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The studies described in this thesis were performed at the Department of Otolaryngology/Head and Neck Surgery, University Hospital Vrije Universiteit, Amsterdam. Financial support for this research was provided by the Dutch "Praeventiefonds".

ISBN 90-9011394-0

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Print: Drukkerij Elinkwijk BV, Utrecht

This doctorate was financially supported by: Abbott, Astra producent van Rhinocort® Nevel en Rhinocort® Turbuhaler®, Carl-Zeiss, Entermed, Glaxo-Wellcome, GN Danavox, Mediprof, Resound, Schering-Plough, UCB Pharma, Veenhuis Medical Audio, Zambon.

VRIJE UNIVERSITEIT

BIOMARKERS IN CHEMOPREVENTION OF SECOND PRIMARY TUMOURS IN HEAD AND NECK CANCER PATIENTS

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
prof.dr. T. Sminia,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der geneeskunde
op vrijdag 20 maart 1998 om 13.45 uur
in het hoofdgebouw van de universiteit,
De Boelelaan 1105

door

Marcel Paul Copper

geboren te 's-Gravenhage

Promotor: prof.dr. G.B. Snow

Copromotor: dr. B.J.M. Braakhuis

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CHAPTER 1

GENERAL INTRODUCTION

HEAD AND NECK CANCER

Squamous cell cancer originating in the mucous membranes of the upper aerodigestive tract accounts for over 95 percent of all malignant tumours of the head and neck, and is worldwide one of the major malignancies (Muir and Weiland 1995). Most patients are over the age of 45 and males are affected at least twice as often as females, (Krolls and Hoffman 1976, Schottenfeld 1992). It has been estimated that there were almost 900,000 new cases of head and neck squamous cell cancer worldwide in 1995, 600,000 in men and 270,000 in women (Parkin *et al.* 1993). The highest reported incidence rates of head and neck squamous cell carcinoma are from parts of south and south-east Asia and Brazil (Parkin *et al.* 1992). In the Western world this type of cancer accounts for approximately five percent of all diagnosed malignancies (Muir and Weiland 1995). The incidence rates for head and neck squamous cell cancer have been increasing during the last decades, as have the mortality rates for patients with this type of cancer (Blot *et al.* 1994).

Etiology

Several factors are known to increase the risk of developing squamous cell cancer in the head and neck. The major risk factors are the life-style factors smoking and alcohol consumption (Wynder *et al.* 1957, Smith 1979, Decker *et al.* 1982). Linear dose-response effects of tobacco smoking have consistently been demonstrated in both prospective and retrospective studies (Moore *et al.* 1971, Rothman *et al.* 1980, Brugère *et al.* 1986). Higher risk for smokers of unfiltered cigarettes and a diminishing risk with increasing time since smoking cessation have also been demonstrated (Wynder *et al.* 1979). Smokeless tobacco, used in parts of the United States, Europe and south Asia, can induce oral squamous cell cancers (International Agency for Research on Cancer 1985), and epidemiological studies suggest a strong association between this habit and oral carcinogenesis (Winn *et al.* 1981, Winn 1992). Alcohol potentiates tobacco-related carcinogenesis and is also an independent risk factor (Talamini *et al.* 1990, Hsu *et al.* 1991). Another life-style factor known to increase head and neck cancer risk is poor oral hygiene (Graham *et al.* 1977).

The relation between dietary deficiency and the development of squamous cell cancer was recognized during the last decades (Bjelke 1975, Byers *et al.* 1984, Fontham 1990). Riboflavin deficiencies produce dysplastic changes of the oral mucosa and may in part explain the relationship between alcoholism and oral cancer (Wynder and Klein 1965). Iron deficiency anemia associated with Plummer-Vinson syndrome produces dysplasia of oral and pharyngeal mucosa, which probably accounts for the increased incidence of oral and pharyngeal cancer in these patients

(Wynder *et al.* 1957). The role of dietary vitamin A and other retinoids is discussed elsewhere in this chapter.

Occupational factors like work in the furniture, asbestos-related industries, and nickel refining are known to increase head and neck cancer risk (Brown *et al.* 1988, Maier *et al.* 1991, Muscat and Wynder 1992). A substantial list of chemical agents, like petroleum products, sulfa, and epoxy paints, has been identified as associated with head and neck cancer (Witek *et al.* 1979).

Viral infections may contribute to the development of head and neck cancer. Human papilloma virus infection, especially infection with genotype 16, has been associated with squamous cell carcinoma of the oral cavity and the oropharynx (Snijders *et al.* 1996, Franceschi *et al.* 1996). However, the majority of head and neck squamous cell cancers show no signs of infection. The role of the human immunodeficiency virus (HIV) in the etiology of squamous cell cancer of the head and neck is not quite clear (Ficarra and Eversole 1994, Singh *et al.* 1996).

In addition individual sensitivity to external carcinogens may be important in the etiology of head and neck squamous cell cancer because so many persons have been exposed to tobacco and alcohol, but only few eventually develop head and neck squamous cell cancer (Li and Montesano 1994). One promising marker to measure individual cancer susceptibility is mutagen sensitivity, which is measured as persistent chromosomal damage in peripheral blood lymphocytes after in vitro challenging with bleomycin (Hsu 1983, Hsu *et al.* 1985). Since this factor is independent of age, sex, alcohol, and tobacco abuse it is proposed to be a constitutional factor which reflects the way genotoxic compounds are dealt with (Cloos *et al.* 1993). It has been shown that mutagen sensitivity is higher in head and neck cancer and in particular in multiple primary cancer patients (Cloos *et al.* 1994). A high mutagen sensitivity score itself does not significantly increase cancer risk. However, a hypersensitive phenotype in combination with high tobacco and alcohol exposure results in a much more accurate assessment of cancer risk (Cloos *et al.* 1996).

The individual susceptibility to external carcinogens may well be a hereditary factor. Several studies have shown an increased lung cancer incidence in relatives of cases with lung cancer (Law 1990, Sellers *et al.* 1990). A few case reports about the familial clustering of oral and laryngeal squamous cell cancer described the simultaneous occurrence of a similar localization of a squamous cell carcinoma in two or three members of a single family (Marlowe 1970, Gencik *et al.* 1986, Tashiro *et al.* 1986, Bhaskar *et al.* 1988). Recently, several case-control studies were published, showing familial clustering of head and neck squamous cell cancer and lung cancer thus providing evidence for a hereditary role in the development of head and neck cancer (Copper *et al.* 1995, Foulkes *et al.* 1995, Foulkes *et al.* 1996). A positive family history of one or more relatives with respiratory and upper digestive tract cancer among patients with initial head and neck squamous cell cancer was established as a risk factor for the development of second primary tumours (Bongers *et al.* 1996).

SECOND PRIMARY TUMOURS

Although early-stage head and neck squamous cell cancer can be treated successfully with surgery or radiation in the great majority of patients, these patients have a relatively high risk of developing second primary tumours in the same organ or organ systems, that is the respiratory tract and the upper digestive tract, including the oesophagus (De Vries *et al.* 1986, Lippman and Hong 1989, De Vries *et al.* 1990). Warren and Gates (1932) provided the classification criteria for second primary tumours, which are still used at present. These criteria require that both tumours are histologically malignant, and that they are geographically separate and distinct and not connected by either submucosal or intraepithelial neoplastic changes. The possibility that the second tumour is a metastasis of the first tumour should be eliminated.

Second primary tumours can be divided into two groups: synchronous second primary tumours if they develop simultaneously or within six months after the initial tumour, and metachronous second primary tumours if they develop more than six months after the first tumour. Most second primary tumours are metachronous and develop during follow-up of head and neck cancer patients after curative treatment of their first tumour. In 1953, Slaughter *et al.* proposed the theory of "field cancerization" to explain the high incidence of multiple primary tumours in environmentally induced carcinomas (Slaughter *et al.* 1953). They coined the term "condemned mucosa" assuming that all or much of the epithelium of the respiratory and upper digestive tract has been damaged, probably as the result of exposure to tobacco products and other carcinogens. It has been postulated that second primary cancers represent new tumours developing from carcinogen-damaged cells in the same epithelial surface as the original tumour. These tumours are proposed to arise independently of each other and are consequently polyclonal.

Recently, an alternative hypothesis for the development of second primary tumours was postulated (Carey 1996). It was hypothesized that multiple primary tumours are monoclonal and the result of micrometastatic foci that have migrated from the original primary site or that have floated away with the saliva and have reimplanted at a secondary site. New methods of examining genetic changes in tumours have made it possible to test this hypothesis. In one study the same pattern of loss of heterozygosity or X-chromosome activation was found in the tumours of eight patients with multiple primary tumours (Bedi *et al.* 1996). These authors concluded that multiple tumours arise in at least a proportion of patients with squamous cell cancer in the head and neck from a single clone. Another research group has found that two synchronous head and neck squamous cell carcinomas shared a clonal Y marker, evidence for the fact that these two tumours arose from the same clone (Worsham *et al.* 1995). However, other researchers found no support for a monoclonal origin of second primary tumours. Several molecular and immunohistochemical studies (Bongers *et al.* 1995a, Shin *et al.* 1996) showed

different p53 expression or different p53 mutations (Nees *et al.* 1993, Chung *et al.* 1993, Zariwala *et al.* 1994, El Naggar 1995) in multiple primary tumours.

According to the larger studies in the literature second primary cancers develop at an annual rate of approximately three percent in head and neck cancer patients (Gluckman *et al.* 1980, Shapsey *et al.* 1980, McGuirt *et al.* 1982, Cooper *et al.* 1989), and the five year cumulative incidence ranges from 15 to 35 percent (Healy *et al.* 1976, De Vries and Snow 1986, Haughey *et al.* 1992). The incidence and localization of the second primary is related to the site of the initial tumour or the so-called "index tumour". With initial tumours of the oral cavity and the pharynx, the second tumour often occurs in the upper digestive tract (Tepperman and Fitzpatrick 1981, De Vries *et al.* 1986), while in laryngeal cancer, the second primary cancer often occurs in the respiratory tract (Wagenfeld *et al.* 1980, De Vries and Snow 1986). The risk of lung cancer is the highest in patients with supraglottic laryngeal cancer (Deleyiannis and Thomas 1997). Second primary tumours occur less frequently in laryngeal cancer than in oral and pharyngeal cancer (Wagenfeld *et al.* 1980, De Vries *et al.* 1986, Haughey *et al.* 1991). Studies on the effect of smoking cessation, once a first cancer has been detected, on the incidence of second primary cancers have been inconclusive. Three studies have found no clear benefit of cessation after cancer diagnosis (Castigliano 1968, Schottenfeld *et al.* 1974, Day *et al.* 1994). In contrast, other studies have reported a reduced risk for second primary cancers among persons who stopped smoking after diagnosis of their first cancer (Moore 1971, Silverman and Griffith 1972, Wagenfeld *et al.* 1980, Silverman *et al.* 1983, Stevens *et al.* 1983). Second primary tumours have in general a poor prognosis, because they arise frequently at notoriously bad sites, such as the lungs or the oesophagus or in previously irradiated or operated areas. The likelihood of death from a second primary tumour even exceeds the chance of death from the first tumour after four years from diagnosis of the primary index tumour (McGarry *et al.* 1992) and this is more prominent in early stage disease (Vikram 1984) (Figure 1). Because the probability of survival in patients with advanced stages of head and neck squamous cell cancer is much less than in patients with early stage disease, the cumulative risk for second primary tumours is highest in patients successfully treated for early stage disease (Cooper *et al.* 1989).

Two approaches can be pursued to reduce morbidity and mortality related to multiple primary cancers: early detection and prevention. Early detection of second primary tumours is associated with many problems (Rachmat *et al.* 1993). There is the question at which intervals patients should be seen and how thorough the examinations should be at each follow-up visit (Snow 1992). Ideally, panendoscopy including bronchoscopy and oesophagoscopy is to be carried out every time, but clearly this is not feasible for practical reasons. However, more accurate cancer risk estimation can define susceptible subgroups who might benefit from intensive screening programs.

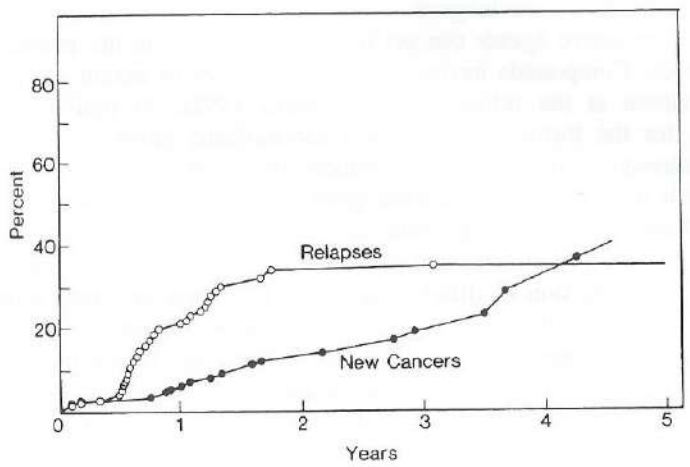

Figure 1: *Rate of appearance of relapses (local, regional, and distant) of head and neck cancer compared with rate of appearance of second malignant neoplasms. (Adapted from Vikram 1984).*

CHEMOPREVENTION

General aspects

Chemoprevention was defined in 1976 as an effort to "arrest or reverse premalignant cells during their progression to invasive malignancy, using physiological mechanisms that are not cytotoxic" (Sporn *et al.* 1976). The chemopreventive approach depends on the ability of certain chemical agents to block mutagenesis and control cellular differentiation and proliferation in epithelial tissues (Hong and Lippman 1995) or to reverse the carcinogenic process. The specific rationales for chemoprevention come from many sources: epidemiological studies, animal studies, and basic studies of carcinogenesis (Willett and MacMahon 1984, Moon and Mehta 1989, DiGiovanni 1991). Epidemiologic data suggest the existence of dietary inhibitors of carcinogenesis. However, it is difficult to sort out from epidemiologic studies which specific compounds within complex foods provide anticarcinogenic effects (Lippman *et al.* 1994). Historically, the first report concerning inhibition of chemical carcinogenesis goes back to the experiment by Berenblum in 1929. In his study, dichloroethyl sulfide inhibited murine skin tumours induced by tar (Berenblum 1929). Theoretically, the chemopreventive approach fits into the biological concepts of field cancerization and multistep carcinogenesis (Farber 1984, Vogelstein *et al.* 1988). The multistep tumorigenic process is thought to be the result of cumulative genetic damage caused by

continuous exposure to carcinogens.

Chemopreventive agents can act in the initiation or in the promotion stage of carcinogenesis. Compounds having the following sites of action are candidates for chemoprevention at the initiation step (Tanaka 1992): 1) inhibition of enzymes responsible for the formation of reactive carcinogenic metabolites or induction of enzyme pathways which produce products of lower carcinogenic potential; 2) detoxification or scavenging of carcinogenic metabolites; 3) enhancement of DNA repair mechanisms. Compounds with the following actions could be antipromotor agents: 1) inhibition of prostaglandin or polyamines synthesis; 2) inhibition of cell proliferation or induction of differentiation; 3) inhibition of signal transduction. In the last few years, the area of chemopreventive research has tremendously progressed from preclinical studies to phase I, II and III clinical trials (De Palo and Formelli 1995). Chemopreventive agents under study in clinical trials now include vitamin A in lung, oral and skin cancer; vitamin C in colon and stomach cancer; synthetic retinoids in breast, cervix, head and neck, lung, and skin cancer; beta-carotene and other carotenoids in breast, cervix, colon, lung, skin, and stomach cancer; fibre in colon cancer; selenium in liver, lung, and skin cancer; calcium in oesophagus and bladder and colon cancer; non-steroidal anti-inflammatory drugs (NSAIDs) in bladder and colon cancer; tamoxifen in breast cancer; vaccination in liver cancer; and dietary regimens in breast, colon, and skin cancer (Osborne *et al.* 1997).

Beta-carotene and the retinoids (which include natural vitamin A, also known as retinol, its esters and synthetic analogues) are the best-studied class of agents in chemoprevention (Lippman *et al.* 1995). The selection of retinoids or beta-carotene in most current chemopreventive studies in the world is based on epidemiological data or animal studies (Schwartz and Shklar 1987, Moon and Metha 1989, Fontham 1990, Boone *et al.* 1990, Battalora *et al.* 1993). There are more than 4,000 different retinoids which have a wide spectrum of preclinical activities, structures, pharmacological profiles, tissue distributions, receptor specifities, and toxicities. Studies on the mechanism of action of retinoids have progressed in the last decade with the discovery of the nuclear retinoic acid receptors (RARs) (Petkovich *et al.* 1987, Giguere *et al.* 1987). The nuclear retinoid receptors are members of the super-family of steroid hormone receptors that act as ligand-activated trans-acting transcription factors. Three different RAR subtypes have been discovered so far: RAR-α, RAR-β and RAR-γ. A second class of retinoid responsive transcription factors was discovered subsequently (Mangelsdorf *et al.* 1990): the retinoid X receptors (RXRs). Three different RXRs have been identified: RXR-α, RXR-β and RXR-γ. Retinoids are ligands for the RARs and RXRs and after binding, homo- or hetero-dimers are formed, and these bind to a specific DNA sequence, the retinoic acid response elements and the retinoid X response elements. These response elements are part of the promotor of certain genes. So in this way up- or downregulation of specific gene transcription is determined by the "retinoid signal transduction pathway". Retinoids have complex biologic effects, including

modulation of differentiation, proliferation, and apoptosis, within both normal and neoplastic tissues (Khuri *et al.* 1997). The pleiotropic effects in the control of gene expression by retinoid signals is generated through complexity at different levels of the signaling pathway, including the type and concentration of both the nuclear receptor and the retinoid (Chambon 1996).

Most clinical randomized chemopreventive trials involve patients with premalignant lesions like leukoplakia (Hong *et al.* 1986, Stich *et al.* 1988a, Stich *et al.* 1988b, Han *et al.* 1990, Chiesa *et al.* 1992, Lippman *et al.* 1993, Costa *et al.* 1994). All these trials, evaluating the efficacy of chemopreventive agents, have used reversal of the premalignant lesion as the study endpoint itself. No study using the reversal of leukoplakia as endpoint has shown to be significantly correlated with the prospective development of cancer. In these trials, it may even be better to speak of chemotherapy of leukoplakia. Therefore, randomized trials using the development of cancer as the primary end point are necessary to evaluate promising chemopreventive agents, as the ultimate goal of chemoprevention is the reduction of cancer development. Most chemoprevention trials are ongoing and results of these trials have to be awaited. Sofar, completed trials did not result in a standard chemopreventive treatment for a specific type of cancer. In breast cancer tamoxifen and fenretinide are promising chemopreventive drugs (Chlebowski *et al.* 1993, Costa 1993). In colon cancer the most favourable agents are the NSAIDs (Marnett 1995). Most cervical cancer chemoprevention trials investigate the effectiveness of retinoids (Mitchell *et al.* 1995).

The results of a few large clinical chemoprevention trials involving beta-carotene using cancer incidence as study end point were published lately and they showed negative effects. The first of these trials was performed in Finland by the alpha-tocopherol, beta-carotene cancer (ATBC) prevention study group. They performed a randomized, double-blind, placebo-controlled primary-prevention trial to determine whether daily supplementation with 50 mg alpha-tocopherol, 20 mg beta-carotene, or both would reduce the incidence of cancer. A total of 29,133 male heavy smokers were randomly assigned to one of four regimens: alpha-tocopherol, beta-carotene, both drugs or placebo. Follow-up continued for five to eight years. At study entry, the men in the cohort averaged 57.2 years of age, smoked an average of 20.4 cigarettes daily, and had smoked for an average of 35.9 years. Unexpectedly, they observed a higher incidence of lung cancer among the men who received beta-carotene. In the beta-carotene group there were 474 cases of lung cancer versus 402 cases in the group not using this compound (Alpha-tocopherol, beta-carotene cancer prevention study group 1994).

Another large trial is the Physicians Health Study in the United States. In this trial 22,071 male physicians were randomized to receive either 50 mg beta-carotene on alternate days or placebo. After a follow-up of 12 years there were no early or late differences in the overall incidence of neoplasms. In the beta-carotene group, 1,273 men had any malignant neoplasm as compared with 1,293 in the placebo group. There also were no significant differences in the number of cases of lung

cancer: 82 in the beta-carotene group versus 88 in the placebo group (Hennekens *et al.* 1996).

The last published study involving beta-carotene and subsequent cancer development is the "beta-carotene and retinol efficacy trial" (CARET). This trial is a multicenter, randomized, double-blind, placebo-controlled primary prevention trial, involving a total of 18,314 smokers, former smokers, and workers exposed to asbestos. The effects of 30 mg of beta-carotene per day and 25,000 IU retinol in the form of retinyl-palmitate per day on the incidence of lung cancer were compared with those of placebo (Goodman *et al.* 1993). The investigators heading the CARET study stopped the intervention 21 months earlier than planned, because 28% more cases of lung cancer developed in the group of participants taking the combination of beta-carotene and retinyl-palmitate than in those on a placebo (Smigel 1996). After an average of four years of supplementation the active-treatment group had a relative risk of lung cancer of 1.28, as compared with the placebo group (Omenn *et al.* 1996a).

No plausible explanation has been offered for the reason why beta-carotene should increase the risk of lung cancer in the ATBC and CARET study. Subgroup analysis indicates that the adverse effects of supplemental beta-carotene in the ATBC study are restricted to persons who smoked one pack of cigarettes or more per day or who drank above-average levels of alcohol (Albanes *et al.* 1996). In the CARET study analysis showed that former smokers who received the supplements were not at increased risk (Omenn *et al.* 1996b). The aggregate data of these additional studies suggest that concurrent cigarette smoke exposure of relatively high intensity is necessary for a promotional effect of supplemental beta-carotene on lung cancer to occur (Mayne *et al.* 1996). A number of investigators have suggested that this increased risk might be associated with the use of synthetic beta-carotene, which consists primarily of the all-*trans* isomer of beta-carotene (Von Eggers Doering 1996). The differences between synthetic all-*trans*-beta-carotene and beta-carotene from natural sources, which also contains the 9-*cis* isomer, may be significant (Challem 1997). There is evidence that the 9-*cis* isomer is far more potent than the all-*trans* isomer as an antioxidant (Ben-Amotz and Levy 1996). The disappointing results of the above mentioned studies indicate that it is important to plan future chemoprevention trials with drugs of which more is known about the mechanism of action.

Chemoprevention of second primary tumours following head and neck cancer

Because squamous cell cancer of the head and neck is likely to remain a significant cause of morbidity and mortality worldwide, it is important to develop effective chemopreventive strategies to decrease its incidence. Furthermore, because the field at risk for this disease includes the oral mucosa, which is easily accessible, studies

of chemoprevention for head and neck squamous cell cancer may serve as a model for other cancer sites (Huber *et al.* 1994). At this moment patients who are cured of head and neck squamous cell cancer form an attractive study population, because of their relatively high risk to develop second primary tumours. However, 65 to 85 % of these patients will never develop another tumour, and thus will not require chemopreventive treatment. Therefore it is essential to identify a subgroup of patients with a higher risk to develop second primary tumours. Beside the consideration of overtreatment of the population that will never develop second primary tumours, we have the opinion that if a subpopulation of head and neck cancer patients could be identified with a much more increased risk, side-effects of chemopreventive agents would be more acceptable.

The first trial showing a beneficial effect of chemopreventive treatment on the development of second primary tumours in cured head and neck cancer patients was the study of the M.D. Anderson Cancer Center in Houston, USA (Hong *et al.* 1990). After treatment with either radiotherapy or surgery or both, 103 patients were randomized to receive either adjuvant 13-*cis*-retinoic acid or placebo. The first 44 patients received 13-*cis*-retinoic acid at a dose of 100 mg/m^2 per day or placebo. Because 13 of these patients required reduction of the dose to 50 mg/m^2 per day due to toxic effects, the protocol was revised to start the remaining 59 patients at a dose of 50 mg/m^2 per day or placebo. The three major study endpoints were primary disease recurrence (local, regional, or distant), the development of a second primary tumour (defined as being at a site more than two centimeter from the previous disease, or occurring more than three years after the initial diagnosis), and survival. Of the 103 patients three were excluded because of protocol violations. At 32 months of follow-up, only two of the 49 patients (4%) receiving 13-*cis*-retinoic acid developed second primary tumours, whereas 12 of the 51 (24%) patients receiving placebo developed second primary tumours. In an update of this trial with 55 months of follow-up, 16 patients (31%) in the placebo group had developed second primary tumours, whereas 7 patients (14%) in the treatment group had developed second primary tumours (Huber *et al.* 1994).

These results were a landmark in head and neck cancer chemoprevention. However, the incidence rate of second primary tumours in the placebo group of 24% is relatively high. After a follow up of 32 months one would expect a rate of approximately eight percent. Furthermore a drawback of 13-*cis*-retinoic acid is its high toxicity. One third of the patients in the 13-*cis*-retinoic acid group did not complete the 12-month course of treatment because of toxicity or non-compliance. These observations, along with evidence from oral leukoplakia studies that low dose 13-*cis*-retinoic acid was both well tolerated and effective, led to a subsequent chemoprevention study now being conducted by the M.D. Anderson Cancer Center. This new trial was designed specifically to determine whether a lower dose of 30 mg/day/m^2 would prevent the formation of new tumours (Benner *et al.* 1994). Results of this study still have to be awaited.

The French Study Group on Head and Neck Tumours (GETTEC) conducted

from 1985 till 1991 a prospective chemoprevention study in patients with T1-2, N0-1 squamous cell cancer of the oral cavity and the oropharynx. 316 patients were randomized to receive either etretinate or placebo during 24 months. Etretinate was given orally at a loading dose of 50 mg/day for the first month followed by a dose of 25 mg/day in the following months. The incidence rate of toxic effects was 51 % in the etretinate group versus 26 % in the placebo group. In the etretinate group in 14 patients the adjuvant treatment was discontinued because of these side-effects versus 5 patients in the placebo group. The five-year survival rates and disease-free survival rates were similar in the two groups. There were no significant differences regarding either local, regional and distant relapses. After a median follow-up of 41 months, 28 patients in the etretinate group and 29 in the placebo group developed a second cancer with, respectively, 12 and 13 in the head and neck region (Bolla *et al.* 1994). The authors concluded that etretinate, a second generation retinoid, does not prevent second primary tumours in patients who have been treated for squamous cell carcinoma of the oral cavity and oropharynx. Two years later the results were updated and after a mean follow-up of 65 months 42 patients in the etretinate group developed a second primary tumour versus 40 patients in the placebo group (Bolla *et al.* 1996).

In 1988 a European chemoprevention study called EUROSCAN was initiated by the head and neck cancer group and the lung cancer cooperative group of the European Organisation for Research and Treatment of Cancer (EORTC). Eligible patients were patients who were previously treated for squamous cancer of the larynx (carcinoma in situ or T1-3, N0-1), squamous cancer of the oral cavity (T1-2, N0-1) or non-small cell lung cancer (T1-2, N0-1 and T3N0). Patients were randomised after surgery and/or completion of radiotherapy to receive retinyl-palmitate 300,000 IU per day for one year and half this dose for a second year, or N-acetylcysteine (NAC) 600 mg daily for two years, or both, or neither of the drugs in a 2x2 factorial design (De Vries *et al.* 1991). When taken orally, NAC is rapidly absorbed, deacetylated, and forms a precursor for the intracellular and extracellular glutathione stores (Bonanomi and Gazzaniga 1980, Meister and Anderson 1983). NAC is a compound that is expected to provide chemopreventive effects by multiple mechanisms, like detoxifying reactive electrophiles and free radicals (De Flora *et al.* 1995). Preliminary data on side effects and toxicity of the drugs used in the Euroscan study have already been published (De Vries *et al.* 1992, De Vries *et al.* 1994). Side-effects were absent in respectively 79.1% of the patients using NAC, in 58.7% of the patients using retinyl-palmitate, and in 53.5% in the patients using both medications. Serious side-effects were noted in respectively 4.0%, 9.4% and 12.2% of the patients (De Vries *et al.* 1993). The most common side effects were mucocutaneous complaints, headache, and dyspepsia. It was concluded from this intermediate analysis of side effects that both the single drugs, as well as the combination treatment in general are well tolerated and that the toxicity is mild as compared with the earlier mentioned protocol as used by Hong (De Vries *et al.* 1993). The results of treatment with the drugs on the development of second primary tumours are not yet available.

Another chemoprevention study with a relatively low dose of 13-*cis*-retinoic acid was initiated in the United States at Stanford. This study is randomizing patients treated for stage I or II head and neck squamous cell cancer to receive 13-*cis*-retinoic acid, 0.15 mg/kg per day for two years, or a placebo. The therapy is initiated as an adjuvant to primary therapy and has begun shortly after the completion of surgery or radiotherapy (Benner *et al.* 1992).

BIOMARKERS

Intermediate endpoint biomarkers

Chemoprevention seems to have potential for reducing the incidence and mortality of second primary tumours in head and neck squamous cell cancer patients. However, because second primary tumours develop at the relatively low annual rate of three percent, chemoprevention trials need large numbers of subjects and a long term follow-up, when cancer incidence is the study endpoint (Shin *et al.* 1994). The use of surrogate endpoint biomarkers in chemoprevention studies, also known as intermediate endpoint biomarkers, would reduce the size, length and cost of clinical prospective randomized trials in high-risk populations.

The multistep tumorigenic process is thought to be the result of an accumulation of genetic damage caused by continuous exposure to carcinogens. Eventually, these genetic alterations give rise to phenotypic changes in the tissues, such as dysregulation of cell proliferation and differentiation. These phenotypic alterations can be driven by alterations of certain specific genes such as tumour suppressor genes (like p53 or rb), oncogenes (like ras and myc), or the genes for growth factors and their receptors (like EGF, EGFR, and TGF-alpha) (Shin *et al.* 1994). It has been estimated that between six and ten separate genetic events must occur before the development of invasive squamous cell cancer (Renan 1993). Therefore, prior to histologically evident invasive disease, one would expect the mucosa to contain detectible genetic aberrations (Lydiatt and Schantz 1996). These genotypic and phenotypic alterations in the mucosa can function as measurable intermediate biomarkers (Wilkinson and Hendricks 1995). They should have the following criteria (Lippman *et al.* 1990a): a) their expression in normal tissue should be different from that in high-risk tissue; b) they can be detected even in small tissue specimens; c) they are expressed in a quantity or pattern that can be correlated with the stage of carcinogenesis. If ideal biomarkers are identified they can be used to monitor trials as early indicators of response or relapse. Biological activity of new chemopreventive agents could be estimated in vivo in short time, and mechanisms of drug activity or resistance could be studied. If preclinical or

early clinical data indicate that the condition represented by a suitable marker can be modulated by study agents, a more rational approach could be provided for long-term phase III trials. At this moment no valuable biomarker with all the above mentioned characteristics has been discovered yet. In table 1, the different classes of biomarkers potentially able to function as intermediate endpoints in chemoprevention trials are listed.

Genomic markers

One of the most extensively studied genomic biomarkers during the last decade is the micronucleus (Shin *et al.* 1994). Micronuclei are chromosome or chromatid fragments formed in proliferating cells during clastogenic events and remain separate after mitosis. The micronuclei in the epithelium of the head and neck area are formed in the proliferating basal cell layer, which gives rise to suprabasal cells that migrate to the epithelial surface and can eventually be detected in easily obtainable exfoliated cells. The presence and frequency of micronuclei in tissue are believed to be quantitative reflections of ongoing DNA damage and/or genetic instability. The frequency of micronuclei was widely studied as a genomic marker in earlier chemoprevention trials (Stich *et al.* 1984, Rosin *et al.* 1987, Stich *et al.* 1988b, Lippman *et al.* 1990b, Stich *et al.* 1991, Prassad *et al.* 1995). Micronucleus frequency, however, indicates an ongoing process of chromosome damage, and not accumulated genetic damage (Papadimitrakopoulou *et al.* 1996). Therefore more

Table 1: *Classes of biomarker candidates in upper aerodigestive tract tumours. (Adapted from the review article of Shin et al., 1994).*

Genomic markers (general):
- Nuclear and chromosomal aberrations (e.g., micronucleus)
- DNA content

Specific genetic markers:
- Oncogene alterations
- Tumour suppressor gene alterations
- Altered gene expression

Proliferation markers:
- Mitotic frequency
- Thymidine labelling index
- Nuclear antigens (e.g., PCNA, Ki-67)
- Polyamines, ornithine decarboxylase

Differentiation markers:
- Cytokeratins
- Transglutaminase type I
- Involucrin

specific alterations of genetic and phenotypic markers resulting from DNA damage by carcinogens are needed (Shin *et al.* 1994). It has been shown in both experimental animal models (Kato *et al.* 1982, Näslund *et al.* 1987) and clinical investigations that the development of squamous cell carcinomas, for example in the bronchial tree (Nasiell *et al.* 1982) and the uterine cervix (Nasiell *et al.* 1979) is paralleled by a progressive increase in nuclear DNA content resulting in a transition of diploid cells in genetically unstable aneuploid cells. It has also been hypothesised that aneuploidy in preinvasive lesions still may be a reversible cellular alteration but nevertheless clearly indicates obligatory precancerous changes in the development of squamous cell carcinoma (Steinbeck *et al.* 1993).

Specific genetic markers

Activation of cellular protooncogenes or inactivation of tumour suppressor genes are the most important genetic alterations in the multistep process of carcinogenesis. The p53 gene is together with the retinoblastoma gene (rb) one of the most extensively studied tumour suppressor genes. The p53 gene, which maps on the short arm of chromosome 17, encodes a nuclear protein. This protein induces cell-cycle arrest at the G_1 checkpoint, allowing DNA repair or induction of cell apoptosis in response to genotoxic damage (Papadimitrakopoulou *et al.* 1996). As such it functions as a "guardian of the genome" (Hartwell and Kastan 1994). Many types of p53 alterations have been observed in a wide variety of cell lines and tumours, the most frequent being point mutations primarily in exons five to eight (Papadimitrakopoulou *et al.* 1996). p53 mutation patterns have been analyzed to identify specific changes associated with particular carcinogens (Greenblatt *et al.* 1994). So far, investigators have found frequent p53 inactivation in head and neck squamous cell carcinomas (Gusterson *et al.* 1991, Field *et al.* 1991, Brachman *et al.* 1992), slightly less frequent inactivation in dysplasias (Boyle *et al.* 1993), and more frequent mutation in smokers and drinkers, versus nonsmokers and nondrinkers (Brennan *et al.* 1995).

Other interesting genomic markers are the RARs and RXRs. On the basis of findings of studies of nuclear retinoid receptors in squamous cell carcinoma, premalignant tissue, and normal tissue by Xu (Xu *et al.* 1994), biopsy specimens of 52 patients with oral leukoplakia were evaluated for the expression of RARs and RXRs, both before and after treatment with 13-*cis*-retinoic acid (Lotan *et al.* 1995). All specimens of healthy controls contained RAR-β messenger RNA, but only 40% of the pretreated leukoplakias had detectable RAR-β messenger RNA levels ($p=0.003$). After treatment with 13-*cis*-retinoic acid during three months 90% of the leukoplakia specimens expressed detectable amounts of RAR-β messenger RNA. The levels of RAR-β increased in 82 % of the patients who had responses to 13-*cis*-retinoic acid and only in 47 % of the patients without responses ($p=0.04$). These results demonstrate that the expression of RAR-β seems to be a promising indicator of retinoid chemopreventive efficacy.

Proliferation markers
It is speculated that only tissues with relatively high levels of proliferative cells are associated with premalignant and malignant tissue changes during tumorigenesis (Shin *et al.* 1994). Several studies indicate that the proliferating cell nuclear antigen (PCNA) could be a useful biomarker for multistep carcinogenesis in head and neck cancer and that its expression could serve as an intermediate end point in chemoprevention trials (Lee *et al.* 1992, Shin *et al.* 1993). Another proliferating markers is Ki-67. Ki-67 is a monoclonal antibody directed against a human nuclear antigen present in normal and neoplastic proliferating cells during all phases of the cell cycle except G_0 and early G_1 (Gerdes *et al.* 1983, Falini *et al.* 1989).

Differentiation markers
The mucosa of the upper aerodigestive tract is formed by squamous epithelium. Keratinization of this epithelium is observed at the dorsum of the tongue, the gingiva and the hard palate. During normal maturation the cells move from the basal layer upwards and obtain characteristics associated with squamous differentiation (Jetten 1987). The cytokeratins, a family of at least 20 intermediate-size filaments, are good differentiation markers because they are differently expressed during squamous differentiation (Moll *et al.* 1982, Papadimitrakopoulou *et al.* 1996). Cytokeratin expression can be aberrant during carcinogenesis of squamous tissue (Jetten 1987). Levels of cytokeratin 16 and 19 were found to be elevated in macroscopically normal mucosal cells of head and neck cancer patients (Bongers *et al.* 1995b). Another marker for squamous cell differentiation is involucrin, one of the major protein components of cornified envelopes (Eckert 1989). This protein undergoes extensive cross-linking by the membrane-associated enzyme type 1 transglutaminase (Thacher and Rice 1985). Squamous differentiation is usually accompanied by increases in the levels of involucrin and transglutaminase 1 (Simon and Green 1985). Involucrin is expressed in premalignant lesions and squamous cell carcinomas (Kaplan *et al.* 1984, Murphy *et al.* 1984). This squamous cell differentiation marker was shown to be modulated by retinoic acid in cell lines (Lotan 1980, Lotan *et al.* 1987).

Biomarkers of compliance

A well known problem in clinical chemoprevention trials is that it is troublesome to monitor patient compliance adequately. Compliance could be a major clinical variable with long prospective trials involving cancer prevention. Monitoring is in most trials realized by asking patients about the intake of their chemopreventive medication or by counting empty pill boxes. In the 13-*cis*-retinoic acid chemoprevention trial of the M.D. Anderson Cancer Center in Houston, compliance was evaluated by means of a pill count at each clinic visit and a daily

calendar completed by each patient (Hong *et al.* 1990). In the GETTEC and the EORTC studies, compliance was assessed by simply asking the patients if they had regularly taken their capsules (Bolla *et al.* 1994). In the ATBC study compliance was assessed by counts of the remaining capsules at each visit of the participant (The Alpha-tocopherol, beta-carotene cancer prevention study group 1994). In the CARET study the subjects compliance was assessed by weighing the returned bottles to estimate the number of capsules remaining or by relying on the subjects own estimates. All these procedures are subjective and the reliability of these methods is questionable. Only in the Physicians Health Study, participants were sent yearly follow-up questionnaires, asking about their compliance with the treatment regimen. To assess the validity of this reported compliance with the assigned treatment, they measured plasma beta-carotene concentrations in blood obtained at unannounced visits to study participants offices in three geographic areas (Satterfield *et al.* 1990, Hennekens *et al.* 1996). Next to monitoring the premalignant condition of the mucosa targeted during or after chemopreventive intervention, a biomarker could be used to monitor patient compliance. Depending on the intervention design, serum levels of specified components might be used as compliance markers (Greenwald *et al.* 1992).

AIM OF THE STUDY AND OUTLINE OF THIS THESIS

Adjuvant chemopreventive medication is a potential modality in the treatment of head and neck cancer patients. Because of the long duration of follow-up that is required to conduct chemoprevention trials, clinical experience on this subject is increasing slowly. The present study focuses on several aspects of research on head and neck cancer chemoprevention, and attempts to increase the efficacy of future head and neck cancer chemoprevention trials. Two chapters of this thesis concentrate on the importance of intermediate endpoint biomarkers. In chapter two several biomarkers in condemned mucosa of the oral cavity in head and neck cancer patients are investigated to identify potential markers to serve as intermediate endpoints. A study on the application of cytomorphometric parameters in the chemoprevention trial Euroscan is described in chapter three. In chapter four a study describing a protocol for counting micronuclei is defined to use this biomarker subsequently as an intermediate endpoint marker in chemoprevention trials. In chapter five of this thesis a new method to measure retinoids in plasma is described. In chapter six the problem to monitor patient compliance in a chemopreventive trial accurately is discussed, and the above mentioned new technique is applied to evaluate several retinoids in the plasma of head and neck cancer patients and controls, and in patients using retinyl-palmitate. Several of these retinoid metabolites can serve as biomarkers, able to control patient compliance in

chemoprevention trials using retinoids as chemopreventive agents. In chapter seven of this thesis the importance of basic research on the subject of chemoprevention is discussed and a study, undertaken to evaluate the effects of retinoic acid on the expression of several genes in head and neck squamous cell cancer cell lines is described.

CHAPTER 2

A PANEL OF BIOMARKERS OF CARCINOGENESIS OF THE UPPER AERODIGESTIVE TRACT AS POTENTIAL INTERMEDIATE END POINTS IN CHEMOPREVENTION TRIALS

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Cancer 1993, 71:825-830.

Abstract

Patients with squamous cell carcinoma of the head and neck have a 15-35% risk of developing a second primary tumour. The concept of "field cancerization" assumes that the whole upper aerodigestive tract is affected and prone to malignant transformation. This study was undertaken to investigate the value of a panel of monoclonal antibodies to identify biomarkers in oral mucosa associated with cancer risk. Such biomarkers may be suitable candidates to serve as intermediate end points in cancer chemoprevention trials. As a model, the expression of antigens was assessed in cytological preparations obtained from macroscopically normal oral mucosa of patients with tongue carcinoma and of controls. The panel consisted of antibodies against cytokeratin 8, 10, 13 and 19 and the monoclonal antibodies designated K931, K984, E48, Ki-67, and UM-A9. Oral mucosa of cancer patients had a more than threefold increased expression of cytokeratin 19 as compared with controls (36.0 versus 11.3 %; $p < 0.01$).

Introduction

The occurrence of multiple primary tumours of the upper aerodigestive tract is an increasingly recognized problem in head and neck oncology. Patients with squamous cell cancer of the head and neck have an increased incidence of second primary tumours of the upper aerodigestive tract, occurring either synchronously or metachronously. Approximately 15-35% of such patients develop second primary tumours (Gluckman *et al.* 1980, Shapsey *et al.* 1980, McGuirt *et al.* 1982, De Vries *et al.* 1986, De Vries *et al.* 1990). A second primary tumour usually carries a bad prognosis because it often occurs either at notorious bad sites, like lung or oesophagus, or within previously treated areas within the head and neck, defying curative treatment. To explain this phenomenon, the concept of "field cancerization" is a generally accepted theory (Slaughter 1946, Slaughter *et al.* 1953, Incze *et al.* 1982, Lippman *et al.* 1990b, Ogden *et al.* 1990, Hittelman *et al.* 1991). Field cancerization is thought to develop in response to carcinogens, such as tobacco and alcohol, affecting the entire area of mucous membranes of the aerodigestive tract.

Prevention of second primary tumours has been gaining much interest during the last decade. Besides eliminating the above-mentioned etiologic factors, cancer prevention through the use of chemical intervention regimens, also called chemoprevention, is becoming a promising new field of clinical oncology. Intervention studies are time consuming, require a large number of subjects, and are expensive to conduct. Although prospective, randomized clinical trials with the occurrence of a second primary tumour or even death as the endpoint are the most desirable means to test the efficacy of a possible chemopreventive agent, the application of biomarkers to predict cancer occurrence would be very advantageous. If good biomarkers could be

found, it would be possible to perform many more trials, with fewer costs and a shorter trial period, with modulation of the biomarker as a surrogate endpoint. Ideally, such a biologic marker should be able to reflect the degree of field cancerization of the upper aerodigestive tract and to respond to improvements in this premalignant tissue, as modulated by chemopreventive agents.

In this study, the value of a panel of potential "biomarkers of risk" was evaluated with regard to their ability to identify mucosa at high risk for cancer of the upper aerodigestive tract. This panel consisted of the following markers, which are associated with proliferation and differentiation of squamous cells:

1. Cytokeratin 8 is one of the 19 intermediate filament proteins characteristic of epithelial cells. This keratin is normally absent in epithelium of the dorsal tongue and is characteristic of simple epithelium (Moll *et al.* 1982, Cooper *et al.* 1985). In moderate and poorly differentiated oral squamous cell carcinomas, keratin 8 may be present (Morgan *et al.* 1987a).
2. Cytokeratin 10 is specific for cornifying squamous epithelia (Moll *et al.* 1982, Morgan *et al.* 1987b). An increasing expression of cytokeratin 1 (which is always coexpressed with cytokeratin 10) in a time-dependent fashion during the development of squamous cell cancer, was described in a previous study (Gimenez-Conti *et al.* 1990).
3. Cytokeratin 13 is usually present in moderate amounts in the suprabasal layer of noncornifying, stratified squamous epithelia (Moll *et al.* 1982, Cooper *et al.* 1985, Nagle *et al.* 1985, Van Muijen *et al.* 1986, Morgan *et al.* 1987a, Morgan *et al.* 1987b). An increase in cytokeratin 13 expression during the development of squamous cell cancer was found in the golden Syrian hamster cheek pouch carcinogenesis model (Gimenez-Conti *et al.* 1990).
4. Cytokeratin 19 is normally present in minor amounts in the basal cell layer of noncornifying regions of oral epithelia (Moll *et al.* 1982, Cooper *et al.* 1985, Nagle *et al.* 1985, Coltrera *et al.* 1990). Other researchers also have found variable degrees of expression in the suprabasal layers of epithelium of the tongue (Bartek *et al.* 1986, Morgan *et al.* 1987b). Recent results suggest increased suprabasal expression of cytokeratin 19 to be a marker related to preneoplastic situations (Lindberg *et al.* 1989, Cintorino *et al.* 1990).
5. K931 is a monoclonal antibody that recognizes simple epithelia. It is normally absent in tongue epithelium but can be present in squamous cell carcinomas (Quak *et al.* 1990a).
6. K984 is a monoclonal antibody that recognizes basal cells in squamous epithelia (Quak *et al.* 1992). Its expression often is found to be extended to suprabasal layers in squamous cell carcinomas.
7. E48 is a monoclonal antibody which is reactive with all cells in squamous epithelium. E48 antigen expression was found to be decreased in a proportion of squamous cell carcinomas (Quak *et al.* 1990b, Schrijvers *et al.* 1991).
8. UM-A9 is a monoclonal antibody that identifies an antigen found on the basal surface of epithelial cells and is expressed by squamous cell carcinomas (Kimmel *et al.* 1986). The UM-A9 antigen was found to be a member of the integrin superfamily of cell-adhesion receptors consisting of $\alpha^6\beta_4$ glycoprotein chains (Van Waes *et al.* 1990). It is likely that A9 antigen has an important role in normal growth regulation of squamous tissue because the expression of this antigen is high in cancers with a propensity for recurrence or metastasis, whereas A9 antigen expression is low or absent in cancers that invade locally, but do not exhibit metastatic behavior (Wolf *et al.* 1990). Evidence was found that changes in UM-A9 expression may occur early in cancer development (Lippman *et al.* 1990a).
9. Ki-67 is a monoclonal antibody directed against a human nuclear antigen present in normal and neoplastic proliferating cells during all phases of the cell cycle except G_0 and early G_1 (Gerdes *et al.* 1983, Falini *et al.* 1989).

Patients and methods

Patient and control population

In 10 consecutive patients presenting with squamous cell carcinoma at the lateral side of the tongue, cytological preparations were obtained from normal, healthy appearing mucosa of the dorsal tongue on the contralateral side of the carcinoma. Laboratory personnel, patients admitted for nonmalignant disease, and spouses of patients served as controls (N=10). Smoking history, current smoking, and alcohol consumption of patients and controls were recorded (table 1).

Cell preparation

After the mouth was moistened with water, cells were collected with a cytobrush (Cytobrush Plus®, Medscand AB, Malmö, Sweden). Mucosal cells from controls were obtained from the lateral part of the dorsal tongue. In patients the contralateral side of the tongue carcinoma was brushed. The brushes were then stirred in 2 ml of Dulbecco's Modification of Eagle's Medium (DMEM) (Flow Laboratories, Irvine, UK), allowing the cells to be released from the brush. Cells were washed with DMEM (1,000 rpm for 10 minutes) and collected in 2 ml DMEM. This way of cell sampling gives a clean cell solution, containing more than 100,000 mucosal cells. Cytocentrifuge preparations were made on poly-L-lysine (Sigma Chemical Co., St. Louis, USA) coated glass slides. Slides were air dried and fixed with 100% methanol for 15 minutes. Slides were again air dried for 60 minutes and then stored at -70°C.

Antibodies

Ten monoclonal antibodies were used in this study. Their specificities and references are listed in table 2. Antibodies M20 and RKSE60 were provided by Dr. F.C.S. Ramaekers (University of Maastricht, the Netherlands). Antibody UM-A9 was provided by Dr. T.E. Carey (University of Michigan, Ann Arbor, USA). Antibodies

K931, K984 and E48 were developed by the departments of Otolaryngology and Pathology of the Free University Hospital, Amsterdam, the Netherlands. Antibodies Ks 13.1 and 170.2.14 were purchased from Boehringer Mannheim Biochemica, Mannheim, Germany. Ki-67 was obtained from Dakopatts, Glostrup, Denmark. Antidesmin was obtained from Sanbio, Uden, the Netherlands.

Table 1: *Characteristics of patients and controls.*

Number	Age in years	Sex	Smoking cig/day	Smoking pack years	Alcohol: units/day	TNM (UICC)
Patients:						
1	82	M	25	66	0	$T_1N_0M_0$
2	42	F	0	0	0	$T_2N_0M_0$
3	62	F	30	56	4	$T_2N_0M_0$
4	31	F	0	0	0	$T_3N_0M_0$
5	57	F	15	24	0	$T_2N_0M_0$
6	62	F	20	34	1	$T_1N_0M_0$
7	62	M	35	46	6	$T_3N_{2b}M_0$
8	67	M	25	50	3	$T_3N_{2a}M_0$
9	65	M	25	40	4	$T_1N_0M_0$
10	45	M	10	12	3	$T_2N_0M_0$
Mean:	58	50%	18.5	32.8	2.1	
SD:	15	male	12.0	23.2	2.2	
Controls:						
1	34	M	0	0	1	
2	28	M	0	0	1	
3	33	M	0	0	1	
4	41	F	0	0	0	
5	53	F	0	0	0	
6	56	F	10	16	0	
7	48	M	40	48	4	
8	60	F	0	0	1	
9	65	F	20	40	2	
10	50	F	0	0	1	
Mean:	47	40%	7.0	10.4	1.1	
SD:	12	male	13.4	18.5	1.2	

M: Male; F: Female
cig: cigarettes
SD: Standard deviation

Immunocytochemistry

Immunocytochemical staining was performed with the immunoperoxidase technique with an avidin-biotin complex (Vectastain ABC-kit, Vector Laboratories, Burlingame, USA). Non-specific staining was blocked with normal horse serum (Vector Laboratories) for 20 minutes, and endogenous peroxidase activity by using 0.006% H_2O_2 in methanol for 10 minutes. After being washed with phosphate-buffered saline, specific monoclonal antibodies were incubated for 60 minutes at room temperature. After being washed three times with phosphate-buffered saline, slides were incubated with biotinylated horse-anti-mouse immunoglobulins (Vector Laboratories), washed again, and incubated with avidin-biotin-peroxidase complex. The peroxidase label was developed with diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). Weak counterstaining was performed with hematoxylin and eosin for 30 seconds. The stained slides finally were dehydrated and mounted with Entellan® (Merck, Darmstadt, Germany). A negative control was made of each person by the use of a monoclonal antibody against desmin, which is normally absent in epithelium.

Table 2: *Monoclonal antibodies used in this study.*

mAb	References	Specificity
M20	Van Muijen *et al.* 1987	Cytokeratin 8
RKSE60	Ramaekers *et al.* 1985	Cytokeratin 10
Ks 13.1	Moll *et al.* 1982 Moll *et al.* 1988	Cytokeratin 13
170.2.14	Moll *et al.* 1982 Quinlan *et al.* 1985 Osborn *et al.* 1986	Cytokeratin 19
K931	Quak *et al.* 1990a	Simple epithelia
K984	Quak *et al.* 1992	Basal squamous cells
E48	Quak *et al.* 1990b	Squamous cells, basal and suprabasal.
UM-A9	Kimmel *et al.* 1986 Wolf *et al.* 1988	$\alpha^5\beta_4$ integrin
Ki-67	Gerdes *et al.* 1983 Falini *et al.* 1989	Nucleus of proliferating cells

mAb: Monoclonal antibody

Quantification and statistical analysis

Evaluation was performed on coded slides. The percentage of immunocytochemically positive cells was scored by light microscopy from at least 250 cells per slide. There was a clear difference between positive and negative cells. Percentages of positive cells were analyzed by way of linear multiple regression to correct for the possibly confounding variables of age, gender, current smoking, and alcohol consumption.

Results

Results are shown in table 3. K931, K984, and UM-A9 were neither expressed in the cells obtained from patients nor in the cells obtained from the controls. In addition, Ki-67 scored negative and did not show to be of value as a biomarker of risk. Scoring of immunoreactivity of this antibody was impossible because of extensive cytoplasmic staining in most cells. This cross-reactivity with an as yet unknown cytoplasmic constituent in squamous epithelia has been reported previously (Falini *et al.* 1989).

Table 3: *Antigen expression of exfoliated cells. Results are shown as percentages of positive cells.*

	CK8	CK10	CK13	CK19	K931	K984	E48	Ki-67	UM-A9
Patients:									
1	0	0.5	9	11	0	0	100	0	0
2	0	1	28	27	0	0	100	0	0
3	1	0.5	6	16	0	0	100	0	0
4	0	0	31	20	0	0	100	0	0
5	0	0	24	40	0	0	100	0	0
6	0	5	44	50	0	0	100	0	0
7	0.5	1	70	64	0	0	100	0	0
8	0	0.5	37	32	0	0	100	0	0
9	0	3	79	79	0	0	100	0	0
10	0	0	17	21	0	0	100	0	0
Mean:	0.15	1.15	34.5	36	0	0	100	0	0
SD:	0.34	1.62	24.2	22.2	0	0	0	0	0
Controls:									
1	0	0.5	11	10	0	0	100	0	0
2	0	0	9	7	0	0	100	0	0
3	0	0	7	8	0	0	100	0	0
4	0	0	2	6	0	0	100	0	0
5	0	0	21	14	0	0	100	0	0
6	0.5	0.5	3	3	0	0	100	0	0
7	0	0	15	7	0	0	100	0	0
8	0	1	26	22	0	0	100	0	0
9	0	0	18	14	0	0	100	0	0
10	0	1	28	22	0	0	100	0	0
Mean:	0.05	0.3	14	11.3	0	0	100	0	0
SD:	0.16	0.42	9.2	6.6	0	0	0	0	0

SD: Standard deviation; CK: Cytokeratin.

Percentages of positive cells were analyzed with linear multiple regression to correct for the possibly confounding factors of age, gender, smoking, and alcohol consumption. An increase in expression in the normal-appearing mucosa of cancer patients was observed with cytokeratin 13 and 19. Statistical evaluation of the data revealed that the increase of cytokeratin 19 expression is a highly significant, independent factor related to tongue cancer ($p<0.01$). Cytokeratin 13 increase just missed statistical significance (p:0.06).

Discussion

The aim of this study was to validate a panel of potential biomarkers able to identify and quantify the process of field cancerization in the upper aerodigestive tract. Such biomarkers may be suitable candidates to serve as intermediate endpoints that predict the effectiveness of chemoprevention of a second primary tumour in head and neck cancer patients. As a model, potentially "condemned mucosa" of tongue cancer patients was investigated for different expression of several biomarkers as compared with mucosal cells of controls without cancer. Since Slaughter's original hypothesis in 1946 (Slaughter 1946), only a few reports are known that found direct evidence in favour of the theory of field cancerization (Incze *et al.* 1982, Ogden *et al.* 1990, Hittelman *et al.* 1991). We believe we have found new evidence to support this theory.

The most promising marker of the investigated panel of monoclonal antibodies seems to be cytokeratin 19 (figure 1).

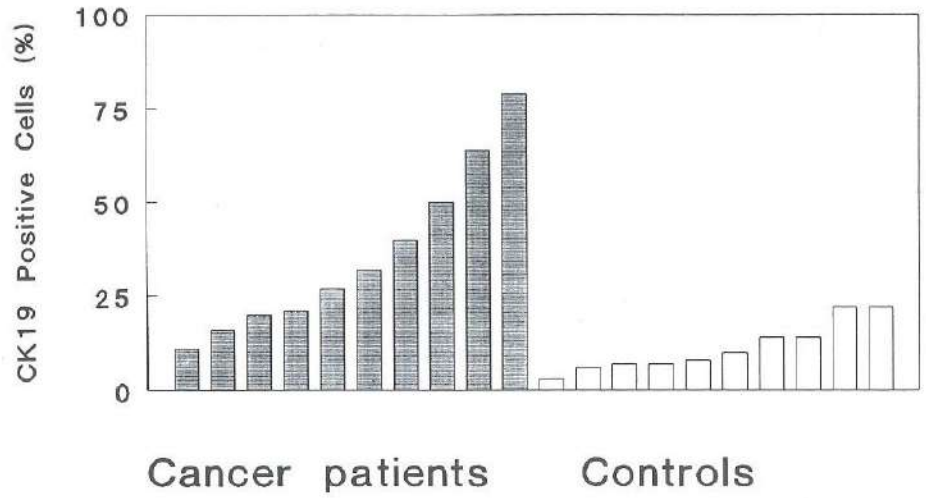

Figure 1: *Percentage of cells with cytokeratin 19 (CK19) expression in macroscopically normal oral mucosa of either patients with a tongue carcinoma or controls without cancer.*

Normally, expression of cytokeratin 19 is scattered throughout the basal cell layer of the noncornified upper aerodigestive tract epithelia (Moll *et al.* 1982, Cooper *et al.* 1985, Nagle *et al.* 1985, Coltrera *et al.* 1990). In dysplastic epithelia as well as in squamous cell carcinomas, the expression of cytokeratin 19 increases and is extended to the suprabasal layers (Morgan *et al.* 1987a, Lindberg *et al.* 1989, Coltrera *et al.* 1990, Cintorino *et al.* 1990). An inverse relation between cellular content of cytokeratin 19 and the terminal differentiation marker involucrin in normal and dysplastic tissue has been described (Lindberg *et al.* 1989), which suggests that keratin 19 expression is linked to retention of the stem cell character. Brushing of the suprabasal layers produced a statistically significant increase in cytokeratin 19 expression in the potentially "condemned mucosa" of the cancer patients. This supports the theory that increased cytokeratin 19 expression is a marker related to premalignancy (Lindberg *et al.* 1989, Cintorino *et al.* 1990). The exact cause of this relationship is unknown. It may represent merely a failure of maturation or increased epithelial turnover kinetics.

The second promising biomarker, cytokeratin 13, is normally present in moderate amounts in the suprabasal layer of noncornifying stratified squamous epithelia (Moll *et al.* 1982, Cooper *et al.* 1985, Nagle *et al.* 1985, Van Muijen *et al.* 1986, Morgan *et al.* 1987a, Morgan *et al.* 1987b). Our findings indicate an increase in expression of this keratin in the normal-appearing mucosa of patients with tongue cancer; however, this value just missed statistical significance (p:0.06).

The noninvasive method of obtaining tissue, as described here, has proved feasible. This is a great advantage in clinical biomarker research. Especially in a population free of disease, it often is not possible to obtain tissue by invasive methods such as biopsy. In addition, many slides can be prepared for analysis with multiple monoclonal antibodies. However, in the case of markers that have a very low expression in epithelial cells (e.g., cytokeratin 8 and 10), the values may be too low to be analyzed.

CHAPTER 3

CYTOMORPHOMETRIC PARAMETERS IN EXFOLIATED CELLS AS BIOMARKERS IN HEAD AND NECK CANCER CHEMOPREVENTION

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International Journal of Oncology 1996, 9:1071-1075.

Abstract

Quantitative DNA analysis has often been proposed as a potential tool capable to detect preneoplastic tissue and as such to function as an intermediate endpoint in cancer chemoprevention trials. The first aim of this study was to test whether cytomorphometric parameters could be used to detect field cancerization characteristics in cytological preparations of oral mucosa. Cytomorphometric parameters in exfoliated cells of apparently normal oral mucosa of head and neck cancer patients were compared with those of healthy controls. The second aim was to assess the value of these parameters subsequently as intermediate endpoint biomarkers in the mucosa of 70 patients receiving N-acetylcysteine and/or retinyl-palmitate as chemopreventive drugs. No differences were detected between "high risk" mucosa and healthy mucosa, nor were differences observed before and after treatment.

Introduction

Squamous cell cancer of the head and neck comprises about 5 % of all cancers in the western world. One of the major problems in patients with early stage head and neck squamous cell carcinoma is the occurrence of second primary tumours after curative therapy of their initial tumour. Patients curatively treated for squamous cell carcinoma of the head and neck have a 15 to 35 percent risk to develop second primary tumours in the respiratory and upper digestive tract (Tepperman and Fitzpatrick 1981, Markman 1981, De Vries *et al.* 1986, De Vries and Snow 1986, Wagenfeld *et al.* 1980, Haughey *et al.* 1992). The large majority of these second primary cancers occurs in the respiratory and upper digestive tract. Because similar risk factors as for head and neck squamous cell carcinoma play a role in the etiology of lung or oesophageal cancer, it is not astonishing that head and neck squamous cell carcinoma patients are predisposed to develop second primary tumours at these sites. This phenomenon of occurrence of multiple primary tumours in a single patient can be explained by the theory of the "field cancerization" (Slaughter 1946, Slaughter *et al.* 1953), which assumes that the entire surface of the mucous membranes of these tracts is affected in response to carcinogens like tobacco and alcohol and on its way to manifest malignancy. It is generally believed that the mucosa of most head and neck squamous cell carcinoma patients is extremely susceptible to external carcinogenic stimuli. Next to these environmentally induced risk factors, the individual, genetically determined susceptibility to develop squamous cell cancer may be of importance as well (Copper *et al.* 1995). This pre-malignant, oversensitive mucosa is known as "condemned mucosa". Previous studies have shown that a change in expression of several markers reflects this process of field cancerization in the mucosa of the head

and neck (Ogden *et al.* 1991, Copper *et al.* 1993).

Prevention of second primary tumours is gaining much interest during the last decades. The elimination of the well known etiologic factors, smoking and alcohol consumption (Moore 1971, Schottenfeld 1979, Brugère *et al.* 1986) is of importance in the primary prevention of tumours (Day *et al.* 1994). Nowadays the use of chemical intervention regimens to prevent new tumour development, also known as chemoprevention, represents a new promising strategy in cancer prevention (De Vries 1990, Mayne and Goodwin 1993). At the moment, the largest chemoprevention study in head and neck squamous cell carcinoma and lung cancer patients is the Euroscan study (De Vries *et al.* 1991, De Vries *et al.* 1993). This study began in 1988 under the responsibility of the European Organization for Research and Treatment of Cancer (EORTC). In this chemoprevention study the possibility to prevent second primary cancers in patients who are cured from early stage head and neck squamous cell carcinoma or lung cancer is investigated. Retinyl-palmitate and/or N-acetylcysteine are being used as chemopreventive drugs in this trial.

A well known problem in chemoprevention research is the long duration of clinical trials and the large number of patients needed to conclude these trials. Although a prospective randomized clinical trial with the occurrence of second primary tumours or even death as study-endpoint is the most agreeable procedure for investigating the efficacy of a potential chemopreventive agent, the application of biomarkers to estimate cancer risk would be very advantageous. In this respect the modulation of the biomarker could serve as a surrogate endpoint instead of classical study endpoints like the incidence of cancer or overall survival. Biomarkers which are able to serve as a surrogate study endpoints are generally referred to as intermediate endpoint biomarkers. If a panel of suitable biomarkers could be established, it would be possible to increase the efficacy of phase II chemoprevention trials (Lippman *et al.* 1990a, Greenwald *et al.* 1992, Schatzkin *et al.* 1993). In patients curatively treated for squamous cell carcinoma in the head and neck area, biomarkers of carcinogenesis should be able to reflect the intensity of field cancerization of the mucosa of their respiratory and upper digestive tracts. Subsequently, if patients are treated with chemopreventive agents in order to improve the condition of their pre-malignant mucosa, the biomarker should respond to this recovery.

Quantitative DNA analysis often has been proposed as a potential tool capable to detect pre-neoplastic tissue (Cowpe *et al.* 1990, Tucker *et al.* 1994) and as such to function as an intermediate endpoint in cancer chemoprevention trials (Kelloff *et al.* 1990, Malone 1991, Kelloff *et al.* 1994, Boone and Kelloff 1994, Palcic 1994). To the best of our knowledge, this study is the first one in which the utility of computerized image analysis in a head and neck squamous cell carcinoma chemoprevention trial is investigated. The first aim of this study was to validate cytomorphometric characteristics in cytological preparations of oral mucosa of head and neck squamous cell carcinoma patients as suitable biomarkers able to detect the

process of field cancerization in their "condemned" mucosa. As a model, potentially "condemned" oral mucosa of head and neck squamous cell carcinoma patients was investigated for different expression of several cytomorphometric features as compared with oral mucosa of controls without cancer. In an earlier study, using the same model, we confirmed that the macroscopically normal mucosa of head and neck squamous cell carcinoma patients showed considerable differences in cytokeratin content as compared with the mucosa of healthy controls (Copper *et al.* 1993), which demonstrated that the use of non-invasive cytological samples was a feasible approach. The second aim of this study was to observe the effect of retinyl-palmitate and N-acetylcysteine on the selected cytomorphometric parameters.

Materials and methods

Patient and control population

The population of cancer patients used to examine whether cytomorphometric parameters could be used to detect condemned mucosa, comprised 5 males and 5 females with cancer of the oral cavity (T1-T3, N0-N2b, M0, according to the TNM classification of the UICC 1987) whose average age was 58 years. Patients who were admitted for non-malignant disease, spouses of patients and laboratory personal served as controls. This population comprised 4 males and 6 females with an average age of 47 years. Cytological preparations were obtained from macroscopically normal, healthy appearing mucosa of the oral cavity.

In order to study the modulation of the cytomorphometric biomarkers by chemopreventive agents, cytological preparations of mucosa of the oral cavity were analyzed in patients, who were randomized for the chemoprevention study Euroscan. These patients used retinyl-palmitate 300,000 IU daily during 1 year and half this dose during a second year, or N-acetylcysteine 600 mg during 2 years, or both drugs or neither in a 2x2 factorial design. Cytological samples were taken from the mucosa of the oral cavity, before the onset of therapy and 2 or 3 months later during therapy. This group of patients comprised 70 persons. 17 patients received retinyl-palmitate during two years, 19 patients used N-acetylcysteine and 18 patients used the combined treatment. 16 patients who used no medication served as control group.

Specimen preparations

After moistening the mouth with water, cells were collected using a Cytobrush Plus® (Medscand AB, Malmö, Sweden). Mucosal cells were obtained from the buccal mucosa in the oral cavity. In patients with an oral squamous cell carcinoma brushes were taken at least 2 cm away from the tumour. The brushes were then stirred in 2 ml of Dulbecco's Modification of Eagle's Medium (DMEM) (Flow Laboratories, Irvine, UK), allowing the cells to be released from the brush. Cells

were washed with DMEM, centrifuged at 1,000 rpm for 10 minutes and collected in 2 ml DMEM. This way of cell sampling gives a clean cell solution, containing more than 100,000 mucosal cells. Cytocentrifuge preparations were made on poly-L-lysine coated glass slides. Slides were air-dried and fixed with 100 % methanol for 15 minutes. Slides were again air-dried for 60 minutes and stored at -70°C.

Cytological examination
One set of cytospins was stained with Giemsa (Merck, Darmstadt, Germany) and evaluated for cytological abnormalities. In this set dysplastic cells were not encountered.

Staining
All specimens were Feulgen stained according to the CAS quantitative DNA staining kit (Becton and Dickinson, Erembodegem, Belgium) in batches of 14 or 15 specimens with 1 or 2 CAS calibration slides. In brief, the specimen was hydrolysed in 5 N HCl for 60 minutes, placed in thionin containing staining solution for 60 minutes, and then rinsed using the CAS reagents. After washing in deionized water for 5 minutes, dehydration in absolute ethanol and xylene, the slides were mounted with Entellan® (Merck, Darmstadt, Germany). All handling was done at room temperature.

DNA cytomorphometry
Determination of the cytomorphometric features was done interactively using the CAS model 200 image analysis system equipped with the quantitative DNA plus software module as described previously for the CAS 100 system (De Cresce 1986, Bacus and Grace 1987, Taylor *et al.* 1989). Using a 40x objective (numerical aperture: 0.66), pixel size was 0.5 x 0.4 μm^2. The CAS system is calibrated for mass measurements in picograms using the predeposited control cells staining a known amount of DNA on each slide. From this calibration slide 50 tetraploid rat nuclei were measured as standard.

The following cytomorphometric markers were analyzed: Integrated optical density, nuclear area, nuclear shape, angular second moment, contrast, inverse difference moment, sum variance, entropy, difference variance, difference entropy, coefficient of variation, peak transition probability, diagonal variance, triangular symmetry, nuclear blobness and standard deviation (Thunnissen *et al.* 1992). Of each specimen 30 nuclei were analyzed.

Statistical analysis
The quantitative features of condemned mucosa and healthy mucosa were compared with univariate analysis. In addition these quantitative features were evaluated for a possible effect of chemoprevention. The Bonferroni correction was not performed.

Results

The first aim of this study was to investigate whether differences in DNA-analysis could be found between apparently normal mucosa of cancer patients and normal mucosa of healthy control individuals. None of the parameters calculated by the computer from the DNA-image proved to be of value to distinguish putative pre-malignant from healthy mucosa (table 1).

Table 1: *Cytomorphometric parameters in macroscopically normal exfoliated mucosal cells.*

Cytomorphometric parameter	Cancer patients (N=10)	Controls (N=10)
Integrated optical density	58.1 ± 3.6	55.6 ± 3.5
Area (μm^2)	63.1 ± 6.0	61.1 ± 5.0
Nuclear shape	14.9 ± 0.4	15.2 ± 0.4
Angular second moment (10^{-3})	44.4 ± 0.9	44.9 ± 1.7
Contrast	9.5 ± 3.0	9.5 ± 3.2
Inverse difference moment (10^{-2})	49.6 ± 2.5	49.5 ± 3.3
Sum variance	97.9 ± 29.3	95.2 ± 29.7
Entropy (10^{-1})	14.7 ± 0.1	14.7 ± 0.1
Difference variance	4.9 ± 1.5	4.9 ± 1.6
Difference entropy (10^{-2})	50.7 ± 1.2	51.1 ± 1.5
Coefficient of variation (10^{-2})	25.4 ± 1.4	25.5 ± 1.3
Peak transition probability (10^{-2})	11.4 ± 0.3	11.6 ± 0.3
Diagonal variance (10^{-4})	12.7 ± 0.5	13.6 ± 1.4
Triangular symmetry (10^{-2})	19.8 ± 1.8	20.5 ± 1.6
Blobness (10^{-2})	38.6 ± 0.9	38.2 ± 1.2
Standard deviation (10^{-3})	50.7 ± 8.5	49.9 ± 8.8

Values are shown as means ± standard deviation.

The second aim of this study was to examine whether the administration of retinyl-palmitate or N-acetylcysteine could alter nuclear area, nuclear shape, DNA-content and several DNA texture features. The results are shown in table 2. It seemed that there were no cytomorphometric differences between the mucosal cells before and after chemopreventive treatment. Besides the two trial-arms in which patients used retinyl-palmitate or N-acetylcysteine exclusively, the other two arms of the Euroscan trial in which patients received both medications or no medication at all were investigated as well (data not shown). In these two groups similar results were obtained as the groups presented in table 2.

Table 2: *Modulation of cytomorphometric parameters in exfoliated cells.*

Cytomorphometric parameter:	Retinyl-palmitate (N=17)	
	Pre-treatment	Post-treatment
Integrated optical density	59.8 ± 8.3	61.1 ± 5.4
Area (μm^2)	79.1 ± 9.3	80.0 ± 5.9
Nuclear shape	16.3 ± 1.0	16.0 ± 1.0
Angular second moment (10^{-3})	47.1 ± 3.9	48.8 ± 3.3
Contrast	5.5 ± 1.8	4.7 ± 1.1
Inverse difference moment (10^{-2})	55.8 ± 3.2	56.8 ± 3.2
Sum variance	57.5 ± 21.6	55.4 ± 14.0
Entropy (10^{-1})	14.5 ± 0.3	14.4 ± 0.4
Difference variance	2.9 ± 0.7	2.5 ± 0.6
Difference entropy (10^{-2})	49.3 ± 2.0	48.4 ± 2.2
Coefficient of variation (10^{-2})	23.1 ± 1.7	22.9 ± 1.4
Peak transition probability (10^{-2})	11.5 ± 0.1	12.0 ± 0.6
Diagonal variance (10^{-4})	13.5 ± 2.4	15.1 ± 1.9
Triangular symmetry (10^{-2})	16.5 ± 2.4	16.2 ± 11.3
Blobness (10^{-2})	39.8 ± 1.5	41.2 ± 1.6
Standard deviation (10^{-3})	36.1 ± 7.2	36.1 ± 5.2
	N-Acetylcysteine (N=19)	
	Pre-treatment	Post-treatment
Integrated optical density	77.3 ± 18.9	75.6 ± 16.8
Area (μm^2)	85.1 ± 10.8	84.9 ± 10.9
Nuclear shape	15.1 ± 0.7	15.1 ± 0.5
Angular second moment (10^{-3})	48.1 ± 1.9	47.6 ± 3.8
Contrast	7.1 ± 3.0	7.1 ± 3.1
Inverse difference moment (10^{-2})	53.7 ± 3.6	53.9 ± 4.3
Sum variance	97.4 ± 48.0	91.4 ± 48.0
Entropy (10^{-1})	14.4 ± 0.2	14.5 ± 0.3
Difference variance	3.7 ± 1.5	3.8 ± 1.6
Difference entropy (10^{-2})	47.0 ± 1.7	47.9 ± 2.5
Coefficient of variation (10^{-2})	25.2 ± 2.9	24.6 ± 2.7
Peak transition probability (10^{-2})	12.0 ± 0.4	12.0 ± 0.6
Diagonal variance (10^{-4})	13.6 ± 1.8	13.8 ± 2.6
Triangular symmetry (10^{-2})	15.1 ± 1.7	15.8 ± 0.7
Blobness (10^{-2})	41.8 ± 1.6	41.3 ± 1.9
Standard deviation (10^{-3})	48.0 ± 13.6	46.2 ± 13.1

Cells were analyzed of patients using retinyl-palmitate or N-acetylcysteine. These patients have been curatively treated for a head and neck squamous cell carcinoma and took part in the Euroscan trial which is aiming to prevent or to delay the development of second primary tumors in the respiratory and upper digestive tract. Samples were taken before and during treatment several months later. Values are presented as mean ± standard deviation.

Discussion

Cancer incidence is usually considered as the definitive endpoint for cancer prevention trials. Clearly, if the field of chemoprevention is to advance more rapidly, measurements that can accurately predict the definitive outcome of the trial at an early stage will be extremely valuable. Generally, such measurements or biomarkers are called intermediate endpoint biomarkers (Meyskens 1992). In addition, we regard biomarkers to be worthwhile when they can be acquired by non-invasive procedures. From an ethical point of view repeated biopsies are felt to be less desirable in a healthy population without current illness. For this reason we prefer to collect target tissue to be analyzed for biomarker expression by means of non-invasive procedures like for example cytological brushes. These brushes were originally developed for cervical smears. By using this method we found in another study that certain proteins which are normally expressed only in the basal layers of oral mucosa, could be detected in the suprabasal layers of condemned mucosa of head and neck squamous cell carcinoma patients (Copper *et al.* 1993). These findings confirmed that this non-invasive procedure was a feasible approach to detect biomarkers in mucosa of head and neck squamous cell carcinoma patients. Another benefit of this strategy is that when valuable biomarkers are being determined in a selected group of patients, it is very easy to use this cytological, non-invasive method subsequently in considerable groups of healthy individuals in order to detect high-risk groups within the general population.

The use of computerized image analysis often has been proposed to discover potential biomarkers capable to detect preneoplastic tissue and as such to function as an intermediate endpoint biomarker in cancer chemoprevention trials. To the best of our knowledge, this study is the first one in which the possibility of quantitative DNA analysis is investigated to serve as biomarker in a head and neck cancer chemoprevention trial. In this population of patients randomized for the chemoprevention trial Euroscan, the use of quantitative DNA analysis of cytological samples by using the CAS 200 system seems not to be of great value in order to detect high-risk tissue in the head and neck area or to detect alterations in mucosal cells as modulated by retinyl-palmitate or N-acetylcysteine. In case of dysplasia of the epithelial cells, DNA histogram abnormalities can be found (Ogden *et al.* 1991, Schulte *et al.* 1991). However, the present study focuses on non-dysplastic epithelial cells. The fact that the results of the present study showed no changes can be explained in three ways: a) cytomorphometry is inappropriate in this setting, b) the chemopreventive medication in Euroscan is not effective, or c) both. The rationale for the choice of N-acetylcysteine and retinyl-palmitate in Euroscan is based on a variety of experimental data showing protective effects. Most convincing anticarcinogenic effects of N-acetylcysteine have been displayed in various animal models (De Flora *et al.* 1986, Wilpart *et al.* 1986, Cesarone *et al.* 1987, Rotstein and Slaga 1988, Boone *et al.* 1992a). In all these animal studies, N-acetylcysteine has shown positive for chemopreventive activity. Previous work on retinyl-palmitate

has shown promising data as well (Pastorino 1991, Pastorino *et al.* 1993). Although the CAS 200 system used in this study is an advanced and sensitive system, the use of other techniques could offer new perspectives with respect to the development of intermediate endpoint biomarkers in head and neck squamous cell carcinoma chemoprevention research.

CHAPTER 4

STANDARDIZATION OF COUNTING MICRONUCLEI: DEFINITION OF A PROTOCOL TO MEASURE GENOTOXIC DAMAGE IN HUMAN EXFOLIATED CELLS

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Carcinogenesis 1995, 16:2395-2400.

Abstract

The proportion of exfoliated buccal mucosal cells with micronuclei gives the opportunity to assess sensitivity to γ-radiation and genotoxic compounds and in addition to monitor the effectiveness of cancer intervention strategies. So far, results on counting micronuclei in various publications are difficult to compare because of differences in methods used, especially with regard to microscopical magnification used and number of cells counted. The aims of this study were 1) to define a protocol for counting micronuclei, 2) to assess the feasibility of manually counting micronuclei and 3) the assessment of inter- and intra-patient variability of the number of micronuclei. We propose the definition of a strict protocol on counting micronuclei, with regard to cytological preparation, definition of micronuclei, instrumentation, sampling of cells in a cytological specimen and sample size. Such a strict protocol is a prerequisite for counting micronuclei in exfoliated cells to get a reproducible and sensitive indicator of exposure and for cancer risk. Although the inter- and intra-observer reproducibility of counting micronuclei per 1,000 cells using such a protocol is well, we show that the variability among 10 assessments of micronuclei per 1,000 cells taken sequentially from a sample size of 10,000 nuclei of the same specimen can be enormous (coefficients of variation varied in 7 individuals studied between 42.1% and 102.9%). Based on the observed low frequencies varying from 1.2 to 5.2 micronuclei per 1,000 cells and the variation found, we conclude that at least 10,000 exfoliated cells should be screened to monitor a significant reduction of 50% in the number of micronuclei (for a patient with an initial frequency in the micronuclei frequency range given). Since it takes about seven hours to evaluate this number of cells, it is also concluded that counting of micronuclei requires automation.

Introduction

Micronuclei are defined as microscopically visible, round or oval cytoplasmic chromatin masses next to the nucleus (Schmid 1975). They are the result of aberrant mitoses and consist of acentric chromosomes, chromatid fragments or aberrant chromosomes. Assessment of the number of micronuclei may be used as a strategy to identify the genotoxic damage in animal or human cells, which are exposed to (organ specific) carcinogens or mutagens. A rise in the number of micronuclei in exfoliated cells indicates an increased risk for cancer of the oesophagus, urinary bladder, cervix (Raafat *et al.* 1984, Stich and Rosin 1984) and oral cavity (Stich *et al.* 1982, International Agency for Research on Cancer 1985, Rosin *et al.* 1987). Therefore the number of cells with micronuclei may be used as an indicator to monitor or to predict the efficacy of cancer intervention strategies. The use of drugs to prevent and delay the development of cancer in high risk persons may have a strong impact on cancer treatment. One such high risk group is the group with patients that have been

curatively treated for a carcinoma in the mucosa of the head and neck. These patients have a relatively high risk to develop another primary tumour in the upper digestive or respiratory track. Hong *et al.* (1990) have shown that by administering 13-*cis*-retinoic acid it is possible to delay or even prevent the occurrence of some of these multiple primary tumours. One big disadvantage of cancer chemoprevention is that it takes a long time before the endpoint, namely the development of a tumour, is attained. Therefore to improve the efficiency of such trials biomarkers are needed that predict or monitor the outcome of such a trial (Boone *et al.* 1992b). So, the number of cells with micronuclei may reflect not only a person's exposure to genotoxic agents (Stich *et al.* 1982, Arlett *et al.* 1989) and X-irradiation, but can also be used to monitor prevention strategies.

At present, the assessment of the number of micronucleated cells is done mainly in a non-automated way. Because the number of cells analyzed influences the sensitivity of the assessment, and the frequency of cells with micronuclei is low, a large number of cells has to be screened to detect significant changes in the total number of micronucleated cells. This is time consuming and tedious. Up to now, the number of cells counted in different studies varies from 100 to 3,000 per patient. The final magnification of the microscope used is not always reported or if it is, varies from 400 and 2,000 times. More important (because of the small size of micronuclei), even the numerical aperture of the objective used is not always given.

In order to obtain reproducible results which are comparable with other studies and laboratories (Baak and Oort 1983), a protocol is required which unambiguously defines: a) standardization of specimen preparation (Wittekind 1985), b) microscope conditions, c) criteria for identifying micronuclei, d) sample size and e) sampling in a cytological specimen. In this chapter we have defined such a protocol for manually counting micronuclei.

Patients and methods

Cytological preparations

In order to obtain high-quality specimens of human exfoliated cells, a previously published staining technique was used and modified (Copper *et al.* 1993). After moistening the mouth with water, cells were collected using a cytobrush plus® (Medscand AB, Malmö, Sweden). Mucosal cells were obtained from the middle part of the inner cheek. The brushes were then stirred by hand in 2 ml of DMEM (Dulbecco's Modification of Eagle's medium, Flow Laboratories, Irvine, UK), allowing the cells to release from the brush. Cells were washed with DMEM (1,000 rpm for 10 minutes) and collected in 1 ml DMEM. This way of cell sampling produces a clean cell solution, containing more than 100,000 mucosal cells. Of each tube four cell dilutions were prepared allowing to choose the slide with the optimal density of cells. Cytocentrifuge preparations were made with a Shandon Cytospin 2 (Shandon Inc. Pittsburg, U.S.A.) at 680 rpm for 5 minutes at 40 g using poly-L-lysine (Sigma Chemical Co., St. Louis, U.S.A.) coated glass slides and thick filtercards (nr. 190005, Shandon). Cytocentrifugation of mucosal cells is an improvement over a smear in that it results in a homogenous spread and flat cell population which is ideal for manual screening and automation. Slides were air-dried (20°C) and fixed using methanol: acetic acid (3:1, v/v) for 15 minutes. Cytocentrifuge preparations were stained with the Feulgen reaction according to a published procedure (Duijndam and van Duin 1975) (with slight modifications): hydrolysis in 5 N HCl for 30 minutes at 27°C, washing in aqua dest for 5 minutes, staining with fresh Schiff reagent (BDH, 19120, Brunschwig Chemie, Amsterdam, the Netherlands) for 45 minutes and again washing in tap water for 15 minutes. Slides were counter-stained with naphthol-yellow 0.1% for 20 seconds. Finally the specimens were dehydrated in alcohol and mounted in Entellan® (Merck, Darmstadt, Germany) and covered by an 0.17 mm thick coverslip.

Patient and specimen selection

Twenty specimens were obtained from three patients (specimen numbers 4, 13 and 14) who received radiotherapy, and 17 curatively treated head and neck cancer patients who were going to take part in the Euroscan cancer chemoprevention trial. This trial investigates whether administration of retinyl-palmitate and/or N-acetylcysteine will prevent or delay the development of second primary tumours (De Vries *et al.* 1991). Eventually we will investigate whether the number of micronuclei can be used to predict and/or monitor the outcome of this kind of trials. These specimens were used to assess the reproducibility of the described protocol. In addition, specimens of seven different patients have been selected from the same trial to study the effect of screening more cells. The specimen with an optimal nuclear density (between 15 and 20 nuclei per square grid line area, see Instrumentation) was selected. Specimens were then examined and selected on having no bacterial or fungoid overgrowth and having not too many injured nuclei as these structures may closely resemble micronuclei. After selection, the specimens were randomized, coded and scored blind by three independent observers.

Instrumentation

A conventional transmission light microscope (Carl Zeiss, Oberkochen, Germany) provided with a 40x dry-lens plan objective with a numerical aperture of 0.65 and 450 μm field diameter at specimen level was used. The specimens were illuminated with a halogen light source, and the fields of vision were firstly filtered with a monochromomatic green filter (λ = 550 nm, $\Delta\lambda \leq$ 10 nm) for which the Feulgen stain shows maximum absorption and secondly with a monochromatic blue filter (λ = 420 nm, $\Delta\lambda \leq$ 10 nm) for which naphthol-yellow shows maximum absorption. In the eyepiece of the microscope a square line grid (1 x 1 cm^2; subdivided in 100 boxes of 1 mm^2) was placed. Using this grid a previously marked area was screened. The area within the cytological specimen was marked with tape (instead of a normal pencil marker to obtain sharp edges), situated approximately 2 or 3 square line grid areas

from the border of the cytological specimen to avoid cytocentrifuge artefacts.

Definition of micronuclei

Criteria for identifying micronuclei were based on those given by Countryman and Heddle (1976) with some additions. An object is classified as micronucleus if it fulfils the following criteria: the object 1) consists of nuclear material (to exclude non-nuclear Feulgen positive objects, e.g. stain particles); 2) is fully separated from the parent nucleus; 3) has an area less than 1/5 of the parent nucleus (to exclude micronuclei originating from spindle disturbances and to exclude binucleated cells as micronuclei); 4) has a light-intensity higher (are lighter) than or equal to that of the parent nucleus (to exclude large stain particles and deep-dark-pink stained bacteria); 5) is not fragmented (to exclude small stain particles and apoptic cells); 6) is round or oval; 7) is located within 4 times the shortest axis of the nearest nucleus; and 8) is associated to the same nucleus with not more than one other micronucleus (to exclude nuclear fragments and staining particles).

To make frequency measurements meaningful, parent nuclei should fulfil criteria 5 and 6 and should not overlap with other neighbouring nuclei.

Sampling: Scanning protocol

To reduce the errors introduced by scanning to a minimum we have applied a slightly adapted scanning protocol, called the "forbidden line method", proposed by Gundersen (1977). The difference with the original method of Gundersen is an additional condition with respect to the square line grid: namely, the area of the square line grid has to be smaller than that of the area of the field of vision so that any micronucleated cell located at an edge remains in the field of vision. This condition is necessary because the criteria for scoring micronuclei require inspection of the complete cell.

Sample size

The number of cells with 0, 1 or 2 micronuclei may confirm with a Poisson distribution as micronuclei have a random distribution between cells in the specimen. The mean and variance of the number of micronucleated cells are approximately the same in a given population. As the mean may be small, a large number of cells has to be screened to obtain reliable results for comparison of negative and positive controls.

The approximate number of exfoliated cells which has to be examined in order to detect a significant result can be determined using the formula for binomial confidence limits (Kirkwood 1988):

$$p \pm z' * \sqrt{\frac{p * (1 - p)}{n}}$$

where p is the proportion of micronucleated cells, n is the number of exfoliated cells to be scored (or analyzed) and z' is the appropriate percentage point of the normal distribution. Given the number of cells analyzed, the approximate confidence limits can

be calculated. This standard significance test has been employed to test a null hypothesis of a difference between the frequencies of the number of micronuclei per 10,000 (and 1,000) cells of two assessments.

Statistical analysis

The number of micronucleated exfoliated cells was assessed on 20 specimens. To obtain the inter- and intra-observer reproducibility, counting was done within the marked area according to the protocol defined in the paragraphs on Cytological preparation, Instrumentation, Definition of micronuclei and Sampling. Three observers scored the 20 specimens blindly and independent of each other to assess the inter-observer reproducibility. One observer scored 10 randomly selected specimens after several weeks again to obtain the intra-observer reproducibility. The inter- and intra-observer reproducibility were assessed by linear regression analysis using BMDP (BMDP Statistical software, Inc., Los Angeles, U.S.A.).

Table 1: *Results from counting micronuclei using described protocol (number of micronuclei expressed per 1,000 exfoliated cells).*

Specimen	Observer 1		Observer 2		Observer 3		Observer 3	
	MN	N	MN	N	MN	N	MN	N
1	0.00	1,278	0.00	1,492	1.50	1,329	nd	
2	0.90	1,112	0.00	1,096	2.47	1,213	nd	
3	2.31	1,292	2.32	1,291	2.49	1,204	nd	
4	32.71	214	37.21	215	31.25	160	nd	
5	0.00	1,515	0.00	1,499	2.11	1,423	nd	
6	0.95	1,049	0.94	1,063	0.00	981	nd	
7	2.94	2,382	1.65	2,419	3.67	2,181	nd	
8	1.78	563	0.00	709	0.00	640	nd	
9	0.54	1,865	0.00	2,547	0.00	2,011	nd	
10	5.40	3,705	1.29	3,891	1.46	4,108	nd	
11	0.00	1,148	0.00	1,261	0.00	1,186	0.00	1,191
12	4.37	458	12.22	573	4.09	733	5.34	749
13	49.08	163	73.06	219	31.75	189	37.04	189
14	79.77	351	92.64	367	66.89	299	69.31	303
15	0.00	368	0.00	466	4.85	412	2.40	417
16	0.00	1,352	1.10	1,826	2.39	1,253	3.13	1,279
17	2.23	897	0.00	1,355	2.19	1,372	2.18	1,378
18	2.28	1,315	1.43	1,402	2.31	1,296	3.07	1,304
19	9.87	304	nd		10.99	364	13.63	367
20	nd		nd		0.00	553	0.00	559

MN: number of micronuclei per 1,000 nuclei; N: number of nuclei counted; nd: not done.

Next the significance and influence of counting of at least 10,000 nuclei on the frequency of micronuclei compared to the counting of circa 1,000 nuclei have been investigated on seven additional specimens selected from patients enrolled in the Euroscan trial (De Vries *et al.* 1991). In order to find out which difference in the number of micronuclei between two specimens is still significant given the total number of nuclei screened (1,000 or 10,000), 95 % confidence limits of difference have been calculated.

Results

Table 1 shows the results of screening all (micro)nuclei within the marked area using the described protocol to obtain the inter- and intra-observer reproducibility. Table 2a and table 2b show the results of linear regression analysis, indicating that correlation between observers was high. However, when the patients who received radiotherapy (who show a very high number of micronuclei) were removed from the analysis, especially the correlation between observer 2 and observer 1 and 3 became worse.

Table 2a: *Results of linear regression analysis of data presented in table 1.*

	Observer 2		Observer 3	
Observer 1	R= 0.988	Y= -0.36+1.24*X	R= 0.986	Y= 0.86+0.79*X
Observer 2			R= 0.962	Y= 1.24+0.61*X
Observer 3			R= 0.997	Y= 0.38+1.05*X

R = correlation coefficient; Y = regression line.

Table 2b: *Results of linear regression analysis of data presented in table 1 but without the patients (4, 13 and 14) who received radiotherapy.*

	Observer 2		Observer 3	
Observer 1	R= 0.568	Y= -0.24+1.04*X	R= 0.732	Y= 0.96+0.77*X
Observer 2			R= 0.430	Y= 1.67+0.21*X
Observer 3			R= 0.955	Y= -0.25+1.18*X

R = correlation coefficient; Y = regression line.

Specimens of seven different patients have been screened to investigate the significance and influence of counting of at least 10,000 nuclei. This on the average took 7 hours (range 5.25 - 9.25) per specimen. Table 3 includes both the number of

Table 3: *Number of micronuclei per 10,000 exfoliated cells and coefficients of variation over samples of 1,000, 3,000 and 5,000 exfoliated cells.*

	Specimen 1		Specimen 2		Specimen 3		Specimen 4	
	#mn	#n	#mn	#n	#mn	#n	#mn	#n
1 - 1,000	2	2	5	5	1	1	6	6
1,001 - 2,000	1	1	9	9	5	5	2	2
2,001 - 3,000	0	0	9	9	2	2	4	4
3,001 - 4,000	2	2	2	2	3	3	2	2
4,001 - 5,000	1	1	2	2	1	1	2	2
5,001 - 6,000	1	1	10	9	2	2	4	4
6,001 - 7,000	0	0	4	4	2	1	4	4
7,001 - 8,000	2	1	3	3	0	0	4	4
8,001 - 9,000	1	1	6	6	3	3	2	2
9,001 - 10,000	2	2	2	2	2	1	0	0
Total number:	12	11	52	51	23	21	30	30
mn:	1.2		5.2		2.1		3.0	
CV^1:	65.73		60.68		65.25		56.66	
CV^2:	27.27		25.30		31.99		22.93	
CV^3:	15.31		13.76		20.98		9.61	

	Specimen 5		Specimen 6		Specimen 7	
	#mn	#n	#mn	#n	#mn	#n
1 - 1,000	2	2	4	4	5	5
1,001 - 2,000	1	1	1	1	3	3
2,001 - 3,000	2	2	2	2	5	5
3,001 - 4,000	2	1	2	2	1	1
4,001 - 5,000	2	2	2	2	2	2
5,001 - 6,000	0	0	2	2	2	2
6,001 - 7,000	0	0	3	3	3	3
7,001 - 8,000	0	0	0	0	5	5
8,001 - 9,000	0	0	2	2	4	4
9,001 - 10,000	4	4	0	0	4	4
Total number:	13	12	18	18	34	34
mn:	1.3		1.8		3.4	
CV^1:	102.88		68.29		42.05	
CV^2:	71.22		29.73		30.39	
CV^3:	46.94		16.13		14.41	

#mn= number of micronuclei; #n= number of nuclei with accompanying micronuclei according to Arlett *et al.* (1989); mn= average number of micronuclei per 1,000 exfoliated cells; CV^1= coefficient of variation of #mn per 1,000 exfoliated cells; CV^2= coefficient of variation of #mn per 3,000 exfoliated cells; CV^3= coefficient of variation of #mn per 5,000 exfoliated cells.

micronucleated cells and the number of micronuclei per cell (as was stated to be reported at a workshop on micronuclei (Arlett *et al.* 1989). The number of micronuclei are presented per 1,000 successively screened exfoliated cells. From these figures the running mean, standard deviation and coefficient of variation per 10,000 nuclei have been calculated. The coefficients of variation were high and as expected decreased with increasing cell number. The running means are shown in figure 1.

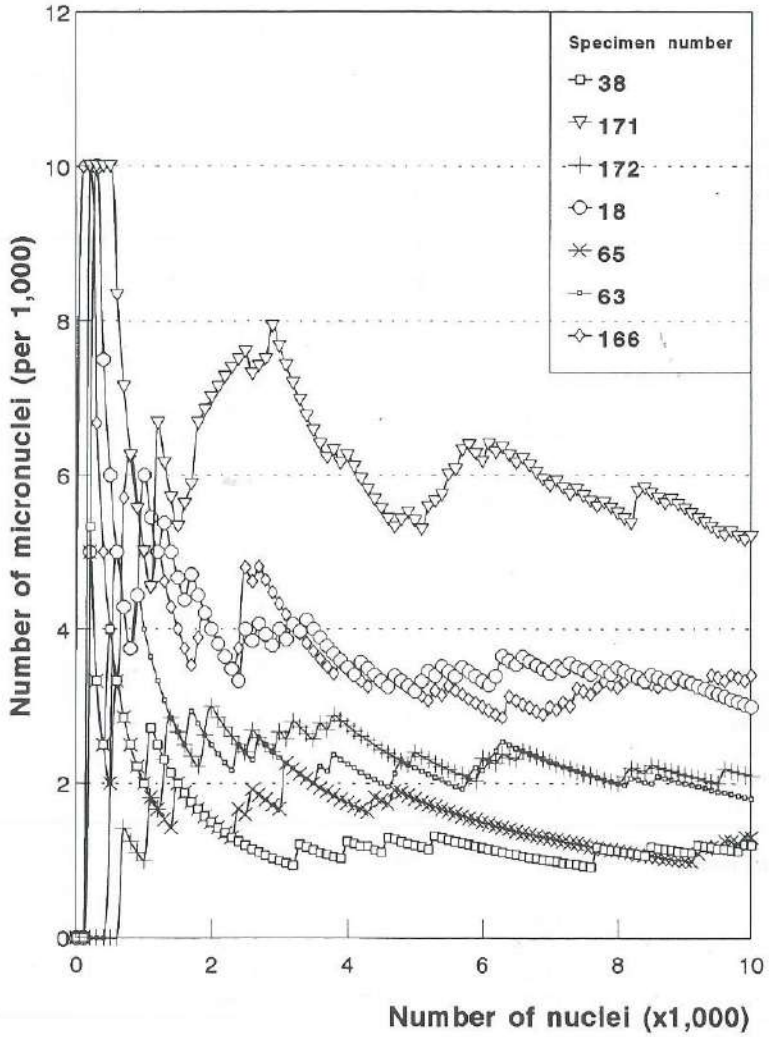

Figure 1: *Running means of the number of micronuclei expressed per 1,000 exfoliated cells, in seven specimens.*

In order to know which difference in micronuclei count between two specimens is still significant, the results obtained from the 7 specimens have been used to calculate the 95% confidence limits of the difference between the frequencies of the number of micronuclei per 10,000 and 1,000 cells for all possible specimen combinations. The confidence intervals and their significance are given in table 4 per 10,000 cells (the confidence intervals per 1,000 cells are not given since none of the differences is significant).

Table 4: *Confidence intervals and significance of difference between two assessments of the number of micronuclei per 10,000 exfoliated cells.*

Confidence intervals:

Spec:	Spec 2	Spec 3	Spec 4	Spec 5	Spec 6	Spec 7
Spec 1:	-40 ± 15.52	-8 ± 10.73	-19 ± 12.53	-1 ± 9.39	-7 ± 10.55	-23 ± 13.13
Spec 2:		31 ± 16.37	21 ± 17.60	39 ± 15.53	34 ± 16.25	18 ± 18.03
Spec 3:			-11 ± 13.70	7 ± 10.90	1 ± 11.91	-15 ± 14.25
Spec 4:				17 ± 12.69	12 ± 13.56	-4 ± 15.65
Spec 5:					-6 ± 10.73	-22 ± 13.28
Spec 6:						-16 ± 14.12

Significance of difference:

Spec:	Spec 2	Spec 3	Spec 4	Spec 5	Spec 6	Spec 7
Spec 1:	****	N.S.	**	N.S.	N.S.	**
Spec 2:		***	*	****	***	N.S.
Spec 3:			N.S.	N.S.	N.S.	N.S.
Spec 4:				**	N.S.	N.S.
Spec 5:					N.S.	**
Spec 6:						*

Spec= Specimen.
Significance levels: N.S.: P >= 0.05; *: P < 0.05; **: P < 0.01; *** P < 0.001; **** P < 0.0001.

Discussion

This article describes the definition of a protocol which can be used as a standard for counting micronuclei, particularly in exfoliated cells. In this protocol definitions are given with respect to cytological preparation, instrumentation, definition of micronuclei and sampling (scanning, sample size). We also have analyzed the reproducibility and confidence intervals of micronuclei counts, using a very large sample number. It was found that the number of exfoliated cells usually counted (i.e., 500 - 2,000), is far too small to detect significant differences between samples with a micronuclei count as different as 1.2 and 5.2 per 1,000 cells.

Heddle *et al.* (1983) stated that a random distribution of cells must be achieved, so that micronuclei can be scored adequately. The slide preparation techniques used so far are nearly all based on making a smear. As smears do not always result in a

completely homogenous spread cell population, the result of scoring micronuclei will strongly depend on the scanning method as discussed by Ashby and Mohammed (1986). Moreover, a smear can be spread out all over the glass slide. As a result, it takes a lot of time to screen all cells present, while a cytospin preparation is located at the centre of the glass slide. This reduces the area to be screened and thus saves time. Therefore, we have applied a slightly altered technique used in our laboratory. With this method we are able to create homogenous spread and flat cell populations which makes it possible to count micronuclei in exfoliated cells not only manually but also by means of image processing.

The criteria for identifying micronuclei given by Countryman and Heddle (1976) have been slightly modified by several authors (Roberts *et al.* 1986, Arlett *et al.* 1989, Krishna *et al.* 1989, Rodilla *et al.* 1990, Heddle *et al.* 1991, Larramendy and Knuutila 1991, Pelt *et al.* 1991). Modifications are allowed as long as simple criteria are used as stated at the workshop on micronuclei (Arlett *et al.* 1989). Sarto *et al.* (1987) described that a distinction between micronuclei arising from chromosome breakage and spindle disturbances should be made by restricting the area of a micronucleus to 1/5 of the parent nucleus. We have used this criterion. Although a number of authors do not explicitly distinguish between the two types of micronuclei they in fact do distinguish them by their restriction on diameter (Yamamoto and Kikuchi 1980, Stich *et al.* 1982, Racine and Matter 1984, Roberts *et al.* 1986, Krishna *et al.* 1989, Rodilla *et al.* 1990, Larramendy and Knuutila 1991) or area (Arlett *et al.* 1989).

Since a micronucleus is a rare event, the number of micronuclei missed by using an inappropriate scanning protocol should be reduced as much as possible, and this heavily depends on the type of protocol. This is supported by Ashby and Mohammed (1986) who state: "It is concluded that the method of slide preparation and assessment can significantly influence the variability of data obtained from a study." and "..., a range of values could be recorded dependent upon where on the slide cells were sampled;...". We therefore have used a (slightly modified) scanning protocol of Gundersen (1977) who states: "A description is given of a family of test-frames for obtaining an unbiased estimate of the numerical density of arbitrary profiles...".

Using the criteria given the definitions so far, the inter- and intra-observer reproducibility were assessed. When all patients are taken into account the inter- and intra-observer assessments are well correlated. However, when the patients who received radiotherapy and therefore show a high number of micronuclei compared to the patients who were going to take part in the Euroscan trial, are removed from the analysis, the correlation between the assessments of especially observer 2 and observers 1 and 3 drops. Although the inter- and intra-observer assessments are well correlated, the variability among 10 assessments of micronuclei per 1,000 cells taken sequentially from a sample size of 10,000 nuclei of the same specimen for 7 patients can be enormous (coefficient of variation of 42.1% - 102.9%, table 3), which is in concordance with the reduced correlation between the independent observations when the patients who received radiotherapy are removed. With such high coefficients of variation for scoring micronuclei, scoring of 1,000 nuclei will not be sufficient to

detect a significant reduction of 50% in the number of micronuclei (e.g. in monitoring effects of cancer chemopreventive agents if the count is between 1.2 and 5.2 per 1,000 cells). The differences found in the number of micronucleated cells could therefore be due to chance alone. Even if micronuclei per 3,000 cells are counted, as suggested by Ashby and Mohammed (1986), the coefficient of variation still would be too high for the samples analyzed (i.e., coefficient of variation of 22.93% -71.22%).

In theory, one should count all cells/nuclei, normally more than 100,000, collected from the middle part of the inner cheek to get an accurate frequency of micronuclei. Doing this manually would be too labour intensive (counting 10,000 cells already took 7 hours per specimen on the average). However, the number of cells screened is especially important for detecting the effectiveness of cancer chemopreventive agents in clinical trials (De Vries *et al.* 1991). The number of cells to be analyzed, given the significance wanted, can be estimated from equation 1. If 1,000 cells would be examined, as most researchers do, to monitor therapy effects the confidence intervals would severely overlap, having no significance and therefore can be justified by chance alone. From figure 1, showing the running means, one might think that screening 6,000-8,000 nuclei will give a stable and reproducible result, however from table 4 it can be concluded that at least 10,000 cells per individual (presenting a low initial frequency) have to be screened to examine a significant reduction of 50% in the number of micronuclei of a given individual as might be important in clinical trials.

In summary, defining and using a strict protocol is a prerequisite for counting micronuclei in exfoliated cells to get a reproducible and sensitive indicator on cancer risk. At least 10,000 cells have to be evaluated, which requires automation.

CHAPTER 5

SIMULTANEOUS ANALYSIS OF RETINOL, ALL-*TRANS*- AND 13-*CIS*-RETINOIC ACID AND 13-*CIS*-4-OXORETINOIC ACID IN PLASMA BY LIQUID CHROMATOGRAPHY USING ON-COLUMN CONCENTRATION AFTER SINGLE-PHASE FLUID EXTRACTION

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Journal of Chromatography B 1997, 694:83-92.

Abstract

A reversed-phase high-performance liquid chromatography method for the simultaneous analysis of retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in human plasma and cell culture medium is described. Sample preparation involves precipitation of proteins and extraction of retinoids with 60% acetonitrile. After centrifugation, the acetonitrile content of the supernatant is reduced to 45%, allowing on-column concentration of analytes. Injection volumes up to 2.0 ml (equivalent to 0.525 ml of sample) can be used without compromising chromatographic resolution of all-*trans*-retinoic acid and 13-*cis*-retinoic acid. Retinoids were stable in this extract and showed no isomerization when stored in the dark in a cooled autosampler, allowing automated analysis of large series of samples. Recoveries from spiked plasma samples were between 95 and 103%. Although no internal standard was used, the inter-assay precision for all retinoids was better than 6% and 4% at concentrations of 30 nmol/l and 100 nmol/l, respectively. The method is a valuable tool for the study of cellular metabolism of all-*trans*-retinoic acid, as polar metabolites of this compound can be detected with high sensitivity in cell culture media.

Introduction

Retinoids are a class of naturally occurring and synthetic compounds that are structurally related to retinol and play a role in a variety of physiological processes, i.e. vision, morphogenesis, growth and differentiation of tissues, reproduction and immune modulation. Nuclear retinoic acid receptors and retinoid X receptors play a pivotal role in retinoid action. These ligand dependent receptors, which form homo- or heterodimers upon retinoid binding, function as transcription factors for a number of genes by binding to retinoic acid responsive elements. Retinoids thus exert their effects on the level of regulation of gene expression. Both natural and synthetic retinoids are used in the treatment of malignancies and skin disorders. Metabolic and clinical aspects and importance of the retinoids have been reviewed extensively (Packer 1990a, Packer 1990b, Goss and McBurney 1992, Sporn *et al.* 1994).

The analysis of retinoids in biological samples is a challenging problem, due to sensitivity of these compounds to light and heat, low endogenous concentrations, and strong protein binding. Separation of retinoids is usually accomplished by HPLC in reversed-phase mode (Wyss and Bucheli 1988a, Creech Kraft *et al.* 1988, Wyss and Bucheli 1988b, Eckhoff and Nau 1990a, Sass and Nau 1990, Periquet *et al.* 1991, Gadde and Burton 1992, Marchetti *et al.* 1994, Guiso *et al.* 1994, Takeda and Yamamoto 1994, Dimitrova *et al.* 1996), although separation in normal-phase mode is also quite possible (Meyer *et al.* 1994, Levebvre *et al.* 1995). Reviews on

chromatographic procedures have appeared in recent years (Wyss 1990, Wyss 1995). Favourable spectral characteristics of most retinoids allow sensitive and selective UV detection. Mass spectrometric detection is also possible (Ranalder *et al.* 1993, Lehman and Franz 1996).

Many procedures for sample pretreatment have been developed (Wyss 1990). Most of these procedures are based on protein precipitation using an organic solvent, followed by liquid-liquid extraction. Usually the final extract is dried under nitrogen and reconstituted in a small volume of mobile phase. To compensate for losses during the various fluid handling steps the use of an internal standard is a prerequisite. The total procedure is laborious and carries the risk of inadvertent oxidation and isomerization of labile retinoids. Another approach is the use of on-line solid-phase extraction in combination with column-switching (Wyss and Bucheli 1988a, Creech Kraft *et al.* 1988, Eckhoff and Nau 1990a, Sass and Nau 1994). This technique minimizes manual sample handling and exposure of samples to light, and excellent results have been reported. However, the required equipment is rather complicated.

For the analysis of large numbers of samples generated by clinical studies we needed a simple and robust system for the simultaneous determination of retinoids, including retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and the polar metabolite 13-*cis*-4-oxoretinoic acid. For the separation of these compounds we used reversed-phase chromatography with gradient elution and UV detection. For sample clean-up we evaluated a single-phase fluid extraction procedure. Acetonitrile was added to plasma samples to effect precipitation of proteins and extraction of retinoids. To avoid a drying step, we performed direct injection of the acetonitrile extract. Peak broadening, caused by the high elution strength of the extract, made large volume injections unfeasible. However, if prior to injection, the acetonitrile content was lowered by addition of water, large volumes could be injected without compromising chromatographic resolution, and in this way adequate overall sensitivity was attained. We have systematically studied the effect of acetonitrile concentration on extraction efficiency, solubility and stability of retinoids in the final extract, and also the relation between injection volume and chromatographic resolution. This led to a final procedure that combines simple and rapid sample preparation with a sensitive and reproducible detection of retinoids, allowing the analysis of large series of samples.

Materials and methods

Materials and reagents

Retinol, 13-*cis*-retinoic acid (isotretinoin), and all-*trans*-retinoic acid (tretinoin) were obtained from Sigma (St. Louis, USA). 13-*cis*-4-oxoretinoic acid and all-*trans*-4-oxoretinoic acid were kindly provided by Hoffmann-La Roche (Basel, Switzerland). Ammonium acetate, and 1.0 mol/l sodium hydroxide solution were supplied by Merck (Amsterdam, Netherlands). HPLC grade acetonitrile was obtained from Biosolve (Barneveld, Netherlands) and acetic acid, methanol (HPLC grade) and absolute ethanol from J.T.Baker (Deventer, Netherlands). HPLC-grade water was prepared from demineralized water using a Milli-Q UF Plus water purification system (Millipore, Milford, MA, USA). To prepare the 1.0 mol/l sodium acetate buffer used for sample extraction, 5.7 ml of acetic acid was diluted to approximately 50 ml with water. After adjustment of the pH to 4.0 with 1.0 mol/l sodium hydroxide, the final volume was brought to 100 ml.

Preparation of standards

Stock solutions of retinoids were prepared in absolute ethanol and stored at -20°C. Concentrations of the stock solutions were determined spectrophotometrically using the following molar extinction coefficients: 52,770 (325 nm) for all-*trans*-retinol; 39,750 (354 nm) for 13-*cis*-retinoic acid; 45,300 (350 nm) for all-*trans*-retinoic acid; 39,000 (361 nm) for 13-*cis*-4-oxoretinoic acid (Furr *et al.* 1994).

For system validation standards with concentrations of 5,000, 1,500, 750, 300, 150, 75, 30, 15, 7.5, 3, 1.5, 0.75 and 0.3 nmol/l were prepared by diluting a mixture of the stock solutions with 45% acetonitrile containing 37.5 mmol/l sodium acetate buffer (pH 4.0). Calibration curves were constructed by linear least-squares regression analysis of peak area (y) versus concentration (x) using a weighting factor of $1/x$.

For routine quantification of retinoids in plasma samples, calibration was performed by triplicate analysis of a single plasma-based standard, containing 13-*cis*-4-oxoretinoic acid (299 nmol/l), 13-*cis*-retinoic acid (290 nmol/l), all-*trans*-retinoic acid (301 nmol/l) and retinol (2,050 nmol/l). This standard was prepared by spiking a plasma pool with a mixture of individual retinoid stock solutions. Final ethanol concentration was below 1%. After thorough mixing in the dark, aliquots were snap-frozen in solid carbon dioxide/acetone and stored at -20°C.

Chromatographic system

The HPLC system consisted of a Model 616 pump and a Model 486 UV detector from Waters (Milford, MA, USA). Mobile phase was passed through a Degasys model DG2410 inline solvent degasser from Uniflows (Tokyo, Japan). A Model 717 plus automatic sample injector with cooling option from Waters was used. To allow the injection of large sample volumes, the auxiliary sample loop was installed and a syringe motor rate of 5.0 µl/s was used. The small window in the door of the sample compartment was covered to shield the samples from light. Column temperature was controlled with a column oven and TCM temperature control module from Waters. Millennium 2010 software (version 2.10) from Waters was used for instrument control and data acquisition and processing. Analyses were performed on a 10 cm x 4.6 mm I.D. Spherisorb ODS2 C18 cartridge column containing 3 µm particles (Phase Separations, Deeside, UK). The analytical column

was protected by an integral 1 cm x 2.0 mm I.D. reversed-phase guard column from the same supplier.

Collection and processing of samples

Blood was obtained by venepuncture and collected in EDTA containing tubes. Tubes were wrapped in aluminium foil to protect the samples from light. Within 1 h after venepuncture plasma was obtained by centrifugation (10 min at 3,000 *g* and 4°C) and stored in cryovials that were flushed with nitrogen before storage at -70°C.

Cell lines

Head and neck squamous cell carcinoma cell lines were obtained from T.E. Carey (University of Michigan, Ann Arbor, USA) and are described elsewhere (Carey 1985). The UM-SCC-35 cell line used in this study originated from a hypopharyngeal tumour. Cells were routinely cultured in DMEM (Dulbecco's modified Eagle's Medium, Flow Laboratories, Irvine, UK) with 10% FCS (Fetal Calf Serum, Flow Laboratories) in 75 cm^2 flasks (Nunc, Roskilde, Denmark). Cellular doubling time was 52 h.

Sample preparation

All operations were performed in a room lit by subdued yellow light. Frozen plasma and cell culture medium samples were thawed at room temperature in the dark.

For routine experiments with 0.4 ml injection volumes, the following extraction procedure was used. To 0.35 ml of plasma, culture medium or plasma based standard, 0.05 ml of 1.0 mol/l sodium acetate buffer (pH 4.0) was added. After mixing, 0.6 ml of acetonitrile was added and samples were immediately vortex-mixed. After centrifugation (5 min at 3,000 *g* and 4°C), 450 μl of the clear supernatant was transferred to a conical plastic autosampler vial (Waters part no. 22476). After addition of 150 μl of water, the vials were capped, mixed by inversion and immediately put in the sample compartment of the autoinjector. The autoinjector was cooled at 7°C.

In experiments where larger injection volumes were used, 1.05 ml of sample was mixed with 0.15 ml of sodium acetate buffer and extracted with 1.8 ml of acetonitrile as described above. 2.1 ml of the supernatant obtained after centrifugation was mixed with 0.7 ml of water and the extracts were stored in 4.0 ml glass vials (Waters part no. 72710).

HPLC conditions

Chromatographic conditions were as described by Eckhoff and Nau (Eckhoff and Nau 1990a) with some modifications. A 40 mmol/l ammonium acetate buffer, adjusted to pH 5.75 with acetic acid was prepared. Mobile phase A consisted of buffer-methanol (50/50, v/v) and mobile phase B was pure methanol. The gradient

conditions are shown in table 1. To avoid column damage by sudden pressure changes during injection of large sample volumes (0.4 to 2.0 ml), flow-rate was kept at 0.1 ml/min during injection. After injection, flow-rate was increased to 0.8 ml/min and maintained at this rate during the analysis. Data acquisition was performed at a rate of 2 Hz for 30 min. After 35 min a next injection was started. Total time between injections was approximately 40 min, depending on the sample volume injected. Column temperature was maintained at 30°C and detection was performed at 340 nm. Retinoids were quantitated on the basis of peak area using external standardization.

Table 1: *Gradient conditions.*

Time (min)	Flow (ml/min)	Percent A	Percent B
0	0.1	85	15
1	0.8	85	15
20	0.8	0	100
22	0.8	0	100
23	0.8	85	15
34	0.8	85	15
35	0.1	85	15

Mobile phase A: 40 mmol/l ammonium acetate (pH 5.75)-methanol (50/50, v/v); mobile phase B: methanol.

Assay performance

For quality control purposes three plasma pools were used. One of these pools was used without fortification (endogenous control). The other pools were spiked with a mixture of retinoids to obtain a low control and a high control plasma, respectively. Aliquots of these control samples were stored at -20°C. For determination of within-day precision, control samples (n=10 at each level) were determined within a single chromatographic series. Between-day precision was assessed by analyzing the control samples on 8 occasions within a two-month period.

Results

Chromatographic conditions

Separation was achieved by reversed-phase chromatography using a binary methanol gradient. We used the separation conditions described by Eckhoff and Nau (1990a) as starting point and modified buffer strength and gradient shape to obtain adequate resolution between 13-*cis*-retinoic acid and all-*trans*-retinoic acid

and allow elution of the more hydrophobic retinol. Gradient conditions are shown in table 1. As we wanted to perform large volume injections, the autoinjector was equipped with a large sample loop. Large sample loops may cause sudden pressure surges in the system during switching of the injection valve, that can reduce column performance. To avoid this potential problem we used flow programming. Flow was kept at 0.1 ml/min during sample injection, subsequently increased to 0.8 ml/min and kept at this rate for the remainder of the analysis.

Column temperature was kept at 30°C. Others have used higher temperatures, e.g. Takeda and Yamamoto heated the column to 50°C (Takeda and Yamamoto 1994) and Eckhoff and Nau even used a temperature of 60°C (Eckhoff and Nau 1990a). Although at elevated temperatures we observed a somewhat better column performance and lower column backpressure, there was an apparent on-column degradation of retinol, resulting in diminished peak height and the appearance of an additional small unresolved peak eluting before the main retinol peak.

Figure 1 shows the chromatogram of a standard solution. The small peak eluting between 13-*cis*-retinoic acid and all-*trans*-retinoic acid is possibly 9-*cis*-retinoic acid present as a contamination due to isomerization.

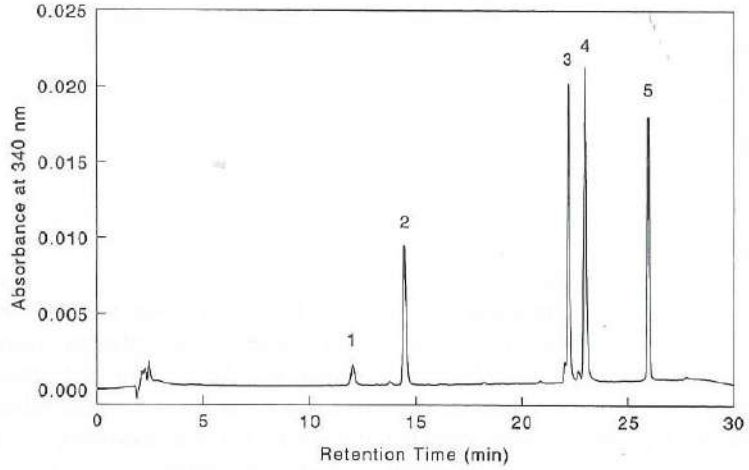

Figure 1: *Chromatogram of a retinoid standard solution. A 5 µl volume of a combined standard in ethanol was injected (total injected amount of each retinoid 15-75 pmol). Peak 1, all-trans-4-oxoretinoic acid; Peak 2, 13-cis-4-oxoretinoic acid; Peak 3, 13-cis-retinoic acid; Peak 4, all-trans-retinoic acid; Peak 5, retinol. Chromatographic conditions as described in the materials and methods section.*

Extraction of retinoids

As we wanted a simple and rapid sample clean-up procedure, we chose to evaluate single-phase fluid extraction with a water miscible organic solvent. We used acetonitrile, that has previously been shown to accomplish effective protein

precipitation and extraction of retinoic acid (Gadde and Burton 1992, Guiso *et al.* 1994, Dimitrova *et al.* 1996). First we determined the minimal concentration of acetonitrile necessary for complete protein precipitation and maximal recovery of retinoids. In preliminary experiments we had observed that lowering the pH of plasma to a value between 4 and 5 before adding acetonitrile, resulted in a more effective protein precipitation. To 0.15 ml of a spiked plasma pool we added 0.05 ml 1.0 mol/l sodium acetate buffer (pH 4.0) and then added water and acetonitrile in various proportions to a final volume of 1.0 ml. The final acetonitrile content varied between 30 and 80%. Only extracts with an acetonitrile content of 50% and higher gave clear supernatants and were evaluated by chromatography. The relation between peak area and acetonitrile concentration is depicted in figure 2.

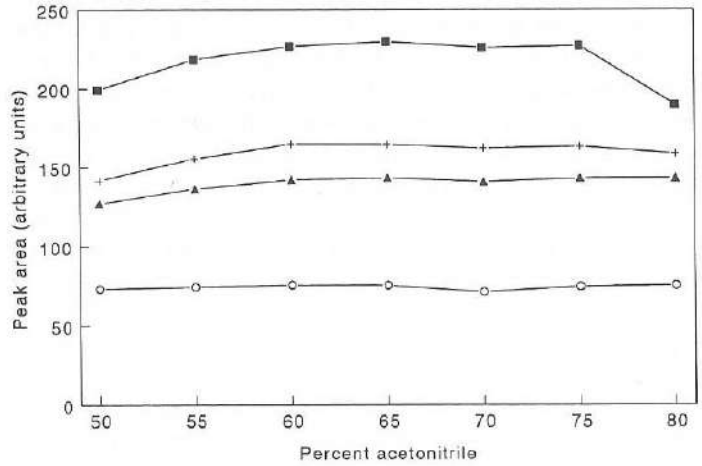

Figure 2: *Dependence of extraction efficiency of retinoids on acetonitrile concentration. Retinoids were extracted from a plasma pool, spiked with a mixture of retinoids. To 0.15 ml of spiked plasma 0.05 ml of 1.0 M sodium acetate buffer (pH 4.0) was added and subsequently retinoids were extracted by addition of water and acetonitrile in various proportions. Final volume was 1.0 ml. After mixing and centrifugation to remove precipitated protein, an aliquot of the supernatant was subjected to chromatography. Chromatographic conditions as described in the materials and methods section. Peak area of 13-cis-4-oxoretinoic acid (○), 13-cis-retinoic acid (▲), all-trans-retinoic acid (+) and retinol (■) was determined and plotted versus acetonitrile concentration.*

For both 13-*cis*-retinoic acid and all-*trans*-retinoic acid peak area slightly increased between 50 and 60% acetonitrile and then remained constant up to 80% acetonitrile. For retinol the same pattern was observed, with the exception that peak area dropped at 80% acetonitrile. This may be explained by the observation that at acetonitrile concentrations between 50 and 75% a finely divided protein precipitate formed, whereas at 80% acetonitrile a lumpy precipitate formed that settled very rapidly even without centrifugation. Inclusion of protein bound retinol in this

precipitate may cause reduced recovery at 80% acetonitrile. Peak area of the polar metabolite 13-*cis*-4-oxoretinoic acid showed no dependence on acetonitrile concentration. From this experiment we concluded that 60% acetonitrile is the minimal amount required to effect complete protein precipitation and maximal extraction of retinoic acid isomers and retinol.

Solubility of extracted retinoids

The elution strength of the extracts containing 60% acetonitrile was too high to allow injection of large volumes without peak broadening. We therefore studied the effect of lowering the acetonitrile content of the extracts by addition of water. As a general rule, capacity factors can be expected to increase 2-3 fold on lowering of the acetonitrile content by 10%. Therefore, loss of sensitivity by dilution of the sample is compensated by the fact that much larger volumes can be injected without loss of resolution, resulting in an overall increased sensitivity. To test solubility of the retinoids in solution as a function of acetonitrile content, a standard mixture of 13-*cis*-4-oxoretinoic acid, 13-*cis*-retinoic acid, all-*trans*-retinoic acid and retinol dissolved in 60% acetonitrile was divided over several tubes. To each tube water and acetonitrile were added in various proportions in such a way that the total volume was kept constant. The final acetonitrile concentrations ranged from 20 to 60%. Peak area was expressed as a percentage of the area observed in the extract containing 60% acetonitrile. When samples were reanalyzed after storage for 6 h in the dark at 7°C in the autosampler, essentially the same peak areas were found, indicating that loss of retinoids is not a time dependent process. It may be that at low acetonitrile content of the extract, retinoids are adsorbed to components of the autosampler during injection. At 45% acetonitrile, relative peak areas of all components were higher than 93%. We therefore decided to use a final percentage of 45% acetonitrile for further experiments. This resulted in the sample preparation procedure as described in the materials and methods section. As can be seen from figure 3 the relative peak area for all components dropped as the content of acetonitrile was lowered. Curves for retinol, 13-*cis*-retinoic acid, and all-*trans*-retinoic acid were nearly identical. The area of 13-*cis*-4-oxoretinoic acid was much less dependent on acetonitrile concentration, in accordance with its higher polarity.

Large volume injection

We subsequently investigated the possibility to inject large volumes of extract, in order to achieve adequate overall sensitivity. A mixture of retinoids was prepared in 45% acetonitrile and volumes from 0.05 up to 2.0 ml were injected. For retinol, 13-*cis*-retinoic acid, and all-*trans*-retinoic acid peak width was completely independent of the injection volume, up to volumes of 2.0 ml. As expected, peak widths for the early eluting compounds all-*trans*-4-oxoretinoic acid and 13-*cis*-4-oxoretinoic acid strongly increased when the injection volume exceeded 0.4 ml. From this experiment we concluded that 0.4 ml injection volumes could be used for the simultaneous analysis of retinol, 13-*cis*-retinoic acid, all-*trans*-retinoic acid and

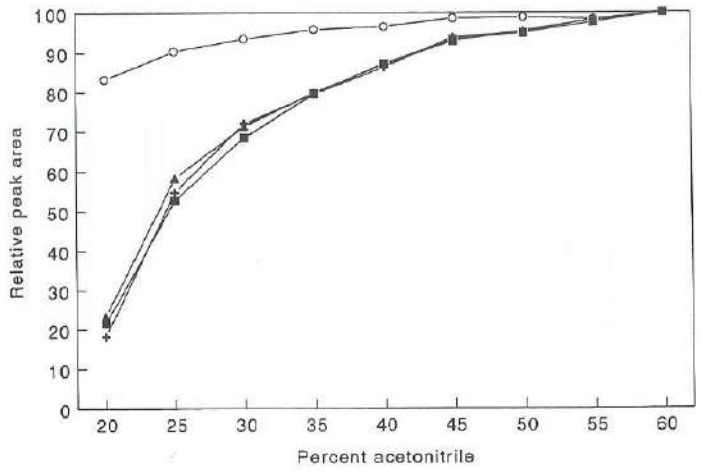

Figure 3: *Effect of acetonitrile content on solubility of retinoids. A mixture of retinoids was prepared in 60% acetonitrile containing 50 mM sodium acetate buffer (pH 4.0). To 1.0 ml aliquots of this mixture, water and acetonitrile were added in various proportions. Final volume was 3.0 ml. All samples were subjected to chromatography using conditions as described in the materials and methods section. Peak area of 13-cis-4-oxoretinoic acid (○), 13-cis-retinoic acid (▲), all-trans-retinoic acid (+) and retinol (■) was determined and relative peak areas were calculated by dividing peak area by the area in the sample containing 60% acetonitrile.*

the polar metabolites all-*trans*-4-oxoretinoic acid and 13-*cis*-4-oxoretinoic acid. If analysis of the polar metabolites is not required, the injection volume can be increased to 2.0 ml without affecting peak width. Figure 4a and 4b show chromatograms of a plasma based standard solution and plasma from a healthy volunteer, respectively, using an injection volume of 0.4 ml. Using this injection volume, endogenous 13-*cis*-4-oxoretinoic acid, 13-*cis*-retinoic acid, all-*trans*-retinoic acid and retinol can be measured. All-*trans*-4-oxoretinoic acid could not be detected in human plasma, in accordance with published observations (Eckhoff and Nau 1990a, Eckhoff *et al.* 1991).

Figure 5 shows the effect of increasing the injection volume from 0.4 ml to 2.0 ml on the chromatogram of an unspiked plasma sample. Increasing the injection volume to 2.0 ml did not lead to peak broadening for 13-*cis*-retinoic acid and all-*trans*-retinoic acid. However, it is clear that quantification is more accurate with the 2.0 ml injection volume. In addition, using the 2.0 ml injection, the chromatogram revealed the presence of additional compounds not visible after a 0.4 ml injection.

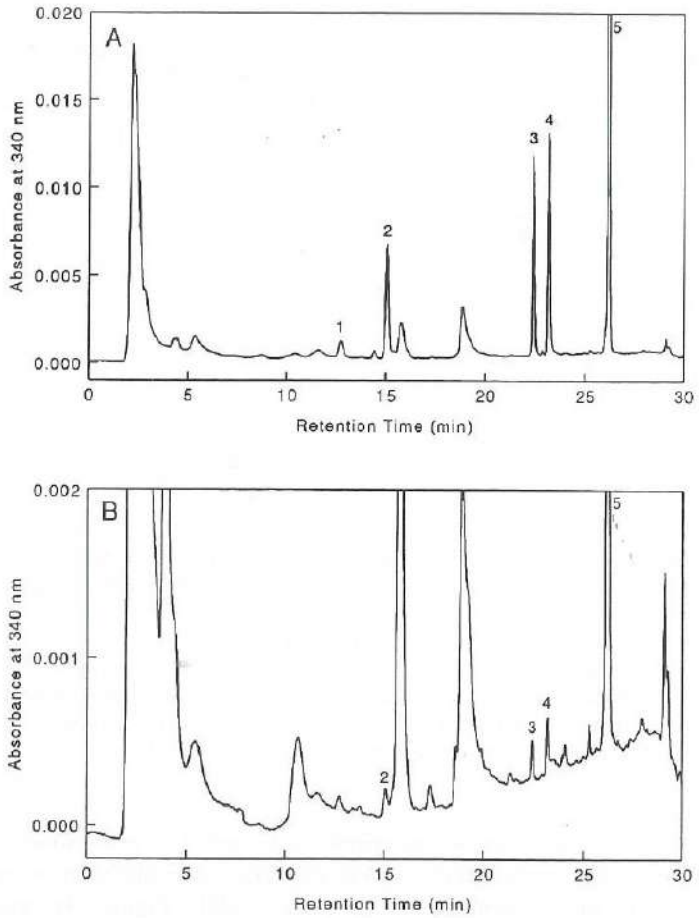

Figure 4: *Chromatograms of a plasma based retinoid standard solution (panel A), containing 13-cis-4-oxoretinoic acid (299 nmol/l), 13-cis-retinoic acid (290 nmol/l), all-trans-retinoic acid (301 nmol/l) and retinol (2050 nmol/l) and non-spiked plasma from a healthy volunteer (panel B), containing 13-cis-4-oxoretinoic acid (6.2 nmol/l), 13-cis-retinoic acid (5.9 nmol/l), all-trans-retinoic acid (7.6 nmol/l) and retinol (2109 nmol/l). To 0.35 ml of standard or plasma 0.05 ml of 1.0 M sodium acetate buffer (pH 4.0) was added, followed by 0.6 ml of acetonitrile. After vortex mixing samples were centrifuged for 5 min at 3,000 g and 4°C. 0.45 ml of the supernatant was mixed with 0.15 ml of water and stored in the dark at 7°C in the sample compartment of the autosampler. An injection volume of 0.4 ml was used. Chromatographic conditions were as described in the materials and methods section. Peak 1, all-trans-4-oxoretinoic acid; Peak 2, 13-cis-4-oxoretinoic acid; Peak 3, 13-cis-retinoic acid; Peak 4, all-trans-retinoic acid; Peak 5, retinol.*

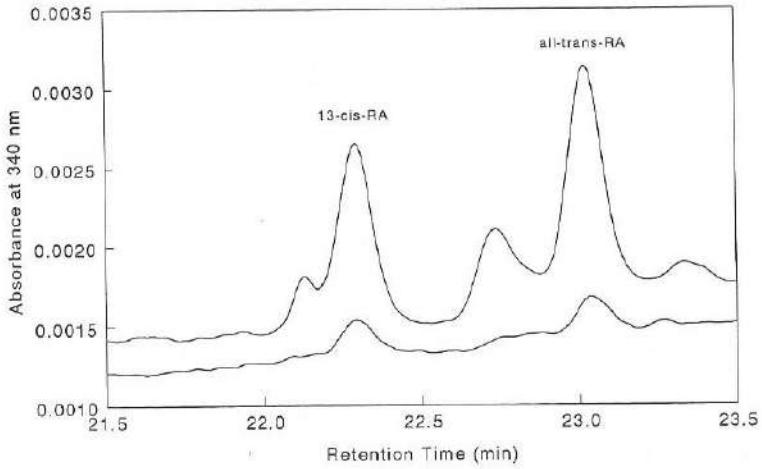

Figure 5: *Effect of injection volume on chromatography of endogenous retinoic acid isomers extracted from human plasma. To 1.05 ml of plasma 0.15 ml of 1.0 M sodium acetate buffer (pH 4.0) was added, followed by 1.8 ml of acetonitrile. After vortex mixing samples were centrifuged for 5 min at 3,000 g and 4°C. The supernatant (2.1 ml) was mixed with water (0.7 ml) and stored in the dark at 7°C in the sample compartment of the autosampler. Chromatographic conditions were as described in the materials and methods section using injection volumes of 0.4 ml (lower trace) and 2.0 ml (upper trace). The plasma concentrations of 13-cis-retinoic acid and all-trans-retinoic acid were 6.2 and 6.9 nmol/l, respectively.*

Isomerization and degradation of extracted retinoids

Although the sample compartment of the autoinjector was shielded from light and kept at a temperature of 7°C, oxidative degradation or isomerization of the retinoids could possibly occur. In fact it has been reported that isomerization of the synthetic retinoid acitretin is enhanced by acetonitrile (Wyss and Bucheli 1992). To test stability, solutions of individual retinoids in 45% acetonitrile were stored in the autosampler in aliquots and injections were performed over a time span of 60 h. To check the effect of plasma components on stability and isomerization we repeated the experiment using extracts from pooled plasma, spiked with individual retinoids. The results are summarized in table 2. Peak area RSD values for individual retinoids were below 1% using standard solutions, and below 2% using spiked plasma. These results indicate that the extracts are very stable, and that no time dependent losses due to adsorption, degradation or isomerization of retinoids occur.

In a separate experiment we determined the extent of isomerization occurring during sample clean-up. This was done by spiking a plasma sample with concentrated stock solutions of either 13-*cis*-retinoic acid or all-*trans*-retinoic acid. After sample clean-up, levels of 13-*cis*-retinoic acid and all-*trans*-retinoic acid were determined to determine the extent of isomerization. Values were corrected for the

Table 2: *Stability of retinoid extract during storage in the autosampler.*

Retinoid	Peak Area RSD (%)	
	Standard (n=14)	Spiked plasma (n=6)
All-*trans*-4-oxoretinoic acid	0.75	1.13
13-*cis*-4-oxoretinoic acid	0.28	0.22
13-*cis*-retinoic acid	0.56	1.02
All-*trans*-retinoic acid	0.54	1.68
Retinol	0.67	1.08

Individual retinoid standard solutions were prepared in 45% acetonitrile containing 37.5 mM sodium acetate buffer (pH 4.0). Pooled plasma was spiked with individual retinoids and extracted as described in the materials and methods section. Standard solutions and plasma extracts were stored in aliquots in closed vials in the dark at 7°C in the autosampler. Injections were performed over a time span of 60 h.

amount of opposite isomer already present in the standard solutions. When plasma was spiked with 13-*cis*-retinoic acid, 0.6% was recovered as all-*trans*-retinoic acid. Conversely, 0.7% of all-*trans*-retinoic acid was recovered as 13-*cis*-retinoic acid after extraction of plasma spiked with all-*trans*-retinoic acid. These results were obtained when sample clean-up was performed under subdued yellow light. Without this precaution isomerization values of 5% or higher were observed. These results confirm published observations (Wyss 1990, Wyss 1995) that extreme care should be taken to shield samples from light during extraction. Once extracted, isomerization of retinoids is negligible, provided the extracts are stored in the dark at 7°C.

Assay performance

Using an injection volume of 0.4 ml, calibration curves showed a linear relation between peak area and concentration up to 1.5 μmol/l for 13-*cis*-4-oxoretinoic acid, 13-*cis*-retinoic acid and all-*trans*-retinoic acid ($r > 0.999$ for all components). As endogenous retinol concentrations are much higher than concentrations of retinoic acid isomers, linearity for retinol was tested over a wider concentration range. The calibration curve for retinol was found to be linear up to 5 μmol/l ($r = 0.9995$). The quantification limit at a S/N ratio of 10 was approximately 0.3 pmol on column for 13-*cis*-retinoic acid, all-*trans*-retinoic acid and retinol, and 0.6 pmol on column for 13-*cis*-4-oxoretinoic acid. Using a 0.4 ml injection volume, equivalent to 0.105 ml of sample, this corresponds to a quantification limit of 3 nmol/l for 13-*cis*-retinoic acid, all-*trans*-retinoic acid and retinol, and 6 nmol/l for 13-*cis*-4-oxoretinoic acid. For 13-*cis*-retinoic acid, all-*trans*-retinoic acid and retinol the quantification limit can be further reduced by increasing the injection volume.

Recovery was determined by spiking five plasma samples with a mixture of

Table 3: *Recovery of retinoids from spiked plasma samples.*

Compound	Spike level (nmol/l)	Recovery Mean ± SD (%)
13-*cis*-4-oxoretinoic acid	18	102.1 ± 3.8
	89	101.4 ± 3.3
	445	103.0 ± 2.2
13-*cis*-retinoic acid	52	96.7 ± 2.3
	259	101.2 ± 0.8
	1,295	99.1 ± 2.0
All-*trans*-retinoic acid	54	96.4 ± 2.6
	269	102.8 ± 2.3
	1,346	96.3 ± 2.4
Retinol	206	102.4 ± 7.1
	1,032	95.5 ± 2.2

Plasma samples (n=5) were spiked with a mixture of retinoids at three concentrations and with retinol at two concentrations. SD: Standard deviation.

retinoids at three concentrations. Because endogenous concentrations of retinol are much higher than the concentrations of retinoic acid isomers, recovery of retinol was only tested at two concentrations. After a 1 h equilibration period, samples were extracted. Recovery of each retinoid was calculated by comparison of peak area in the spiked plasma minus the peak area of the unspiked plasma with the peak area obtained after direct injection of the respective amounts of retinoid. The results presented in table 3, show that the mean recoveries ranged from 95 to 103%, and were independent of the concentration used for spiking. For quality control purposes, three plasma pools were used, one unfortified (endogenous pool), and two spiked with 30 nmol/l (low control plasma) and 100 nmol/l (high control plasma) of each retinoid, respectively. Intra-assay precision was determined by analyzing the quality control samples (n=10 each) in a single chromatographic analysis series. The results are shown in table 4. Inter-assay precision and accuracy were determined by analyzing the control samples on 8 different days over a period of two months. The same plasma based standard solution was used during this period for calibration. The results shown in table 5 indicate that although no internal standard was used in our method, between-run precision was very acceptable, and comparable to other published methods (Wyss and Bucheli 1988b, Creech Kraft 1988, Eckhoff and Nau 1990a, Periquet *et al.* 1991, Gadde and Burton 1992, Sass and Nau 1994, Meyer *et al.* 1994, Takeda and Yamamoto 1994, Guiso *et al.* 1994, Levebvre *et al.* 1995, Dimitrova *et al.* 1996). It should be noted that in order to allow quantitation of the polar metabolite 13-*cis*-4-oxoretinoic acid,

Table 4: *Intra-assay precision for assay of retinoids in plasma (n=10).*

	Mean ± standard deviation (nmol/l)	Coefficient of variation (%)
Endogenous control		
13-*cis*-4-oxoretinoic acid	8.30 ± 0.64	7.8
13-*cis*-retinoic acid	7.08 ± 0.28	4.0
All-*trans*-retinoic acid	8.38 ± 0.31	3.7
Retinol	1,920 ± 7.3	0.4
Low control		
13-*cis*-4-oxoretinoic acid	30.3 ± 1.3	4.3
13-*cis*-retinoic acid	34.7 ± 0.3	1.0
All-*trans*-retinoic acid	32.2 ± 0.4	1.3
Retinol	1,925 ± 15.1	0.8
High control		
13-*cis*-4-oxoretinoic acid	103.2 ± 1.1	1.0
13-*cis*-retinoic acid	108.1 ± 1.3	1.2
All-*trans*-retinoic acid	96.1 ± 2.5	2.6
Retinol	2,022± 25.3	1.3

we used a 0.4 ml injection volume. Under these circumstances endogenous levels of the retinoids are rather close to the lower quantification limit of the method, explaining the relatively large inter-assay coefficient of variation in the unspiked control sample. As already shown in figure 5, 13-*cis*-retinoic acid and all-*trans*-retinoic acid can be analyzed with higher sensitivity using a 2.0 ml injection volume. Although we did not determine precision using 2.0 ml injection volumes, the coefficient of variation for inter-assay precision of endogenous 13-*cis*-retinoic acid and all-*trans*-retinoic acid can be expected to drop to values of approximately 3% using this injection volume, as observed for the low control quality control sample using an injection volume of 0.4 ml.

Analysis of retinoids in cell culture medium

The method was developed for the analysis of retinoids in plasma samples. We currently investigate the cellular metabolism of retinoic acid by squamous cell carcinoma cell lines and we routinely use the plasma procedure for the analysis of retinoids in cell culture medium. A typical chromatogram is shown in figure 6, demonstrating the possibility to analyze polar metabolites of all-*trans*-retinoic acid that appear in the medium after incubation for 24 h with the cell line UM-SCC-35. As can be seen from figure 6a a large number of compounds that are eluted early in the chromatogram (between 4 and 18 min), are not seen in control incubations without cells (figure 6b). Although none of the early eluting components were identified as yet, this experiment clearly demonstrates the potential of the current chromatographic method to study cellular metabolism of retinoids.

Table 5: *Between-day precision and accuracy for assay of retinoids in plasma (n=8).*

	Concentration (nmol/l) Nominal	Found	Coefficient of variation (%)	Accuracy (%)
Endogenous control				
13-*cis*-4-oxoRA		5.60 ± 0.97	17.3	
13-*cis*-RA		6.18 ± 0.39	6.2	
All-*trans*-RA		7.00 ± 0.77	11.0	
Retinol		2,193 ± 53	2.4	
Low control				
13-*cis*-4-oxoRA	35.6	35.1 ± 2.0	5.8	98.6 ± 5.6
13-*cis*-RA	36.2	36.2 ± 0.9	2.5	100.0 ± 2.5
All-*trans*-RA	37.0	34.3 ± 1.0	2.8	92.7 ± 2.7
Retinol	2,223	2,175 ± 59	2.7	98.1 ± 2.7
High control				
13-*cis*-4-oxoRA	105.6	112.4 ± 3.9	3.5	106.4 ± 3.7
13-*cis*-RA	106.2	112.7 ± 3.4	3.0	106.1 ± 3.2
All-*trans*-RA	107.0	105.2 ± 2.7	2.6	98.3 ± 2.5
Retinol	2,293	2,227 ± 80	3.6	97.1 ± 3.5

RA: retinoic acid.

Discussion

A very simple and rapid procedure for the simultaneous extraction of retinoic acid isomers and retinol from plasma and cell culture medium was developed. By reducing the acetonitrile concentration of the extract obtained, very large injection volumes could be used, without loss of chromatographic resolution, allowing quantification of endogenous levels of retinoids in plasma. In this way concentration of the extracts by time consuming solvent evaporation, with the inherent risk of oxidation and isomerization, is avoided. The extracts can be stored in the autoinjector in the dark at 7°C for up to 60 hours without any change in peak area or isomerization. This allows the analysis of large series of samples. We routinely analyze series of 96 samples. Because sample preparation is very straightforward, excellent precision is obtained without the use of an internal standard. Another advantage of the current procedure is that in a single run both all-*trans*-retinoic acid and 13-*cis*-retinoic acid are analyzed, together with the more apolar retinol, as well as more polar metabolites like 13-*cis*-4-oxoretinoic acid. The combination of high sensitivity and the ability to quantitate polar metabolites make the method a valuable tool for the study of cellular metabolism of retinoids.

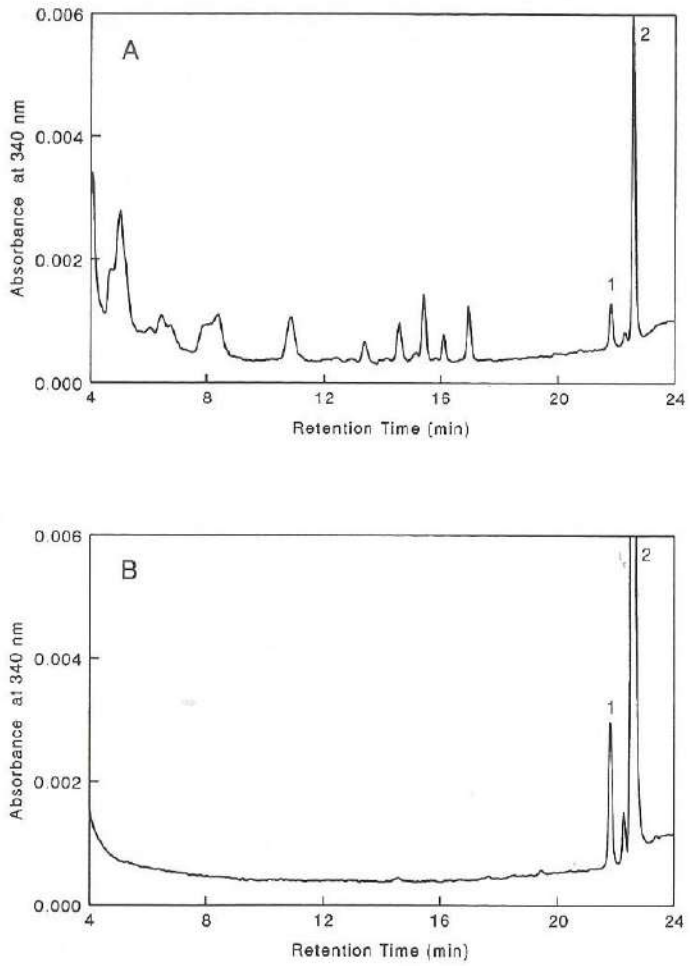

Figure 6: *Metabolism of all-trans-retinoic acid by the UM-SCC-35 cell line. Cells were grown in culture medium supplemented with 1 μM all-trans-retinoic acid. After 24 h the medium was analyzed for retinoic acid metabolites (panel A). In a parallel experiment culture medium supplemented with 1 μM all-trans-retinoic acid without cells was also analyzed after 24 h (panel B). To 0.35 ml of cell culture supernatant or control medium 0.05 ml of 1.0 M sodium acetate buffer (pH 4.0) was added, followed by 0.6 ml of acetonitrile. After vortex mixing samples were centrifuged for 5 min at 3,000 g and 4°C. 0.45 ml of the supernatant was mixed with 0.15 ml of water and stored in the dark at 7°C in the sample compartment of the autosampler. An injection volume of 0.4 ml was used. Chromatographic conditions were as described in the materials and methods section. Only a part of the chromatogram is shown to illustrate the appearance of polar metabolites of all-trans-retinoic acid after incubation with cells. Peak 1, 13-cis-retinoic acid; Peak 2, all-trans-retinoic acid.*

CHAPTER 6

PLASMA RETINOID LEVELS IN HEAD AND NECK CANCER PATIENTS: A COMPARISON WITH HEALTHY CONTROLS AND THE EFFECT OF RETINYL-PALMITATE TREATMENT

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Submitted.

Abstract

Vitamin A and related compounds, also known as retinoids are thought to play a role in the development of head and neck cancer. We measured levels of the major retinoids, retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in plasma of head and neck cancer patients in comparison with controls without cancer. No differences were found between plasma levels of these retinoids between 25 head and neck cancer patients and 21 controls. Mean baseline levels for the patients were 2,458, 6.0, 6.4 and 8.6 nmol/l for retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid, respectively. In addition, we selected 10 patients from the chemoprevention trial Euroscan and measured the effect on retinoid levels of 300,000 IU daily retinyl-palmitate intake during one month. Medication caused significant elevations in plasma levels of retinol (1.2 fold), all-*trans*-retinoic acid (2.2-fold) and its metabolites 13-*cis*-retinoic acid (5.8 fold) and 13-*cis*-4-oxoretinoic acid (8.9 fold). Because of its high increase in levels, 13-*cis*-4-oxoretinoic acid seems a good candidate to serve as a suitable marker to monitor patient compliance in future chemoprevention trials involving retinoids. No relations were found between the occurrence of side-effects of retinyl-palmitate and retinoid levels during treatment. However, the two patients who developed side-effects had the highest pre-treatment levels of 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid, suggesting that retinoid toxicity is associated with relatively high basal retinoid metabolism.

Introduction

Vitamin A or retinol is an essential micronutrient for several processes in man including growth, differentiation, vision and reproduction. The relation between dietary intake of retinol and the development of squamous cell cancer in several tissues of the body was recognized during the last two decades (Bjelke 1975, Byers *et al.* 1984, Fontham 1990). Epidemiological studies on the relation between low serum concentrations of retinol and subsequent cancer risk were not conclusive. A number of these studies showed a positive correlation between low serum retinol concentrations and the development of cancer (Wald *et al.* 1980, Zheng *et al.* 1993), but other studies failed to confirm this (Willett *et al.* 1984, Wald *et al.* 1986, Coates *et al.* 1988, Knekt *et al.* 1990). From the clinical point of view it was recognized that retinoids have activity in leukoplakia, the most common premalignant form of oral cancer (Lippman *et al.* 1993). Oral suppletion of retinoic acid in the form of 13-*cis*-retinoic acid was also able to prevent the formation of second primary tumours in head and neck cancer patients (Hong *et al.* 1990). At this moment it is not clear how retinol and its metabolites (termed 'retinoids') are able to inhibit the development of cancer. In the late eighties more insight in the molecular mechanisms of the action of vitamin A was acquired by the discovery of

the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Petkovich *et al.* 1987, Giguere *et al.* 1987, Mangelsdorf *et al.* 1990). Natural metabolites of retinol, like all-*trans*-retinoic acid and 9-*cis*-retinoic acid bind to these factors and form hetero or homo dimers. These dimers are transcription factors and regulate gene expression after binding to a specific DNA sequence in the promotor region of various genes (Yu *et al.* 1992). Both receptor type as well as type of retinoid are of importance in this regulation (Mangelsdorf *et al.* 1994).

The epidemiologic data supporting a protective effect of retinoids on cancer development and the data of animal studies which showed several retinoids to prevent cancer, resulted in the initiating of clinical chemoprevention trials in the last decade. One of the largest head and neck cancer and lung cancer chemoprevention trials conducted at the moment is the Euroscan trial (De Vries *et al.* 1991, De Vries *et al.* 1993). Cured head and neck cancer or lung cancer patients were randomized in a 2x2 factorial design to receive either retinyl-palmitate or N-acetylcysteine or both or nothing during a period of two years. We were treating these patients in our clinic and we were interested to know whether: 1) retinyl-palmitate treatment had influence on the plasma levels of the several retinoids 2) retinoid levels could be used to monitor patient compliance and 3) it was possible to predict retinoid-induced toxic side-effects.

Materials and methods

Patients and controls

Plasma was obtained from 25 head and neck cancer patients (20 males and 5 females, mean age of 60 years): eight patients with oral cancer, three patients with oropharyngeal cancer and 14 patients with laryngeal cancer (T_1-T_4, N_0-N_{2c}, according to the TNM classification of the UICC (TNM classification of malignant tumours). Nine of the 25 patients were treated for head and neck cancer in the past and had no tumour at the time of blood collection. 21 patients (12 males and 9 females, mean age of 47 years) without cancer in their history and who were admitted at our department or the department of maxillofacial surgery served as control group. The mean values of the smoking habits of the cancer patients were 8.0 cigarettes per day (range: 0-40) versus 7.2 cigarettes per day (range: 0-30) for the control population. The mean values of alcohol consumptions per day of the cancer patients were 2.3 (range: 0-7) versus 1.1 (range: 0-6) for the control population. For the evaluation of the influence of high dose retinyl-palmitate administration on retinoid levels, plasma samples of ten patients participating in the chemoprevention trial Euroscan were examined. Patients used 300,000 IU retinyl-palmitate per day during the first year of treatment, followed by a dose of 150,000 IU retinyl-palmitate per day during the second year. The objective of the Euroscan trial is to study the effect of retinyl-palmitate, N-acetylcysteine or both in preventing or delaying the occurrence of second primary tumours in patients

curatively treated for oral, laryngeal or lung cancer or carcinoma in situ of the larynx. Patients were randomized in agreement with the Euroscan protocol of the European Organization for Research and Treatment of Cancer (EORTC study number 24871). Patients were seen every month in the outpatient clinic where side-effects were recorded. Routine liver function tests were performed (serum levels of SGOT, SGPT, LDH, gamma-GT and bilirubin) as well as serum cholesterol levels. Blood for retinoid analysis was collected immediately following randomization and one month afterwards when patients were visiting our outpatient clinic and 13 months afterwards (N=4). Samples were taken from non-fasting individuals two to five hours after retinyl-palmitate intake.

Blood collection, sample preparation and HPLC procedure

Blood was obtained by venepuncture using vacuum tubes containing EDTA. These tubes were covered with aluminium foil to protect them from light and stored in a 4° C refrigerator. Within one hour after venepuncture, plasma was obtained by centrifugation (10 minutes at 3,000 x g and 4° C). Subsequently, plasma was collected in cryovials that were flushed with nitrogen, before closing. Cryovials were again covered with aluminium foil and then stored in the dark at -70° C. Sample preparation and HPLC procedure were performed as described earlier (Teerlink *et al.* 1997). This method has a high sensitivity and reproducibility.

Results

Cancer patients and controls

No differences were observed between retinoid levels of cancer patients as compared with control individuals (table 1).

Table 1: *Retinoid levels of cancer patients and control individuals.*

Group	N	Retinol	all-*trans*-RA	13-*cis*-RA	13-*cis*-4-oxoRA
HNSCCP	25	2,458 ± 851	6.0 ± 2.5	6.4 ± 2.2	8.6 ± 5.3
Controls	21	2,244 ± 798	4.9 ± 2.2	6.1 ± 2.6	7.4 ± 4.5
p-value*:		0.40	0.16	0.66	0.39

Retinoid plasma levels ± standard deviation in head and neck squamous cell cancer patients (HNSCCP) and controls (levels in nmol/l). *: 2-sample t-test with equal variance. RA: retinoic acid.

Patients using daily retinyl-palmitate

Plasma retinol levels in patients increased slightly, but significantly after a month treatment with 300,000 IU retinyl-palmitate per day. Mean values of plasma levels of all-*trans*-retinoic acid and its metabolites 13-*cis*-retinoic acid and 13-*cis*-4-

oxoretinoic acid were significantly increased two to nine times (table 2). After one year of treatment with 300,000 IU retinyl-palmitate the daily dose of retinyl-palmitate was reduced to 150,000 IU retinyl-palmitate according to the protocol of the Euroscan trial. The plasma retinoid levels of four patients were evaluated in this second year. After one month of treatment with the lower dose retinyl-palmitate (which is 13 months after randomization for the trial) plasma samples were investigated. As expected, retinoid levels in the second year were lower than in the first year, when patients were using the double dose, but higher than before treatment. Surprisingly, in three of the four evaluated patients, retinol levels were even lower at this time point than before treatment (table 3). This difference was not significant ($p=0.18$, 2-sample t-test with equal variance).

Table 2: *Retinoid levels during retinyl-palmitate treatment.*

Patients	Retinol	all-*trans*-RA	13-*cis*-RA	13-*cis*-4-oxoRA
Pre-treatment	2,608 ± 622	5.7 ± 2.1	6.6 ± 2.0	10.3 ± 5.3
During treatment	3,242 ± 445	12.8 ± 8.9	38.2 ± 12.9	92.1 ± 22.0
p-value*:	0.01	0.01	$<10^{-5}$	$<10^{-5}$

Retinoid plasma levels ± standard deviation in 10 patients before and 1 month after starting a daily intake of 300,000 IU retinyl-palmitate (levels in nmol/l).
*: paired 2-sample t-test. RA: retinoic acid.

Table 3: *Retinoid levels after long-term retinyl-palmitate treatment.*

Patients	Retinol	all-*trans*-RA	13-*cis*-RA	13-*cis*-4-oxoRA
Pre-treatment	2,235 ± 632	5.2 ± 1.7	7.1 ± 2.2	11.8 ± 4.3
After 1 month	3,077 ± 375	13.9 ± 7.0	40.0 ± 4.5	100.1 ± 21.8
After 13 months	1,709 ± 687	8.7 ± 4.6	21.3 ± 11.4	82.7 ± 35.1

Retinoid plasma levels ± standard deviation in four patients before treatment, after 1 month daily treatment with 300,000 IU retinyl-palmitate, and after 12 months treatment with 300,000 IU retinyl-palmitate followed by 1 month treatment of 150,000 IU retinyl-palmitate (levels in nmol/l).
RA: retinoic acid.

Side-effects and safety parameters

Two of the ten patients using retinyl-palmitate reported side-effects. These side-effects were well tolerated complaints of mucosal dryness in one patient and poorly tolerated skin desquamation in another patient. In this last patient, the daily dose of retinyl-palmitate was reduced to 150,000 IU, resulting in moderate and bearable side-effects. The additional eight patients did not report to have side-effects. The pre-treatment levels of 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid were noted to be the highest in the patients with side-effects. The levels of 13-*cis*-retinoic acid

in the patients with side-effects were 10.8 and 9.6 nmol/l compared to a mean level of 5.8 nmol/l (range: 4.1 to 7.2 nmol/l) in the patients without side-effects. The levels of 13-*cis*-4-oxoretinoic acid in the patients with side-effects were 18.9 and 20.4 nmol/l compared to a mean level of 7.9 nmol/l (range: 3.3 to 11.6 nmol/l) in the patients without side-effects. The levels of retinol and all-*trans*-retinoic acid showed no differences between the patient with and without side-effects. None of the 10 patients had liver function disturbances as checked by blood tests. One patient had an increase in cholesterol level from 4.7 to 10.2 mmol/l. Plasma retinoid levels after treatment of this patient were within the range of those of the other patients without cholesterol level increase.

Discussion

During the last decades epidemiological studies revealed a relationship between vitamin A intake and subsequent cancer risk. With the discovery of the retinoid receptors a molecular basis was provided for a possible working mechanism of retinol which could explain these epidemiological observations. Retinol itself does not bind to the RARs or RXRs and it has to be converted into all-*trans*-retinoic acid or 9-*cis*-retinoic acid before it can induce or inhibit gene-expression. In most epidemiological studies no or minor differences in blood retinol concentrations were found in cancer patients compared with controls, not explaining an increased cancer risk of specific individuals. Besides these findings, oral supplementation of 13-*cis*-retinoic acid showed to have a protective effect on the development of second primary tumours in head and neck cancer patients (Hong *et al.* 1990) and a therapeutic effect was found of this retinoic acid metabolite and also of all-*trans*-retinoic acid on the development of oral leukoplakia (Lippman *et al.* 1993). These two findings suggest a metabolic defect which could be restored by supplementing 13-*cis*- and all-*trans*-retinoic acid rather than retinol. We were therefore interested in the plasma concentrations of all-*trans*-retinoic acid and its metabolites 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in head and neck cancer patients, assuming that plasma levels reflect intracellular processes. The present study showed no difference in the plasma levels of retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid between head and neck cancer patient and healthy controls. As for all-*trans*-retinoic acid and 13-*cis*-retinoic acid, these results were in agreement with the findings of a Swedish group (Wahlberg and Fex 1996), although these investigators reported somewhat lower values for the patient and control group. So, the combined data of both studies show that a relatively poor nutritional status, that may be expected in head and neck cancer patients, is not reflected in an alteration of the plasma levels of the most important retinoids. Consequently, it seems that the beneficial effect of 13-*cis*-retinoic acid on tumour-development and leukoplakia-advancement can not be explained by correcting a deficiency of this metabolite. Apparently, the levels of retinoids in plasma are a too crude indicator of the intracellular retinoid levels in epithelial cells influencing the

process of gene transcription.

We investigated the plasma levels of several retinoids in samples of patients randomized to use retinyl-palmitate. We found a considerable increase of blood retinoids in all patients using retinyl-palmitate. The retinoic acid metabolite 13-*cis*-4-oxoretinoic acid showed the highest increase in plasma concentration of about nine times the pre-treatment levels. 13-*cis*-retinoic acid showed a mean plasma level increase of about six times the pre-treatment levels. Another study showed stationary plasma concentrations of 13-*cis*-4-oxoretinoic acid during the day independent of the interval after retinoid intake, while 13-*cis*-retinoic acid showed considerable fluctuations of plasma levels during the day after retinoid intake (Eckhoff *et al.* 1991). A well known problem in clinical chemoprevention trials is that it is troublesome to monitor patient compliance adequately. This monitoring is in most trials realized by questioning patients about taking their chemopreventive medication or by pill count. Both methods are subjective and the reliability of these methods is questionable. Earlier studies already showed increasing levels of retinoic acid and its metabolites after supplementation of retinol or retinyl-palmitate (Eckhoff and Nau 1990b, Tang and Russell 1991). Because of its high increase of levels 13-*cis*-4-oxoretinoic acid seems a very suitable candidate to serve as an objective plasma marker to monitor patient compliance in future chemoprevention trials involving retinyl-palmitate and possibly other retinoids.

Four patients were evaluated more than one year after the onset of retinyl-palmitate treatment. Patients randomized in one of the retinyl-palmitate arms of the Euroscan trial begin to use 300,000 IU retinyl-palmitate daily during one year. In the second year this dose is reduced to 150,000 IU retinyl-palmitate daily. As expected, plasma levels of all-*trans*-retinoic acid and its metabolites 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid were lower in the second year in comparison with the plasma levels in the first year of treatment. Surprisingly, retinol levels in the patients still receiving the relatively high dose of 150,000 IU retinyl-palmitate daily were reduced compared with the pre-treatment levels in three of these patients suggesting an elevated metabolism of retinyl-palmitate in the body after a prolonged period of retinyl-palmitate supplementation.

A well known problem in chemoprevention trials using retinoids is the occurrence of serious side-effects in a number of patients. In our group of patients using 300,000 IU retinyl-palmitate daily, side-effects were present in two patients. Plasma levels during retinyl-palmitate treatment of patients who experienced side-effects were comparable with the levels of the patients without side-effects. An explanation of this observation could be that plasma levels of retinoids do not correlate with the intracellular retinoid levels of epithelial cells. The pre-treatment levels of 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid were the highest in the two patients with side-effects. Both are metabolites of all-*trans*-retinoic acid, which suggests an increased basal retinoid metabolism in these two patients. The numbers of patients with side effects is small in this study and more research is required to confirm this observation.

CHAPTER 7

ALL-*TRANS*-RETINOIC ACID INDUCED GENE-EXPRESSION AND GROWTH INHIBITION IN HEAD AND NECK CANCER CELL LINES

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European Journal of Cancer Part B: Oral Oncology 1997, 33:270-274.

Abstract

Retinoids are natural and synthetic analogues of vitamin A and have proven activity in various types of cancer. As for head and neck squamous cell cancer, retinoids are especially active in leukoplakia and in preventing second primary cancers. The aim of this study was to assess the growth inhibiting activity of all-*trans*-retinoic acid in a panel of six head and neck squamous cell cancer cell lines and to correlate this response to the mRNA expression of factors related to differentiation and receptor mediated signal transduction. Three lines showed minimal, two moderate and one strong growth inhibition after 72 hours exposure to all-*trans*-retinoic acid. Three lines with a dissimilar response were selected for further studies, the measurement of mRNA expression by northern blotting. It was found that neither the expression nor the induction of Retinoic Acid Receptor-α, -β and -γ and Retinoic X Receptor-α mRNA was related to sensitivity. The mRNA expression of RAR-β was too low to be measured in the three cell lines. The most sensitive cell line was, however, the only one that expressed mRNA of squamous differentiation markers. These data suggest a relationship between the retinoid sensitivity profile and the degree of cellular differentiation.

Introduction

One of the major problems in patients with early stage head and neck cancer is the occurrence of second primary tumours in the respiratory and upper digestive tract after curative therapy of the initial tumour (Tepperman and Fitzpatrick 1981, Haughey *et al.* 1992). In patients with early stage head and neck cancer the prognosis is more determined by the development of these second primary tumors than by their initial tumor. The occurrence of multiple primary tumours in a single patient can be explained by the phenomenon of "field cancerization" (Slaughter 1946, Copper *et al.* 1993). Prevention of multiple primary tumours is gaining more and more interest during the last decade. Next to the elimination of the well-known etiological factors smoking and alcohol consumption (Brugère *et al.* 1986, Day *et al.* 1994), the use of drugs to block or inhibit new tumour development, also known as chemoprevention, represents a new strategy in cancer prevention. Numerous epidemiological studies have demonstrated that nutritional factors are associated with a decreased risk to develop squamous cell cancer (Stähelin *et al.* 1984, Salonen *et al.* 1985, Zheng *et al.* 1993) and one of the most promising class of compounds involved are retinoids (Bollag and Holdener 1992). Several studies showed a chemopreventive effect in patients using retinoids. 13-*cis*-retinoic acid was shown to have a protective effect in curatively treated head and neck cancer patients on the development of second primary tumours (Hong *et al.* 1990), and also had a therapeutic effect on a proportion of patients with leukoplakia (Lippman

et al. 1993). In addition, retinyl-palmitate has some activity in the prevention of second primary tumours and recurrences in patients who had been surgically treated for stage I lung cancer (Pastorino *et al.* 1993).

Retinoids are a class of compounds consist of the natural vitamin A derivatives, such as retinol, retinal, retinoic acid, their various metabolites and in addition a large number of synthetic analogues. At the cellular level it is not exactly known how retinoids can inhibit or reverse the process of carcinogenesis. Development and maintenance of normal tissue depends on an adequate balance between growth and differentiation as well as cell renewal and cell loss. In cancer this equilibrium is disturbed. Retinoids could be effective in restoring this disturbed balance by induction of cellular differentiation and inhibition of cellular proliferation (Strickland and Mahdavi 1978, Lotan 1980, Warrell 1993).

The selection of retinoids or beta-carotene in most current chemopreventive studies in the world is based on epidemiological data or animal studies (De Vries *et al.* 1991). However, two chemopreventive studies recently showed an adverse effect of beta-carotene as a single agent or combined with retinol on the prevention of the development of lung cancer (Alpha-tocopherol, beta carotene cancer prevention study group 1994, Omenn 1996a). These dissatisfying results indicate that it is important to plan future chemoprevention trials with drugs of which the mechanism of action is better known. Studies on the mechanism of action of retinoids have progressed in the last decade with the discovery of the nuclear retinoic acid receptors (RARs) (Petkovich *et al.* 1987, Giguere *et al.* 1987). The nuclear retinoid receptors are members of the super-family of steroid hormone receptors that act as ligand-activated trans-acting transcription factors. Three different RAR subtypes have been discovered so far: RAR-α, RAR-β and RAR-γ (Brand *et al.* 1988, Krust *et al.* 1989). A second class of retinoid responsive transcription factors was discovered subsequently: the retinoid X receptors (RXRs) (Mangelsdorf *et al.* 1990). Three different RXRs have been identified: RXR-α, RXR-β and RXR-γ. Retinoids are ligands for the RARs and RXRs and after binding, homo- or hetero-dimers are formed, and these bind to a specific DNA sequence, the retinoic acid response elements (RARE) or the retinoid X response elements (RXRE). So in this way up- or down-regulation of specific gene transcription is determined by the "retinoid signal transduction pathway". It is evident that a number of factors, i.e. the availability of the various retinoids and receptors are determinants in these processes.

This study was undertaken to see whether a variation in all-*trans*-retinoic acid induced growth inhibition between head and neck squamous cell carcinoma cell lines exists and which factors are related to this process. Three head and neck squamous cell carcinoma cell lines with a different pattern of sensitivity to all-*trans*-retinoic acid were selected and further characterised with respect to mRNA expression of putative relevant genes, including RARs and RXRs and the ones related to squamous differentiation.

Materials and methods

Cell culture and all-trans-retinoic acid treatment procedures

The characteristics of the cell lines are presented in table 1. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Flow Laboratories, Irvine, UK) with 5% fetal calf serum (FCS) (Hyclone, Utah, USA).

Table 1: *Characteristics of head and neck squamous cell carcinoma cell lines.*

Cell line	Site	Degree of differentiation*	Doubling time (hour)
UM-SCC-11B	Larynx	Unknown	31
UM-SCC-14A	Larynx	Unknown	26
UM-SCC-14C	Oral cavity	Poor	26
UM-SCC-22A	Hypopharynx	Well	36
UM-SCC-22B	Hypopharynx	Well	34
UM-SCC-35	Oropharynx	Well	53

The cell lines were obtained from Dr T. Carey, Ann Arbor, USA.
*: As established in xenografts transplanted into nude mice.

Sensitivity testing

The cell lines were tested for growth inhibition with a microtiter 96 well cell proliferation assay that is based on the staining of cellular protein with Sulphorodamine B (Braakhuis *et al.* 1993). Cell lines were cultured in the presence of different all-*trans*-retinoic acid concentrations of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/l. The stock solution was made in dimethyl sulfoxide (DMSO) (JT Baker, Deventer, Holland) at a concentration of 10^{-2} mol/l. For each experiment, this stock was further diluted with cell culture medium. The exposure of the cell lines to all-*trans*-retinoic acid was for 72 hours. Before the retinoids were added a latency time of 72 hours was allowed. The amount of cells plated was chosen as such that exponential control growth during the exposure period was ensured.

Probes

cDNA clones for the experiments were kindly provided by dr P. Chambon (RAR-α, -β, -γ and RXR-α), dr. A. Åstrom (CRABP-I), dr C. Backendorff (SPRR1, 2 and 3), dr W. Franke (Cytokeratin 10) and dr H. Green (Cytokeratin 19). Plasmids were propagated in *Escherichia coli* according to Sambrook *et al.* (1989). The following DNA fragments were used: a 1918 bp EcoRI RAR-α fragment, a 1440 bp SacI/BamHI RAR-β fragment, a 670 bp BamHi/SacI RAR-γ fragment, a 1.5 kb EcoRI RXR-α fragment, a 590 bp XbaI/BamHI CRABP-I fragment, a 330 bp BamHI cytokeratin 10 fragment, a 745 bp SmaI cytokeratin 19 fragment, a 440 bp EcoRI SPRR1 fragment, a 680 bp RsaI SPRR2 fragment and a 250 bp HaeIII/HinfI

SPRR3 fragment. The fragments were excised from the vector by the appropriate enzymes and separated by agarose gel electrophoresis (Pharmacia Benelux, Roosendaal, the Netherlands). The band was cut from the gel and frozen. The DNA was squeezed out between parafilm, purified by phenol chloroform extraction and collected by ethanol precipitation. The DNA fragments were labelled by multiprimed elongation using [^{32}P]dCTP (3,000 Ci/mmol: DuPont Co., Boston, USA) to a specific activity of about 10^9 dpm/μg (Feinberg and Vogelstein 1983).

RNA isolation

Total RNA was isolated from cultured cells according to Gough (1988). Total RNA (20 μg) was loaded on to a 1% agarose formaldehyde gel and electrophoresed in 3-(N-morpholine-)propane sulfonic acid (MOPS) buffer essentially as described by Sambrook *et al.* (1989). The RNA was blotted by capillary transfer in 10xSSC on to genescreen plus filters (DuPont Co.). The RNA was cross-linked to the filter by heating for 2 hours at 80°C, prehybridised in 7% SDS, 0.5 mol/l sodiumphosphate buffer, 1mM ethylene-diamine-tetra-acetic acid (EDTA), pH 7.0 for 2 hours at 65°C, and after addition of the denatured probe hybridised at 65°C for 16 hours. After hybridisation the filters were washed twice with 2xSSC, 0.1% SDS and twice with 0.2 SSC, 0.1% SDS at 65°C for 15 minutes. The bands were visualised by autoradiography with Kodak X-AR 5 film using intensifying screens.

Quantification of RNA

The bands on the autoradiographs were quantified using a charge coupled device video camera coupled to an image processing system (Cybertech, Berlin, Germany). 18S rRNA was used as an internal standard to correct for the amount of total RNA loaded on the gel. The amount of RNA was expressed as a percentage of the highest measured value.

Results

Inhibition of cell proliferation

Growth inhibition by all-*trans*-retinoic acid was tested in six head and neck squamous cell carcinoma cell lines. UM-SCC-35 was the most all-*trans*-retinoic acid sensitive cell line, with a growth inhibitory effect occurring at 10^{-9} mol/l all-*trans*-retinoic acid. UM-SCC-14A, -14C and -11B did not show any inhibitory growth at all-*trans*-retinoic acid concentrations between 10^{-9} and 10^{-6} mol/l and some inhibition at the relatively high concentration of 10^{-5} mol/l all-*trans*-retinoic acid. UM-SCC-22A and 22B showed an intermediate type of response, with a moderate growth inhibition at concentrations between 10^{-5} through 10^{-9} mol/l all-*trans*-retinoic acid. Three lines were chosen on the basis of their difference in sensitivity. Details in the pattern of inhibition of these six lines are shown in table 2.

Table 2: *The inhibition of growth after exposure to 10^{-6} and 10^{-8} mol/l all-trans-retinoic acid in six head and neck squamous cell carcinoma cell lines.*

Cell line	Percentage growth with 10^{-6} mol/l all-*trans*-RA	Percentage growth with 10^{-8} mol/l all-*trans*-RA
UM-SCC-14C:	97 ± 8.0	106 ± 7.3
UM-SCC-22A:	73 ± 5.0	73 ± 5.7
UM-SCC-35:	9.0 ± 10	44 ± 22
UM-SCC-11B:	95 ± 8.0	101 ± 7.3
UM-SCC-22B:	78 ± 6.8	68 ± 12
UM-SCC-14A:	76 ± 13.3	69 ± 10

Results are from three separate experiments and expressed as the mean ± standard deviation. Results from untreated control cells are set at 100%. RA: retinoic acid.

Expression of mRNA in cell lines

In three head and neck squamous cell carcinoma cell lines the mRNA expression of several genes and its regulation by all-*trans*-retinoic acid was investigated by northern blotting. Results are presented in table 3. The mRNA levels of CRABP-I and RAR-β appeared to be too low to be measured by northern blotting. Expression levels for all other receptors tested (RAR-α, -γ and RXR-α) varied per cell line. RAR-α and RXR-α mRNA levels were the lowest for UM-SCC-35, but still present in a significant amount. It appeared that in most cases the expression of RAR-α and -γ and of RXR-α was increased by all-*trans*-retinoic acid. There were two exceptions: RAR-γ expression was down-regulated in UM-SCC-14C (figure 1) and RAR-α in UM-SCC-22A.

Table 3: *mRNA expression in three head and neck squamous cell carcinoma cell lines.*

	SCC-14C		SCC-22A		SCC-35	
	Untreated	all-*trans*-RA	Untreated	all-*trans*-RA	Untreated	all-*trans*-RA
RAR-α	94	100	84	45	12	24
RAR-γ	95	51	83	100	47	84
RXR-α	39	47	39	100	6	9
CK 10	1	0	0	0	100	43
CK 19	4	0	86	100	3	3
SPRR1	0	0	0	0	94	100
SPRR2	0	0	4	0	100	91
SPRR3	0	0	0	0	67	100

Data on expression are given as percentage of the highest value. Exposure to all-*trans*-retinoic acid was for 24 hours at a concentration of 10^{-6} mol/l. 18S rRNA was used as an internal standard to correct for the amount of RNA loaded on to the gel. CRABP-I and RAR-β expression was too low to measure. CK: Cytokeratin. RA: retinoic acid.

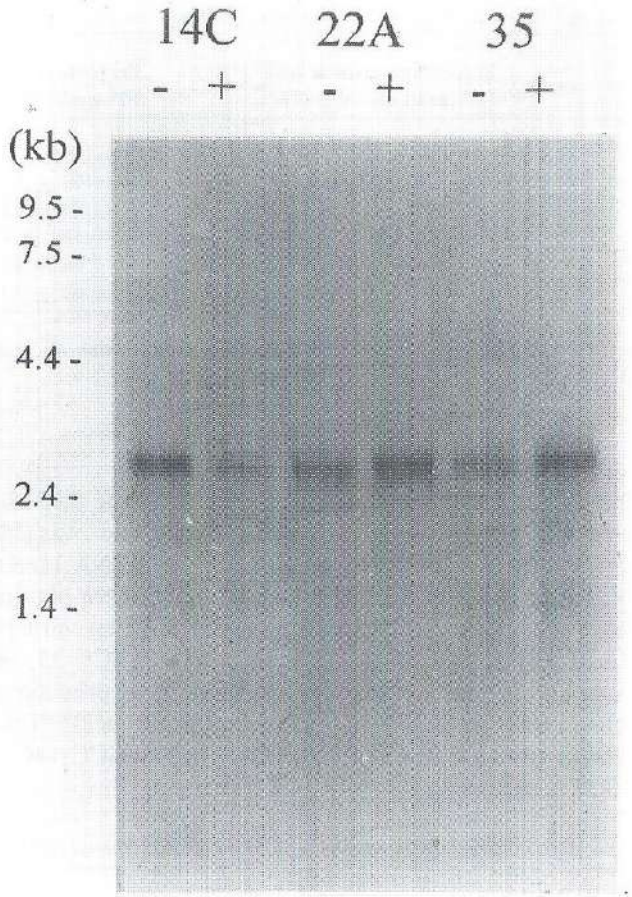

Figure 1: Northern blot analysis of expression of RAR-γ mRNA in untreated (-) and all-*trans*-retinoic acid treated (+) head and neck squamous cell carcinoma cells. 18S rRNA was included as a control and showed minimal or no difference between the cell lines.

Cytokeratin 10 mRNA was detected only in UM-SCC-35 where it showed a decrease in expression after all-*trans*-retinoic acid exposure. Cytokeratin 19 mRNA was detected in UM-SCC-22A only, in which it showed a slight upregulation after all-*trans*-retinoic acid exposure. The small proline-rich proteins SPRR1, SPRR2 and SPRR3 were expressed in UM-SCC-35 exclusively. In this cell line, SPRR3 appeared to be upregulated by all-*trans*-retinoic acid, whereas SPRR1 and SPRR2 showed no obvious difference in expression after exposure to all-*trans*-retinoic acid.

Discussion

The observed difference of all-*trans*-retinoic acid sensitivity in different head and neck squamous cell carcinoma cell lines could depend on the availability of the retinoids or retinoid receptors or to the commitment to differentiation. UM-SCC-35 is a remarkably all-*trans*-retinoic acid sensitive cell line. This cell line exhibited several features that were not present with the other two less sensitive cell lines. First, RNA coding for cytokeratin 10 was only present in UM-SCC-35. Cytokeratin 10 is a protein which is present in terminally differentiated cornifying squamous epithelia (Morgan *et al.* 1987b). Second, UM-SCC-35 was the only cell line that expressed SPRR RNA. SPRR genes encode a class of polypeptides that are strongly induced during end-stage differentiation of human epidermal keratinocytes and are involved in the cross-linking of the cornified envelopes (Gibbs *et al.* 1993). The last two findings indicate that the UM-SCC-35 cell line has a more differentiated phenotype than the other cell lines. Although carcinogenesis is often linked to an abnormal expression of differentiation related genes and a deviant response to differentiation inducing stimuli, some malignant cells are able to conserve the expression of various squamous-cell differentiation markers (Parkinson 1985, Jetten *et al.* 1990). The results of the present study suggest that a more differentiated phenotype is associated with all-*trans*-retinoic acid sensitivity. Assessment of the differentiation status of a squamous cell carcinoma (or a precursor lesion) may be important to predict a retinoid response. It must be stressed that the present results are preliminary and that further studies should be performed to substantiate the findings.

RAR-β mRNA levels were found to be very low in all three head and neck cancer cell lines. It was already known that RAR-β expression is selectively lost in premalignant oral mucosa (Lotan *et al.* 1995) and our findings are consistent with earlier studies that showed a low RAR-β expression in head and neck squamous cell cancers (Xu *et al.* 1994). In breast cancer and renal cancer it ha been found that RAR-β expression is correlated with all-*trans*-retinoic acid sensitivity (Hoffman *et al.* 1996, Jing *et al.* 1996).

RAR-α, RAR-γ and RXR-α showed variable baseline RNA expressions and various responses after all-*trans*-retinoic acid exposure in all three cell lines, suggesting they are not significant markers of all-*trans*-retinoic acid sensitivity. The present results confirm the study of Xu *et al.* (1994) that also showed a lack of correlation between the mRNA expression of retinoid receptors and sensitivity.

Another factor that may be important in explaining sensitivity to retinoids is the expression of CRABP-II mRNA, which encodes a protein probably involved in regulating the concentration of free all-*trans*-retinoic acid in the cell (Åström *et al.* 1991, Delva *et al.* 1993). It shows a very low basic mRNA level in UM-SCC-35, the most sensitive cell line, and an increased expression could not be induced by all-*trans*-retinoic acid (Braakhuis *et al.* 1997). This suggests that CRABP-II provides protection to the cells against toxic effects of supraphysiological levels of all-*trans*-retinoic acid and other retinoids.

CHAPTER 8

SUMMARY, CONCLUSIONS AND PERSPECTIVES

SUMMARY

In this thesis the application of biomarkers in chemoprevention of second primary cancers in head and neck cancer is investigated.

Chapter 1

A general introduction concerning the problem of second primary tumours in head and neck cancer patients is given. Early stage head and neck cancer patients have a relatively high risk of 15 - 35 percent to develop second primary tumours some time after treatment of their first cancer. Chemoprevention is a new treatment modality intended to decrease the incidence of multiple primary tumours in these patients. Chemoprevention means to stop or to delay the process of carcinogenesis in target tissue by the administration of chemical compounds. Worldwide much research is being performed in order to find effective substances with little side-effects. Beta-carotene and the retinoids, which include retinol and its natural and synthetic analogues, are the best-studied class of agents in chemoprevention. Major problems in clinical chemoprevention trials are the long duration and the large number of subjects needed to conduct this kind of trials. If other, earlier end points than the occurrence of second primary tumours could be used to obtain study results, potential chemopreventive drugs could be screened much faster. One of the possibilities is the application of so called intermediate endpoint biomarkers. A review of the literature on the current status of head and neck cancer chemoprevention and biomarker research is reported in this chapter.

Chapter 2

The phenomenon of second primary tumours can be explained by the concept of field cancerization, which assumes that the mucosa of the whole respiratory and upper digestive tract is affected and prone to malignant transformation as a result of repeated exposure to tobacco products and other carcinogens. It can be hypothesized that the patients with the most severe manifestations of field cancerization are the ones who will develop a second primary tumour. At the molecular level support for the theory of field cancerization is still scarce. In this chapter a study is described which was undertaken to investigate the value of a panel of nine monoclonal antibodies to identify biomarkers in oral mucosa associated with cancer risk. An immunocytochemical study was performed on macroscopically normal oral mucosa of patients with carcinoma of the tongue and that of healthy controls without cancer. The panel consisted of antibodies against cytokeratin 8, 10, 13 and 19, the proliferation marker Ki-67 and the monoclonal

antibodies designated K928, K931, K984 and UM-A9. The results showed that cancer patients had a more than three times increased expression of cytokeratin 19 in their macroscopically normal mucosa as compared to controls, which makes it an interesting candidate to quantify the process of field cancerization.

Chapter 3

Quantitative DNA analysis has often been proposed as a potential tool capable to detect preneoplastic tissue. As such it may function as an intermediate endpoint in cancer chemoprevention trials. In chapter three a study is described which was undertaken to test whether cytomorphometric parameters could be used to detect characteristics associated with field cancerization in cytological preparations of oral mucosa. Cytomorphometric parameters in exfoliated cells of apparently normal oral mucosa of head and neck cancer patients were compared with those of healthy controls. Several markers were determined by using the CAS model 200 image analysis system. No differences were detected between cancer patients and controls. The second aim of the study described in this chapter was to assess the value of these parameters subsequently as intermediate endpoint biomarkers in the mucosa of patients receiving N-acetylcysteine and/or retinyl-palmitate as chemopreventive drugs. Cytological samples of the oral mucosa were taken before the onset of therapy and two or three months later during therapy. This group of patients comprised 70 persons. 19 patients received N-acetylcysteine, 17 patients used retinyl-palmitate and 18 patients used the combined treatment. 16 patients who used no medication served as a control group. In these groups, no differences were observed before and after treatment with the chemopreventive drugs. Consequently, in this population of patients the use of quantitative DNA analysis of cytological samples by using the CAS 200 system seems not to be of value in order to detect tissue at high risk for cancer in the head and neck area or to detect alterations in mucosal cells as modulated by retinyl-palmitate or N-acetylcysteine.

Chapter 4

One of the most extensively studied intermediate end point biomarkers during the last decade is the micronucleus. Micronuclei are chromosome or chromatid fragments formed in proliferating cells during clastogenic events and remain separate after mitosis. The micronuclei in the epithelium of the head and neck area are formed in the proliferating basal cell layer, which gives rise to suprabasal cells that migrate to the epithelial surface and can eventually be detected in easily obtainable exfoliated cells. The presence and frequency of micronuclei in tissue are believed to be quantitative reflections of ongoing DNA damage and/or genetic instability. A strict protocol for counting micronuclei is a prerequisite to get a reproducible and

sensitive indicator of exposure to carcinogens and for cancer risk. In this chapter a study is described in which such a protocol is defined. In this study it appeared that, in contrast to almost all performed clinical studies so far, at least 10,000 exfoliated cells per individual should be screened to monitor significant reductions in the number of micronuclei during and/or after chemopreventive intervention.

Chapter 5

The analysis of retinoids in biological samples is a challenging problem, due to sensitivity of these compounds to light and heat, low endogenous concentrations and strong protein binding. For the analysis of large numbers of samples in chemoprevention trials a simple and reliable system was needed. The method reported in chapter five is a new, very simple and rapid procedure for the simultaneous determination of the most important retinoids in plasma and cultured cells. The basis of the system was a new sample extraction process and the detection method proved to be sensitive and reproducible.

Chapter 6

There is some epidemiological evidence that retinoids might play a role in the development of head and neck cancer. In this chapter a study is described, in which retinoids in head and neck cancer patients are measured, by using the new developed method described in the previous chapter. Levels of retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid were analyzed in plasma of head and neck cancer patients in comparison with controls without cancer. In addition ten patients from the chemoprevention trial Euroscan were selected and the effect on retinoid levels of daily retinyl-palmitate treatment during one month was observed. No differences were found between plasma levels of these retinoids between 25 head and neck cancer patients and 21 controls, but retinyl-palmitate treatment caused significant elevations in plasma levels of retinol, all-*trans*-retinoic acid and its metabolites 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid. Because of its high increase in levels, 13-*cis*-4-oxoretinoic acid seems a good candidate to serve as a suitable marker to monitor patient compliance in future chemoprevention trials involving retinoids.

Chapter 7

The selection of retinoids or beta-carotene in most chemoprevention studies is based on epidemiological data or animal studies. However, several studies recently showed an adverse effect of chemopreventive intervention on the development of

lung cancer. These dissatisfying results indicate that it is important to plan future chemoprevention trials with drugs of which the basic molecular mechanism of action is better understood. The study reported in chapter seven was undertaken to see whether a variation in all-*trans*-retinoic acid induced growth inhibition between head and neck squamous cell carcinoma cell lines exists and which factors are related to this process. Three lines showed minimal, two moderate and one strong growth inhibition after 72 hours exposure to all-*trans*-retinoic acid. Three lines with a variation in response were selected for further studies. It was found that the expression of retinoic acid receptor-α, -β and -γ and retinoid X receptor-α mRNA with and without induction by retinoic acid was not related to sensitivity. The most sensitive cell line, however, had the highest mRNA levels of squamous differentiation markers, which suggests a relationship between the retinoid sensitivity profile and the degree of cellular differentiation.

CONCLUSIONS AND PERSPECTIVES

Second primary tumours remain a threat for head and neck cancer patients for many years after curative treatment of their first cancer. Chemoprevention is one of the few potential options to reduce the morbidity and the mortality of second primary cancers in these patients. Recently, negative results were published from two large primary prevention trials showing an adverse effect of beta-carotene on the development of lung cancer in heavy smokers (Alpha-tocopherol, beta-carotene cancer prevention study group 1994) and ex-smokers (Omenn *et al.* 1996a). As for prevention of second primary tumours after head and neck cancer both a positive and a negative (no effect) result have been reported. The M.D. Anderson chemoprevention study showed a beneficial effect of 13-cis-retinoic acid in reducing second primary tumours in head and neck cancer patients (Hong *et al.* 1990). The French GETTEC study showed no cancer preventive effect of etretinate (Bolla *et al.* 1994). Results will soon be available from six more trials.

Animal studies provide many promising potential chemopreventive agents to be tested in clinical trials, but it is impossible to test them all in phase II and phase III clinical chemoprevention studies. Because second primary tumours develop at the relatively low annual rate of three percent, these trials need large numbers of subjects and a long term follow-up, when cancer incidence is the study endpoint. The use of suitable intermediate endpoint biomarkers would reduce the size, length and costs of clinical prospective randomized trials in high-risk populations. Biomarkers can also be used to obtain a better cancer risk assessment of individuals. More accurate risk estimation, for instance with the mutagen sensitivity test, can define susceptible subgroups who might benefit from intensive screening and chemoprevention programs.

The verification of an intermediate endpoint is faced with the same problems

as chemoprevention trials themselves, because the ultimate goal of an intermediate endpoint is that the modulation of the biomarker correlates with the clinical outcome of the trial. So, a biomarker can only be validated if the clinical results are available of the chemoprevention trial in which the biomarker was evaluated. In our study cytokeratins 13 and 19 showed promising results in order to identify condemned mucosa. Subsequently, both proteins were evaluated in 70 patients, who were randomized for the Euroscan trial and who used retinyl-palmitate and/or N-acetyl-cysteine. No decreased expression of both cytokeratins was observed during and after this chemopreventive intervention. The fact that the results of that study showed no changes can be explained in three ways: a) cytokeratin 13 and 19 are inappropriate to serve as intermediate endpoints, b) the chemopreventive medication in Euroscan is not effective, or c) both. Which of these three explanations is correct will hopefully be known when the clinical results of the Euroscan trial will become available. The cytomorphometric study described in this thesis deals with the same validation problem. Both studies illustrate that the validation of a biomarker takes at least as much time as a chemoprevention trial itself. However, once good intermediate endpoints are available, the clinical testing of new chemopreventive drugs can be performed so much faster, that this justifies all future efforts to determine appropriate intermediate endpoint biomarkers. The only way to progress in cancer chemoprevention is to perform well designed phase II and III clinical trials in which biomarker research is incorporated. In phase II trials potential biomarkers should be selected on aspects like dose biomarker-response modulation. Especially when short phase II trials are being performed in patients with intra-epithelial neoplasia, tissue is easily available to be analyzed on various biomarkers. Subsequently, in phase III trials significant reductions in incidence or delay in occurrence of cancer should be evaluated, and the biomarkers selected in the phase II trials should be validated as surrogate study end points.

A pitfall in most chemoprevention trials is that drugs are used of which the exact working mechanism is not known. There still is no plausible explanation why for example beta-carotene, etretinate or retinol are not effective in preventing cancer, in contrast to 13-*cis*-retinoic acid, which seems to be active. It appears that the selection of drugs in most trials is based on trial and error rather than on fundamental scientific knowledge. Uncovering the molecular mechanisms of action of for example retinoids could perhaps provide more rational choices in deciding which drugs should be tested in upcoming trials.

In conclusion, much effort should be put into basic chemopreventive research, like biomarkers and working mechanisms of drugs, because at present cancer chemoprevention seems to be one of the few modalities that theoretically could decrease the morbidity and mortality of second primary cancers in head and neck cancer patients.

REFERENCES

References

Albanes D, Heinonen OP, Taylor PR, Virtamo J, Edwards BK, Rautalahti M, Hartman AM, Palmgren J, Freedman LS, Haapakoski J, Barrett MJ, Pietinen P, Malila N, Tala E, Liippo K, Salomaa ER, Tangrea JA, Teppo L, Askin FB, Taskinen E, Erozan Y, Greenwald P, Huttunen JK. α-Tocopherol and β-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance. J Natl Cancer I 1996; 88:1560-1570.

Alpha-tocopherol, beta-carotene cancer prevention study group. The effect of vitamin E and beta-carotene on the incidence of lung cancer and other cancers in male smokers. New Engl J Med 1994; 330:1029-1035.

Arlett CF, Ashby J, Fielder RJ, Scott D. Micronuclei: origins, applications and methodologies - a workshop sponsored by the Health and Safety Executive held in Manchester, May 23-25 1988. Mutagenesis 1989; 4:482-485.

Ashby J and Mohammed R. Slide preparation and sampling as a major source of variability in the mouse micronucleus assay. Mutat Res 1986; 164:217-235.

Åström A, Tavakkol A, Petterson U, Cromie M, Elder JT, Voorhees JJ. Molecular cloning of two human cellular retinoic acid-binding proteins (CRABP). Retinoic acid-induced expression of CRABP-II but not CRABP-I in adult human skin in vivo and in skin fibroblasts in vitro. J Biol Chem 1991; 266:17662-17666.

Baak JPA and Oort J. Obtaining quantitative data. In: Baak JPA and Oort J (eds): A Manual of morphometry in diagnostic pathology. Springer Verlag, Berlin-Heidelberg-New York-Tokyo 1983.

Bacus TW and Grace LJ. Optical microscope system for standardized cell measurements and analysis. Appl Optics 1987; 26:3249-3257.

Bartek J, Bartkova J, Taylor-Papadimitriou J, Rejthar A, Kovarik J, Lukas Z, Vojtesek B. Differential expression of keratin 19 in normal human epithelial tissues revealed by monospecific monoclonal antibodies. Histochem J 1986; 18:565-575.

Battalora MSJ, Kruszewski FH, DiGiovanni J. Inhibition of chrysarobin skin tumor promotion in SENCAR mice by antioxidants. Carcinogenesis 1993; 14:2507-2512.

Bedi GC, Westra WH, Gabrielson E, Koch W, Sidranski D. Multiple head and neck tumors: evidence for a common clonal origin. Cancer Res 1996; 56:2484-2487.

Ben-Amotz A and Levy Y. Bioavailability of a natural isomer mixture compared with synthetic all-trans beta-carotene in human serum. Am J Clin Nutr 1996; 63:729-734.

Benner SE, Lippman SM, Hong WK. Current status of chemoprevention of head and neck cancer. Oncology 1992; 6:61-66.

Benner SE, Pajak TF, Stetz J, Lippman SM, Hong WK, Schantz SP, Gallagher MJ, Shenouda G. Toxicity of isotretinoin in a chemoprevention trial to prevent second primary tumors following head and neck cancer. J Natl Cancer I 1994; 86:1799-1800.

Berenblum I. The modifying influence of dichloroethyl sulphide on the induction of tumours in mice by tar. J Pathol Bacteriol 1929; 32:425-434.

Bhaskar PB, Smith RG, Baughman RA. Oral squamous cell carcinoma in identical twins: Report of a case. J Oral Maxil Surg 1988; 46:1096-1098.

Bjelke E. Dietary vitamin A and human lung cancer. Int J Cancer 1975; 15:561-565.

Blot WJ, Devesa SS, McLaughlin JK, Fraumeni JF Jr. Oral and pharyngeal cancers. Cancer Surv 1994; 20:23-42.

Bolla M, Lefur R, Ton Van J, Domenge C, Badet JM, Koskas Y, Laplanche A. Prevention of second primary tumours with etretinate in squamous cell carcinoma of the oral cavity and oropharynx. Results of a multicentric double-blind randomised study. Eur J Cancer 1994; 30A:767-772.

Bolla M, Laplanche A, Lefur R, Ton Van J, Domenge C, Lefebvre JL, Luboinski B. Prevention of second primary tumours with a second generation retinoid in squamous cell carcinoma of oral cavity and oropharynx: Long term follow-up. Eur J Cancer 1996; 32A:376-377.

Bollag W and Holdener EE. Retinoids in cancer prevention and therapy. Ann Oncol 1992; 3:513-526.

Bonanomi L and Gazzaniga A. Toxicological, pharmacokinetic and metabolic studies on acetylcysteine. Eur J Respir Dis 1980; 61:S45-S51.

Bongers V, Snow GB, Van der Waal I, Braakhuis BJM. Value of p53 expression in oral cancer and adjacent normal mucosa in relation to the occurrence of multiple primary carcinomas. Eur J Cancer 1995a; 31B:392-395.

Bongers V, Snow GB, De Vries N, Braakhuis BJM. Potential early markers of carcinogenesis in the mucosa of the head and neck using exfoliative cytology. J Pathol 1995b; 178:284-289.

Bongers V, Braakhuis BJM, Tobi H, Lubsen H, Snow GB. The relation between cancer incidence among relatives and the occurrence of multiple primary carcinomas following head and neck cancer. Cancer Epidem Biom 1996; 5:595-598.

Boone CW, Kelloff GJ, Malone WE. Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trials: a review. Cancer Res 1990; 50:2-9.

Boone CW, Steele VE, Kelloff GJ. Screening for chemopreventive (anticarcinogenic) compounds in rodents. Mutat Res 1992a; 267:251-255.

Boone CW, Kelloff GJ, Steele VE. Natural history of intraepithelial neoplasia in humans with implications for cancer chemoprevention strategy. Cancer Res 1992b; 52:1651-1659.

Boone CW and Kelloff GJ. Development of surrogate endpoint biomarkers for clinical trials of cancer chemopreventive agents: Relationships for fundamental properties of preinvasive (intraepithelial) neoplasia. J Cell Biochem 1994; 19:S10-S22.

Boyle JO, Koch W, Hruban RH, Van der Riet P, Sidranski D. The incidence of p53 mutations increase with progression of head and neck cancer. Cancer Res 1993; 53:4477-4480.

Braakhuis BJM, Jansen G, Noordhuis P, Kegel A, Peters GJ. Importance of pharmacodynamics in the in vitro antiproliferative activity of the antifolates methotrexate and 10-ethyl-10deazaaminopterin against human head and neck squamous cell carcinoma. Biochem Pharmacol 1993; 46:2155-2161.

Braakhuis BJM, Klaassen I, Van der Leede BM, Cloos J, Brakenhoff RH, Copper MP, Teerlink T, Hendriks HFJ, Van der Saag PT, Snow GB. Retinoid metabolism and all-trans retinoic acid induced growth inhibition in head and neck squamous cell carcinoma cell lines. Brit J Cancer 1997; 76:189-197.

Brachman DG, Graves D, Vokes E, Beckett M, Haraf D, Montag A, Dumphy E, Mick R, Yandell D, Weichselbaum RR. Occurrence of p53 gene deletion and human papilloma virus infection in head and neck cancer. Cancer Res 1992; 52:4382-4386.

Brand N, Petkovich M, Krust A, Chambon P, de The H, Marchio A, Tiollais P, Dejean A. Identification of a second human retinoic acid receptor. Nature 1988; 332:850-853.

Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, Goodman SN, Sidranski D. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. New Engl J Med 1995; 332:429-435.

Brown LM, Mason TJ, Pickle LW, Stewart PA, Buffler PA, Burau K, Ziegler RG, Fraumeni JF jr. Occupational risk factors for laryngeal cancer on the Texas Gulf Coast. Cancer Res 1988; 48:1960-1964.

Brugère J, Guenel P, Leclerc A, Rodriguez J. Differential effects of tobacco and alcohol in cancer of the larynx, pharynx and mouth. Cancer 1986; 57:391-395.

Byers T, Vena J, Mettlin C, Swanson M, Graham S. Dietary vitamin A and lung cancer risk: an analysis by histologic subtypes. Am J Epidemiol 1984; 120:769-776.

Carey TE. In: Wittes RE (ed): Head and neck cancer. John Wiley and Sons, New York, 1985, p287.

Carey TE. Field cancerization: are multiple primary cancers monoclonal or polyclonal? Ann Med 1996; 28:183-188.

Castigliano SG. Influence of continued smoking on the incidence of second primary cancers involving mouth, pharynx, and larynx. J Am Dent Assoc 1968; 77:580-585.

Cesarone CF, Scarabelli L, Orenesu M, Bagnasco M, De Flora S. Effects of aminothiols in 2-acetylaminofluorone-treated rats. I. Damage and repair of liver DNA, hyperplastic foci, and zymbal gland tumors. In Vivo 1987; 1:85-91.

Challem JJ. Re: risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. J Natl Cancer I 1997; 89:325.

Chambon P. A decade of molecular biology of retinoic acid receptors. FASEB 1996; 10:940-954.

Chiesa F, Tradati N, Marazza M, Rossi N, Boracchi P, Mariani L, Formelli F, Giardini R, Costa A, De Palo G, Veronesi U. Fenretinide (4-HPR) in chemoprevention of oral leukoplakia. J Cell Biochem 1993; 17:S255-S261.

Chung KY, Mukhopadhyay T, Kim J, Casson A, Ro JY, Goepfert H, Hong WK. Discordant p53 gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. Cancer Res 1993; 53:1676-1683.

Cintorino M, Petracca R, Vindigni C, Tripodi SA, Leoncini P. Topography-related expression of individual cytokeratins in normal and pathological (non-neoplastic and neoplastic) human oral mucosa. Virchows Arch A 1990; 417:419-426.

Cloos J, Steen I, Joenje H, Ko JY, De Vries N, Van der Sterre MLT, Nauta JJP, Snow GB, Braakhuis BJM. Association between bleomycin genotoxicity and non-constitutional risk factors for head and neck cancer. Cancer Lett 1993; 74:161-165.

Cloos J, Braakhuis BJM, Steen I, Copper MP, De Vries N, Nauta JJP, Snow GB. Increased mutagen sensitivity in head and neck squamous cell carcinoma patients, particularly those with multiple primary tumors. Int J Cancer 1994; 56:816-819.

Cloos J, Spitz MR, Schantz SP, Hsu TC, Zhang ZF, Tobi H, Braakhuis BJM, Snow GB. Genetic susceptibility to head and neck squamous cell carcinoma. J Natl Cancer I 1996; 88:530-535.

Coates RJ, Weiss NS, Daling JR, Morris JS, Labbe RF. Serum levels of selenium and retinol and the subsequent risk of cancer. Am J Epidemiol 1988; 128:515-523.

Coltrera MD, Zarbo RJ, Gown AM. Comparison of two putative markers of premalignant change in the oral cavity: suprabasal expression of CK-19 and proliferating cell nuclear antigen. In: Wolf GT and Carey TE (eds): Proceedings of the Third International Head and Neck Oncology Research Conference: 1990 September 26-28; Las Vegas: The American Society for Head and Neck surgery.

Cooper D, Schermer A, Sun T-T. Biology of Disease. Classification of human epithelia and their neoplasms using monoclonal antibodies to keratins: Strategies, applications, and limitations. Lab Invest 1985; 52:243-256.

Cooper JS, Pajak TF, Rubin P, Tupchong L, Brady LW, Leibel SA, Laramore GE, Marcial VA, Davis LW, Cox JD. Second malignancies in patients who have head and neck cancers: incidence, effect on survival and implications based on the RTOG experience. Int J Radiat Oncol 1989; 17:449-456.

Copper MP, Braakhuis BJM, De Vries N, Van Dongen GAMS, Nauta JJP, Snow GB. A panel of biomarkers of carcinogenesis of the upper aerodigestive tract as potential intermediate end points in chemoprevention trials. Cancer 1993; 71:825-830.

Copper MP, Jovanovic A, Nauta JJP, Braakhuis BJM, De Vries N, Van Der Waal I, Snow GB. Role of genetic factors in the etiology of squamous cell carcinoma of the head and neck. Arch Otolaryngol 1995; 121:157-160.

Costa A. Breast cancer chemoprevention. Eur J Cancer 1993; 29A:589-592.

Costa A, Formelli F, Chiesa F, Decensi A, De Palo G, Veronesi U. Prospects of chemoprevention of human cancers with the synthetic retinoid fenretinide. Cancer Res 1994; 54:S2032-S2037.

Countryman PI and Heddle JA. The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. Mutat Res 1976; 41:321-332.

Cowpe JG, Green MW, Ogden GR. Quantitative cytology of oral smears. A comparison of two methods of measurement. Anal Quant Cytol 1990; 13:11-15.

Creech Kraft J, Eckhoff Ch, Kuhnz W, Löfberg B, Nau H. Automated determination of 13-cis- and all-trans-retinoic acid, their 4-oxo metabolites and retinol in plasm, amniotic fluid and embryo by reversed-phase high performance liquid chromatography with a precolumn switching technique. J Liq Chromatogr 1988; 11:2051-2069.

Day GL, Blot WJ, Shore RE, McLaughlin JK, Austin DF, Greenberg RS, Liff JM, Preston-Martin S, Sarkar S, Schoenberg JB, Fraumeni JF jr. Second cancers following oral and pharyngeal cancers: role of tobacco and alcohol. J Natl Cancer I 1994; 86:131-137.

Decker J and Goldstein JC. Risk factors in head and neck cancer. New Engl J Med 1982; 306:1151-1155.

De Cresce R. The CAS 100: A computerized microscope image analysis system. Lab Med 1996; 17:163-165.

De Flora S, Astengo M, Serra D, Benicelli C. Prevention of induced lung tumors in mice by dietary N-acetylcysteine. Cancer Lett 1986; 32:235-241.

De Flora S, Cesarone CF, Balansky RM, Albini A, D'Agostini F, Bennicelli C, Bagnasco M, Camoirano A, Scatolini L, Rovida A. Chemopreventive properties and mechanisms of N-Acetylcysteine. The experimental background. J Cell Biochem 1995; 22:S33-S41.

Deleyiannis FWB and Thomas DB. Risk of lung cancer among patients with head and neck cancer. Otolaryngol Head Neck 1997; 116:630-636.

Delva L, Cornic M, Balitrand N, Guidez F, Miclea JM, Delmer A, Teillet F, Fenaux P, Castaigne S, Degos L. Resistance to all-trans-retinoic acid (ATRA) therapy in relapsing acute promyelocytic leukemia- study of in vitro ATRA sensitivity and cellular retinoic acid binding protein levels in leukemic cells. Blood 1993; 82:2175-2181.

De Palo G and Formelli F. Risks and benefits of retinoids in the chemoprevention of cancer. Drug Safety 1995; 12:245-256.

De Vries N, Van Der Waal I, Snow GB. Multiple primary tumours in oral cancer. Int J Oral Maxillof 1986; 15:85-87.

De Vries N and Snow GB. Multiple primary tumours in laryngeal cancer. J Laryngol Otol 1986; 100:915-918.

De Vries N. Magnitude of the problem. In: De Vries N and Gluckman JL (eds): Multiple primary tumors in the head and neck. George Thieme Verlag, Stuttgart-New York, 1990, p1-29.

De Vries N, Van Zandwijk N, Pastorino U. The Euroscan trial. Brit J Cancer 1991; 64:985-989.

De Vries N, Van Zandwijk N, Pastorino U. Chemoprevention in the management of oral cancer: Euroscan and other studies. Eur J Cancer 1992; 28B:153-157.

De Vries N, Van Zandwijk N, Pastorino U. The Euroscan study: A progress report. Am J Otolaryngol 1993; 14:62-66.

De Vries N, Van Zandwijk N, Pastorino U. Chemoprevention of second primary tumours in head and neck cancer in Europe: Euroscan. Eur J Cancer 1994; 30B:367-368.

DiGiovanni J. Multistage carcinogenesis in mouse skin. Pharmacol Ther 1992; 54:63-128.

Dimitrova B, Poyrè M, Guiso G, Badiali A, Caccia S. Isocratic reversed-phase liquid chromatography of all-trans-retinoic acid and its major metabolites in new potential supplementary test systems for developmental toxicology. J Chromatogr B 1996; 681:153-160.

Duijndam WAL and Van Duin P. The interaction of apurinic acid aldehyde groups with pararosanilin in the Feulgen-Schiff and relating staining procedures. Histochem 1975; 44:67-85.

Eckert RL. Structure, function, and differentiation of the keratinocyte. Physiol Rev 1989; 69:1316-1346.

Eckhoff C and Nau H. Identification and quantitation of all-trans- and 13-cis-retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. J Lipid Res 1990a; 31:1445-1454.

Eckhoff C and Nau H. Vitamin A supplementation increases levels of retinoic acid compounds in human plasma: possible implications for teratogenesis. Arch Toxicol 1990b; 64:502-503.

Eckhoff C, Collind MD, Nau H. Human plasm all-trans-, 13-cis- and 13-cis-4-oxoretinoic acid profiles during subchronic vitamin A supplementation: Comparison to retinol and retinyl plasma levels. J Nutr 1991; 121:1016-1025.

El Naggar AK, Lai S, Luna MA, Zhou XD, Weber RS, Goepfert H, Batsakis JG. Sequential p53 mutation analysis of pre-invasive and invasive head and neck squamous carcinoma. Int J Cancer 1995; 64:196-201.

Falini B, Flenghi L, Fagioli M, Stein H, Schwarting R, Riccardi C, Manocchio I, Pileri S, Pelicci PG, Lanfrancone L. Evolutionary conservation in various mammalian species of the human proliferation-associated epitope recognized by the Ki-67 monoclonal antibody. J Histochem Cytochem 1989; 37:1471-1478.

Farber E. The multistep nature of cancer development. Cancer Res 1984; 44:4217-4223.

Feinberg AP and Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1983; 132:6-13.

Ficarra G and Eversole LE. HIV-related tumors of the oral cavity. Crit Rev Oral Biol M 1994; 5:159-185.

Field JK, Spandidos SA, Malliri A, Gosney JR, Yiagnisis M, Stell PM. Elevated p53 expression correlates with a history of heavy smoking in squamous cell carcinomas of the head and neck. Brit J Cancer 1991;64:573-577.

Fontham ET. Protective dietary factors and lung cancer. Int J Epidemiol 1990; 19:S32-S42.

Foulkes WD, Brunet JS, Kowalski LP, Narod SA, Franco EL. Family history of cancer is a risk factor for squamous cell carcinoma of the head and neck in Brazil: a case-control study. Int J Cancer 1995; 63:769-773.

Foulkes WD, Brunet JS, Sieh W, Black MJ, Shenouda G, Narod SA. Familial risk of squamous cell carcinoma of the head and neck: retrospective case-control study. Brit Med J 1996; 313:716-721.

Franceschi S, Munoz N, Bosch XF, Snijders PJ, Walboomers JM. Human papillomavirus and cancers of the upper aerodigestive tract: a review of epidemiological and experimental evidence. Cancer Epidem Biomar 1996; 5:567-575.

Furr HC, Barua AB, Olson JA. In: Sporn MB, Roberts AB, Goodman DS (eds): The Retinoids: Biology, Chemistry and Medicine (2nd ed). Raven Press, New York, 1994, p179.

Gadde RR and Burton FW. Simple reversed-phase high-performance liquid chromatographic method for 13-cis-retinoic acid in serum. J Chromatogr 1992; 593:41-46.

Gencik A, Wey W, Müller Hj. High incidence of laryngeal carcinoma in a Swiss family. ORL 1986; 48:162-166.

Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983; 31:13-20.

Gibbs S, Fijneman R, Wiegand J, Geurts van Kessel A, Van De Putte P, Backendorf C. Molecular characterization and evolution of the SPRR family of keratinocyte differentiation markers encoding small proline-rich proteins. Genomics 1993; 16:630-637.

Giguere V, Ong E, Segui P, Evans RM. Identification of a receptor for the morphogen retinoic acid. Nature 1987; 330:624-629.

Gimenez-Conti IB, Shin DM, Bianchi AB, Roop DR, Hong WK, Conti CJ, Slaga TJ. Changes in keratin expression during 7,12-dimethylbenz[a]anthracene-induced hamster cheek pouch carcinogenesis. Cancer Res 1990; 50:4441-4445.

Gluckman JL, Crissman JD, Donegan JO. Multicentric squamous cell carcinoma of the upper aerodigestive tract. Head Neck-J Sci Spec 1980; 3:90-96.

Goodman GE, Omenn GS, Thornquist MD, Lund B, Metch B, Gylys-Colwell I. The carotene and retinol efficacy trial (CARET) to prevent lung cancer in high-risk populations: pilot study with cigarette smokers. Cancer Epidem Biomar 1993; 2:381-387.

Goss GD and McBurney MW. Physiological and clinical aspects of vitamin A and its metabolites. Critical Rev Clin Lab Sciences 1992; 29:185-215.

Gough NM. Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. Anal Biochem 1988; 173:93-95.

Graham S, Dyal H, Rohrer T, Swanson M, Sultz H, Fischman S. Dentition, diet, tobacco and alcohol in the epidemiology of oral cancer. J Natl Cancer I 1977; 59:1611-1618.

Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. Cancer Res 1994; 54:4855-4878.

Greenwald P, Witkin KM, Malone WF, Byar DP, Freedman LS, Stern HR. The study of markers of biological effect in cancer prevention research trials. Int J Cancer 1992; 52:189-196.

Guiso G, Rambaldi A, Dimitrova B, Biondi A, Caccia S. Determination of orally administered all-trans-retinoic acid in human plasma by high-performance liquid chromatography. J Chromatogr B 1994; 656:239-244.

Gundersen HJG. Note on the estimation of the numerical density of arbitrary profiles: the edge effect. J Microscopy 1977; 111:219-223.

Gusterson BA, Anbazhagan R, Warren W, Midgely C, Lane DP, O'Hare M, Stamps A, Carter R, Jayatilake H. Expression of p53 in premalignant and malignant squamous epithelium. Oncogene 1991; 6:1785-1789.

Han J, Jiao L, Lu Y, Sun Z, Gu QM, Scanlon KJ. Evaluation of N-4-(hydroxycarbophenyl) retinide as a cancer prevention agent and as a cancer chemotherapeutic agent. In Vivo 1990; 4:153-160.

Hartwell LH and Kastan MB. Cell cycle control and cancer. Science 1994; 266:1821-1828.

Haughey BH, Arfken CL, Gates GA, Harvey J. Meta-analysis of second malignant tumors in head and neck cancer: the case for an endoscopic screening protocol. Ann Oto Rhinol Laryn 1992; 101:105-112.

Healy GB, Stuart-Strong M, Uchmahli A, Vaughan GW, Di Troia JF. Carcinoma of the palatine arch. Am J Surg 1976; 132:498-503.

Heddle JA, Hite M, Kirkhart B, Mavournin K, MacGregor JT, Newell GW, Salamone MF. The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat Res 1983; 123:61-118.

Heddle JA, Cimino MC, Hayashi M, Romagna F, Shelby MD, Tucker JD, Vanparys Ph, MacGregor JT. Micronuclei as an Index of Cytogenetic Damage: Past, Present, and Future. Environmental and Molecular Mutagenesis 1991; 18:277-291.

Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, Belanger C, LaMotte F, Gaziano JM, Ridker PM, Willett W, Peto R. Lack of effect of long-term supplementation with beta-carotene on the incidence of malignant neoplasms and cardiovascular disease. New Engl J Med 1996; 334:1145-1149.

Hittelman WN, Lee JS, Cheong N, Shin D, Hong WK. The chromosome view of "field cancerization" and multistep carcinogenesis. Implications for chemopreventive approaches. In: Pastorino U and Hong WK (eds): Chemoimmuno Prevention of Cancer. George Thieme Verlag, Stuttgart-New York, 1991, p41-47.

Hoffman AD, Engelsten D, Bogenrieder T, Papandreou CN, Steckelman E, Dave A, Motzer RJ, Dmitrovsky E, Albino AP, Nanus DM. Expression of retinoic acid receptor-β in human renal cell carcinomas correlates with sensitivity to the antiproliferative effects of 13-*cis*-retinoic acid. Clin Cancer Res 1996; 2:1077-1082.

Hong WK, Endicott J, Itri LM, Doos W, Batsakis JG, Bell R, Fofonoff S, Byers R, Atkinson EN, Vaughan C, Toth BB, Kramer A, Dimery IW, Skipper P, Strong S. 13-cis-retinoic acid in the treatment of oral leukoplakia. New Engl J Med 1986; 315:1501-1505.

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, Goepfert H. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. New Engl J Med 1990; 323:795-801.

Hong WK and Lippman SM. Cancer chemoprevention. J Natl Cancer I Monogr 1995; 17:49-53.

Hsu TC. Genetic instability in the human population: a working hypothesis. Hereditas 1983; 98:1-9.

Hsu TC, Cherry LM, Samaan NA. Differential mutagen susceptibility in cultured lymphocytes of normal individuals and cancer patients. Cancer Genet Cytogenet 1985; 17:307-313.

Hsu TC, Furlong C, Spitz MR. Ethyl alcohol as a cocarcinogen with special reference to the aerodigestive tract: a cytogenetic study. Anticancer Res 1991; 11:1097-1102.

Huber MH, Lippman SM, Hong WK. Chemoprevention of head and neck cancer. Semin Oncol 1994; 21:366-375.

Incze J, Vaughan CW, Lui P, Strong MS, Kulapaditharom B. Premalignant changes in normal appearing epithelium in patients with squamous cell carcinoma of the upper aerodigestive tract. Am J Surg 1982; 144:401-405.

International Agency for Research on Cancer. Tobacco habits other than smoking; betel-quid and areca-nut chewing; and some related nitrosamines. Monographs on the evaluation of the carcinogenic risk of chemicals to humans. IARC, Lyon, 1985, Vol 37, p1-291.

Jetten AM. Multistep process of squamous differentiation of tracheobronchial epithelial cells. Role of retinoids. Dermatologica 1987; 175:37-44.

Jetten AM, Kim JS, Sacks PG, Rearick JI, Lotan D, Hong WK, Lotan R. Inhibition of growth and squamous-cell differentiation markers in cultured human head and neck squamous carcinoma cells by β-all-trans retinoic acid. Int J Cancer 1990; 45:195-202.

Jing Y, Zhang J, Bleiweiss IJ, Waxman S, Zelent A, Mira-Y-Lopez R. Defective expression of cellular retinol binding protein type I and retinoic acid receptors $\alpha 2$, $\beta 2$, and $\gamma 2$ in human breast cancer cells. FASEB J 1996; 10:1064-1070.

Kaplan MJ, Mills SE, Rice RH, Johns ME. Involucrin in laryngeal dysplasia. Arch Otolaryngol 1984; 110:713-716.

Kato H, Konaka C, Hayata Y, Ono J, Matsushima Y, Tahara M, Lei J, Nasiell M, Auer G. Lung cancer histogenesis following in vivo bronchial injections of 20-methylcholanthrene in dogs. Recent Results Cancer Res 1982; 82:69-86.

Kelloff GJ, Malone WF, Boone CW, Sigman CC, Fay JR. Progress in applied chemoprevention research. Semin Oncol 1990; 17:438-455.

Kelloff GJ, Boone CW, Crowell JA, Steele VE, Lubet R, Doody LA. Surrogate endpoint biomarkers for phase II chemoprevention trials. J Cell Biochem 1994; 19:S1-S9.

Khuri FR, Lippman SM, Spitz MR, Lotan R, Hong WK. Molecular epidemiology and retinoid chemoprevention of head and neck cancer. J Natl Cancer I 1997; 89:199-211.

Kimmel KA and Carey TE. Altered expression in squamous carcinoma cells of an orientation-restricted epithelial antigen detected by monoclonal antibody A9. Cancer Res 1986; 46:3614-3623.

Kirkwood BR. Essentials of medical statistics. Blackwell Scientific Publications Ltd, Oxford-London-Edinburgh-Boston-Melbourne-Paris-Berlin-Vienna, 1988.

Knekt P, Aromaa A, Maatela J, Aaran RK, Nikkari T, Hakama M, Hakulinen T, Peto R, Teppo L. Serum vitamin A and subsequent risk cancer: cancer incidence follow-up of the Finnish Mobile Clinic Health Examination Survey. Am J Epidemiol 1990; 132:857-870.

Krishna G, Kropko ML, Theiss JC. Use of the cytokinesis-block method for the analysis of micronuclei in V79 Chinese hamster lung cells: results with mitomycin C and cyclophosphamide. Mutat Res 1989; 222:63-69.

Krolls SO and Hoffman S. Squamous cell carcinoma of the oral soft tissues: a statistical analysis of 14,253 cases by age, sex and race. J Am Dent Assoc 1976; 92:571-574.

Krust A, Kastner P, Petkovich M, Kastner P, Chambon P. A third human retinoic acid receptor, hRAR-γ. Proc Natl Acad Sci 1989; 86:5310-5314.

Larramendy ML and Knuutila S. Increased frequency of micronuclei in B and T8 lymphocytes from smokers. Mutat Res 1991; 259:189-195.

Law MR. Genetic predisposition to lung cancer. Brit J Cancer 1990; 61:195-206.

Lee JS, Lippman SM, Hong WK, Ro JY, Kim SY, Lotan R, Hittelman WN. Determination of biomarkers for intermediate end points in chemoprevention trials. Cancer Res 1993; 52:S2707-S2710.

Lehman PA and Franz TJ. A sensitive high-pressure liquid chromatography/particle beam/mass spectrometry assay for the determination of all-trans-retinoic acid and 13-cis-retinoic acid in human plasma. J Pharmaceutical Sci 1996; 85:287-290.

Levebvre P, Agadir A, Cornic M, Gourmel B, Hue B, Dreux C, Degos L, Chomienne C. Simultaneous determination of all-trans and 13-cis retinoic acids and their 4-oxo metabolites by adsorption liquid chromatography after solid-phase extraction. J Chromatogr B 1995; 666:55-61.

Li FP and Montesano R. Interactions of cancer susceptibility genes and environmental carcinogens-American Association for Cancer Research (AACR) International Agency for Research on Cancer (IACR) joint conference. Cancer Res 1994; 54:4243-4247.

Lindberg K and Rheinwald JG. Suprabasal 40 kd keratin (K19) expression as immunohistological marker of premalignancy in oral epithelium. Am J Pathol 1989; 134:89-98.

Lippman SM and Hong WK. Second malignant tumors in head and neck squamous cell carcinoma: the overshadowing threat for patients with early-stage disease. Int J Radiat Oncol 1989; 17:691-694.

Lippman SM, Lee JS, Lotan R, Hittelman WN, Wargovich MJ, Hong WK. Biomarkers as intermediate end points in chemoprevention trials. J Natl Cancer I 1990a; 82:555-560.

Lippman SM, Peters E, Wargovich M. Bronchial micronuclei as a marker of an "early" stage of carcinogenesis in human tracheobronchial epithelium. Int J Cancer 1990b; 45:811-815.

Lippman SM, Batsakis JG, Toth BB, Weber RS, Lee JJ, Martin JW, Hays GL, Goepfert H, Hong WK. Comparison of low-dose isotretinoin with beta-carotene to prevent oral carcinogenesis. New Engl J Med 1993; 328:15-20.

Lippman SM, Benner SE, Hong WK. Cancer chemoprevention. J Clin Oncol 1994; 12:851-873.

Lippman SM, Heyman RA, Kurie JM, Benner SE, Hong WK. Retinoids and basic studies. J Cell Biochem 1995; 22:S1-S10.

Lotan R. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochem Biophys Acta 1980; 605:33-91.

Lotan R, Sacks PG, Lotan D, Hong WK. Differential effects of retinoic acid on the in vitro growth and cell-surface glycoconjugates of two human head and neck squamous cell carcinomas. Int J Cancer 1987; 40:224-229.

Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ, Hong WK. Suppression of retinoic acid receptor-beta in premalignant oral lesions and its upregulation by isotretinoin. New Engl J Med 1995; 332:1405-1410.

Lydiatt WM and Schantz SP. Biological staging of head and neck cancer and its role in developing effective treatment strategies. Cancer Metast Rev 1996; 15:11-25.

Maier H, De Vries N, Snow GB. Occupational factors in the aetiology of head and neck cancer. Clin Otolaryngol 1991; 16:406-412.

Malone WF. Studies evaluating antioxidants and β-carotene as chemopreventives. Am J Clin Nutr 1991; 53:S305-S313.

Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 1990; 345:224-229.

Mangelsdorf DJ, Umesono K, Evans RM. The retinoid receptors. In: Sporn MB, Roberts AR, Goodman DS (eds): The retinoids. Biology, chemistry and medicine (2nd ed). Raven Press, New York, 1994, p319-349.

Marchetti MN, Bun H, Geiger JM, Durand A. Anal Lett 1994; 27:1847.

Markman M. Second primary respiratory tract and oesophageal cancers following head and neck malignancies. Lancet 1981; II:230.

Marlowe FI. Simultaneous laryngeal tumors in sisters. Arch Otolaryngol 1970; 92:195-197.

Marnett LJ. Aspirin and related nonsteroidal anti-inflammatory drugs as chemopreventive agents against colon cancer. Prev Med 1995; 24:103-106.

Mayne ST and Goodwin WJ. Chemoprevention of head and neck cancer. Curr Opin Otolaryngol Head Neck Surg 1993; 1:126-132.

Mayne ST, Handelman GJ, Beecher G. β-carotene and lung cancer promotion in heavy smokers-a plausible relationship. J Natl Cancer I 1996; 88:1513-1515.

McGarry GW, Mackenzie K, Periasamy P, McGurk F, Gatehouse S. Multiple primary malignant tumours in patients with head and neck cancer: the implications for follow-up. Clin Otolaryngol 1992; 17:558-562.

McGuirt WF, Matthews B, Koufman JA. Multiple simultaneous tumors in patients with head and neck cancer. Cancer 1982; 50:1195-1199.

Meister A and Anderson M. Glutathione. Ann Rev Biochem 1983; 52:711-760.

Meyer E, Lambert WE, De Leenheer AP. Simultaneous determination of endogenous retinoic acid isomers and retinol in human plasma by isocratic normal-phase HPLC with ultraviolet detection. Clin Chem 1994; 40:48-57.

Meyskens FL. Biomarker Intermediate Endpoints and Cancer Prevention. J Natl Cancer I Monogr 1992; 13:177-181.

Mitchell MF, Hittelman WK, Lotan R, Nishioska K, Tortelero-Luna G, Richards-Kortum R, Wharton JT, Hong WK. Chemoprevention trials and surrogate end point biomarkers in the cervix. Cancer 1995; 76:1956-1977.

Moll R, Franke WW, Schiller DL. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982; 31:11-24.

Moll R, Achtstätter T, Becht E, Balcarova-Ständer J, Ittensohn M, Franke WW. Cytokeratins in normal and malignant transitional epithelium. Am J Path 1988; 132:123-144.

Moon RC and Mehta RG. Chemoprevention of experimental carcinogenesis in animals. Prev Med 1989; 18:576-591.

Moore C. Smoking and cancer of the mouth, pharynx and larynx. JAMA 1971; 218:553-558.

Morgan PR, Shirlaw PJ, Johnson NW, Leigh IM, Lane EB. Potential applications of anti-keratin antibodies in oral diagnosis. J Oral Pathol 1987a; 16:212-222.

Morgan PR, Leigh IM, Purkis PE, Gardner ID, Van Muijen GNP, Lane EB. Site variation in keratin expression in human oral epithelia - an immunocytochemical study of individual keratins. Epithelia 1987b; 1:31-43.

Muir C and Weiland L. Upper aerodigestive tract cancers. Cancer 1995; 75:147-153.

Murphy GM, Flynn TC, Rice RH, Pinkus GS. Involucrin expression in normal and neoplastic human skin: a marker for keratinocyte differentiation. J Invest Dermatol 1984; 82:453-457.

Muscat JE and Wynder EL. Tobacco, alcohol, asbestos, and occupational risk factors for laryngeal cancer. Cancer 1992; 69:2244-2251.

Nagle RB, Moll R, Weidauer H, Nemetschek H, Franke WW. Different patterns of cytokeratin expression in the normal epithelia of the upper respiratory tract. Differentiation 1985; 30:130-140.

Nasiell K, Auer G, Nasiell M, Zetterberg A. Retrospective DNA analysis in cervical dysplasia as related to neoplastic progression or regression. Analyt Quant Cytol 1979; 1:103-106.

Nasiell M, Carlens E, Auer G, Hayata Y, Kato H, Konaka C, Roger V, Nasiell K, Enstad I. Pathogenesis of bronchial carcinoma with special reference to morphogenesis and the influence on the bronchial mucosa of 20-methylcholanthrene and cigarette smoking. Recent Results Cancer Res 1982; 82:53-68.

Näslund I, Rubio C, Auer G. Nuclear DNA changes during pathogenesis of squamous carcinoma of the cervix in 3,4-benzoepyrene-treated mice. Analyt Quant Cytol 1987; 9:411-418.

Nees M, Homann N, Discher H, Andl T, Enders C, Herold-Mende C, Schuhmann A, Bosch FX. Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumors. Cancer Res 1993; 53:4189-4196.

Ogden GR, Cowpe JG, Green MW. Evidence of field change in oral cancer. Brit J Oral Max Surg 1990; 28:390-392.

Ogden GR, Cowpe JG, Green MW. Detection of field change in oral cancer using oral exfoliative cytologic study. Cancer 1991; 68:1611-1615.

Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Hammar S. Effects of a combination of beta-carotene and vitamin A on lung cancer and cardiovascular disease. New Engl J Med 1996a; 334:1150-1155.

Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Cherniack MG, Brodkin CA, Hammar S. Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. J Natl Cancer I 1996b; 88:1550-1559.

Osborn M, Van Lessen G, Weber K, Klöppel G, Altmannsberger M. Differential diagnosis of gastrointestinal carcinomas by using monoclonal antibodies specific for individual keratin polypeptides. Lab Invest 1986; 55:497-504.

Osborne M, Boyle P, Lipkin M. Cancer prevention. Lancet 1997; 349:S27-S30.

Packer L. Retinoids, Part A: Molecular and Metabolic Aspects. Methods Enzymol 1990a; 189.

Packer L. Retinoids, Part B: Cell Differentiation and Clinical Aspects. Methods Enzymol 1990b; 190.

Palcic B. Nuclear texture: Can it be used as a surrogate endpoint biomarker? J Cell Biochem 1994; 19:S10-S22.

Papadimitrakopoulou VA, Shin DM, Hong WK. Molecular and cellular biomarkers for field cancerization and multistep process in head and neck tumorigenesis. Cancer Metast Rev 1996; 15:53-76.

Parkin DM, Muir CS, Whelan SL, Gao YT, Ferlay J, Powell J (eds). Cancer incidence in five continents. Volume VI. International Agency for Research on Cancer (WHO). Lyon, 1992.

Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of eighteen major cancers in 1995. Int J Cancer 1993; 54:594-606.

Parkinson EK. Defective responses of transformed keratinocytes to terminal differentiation stimuli. Their role in epidermal tumor promotion by phorbol esters and by deep skin wounding. Brit J Cancer 1985; 52:479-493.

Pastorino U. Lung cancer chemoprevention: facts and hope. Lung cancer 1991; 7:133-150.

Pastorino U, Infante M, Maioli M, Chiesa G, Buyse M, Firket P, Rosmentz N, Clerici M, Soresi E, Valente M, Belloni P, Ravasi G. Adjuvant treatment of stage I lung cancer with high-dose vitamin A. J Clin Oncol 1993; 11:1216-1222.

Pelt FNAM, Haring RM, Overamp MJI, Weterings PJJM. Micronucleus formation in cultured human keratinocytes following exposure to mitomycin C and cyclophosphamide. Mutat Res 1991; 252:45-50.

Periquet B, Lambert W, Garcia J, Lecomte G, De Leenheer AP, Mazieres B, Thouvenot JP, Arlet J. Increased concentrations of endogenous 13-cis- and all-trans-retinoic acids in diffuse idiopathic skeletal hyperostosis, as demonstrated by HPLC. Clin Chim Acta 1991; 203:57-65.

Petkovich M, Brand NJ, Krust A, Chambon P. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 1987; 330:444-450.

Prassad MPR, Mukundan MA, Krishnaswamy K. Micronuclei and carcinogen DNA adducts as intermediate end points in nutrient intervention trial of precancerous lesions in the oral cavity. Eur J Cancer 1995; 31B:155-159.

Quak JJ, Van Dongen GAMS, Brakkee JPG, Hayashida DJ, Balm AJM, Snow GB, Meijer CJ. Production of a monoclonal antibody (K 931) to a squamous cell carcinoma antigen identified as the 17-1a antigen. Hybridoma 1990a; 9:377-387.

Quak JJ, Balm AJM, Van Dongen GAMS, Brakkee JGP, Scheper RJ, Snow GB, Meijer CJLM. A 22 Kd surface antigen detected by monoclonal antibody E 48 is exclusively expressed in stratified squamous and transitional epithelia. Am J Pathol 1990b; 136:191-197.

Quak JJ, Schrijvers AHGJ, Brakkee JGP, Davis HD, Scheper RJ, Meijer CJLM, Snow GB, van Dongen GAMS. Expression and characterization of two differentiation antigens in stratified squamous epithelia and carcinomas. Int J Cancer 1992; 50:507-513.

Quinlan RA, Schiller DL, Hatzfeld M. Pattern of expression and organization of cytokeratin intermediate filaments. Ann NY Acad Sci 1985; 455:282-260.

Raafat M, El-Gerzawi S, Stich HF. Detection of mutagenicity in urothelial cells of bilharzial patients by "the micronucleus test." J Egypt Natl Cancer I 1984; 1:63-73.

Rachmat L, Vreeburg GC, De Vries N, Hordijk GJ, Lubsen H, Manni J, Snow GB. The value of twice yearly bronchoscopy in the work-up and follow-up of patients with laryngeal cancer. Eur J Cancer 1993; 29A:1096-1099.

Racine RR and Matter BE. The micronucleus test as indicator of mutagenic exposure. In: Ansari AA and Serres FJ (eds): Single-Cell Mutation Monitoring Systems, Plentum, New York, 1984, p217-232.

Ramaekers FCS, Moesker O, Huysmans A, Schaart G, Westerhof G, Wagenaar SS, Herman CJ, Vooijs GP. Intermediate filament proteins in the study of tumor heterogeneity: an in-depth study of tumors of the urinary and respiratory tracts. Ann NY Acad Sci 1985; 455:614-634.

Ranalder UB, Lausecker BB, Huselton C. Micro liquid chromatography-mass spectrometry with direct liquid introduction used for separation and quantification of all-trans- and 13-cis-retinoic acids and their 4-oxo metabolites in human plasma. J Chromatogr 1993; 617:129-135.

Renan MJ. How many mutations are required for tumorigenesis? Implications from human cancer data. Mol Carcinog 1993; 7:139-146.

Roberts CJ, Morgan GR, Holt PD. A critical comparison of the micronucleus yield from high and low LET irradiation of plateau-phase cell populations. Mutat Res 1986; 160:237-242.

Rodilla V, Pellicer JA, Pertusa J, Mothersill C. Induction of micronucleated and binucleate cells in Chinese hamster ovary (CHO) cells by *cis*-diamminedichloroplatinum (II): a morphological and morphometric study. Mutat Res 1990; 241:115-124.

Rosin MP, Dunn BP, Stich HF. Use of intermediate endpoints in quantitating the response of pre-cancerous lesions to chemopreventive agents. Can J Physiol Pharmacol 1987; 65:483-487.

Rothman KJ, Cann CI, Flanders D, Fried MP. Epidemiology of laryngeal cancer. Epidemiol Rev 1980; 2:195-209.

Rotstein JB and Slaga TJ. Effects of exogenous glutathione on tumour progression in the murine skin multistage carcinogenesis model. Carcinogenesis 1988; 9:1547-1551.

Salonen JT, Salonen R, Lappeteläinen R, Maenpää PH, Alfthan G, Puska P. Risk of cancer in relation to serum concentrations of selenium and vitamins A and E: matched case-control analysis of prospective data. Brit Med J 1985; 290:417-420.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, 1989.

Sarto F, Finotto S, Giacomelli L, Mazzotti D, Tomanin R, Levis AG. The micronucleus assay in exfoliated cells of the human buccal mucosa. Mutagenesis 1987; 2:11-17.

Sass JO and Nau H. Single-run analysis of isomers of retinoyl-beta-D-glucuronide and retinoic acid by reverse-phase high-performance liquid chromatography. J Chromatogr A 1994; 685:182-188.

Satterfield S, Greco PJ, Goldhaber SZ, Stampfer MJ, Schwartz SL, Stein EA, Kaplan L, Hennekens CH. Biochemical markers of compliance in the Physicians' Health Study. Am J Prev Med 1990; 6:290-294.

Schatzkin A, Freedman l, Schiffmann M. An epidemiologic perspective on biomarkers. J Int Med 1993; 233:75-79.

Schmid W. The micronucleus test. Mutat Res 1975; 31:9-15.

Schottenfeld D, Gantt RC, Wynder EL. The role of alcohol and tobacco in multiple primary cancers of the upper digestive system, larynx, and lung: a prospective study. Prev Med 1974; 3:277-293.

Schottenfeld D. Alcohol as a co-factor in the etiology of cancer. Cancer 1979; 43:1962-1966.

Schottenfeld D. The etiology and prevention of aerodigestive tract cancers. In: Newell GR, Hong WK (eds): The biology and prevention of aerodigestive tract cancers. Plenum Press, New York, 1992, p1-19.

Schrijvers AHGJ, Gerretsen M, Fritz JM, Van Walsum M, Quak JJ, Snow GB, van Dongen GAMS. Evidence for a role of the monoclonal antibody E48 defined antigen in cell-cell adhesion in squamous epithelia and head and neck squamous cell carcinoma. Exp Cell Res 1991; 196:264-269.

Schulte EKW, Joos U, Kasper M, Eckert HM. Cytological detection of epithelial dysplasia in the oral mucosa using Feulgen DNA-image cytometry. Diagn Cytopathol 1991; 7:436-441.

Schwartz J and Shklar G. Regression of experimental hamster cancer by beta carotene and algae extracts. J Oral Maxil Surg 1987; 45:510-515.

Sellers TA, Bailey-Wilson JE, Elston RC, Wilson AF, Elston GZ, Ooi WL, Rothschild H. Evidence for Mendelian inheritance in the pathogenesis of lung cancer. J Natl Cancer I 1990; 82:1272-1279.

Shapshay SM, Hong WK, Fried MP, Sismaris A, Vaughan SW, Strong MS. Simultaneous carcinomas of the esophagus and upper aerodigestive tract. Otolaryngol Head Neck 1980; 88:373-377.

Shin DM, Voravud N, Ro JY, Lee JS, Hong WK, Hittelman WN. Sequential increase in proliferating cell nuclear antigen in head and neck tumorigenesis: a potential biomarker. J Natl Cancer I 1993; 85:971-978.

Shin DM, Hittelman WN, Hong WK. Biomarkers in upper aerodigestive tract tumorigenesis: a review. Cancer Epidem Biomar 1994; 3:697-709.

Shin DM, Lee JS, Lippman SM, Lee JJ, Tu ZN, Choi G, Heyne K, Shin HJ, Ro JY, Goepfert H, Hong WK, Hittelman WN. p53 expressions: predicting recurrence and second primary tumors in head and neck squamous cell carcinoma. J Natl Cancer I 1996; 88:519-529.

Silverman S and Griffith M. Smoking characteristics of patients with oral carcinomas and the risk for second oral primary carcinomas. J Am Dent Assoc 1972; 85:637-640.

Silverman S, Gorsky M, Greenspan D. Tobacco usage in patients with head and neck carcinomas: a follow-up study on habit changes and second primary oral/oropharyngeal cancers. J Am Dent Assoc 1983; 106:33-35.

Simon M and Green H. Enzymatic cross-linking of involucrin and other proteins by keratinocyte particulates in vitro. Cell 1985; 40:677-683.

Singh B, Balwally AN, Shaha AR, Rosenfeld RM, Har-El G, Lucente FE. Upper aerodigestive tract squamous cell carcinoma. The human immunodeficiency virus connection. Arch Otolaryngol 1996; 122:639-643.

Slaughter DP. Multicentric origin of intraoral carcinoma. Surgery 1946; 20:133-146.

Slaughter DP, Southwick HW, Smejkal W. "Field cancerization" in oral stratified squamous epithelium. Cancer 1953; 6:963-968.

Smigel K. Beta-carotene fails to prevent cancer in two major studies; CARET intervention stopped. J Natl Cancer I 1996; 88:145.

Smith EM. Epidemiology of oral and pharyngeal cancers in the United States: review of recent literature. J Natl Cancer I 1979; 63:1189-1198.

Snijders PJ, Scholes AG, Hart CA, Jones AS, Vaughan ED, Woolgar JA, Meijer CJ, Walboomers JM, Field JK. Prevalence of mucosotropic human papillomaviruses in squamous-cell carcinoma of the head and neck. Int J Cancer 1996; 66:464-469.

Snow GB. Follow-up in patients treated for head and neck cancer: how frequent, how thorough and for how long? Eur J Cancer 1992; 28B:315-316.

Sporn MB, Dunlop NM, Newton DL, Smith JM. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed Proc 1976; 35:1332-1338.

Sporn MB, Roberts AB, Goodman DS (eds). In: The Retinoids: Biology, Chemistry and Medicine (2nd ed). Raven Press, New York, 1994.

Stähelin HB, Rösel F, Buess E, Brubacher G. Cancer, vitamins and plasma lipids: prospective Basel study. J Natl Cancer I 1984; 73:1463-1468.

Steinbeck RG, Moege J, Heselmeyer KM, Klebe W, Neugebauer W, Borg B, Auer GU. DNA content and PCNA immunoreactivity in oral precancerous and cancerous lesions. Eur J Cancer 1993; 29B:279-284.

Stevens MH, Gardner JW, Parkin JL, Johnson LP. Head and neck cancer survival and life-style change. Arch Otolaryngol 1983; 109:746-749.

Stich HF, Stich W, Parida BB. Elevated frequency of micronucleated cells in the buccal mucosa of individuals at high risk for oral cancer: betel quid chewers. Cancer Lett 1982; 17:125-134.

Stich HF, Rosin MP, Hornby AP. Reduction with vitamin A and beta-carotene administration of the proportion of micronucleated buccal mucosal cells in Asian betel nut and tobacco chewers. Lancet 1984; I:1204-1206.

Stich HF and Rosin MP. Micronuclei in exfoliated human cells as a tool for studies in cancer risk and cancer intervention. Cancer Lett 1984; 22:241-253.

Stich HF, Hornby AP, Mathew B, Sankaranarayanan R, Nair MK. Response of oral leukoplakia to the administration of vitamin A. Cancer Lett 1988a; 40:93-101.

Stich HF, Rosin MP, Hornby P, Methew B, Sankaranarayanan R, Nair MK. Remission of oral leukoplakias and micronuclei in tobacco/betel quid chewers treated with beta-carotene and with beta-carotene plus vitamin A. Int J Cancer 1988b; 42:195-199.

Stich HF, Mathew B, Sankaranarayanan R, Nair MK. Remission of precancerous lesions in the oral cavity of tobacco chewers and maintenance of the protective effect of beta-carotene or vitamin A. Am J Clin Nutr 1991; 53:S298-S304.

Strickland S and Mahdavi V. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. Cell 1978; 15:393-403.

Takeda N and Yamamoto A. Simultaneous determination of 13-cis- and all-trans-retinoic acids and retinol in human serum by high-performance liquid chromatography. J Chromatogr B 1994; 657:53-59.

Talamini R, Franceschi S, Barra S, La Vecchia C. The role of alcohol in oral and pharyngeal cancer in non-smokers, and of tobacco in non-drinkers. Int J Cancer 1990; 46:391-393.

Tanaka T. Cancer chemoprevention. Cancer J 1992; 5:11-16.
Tang G and Russell RM. Formation of all-trans-retinoic acid and 13-cis-retinoic acid from all-trans-retinyl palmitate in humans. J Nutr Biochem 1991; 2:210-213.
Tashiro H, Abe K, Tanioka H. Familial occurrence of cancer of the mouth: Report of cases. J Oral Maxil Surg 1986; 44:322-323.
Taylor SR, Titus-Ernstoff L, Stitely S. Central values and variation of measured nuclear DNA content in imprints of normal tissues determined by image analysis. Cytometry 1989; 10:382-387.
Teerlink T, Copper MP, Klaassen I, Braakhuis BJM. Simultaneous analysis of retinol, retinoic acid isomers and polar metabolites in biological fluids by HPLC using on-column concentration after single-phase fluid extraction. J Chromat B 1997; 694:83-92.
Tepperman BS and Fitzpatrick PJ. Second respiratory and upper digestive tract cancers after oral cancer. Lancet 1981; II:547-549.
Thacher SM and Rice RH. Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. Cell 1985; 40:685-695.
Thunnissen FBJM, Perdaen H, Forrest J. Influence of different cell extraction methods on cytometric features. Cytometry 1992; 13:485-489.
TNM classification of malignant tumours (4th ed). Springer-Verlag, New York, 1987.
Tucker JH, Cowpe JG, Ogden GR. Nuclear DNA content and morphometric characteristics of normal, premalignant and malignant oral smears. Anal Cell Pathol 1994; 6:117-128.
Van Muijen GNP, Ruiter DJ, Franke WW, Achtstätter T, Haasnoot WHB, Ponec M, Warnaar SO. Cell type heterogeneity of cytokeratin expression in complex epithelia and carcinomas as demonstrated by monoclonal antibodies specific for cytokeratins nos. 4 and 13. Exp Cell Res 1986; 162:97-113.
Van Muijen GNP, Warnaar SO, Ponec M. Differentiation-related changes of cytokeratin expression in cultured keratinocytes and in fetal, newborn, and adult epidermis. Exp Cell Res 1987; 171:331-345.
Van Waes C, Kozarsky KF, Warren AB, Kidd L, Paugh D, Liebert M. Identity of the newly defined integrin $\alpha^6\beta_4$ with the A9 antigen associated with highly malignant squamous cell carcinoma. Proc Am Ass Cancer Res 1990; 31:257.
Vikram B. Changing patterns of failure in advanced head and neck cancer. Arch Otolaryngol 1984; 110:564-565.
Vogelstein B, Fearon ER, Hamilton SR. Genetic alterations during colorectal tumor development. New Engl J Med 1988; 319:525-532.
Von Eggers Doering W. Antioxidant vitamins, cancer, and cardiovascular disease. New Engl J Med 1996; 335:1065.
Wagenfeld DJH, Harwood AR, Bryce DP, Von Nostrand P, De Boer G. Second primary respiratory tract malignancies in glottic carcinoma. Cancer 1980; 46:1883-1886.
Wagenfeld DJH, Harwood AR, Bryce DP, Von Nostrand P, De Boer G. Second primary respiratory tract malignant neoplasms in supraglottic carcinoma. Arch Otolaryngol 1981; 102:135-137.
Wahlberg P and Fex G. Retinoic acid concentrations in patients with squamous cell carcinoma of the head and neck. Eur J Cancer 1996; 32A:366-367.
Wald N, Idle M, Boreham J, Bailey A. Low serum-vitamin-A and subsequent risk of cancer. Preliminary results of a prospective study. Lancet 1980; II:813-815.
Wald N, Boreham J, Bailey A. Serum retinol and subsequent risk of cancer. Brit J Cancer 1986; 54:957-961.
Warrell RP. Retinoid resistance. Lancet 1993; 341:126.

Warren S and Gates O. Multiple primary malignant tumors: a survey of the literature and a statistical study. Am J Cancer 1932; 16:1358-1414.
Wilkinson EJ and Hendricks JB. Role of pathologist in biomarker studies. J Cell Biochem 1995; 23:S10-S18.
Willett WC and MacMahon B. Diet and cancer - An overview. New Engl J Med 1984; 310:697-703.
Willett WC, Polk BF, Underwood BA, Stampfer MJ, Pressel S, Rosner B, Taylor JO, Schneider K, Hames CG. Relation of serum vitamins A and E and carotenoids to the risk of cancer. New Engl J Med 1984; 310:430-434.
Wilpart M, Speder A, Roberfroid M. Anti-initiation activity of N-acetylcysteine in experimental colonic carcinogenesis. Cancer Lett 1986; 31:319-324.
Winn DM, Blot WJ, Shy CM, Pickle LW, Toledo A, Fraumeni JF jr. Snuff dipping and oral cancer among women in the southern United States. New Engl J Med 1981; 304:745-749.
Winn DM. Smokeless tobacco and aerodigestive tract cancers: recent research directions. In: Newell GR and Hong WK (eds): The biology and prevention of aerodigestive tract cancers. Plenum Press, New York, 1992, p39-46.
Witek E, Kwapisz H, Gora B, Pokrant H, Romankiewicz G, Kreglewska B. Clinical changes of the oral mucosa and parodontium in workers exposed to certain toxic chemicals. Pol Tyg Lek 1979; 34:1709-1712.
Wittekind D. Standardization of dyes and stains for automated cell pattern recognition. Anal Quant Cytol 1985; 7:6-31.
Wolf GT, Carey TE, Hayashida DJS, Poore J, Davis L, McClatchey KD. Monoclonal antibodies as prognostic indicators in patients with squamous cancer of the oral cavity and oral pharynx. Acta Otolaryngol 1988; 449:229-234.
Wolf GT, Carey TE, Schmaltz SP, McClatchey KD, Poore J, Glaser L, Hayashida DJ, Hsu S. Altered antigen expression predicts outcome in squamous cell carcinoma of the head and neck. J Natl Cancer I 1990; 82:1566-1572.
Worsham MJ, Wolman SR, Carey TE, Zarbo RJ, Benniger MS, Van Dyke DL. Common clonal origin of synchronous primary head and neck squamous cell carcinomas: analysis by tumor karyotypes and fluorescence in situ hybridization. Hum Pathol 1995; 26:251-261.
Wynder EL, Bross IJ, Feldman RM. A study of etiologic factors in cancer of the mouth. Cancer 1957; 10:1300-1323.
Wynder EL and Klein UA. The possible role of riboflavin deficiency in epithelial neoplasia. Cancer 1965; 18:167-171.
Wynder EL and Stellman SD. Impact of long-term filter cigarette usage on lung and larynx cancer risk: a case-control study. J Natl Cancer I 1979; 62:471-477.
Wyss R and Bucheli F. Quantitative analysis of retinoids in biological fluids by high-performance liquid chromatography using column switching. I. Determination of isotretinoin and tretinoin and their 4-oxo metabolites in plasma. J Chromatogr 1988a; 424:303-314.
Wyss R and Bucheli F. Determination of highly protein bound drugs in plasma using high-performance liquid chromatography and column switching, exemplified by the retinoids. J Chromatogr 1988b; 456:33-43.
Wyss R. Chromatography of retinoids. J Chromatogr 1990; 531:481-508.
Wyss R and Bucheli F. Use of direct injection precolumn techniques for the high-performance liquid chromatographic determination of the retinoids acitretin and 13-cis-acitretin in plasma. J Chromatogr 1992; 593:55-62.
Wyss R. Chromatographic and electrophoretic analysis of biomedically important retinoids. J Chromatogr B 1995; 671:381-425.

Xu XC, Ro JY, Lee JS, Shin DM, Hong WK, Lotan R. Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. Cancer Res 1994; 54:3580-3587.

Yamamoto KI and Kikuchi Y. A comparison of diameters of micronuclei induced clastogens and by spindle poisons. Mutat Res 1980; 71:127-131.

Yu VC, Näär AM, Rosenfeld MG. Transcriptional regulation by the nuclear receptor superfamily. Curr Opin Biotech 1992; 3:597-602.

Zariwala M, Schmid S, Pfaltz M, Ohgaki H, Kleihues P, Schäfer R. p53 Gene mutations in oropharyngeal carcinoma: a comparison of solitary and multiple primary tumours and lymph-node metastases. Int J Cancer 1994; 56:807-811.

Zheng W, Blot WJ, Diamond EL, Norkus EP, Spate V, Morris JS, Comstock GW. Serum micronutrients and the subsequent risk of oral and pharyngeal cancer. Cancer Res 1993; 53:795-798.

SAMENVATTING

Biomarkers bij chemopreventie van tweede primaire tumoren in patiënten met een hoofd-hals carcinoom.

In dit proefschrift wordt onderzoek beschreven dat beoogt de efficiëntie te verbeteren van chemopreventie van tweede primaire tumoren bij patiënten die genezen zijn van een hoofd-hals carcinoom.

Hoofdstuk 1
Patiënten die genezen zijn van een carcinoom in het hoofd-hals gebied hebben een relatief grote kans van 10 tot 35 procent om binnen een aantal jaren een nieuw carcinoom te ontwikkelen in het hoofd-hals gebied, de longen of de slokdarm. Chemopreventie is een nieuwe behandelingsmodaliteit voor deze groep patiënten, waarbij middels medicijnen getracht wordt het ontstaan van dergelijke tweede primaire tumoren af te remmen of te voorkomen. Wereldwijd wordt veel onderzoek verricht naar medicijnen met een mogelijk chemopreventieve werking. Twee meest onderzochte groepen zijn carotenoiden en retinoiden. Onder deze laatste wordt verstaan retinol met zijn natuurlijke en synthetische analoga. Een bekend probleem bij het testen of bepaalde stoffen inderdaad een preventieve werking hebben is dat het resultaat (d.w.z. het niet ontstaan van een nieuwe tumor) pas na vele jaren gemeten kan worden. Bovendien zijn voor dergelijke studies zeer grote groepen patiënten nodig met daaraan gekoppeld hoge kosten. Wanneer andere, vroegere eindpunten, dan het ontstaan van een nieuwe tumor, voor chemopreventie studies kunnen worden gebruikt, dan zouden in veel kortere tijd veel meer stoffen getest kunnen worden op hun preventieve waarde. Bij een dergelijk surrogaat eindpunt denke men aan een biologische marker in het slijmvlies "at risk" of in het bloed van patiënten, die al na korte tijd onder invloed van de chemopreventieve medicatie en parallel aan de chemopreventieve werking daarvan, een verandering vertoont. In de engelse literatuur wordt wel gesproken van een "intermediate endpoint biomarker". In dit hoofdstuk wordt nader ingegaan op deze problematiek en wordt tevens de huidige stand van zaken in de literatuur met betrekking tot chemopreventie en het gebruik van biomarkers beschreven.

Hoofdstuk 2
Het ontstaan van tweede primaire tumoren kan worden verklaard door de volgende theorie: Op het moment dat een eerste tumor in het hoofd-hals gebied manifest wordt, heeft het overige slijmvlies in het hoofd-hals gebied, alsmede het slijmvlies van de longen en de slokdarm, reeds DNA-schade opgelopen als gevolg van een langdurige blootstelling aan alcohol, tabaksproducten en andere carcinogene stoffen.

Het gehele slijmvliesveld is aldus door carcinogenen beschadigd. In de engelstalige literatuur wordt dit fenomeen "field cancerization" genoemd. Wanneer bepaalde cellen in dit slijmvlies multipele specifieke genetische veranderingen hebben ondergaan kunnen daaruit nieuwe tumoren ontstaan.

Hypothetisch is het mogelijk dat op het moment dat een eerste tumor manifest wordt, cellen in het slijmvlies van de bovenste voedsel- en luchtweg reeds in meer of mindere mate veranderd zijn als gevolg van in het verleden opgetreden DNA-schade. Deze DNA-schade kan tot gevolg hebben dat cellen fenotypische veranderingen vertonen. In dit hoofdstuk wordt een studie beschreven waarbij onderzocht wordt of bepaalde eiwitten meer of minder aanwezig zijn in normaal ogend slijmvlies van tien patiënten met een tongcarcinoom in vergelijking met slijmvlies van tien controle personen. De bepalingen werden verricht met behulp van monoclonale antilichamen specifiek gericht tegen de cytokeratines 8, 10, 13 en 19, het proliferatie eiwit Ki-67 en met behulp van de antilichamen genaamd K928, K931, K984 en UM-A9. Er werd gebruik gemaakt van een non-invasieve methode waarbij met behulp van kleine borstels slijmvliescellen verkregen werden. Het bleek dat het eiwit cytokeratine 19 een meer dan drievoudige expressie vertoonde in het slijmvlies van de patiënten vergeleken met het slijmvlies van controlepersonen. Dit betekent dat cytokeratine 19 mogelijk gebruikt kan worden om het proces van "field cancerization" te meten en dat het als zodanig zou kunnen functioneren als een "intermediate endpoint biomarker" in chemopreventie studies.

Hoofdstuk 3

In de literatuur wordt kwantitatieve DNA-analyse of cytomorfometrie vaak genoemd als een mogelijke methode om premaligne slijmvlies te detecteren. Als zodanig zou het kunnen functioneren als een "intermediate endpoint biomarker" in chemopreventie onderzoek. In hoofdstuk drie wordt een studie beschreven, welke verricht werd om de waarde te bepalen van de geautomatiseerde cytomorfometrie voor het detecteren van premaligne mondholte slijmvlies. In cytologische preparaten van mondholteslijmvlies van patiënten met een tongcarcinoom en van controlepersonen zonder maligniteit werden verscheidene cytomorfometrische parameters bepaald waaronder DNA-index, kern-oppervlakte en kern-vorm. Er werd gebruik gemaakt van een CAS 200 beeld analyse systeem. Geen van de onderzochte parameters vertoonde een statistisch significant verschil tussen de cellen van de beide onderzochte groepen.

In vervolg hierop werd bestudeerd of deze methode van beeldanalyse van nut was om veranderingen te detecteren in slijmvliescellen van patiënten die chemopreventieve medicatie gebruiken. Hiervoor werden cytologische preparaten van mondholteslijmvlies vervaardigd van patiënten die in het kader van de chemopreventie studie Euroscan retinyl-palmitaat en/of N-acetylcysteine gebruikten. Preparaten werden afgenomen voor aanvang van de interventie met deze middelen en twee à drie maanden later. In totaal participeerden 70 patiënten aan dit

onderzoek: 19 patiënten gebruikten 600 mg N-acetylcysteine per dag, 17 patiënten gebruikten dagelijks 300.000 IU retinyl-palmitaat en 18 patiënten gebruikten de combinatie van beide middelen. 16 patiënten, die geen medicatie gebruikten dienden als controlepersonen. Geen van de onderzochte cytomorfometrische parameters vertoonde een verandering gedurende de interventie met de medicijnen. Geconcludeerd kan worden dat de beschreven methode met het CAS 200 beeld analyse systeem geen waarde lijkt te hebben om in deze patiëntengroep premaligne slijmvlies, dan wel veranderingen geïnduceerd door retinyl-palmitaat en/of N-acetylcysteine, aannemende dat deze aanwezig zijn, te detecteren.

Hoofdstuk 4

Een van de meest onderzochte "intermediate endpoint biomarkers" gedurende de laatste tien jaar is de micronucleus. Een micronucleus is een chromosoom of chromatide fragment, dat ontstaan is als gevolg van DNA-schade in delende cellen en dat zich na een celdeling als een afzonderlijk stukje DNA in de cel ophoudt. De micronuclei in slijmvliesepitheel in het hoofd-hals gebied worden gevormd in de basale laag van het slijmvlies. Deze cellen migreren uiteindelijk naar de oppervlakkige laag van het slijmvlies en kunnen hier dan eenvoudig van worden afgeschraapt voor nadere analyse. Het aantal micronuclei in slijmvliescellen kan dienen als een maat voor DNA-schade. In dit hoofdstuk wordt een gedetailleerd en strak omschreven protocol beschreven voor het tellen van micronuclei in slijmvliescellen van het hoofd-hals gebied. Statistische analyse liet zien dat minstens 10.000 cellen per patiënt beoordeeld moesten worden, om veranderingen te kunnen detecteren tijdens chemopreventieve interventie. Dit is in tegenspraak met de veel lagere aantallen cellen, die in de tot op heden gerapporteerde analyses vermeld worden.

Hoofdstuk 5

De analyse van retinoiden in plasma is een complexe zaak als gevolg van de gevoeligheid van deze stoffen voor licht, warmte en zuurstof, de lage concentraties in plasma en de sterke binding aan eiwitten. Voor de analyse van grote aantallen monsters in bijvoorbeeld chemopreventie studies is een eenvoudige en betrouwbare methode noodzakelijk. In hoofdstuk vijf wordt een nieuw ontwikkelde analyse methode beschreven, die gebruik maakt van een "reverse phase HPLC", die opvalt door zijn snelheid en eenvoud. De basis van het systeem is een nieuwe monster extractie techniek. Deze nieuwe techniek bleek betrouwbaar en reproduceerbaar.

Hoofdstuk 6

Epidemiologisch onderzoek toont aan dat een tekort aan vitamine A en andere retinoiden een rol zou spelen bij het ontstaan van plaveiselcelcarcinomen in het

hoofd-hals gebied. In hoofdstuk zes wordt een studie beschreven, waarin verscheidene retinoiden in het plasma van hoofd-hals kanker patiënten werden bepaald, gebruik makend van de nieuwe methode die in hoofdstuk vijf wordt beschreven. De concentraties in het plasma van vitamine A, all-*trans* vitamine A zuur, 13-*cis* vitamine A zuur en 13-*cis*-4-oxo vitamine A zuur werden bepaald en vergeleken met de waarden in het plasma van controle personen zonder maligniteit. Er bleken geen significante verschillen in plasma concentraties van de genoemde retinoiden te zijn tussen 25 patiënten met een maligniteit in het hoofd-hals gebied en 21 controle personen.

Tevens werd gekeken naar de invloed van het dagelijks innemen van 300.000 IU retinyl-palmitaat op de retinoid concentraties in het plasma van patiënten die participeerden aan de chemopreventie studie Euroscan. Eén maand na het starten van interventie met retinyl-palmitaat bleken de concentraties van all-*trans* vitamine A zuur, 13-*cis* vitamine A zuur, 13-*cis*-4-oxo vitamine A zuur en retinol significant te zijn gestegen. Van deze stoffen lijkt met name 13-*cis*-4-oxo vitamine A zuur een geschikte kandidaat om patiënten, die participeren aan studies waarbij retinoiden op hun chemopreventieve werking worden onderzocht, te controleren op adequate inname van hun medicatie.

Hoofdstuk 7

De selectie van bepaalde retinoiden om in grote klinische onderzoeken getest te worden op hun chemopreventieve werking is vaak gebaseerd op epidemiologische gronden of positieve resultaten in dierproeven. Enkele recente studies bij zware rokers en ex-rokers bij wie de stof beta-caroteen werd getest vertoonden echter een negatieve invloed daarvan op de ontwikkeling van het longcarcinoom. Dit onverwachte effect laat zien, dat het van belang is dat medicijnen die in dergelijke trials gebruikt worden beter onderzocht worden op hun werkingsmechanisme. In dit hoofdstuk wordt een studie beschreven, waarin het effect van vitamine A zuur op de expressie van verscheidene genen in drie verschillende cellijnen van hoofd-hals carcinomen wordt bekeken. Het bleek dat er geen relatie was tussen de mate van groeiremming door vitamine A zuur van de verschillende cellijnen en de expressie van vitamine A zuur receptor-α, -β and -γ mRNA and retinoid X receptor-α mRNA voor en na inductie door vitamine A zuur. De cellijn die de meeste groeiremming door vitamine A zuur vertoonde, had echter de hoogste mRNA expressie van genen gerelateerd aan plaveiselcellige differentiatie. Dit suggereert een relatie tussen de gevoeligheid van maligne plaveiselcellen voor vitamine A zuur en de mate van plaveiselcellige differentiatie.

DANKWOORD

Op deze plaats wil ik graag al diegenen bedanken, die op directe of indirecte wijze hebben bijgedragen aan het tot stand komen van dit proefschrift. Een aantal personen wil ik hier in het bijzonder noemen.

Mijn promotor prof.dr. G.B. Snow. U wil ik bedanken voor uw getoonde interesse en de waardevolle kritiek tijdens de ontwikkeling van het proefschrift alsmede voor de mogelijkheid die U mij geboden heeft mijn opleiding tot KNO-arts in uw kliniek te volgen.

Mijn copromotor dr. B.J.M. Braakhuis. Beste Boudewijn, jij hebt door je enthousiasme, steun en inzet een belangrijke rol gespeeld bij het tot stand komen van dit proefschrift. Jouw advies en nauwgezetheid heb ik zeer gewaardeerd.

De leden van de beoordelingscommissie, dr. F.E. van Leeuwen, prof.dr. C.J.L.M. Meijer, prof.dr. H.P. Sauerwein, prof.dr. R.B.H. Schutgens, prof.dr. H.J. Tanke, prof.dr. J.B. Vermorken en prof.dr. I. van der Waal dank ik voor hun bereidwilligheid om het manuscript te beoordelen.

Dr. T. Teerlink. Beste Tom, ik wil je bedanken voor je inzet een geschikt HPLC systeem te ontwikkelen voor het bepalen van retinoid-concentraties teneinde enkele van onze vraagstellingen te kunnen beantwoorden.

Drs. J.A.M. Beliën. Beste Jeroen, jou ben ik erkentelijk voor je vele inspanningen software te ontwikkelen ter kwantificering van de micronucleus.

Dr. F.B.J.M. Thunnissen. Beste Erik, jou wil ik bedanken voor de prettige samenwerking alsmede de gastvrijheid die je me op je afdeling verleende en die resulteerde in een hoofdstuk van dit proefschrift.

Dr. R.H. Brakenhoff. Beste Ruud, jou wil ik bedanken voor de scholing die je me hebt gegeven op het gebied van de moleculaire biologie.

Dr. N. de Vries. Beste Nico, jou ben ik erkentelijk voor je begeleiding gedurende de eerste fase van het biomarker onderzoek.

Zr. A. Beerthuis en de administratieve medewerkers van de polikliniek KNO dank ik voor hun inspanningen bij het verkrijgen van de patiëntengegevens.

Alle medewerkers van het tumorbiologisch laboratorium wil ik bedanken voor de prettige werksfeer die ze me boden op het lab.

Alle stafleden, arts-assistenten, Fred Snel en de medewerksters van het secretariaat en de polikliniek van de afdeling KNO dank ik voor hun belangstelling voor mijn onderzoek.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 7 maart 1963 geboren in Den Haag. Na het behalen van het diploma VWO aan het Caland Lyceum in Rotterdam werd in 1981 begonnen met de studie geneeskunde aan de Rijksuniversiteit Leiden. In augustus 1988 slaagde hij voor het arts-examen. Na de algemene opleiding tot officier-arts werkte hij van december 1988 tot maart 1990 als dienstplichtig arts-assistent keel-, neus- en oorheelkunde in het militair hospitaal dr. A. Mathijsen te Utrecht. Hierna was hij tot september 1990 werkzaam als arts-assistent algemene heelkunde in het Diaconessenhuis te Leiden. Vanaf die datum tot oktober 1992 werkte hij als assistent in opleiding aan de Vrije Universiteit op het tumorbiologisch laboratorium van de afdeling keel-, neus- en oorheelkunde. In deze periode werd de basis gelegd voor dit proefschrift. In april 1992 startte hij met de opleiding tot keel-, neus- en oorarts in het academisch ziekenhuis van de Vrije Universiteit (opleider: prof.dr. G.B. Snow). Van april 1995 tot november 1995 werd een deel van deze opleiding volbracht in het Westeinde Ziekenhuis in Den Haag (opleider: dr. I.B. Tan). Na het afsluiten van deze opleiding in april 1997 aanvaardde hij de functie van academisch specialist in het Academisch Medisch Centrum te Amsterdam. Sedert september 1997 is hij lid van de International Society of Cancer Chemoprevention.

LIST OF PUBLICATIONS

C.F. Smit, M.P. Copper, J.M.G. Urlings. Twee HIV-seropositieve dienstplichtigen met KNO-klachten. NMTG 1990; 5:163-165.

M.P. Copper, B.J.M. Braakhuis, N. de Vries, G.A.M.S. van Dongen, J.J.P. Nauta, G.B. Snow. A panel of biomarkers of carcinogenesis of the upper aerodigestive tract as potential intermediate end points in chemoprevention trials. Cancer 1993; 71:825-830.

J. Cloos, I. Steen, B.J.M. Braakhuis, M.P. Copper, N. de Vries, J.J.P. Nauta, G.B. Snow. Increased mutagen sensitivity in head-and-neck squamous-cell carcinoma patients, particularly those with multiple primary tumors. International Journal of Cancer 1994; 56:816-819.

M.P. Copper, A. Jovanovic, J.J.P. Nauta, B.J.M. Braakhuis, N. de Vries, I. van der Waal, G.B. Snow. Role of genetic factors in the etiology of squamous cell carcinoma of the head and neck. Archives of Otolaryngology Head and Neck Surgery 1995; 121:157-60.

L.J.J.M. Bauwens, M.P. Copper, J.T. Schmidt. Levensbedreigend angio-oedeem als bijwerking van angiotensine-converting-enzym (ACE)-remmers. NTVG 1995; 139:674-677.

J.A.M. Beliën, M.P. Copper, B.J.M. Braakhuis, G.B. Snow, J.P.A. Baak. Standardization of counting micronuclei: definition of a protocol to measure genotoxic damage in human exfoliated cells. Carcinogenesis 1995; 16:2395-2400.

M.P. Copper, F.B.J.M. Thunnissen, N. de Vries, G.B. Snow, B.J.M. Braakhuis. Cytomorphometric parameters in exfoliated cells as biomarkers in head and neck cancer chemoprevention. International Journal of Oncology 1996; 9:1071-1075.

J.W. Wirds, E.S. Boon, M.P. Copper, G.J. Scheffer, H.F. Mahieu. Percutane tracheotomie met behulp van een dilaterende forceps: een snelle en veilige techniek. Intensive Care Reviews 1997; 12:9-14.

B.J.M. Braakhuis, I. Klaassen, B.M. van der Leede, J. Cloos, R.H. Brakenhoff, M.P. Copper, T. Teerlink, H.F.J. Hendriks, P.T. van der Saag, G.B. Snow. Retinoid metabolism and all-trans retinoic acid induced growth inhibition in head and neck squamous cell carcinoma cell lines. British Journal of Cancer 1997; 76:189-197.

M.P. Copper, R.H. Brakenhoff, J. Cloos, G.B. Snow, B.J.M. Braakhuis. Retinoid induced gene-expression and growth inhibition in head and neck cancer cell lines. European Journal of Cancer Part B: Oral Oncology 1997; 33:270-274.

T. Teerlink, M.P. Copper, I. Klaassen, B.J.M. Braakhuis. Simultaneous analysis of retinol, retinoic acid isomers and polar metabolites in biological fluids by HPLC using on-column concentration after single-phase fluid extraction. Journal of Chromatography B 1997; 694:83-92.

M.P. Copper, T. Teerlink, I. Klaassen, G.B. Snow, N. de Vries, B.J.M. Braakhuis. Plasma retinoid levels in head and neck cancer patients: a comparison with healthy controls and the effect of retinyl-palmitate treatment. Submitted.

I. Klaassen, R.H. Brakenhoff, S.J. Smeets, M.P. Copper, G.B. Snow, B.J.M. Braakhuis. Suitability of messenger RNA in oral exfoliated cells as biomarker of head and neck carcinogenesis. Submitted.

H.A.J. Brok, R.J. Stroeve, M.P. Copper, P.F. Schouwenburg. The treatment of the pharyngo-esophageal segment in laryngectomized patients: a review. Submitted.

H.A.J. Brok, R.J. Stroeve, M.P. Copper, B.W. Ongerboer De Visser, A.J. Venker-van Haagen, P.F. Schouwenburg. Recurrent laryngeal nerve contribution in motor innervation of the human cricopharyngeal muscle. Submitted.