

# Topical mitomycin C and radiation induce conjunctival DNA-polyploidy

Olaf Carlsburg<sup>a,\*</sup>, Christopher Kallen<sup>a</sup>,  
Jost Hillenkamp<sup>a</sup>, Rainer Sundmacher<sup>a</sup>,  
Natalia Pomjanski<sup>b</sup> and Alfred Böcking<sup>b</sup>

<sup>a</sup> *Department of Ophthalmology, Heinrich-Heine University, Düsseldorf, Germany*

<sup>b</sup> *Institute of Cytopathology, Heinrich-Heine University, Düsseldorf, Germany*

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**Introduction:** Atypical cell changes often occur following treatment of premalignant or malignant conjunctival neoplasias with topical mitomycin C (MMC) and/or radiation. These reactive, non-neoplastic alterations of the conjunctival epithelium can be a differential diagnostic problem. Our aim was to investigate changes in the nuclear DNA-distribution of conjunctival epithelial cells after MMC- and radiation therapy by DNA-image-cytometry. **Methods:** Conjunctival brush smears were obtained from 13 patients (13 eyes) with squamous cell carcinomas and six patients (6 eyes) with conjunctival malignant melanomas *in situ* before, during and after treatment. The patients were treated with MMC-drops (0.02% or 0.04%) alone ( $n = 12$ ), with radiation therapy ( $n = 3$ ) or both ( $n = 4$ ). At first, the obtained brush smears were evaluated by cytology. Secondly, after Feulgen restaining, the DNA content of reactively changed cells was determined using the AutoCyte-QUIC-DNA<sup>®</sup> workstation. **Results:** We observed euploid DNA-polyploidy and cytomorphological changes in all patients (19/19). We considered these alterations as reactive to treatment. Four patients showed their greatest DNA-stemline at 4c and 15 patients at 8c. This effect was observed during and following MMC-drops and/or radiation and remained stable in 94% of all patients after a mean follow-up of 22.5 months (SD 15.4). In five cases image cytometry additionally demonstrated DNA-stemline aneuploidy as an evidence of tumor recurrence. **Conclusion:** Measurements of DNA-content revealed euploid polyploidisation of morphological suspicious

but benign squamous cells which is the biologic correlate of well known secondary morphologic changes following topical chemotherapy and/or radiation. DNA-image-cytometry is a useful tool in the differentiation of euploid polyploidization as a sign of reactive cell changes following treatment and tumor recurrences.

**Keywords:** DNA polyploidy, mitomycin C, radiation, DNA image cytometry, cytology

Alkylating mitomycin C (MMC) has been well described as a topical chemotherapeutic agent for conjunctival tumors such as intraepithelial neoplasia or carcinoma *in situ* (Cis) [6,9,16,17,20,27,37], primary acquired melanosis with atypia (PAM with atypia) and non-invasive conjunctival malignant melanoma (MM) [10–13,15,25,36]. Furthermore, its fibroblast-inhibiting properties make MMC a useful agent in the prevention of recurrences following pterygium surgery [14] and as an adjunctive agent in trabeculectomy [30]. Any treatment of conjunctival neoplasia requires careful post-treatment monitoring. Conventional monitoring methods include histological and cytological evaluation of regularly obtained biopsies.

The monitoring after MMC-therapy is associated with specific diagnostic problems. Difficulties in the differentiation between harmless changes caused by MMC and neoplastic recurrences following topical chemotherapy of bladder cancer with MMC have been documented [26,28,29]. In spite of the meanwhile established use of topical MMC in ophthalmology this diagnostic problem has not yet been widely recognised. In 1999 Salomão et al. first reported conjunctival morphological cell alterations following topical MMC-therapy mimicking malignant cell changes [33].

Since 1995, we have treated premalignant and malignant conjunctival neoplasia such as Cis, PAM with atypia and MM with MMC-eyedrops [6,25,27]. Consecutive cytomorphological cell changes were observed in all patients [30]. We found similar changes following radiation in conjunctival neoplasia [6]. Histopathological diagnostic problems caused by reactive

\*Corresponding author: Dr. Olaf Carlsburg, Department of Ophthalmology, Heinrich-Heine-University, Moorenstr. 5, D-40225 Düsseldorf, Germany. Tel.: +49 211 811 7333; Fax.: +49 211 811 6241; E-mail: carlsbur@uni-duesseldorf.de.

cell changes following treatment of conjunctival intraepithelial and invasive neoplasia with ionising radiation have also been reported [8,11].

The aim of this study was to compare cytology combined with DNA-cytometry as a diagnostic tool in the monitoring of conjunctival cell changes during and after topical chemotherapy with MMC and its usefulness for the differentiation between non-malignant, reactive cell changes and tumor recurrences with the conventional method of histological evaluation of conjunctival biopsies.

## 1. Patients and methods

Clinical, therapeutic, histological, cytological and DNA-cytometric data of our patients are summarised in Table 1 (conjunctival carcinomas) and Table 2 (malignant melanomas). Since 1995 we included 19 patients (19 eyes) in this study with a mean follow-up period of 22.5 months (SD 15.4). The mean age was 53.2 years (range 21–77). 13 patients presented with a carcinoma *in situ* (Cis), 6 with a malignant melanoma (MM). One case of Cis was a Cis with recurrence and two cases of MM also represented recurrences. We confirmed the diagnoses by histological evaluation of conjunctival biopsies in all patients. After closure of the epithelial defect caused by the biopsy, either topical chemotherapy or radiation was started. In 11 of 13 patients with conjunctival Cis we initially applied 1–6 cycles of MMC 0.02% eye-drops (MMC). One patient was treated with 3 cycles of MMC 0.02% having suffered a recurrence after initial radiation. Each cycle consisted of an application of MMC-eyedrops 4 times/day over a period of 2 weeks with a pause of 2 weeks between cycles. One patient with Cis was treated with radiation only. Four of the six patients with MM were initially treated with 4–6 cycles of MMC 0.04% 4 times/day over a period of one week. After a pause of 3 weeks 2 of the 6 MM-patients were treated with radiation alone and one patient was treated with a combination of chemotherapy and radiation after a recurrence following chemotherapy alone. Since 1995, we have routinely obtained conjunctival brush smears in most patients (16/19) before having confirmed the primary diagnosis of conjunctival neoplasia by biopsy. In two patients with Cis (Table 1, patients 5 and 7) and one patient with MM (Table 2, patient 2) we only obtained biopsies for histological evaluation. We regularly obtained conjunctival brush smears to fol-

low up treatment with topical MMC and/or radiation in all patients (19/19). After topical anaesthesia with proxymetacaine-hydrochloride 0.5%, superficial cells from macroscopically suspicious sites of the conjunctiva and cornea were harvested with tiny brushes (Accellon™ Multi, Medscan Medical AB, Malmö, Sweden). The slides were fixed immediately with an alcoholic spray (Merckofix™, E. Merck, Darmstadt, Germany), stained according to Papanicolaou and evaluated cytopathologically [18,35] in the Institute of Cytopathology, Heinrich-Heine-University, Düsseldorf. The probability of the observed cell changes to represent malignant cells was expressed in four diagnostic categories [4]:

- Unequivocal evidence for malignant cells: positive
- Malignant cells likely: suspicious
- Malignant cells cannot be excluded: doubtful
- No evidence for malignant cells: negative

When the morphological evaluation of cells did not allow an unequivocal diagnosis concerning the presence of malignant cells we additionally investigated these by DNA image-cytometry. We then removed the cover slips of the slides in xylene, refixed with buffered 10% formaline, restained according to Feulgen [22] and covered the slides with Entelan® (E. Merck, Darmstadt, Germany). DNA-image-cytometry was performed using a TV-image-analysis system (AutoCyte-Quick-DNA®, AutoCyte, Burlington, NC, USA) according to the standards of the European consensus on diagnostic DNA-image-cytometry [3,19,21,22,24]. For internal calibration and determination of the normal DNA-content of 2c (equivalent to a normal twofold set of chromosomes) we measured the integrated optical density (IOD) of 30 cytologically normal appearing squamous cells (Fig. 1). Their coefficient of variation was less than 5%. In all cases we further measured the IODs of 300 cytologically abnormal or atypical squamous cells or melanocytes (Figs 2–4). Standardised algorithms were used to diagnostically evaluate DNA-histograms according to the European consensus on DNA image-cytometry [3,19,21,22] (Figs 4, 5). DNA-aneuploidy was assumed in cell populations which had not previously been treated with either MMC or radiation if stemlines occurred outside of 2c, 4c or 8c  $\pm 10\%$  or if cells with a DNA content of more than  $>9c$  were observed (Figs 1, 4). DNA-aneuploidy was regarded as an indicator of neoplastic cell transformation [2,5,23]. Euploid DNA-polyploidy was defined as the occurrence of stem-

Table 1  
Summary of clinical data, diagnoses, treatment and follow ups of patients with carcinoma *in situ* of the conjunctiva

No	Pretherapeutical diagnosis			Treatment		Cytologic/cytometric follow-up				
	Biopsy	Cytol.	DNA-Cytometry	MMC/Radiation	Duration	Date	Cytol.	DNA content of STL		
1	Cis	pos	Aneupl.: STL3.82c	2×MMC 0.02%	10.95–01.96	01.96	doubtf	2c,4c		
						10.97	neg	2c		
2	Cis	pos	Aneupl.: STL 2.3c, 3.4c, 4.4c	2×MMC 0.02%	09.95–12.96	09.95	neg	2c,4c		
						01.96	neg	2c,4c		
						04.97	neg	2c,4c		
						Radiation (30 Gy)	05.97	09.97	neg	2c,4c
						03.99	neg	2c,4c		
04.00	neg	2c,4c,8c								
3	Cis	pos	Aneupl.: 9c EE	1×MMC 0.02%	08.95	01.96	neg	2c,4c		
						11.96	neg	2c,4c		
						07.99	neg	2c,4c		
						09.00	doubtf	2c,4c,8c		
4	Cis	susp	Aneupl.: 9c EE	6×MMC 0.02%	04.97–10.97	05.97	neg	2c,4c		
						06.97	neg	2c,4c,8c		
						10.97	neg	2c,4c		
						12.97	neg	2c,4c		
5	Cis	–	–	6×MMC 0.02%	06.97–12.97	07.97	neg	2c,4c		
						11.97	neg	2c,4c,8c		
						01.98	neg	2c,4c		
						12.98	doubtf	recurr. STL 3.27c		
						11.99	neg	2c,4c,8c		
6	Cis	pos	Aneupl.: STL1.87c 13c EE	6×MMC 0.02%	06.97–02.98	10.97	neg	2c,4c		
						11.97	neg	2c,4c,8c		
						02.98	neg	2c,4c,8c		
						10.98	neg	2c,4c		
7	Cis	–	–	6×MMC 0.02%	01.98–06.98	03.98	neg	2c,4c		
						10.98	neg	recurr. STL 3.54c		
						Radiation (30 Gy)	12.98	02.99	neg	2c,4c
03.00	neg	2c,4c								
8	Cis	pos	Aneupl.: 9c EE	4×MMC 0.02%	03.98–07.98	03.98	neg	2c,4c,8c		
						08.99	doubtf	2c,4c,8c		
9	Cis	pos	Euploid: STL 2c,4c highly diff.	Radiation (30 Gy)	04.98	05.98	neg	2c,4c,8c		
						01.99	neg	2c,4c		
						10.99	neg	2c,4c		
10	Cis	pos	Aneupl.: STL3.58c	4×MMC 0.02%	08.98–12.98	10.98	doubtf	2c,4c,8c		
						12.98	neg	2c,4c		
						01.99	neg	2c,4c		
						07.99	neg	2c,4c		
						10.00	neg	2c,4c		

Table 1  
Continued

No	Pretherapeutic diagnosis			Treatment		Cytologic/cytometric follow-up		
	Biopsy	Cytol.	DNA-Cytometry	MMC/Radiation	Duration	Date	Cytol.	DNA content of STL
11	Cis	pos	Aneupl.: STL 3.23c	4×MMC 0.02%	05.99–08.99	07.99	neg	2c,4c
						08.99	doubtf	2c,4c,8c
						09.00	doubtf	2c,4c,8c
12	Cis	pos	Aneupl.: STL 4c, 6c,7c	4×MMC 0.02%	12.99–03.00	01.00	neg	2c,4c,8c
						02.00	neg	2c,4c,8c
						03.00	doubtf	2c,4c,8c
						07.00	doubtf	2c,4c,8c
						12.00	doubtf	2c,4c,8c
13	Cis recurr.	doubtf	Aneupl.: 9c EE	Radiation (50 Gy)	10.93	02.99	neg	2c,4c
						02.00	doubtf	2c,4c,8c
				3×MMC 0.02%	06.00–08.00	05.00	pos	2c,4c,8c+recurr. 9c EE
						07.00	doubtf	2c,4c,8c
						08.00	pos	2c,4c,8c

m = male; f = female; OD = right eye; OL = left eye; Cytol. = cytological diagnosis; Cis = carcinoma *in situ* of the conjunctiva; MMC = mitomycin C-drops; Aneupl. = DNA-aneuploidy; STL = DNA-stemline; EE = exceeding events; recurr. = recurrence; pos = positive; suspic = suspicious; doubtf = doubtful; neg = negative; – = not done.

lines at 2c, 4c and/or at 8c and/or 16c ±10% (Figs 4, 6) [24]. All other DNA-histograms were defined as DNA-diploid:

DNA-diploid: 1.8c < STL < 2.2c  
DNA-polyloid: 1.8c < STL < 2.2c  
and 3.6c < STL < 4.4c  
and/or 7.2c < STL < 8.8c  
DNA-aneuploid: 1.8c > STL > 2.2c  
or 3.6c > STL > 4.4c  
or 7.2c > STL > 8.8c  
and/or 9c exceeding  
events >0

## 2. Results

Our data on clinical diagnoses, treatment and monitoring are summarised in Tables 1 and 2. Data on 13 patients with Cis are summarised in Table 1 and those on 6 patients with MM in Table 2.

We clinically evaluated suspicious conjunctival findings by cytology and DNA-image-cytometry applied to conjunctival swabs in 16/19 patients. Both methods were successful in identifying malignant cells. Malignant cells could be clearly identified by cytology alone in 13 patients (6 Cis, 1 Cis-recurrence, 3 MM, 2 MM-recurrences). In patients who had previously been treated with either chemotherapy or radiation the cy-

tological diagnosis was confirmed by the cytometric finding of DNA-stemline-aneuploidy. In patients who had not previously been treated by either chemotherapy or radiation the diagnosis was confirmed by a finding of cells >9c (EE > 9c).

In one case (Table 1, patient 9) we found cytological evidence of a squamous cell carcinoma and a peridiploid DNA-stemline. This finding suggests the diagnosis of a highly differentiated squamous cell carcinoma. DNA-cytometry allowed to establish the diagnosis of conjunctival neoplasia in two patients with a suspicious cytological (Table 1, patient 4) and one doubtful cytological finding (Table 1, patient 13).

We confirmed all clinical, cytological and cytometric findings by histological evaluation of conjunctival biopsies.

During and after treatment with topical MMC (0.02% or 0.04%) and/or radiation we found numerous morphologically abnormal squamous cells. Our findings included variability of nuclear size, hyperchromasia and coarse chromatin, yet with a normal nuclear cytoplasmic relation. These changes can also be found in carcinomas. In order to rule out a malignant transformation we therefore additionally investigated these cells by DNA-image-cytometry. We found DNA-stemlines at 2c, 4c, 8c as quantitative equivalent of the mentioned cytomorphological changes. These findings cytometrically represent euploid polyploidy. In four patients polyploidy resulted in a greatest stemline at 4c. In 15 patients it was equal to 8c.



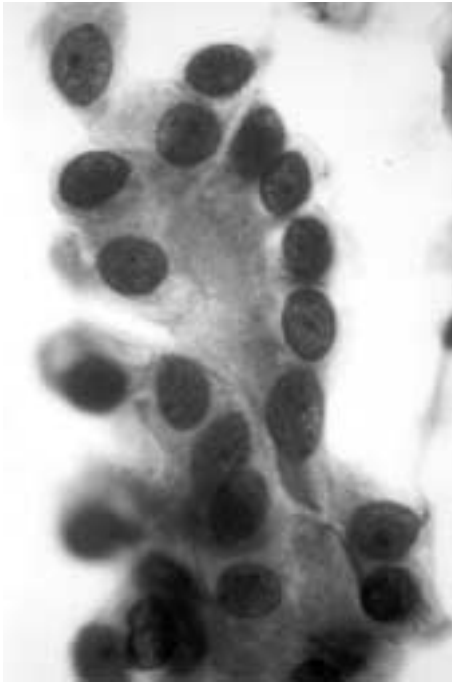


Fig. 1. Brushing smear from benign and unaffected squamous cells of conjunctiva. Magnification 63 $\times$ .

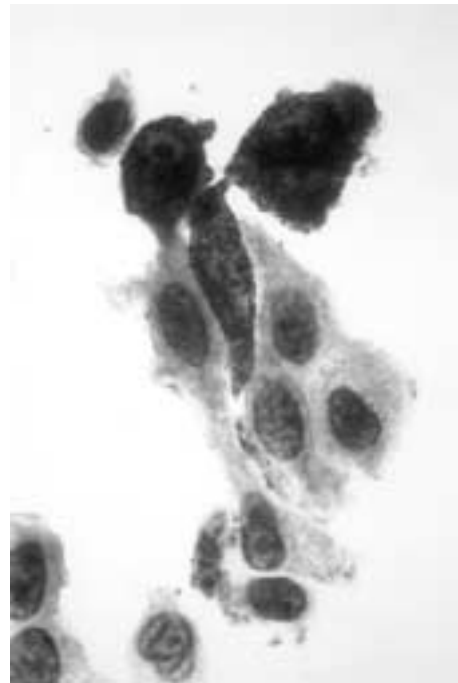


Fig. 2. Brushing smear from a conjunctival malignant melanoma. Polymorphous tumor cells containing cytoplasmic melanin. Magnification 63 $\times$ .

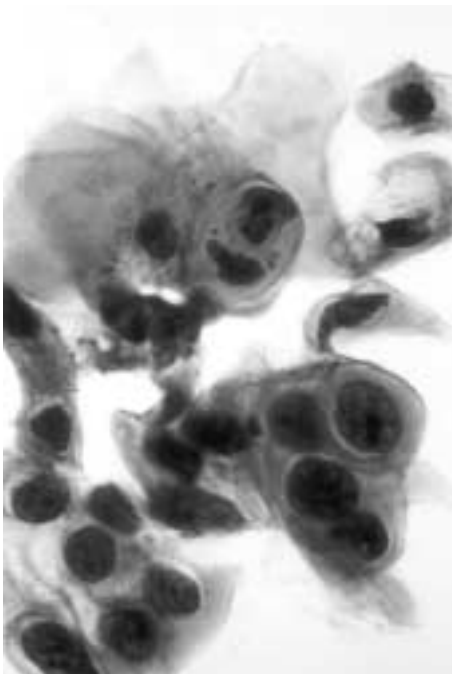


Fig. 3. Brushing smear of a carcinoma *in situ* of the conjunctiva. Polymorphous abnormal squamous cells revealing keratinisation. Magnification 63 $\times$ .

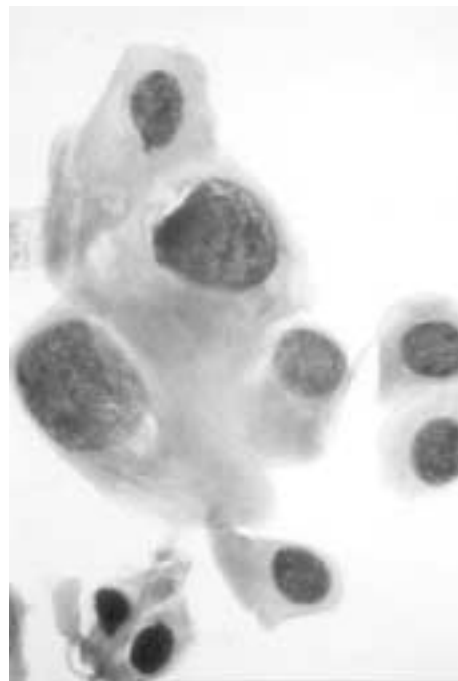


Fig. 4. Brushing smear obtained months after MMC-treatment of a carcinoma *in situ* of the conjunctiva showing drug-associated anisonucleosis of non-neoplastic squamous cells. Magnification 63 $\times$ .

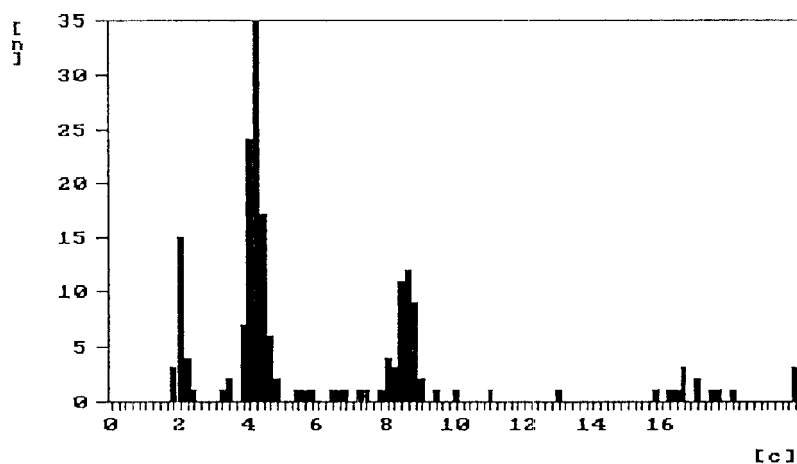


Fig. 5. DNA-histogram of brush-harvested carcinoma *in situ* cells (Fig. 2) reveals DNA-aneuploidy as evidence of malignancy (abnormal stemlines at 4.4c and 8.8c and 17 cells >9c).

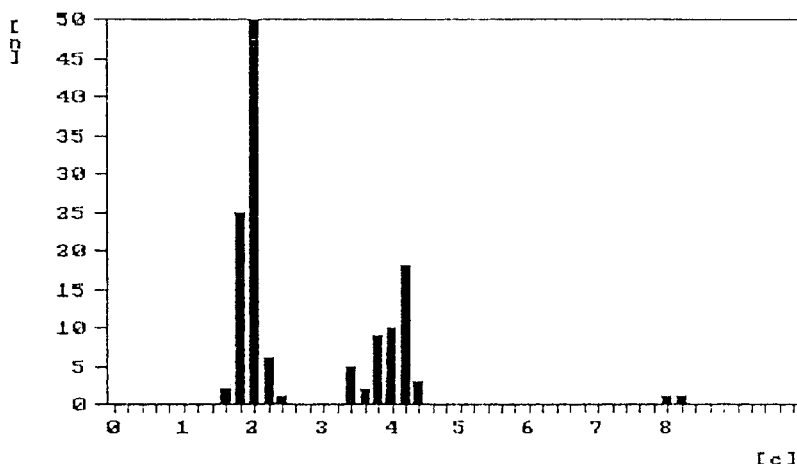


Fig. 6. DNA-histogram of brush-harvested squamous cells reveals drug-associated DNA-polyploidy (DNA-stemlines at 2c and 4c) without signs of DNA-aneuploidy (Fig. 3).

### 3. Discussion

Mitomycin C is well established as topical chemotherapy for PAM with atypia, MM and Cis [6,9–13, 15–17,20,25,27,36,37]. MMC as an alkylating agent induces cell death (apoptosis) by forming covalent bridges between neutrophilic DNA. This effect leads to an increased turnover of cells and to an reduced tumor growth by decreasing DNA-synthesis. It has been described that MMC not only affects malignant but also normal cells. This leads to specific morphological changes [18,26,28–30,33]. Harmless reactive changes following treatment of bladder carcinoma with topical MMC are difficult to clearly distinguish from tumor recurrences [28,29]. The same effects have been

observed following radiation of the bladder [8,11,35]. We found no difference in cellular changes caused by radiation and chemotherapy with topical MMC.

Salomão et al. reported morphological changes of conjunctival squamous cells in 9 of 10 patients with PAM with atypia who had been treated with MMC-eyedrops [33]. They described enlargement of the nucleus, polychromasia and apoptotic cells. These changes could morphologically not be distinguished from malignant cells. In order to be able to differentiate between reactive cell changes and tumor recurrences Salomão and co-workers intentionally included only patients with treated PAM but no patients with squamous carcinoma in their study [33]. Recurrences of PAM with atypia can be clearly diagnosed on morphological grounds because besides squamous

cell changes melanocytes also appear pathologically altered. It is more difficult to differentiate between tumor recurrences and reactive cell changes in squamous malignancies (Cis) as demonstrated by 13 patients with this diagnosis included in our study. In these cases, it was impossible to establish a clear diagnosis by evaluation of cytomorphological criteria only. We could demonstrate that DNA-image cytometry is a useful tool in the evaluation of cell changes suspicious for cancer. Grossniklaus developed criteria for the differentiation of reactive and malignant cell changes based on cytomorphological evaluation of the chromatin structure only [33]. According to his criteria an uneven distribution of chromatin indicates malignant cell changes whereas an even distribution indicates reactive changes [20]. In our opinion, the evaluation of morphological criteria alone is not only subjective but unreliable and not sufficiently reproducible. Yet, DNA-image-cytometry can be helpful in the evaluation of doubtful cases by adding objective criteria. Measurement of the DNA-content of morphologically suspicious cells mostly allows to clearly classify the dignity of the observed cell changes. Most malignant tumor cells reveal numeric or structural chromosomal aberrations (chromosomal aneuploidy) that differ from normal or reactively altered cells [34]. Evidence of clonal chromosomal aneuploidy is accepted as a marker of neoplastic cell transformation. Yet, cytogenetic investigations are too time consuming and too expensive for routine diagnostics and most samples will not grow in cell culture. We therefore use DNA-image-cytometry to measure the net effect of chromosomal aberrations on the DNA-content of evaluated cells [2,5]. The finding of DNA-aneuploidy as a quantitative equivalent of chromosomal aneuploidy may thus be taken as proof of neoplastic cell transformation. DNA-image-cytometry therefore allows to clearly differentiate between malignant and reactive cell changes to either chemotherapy or radiation in both squamous cell carcinomas or melanocytic tumors. The finding of a DNA-distribution that statistically significantly differs from that of normal (resting, proliferating or polyploid) cell populations proves DNA-aneuploidy and thereby allows to identify malignant cells or tumor recurrences in squamous epithelial and melanocytic cells.

DNA-image-cytometry was particularly helpful when the cytological diagnosis was tumor cell-negative and DNA-aneuploidy was found indicating tumor recurrence or when cytometry showed euploid DNA-polyploidy following a doubtful or even tumor cell-positive diagnosis indicating harmless reactive epithe-

lial changes. Euploid polyploidy in morphologically suspicious cells with DNA-stemlines at 2c, 4c or 8c indicates non-malignant reactive cell changes secondary to treatment.

Biesterfeld et al. reported the finding of DNA-polyploidy in non-neoplastic tissue in more than 20 different types of tissue [1]. DNA-polyploidy can be interpreted as a sign of tissue differentiation or as a phenomenon secondary to an increased functional demand [1,2]. For example, left ventricular cardiac muscle cells show a higher degree of DNA-polyploidy than those of the right ventricle. A comparatively high degree of DNA-polyploidy can also be observed in lymphocytes during the early stages of an infection with HIV or in adult epithelial hepatic cells but not in hepatic cells of newborns [1]. Reactive conjunctival cell changes following topical chemotherapy or radiation may equally be interpreted as a functional adaptation or as a sign of cellular regeneration following treatment induced cell death [2]. The cells revealing DNA-polyploidy did not reveal morphological signs of malignancy (chromatin pattern changes, irregularities of nuclear membrane, changed nuclear cytoplasmic ratio). Clinically there was never any sign of malignancy in these cases where DNA-polyploidy was observed. This was also true for a mean follow-up period of 22.5 months. The polyploidization was also found at sites beneath the original tumor, which never showed evidence of malignancy.

So far it is unknown for how long reactive cell changes following treatment persist in epithelial cells. Reactive changes in epithelial cells of the urinary tract have been observed up to months after treatment [28, 29]. The described reactive morphological cell changes and the euploid DNA-polyploidy following treatment appear to regress slowly. We observed persisting cell changes after a mean follow-up period of 22.5 months (SD 15.4) in 94% of all cases.

Table 3

Factors influencing nuclear DNA content [3]	
<i>Physiologic</i>	
	Replication
	Euploid polyploidization
	Apoptosis
	Necrosis
<i>Nonphysiologic</i>	
	Viral infections (e.g., HPV, CMV, Herpes, HIV)
	Radiotherapy
	Cytostatic therapy
	Vitamin B <sub>12</sub> deficiency



Conjunctival biopsies for histological evaluation cannot be obtained from patients in an unlimited number, especially when the course of disease stretches over several years. Cytology in combination with DNA-image-cytometry therefore represents a useful tool in the non-invasive postoperative monitoring of conjunctival neoplasia. Cytology and DNA-image-cytometry may become the methods of choice in the primary differentiation between benign cell changes, premalignant dysplasias, carcinomas and melanomas [7]. Both methods prove to be particularly useful in the regular long-term non-invasive monitoring of cell changes following surgical tumor excision and/or topical chemotherapy with MMC or radiation. DNA-image-cytometry, applied additionally to cytology may represent a safe alternative superior to the conventional invasive method of histological evaluation of conjunctival biopsies.

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