either alone or in combination). It will be discussed later in this chapter why it makes sense to keep special attention on tetrasomy.

The combination of four probes was also tested by Luhn and colleagues, who aimed to examine the feasibility of an automated scanning method to simultaneously detect gains at 3q26, 5p15, 20q13 and centromere 7 (Luhn et al., 2013). Counting the huge number of 2000 cells per specimen and using a different cutoff for each probe (gain ≥3 signals: 3q26- ≥39 cells; 5p15- ≥24 cells; 20q13- ≥15 cells; and cen7- ≥23 cells), the accuracy of the individual probes and varying combinations of two, three or four probes for the detection of CIN2+ and/or HSIL lesions was tested. Evaluating gains for 3q26 or 20q13 or cen7 as positive, the rates for sensitivity and specificity were 47.1% and 86.7%, respectively. If all four probes were required as positive, sensitivity decreased to 29.4%, and specificity increased to 90.0%. This study has a similar approach to the current study regarding the discussion of different evaluation methods.

Obermann and colleagues tested probes for HPV (HPV types 16, 18, 26, 31, 33, 35, 39, 45, 52, 53, 56, 58, 59, 66, and 82), hTERC and the common HPV-integration-site MYC as markers to predict progression for LSIL cases (Obermann et al., 2013). Regression of LSIL was stated after two negative Papanicolaou smears and/or negative histology over a period of at least 6 months. Persistence was assumed, if there was the repeated detection of LSIL over a period of at least 6 months on cytology and/or histology and progression, if the initial LSIL cytology was followed by two Pap smears with a diagnosis of high-grade squamous intraepithelial lesions (HSIL) or one histology with a diagnosis of CIN2+. Only the gain of hTERC and the combined evaluation of hTERC and MYC was statistically significant to predict regression/persistence and progression with 70% sensitivity and 76% specificity. In a subset of the patients, an additional high-risk HPV-test was available. Evaluation of the HPV-tested LSIL cases only (different evaluations for either HPV-PCR or HPV-FISH) interestingly showed no significant difference between regression/persistence and progression. This result raises the question if a gain of at least hTERC may more probably be an effect of the HPV-infection than a hint for a transforming process with the increased risk of a malignant transformation.

Table 49 gives an overview of selected studies that use three or more FISH probes in a batch. Some studies, despite of the adoption of more than two probes, evaluated only just on two of them (Kudela et al., 2018) or performed only individual analyses for each probe without a combined evaluation (Visnovsky et al., 2014).

| Study | Specimen | Probes | Cutoff | Main results | Study Endpoint |
|---|---|---|---|---|---|
| **Zhang et al. 2002** | Formalin-fixed paraffin-embedded (FFPE) | 3q26.3, 5p15.33, 8q24, 11q13.3, 17q21.2 and 20q13.2. | Samples with 3 or more copies per nucleus of the given genes in more than 50% of counted cells were considered to contain gene amplification. | Gain of 3q26.3, more often in G1 than G2 tumors, 64% and 33%, respectively ($p < 0.05$). No significant correlation with G2 tumours and gain of the other 5 individual genes. | Frequency of amplification |
| **Marzano et al. 2004** | Touch preparations (imprints) | Cen3,<br>cen 7<br>X<br>7p12 | Polysomy of at least 20% of the cells for each centromere probe.<br>7p12 amplification:<br>7p12/CEP7 ratio >2. | Cen3 and X showed significant differences between HSIL and cervical carcinoma ($P < 0:0001$). | Frequency of gain |
| **Sokolova et al. 2007** | ThinPrep slides | 3q26<br>8q24<br>HPV (16, 18, 30, 45, 51, 58) | >4 HPV-postive cells with Gain of 3q26 or 8q24 (> 2 signals).<br>HPV - punctate and/or diffuse staining was accepted as positive. | the average number of double-positive (HPV + gene) cells increased from 2 in ASCUS to 22 in LSIL and 99 in HSIL samples | Correlation of FISH and cytological/ histological diagnoses |
| **Obermann et al. 2013** | Liquid-based cytology | 3q26<br>8q24.2<br>HPV (16, 18, 26, 31, 33, 35, 39, 45, 52, 53, 56, 58, 59, 66, 82) | Gain of MYC or TERC: >2 locus-specific signals in at least 10% of cells.HPV - punctate and/or diffuse staining were accepted as positive. | Increased 8q24 and/or 3q26 gene copy number was more frequent in HSIL (85%) than in LSIL (33%) (HSIL vs LSIL: P<.0001). 3q26 gene copy number was significantly correlated with progression of LSIL (P<.01; odds ratio, 7.44) | Prediction of the clinical course of LSIL (regression/persistence, and progression) |
| **Luhn et al. 2013** | ThinPrep liquid-based | 3q26<br>5p15<br>20q13<br>cen7 | Gain: >2 signals per cell<br>Tetraploidy: patterns of 4-4-4-4, 4-4-4-3 4-4-4-5 or 4-4-3-3.<br>Cutoffs (automated screening of 2000 cells per specimen): ≥39 cells for 3q26, ≥24 cells for 5p15, ≥15 cells for 20q13 ≥23 cells for cen7 | 3q26 or 20q13 or cen7 with gain: 47.1% sensitivity and 86.7% specificity<br><br>Positive for all four probes - 29.4% sensitivity and 90.0% specificity | Underlying CIN2+ and/or HSIL |
| **Visnovsky et al. 2014** | LiquiPrep™ | 3q26,<br>5p15<br>cen7 | gain of 3q26 in ≥ 2.03 cells<br>gain of 5p15 in ≥ 2.05 cells | The specificity and sensitivity of 3q26 for CIN2+ lesions was 88.2% and 95.5% respectively. 5p15 showed 94.1% and 77.3% respectively. | Underlying CIN2+ |

| | | | | | |
|---|---|---|---|---|---|
| **Kuglik et al. 2015** | Conventiona l smears | 3q26<br>8q24.2<br>HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68) | >4 HPV positive and chromosomally abnormal cells (> 2 signals of hTERC and/or MYC). HPV - punctate and/or diffuse staining was accepted as positive | 3q26 and 8q24 genes<br>Gain was more frequent in cervical carcinomas than in premalignant<br>lesions (62.5% vs. 33.3%; p=0.008). Solely 3q26 gene gain was significant | Progression to Ca in situ |
| **Hopley et al. 2016** | ThinPrep liquid-based | 3q26<br>5p15<br>20q<br>Cep7 | No information | Case 1: 6.6% of cells showed gain of the 3q26 region<br>Case 2: 14.6% cells with a gain of 3q26 and 5p15 regions | Case report: 1. Detection of underlying ≥ CIN2 lesion in ASC-US; 2. Detection of underlying adenocarcinoma in situ (AIS) in cytologically detected atypical glandular cells of undetermined significance (AG-US) |
| **Upendram et al. 2017** | Convetional smears | 3q26<br>5p15<br>20q13<br>cen7 | Percentage of cells with gain (> 2 signals, automated screening of 2000 cells per specimen): 3q26 >0.62%, 5p15 > 0.32%, 20q13 >0.22%, cen7 >0.08% | Combined hr-HPV virus and fluorescent in situ hybridization had 94% sensitivity, specificity, and negative predictive value | Progression to CIN2+ |
| **Kudela et al. 2018** | LiquiPrep™ | 3q26<br>5p15 | > 11 cells with gain (>2 signals) for 3q26<br>> 3 cells with gain (>2 signals) for 5p15 | 3q26 gain related to CIN2+ lesions ($p < 0.01$), 5p15 gain was shifted up towards CIN 3/CIS ($p < 0.001$) and cervical cancer. | Underlying CIN2+ |

**Table 49: Resume of studies with analysis of more than two probes. CIN: Cervical intraepithelial neoplasia. SCC: squamous cell carcinoma. ASC-US: Atypical squamous cells of undetermined significance. LSIL: Low-grade squamous intraepithelial lesion. HSIL: High-grade squamous intraepithelial lesion. CIS: Carcinoma in situ. AG-US: Atypical glandular cells of undetermined significance.**

### *4.4.4 Detection of chromosomal aneuploidy*

The combined evaluation of several FISH probes in a batch opens the alternative to examine chromosomal aneuploidy. This is based on the observation that nearly every solid tumor and half of all hematologic malignancies are aneuploid (Santaguida and Amon, 2015). There is some debate whether aneuploidy is the origin of malignant transformation or just a bystander effect. This question may be essential for the investigation of tumorigenesis. For a diagnostic application, however, this question is subordinate. Aneuploidy in this context is defined as a chromosomal set $\neq 2^n$, which is because integrated-value multiples of single chromosomal sets, apart from $2^n$, do not occur in non-neoplastic tissues (Schramm et al., 2011).

The investigation of DNA aneuploidy with DNA image cytometry has been worldwide used in the past in diagnostic cytology, including cervical cytology (Bocking and Nguyen, 2004), mainly as a triage of atypical or suspicious cytology to confirm a malignant transformation or for grading of malignant tumors (Demirel et al., 2013, Lorenzato et al., 2008). Grote and co-workers have prospectively investigated DNA-aneuploidy in cytological specimens from the uterine cervix either with LSIL or atypical squamous or glandular cells. The reference standard was a histologically confirmed CIN 2+ or 3+ or "cytologic follow-up of at least 6 months if at least 2 consecutive Pap smears agreed with respect to the presence or absence of progressive disease" (Grote et al., 2004). They found a PPV of 65.9% and a NPV of 85% for prediction of CIN2+. If only so-called stemline aneuploidy was considered, the PPV to predict CIN2+ was 92.3%.

Although the complete DNA contents of whole chromosomal sets cannot be simultaneously investigated easily, the paralleled evaluation of four different FISH probes, either CEP- or LSI-probes to determine chromosomal aneuploidy, is widespread in diagnostic cytology of solid organs, especially lung, pancreas, bile ducts, bladder, and urinary tract:

UroVysion is a 4-target, multicolour FISH probe set that contains differently fluorescent-labeled CEP probes for chromosomes 3, 7, and 17, and an LSI probe for the 9p21 band. The assay was designed to detect common chromosome abnormalities observed in urothelial cell carcinomas, i.e. polysomy for chromosomes 3, 7, and/or 17 and homozygous loss 9p21in voided urine samples (Sokolova et al., 2000). At least 25 morphologically abnormal cells have to be scored. A positive result is defined when five or more cells display gains for two or more chromosomes (3, 7 or 17) or > 12 cells with a homozygous deletion of 9p21 are detected (Laudadio et al., 2005).

The UroVysion probe set received FDA approval in 2001 for monitoring recurrence in patients with bladder cancer and for assessing patients with hematuria (gross and microscopic) for bladder cancer in 2005 (Sarosdy et al., 2006). In a meta-analysis of 14 studies that together comprised 878 patients, Hajdinjak and co-workers found a pooled sensitivity and specificity of 72% (range 69%–75%) and 83% (range 82%–85%), respectively, for detecting urothelial cancers in urine (Hajdinjak, 2008).

There are some studies that investigate the application of UroVysion as a diagnostic tool for detecting pancreatic adenocarcinoma and malignant bile tract strictures, mainly caused by pancreatic and cholangiocellular carcinomas. Barr Fritcher and colleagues found 44% sensitivity and 98% specificity for the detection of malignant pancreatobiliary strictures with

UroVysion in a study of 284 brushings (Barr Fritcher et al., 2007). Gain >2 of at least two of the four probes, for example, 2-2-3-4, defined a cell as “polyploidy”. In another study which evaluated the performance of UroVysion for the detection of malignant bile duct brushing specimens, the sensitivity of routine cytology and FISH was 14% (3/22) and 55% (12/22), the specificity was 100% (13/13) and 62% (8/13), respectively (Zhai, 2018).

Ribeiro and colleagues investigated the application of UroVysion in 104 fine needle aspiration biopsies of pancreatic masses in combination with the cytological result (Ribeiro et al., 2014). A positive FISH result was defined by several conditions: “polysomy” (gain >2 of at least two of the four probes) in 5 or more cells, homozygous or heterozygous deletion of 9p21 in 5 or more cells or tetrasomy of chromosome 7 in 10 or more cells. The combined evaluation of cytology and FISH detected pancreatic cancer with 89% sensitivity and 100% specificity.

Several studies have assessed the sensitivity and specificity of a multicolour probe set (LAVysion, Vysis, Downers Grove, IL), that contains locus-specific probes of 5p15, 7p12 (EGFR), 8q24 (C-MYC) and a centromeric probe of chromosome 6. They tested the application on bronchial brushings, washings and sputum for the detection of lung cancer and have found that the test increases the sensitivity of cytology for the detection of peripheral NSCLC from 30 to 70% (for bronchial brushings), albeit at the cost of decreased specificity with a 6–13% false-positive rate (Varella-Garcia et al., 2004, Savic and Bubendorf, 2012). Sokolova and colleagues, comparing FISH and cytology to detect genetic changes in bronchial washing specimens of lung carcinoma patients, found a sensitivity of 82% for FISH and 54% for cytology. The specificity of FISH and cytology were 82% and 100%, respectively, and were not significantly different ($P<0.993$) (Sokolova et al., 2002). All of the studies utilized a combined evaluation of the four probes in the batch. A cell was positive if 2 of the four probes showed more than two signals. This could be either cells in the S-phase of the cell cycle or cells, displaying chromosomal aneuploidy. To prevent false positive results due to counting cells in the S-phase, a cutoff of several cells is usually introduced to render the specimen “positive” as a whole.

Schramm and colleagues from our own lab, working with bronchoscopically obtained diagnostic material (including transbronchial fine needle aspiration biopsies from mediastinal and pulmonary lymph nodes) of patients with suspected lung cancer from the Florence Nightingale Hospital in Düsseldorf, showed that the application of LAVysion multicolor FISH probe improved the diagnostic accuracy to a sensitivity of 78% and a specificity of 98.2%

(Schramm et al., 2011) over cytology, that had 69,8% specificity. This low specificity of cytology was related to the fact that equivocal cytological diagnoses were interpreted as positive for the purpose of the study to prevent diagnostic losses. An additional application of FISH, especially in these cases, clarified 46 out of 66 equivocal cytological diagnoses as truly positive or negative for malignancy. In this study, again a cell was defined as chromosomally aneuploid with a gain of 2 or more of the 4 probes. A specimen was considered positive for malignancy when 6 or more cells on a slide exhibited chromosomal aneuploidy. Moreover, tetrasomy or even octasomy, defined as the presence of 4 or 8 signals of 3 or more probes, was not considered abnormal, because this phenomenon is often caused by benign euploid polyploidization in bronchial epithelia.

### *4.4.5 Euploid polyploidization*

It is important to distinct aneuploidy from euploid polyploidization, in which cells contain more than two complete sets of chromosomes, but always an exact multiple of the diploid number ($2^n$ sets of chromosomes) so that the chromosomes remain balanced. Tetraploidy is a special form of euploid polyploidization, which, for example, occurs in normal, nonneoplastic tissues (for example thyroid, liver, lung….) or regeneration (Biesterfeld et al., 1994). There are several mechanisms that promote the genesis of tetraploid cells in otherwise euploid tissues. For instance, endoreplication, which is the process by which DNA replication occurs without cell division. This is a normal, programmed cellular process that leads cytokinesis failure and to the creation of terminally differentiated non-dividing polyploid cells (Zimmet and Ravid, 2000, Ganem et al., 2007, Eggert et al., 2006). Thus, an additional algorithm for cervical FISH with special attention of tetraploid cells is important to eliminate the influence of microscopically abnormal but non-neoplastic cells on the results. In the present study, this was achieved by the exclusion of tetraploid cells during the FISH procedure. Similarly, to the work of Luhn et al., for the detection of tetrasomy we considered also the possibility of a false interpretation of some fluorescent signals (Luhn et al., 2013). Not only a pattern of four signals each was considered as tetrasomic (4-4-4), but a deviation in one of the probes was accepted as a tetrasomic patter, too (for example 4-4-3 or 4-4-5).

The possible disadvantage of this approach is the missing of (pre)neoplastic cells that may show a 4-4-4, 4-4-3 or 4-4-5 pattern.

There is an additional argument to take special attention to euploid polyploidization. HPV infections of the uterine cervix are often inducing polyploidization of whole chromosomal sets. The equivalent of the resulting euploid polyploid DNA pattern was observed with DNA image cytometry. In cases with cervical HPV-infections, the obtained smears showed DNA stemlines in the 2c, 4c and even 8c region (the DNA equivalent of diploid, tetraploid or octaploid cells) (Bibbo et al., 1989, Fujii et al., 1984). This phenomenon may be attributed as an effect of HPV-infection and has to be differentiated from a (pre)malignant transformation with the occurrence of chromosomal instability (aneuploidy ) (Mehes et al., 2004).

## 4.5 Correlation of Cytology and Histology in the current study

Before the FISH-results of the current study are embedded in the context of the literature, the correlation of cytological and histological results is discussed. Since in the current study FISH is intended as a triage of cytology, the accuracy of the latter and the histological reference standard are expected to have an impact on the FISH-results.

### *4.5.1 Biopsy or Conization specimen as the reference standard; significance of CIN2*

For 101 cervical smears of groups II-p to V according to MN III, a biopsy or a conization specimen was available for reference standard in this study. The cytological diagnoses were compared to any available histology or to conization specimens only (32 women) in two independent analyses. This was performed due to the putative better agreement between cytology and conizations as compared to cytology and biopsies (Massad et al., 1996, Boonlikit et al., 2006). Cervical conization is both a diagnostic and treatment tool used to detect and treat abnormalities of the uterine cervix. Cold knife surgery, that is the most adopted conization method in the treatment of FIGO stage IA1 cervical cancer, produces an excellent specimen for diagnosis (Paraskevaidis et al., 2002). Some data show an overall agreement with a colposcopic diagnosis of around 85% (Duesing et al., 2012, Muller et al., 2016).

There is a debate about the best reference standard for cervical cytological - histological correlations. Some authors argue that the use of colposcopic-directed biopsies as the gold standard in cervical cancer screening studies may underestimate the prevalence of CIN 2 or worse (Pretorius et al., 2007). The conventional biopsy, as demonstrated in some works, needs

to be performed more than two times in the same lesion to achieve an agreement rate around 80% (Stoler et al., 2011a, Gage et al., 2006). Wentzensen and colleagues hypothesized that the collection of additional lesion-directed biopsies during colposcopic evaluation of the uterine cervix subsequent abnormal cytology will increase the detection of a high grade squamous intraepithelial lesion. Biopsies were taken from distinct acetowhite lesions of 690 women. They observed an increase in the sensitivity for detecting HSIL from 60.6% from a single biopsy to 85.6% after two biopsies and to 95.6% after three biopsies. Only 2% of HSILs were detected from a biopsy of normal appearing mucosa (Wentzensen et al., 2015). These data indicate problems for the evaluation of cytological results if a histological reference standard consisting of only one biopsy is applied.

A conization specimen as the reference standard is expected to be much more representative of a given lesion but is invasive and more expensive. Apart from the financial aspect, it is associated with the risk of long-term complications, such as cervical stenosis, increased rates of premature delivery and mid-trimester abortion, increased cesarean section rates, low birth weight, and rapid labor (Paraskevaidis et al., 2002, Leiman et al., 1980, Jones et al., 1979). The invasiveness of conization prohibits its usual application as the reference standard for determining the accuracy of cytology. Nevertheless, conization is sometimes necessary for therapeutical reasons. Some recent data suggest that the risk factors for dysplasia and preterm birth are shared and that conization by itself may not be an independent risk factor for preterm birth (Conner et al., 2014, Poon et al., 2012).

Choosing CIN2 and CIN3 for reference is also a controversial subject. In Germany, the target of cervical cancer screening is CIN3. Unlike CIN1 and CIN3, the biological significance of CIN2 is ambiguous due to the fact that this lesion could represent an intermediate step in the transition from HPV infections to cervical cancer precursors or simply a reflection of the imprecision of a histological diagnosis (Carreon et al., 2007). In a concept developed by US-based Lower Anogenital Squamous Terminology (LAST) project, broad agreement was reached to replace the three-tier (CIN1, CIN2, and CIN3) terminology with a two-tier system of low- and high-grade intraepithelial lesions. In Germany, the concept of CIN1-3 is mandatory according to the national guidelines for early cancer detection (Qualitätssicherungs-vereinbarung Zervix-Zytologie, 2015). These different circumstances and in addition the different reporting systems for cervical cytology in Germany (MN III) and international (Bethesda) were the basis for the different evaluations of the cytological-histological correlations of this study, reported in tables 11 to 18. The most relevant difference between the Bethesda system and MN III is the division of the HSIL group (Bethesda) into groups IIID2

(moderate dysplasia analogous to CIN2) and IVa-p (severe dysplasia/carcinoma in situ analogous to CIN3).

The division of HSIL into two different groups is attributed to the fact that moderate grade changes are more often reversible than high-grade ones and that the presence of an underlying invasive carcinoma in a patient with cytologically diagnosed moderate-grade dysplasia is very rare. Marquardt und Ziemke, corroborated with these assumptions, showed a significant difference in the risk of underlying high-grade lesions for the groups IIID1, IIID2 and IVa-p (Marquardt and Ziemke, 2018) during an observation period of 36 months. Reporting data of 3396 patients from 2014 until 2016, they found that the cumulative risk of an initial IIID2 diagnosis for a later occurring CIN2+ and CIN3+ was 62,44% and 45,80%. For patients diagnosed initially with IVa-p, the risk was 98,07% and 87,47%, respectively. Additionally, within the group IIID2 the overwhelming majority of CIN3+ lesions were classified as CIN3 (45,14%), only very rare cases as squamous cell carcinoma (0,66%). Also in the group IVa, most of the CIN3+ cases in the clinical course were classified as non invasive lesions, i.e. as CIN3 (84,82%) or AIS (0,24%). However, 2,41% of the IVa cases developed to invasive carcinoma - which means a 3.6-fold higher risk for carcinoma as compared to the patients with an initial cytological diagnosis of IIID2.

### *4.5.2 Comparing cytology and histology in a colposcopy referral population*

In the current study, we selected abnormal smears from women referred to the colposcopy unit at the university hospital in Düsseldorf. The cytological diagnoses were reported according to the Münchner Nomenklatur III (MN III) and in addition according to the Bethesda system. To compare the results with the histological diagnoses, the latter are reported according to the two- or three-tier classifications of dysplastic lesions, provided by the WHO classifications of tumors 2003 and 2014, respectively. Therefore, in this study, we contrast these diagnoses with the corresponding high grade squamous intraepithelial lesion or CIN3 lesion as classified by the WHO. For comparing with the two-tier WHO 2014 classification, group IIID2 of MN III was considered additionally.

Depending on the kind of classification used (refer to tables 11-14), 62.5%-82% of the group IVa-p or HSIL diagnoses corresponded to ≥ CIN3 or ≥ high-grade lesions. The best correlation was achieved using MN III and the WHO 2003 classification, what represents the current standard in Germany. 60.9%-73% of the ≥CIN3 or high-grade lesions were preceded

by a group ≥IVa-p or HSIL cytological diagnosis. The better correlation in this latter instance was achieved with the Bethesda classification because moderate dyskaryoses in this study correlated better with a CIN3 (7 out of 22) than a CIN2 (3 out of 22).

If only the histological diagnosis of a conization specimen was used as a reference, 53.8%-69.3% of group IVa-p (HSIL) or higher cytology corresponded to a ≥CIN3 or high-grade lesion (refer to tables 15-18). This evaluation was hampered by a low number of cases (n=32). The poorest correlation was provided by MN III and the WHO 2003 classification.

The abovementioned data refer to the cytologic accuracy to detect high-grade lesions, that have to be definitely treated to prevent progression to invasive cancer. Beside this analysis of a right indication for therapy, an evaluation of the overall agreement of cytological and histological diagnostic categories was done in the current study. Except for the analysis between MN III and the 2014 WHO classification of tumors, the overall agreement was the same independent of the classification used. The overall agreement rate for cytology and histological biopsies/conizations was 51.6%. Considering only conizations, it was 63.3%. The lower overall agreement rate between the Münchner Nomenklatur III and the WHO 2003 classification (45.2%-50%, refer to table 19) was probably caused by the subdivision HSIL group in both systems, that is merged in the Bethesda system and the WHO 2014 classification. The IIID2 group, for example, had a corresponding CIN2 histological result just in 13.6% (3 out of 22) of the samples, while 31.8% (7 out of 22) of the samples corresponded to CIN3. Carreon and coworkers revealed that CIN3 was a more reproducible and more specific marker of cervical cancer; further, CIN3 was more frequently associated with high-risk HPV types than CIN2 (Carreon et al., 2007).

Massad and coworkers analyzed samples from 2263 women who underwent colposcopy for abnormal cytology at an urban teaching hospital between 1996 and 1999. They compared the cytological results, using the Bethesda System, with the worst histological biopsy reported within an 8–26 months follow-up interval. Among the 1842 women with squamous cytologic abnormalities, a biopsy revealed a lesion more severe than that suggested by referral cytology in 577 (31,3%) and a less severe lesion in 648 (35,2%). Exact correspondence was found in 646 (35,1%) (Massad et al., 2001). LSIL had an exact histological correspondent in 28% of cases, an 11.3% higher rate than the value found in our current study (16.7%). This result is of course not as clinically important as the correlation of high-grade lesions: The concordance between HSIL and CIN2/CIN3 was 48%, while in our study this rate was 62.2% (table 13).

Bergeron and coworkers carried out a study using conventional smears and AutoCyte PREP® (liquid-based preparations) specimens of 500 consecutive women referred for loop electrosurgical excision proceduree (LEEP) of the cervix (Bergeron et al., 2001). LSIL cytology corresponded to 46.2% of the low-grade SIL LEEP specimens, while 56.4% of HSIL cytology corresponded to high-grade SIL LEEP specimens. In our study, these rates were 11.1% and 89.5%, respectively (table 18). 26% of LSIL cytology corresponded to a high-grade SIL LEEP diagnosis, compared to 77.8% in the current study, the latter most probably caused by inadequate sampling of the colposcopically suspect lesion. 43.6% of the high-grade LEEP lesions were under-diagnosed by the preceding cytology. In the current study, only 10.5% of the conizations with high-grade SIL were preceded by an LSIL cytology. For the interpretation of the results, the difference between the number of samples between these two studies has to be considered since in the current work only 32 LEEP specimens were available.

The disagreement between cytology and histology can occur essentially due to interpretive or sampling errors. A sampling error is made when abnormal cells are not collected or are not transferred from the brush to the slide. Another possibility is the inadequate diagnostic sampling of the cervical tissue as mentioned above (Wentzensen et al., 2015). The most common cytological sampling error is a lack of cells from the cervical transformational zone (Nanda et al., 2000). In order to improve the sampling quality, the use of an endocervical cytobrush or a spatula instead of a cotton swab is recommended. In addition, the implementation of new technologies in the conservation and preparation of the slides as liquid-based cervical cytology (Thin Prep) is proposed. However, the broad introduction of the expensive liquid-based cytology is currently not recommended, because initial promising improvements have not shown to significantly enhance the diagnostic accuracy of cytology. Halford et al., using 1.083 biopsy-confirmed high-grade lesions, revealed that there was no statistically significant difference between conventional cytology and Thinprep imaged slides when used for the diagnosis of high-grade lesions (Halford et al., 2010). The correct diagnosis of high grade or possibly high-grade squamous epithelial changes was made on the ThinPrep imaged slides in 61.0% (661/1.083) of cases and on the conventional slides in 59.4% (643/1.083). According to the recent German national guidelines for early cancer detection (Qualitätssicherungs-vereinbarung Zervix-Zytologie, 2015), a cytobrush and a cotton swab have to be used for sampling.

It is too complex to explain the low agreement rate between cytology and histology in the current study since not all stages of the diagnostic workup of the patients, especially the histology, were made by our laboratory and could only be obtained from the patients' charts.

But at least regarding the high-grade lesions, we are in line with some published studies as mentioned above. A hint for some problems with the histological reference, either sampling or interpretation, is the better agreement of cytology with the current results of the FISH analysis. There is a concomitant increase in the number of cells with gain of the hybridized genetic regions with the degree of dysplastic/neoplastic changes in cytology, as demonstrated in figure 1.

### *4.5.3 Benchmarking reports: Correlation of cytology and histology in the cervical cancer screening program in Germany*

It would be interesting to elucidate the results of cytological-histological correlation of the population-based cervical cancer screening in comparison to our current study that used selected specimens obtained in a colposcopy referral unit.

In a study that gathered cytological and histological findings from 2005 to 2009 in the German federal state Mecklenburg-Vorpommern with approximately 360.000 women examined per year, the group IIID (according to MN II) corresponded to a CIN1 in 22%, CIN2 in 40%, CIN3 in 34% and carcinoma in <1% of the cases, while 3% were histologically negative for intra-epithelial neoplasia (Marquardt, 2011). In conclusion, a major portion of the IIID diagnoses corresponded to a lesion worth to treat and not to follow cytologically as usually practiced. A similar observation was made in the triage: Duesing et coworkers, examining 266 consecutive patients with cytologically suspected cervical intraepithelial lesions, found a similar result (Duesing et al., 2012): The group IIID agreed more often with a CIN3 diagnosis (45.3%) than with a CIN2 (20.9%) or a CIN1 (23.3%). 9 cases (10.46%) were histologically diagnosed as negative for intra-epithelial neoplasia. All cytologic diagnoses in this study were based on conventional smears according to the proposals of the German screening program.

Meanwhile, there are some statistics available, that use the MN III, which was mandatorily introduced in 2015 after a 6-month voluntary period:

On occasion of the 25th Conference on Clinical Cytology in Munich in November 2015, Marquardt presented an early benchmarking report summing up results from eight cytology labs using the MN III in Mecklenburg-Vorpommern, The correlation of the cytological diagnoses with the available histological reports from 330.814 examined women showed an approx. 10% higher agreement of group IIID2 with CIN3 than with CIN2, being 46.1% and 36.7%, respectively.

In the statistics of the results of cervical screening in the federal state of Nordrhein-Westfalen for the year 2015, 35.21% of the patients with IIID2 cytological results had a corresponding CIN2 histological diagnosis, while 43.05% had a CIN3 result. Patients with a IVa-p diagnosis in cytology, however, revealed an even higher agreement rate with CIN3 of 83.55%, while just 7.4% of group IVa-p had a corresponding CIN2 result.

Meanwhile, also data from all over Germany are available, enclosing 18.340.028 specimens (Marquardt et al., 2017): 200.633 specimens were classified as IIID1, IIID2, IVa-p, and IVb-p, respectively, 37.650 of them with corresponding histology. 37.95% of the patients with IIID2 cytological results had a CIN2 correspondent histological diagnosis, while 39.76% and 12.8% had a CIN3 and CIN1 result, respectively. 82.24% of the group IVa-p diagnoses corresponded to a CIN3 lesion and 2.48% to a squamous cell carcinoma. Group IVb-p is a rare cytological diagnosis that indicates a higher risk of an underlying invasive squamous cell carcinoma of the cervix uteri. In the statistics of 2015, a high rate of histological evaluation, 78.78%, is reported. 65.14% of group IVb-p diagnoses corresponded to a CIN3 lesion and 22.26% to squamous cell carcinoma. The latter aspect confirms the higher risk of invasive carcinoma of group IVb-p compared to group IVa-p. The approx. 40% difference of an underlying CIN3 lesion after a preceding cytological diagnosis of group IIID2 or IVa-p indicates the prognostic significance of these distinct groups in the new MN III. Both groups are summarized to the HSIL diagnosis in the Bethesda system. Since the further management procedure of the patients with IIID2 and IVa-p differ from each other, the new Münchner Nomenklatur would have an advantage in comparison with the Bethesda nomenclature. Over-therapy in the form of too early conization can be avoided in group IIID2 and conversely subsequent a group IVa-p diagnosis, the patients could be earlier referred to colposcopy and conization. There are divergent opinions about this division. Some authors defend the idea that the division of the HSIL group in IIID2 and IVa-p in cytology, and in CIN2 and CIN3 in histology does not yield a significant improvement to the agreement between these two types of diagnosis. Marquardt and Ziemke however, in a study with 4162 cytological cases, found that the rate of CIN2+ cases diagnosed as IIID2 in cytology rose from 27% to 62.4% after 36 months, while in the same period of time the rate of CIN2+ cases diagnosed as IVa-p rose from 93.25% to 98.07% (Marquardt and Ziemke, 2018). This indicates a significant difference in the specificity of the two cytological diagnostic groups at the time of the first diagnosis and a possible value of the IIID2 group as a prognostic tool.

### *4.5.4 Is there a need for adjuvant methods in addition to conventional cytology?*

Nowadays the discussion about the improvement of cervical cancer screening is in vogue. The emergence of adjuvant techniques as hr-HPV tests, FISH, immunocytochemistry, or DNA cytometry, and the implementation of the HPV vaccine brought the necessity to rethink the strategies of early cervical cancer detection. Namely, it has to be discussed if those adjuvant applications could provide additional, more precise information to confirm the physician and patient about the biologic character (progression, persistence or regression) of a given cervical lesion. It has to be noted, however, that the definition of a method as a screening- or confirmation tool depends on many factors. The cost, complexity, sensitivity, and specificity of the test are some of the main characteristics to be taken into consideration.

The numbers given in the last chapter prove that a cytological finding of a group IVa-p is a clear indication for further clarification by colposcopy, biopsy and/or conization since the number of serious histological diagnoses (CIN3 and higher) is very high. Accordingly, an application of additional diagnostic methods in advance of colposcopy is not necessary. The situation is different for the groups IIID1 and IIID2. Today, there is no discussion about the fact that there is a need to supplement the cytological diagnoses with additional methods. It is quite obvious: the numbers presented above show that in fact many of these diagnoses correlate with histological changes of CIN1 or CIN2, but not infrequently no lesion is found or a CIN3 lesion is diagnosed.

DNA image cytometry that should be mentioned briefly in this context, is an important adjuvant method because it has a high negative predictive value (95%) in cervical smears with ASCUS and LSIL diagnoses revealing a diploid DNA distribution, allowing patients to return to normal screening intervals. Moreover, the high positive predictive values for patients who have CIN3 or higher grade lesions after 2 months and up to 100% after 3 years for patients who have ASCUS and LSIL with additional DNA aneuploidy allows the removal of these lesions by conization or loop electrical excision procedure (LEEP) (Bocking and Nguyen, 2004). However, this method is unfortunately only rarely used outside of specialized morphologic institutions like the cytopathology department of the Heinrich Heine University and thus plays no important role in diagnostics from a general point of view.

More important is the analysis of the genital HPV status of the patient that can be made by different approved test applications. In the present study, results of the Hybrid Capture 2 HPV DNA Test (hc2) (Digene Corporation, Gaithersburg, MD), performed at the same time or

within 6 months after the cytological smear, were available in many of the cases (77 - 61.14%), being hr-HPV positive in 56 (72.7%) cases and hr-HPV negative in 21 (27.3%) cases.

In concordance to the literature, the sensitivity of the hr-HPV test for CIN3+ diagnosis was around 90% (table 47). The specificities, however, were below 40% (table 47), possibly caused by the low number of samples, mainly by the groups IIp and IIID1. In a review from 2017, which involved 25 cross-sectional studies assessing HC2 for the detection of CIN2+ with 138,230 participants, the sensitivity values of HC2 ranged from 61% to 100%, and specificity values ranged from 64% to 95%. For the detection of CIN3+, from the 19 cross-sectional studies assessed, HC2 showed sensitivities from 81% to 100% and specificities from 69% to 95% (Koliopoulos et al., 2017, Yin et al., 2014, Luu et al., 2013, Arbyn et al., 2013). The Athena Study from 2011, using samples obtained from screening programs, found a sensitivity for CIN3+ with the hr-HPV test using HC2 of 91.3% (79.7-96.6) and a specificity of 70.0% (67.7-72.3) (Stoler et al., 2011b).

Since the accuracy of the conventional Pap test or HPV test is not high enough to confirm a diagnosis, many lesions demand more than one examination after some months, causing thereby emotional suffering in the patient due to the uncertainty about the natural course of the lesion. Thus, FISH applications might be an alternative: As a molecular method, it can be applied to cytologic smears in order to detect early genetic alterations which could lead to cancer development with high sensitivity and specificity, as outlined in chapter 4.4. This means that the development of a FISH protocol as an adjunct to suspicious lesions in cervix could, along with cytology and/or hr-HPV Test, provide better prognostic information about the final outcome, or may even be used as a first-line diagnostic test.

It is important to state that this study used samples from women who had previously some kind of cervical atypia, no sample was originating from screening programmes. Thus, besides the fact that FISH is an expensive method (see chapter 4.7), this study uses FISH as a confirmational diagnostic test for underlying CIN2 or higher lesions.

## 4.6 FISH analysis

In the current study, we aimed to test a new FISH protocol on archived smears of women referred to the colposcopy unit at the University Hospital of Düsseldorf (UKD). We analyzed whether this protocol could add information to conventional cytology about the risk of

progression of a given lesion. Therefore, the cytological diagnoses and different algorithms of possible FISH applications were compared to corresponding near-term tissue biopsies or conization specimens and/or to clinical follow-up. Whenever possible, hr-HPV test results were extracted from the patients' charts. Moreover, it was verified the accuracy of FISH protocol through the gain analysis of three LSI probes for the diagnosis of CIN2+ or CIN3+ cervical lesions.

FISH being a technique that uses fluorescently labeled DNA probes to detect chromosomal alterations in cells, can detect various types of cytogenetic alterations including aneusomy, duplication, amplification, deletion, and translocation. It is important to observe that in the present work the term "gain" appeared more suitable to describe the findings observed by FISH analysis. Terms as amplification, deletion and aneuploidy could just be adopted when the work uses centromeric probes (CEP) and thus the number of chromosomal copies is known. If the number of chromosomal copies of the respective gene probes (chromosome 3 for the hTERC probe, chromosome 8 for the MYC probe and chromosome 7 for the EGFR probe) is unclear, however, it is impossible to determine if genes were amplified or if the chromosome was present in a higher copy number than the diploid normal.

In the following, our results will be presented and interpreted comparing them with the results of studies from the literature. When we prepared this chapter, however, it became obvious that the design, the methodology and the way to statistically analyze and interpret the results were quite different from study to study. Thus, as many details had to be considered, a comprehensive way for comparison had to be found. In the next chapters (4.6.1 - 4.6.4) several aspects of FISH application in cervical pathology will be discussed focusing on some most important points.

### *4.6.1 ANOVA Analysis*

In the ANOVA analysis of our data, there was a significant difference between the low-grade (group IIp and IIID1) or negative (group I) lesions cytologically diagnosed and all moderate or high-grade lesions (IIID2, IVa-p, and V-p) for all individual gene probes and combinations (table 24). In the analysis between the low-grade/negative (CIN1 and negative) and moderate/high-grade (CIN2, CIN3, and SCC) histological follow-up groups, however, there was only statistically significant difference in a few cases (table 24). This, in conjunction with other factors, could possibly contribute to the low accuracy of FISH in the detection of

high-grade lesions, since the reference standard adopt for the present study was the correspondent histological result.

In the literature, some data about the relation between the cytologic interpretation and number of cells with gain may be found, which corroborate with our results. Heselmeyer-Haddad et al., working on 68 residual PreservCyt (Cytyc) specimens reported that the number of cells with more than two 3q signals, the percentage of cells with more than two 3q signals, and the relative 3q gain (compared to the gain in the chromosome 7) increased significantly with the severity of cytologic interpretation (PTrend >0.005). Caraway et al. found that patients with an HSIL or SCCA cytologic diagnosis had a significantly higher percentage of cells with gain of 3q26 (from Cervixcyte probe panel) than did patients with a NILM or ASC-US cytologic diagnosis ($p<0.0001$) (Caraway et al., 2008).

In relation to the comparison with histological follow-up results, Chen et al. showed that the TERC and C-MYC positive rates were similar between normal and CIN1 but higher in CIN2+ than in CIN1 lesions ($p <0.01$) (Chen et al., 2012). Besides that, significant differences were also observed between CIN1/ lower and CIN2/higher lesions ($p < 0.01$). The sensitivity and specificity of the combination of hTERC and c-MYC for the diagnosis of CIN2+ lesions in cytologically ASC-US+ in this work were 78.0% and 95.3%.

### *4.6.2 Cutoffs based on Youden indices and clinical experience*

Henceforth, the discussion will be focused on the cutoff analyses. The sequence of results will follow the chronological order of the work, i.e., first the performance of the cutoffs based on the Youden indices and clinical experience, then the results obtained with predefined cutoffs and/or using just LEEP conizations as reference standard, and at last the results of the analysis using the predefined cutoff for the groups IIp, IIID1, IIID2 and IVa-p.

Another important aspect to observe is that the follow-up was made in order to analyze the progression or regression of the lesions and a possible better agreement with the FISH results. Unlike other studies, the follow-up was not planned to indicate a predictive feature of FISH, but to be used as a more reliable reference standard. The limit of 18 months for the collection of histological and/or hr-HPV results was defined based on the average time of a transient HPV infection. In other words, if a patient had an HPV infection at the time of the first

cytological diagnosis, it could cause a genetic alteration without necessarily a morphological alteration on the cell. The follow-up results would be probably more suitable for FISH.

Initially, it was performed an analysis based on the Youden indices and for all cytological groups together (IIP, IIID1, IIID2, IVa-p, and V). This means that the sensitivity, specificity, AUC, AUC (IC) and AUC (p) of each probe or combination were derived from the optimal cutoff indicated by the Youden indices and not related to a specific cytological group. In some cases, when the cutoffs were not extremely high, it was possible to change it to a one more suitable to the clinical routine. When the cutoffs were much higher than the maximum desired (< 15 cells), the variation to an accepted value would consequently cause a critical reduction on the accuracy values (sensitivity and/or specificity).

Based on our results, it is possible to observe that none of the analyses showed an optimal result for the clinical routine (specificity > 90%, cutoff < 15 cells and a reasonable sensitivity). For the detection of CIN2 or CIN3 lesions, excluding or not the cells with tetrasomy pattern, the analyses which demonstrated an optimal specificity had a high cutoff. The individual analysis of MYC, for example, using CIN3 as the gold standard, excluding cells with a tetrasomic pattern (Table 31), showed a sensitivity and specificity of 59.6% and 91.3%, respectively. However, the cutoff presented (>19) is not suitable for the clinical routine.

In a study which retrospectively investigated 140 Thinprep cytologic tests (TCT) specimens, MYC had a sensitivity of 64.5% and specificity 93.8% for CIN2+, using a threshold of 4.83% (Zhao et al., 2016). For CIN2/3 lesions the sensitivity was 58.6% and the specificity of 88.5%. For signal identification, abnormal c-MYC gene amplification was indicated by a cell nucleus signal greater than 2.

Working with residual PreservCyt (Cytyc) cytological specimens from 243 outpatients (NILM, ASC-US, LSIL, ASC-H, HSIL and SCC), Chen et al., found a sensitivity of 80.0% (68.9-91.2) and specificity of 77.7% (71.8-83.6) for CIN2+ diagnosis using a cutoff of ≥ 3% aberrant cells (Chen et al., 2012).

Another trend observed in the detection of CIN2 and CIN3 was the increase of the specificity and the decrease of the sensitivity by combining the probes (tables 28, 29, 30 and 31). Except in some cases, the combination of the probes it is a more rigorous criterion that comprises fewer positive cases. This is, however, necessary for our purpose, since it is not desired to create an exploratory screening test but a confirmatory one that can rule out false-positive cases. The analysis with 3 probes, at least two of them with gain, at least 2 in the same

nucleus, for example, showed a sensitivity of 50.80%, specificity of 90.90% and a cutoff of >36 cells using CIN2 as the gold standard (table 28). On the same table, it may be observed that the highest specificity found by the individual analyses was 69.80% with the probe for EGFR (table 28). Moreover, this trend was observed by the works of some other authors (Chen et al., 2012, Luhn et al., 2013). In the study of Luhn et al., for CIN2+ or HSIL, the probes individually showed sensitivities of 82.4% for 3q26, 76.5% for 5p15, 76.5% for cen7 and 76.5% for 20q13 (Luhn et al., 2013). The specificities were 53.3% for 3q26, 56.7% for 5p15, 65.4% for cen7 and 56.7% for 20q13. Combined, the probes for 3q26, 20q13 and cen7 showed a sensitivity of 47.1% and a specificity of 86.7%.

The best cutoff of the individual analysis of hTERC based on the Youden indices was found in the analysis with CIN3 as the reference standard and without the cells with tetrasomy pattern, with a sensitivity of 91.7%, a specificity of 61.5% and a cutoff of >13 cells with gain (Table 31). In comparison to the other gene probes (EGFR and MYC), the probe for hTERC showed the worst result since the specificity was at maximum 68.5% (Table 27). The activation of telomerase in cervical carcinogenesis process is considered a relatively early process by some authors (Jiang et al., 2010, Heselmeyer-Haddad et al., 2003) and this could be the reason for the higher sensitivity demonstrated, as hTERC amplification comprises all grades of histological results. As already described in this work, the probe for hTERC showed an earlier high number of gains in comparison to the probes for MYC and EGFR (table 22).

Li et al. found a sensitivity of 87.50% and a specificity of 76.77% for the diagnosis of CIN3+ lesion with a cutoff of >10 abnormal cells using a probe for hTERC (>2 signals). They used ThinPrep samples with exfoliated cells from 171 patients who were diagnosed with ASCUS, LSIL, ASC-H, HSIL or SCC (Li et al., 2014c). Luhn et al., using Thinprep samples from 168 women referred to colposcopy, found a sensitivity of 82.4% and a specificity of 53.3% with a cutoff of ≥39 abnormal cells (>2 signals) (Luhn et al., 2013). Similarly to our work, the reference standard was based on the worst histologic diagnosis including biopsy diagnoses and LEEP outcomes, and the cutoff was defined based on the Youden index.

The Youden Index is useful method to perform a coprehensive analysis of the maximum potential effectiveness of a biomarker. Notwithstanding, the high number of cells necessary to perform a diagnosis found on our and other studies shows that the value found in this kind of analysis won´t necessarily be the most appropriate for the labor daily routine.

The cutoffs values, however, can sometimes be subtly altered without provoking drastic changes in the sensitivity and specificity values. A more comprehensive analysis of the values provided by the Youden Index is important to find cases which this adjustment can be made.

### *4.6.3 Pre-defined cutoffs and/or LEEP conization as the unique reference standard*

Based on the initial outcomes, it was decided to perform additional analyses with a pre-defined cutoff of > 10 cells. This cutoff value would be optimal for the clinical routine and could, possibly, provide a reasonable specificity. Moreover, using the same cutoff value we investigated the influence of the reference standards on our results. Based on the assumption that LEEP conization provides a more accurate diagnosis (Ueda et al., 2006), and on the fact that our cytological results had poor correlation with the histological outcomes, some tests were made using just the conization results as the reference standard. Since the aim of the study was to create a FISH protocol which could detect high-grade lesions of the cervix with a high specificity, just some combinations of three probes were adopted on this step.

The adoption of the conization as the unique reference standard and the exclusion of the cell with tetrasomy pattern proved to be useful for increasing the specificity (tables 33 to 44). Thus, the analysis with a predefined cutoff of >10 and conization as the reference standard had provided the best result with a sensitivity 78.60% and a specificity 92.00% for the detection of ≥ CIN2 lesions (table 34). In comparison, the same analysis but including the tetrasomy cells found a sensitivity of 85.70% and specificity of 84.00% ≥ CIN2 lesions (table 34).

As mentioned previously, this work presents a distinct methodology in comparison to other multicolor FISH studies found in the literature, being the comparison of the results not perfectly fair. Nevertheless, our best results for the detection of CIN2+ lesions was similar to those reported by Luhn et al., who worked with the combination 3q26, 20q13 and cen7 (Luhn et al., 2013) with a sensitivity of 47.1% and specificity of 86.7%. The cutoffs obtained by the Youden index were ≥39 cells for 3q26, ≥15 cells for 20q13, and ≥23 cells for cen7, requiring the analysis of ≥1000 cells per sample and employing an automated system method (Metafer4, Metasystems, Altlussheim, Germany).

Some studies have considered the tetraploid pattern on the FISH analysis. Heselmeyer-Haddad et al. concluded that 3q gain including tetraploid cells provides the best method for distinguishing HSIL, with a sensitivity of 92% and a cutoff of ≥ 2.5% cells for CIN2/CIN3

detection (Heselmeyer-Haddad et al., 2003). There was no great difference between the results with or without tetraploid cells. When the gain in a cell was accepted if ≥ 5 signals were found and the cutoff of ≥1% positive cells was defined, the analysis without tetraploid cells showed the same sensitivity rate. The criteria for the tetraploid pattern were, however, more strict. Just cells with four signals for each probe (pattern 4-4-4), 3 (CEP3) and 7 (CEP7) and a set of four overlapping BAC clones that contain sequences for the TERC gene, were considered tetraploid.

Luhn et al. used similar patterns for tetraploidy compared to our work (Luhn et al., 2013). For their four probes (3q26, 5p15, cen7, 20q13), the two sets of patterns to define tetraploid cells were: (4-4-4-4 or 4-4-4-3) and (4-4-4-5 or 4-4-3-3). They concluded that exclusion of these sets of cells, using either definition, did not alter the results.

### *4.6.4 Pre-defined cutoff analyses with the cytological groups*

The analyses using all cytological results together provide interesting results important if the development of a primary screening method is planned. For the handling of patients who have already a lesion detected, however, it is more important to collect data about the behavior of the probes when applied to a specific group of lesions. For lesions diagnosed as IIp, IID1 or IID2, for example, would be important to confirm or discard this diagnosis with a non-invasive method instead of wait 3 or 6 moths for a new cytological control. It would avoid the psychological distress usually involved in this process.

Thus, based on the previous results, it was performed analyses individually on the cytological groups IIp, IIID1, IIID2, and IVa-p, with a pre-defined cutoff of >10 abnormal cells for CIN2+ and CIN3+ lesions. The best result was obtained by the analysis of gain of 3 probes, at least 2 in the same nucleus [(+++) or [(++)+], with a sensitivity of 42.90% and specificity of 94.10%, for the detection of CIN3+ lesions, using just LSIL samples and excluding the tetraploid cells (table 40).

The triage of women diagnosed with ASCUS or LSIL is frequently a challenging process. Almost 35% of all LSIL diagnoses are over- or underrated with respect to an inter-observer agreement (Stoler et al., 2001). Moreover, it is estimated that nearly 20% of the LSIL cytology represent CIN2/3 at colposcopy (Solomon et al., 2001, Arbyn et al., 2006), however not the CIN 1. The development of new methods which can diagnose underlying CIN2+ or CIN3+ lesions in LSIL cases with high accuracy is necessary since the current protocol with cytological controls in three- or six-monthly intervals creates a stressful environment for the patients (Coker et al., 2003).

Heitmann et al. analyzed the accuracy of the probe for 3q26 for the diagnosis of underlying CIN2+ lesions in LSIL women (Heitmann et al., 2012). Combining manual and automated scanning methods, they found a sensitivity of 70% and a specificity of 91%. The test, in this case, was positive if two or more cells with more than four 3q26 FISH signals were detected.

In a multicenter study in China, Jiang et al. found a sensitivity of 80.7% and a specificity of 83.8% for the diagnosis of cytological high-grade cervical lesions and invasive cancer in LSIL samples (n = 2316) using probe for TERC and the chromosome 3 centromere-specific probe (CSP 3) (Jiang et al., 2010). The signal ratio of CSP3 to TERC at 2:2 in a cell indicated a normal signal pattern, whereas ratios of 2:3, 2:4, 2:5, 3:3, 4:4, and so on, represented abnormal signal patterns.

## 4.7 Perspective

The number of cells with gain increased in a similar proportion to the severity of the lesion, suggesting a good agreement between FISH and cytology. Moreover, the low grade of agreement between cytology and histology predicts some problems to the accuracy of FISH. Besides that, the German reporting system for both cytology and histology demonstrated a lower level of agreement in comparison to the Bethesda Report System and 2014 WHO histological classification of tumors of the uterine cervix. It is important to notice that due to the different number of groups of each report system or classification, the comparison is not completely unbiased.

Another problem was the initial impossibility of the application of the three probes on the same area, what in some cases decreased the number of samples available for analysis. However, it was always possible to analyze a reasonable number of cases, comparable to the other similar studies found in the literature.

Our work has also explored alternative methods of definition of cutoffs. In a first step, the Youden Index was applied which is very useful to provide a general vision of the cutoff possibilities. Based on this information a practical cutoff of >10 abnormal cells was used for the posterior analysis. This cutoff could be adopted in the routine and provided a decent sensitivity and specificity in many cases.

Thus, the study presented a broad view of the use of probes for MYC, hTERC, and EGFR in suspicious cervical smears. The results suggest that combinations of two or three of them could be useful for diagnosis of cervical carcinoma as a confirmation test for doubtful cases with low numbers of suspicious cells. Furthermore, the information collected gives us the necessary knowledge to develop new studies with higher potential in order to create more sensitive and specific protocols.

The addition of another probe more specific for HSIL diagnosis and the use of Liquid Based Cytology (LBC) could provide better results.

Liquid Based Cytology could bring some advantages for the FISH performance since the concentration of a high number of cells in a small area, the inexistence of mucus and the better conservation of the morphological and molecular apparatus of the cells could solve some technical issues of this work. This assumption is based on the good results of works which used liquid-based samples for FISH, with specificity around 90%, and on our own laboratory experience (Zhao et al., 2016, Li et al., 2014c, Zhang et al., 2009, Yin et al., 2012).

The early gain of the probe for hTERC gene possible contributed to low specificity of the analysis since many cytological samples with correspondent < CIN2 lesions had already a significant number of cells with gain. The substitution of hTERC and/or the addition of another probe which demonstrates to be more related to high-grade lesions could bring better results. Some studies showed a late expression of the CDC6 protein in cervical cancer development. Bonds et al. suggested a use of CDC6 as a molecular marker to identify underlying high-grade lesions (Bonds et al., 2002). Murphy et al. showed that CDC6 protein was expressed preferentially in high-grade lesions and in invasive carcinomas (Murphy et al., 2005). This protein is encoded by the gene CDC6 located on the cytogenetic band 17q21.2, which has already been demonstrated to be related to cervical carcinoma and to be an integration site for HPV (Network, 2017, Lyng et al., 2006, Schmitz et al., 2012). Zhang et al. showed a low-level amplification (3 to 7 signals) of the probe for ERBB2 at cytogenetic band 17q21.2 in 24 from the 84 (29%) cervical malignant lesions cases (Zhang et al., 2002)

Some of the chromosomal aberration related specifically to high-grade cervical lesions are the gains 1q, 5p arms and the loss of chromosomal bands 2q36-37 (Rao et al., 2004, Allen et al., 2000). Analysing 100 atypical cells per slide of 131 patients a study demonstrated that while the increase in the 3q26 amplification (Cutoff >11 cells with >2 signals) was evident even at CIN 2+ lesions ($p < 0.01$), 5p15 amplification (Cutoff >3 cells with >2 signals) was preferably present in CIN3+ lesions ($p < 0.001$) (Kudela et al., 2018).

The relatively low sensitivity could be overcome with the concomitant use of another adjuvant method. Upendram et al. combined hr-HPV test and fluorescent in situ hybridization (3q26, 5p15, 20q13, and CEP7) and found a 94% sensitivity, specificity, and negative predictive value for CIN2+ diagnosis (Upendram et al., 2017).

As mentioned before, there are some studies with multicolor FISH that show a very high specificity (over 90%) (Skacel et al., 2003, Schramm et al., 2011, Varella-Garcia et al., 2004). Considering that some of these studies reached these results by performing a long follow-up, the insufficient accuracy of FISH in cervical smears could have been caused by the low accuracy of the conventional histology methods.

In summary, our study showed many diagnostic alternatives for the application interpretation of FISH in cervical carcinoma and its precursor lesions in conventional PAP smears. Despite the methodological issues, in many situations, the results were similar to those in recent literature. However, to be accepted as a method for clinical routine, additional FISH studies with newly developed probes with higher sensitivity and preferably more suitable samples are needed in order to create a FISH methodology which could overcome incisively the limited accuracy of the current confirmation tests. Nevertheless the present study was effective in bringing important information about the application of FISH on cervical smears which could be applied in future works.

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