Etiology of Oral Cancer

Elsy-Britt Schildt

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AKADEMISK AVHANDLING

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Abstract

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Oral cancer is a disease with increasing incidence in most West European countries. In Sweden in 1995, intraoral cancer accounted for 1.4% and 0.8% of malignant tumours among men and women respectively. The disease has a bad prognosis and approximately half of the patients will die of their disease.

The aim of this investigation was to focus on the relationship between oral cancer and a suite of potential risk factors. The prime focus was to investigate the risk of snuff use but smoking tobacco, alcohol consumption, infections, dental factors, dental X-ray, iron deficiency, occupations and occupational exposures were also studied using the case-control design. The study was conducted in Northern Sweden and encompassed 410 cases and as many controls. Furthermore, two molecular epidemiological studies were done regarding the relationship between exposure factors and certain biological parameters of tumours.

The use of oral snuff is an increasingly common habit in Sweden. Contrary to previous American studies of cancer risk from oral snuff use, this study showed no elevated risk for oral cancer. Smoking was an important risk factor for oral cancer, but this was restricted to current smokers. Alcohol consumption showed a clear dose-response relationship to oral cancer, and the combination with smoking increased the risk further.

Moreover, the case-control study strongly indicated recurrent HSV-1 infections as an independent causative factor in oral cancer, particularly when the infection was on the lip. Dental factors like different kinds of fillings, fixed prosthesis or removable dentures were not associated with increased risks, nor were dental X-rays.

Pulp industry workers and wood or wood product workers were found to have increased risk for oral cancer. Smoking and alcohol in addition increased the risk. Exposure to phenoxyacetic acids indicated an increased risk.

Oral infections, and HSV-1 infections in particular, were associated with an increased risk for oral cancer in patients with p53 positive tumours as demonstrated by immunohistochemistry (IHC). However, this relation was not found in patients with p53 mutated tumours confirmed by PCR techniques. This suggests that HSV-1 infection, directly or indirectly, can inactivate p53 function by binding of wild type protein. No association between smoking and p53 positive tumours was found.

A new non-random deletion in exon 8 in oral squamous cell carcinoma was found. The cause and clinical significance of this new 14 base pair deletion is unknown. Eighty percent of the patients with the deletion were women. No correlation was found for the deletion group with the known risk factors for oral cancer such as smoking.

Key words: Oral cancer, case-control study, snuff use, smoking, alcohol, HSV-infections, dental factors, occupations, molecular epidemiology, p53.
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To Sara, Henrik and Maja
Livet är kort, konsten är lång, 
rätta tillfället flyktigt, 
erfarenheten bedräglig, 
omdömet svårt.

Hippokrates
Abstract

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
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<tr>
<td>del</td>
<td>Deletion</td>
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<tr>
<td>EGRET</td>
<td>Epidemiological Graphics Estimation and Testing package</td>
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<td>HPV</td>
<td>Human papillomavirus</td>
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<td>HSV</td>
<td>Herpes Simplex virus</td>
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<td>IdUrd</td>
<td>Iododeoxyuridine</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>LI</td>
<td>Labelling index</td>
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<td>NYK</td>
<td>Nordic Working Classification</td>
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<td>OR</td>
<td>Odds Ratio</td>
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<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<td>SCCHN</td>
<td>Squamous cell carcinoma of the head and neck</td>
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<td>SEI</td>
<td>Swedish Socioeconomic Classification</td>
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<td>SSCP</td>
<td>Single strand conformation polymorphism</td>
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<td>TSNA</td>
<td>Tobacco-specific N-nitrosamines</td>
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III. Occupational exposures as risk factors for oral cancer evaluated in a Swedish case-control study.

Schildt E-B., Eriksson M., Hardell L., Magnusson A. (submitted)


V. A non-random deletion in the p53 gene in oral squamous cell carcinoma.


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Abstract

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   Paper I-V
1. Background

1.1 Oral cancer

Oral cancer is a disease with increasing incidence in most West European countries but the incidence varies widely. It is most common in France and India but the incidence is also high in central European countries e.g., Slovakia, Slovenia, Switzerland and Hungary (Boyle et al., 1995). In some parts of India oral cancer accounts for approximately 40% of all cancers registered (Pindborg 1980). Low rates are reported from Japan, China and countries of northern Europe. For women the incidence is lower in general but high rates are found in India, Southeast Asia and the United States (Boyle et al., 1995).

In the Nordic countries, an increasing trend is reported from both Sweden and Norway and in Denmark there has been a steep rise in incidence during the past decades (Hakulinen et al., 1986, Bundgaard et al., 1995). However, no increase in incidence is reported from Finland (Hakulinen et al., 1986).

In Sweden during 1995, intraoral cancer accounted for 1.4% and 0.8% of malignant tumours among Swedish men and women respectively (The National Board of Health and Welfare 1995). As for most cancers, the risk for developing oral cancer increases with increasing age. However, an increasing incidence in younger ages has also been reported (Bundgaard et al., 1995).

Mortality of oral cancer varies between subsites and morphologically similar tumours show a wide and often unpredictable range in clinical behaviour (Carter 1992). When all sites are considered in the larger group head and neck cancers, approximately half of the patients will die of their disease. This rather bad prognosis, shown in several studies, is due to loco-regional failure which occurs in 28-73% of all patients despite adequate treatment. Approximately 90% of all relapses appear within the first two years (Langdon 1991).

1.2 Risk factors

1.2.1 Smokeless tobacco

Indians in South-America were the first people known to use snuff. This was as early as in the fifteenth century. A Franciscan monk, Friar Ramón Pané who travelled with Christopher Columbus on his second voyage to the New World in 1493, brought the tobacco plant to Europe from the West Indies, where the natives smoked tobacco in pipes for medicinal and ceremonial purposes (Stewart 1967). In the eighteenth century, King Gustav III made snuff use really popular in Sweden, but snuff was mentioned for the first time in Swedish history in 1637. Several local snuff producers started their business in the eighteenth century, but in 1914 the Swedish Tobacco Monopoly (a governmental institution) took over the production of snuff (Andersson 1991). Even though snuff use is very common in some developing countries, e.g., Sudan, Sweden has the highest per capita consumption of smokeless tobacco in the Western world (M. Curwall, pers.com.).

The importance of smokeless tobacco has been investigated in numerous studies. Tobacco chewing, a common habit especially in Asian countries and in USA, is well known as a strong risk factor for oral cancer.

In the Orient and especially in India, the chewing of Betel quid is widespread. The betel quid consists of a betel leaf, areca nut and lime and it is used with or without the addition of crushed tobacco leaves (IARC 1985). There is sufficient evidence that the habit of chewing betel quid containing tobacco is carcinogenic to humans (IARC 1985).
The association between oral snuff use and oral cancer is widely debated. In the United States there have been reports of oral snuff as a risk factor for oral cancer (IARC 1985). In particular, the case-control study made by Winn et al. (1981) of snuff use in women in rural southern United States (with a nearly five-fold increased risk for oral cancer) has received great attention. For the use of Swedish moist snuff, an association with oral cancer has not been established.

The snuff used in Sweden is a moist, non-fermented tobacco product. The ground tobacco, after addition of salt and water, undergoes a heat treatment which renders it practically free from micro-organisms, lowering the risk of nitrate formation and subsequent formation of nitrosamines with carcinogenic potential. The "American" moist snuff is a fermented product. The fermentation is a spontaneously occurring biochemical process in the moistened tobacco which causes chemical changes (IARC 1985). This difference in manufacturing may be one possible reason for the differences among studies of cancer risk.

The most common snuff-type in Sweden is a loose moist snuff used as a 1-2 g quid, formed by the fingers and placed under the upper lip. Portion-bags of moist snuff have become increasingly popular, especially among women. The habit is most common in North Sweden. About 20% of all males and 8% of all women use moist snuff regularly in Sweden (Ahlbom et al., 1997). The use of moist snuff is especially common among young males and the age of onset seems to be decreasing (Bolinder 1997). The increase in snuff use has not been followed by a similar increase in incidence in oral cancer. The incidence is slowly increasing, however, (Hakulinen et al., 1986) and as the prevalence of smoking now decreases in Sweden, it is indeed interesting to evaluate the risk for oral cancer from moist snuff use. Parallel to the present study, another Swedish study was performed on oral snuff and cancer risk (Lewin et al., 1998). This study did not reveal any association between the use of oral snuff and oropharyngeal cancer.

Other local health effects of snuff use have been the subject of many studies. "Snuff dipper's lesion" is the common term for clinically observable changes in oral mucosa. It is characterised by a local thickening and hyperkeratosis of the epithelium (Andersson et al., 1989a) and usually reversible when snuff use is interrupted. Out of several reports from Swedish histopathological materials, none shows a transformation of lesions into premalignant leucoplasias (Axell et al., 1976, Andersson et al., 1989b, Larsson et al., 1991). Gingival recession and discoloration of the teeth are generally not reversible to the same extent.

1.2.2 Smoking tobacco
Smoking of cigarettes as well as pipe smoking are well-known strong risk factors for lung cancer. It has also been established that smoking is important for development of cancer in the oral cavity (Blot et al., 1988, Franceschi et al., 1992, Mashberg et al., 1993, Bundgaard et al., 1995) and pharynx among other sites. Associations between pipe smoking and lip cancer has also been reported (Spitzer et al., 1975).

Use of cigarettes have continually increased during the last century but in contrast to lung cancer, there has not been a corresponding increase in oral cancer. Tobacco products are carcinogenic but probably there has to be a promoter for carcinogenesis in the oral cavity. The interaction between smoking and alcohol consumption is interesting. The almost multiplicative effect of combined exposure to tobacco smoking and alcohol use has been described by several authors (Blot et al., 1988, Bundgaard et al., 1995, Lewin et al., 1998). On the other hand, the interaction between snuff use and other factors as smoking and alcohol has rarely been studied.

1.2.3 Alcohol
Heavy alcohol consumption seems to be a strong risk factor for oral cancer. The most probable reason for the lack of conclusive evidence for the carcinogenic capacity of
alcohol is the difficulty in isolating heavy alcohol consumption from smoking. Several studies have reported increased risk for oral cancer among consumers with high intake of alcohol (Blot et al., 1988, Mashberg et al., 1993, Boyle et al., 1995, Bundgaard et al., 1995) but this does not imply a direct association with ethanol. Other potential risk factors are possible carcinogens in the form of contaminants or congeners in the alcoholic beverage and the associated health risks of smoking and poor nutrition (Rothman 1978). The importance of different kinds of beverages is described by Blot et al. (1988) who reported higher risk for oropharyngeal cancer and a stronger dose-response effect among those consuming hard liquor or beer than wine.

1.2.4 Infections
A relationship between different infectious agents and oral cancer has been suggested. Most of the studies are based on experimental research. Few epidemiological studies have focused on clinical infections (Maden et al., 1992).

Evidence from in vitro studies associates infections by Herpes simplex virus type 1 (HSV-1) with oral cancer, particularly studies on HSV antibodies (Scully 1983). Herpes lesions of the oral cavity are generally considered benign, but anecdotal reports of oral herpes lesions undergoing malignant transformation exist (Wyburn-Mason 1957, Gecht 1980, Eskinazi 1987) and HSV-1 RNA have been detected in oral squamous carcinoma (Eglin et al., 1983).

Human papilloma virus (HPV) is established as a risk factor for cervical cancer. It is also a suspected carcinogen in other malignancies and has been associated with possible oral carcinogenicity (Gissman 1992, Lakshmi et al., 1993).

Regarding bacterial infections, the only described association is between late stage syphilis and lingual carcinoma (Clemmesen 1965). It is possible, however, that various preparations of arsenicals and heavy metals used in the treatment of that disease may have acted as the most important carcinogenic agents (Smith et al., 1990).

Chronic candidiasis has been suspected as an oral carcinogen based on the fact that it causes leukoplakias. Thus, it seems likely that chronic candidiasis may be a precancerous condition, although the possibility that candidal invasion may occur as a superimposed infection has been discussed (Cawson and Binnie, 1980).

1.2.5 Dental status
Some epidemiological studies report poor dentition, indicated by missing teeth, bad oral hygiene and bad condition of dentures as risk factors for oral cancer independent of smoking and drinking habits (Zheng et al., 1990, Graham et al., 1977).

1.2.6 Host factors
Host factors such as iron deficiency connected with Plummer-Vinson syndrome are related to an increased risk for oral cancer (Ahlbom 1937). Increased risk for pharyngeal and lingual cancer was suggested in a study of an American cohort of white males with pernicious anaemia, possibly related to dysplastic changes related to the disease (Brinton 1989).

1.2.7 Occupations and occupational exposures
Little is known about occupational hazards and the risk for oral cancer. However, in some studies certain professions have been associated with this disease, e.g., electrical and electronics workers (Winn et al., 1982, Vågerö et al., 1983, Merletti et al., 1991, Pukkala et al., 1994), painters (Engholm et al., 1982, Matanoski et al., 1986, Skov et al., 1993), workers with access to alcohol e.g., bartenders and restaurant workers (Pearce et al., 1986, Olsen et al., 1987) and cellulose fibre production workers (Lanes et al., 1990).

1.3 Molecular epidemiology
By combining molecular biological and epi-
to investigate a potential association between specific exposure factors and genetic aberrations with possible effects on tumour growth and progression.

To understand the impact of p53 in the etiology and pathogenesis of cancer it is important to characterise p53 gene status and correlate that as well as expression of the p53 protein to different exposure factors.

The normal p53 functions as "guardian of the genome" (Lane 1992) and the p53 gene is located on the short arm of chromosome 17. The p53 protein consists of 393 amino acids and can be divided into three regions with different functions (Greenblatt et al., 1994). Mutation in the p53 tumour suppressor gene is the most frequently observed genetic aberration in human cancer, and in SCCHN p53 mutations have been found in 40-50% of the tumours (Brachman et al., 1992, Boyle et al., 1993, Nylander et al., 1995a,b). For SCCHN approximately 98% of all p53 mutations are found within exons 5-8. The most common type of mutation in the midregion is a missense mutation, constituting up to 79% of all mutations (Greenblatt et al., 1994).

Expression of markers such as PCNA and Ki-67 as well as bcl-2 protein related to different kinds of exposures has so far not been investigated.

The proliferating cell nuclear antigen (PCNA) has a dual function in that it is involved in DNA replication as well as DNA repair. Wild type p53 protein can indirectly and selectively inhibit the activity of PCNA in DNA replication, whereas PCNA function in nucleotide excision-repair is unaffected (Shivji et al., 1994).

The Ki-67 protein is a non-histone protein present in G1, S, G2 and M-phases of the cell cycle but absent in G0 cells (Gerdes et al., 1984; 1991). Expression of Ki-67 provides information whether cells are in the cell cycle or not, whereas no information regarding the length of the cell cycle or whether cells are actively cycling is achieved.

The bcl-2 protein, a product of the oncogene bcl-2 located on chromosome 18, is a key factor to hamper cell loss by apoptosis and can inhibit most types of apoptotic cell death.
2. Aims

The overall aim of the studies was to evaluate the importance of different risk factors for oral cancer in Sweden and to investigate the relationship between exposure factors and biological tumour factors with the main focus on the \textit{p53} gene and p53 protein status.

**Paper 1**

The aim of this part of the case-control study was to investigate the importance of snuff use, smoking tobacco and alcohol to oral cancer, as well the interaction of these factors.

**Paper 2**

This second part of the case-control study aimed to investigate the importance of other local factors in relation to the risk for oral cancer, such as different kinds of infections, dental status, dental X-ray and iron deficiency.

**Paper 3**

In the third part of the case-control study the aim was to investigate the role of occupations and occupational exposures as risk factors for oral cancer.

**Paper 4**

The aim of this study was to investigate the association between p53 protein expression, p53 mutations and risk factors investigated in paper 1 and 2. Tumours were also evaluated using immunohistochemistry (IHC) for expression of PCNA, Ki-67 and bcl-2 proteins related to different exposure factors.

**Paper 5**

During the course of this work a novel non-random deletion, located in exon 8, was unexpectedly found in a subset of material in the case-control study. The aim of this paper was to describe this deletion and test for any possible correlations with the epidemiological data.
3. Materials and methods

This investigation was based on a case-control study (papers I, II and III) and a subgroup from the case-control study (paper IV and V). The different methods and the materials used in the separate studies are presented below.

3.1 Case-control study
The case-control study was composed of three different parts in which assessment of exposure was performed in the same way.

**Cases**
The study base was defined as all persons living in the four most northern counties in Sweden; Norrbotten, Västerbotten, Jämtland and Västernorrland, during 1980-1989. The persons defined as eligible were reported to the Swedish Cancer Registry as having oral cancer including all histopathologically verified squamous cell cancer (ICD-7 codes 140=lip, 141=tounge, 143=floor of mouth, 144=gingiva, 145=tonsill/mesopharynx) diagnosed during the study period.

Out of 419 identified patients one was excluded because of wrong coding of diagnosis and eight deceased cases due to lack of relatives who could provide required information. Thus, the study comprised in total 410 cases; 175 (42.7%) alive and 235 (57.3%) deceased. No restrictions regarding sex or age were applied. Out of the 410 cases, 134 were women and 276 were men. The mean age was 72.3 years for women and 69.6 years for men.

**Controls**
For each of the 175 living cases, one living control was drawn from the National Population Registry. The person closest in age i.e., the one with the closest personal identification number, with the same sex and living in the same county was used. For each of the 235 deceased cases one deceased control was selected from the National Registry for Causes of Death. The same matching criteria were used (age, sex, county) and furthermore, deceased controls were matched on year of death. The case-control study thus involved 820 persons: 410 cases and 410 controls.

3.2 Assessment of exposure
All living subjects in the study, cases and controls, received a mailed questionnaire. To obtain information concerning deceased persons, the questionnaire was sent to the next-of-kin defined in the order of husband or wife, child, parent, sibling or other. The specific nature of the investigation was not disclosed, and there was no reference to the disease under study. Instead, the general information given was that different factors of potential importance for health were studied.

If the questionnaire was incomplete or a question was obviously misunderstood the subject was contacted by telephone by a specially-trained interviewer who did not know whether the person under investigation was a case or a control, and the data were supplemented according to written instructions. Some persons, or next-of-kins, did not answer the questionnaire, but were willing to participate in a full telephone interview.

After the questionnaire had been completed by the interviewer, the front page including name, personal identification number and address was removed, thus enabling a blind coding of the answers.

3.2.1 Paper I
**Tobacco exposure**: Use of moist snuff, cigarettes, cheroots, cigars and pipe tobacco was covered. The questionnaire mapped both the daily consumption and the time period of smoking. In the analysis, lifetime consumption (kg tobacco) was assessed. All tobacco exposure was expressed in grams of
tobacco/day. One cigarette is equivalent to 1 g of tobacco, one cheroot to 3 g and one cigar to 5 g. One pack of pipe tobacco is equivalent to 50 g of tobacco and one pack of moist snuff to 50 g. One quid of moist snuff is estimated to contain 1 g of tobacco. The brand of snuff was also recorded.

An ex-smoker or ex-snuff user was defined as a person who had quit the habit at least one year before the diagnosis; for controls the corresponding year was the year of diagnosis for the respective case. Subjects who had stopped smoking or stopped using moist snuff within the year before diagnosis were coded as current users of tobacco and with a daily consumption corresponding to the actual consumption by the time they quit.

Alcohol exposure: These questions covered the use of light beer (alcohol content less than 4.5 volume %), beer (alcohol content minimum 4.5 volume %), wine and liquor. The questionnaire asked for weekly consumption and if there was any substantial change over the years.

The subjects were asked to estimate the light beer consumption according to four alternatives: 1. No light beer at all; 2. 1-9 light beer bottles (33 cl)/week; 3. 10-19 bottles/week; 4. At least 20 bottles/week. Beer consumption was surveyed in a corresponding way with the following four alternatives: 1. No beer at all; 2. 1-4 beer bottles/week; 3. 5-9 bottles/week; 4. At least 10 bottles/week.

Estimation of wine and liquor exposure was made with regard both to how often the subjects drank and to the average amount each time. Regarding the frequency of wine drinking, the subjects had to choose between five alternatives: 1. never; 2. seldom; 3. about once a month; 4. about once a week; 5. daily. As for the approximate quantity on each occasion, there were three alternatives: 1. not more than one glass; 2. about two glasses; 3. one bottle or more. The results of these answers were transformed into a score taking amount and frequency into account. Concerning liquor consumption, the same frequency alternatives were used as for wine, but regarding the quantity per occasion four alternatives were given: 1. not more than one glass; 2. about two glasses; 3. about 37 cl; 4. more than 37 cl. The answers about liquor consumption were also transformed to an exposure score.

3.2.2 Paper II
Oral infections: The subjects were asked if and when they had had infections in the oral cavity or on the lips, and if so, they were asked to describe the localisation and type of infection, and if the infection was chronic or intermittent.

Because of the suggestion that oral infections might be a tumour related problem, it was decided to exclude all subjects with exposure to an infection within a year before tumour diagnosis. For controls the year of diagnosis of the matched case was used.

Dental factors: The subjects were asked questions about their use of removable dentures and fixed prostheses, their number of dental X-rays, and if they had tooth-fillings with dental amalgam, gold, or plastic material. They were also asked if they have had caries, tooth loss or dental calculus. Concerning dental care, a specific category was constructed with all subjects who reported either fillings or removable denture/fixed prosthesis, in order to evaluate if care about dental health affected the risk for oral cancer. This category is denominated "ever dental care".

Iron supplements: The subjects were asked if they have got iron supplement, and if so, for what reason and for how long.

3.2.3 Paper III
Occupations: The questionnaire included a lifetime occupational history. Many subjects had several different occupations during their lifetime. Only occupations with a duration of at least one year were included. All occupations were classified according to the Nordic Working Classification system.
Elsy-Britt Schildt
(NYK) 1989.

Occupational exposures: The subjects were asked if they have been exposed to chemical agents, an if so, what type of agents, how often and if in occupation or during leisure time. All persons who had worked with pesticides, as well in farming as in forestry, were phoned to ensure uniform assessment to such chemicals.

Socioeconomic status: The subjects were classified according to their socioeconomic status using the Swedish Socioeconomic Classification (SEI) 1989 and all subjects were also classified according to place of residence.

3.3 Immunohistochemistry (IHC)
Immunohistochemical studies were performed on a subset of cases from the case-control study described above. In paper IV all 133 primary oral squamous cell carcinomas diagnosed at the Department of Pathology in Umeå, Sweden, with representative formalin-fixed and paraffin-embedded samples were included. In paper V, a subset of 80 of these 133 patients were studied, namely cases with tumours located in the oral cavity, excluding lip carcinoma. The findings from the immunohistochemical (IHC) and polymerase chain reaction (PCR) analysis (described below) were then correlated to exposure data.

In the immunohistochemical analysis monoclonal antibodies against p53, PCNA, Ki-67 and bcl-2 were used. Five µm sections were cut and left to dry at room temperature for at least 24 hours. In all stainings the alkaline-phosphatase-anti-alkaline phosphatase (APAAP) technique was used (Mason et al. 1981, Cordell et al. 1984).

The immunohistochemical evaluation was performed for p53 and bcl-2, by grading the tumours positive or negative, where tumours with only occasional p53 positive cells were considered negative. For PCNA and Ki-67 stained slides, a labelling index (LI) was calculated. In brief a 10x10 square grid comprising 121 cross points was fitted into the eyepiece of the microscope with an objective lens of x40, and cells showing a distinct nuclear staining were counted in 5-10 randomly chosen fields, in most cases covering the whole tumour. The average LI was calculated as the percentage of positively stained nuclei falling in the crossing between two lines of the grid.

3.4 p53 mutation analyses
Hematoxylin/eosin stained slides were available from all samples, and by referring to these, normal tissue was removed from the blocks using a scalpel. When extracting DNA from the blocks an extraction buffer consisting of 100 mM Tris-HCL, 1mM EDTA (pH 8.0) and 0.4 µg/µl proteinase K was used (Shibata et al., 1992). For samples that were resectioned due to poor or no amplification in the PCR-reaction, an extraction buffer consisting of 50mM Tris-HCL 1 mM EDTA, 0.5% Tween 20 and 0.2 µg/µl proteinase K was used instead (Wright et al., 1990). By using tissue fixed in 4% formaldehyde for DNA extraction, the mean DNA concentration is known to be reduced with longer fixation time (Karlsen et al., 1994), but this does not confound our statistical analysis because all samples were treated identically.

The procedure for detection of base changes was based on a method (Single Strand Conformation Polymorphism analysis, SSCP) described earlier (Orita et al., 1989). Using this technique a specific region of a gene is amplified, denatured and run on a non-denaturating polyacrylamide gel allowing single strands to form different conformations. The mobility of the single strands in the gel thus depends on their size and sequence. Single strands with the same size and sequence that have formed similar conformations move at the same pace, whereas strands with mutations form other conformations, causing aberrant mobility in the gel (Nylander 1995). The sensitivity of the technique is 70-95% for detection of mutations
in PCR products of 200 bp or less (Grompe et al., 1993). A reduction in mutation induced mobility change has been noted for amplified products exceeding 180 bp (Moyret et al., 1994). When analysing mixed cell populations, like solid tumours, an admixture of as much as 85-95% normal tissue allows detection of p53 mutations (Wu et al., 1993).

After PCR amplification of the exon in question, ligation into a vector and cloning in bacteria was performed in order to sequence DNA. After PCR/SSCP control of 16-48 different clones per exon, both strands of a minimum of 2 clones were sequenced using the dideoxy sequencing method.

3.5 Statistical methods

Paper I-IV: Matched analyses were performed and the exposure factors in the questionnaire were analysed with univariate, and when fulfilling certain criteria, multivariate methods (conditional logistic regression). The calculations were performed using the EGRET program (Epidemiological Graphics Estimation and Testing package, SERC, Seattle, USA). The variables were expressed in categorical forms and the results are presented as the odds ratio (OR) and 95% confidence interval (CI) in particular category compared with the reference category.

Regarding the IHC and PCR/SSCP analyses of p53, five different OR’s were calculated for every exposure factor: all cases, p53 IHC positive, p53 IHC negative, p53 mutation positive, and p53 mutation negative cases respectively.

Regarding PCNA the subjects were subdivided into three equally sized groups according to the labelling index (LI) of this proliferation marker: PCNA (1): 14-47%, PCNA (2): 48-65%, PCNA (3): 66-93%. As a consequence, regarding PCNA, four OR’s where calculated for every exposure factor; one including all matched pairs in the PCNA analysis (n=106), and one for each category as stated above. As for PCNA, a subdivision into three equally sized groups was also done for the LI of the proliferation marker Ki-67: Ki-67 (1): 11-24%, Ki-67 (2): 25-38%, Ki-67 (3): 39-60%.

Paper V: The exposure factors in the questionnaire were analysed for group A (tumours with deletion) and B (all other tumours). The p-value for differences between group A and B was calculated using the chi-square test according to Pearson.
4. Results

4.1 Paper I: Oral snuff, smoking habits and alcohol consumption in relation to oral cancer evaluated in a Swedish case-control study

The response rates were 96% and 91% for cases and controls, respectively.

**Snuff:** In the study, 20% were active or ex-users of snuff. Only one woman reported use of moist snuff. No increased risk for oral cancer was found among active snuff users, OR 0.7 (CI 0.4-1.1), Table 1. A slight but not significantly increased risk was found for ex-snuff users, OR 1.5 (CI 0.8-2.9).

When snuff users, active and ex-users respectively, were analysed according to if they had smoked or not, increased risks were observed for ex-snuff users regardless of smoking habits, with the highest risk for ex-users who also were active smokers, OR 3.1 (CI 1.4-6.8). On the other hand, active snuff users did not experience any significantly increased risk regardless of smoking habits.

When analysing different tumour localisations separately, an increased risk was found for lip cancer among ex-snuff users, OR 1.8, CI 0.9-3.7. For current users the risk was close to unity.

To investigate if a dose-response effect existed, we divided the snuff-users in two groups according to lifetime consumption if we were able to assess duration of use. The median value among the controls in kg was calculated to be 156.0 kg, which corresponds to about 2 packages (100 g) of snuff per day during roughly 30 years. Lifetime consumption of over 156.0 kg yielded an OR of 1.1 (CI 0.5-2.0); less than that an OR of 0.8 (CI 0.4-1.5).

No difference in risk was found among the different snuff brands used.

**Smoking:** Of the subjects in the study, 30% were active smokers and 25% were ex-smokers. Among the cases, 34% were active smokers and 23% ex-smokers. Corresponding numbers among controls were 25% and 27% respectively. In the group of active or ex-smokers, 76% used cigarettes, 55% used pipes and only 5% cigars or cheroots.

Active smokers experienced a significantly increased risk (OR 1.8, CI 1.1-2.7) whereas no increased risk was found for ex-smokers, Table 1. A division of ex-smokers in two groups according to if they had stopped smoking for more or less than ten years did not reveal any increased risk either.

To investigate if a dose-response effect exists in the group who could state their consumption, we divided tobacco smokers in two groups according to lifetime consumption. The median value among the controls was calculated to be 124.8 kg, which corresponds to about 1 package of cigarettes per day during 17 years. Current smokers with >124.8 kg lifetime consumption of tobacco had a significantly increased risk for oral cancer (OR 1.8, CI 1.2-2.8). Lower consumption produced a risk around unity.

When dividing the material according to localisation, an increased risk was found among smokers for cancer in the floor of the mouth, OR 8.0 (CI 1.0-64.0).

Pipe-smokers were also analysed separately, as well the whole group as divided according to cancer localisation in the lip or at other sites. The analysis for all localisations together showed a non-significant OR of 1.2 (CI 0.7-1.9) among ever pipe smokers. The group of pipe smokers was also divided into current and ex-smokers. Among current pipe-smokers, significantly increased OR's were produced for all localisations together.
Etiology of Oral Cancer

(OR 2.0, CI 1.1-3.4). For all localisations excluding lip combined, an increased risk was found (OR 3.1, CI 1.3-7.5), whereas no significantly increased risk was found for lip cancer (OR 1.5, CI 0.7-3.1).

**Alcohol**: Alcoholic beverages turned out to be the strongest risk factor for oral cancer in this study. The increased risk was confined to beer (OR 1.9 CI 0.9-3.9), and liquor (OR 1.6 CI 1.1-2.3), Table 1.

Wine consumption overall did not show a statistically-significant increased risk for oral cancer, OR 1.3 (CI 0.9-1.8), but appeared to constitute a risk factor for high consumers. Using a score system taking amount and frequency into account, high consumption gave an OR of 8.6 (CI 1.0-70).

When using the same score system for liquor, significantly increased risks for oral cancer was found among medium and high consumers of liquor. The individuals in the medium group, showed an OR of 1.6 (CI 1.0-2.7) and for those with the highest consumption, an OR of 3.6 (CI 1.8-7.2). When analysing only alive individuals reporting liquor consumption, a decrease in the OR to 1.1 was observed (CI 0.6-1.8).

The score system described for wine and liquor does not correspond exactly to the amount of beverages consumed. Thus, persons who have consumed relatively small amounts daily tend to cumulate larger amounts during lifetime than persons who drink much and seldom. It is unclear whether the frequency of drinking or the total amount consumed is of greatest importance in cancerogenesis. We have also analysed the total volume consumed without considering the frequency of consumption. In this analysis, we found a similar dose-response effect as in the score analyses.

A multivariate analysis was performed considering snuff use, smoking and alcohol. The most important risk factors were beer and liquor consumption.

To further evaluate if there existed any important interaction between the three factors smoking, oral snuff and alcohol consumption, pairwise analyses were done. Smoking tobacco seemed to be a risk factor independent of oral snuff use. Regarding snuff use and liquor consumption the risk decreased with dose of oral snuff in the group with high consumption of alcohol, a tendency not seen in the other alcohol consumption groups. Smoking tobacco and liquor seemed to interact with the highest risk in the highest consumption group of both exposures.
**Table 1.** ORs and 95% CIs for the different variables concerning tobacco and alcoholic consumption, univariate analysis.

<table>
<thead>
<tr>
<th>Exposure factors</th>
<th>Ca/Co</th>
<th>OR</th>
<th>CI (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oral snuff</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>never user of snuff</td>
<td>287/282</td>
<td>1.0</td>
<td>---</td>
</tr>
<tr>
<td>active</td>
<td>39/54</td>
<td>0.7</td>
<td>0.4-1.1</td>
</tr>
<tr>
<td>ex-user</td>
<td>28/18</td>
<td>1.5</td>
<td>0.8-2.9</td>
</tr>
<tr>
<td>ever user</td>
<td>67/72</td>
<td>0.9</td>
<td>0.6-1.4</td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>never smoker</td>
<td>152/171</td>
<td>1.0</td>
<td>---</td>
</tr>
<tr>
<td>active</td>
<td>122/88</td>
<td>1.8</td>
<td>1.1-2.7</td>
</tr>
<tr>
<td>ex-smoker</td>
<td>80/95</td>
<td>1.0</td>
<td>0.6-1.6</td>
</tr>
<tr>
<td>ever smoker</td>
<td>202/183</td>
<td>1.3</td>
<td>0.9-1.9</td>
</tr>
<tr>
<td><strong>Chewing tobacco</strong></td>
<td>5/8</td>
<td>0.6</td>
<td>0.2-2.0</td>
</tr>
<tr>
<td><strong>Light beer</strong></td>
<td>148/120</td>
<td>1.4</td>
<td>1.0-2.0</td>
</tr>
<tr>
<td><strong>Beer</strong></td>
<td>27/16</td>
<td>1.9</td>
<td>0.9-3.9</td>
</tr>
<tr>
<td><strong>Wine</strong></td>
<td>188/168</td>
<td>1.3</td>
<td>0.9-1.8</td>
</tr>
<tr>
<td><strong>Liquor</strong></td>
<td>234/202</td>
<td>1.6</td>
<td>1.1-2.3</td>
</tr>
</tbody>
</table>

*Ca/Co = cases/controls

4.2 Paper II: Oral infections and dental factors in relation to oral cancer - a Swedish case-control study

Oral infections as stated by the subjects were a strong risk factor for oral cancer (OR 3.8, CI 2.1-6.9). In particular, recurrent herpes infection (HSV-1) in the oral cavity and on the lip showed increased risks. Several individuals reported recurrent herpes infection (HSV-1) as the specific type. This motivated a sub-analysis of HSV-1 infections only, which turned out to be a non-significant risk factor, with an OR of 1.9 (CI 0.7-4.5). Furthermore, in 27 subjects the oral infection was highly suspected to be HSV-1, but was not stated specifically. When these were included in the group of certain HSV-1, and re-analysed, the risk increased to a significant OR of 3.3 (CI 1.6-6.5).

Because of the usual localisation of HSV-1 on the lip we made a subanalysis for that localisation, which showed an increased OR for both certain (OR 2.3, CI 0.6-9.1) and probable (OR 4.6, CI 1.7-13) HSV-1 infection.

Neither different kinds of fillings nor fixed prosthesis or removable dentures were associated with increased risks. However, eight cases but no control stated rubbing removable denture in relation to infections, but for this obviously increased risk no OR could be calculated because of no control with such exposure.

Dental X-rays or the use of iron supplements did not produce increased risks either.

Two separate multivariate analysis were performed to evaluate any interactions between different factors of interest. Firstly, infections were analysed with regard to each of the factors smoking, oral snuff and alcohol habits. Regarding smoking and oral snuff, a division was made according to present or former use. In these analyses, infec-
etiology remained an important risk factor of about the same magnitude in all categories, Table 2.

Secondly, oral infections, oral snuff, smoking and liquor consumption were all included in a multivariate analysis, Table 2.

This analysis revealed that oral infections were an independent risk factor, together with liquor consumption. Increased risk was also found for active smoking, although not significant. For oral snuff use no pattern of an association was found.

Table 2. ORs and 95% CIs for the different exposure factors, univariate and multivariate outcome.

<table>
<thead>
<tr>
<th>Exposure factors</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>Cl (95%)</td>
</tr>
<tr>
<td>Oral snuff</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ex-use</td>
<td>1.4</td>
<td>0.7-2.8</td>
</tr>
<tr>
<td>active use</td>
<td>0.6</td>
<td>0.3-1.1</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ex-smoking</td>
<td>1.0</td>
<td>0.6-1.5</td>
</tr>
<tr>
<td>active smoking</td>
<td>1.7</td>
<td>1.0-2.6</td>
</tr>
<tr>
<td>Liquor</td>
<td>1.6</td>
<td>1.1-2.3</td>
</tr>
<tr>
<td>Infections</td>
<td>3.7</td>
<td>2.0-6.8</td>
</tr>
</tbody>
</table>

4.3 Paper III: Occupational exposures as risk factors for oral cancer evaluated in a Swedish case-control study

A significantly increased risk was found for oral cancer among pulp industry workers (OR 4.0, Cl 1.3-12). Smoking and alcohol in this occupational group added to the risk.

An increased risk, (OR 5.5, Cl 1.2-25), was also found for wood or wood products workers. This category is comprised of persons working with wood and wood products within manufactures of furniture/fixtures. Smoking in addition increased the risk. The influence of liquor was not possible to calculate because all individuals reported liquor consumption in the control group.

When analysing exposure to various types of chemicals, phenoxyacetic acids caused an increased risk with OR 1.7 (Cl 0.8-3.5). Mercurial seed dressing yielded an OR of 1.5 (Cl 0.4-5.3). Exposure for impregnating agents did not produce an increased risk, OR 1.1 (Cl 0.5-2.3). Creosote exposure, as well as other specific impregnating agents, were analyzed separately. Exposure to creosote did not produce an increased risk (OR 0.5, Cl 0.1-2.0) but the exposed individuals were very few (3 cases and 6 controls).

No significant differences were found when analyzing socio-economic factors, as defined by the SEI codes, different educational levels, or place of residence.

Figure 1 presents an overview of some of the results from paper I-III.
Figure 1. ORs and 95% CIs for different variables of interest for oral cancer presented in paper I-III, univariate analysis.
**4.4 Paper IV: Expression of p53, PCNA, Ki-67 and bcl-2 in relation to risk factors in oral cancer - a molecular epidemiological study**

*p53 analysis:* Regarding p53 analysis two different methods were used (IHC and PCR/SSCP) each including 114 cases. Seventy-two out of 114 tumours (63%) were IHC positive, and 41 out of 114 tumours (36%) had mutations within exons 5-9. Only infections, and HSV-infections in particular, gave statistically significant ORs for the whole group of tumours as well as for p53 positive tumours only. Results from the PCR analysis showed statistically significant OR for HSV-infections in non-mutated tumours. Since there was a discordance between p53 protein expression and presence of mutation in the p53 gene, we also compared the exposure patterns between subjects with any sign of p53 aberration, i.e. IHC and/or PCR positivity, and subjects lacking both p53 expression and mutation. In this analysis infections and specifically HSV infections gave statistically significant ORs for the group of tumours with p53 aberration shown by IHC and/or PCR. Only very few subjects with exposure to oral infection were negative with both methods. No association between smoking and p53 positivity was found.

The most common single exon mutated was exon 8, with 34 cases in total. We also made an analysis restricted to these cases and their controls. Because of the rather small numbers in different exposure categories analysed only a few ORs could be calculated.

*PCNA analysis:* Due to technical problems only 106 cases were included in the analysis of PCNA expression. Oral infection turned out to be a risk factor for cases with cancers belonging to PCNA group 1 and 2. Regarding smoking, it was observed that ORs decreased with increasing PCNA category, both for active smokers and ex-smokers.

*Ki-67 analysis:* In this IHC analysis 111 cases could be evaluated. Oral infections were significantly associated only in the combined analysis, not in the subgroups. Notably, there was rather an inverse trend regarding smoking between the subgroups as compared with PCNA, with increasing OR with increasing Ki-67 category.

*bcl-2 analysis:* Only 9 cases expressed the bcl-2 protein and therefore this group was not further analysed.

**4.5 Paper V: A non-random deletion in the p53 gene in oral squamous cell carcinoma**

Out of the 80 primary oral cell carcinomas (lip cancer excluded) the majority of the mutations (62%) were found in exon 8. This finding was unexpected and therefore investigated in greater detail. Women were significantly over-represented in group A (=tumours with the 14 bp deletion), 80%, compared with group B (=all other tumours), 47%. The majority of the patients in group A (60%) were over 70 years at diagnosis compared with only 47% in group B. Thirty-three % in group A reported tobacco smoking, compared with 55% in group B.

No correlation was found for the deletion group to the known risk factors for oral cancer such as smoking.
5. General discussion

5.1 Methodological considerations

5.1.1 Epidemiological study design

A case-control study has been defined as "an investigation of the exposure frequencies of at least two groups of subjects selected on the basis of their status with respect to a particular disease entity" (Cole 1979).

The case-control study is particularly suitable for rare diseases, and permits the study of several different exposure factors with potential etiological impact.

Thanks to the population registries and the national cancer registration in Sweden, this country is particularly suitable for population-based case-control studies. The cases in our study were obtained from the Regional Cancer Registry of Northern Sweden. The compulsory reporting system for malignant diseases makes it very likely that practically all incident cases in the study base were included.

In Sweden complete population registries cover the whole population, which permits the use of a control group from the general population, thereby avoiding selection bias. Efficacy in the analyses was increased by using a matched study design controlling for sex, county, year of death and age. Since the response rate was very high, little information was lost in the analysis through the matching procedure.

An important matter concerns the power of the statistical analysis. The power of our tests is the probability that when the alternative hypothesis is true (i.e., increased risk), this will be detected by the test in question. High power is achieved by the design of the experiment (in our case data collection) or by increasing sample size. The consequent conditional (matched) analyses enhances the design but lowers sample size somewhat. Refraining from consequent matched analyses impairs the design but keeps sample size large.

The reason for using dead controls for deceased cases was to ensure a similar quality in data assessment thereby avoiding recall bias (as described in Gordis 1982). However, a potential effect when using dead controls is that a greater proportion in this group might be exposed to life-threatening factors, e.g., smoking, thereby decreasing OR for such factors. Therefore we also analysed the main potential risk factors separately for alive subjects only, i.e., when exposure information were given from the living subjects themselves. This did not lead to significantly different odds ratios for tobacco exposure compared to the whole study, but lower odds ratios were found for alive subjects admitting liquor consumption indicating at least some recall bias for this group.

In case-control studies there is always a possibility of recall bias, e.g. a tendency for the cases to remember or express more hazardous exposures than controls. In order to reduce this risk the aim of the study was concealed and no mention of the disease under study was made to the subjects.

There were no differences in information letters or questionnaires sent to cases or to controls. All non-respondents were sent similar reminder letters, with no difference in the effort to obtain data from cases or controls.

The study has had a "blind" design, e.g., the interviewer did not know if the subject was a case or a control, to minimise any potential observational bias during telephone interviews. Furthermore, the final coding of the answers was also done blind in this respect.

Since the number of cases available for this study was rather high, and the cost to assess
data from cases and controls did not differ, it was decided to choose one control for each case.

5.1.2 Data analysis
For description of results the variables were expressed in categorical forms and the results are presented as the odds ratio (OR) and 95% confidence interval (CI) in the particular category compared with the reference category. In the last paper probability values have been used but otherwise p-values have been avoided because of their limitations in relation to negative or nearly negative results (Ahlbom et al., 1990).

In the univariate and the multivariate analyses, conditional logistic regression was used. The multivariate analyses in paper I-III were performed to control for confounding factors and to evaluate any interactions between different factors of interest.

5.1.3 Molecular biology
Formaldehyde fixed and paraffin embedded samples were used. The effect of formaldehyde fixation on human DNA has been extensively studied (Haeselkorn et al., 1960, McGhee et al., 1977, Karlsen et al., 1994) showing formation of DNA-protein cross-linking (Freifelder et al., 1963), which is almost reversible by the use of enzymes such as proteinase K (Karlsen et al., 1994). An advantage with paraffin embedded material for extraction of DNA is the possibility to cut away normal tissue before sectioning the sample for DNA extraction (Neubauer et al., 1992).

In this study, we have correlated different exposure factors to the expression of p53 protein and compared these results with the outcome from gene mutation analysis with correlation to the same exposure factors, i.e., the outcome of two different techniques compared with same exposure factors. The discrepancy in results will be discussed below.

5.2 Implications

5.2.1 Oral snuff
Oral snuff was not found to be a risk factor for oral cancer in this study. Former snuff users showed a tendency for an increased risk, compared with active snuff users who had instead a decreased risk. These results were confirmed when analysis was done including only active or former snuff users who never had smoked. An analysis was also performed for only living snuff users. The OR for active use decreased further and increased for ex-users. This rather paradoxical finding for ex-users was also found by Lewin et al. (1998). The possibility that ex-snuff users tend to be current smokers was considered. However, only 1 person had begun smoking after having quit using snuff so this explanation does not seem likely. Another possible explanation could be that this group of former snuff users consists of persons who experienced mucosal problems and perhaps pre-malignant changes while using snuff and therefore stopped the habit. A further possibility could be that ex-users of snuff had used more snuff before they quit the habit. However, this hypothesis was not confirmed in separate analyses.

When combining lip cancer with other oral cancer localisations it is possible to obscure the risk factors for either type. To avoid this we made separate analyses of the different cancer localisations in the oral cavity. An increased risk was found for lip cancer among ex-snuff users, however this was not significant. For current snuff users the risk was close to unity.

The results from this study leads inevitably to the question whether snuff is a more healthy alternative to smoking. From a cancer perspective the question must be answered with a “yes”. However, according to studies on other health effects from snuff use the answer probably is “no”. Nicotine is an addictive drug and use of oral snuff or smoking tobacco leads to chemical addiction. The average blood nicotine levels are similar in habitual smokers and smokeless
tobacco users (Gritz et al., 1981; Rusell et al., 1981 Benowitz et al., 1988). Users of smokeless tobacco seem, however, to absorb a larger total amount of nicotine through the gastrointestinal mucosa by swallowing, followed by a first-pass metabolism of nicotine to pharmacologically inactive metabolites in the liver (Benowitz et al., 1989, Holm et al., 1992). This results in equal levels of blood nicotine, but significantly higher levels of cotinine in smokeless tobacco users (Bolinder 1997). Smokeless tobacco users tend to keep their nicotine blood levels constant by changing the quid regularly during the day, and a number of subjects also use it during sleep (Bolinder 1997). Cessation of smokeless tobacco use is, in many subjects, even more difficult than cessation of smoking (Bolinder 1997). The average usage of smokeless tobacco in Sweden is 13 hours per day (Andersson et al., 1994). Exposure to nicotine leads to continuous circulatory stress which is associated with an increase in cardiovascular events, compared with non-users of tobacco (Bolinder 1997).

Risk factors are statistical associations. The matter of assessing health risks results in obvious information problems. It is evident that smoking involves far more health hazards than use of smokeless tobacco but there is a risk that a young snuff user becomes a smoker later in life and the health hazard with smoking is substantial.

The difference in results between American and Swedish studies regarding snuff use is evident and has been discussed extensively. One possible explanation is differences in manufacturing processes which result in different concentrations of tobacco-specific N-nitrosamines (TSNA). The concentration of nitrosamines in both types of snuff were analysed in 1980, and much higher concentrations of TSNA were found in American snuff (up to 18-fold higher) compared with Swedish snuff (IARC 1985). This difference, as well as 1.5-2-fold higher concentration of nicotine in American snuff (Djordjevic et al., 1993), may explain why our results differ from American studies on cancer risk from smokeless tobacco use.

5.2.2 Smoking
Our study confirmed earlier findings of smoking as a clear risk factor for oral cancer. The risk was however rather low compared with other studies from Europe, e.g., in Italy where much higher risk compared with our results have been found (Franceschi et al., 1992). Perhaps the difference lies in different drinking and smoking habits, different types of cigarettes or perhaps even genetic differences.

In this study we actually found that ex-smokers (individuals who had quit the habit at least one year before diagnosis) did not have an increased risk for oral cancer which leads us to conclude that quitting smoking results in a rapid and clear decrease in risk within one year. This short cut-off time is in contrast to the finding in a study from Florida (Stockwell et al., 1986) but in line with other studies, however (Blot et al., 1988, Mashberg et al., 1993).

5.2.3 Alcohol
Alcohol showed the strongest association with oral cancer in this study. When analysing different kinds of alcoholic beverages, liquor produced the highest OR. When evaluating the importance of interaction, it appeared that the combination of smoking and liquor consumption revealed the highest risk in the highest consumption group of both exposures. Wine drinking appeared to be a risk factor only in the high consumption group which is in line with other studies in the field (e.g. Franceschi et al., 1992).

5.2.4 Infections
The results in this study gives strong support to experimental studies indicating infectious agents to be involved in the carcinogenesis of oral cancer. Our findings concerning HSV-infections and especially the combination HSV-infection and smoking in relation to oral cancer is indeed striking. Both HSV and tobacco have carcinogenic capacity. Exposure to the combination of HSV and smokeless tobacco use results in a significant increase of tu-
mourn development in rats (Larsson et al., 1989). Serum IgA antibodies to HSV-1-induced antigens are increased in smokers, suggesting that cigarette smoking might lead to a predisposition to mucosal HSV infection (Smith 1996). The distinction between an etiologic association of a virus with a tumor and a secondary (passenger) association is difficult to establish (Scully 1983). However, the regular presence of the viral genome or viral products in a tumor, irrespective of geography, is regarded as fairly conclusive evidence of viral oncogenesis (Klein 1971). Most likely complex interactions between tobacco chemicals and HSV are involved in the tumorigenic mechanisms (Larsson 1990).

Further studies are needed to clearly establish the association in oral squamous cell carcinoma and if further studies verify our findings of a relation between HSV-infections and oral cancer this will raise the question of early antiviral treatment as standard for HSV-infection.

5.2.5 Dental factors
Sweden is a country with quite a long history of general socialised dental health care. As early as 1938, the Swedish government instituted community responsible dental health care, especially for children and youths. As a result, dental health has improved in all aspects (Hugosson et al., 1991). Thus, it was impossible to confirm a protective effect by dental care in this material.

5.2.6 Occupational factors
Concerning the risk for oral cancer in different occupational groups this investigation was made without any a priori hypotheses. Since there were 101 different occupations reported in this study and two occupations yielded significantly increased risks, the findings must be interpreted with caution and regarded as hypothesis generating rather than indicative. However, the findings for pulp workers and wood or wood product workers are interesting especially as they are in line with earlier studies (Lanes et al., 1990, Huebner et al., 1992).

5.2.7 Other exposures
The increased risk for oral cancer after exposure to phenoxy herbicides in this study is in accordance with a German study (Becher et al., 1996) where workers from factories involved in the production of phenoxyacetic acid herbicides and the related chlorophenols were investigated. In earlier studies from Sweden exposure to 2,4-D and 2,4,5-T has been associated with increased risk for soft-tissue sarcoma and non-Hodgkin lymphoma, findings later verified in other countries (Hardell et al., 1994). Almost all of the exposed cases and controls in this study had sprayed in forestry to control hardwood during the 50’s and 60’s with a combination of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-dichlorophenoxyacetic acid (2,4-D). This combination was known as Agent Orange during the Vietnam war.

5.3 Molecular correlations
The lack of association between results from IHC detection of p53 protein and mutation analysis of the p53 gene might be explainable in several ways, e.g., that mutations were present outside the regions analysed (exon 4-9), that the immunopositive but mutation negative tumours contained wild type protein retained in the tissue by binding to other proteins or that there could be a defect in degradation of the p53 protein. The immunonegative but mutation positive tumours on the other hand, showed by sequencing different nonsense mutations causing synthesis of non-functional proteins. This discrepancy between immunoreactivity for the p53 protein and presence of mutations in the p53 gene has been discussed in other studies and in the literature there are studies showing good correlations (Ahomadegbe 1995) as well as a lack thereof (Nylander et al., 1995b, Xu et al., 1998).

In the literature there are several studies reporting data concerning expression of p53 (IHC and mutation analysis) related to tobacco and alcohol exposure but very little is known about the risk of tumour development after other kinds of exposures in relation to
p53 status. In this study we found interesting correlations regarding HSV-1 and oral cancer, with an increased risk for tumours expressing p53 protein as demonstrated by IHC in patients with HSV infection. No increased risk was seen in patients with HSV infection and tumours with a p53 mutation shown by PCR. The most probable interpretation is that HSV-1 in some way inactivates p53 function by binding to the wild type protein thereby allowing detection with IHC, whereas no influence on the gene status was detected. Further studies on this condition may increase our knowledge on the pathogenetic role of HSV-1 in cancer.

The finding of a new random deletion was surprising. The validity of this finding was confirmed by extensive control experiments. Of patients with the deletion, 80% were women, and there was a tendency for later onset of the disease. In order to characterise this group of patients as thoroughly as possible, exposure to different factors was investigated. There is, to our knowledge, no scientific basis for the association between dental treatment and p53 mutation, but the deletion group differed significantly from the other patients concerning filling material. Our data for this group furthermore implicated a possible independence of known risk factors for SCCHN such as smoking. It seems like there might be an unknown exposure factor leading to this specific mutation, that might be of importance for older women in particular.
6. Summary

This epidemiological investigation dealt with risk factors for oral cancer. The importance of each suspected etiological risk factor was evaluated in a population-based case-control study in northern Sweden. In addition, two molecular epidemiological studies were performed, focused on the relationship between exposure factors and tumour biological parameters.

The findings and conclusions from the studies are summarised below:

• Oral snuff was not found to be a risk factor for oral cancer.

• A non-significant increased risk for lip cancer was found for snuff users when different tumour sites were analysed separately.

• Smoking and alcohol consumption were verified as risk factors.

• The results strongly indicated recurrent HSV-1 infections as an independent causative factor in oral cancer, especially when the infection was localised on the lip.

• Dental factors like different kind of fillings, fixed prostheses or removable dentures were not associated with increased risks. Dental X-rays did not produce an increased risk either.

• Pulp industry workers and wood or wood product workers were found to have an increased risk for oral cancer. Smoking and alcohol consumption increased the risk further.

• Exposure to phenoxyacetic acids indicated an increased risk.

• Oral infections, and HSV-1 infections in particular, were associated with an increased risk for oral cancer in the p53 IHC positive group, but not in the p53 mutation group. This suggests that HSV-1 infection, directly or indirectly, can inactivate p53 function by binding of wild type protein.

• No association between smoking and p53 positive tumours was found.

• A new non-random deletion in exon 8 in oral SSC was found. The cause and clinical significance of this new 14 bp deletion is so far not known. Of the patients with the deletion 80% were women. No correlation was found for the deletion group with the known risk factors for oral cancer such as smoking.
7. Summary in Swedish

**Orsaker till cancer i munhålan**

Cancer i munhålan är en ovanlig cancerform i Sverige. Den utgjorde 1995 1,4% av all cancer hos män, och 0,8% hos kvinnor (Socialstyrelsen 1995). Sjukdomen är vanligast i Frankrike och Indien där antalet individer som nyinsjuknar ligger på ca 20-50 / 100.000 invånare årligen. Den har en dålig prognos och ca hälften av alla som insjuknar dör i sjukdomen trots behandling. Antalet fall som insjuknar varje år ökar nu i större delen av västvärlden, så även i Sverige. Detta faktum förbryllar eftersom rökning, som är en av de starkaste riskfaktorerna, minskar. En annan känd riskfaktor för cancer i munhålan är alkoholbruk. Andra riskfaktorers betydelse är mindre väl belysta. Snusets betydelse för uppkomst av munålecancer är mycket omdiskuterat.

Syftet med denna avhandling har varit att försöka belysa olika potentiella riskfaktorers betydelse för cancer i munhålan, liksom eventuell interaktion mellan dessa faktorer.

De första tre delarbetena utgår från en och samma fall-kontrollstudie, medan resultaten i delarbete IV och V utgår från delmaterial.

**Avhandlingens fem delarbeten**

**I: Snus, rökning och alkoholbruk i relation till munhålecancer.**


**Slutsats:** Någon överskatt för munhålecancer hos snusare kunde inte påvisas. Våra resultat styrker tidigare resultat beträffande rökning och alkoholanvändning som riskfaktorer för munhålecancer.
II: Infektioner i munhålan och tandvårdsfaktorer i relation till munhålecan­
cer.


I de vidare analyserna av fall-kontrollstudien beskriven i delarbete I har vi analyserat andra riskfaktorer för munhålecan­
cer än tobak och alkohol. I detta delarbete har infektioner i munnen, tandproteser, tandbrygggor, tand­
sten, olika typer av tandfyllningar samt rönt­
gen av tänderna studerats. Resultaten visar ett statistiskt signifikant samband mellan återkommande infektioner, såväl i munhålan som på läppen, och munhålecan­
cer (oddskwot 3,8). Separata analyser utfördes för de individer som kunde rapportera anam­
nestiskt säkra eller mycket sannolika her­
pesinfektioner i munnen eller på läppen. För gruppen säkra herpesinfektioner blev risken signifikant förhöjd med oddskwot 1,9 och för gruppen säkra och sannolika herpesin­
fektioner var oddskwoten 3,3. Infektioner analyser­
rades även med hänsyn tagen till samtidig tobak/alkoholanvändning. Tandproteser, tandbrygggor eller olika tandfyllningar gav inga överrisker för munhålecan­
cer, ej heller upprepad röntgen av munhålan.

Slutsats: Denna studie indikerar starkt att återkommande herpesinfektioner i munhålan och på läppen är en oberoende riskfaktor för cancerutveckling i munhålan och framför allt när infektionen är belägen på läppen.

III: Yrkesexponering som riskfaktor för munhålecan­
cer.

Schildt E-B., Eriksson M., Hardell L., Magnusson A. Occupational exposures as risk factors for oral cancer evaluated in a Swedish case-control study. (submitted).

I detta delarbete undersöktes risken för can­
cer i munhålan hos olika yrkesgrupper liks­
om olika yrkesexponeringar, bl a lösnings­
medel, bekämpningsmedel, plastkemikalier, asbest mm. Vi beaktade även socioekono­
miska faktorer liksom boendesortens/arbetsortens betydelse, men ingetdra­
var av betydelse för munhålecan­
cer. Vi fann ökad risk för munhålecan­
cer hos pappers­
massearbetare och verkstadssnickare (oddskwot 4,0 respektive 5,5). Bland olika kemiska medel märktes en ökad men ej sig­
nifikant risk för de individer som var expo­
erade för fenoxisyror, en typ av ogräsbe­
kämpningsmedel (oddskwot 1,7).

Slutsats: De positiva fynden i denna studie kan förklaras av exponering för olika kemi­
kalier men på grund av få ingående fall och kontroller i vissa grupper och samvariation med bl a tobak och alkohol måste resultaten tolkas med stor försiktighet.


V: En icke slumpmässig deletion i p53-genen vid munhålecancer.


I ett delmaterial av fall- kontrollstudien har 80 fall av munhålecancer undersökt avseende korrelation mellan exponeringsfaktorer undersökta i arbete I, II och III och förekomst av en oväntad och tidigare ej beskrev en specifik deletion (förlust av kromosomdel) i p53-genen i denna grupp av cancerfall. p53-genen undersöks med mutationalys (PCR/SSCP) och exon 5-9 amplifierades med s.k. primers. Majoriteten av mutationerna (62%) var belägna i exon 8. Detta var oväntat och undersöks därför vidare. 17 av de 80 inkluderade tumörerna uppvisade den specifika deletion. kvinnor var överrepresenterade, 80%, jämfört med 47% för gruppen tumörer utan deletion.

Slutsats: Vi har beskrivit en ny oväntad ej slumpmässig deletion i exon 8 i p53-genen hos en grupp av de inga cancerfallen. Orsaken och värdet av detta fynd kan vi ännu ej bedöma. Ingen korrelation kunde påvisas för tumörgen i relation och kända riskfaktorer för munhålecancer som t.ex röknings.
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