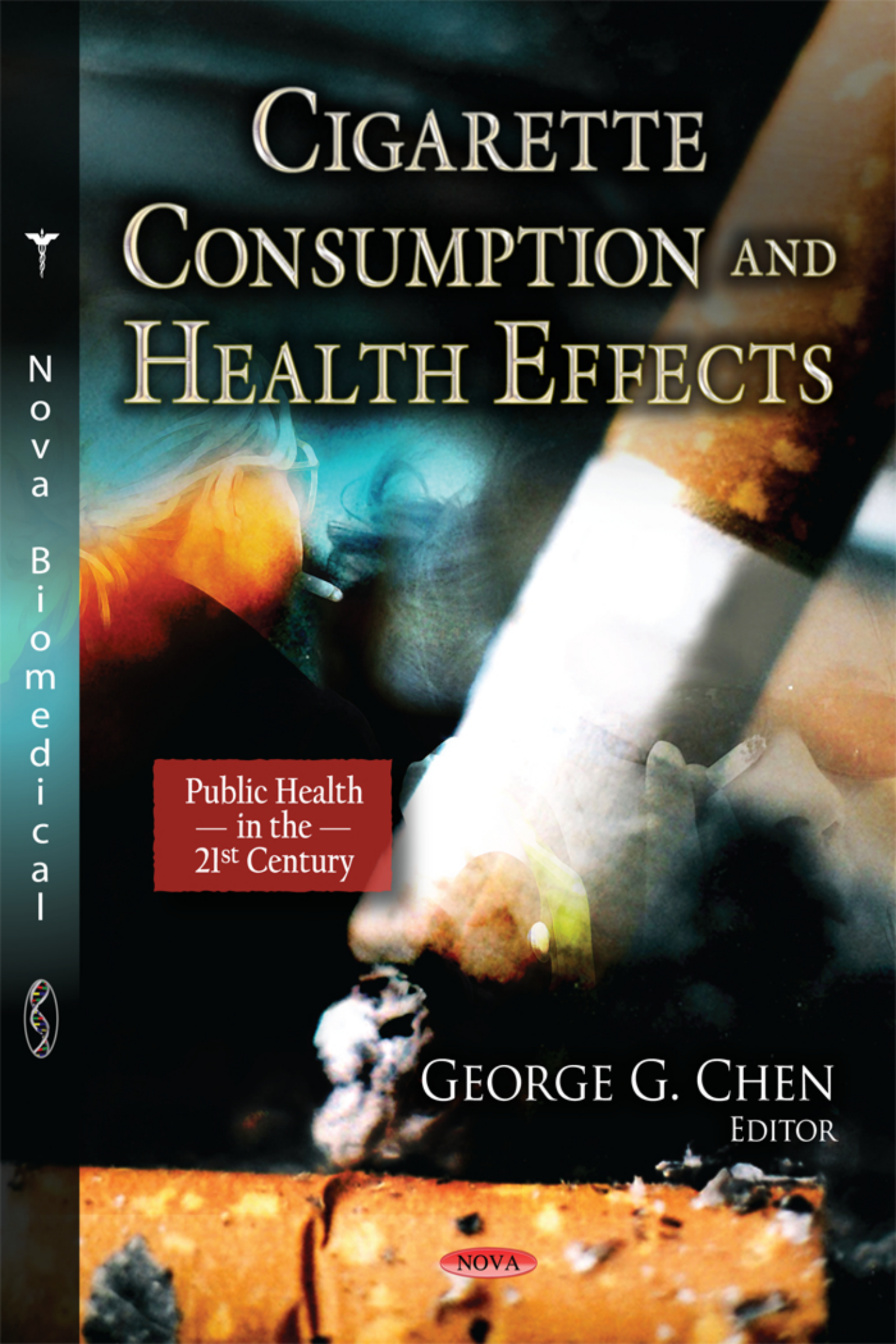
Nova Biomedical
Cigarette Consumption and Health Effects
Public Health — in the — 21st Century
George G. Chen
Editor
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PUBLIC HEALTH IN THE 21ST CENTURY

CIGARETTE CONSUMPTION AND HEALTH EFFECTS

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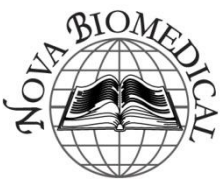

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Contents

Preface

The Impact of Cigarette Smoking on Health, an Unfolding Battle

An association between cigarette smoking and the health hazards has been scientifically confirmed for decades. However, it is astonished that today cigarette smoking still kills approximately 5 - 6 million people annually worldwide and furthermore, it is estimated that the number of annual deaths due to smoking will rise to around 10 million by 2030 [1]. People may puzzle - why cigarette smoking, a preventable risk to death can continue to be a major contributor to morbidity and mortality from cancers, cardiovascular and respiratory diseases. Factors that enable this most serious pandemic to continue are multiple, involving social, political, commercial and scientific aspects. For example, in some societies, the practices of gifting and sharing cigarettes have historical and cultural roots which stubbornly influence tobacco control efforts [2]. From the scientific point of view, academic studies on the relationship between cigarettes and health effects still face a lot of unclear and unanswered questions, uncovering which should provide solid and scientific evidence for policy agencies to make better measurements to control cigarette production, sale and smoking, for the public to understand the risk associated with cigarette smoking, and for clinics to offer effective treatments against the diseases associated with cigarette smoking.

The cigarette smoking can cause a broad spectrum of diseases that extend to virtually every organs and systems in humans. Although the cardiovascular disorders, pulmonary diseases and cancers are the major subjects of study [3-5], research on the cigarette smoking and the health hazards has extended to other areas, for example, sex hormones and hearing system [6,7]. Obviously, it is beyond a single author to address adequately the complexity of the harmful health effects induced by cigarette smoking. We have therefore opted for a team of scholars around the world, each an expert in his/her own field, to present this volume of the book with 12 chapters to address cigarette consumption and health effects. It is my hope that the experience of our contributors, as imparted in the pages of this book, will assist policy makers, investigators, and the public by providing a source of information to which they may refer when dealing with cigarette smoking issues.

I wish to express my gratitude to all those who have shown their supports in the publication of this book, particularly the contributors who have devoted their great efforts, patience and tolerance in the face of an extensive amount of preparing and editing. I would

also like to thank Ms Christina Lou who has provided excellent secretary work to make this publication possible.

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Chapter 1

Tobacco Smoke and Breast Cancer Risk: Rapid Evolution of Evidence and Understanding in the Early 21st Century

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Abstract

Over the last decade more than 35 new epidemiologic studies and a dozen meta-analyses and reviews have been published on the relationship between tobacco smoke and breast cancer risk. This broad new evidence base and its implications present a paradigm shift away from the interpretation of earlier epidemiologic evidence that there was little relationship between smoking and breast cancer. In 2009 a Canadian expert panel was the first to conclude that the weight of evidence from epidemiological and toxicological studies, and understanding of biological mechanisms, was consistent with a causal relationship between active smoking and breast cancer. The key epidemiologic evidence came from: 1) the eight large, high-quality cohort studies with detailed active smoking metrics, that suggested early age of smoking commencement, longer duration of smoking before first full-term pregnancy, higher lifetime pack-years and longer duration of smoking were associated with increased breast cancer risks of 15 to 40%; and 2) three recent meta-analyses which reported 35% to 50% increases in breast cancer risk for long-term smokers with *N-acetyltransferase 2* (*NAT2*) slow acetylation genotypes. In 2011, in the largest cohort analysis to date (8,772 breast cancer cases) with the most precise analysis of active smoking risk, researchers reported strong dose-response evidence that the critical active smoking exposure period was from menarche to the first birth and that breast cancer risk was limited for smoking after the first childbirth. The interpretation of evidence linking secondhand smoke (SHS) and breast cancer risk has been particularly controversial — three major reviews of SHS and breast cancer published between 2004 and 2006 each came to a different conclusion. Among younger, primarily premenopausal women, increased breast cancer risk was consistently observed in six case-control studies

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with comprehensive lifetime measures of SHS exposure, but generally not in studies with less comprehensive SHS exposure assessment. Studies since 2006 have continued in the same pattern. The first two cohort studies with comprehensive lifetime measures of SHS were recently published: higher levels of lifetime SHS exposure were associated with estimated 26% and 32% increases in postmenopausal breast cancer risk. The WHO Framework Convention on Tobacco Control calls for more education, communication, and increasing of public awareness about the dangers of tobacco as part of comprehensive tobacco control. Communicating the new understanding of the breast cancer risks associated with active smoking and SHS, especially to girls and young women, should be an important component of that plan.

Introduction

More than 120 studies have been published that provide estimations of risk of breast cancer associated with active smoking or secondhand smoke (SHS).[1] In many of the studies of active smoking published prior to the year 2000, the analysis was limited to risks associated with ever/never smoking, ex/current smoker and in some, number of cigarettes per day and/or age of smoking initiation.[2]

More recent studies have generally included reporting of risks associated with age of initiation of smoking, duration of smoking before first pregnancy, total years and/or total pack-years of smoking, allowing for much more precise risk estimation.[1] In studies of breast cancer and SHS, also referred to as passive smoking, involuntary smoking, and environmental tobacco smoke (ETS), the metrics have varied widely. Studies with more comprehensive SHS measures have facilitated more precise estimates of SHS-breast cancer risk. This article examines breast cancer risk, first for active smoking and then for passive smoking, demonstrating the strong impact of improving exposure precision on observation and understanding of risk.

A. Active Smoking and Breast Cancer Risk

There have been a number of reviews of smoking and breast cancer over the last two decades. Palmer and Rosenberg[3] reviewed 50 studies published up until 1993, but found only five cohort and 10 case-control studies that met basic quality criteria. The results of the better-quality studies were equivocal, and the authors found little evidence that cigarette smoking materially increased breast cancer risk based on risks reported for ever smoking, current smoking or heavy smoking.[3]

In 2002 Terry and Rohan reviewed the much-expanded literature and concluded that there may be an increased breast cancer risk with smoking of long duration, smoking before a first full-term pregnancy and passive smoking.[2] Major reports by the International Agency for Research on Cancer (IARC) and United States Surgeon General's office also reviewed the active smoking evidence published up until about 2002.[4,5] Between 2002 and 2008 at least 30 more original epidemiologic studies and at least 10 meta-analyses[6-15] as well as two major government reports on passive smoking[16,17] were published.

To consolidate the extensive new evidence base, in 2008 a Canadian expert panel was struck by four Canadian health agencies and charged with the task of providing an up-to-date synthesis of evidence of active and passive smoking and breast cancer risk. In their 2009 report, the Canadian Expert Panel on Tobacco Smoke and Breast Cancer Risk summarized breast cancer risks observed for smoking metrics beyond ever/never smoker and current/ex-smoker for cohort studies and for case-control studies.[1,18] Key epidemiologic findings focused on the fact that the eight cohort studies with more precise smoking metrics[19-26] consistently observed small elevations in risk associated with earlier age at smoking initiation, longer duration of smoking before first full-term pregnancy, and for longer lifetime duration and pack-years of smoking.[18,27] (See Table 1.1.) More recently Luo *et al.* published similar findings from the Women's Health Initiative Cohort[27] and Xue *et al.* found similar results for an updated analysis of the Harvard Nurses' Health Study.[28] (See Table 1.1.) Egan *et al.* had originally published on the Nurses' Health Study cohort in 2002.[20]

The many case-control studies examining the same metrics demonstrated a much less consistent risk pattern, with some studies reporting increased risk, some no risk and a few reduced risk.[1] Given the consistency of the cohort studies in suggesting increased risk, the case-control pattern suggests that some of the case-control studies suffer from recall and/or response bias which is likely attenuating risks. [1,18]

Smoking Risk before First Birth Appears to Be the Critical Window for Exposure

An analysis by Ha *et al.*[26] published in 2007 of 906 incident breast cancer cases from a US nationwide cohort of 56,042 radiologic technologists, was the first to closely examine risks associated with smoking in different periods of reproductive life. Among parous women Ha *et al.* found a statistically significant dose-response relationship between the number of pack-years of smoking between menarche and first childbirth and breast cancer risk. Risk increased an average of 3% per pack-year of smoking (RR = 1.03 95% CI 1.02-1.05). (See Figure 1.1.)

An independent effect of age at smoking initiation (after adjustment for pack-years of smoking before first childbirth) was also observed with RR's compared to never smokers of 0.97, 1.09, 1.19 and 1.48 (95% CI 0.77-2.84) for smoking initiation at age >20, 18-20, 15-17 and <15 (*p* for trend 0.06). For cigarettes smoked after first childbirth, risk actually decreased with increasing pack-years although the trend was not statistically significant (Figure 1.1.).

An extended analysis of the Harvard Nurses' Health Study cohort published in 2011[28] provides the best epidemiologic evidence to date suggesting that the critical period of smoking exposure is the time before first childbirth. Xue *et al.*'s analysis is the largest cohort analysis to date (8,772 incident cases of invasive breast cancer) and likely has the most thorough assessment of active smoking history of any of the cohort studies, as the participants were asked about their smoking status every two years throughout the 30 year follow-up period (1976 to 2006). The study goes a long way towards disentangling the overlapping measures suggesting increased risk in other cohort studies for initiation age, smoking before first full-term pregnancy, total smoking duration and total pack-years of smoking.

Table 1.1. Cohort Studies (>500 cases*) of Active Smoking and Breast Cancer Risk by Highest Exposure Categories

First author, year	Years of data collection	No. of incident cases/ no. in cohort	Age range at enrollment (years)	Youngest age[1] of initiation RR (95% CI)	Longest duration before[2] first full-term pregnancy RR (95% CI)	Longest duration[3] RR (95% CI)	Highest pack-years[4] RR (95% CI)
Calle (1994)[5]	1982-1986	800 (deaths)/ 604,412	30-70+	1.59 (1.17-2.15)			1.38 (1.05-1.83)
Al-Delaimy (2004)	1989-1999	1,009/ 112,844	25-42	1.29 (0.97-1.71)	1.10 (0.80-1.52)	1.21 (1.01-1.45)	
Reynolds (2004)	1995-2000	2,005/ 116,544	<75+	1.17 (1.05-1.30)	1.13 (1.00-1.25)	1.15 (1.00-1.33)	1.25 (1.06-1.47)
Gram (2005)	1991-2000	1,240/ 102,098	30-50	1.48[6] (1.03-2.13)	1.27 (1.07-1.37)	1.36 (1.06-1.74)	1.46 (1.11-1.93)
Olson (2005)	1986-1999	2,017/ 41,836	55-69	1.12 (0.92-1.36)	1.21 (1.01-1.25)	1.18 (1.00-1.38)	1.15 (0.96-1.37)
Cui (2006)[7]	1980-2000	4,445/89,835	40-59	1.11 (0.97-1.28)	1.13 (1.01-1.25)	1.50 (1.19-1.89)	1.17 (1.02-1.34)
Ha (2007)	1983-1998	906/ 56,042	22-92	1.48 (0.77-2.84)	1.78 (1.27-2.49)[8]		
Luo (2011)	1993-2009	3,520/ 79,990	50-79	1.12 (0.92- 1.36)	1.21 (1.11-1.33)[9]	1.35 (1.03-1.77)	1.18 (1.02-1.37)
Xue (2011)	1976-2006	8,772/ 111,140	30-55	1.04 (0.98-1.09)	1.25 (1.11-1.40)[10]	1.15 (1.04-1.27)	1.27 (1.16-1.38)

Source: Adapted and expanded from the Canadian Expert Panel on Tobacco Smoke and Breast Cancer Risk [1,18].

All relative risks and 95% confidence intervals [RR (95% CI)] are relative to never (active) smokers unless indicated otherwise.

* Goodman *et al.* (1997), Lawlor (2004) and Lin (2008) are not reported here because of small numbers of observed cases resulting in unstable risks and low statistical power (Goodman: only 21 smokers among the 156 breast cancer cases and Lin only 12 ever smokers among 208 breast cancer cases); Lawlor (2004) reported only on timing of smoking relative to first birth (only 45 smokers before first birth among 139 breast cancer cases who gave birth and reported age at first birth).

[1] All risk estimates based on young women starting at age <20 years; cutoff varied from <15 to <20 depending on the study.

[2] All risk estimates based on smoking ≥5 years before first birth; except Gram (2005) and Olson (2005) where years not reported.

[3] All risk estimates based on smoking >20 years; with most women smoking >40 years.

[4] All risk estimates based on smoking >10 pack-years; with most women smoking >40 pack-years.

[5] The endpoint examined in this one cohort study was breast cancer mortality.

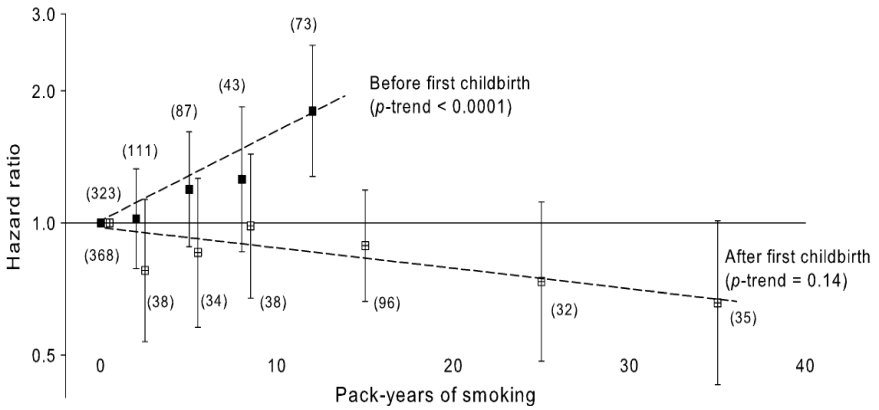
[6] Risk estimate based on ever smokers, who smoked 20+ years and started smoking at 10-14 years.

[7] Extended follow-up for same cohort as Terry (2002).

[8] Risk estimate based on 10+ pack-years of smoking before first childbirth after adjusting for smoking after first birth and other covariates, compared with not smoking before. The trend for smoking before first birth remained significant after additionally adjusting for age at smoking initiation.

[9] Risk estimate is for all women who started to smoke before their first full-term pregnancy. Comparison is among the 69,533 women who had at least one full term pregnancy.

[10] Risk estimate based on ≥16 pack-years of smoking from menarche to before first birth, after adjusting for smoking in other life periods and 13 other risk factors.



Source: Ha *et al.*[26]

Figure 1.1. Breast cancer risk by pack-years of smoking before and after first childbirth among parous women, US Radiologic Technologists Health Study, 1983-1998. Hazard ratios and 95% confidence intervals were estimated from one multivariate proportional hazards model including separate variables for pack-years of smoking before and after first childbirth, with age as the time scale, stratified for birth cohort in 5-year intervals and adjusted for alcohol intake, age at menarche, age at first childbirth, parity, family history of breast cancer, hormone replacement therapy, year that a woman first worked as a radiologic technologist, body mass index, and time-dependent menopausal status. The numbers of breast cancer cases in each category are provided in parentheses.

It overcomes "well-recognized limitations of most cohort studies including cohort size, duration of follow-up, and number of incident cases, which may reduce statistical power and limit the availability of covariate data and the opportunity to update information for time-dependent exposures and confounders."[5]

The Nurses' Health Study analysis controlled for known risk factors and for smoking in other time periods, thus isolating the pre-pregnancy risk. The authors reported increasing adjusted relative risks, each statistically significant, of 1.11 (95% CI 1.04-1.20), 1.19 (95% CI 1.09-1.30), 1.21(95% CI 1.07-1.36) and 1.25 (95% CI 1.11-1.40), for 1-5, 6-10, 11-15 and ≥16 pack-years of smoking before first birth (p for trend <0.001). (See Table 1.2, a reproduction of part of Xue *et al.*'s Table 4.) The Harvard Nurses' Study results are an important replication of the pattern reported by Ha *et al.*[26] that smoking before first childbirth increased risk in a dose-response manner based on pack-years of exposure and that smoking after menopause may be associated with small dose-response reductions in breast cancer risk. These findings may be a key to understanding the apparently erratic results seen in earlier studies of the smoking risks for current and ex-smokers and dose-response results for total pack-years and total duration.

Although the Nurses' Health Study did not find an independent effect for younger age of initiation of smoking similar to that observed in the radiation technologists cohort, the youngest age at smoking initiation cutoff was under age 18 in the Harvard Nurses' Study, whereas the radiation technicians study saw increasing risks with decreasing age of initiation, with the highest risk for women <15. Other cohorts have tended to see higher risk with lower age at initiation,[1] but the other cohorts have not done the analyses controlling for smoking in different reproductive periods as in these two studies, so the role of younger age at smoking initiation remains less certain.

The study by Innes *et al.*[29] provides additional support for a risk for smoking before first birth. Innes *et al.* found that smoking during pregnancy was associated with increased breast cancer risk. Although a small case-control study, the investigation was population-based and the smoking data were from records collected during pregnancy (i.e. prospective exposure assessment).

Table 1.2.

Table 4. Pack-years of Smoking Relevant to Menarche, Age at First Birth, and Menopause in Relation to Incidence of Breast Cancer Among 111 140 Participants in the NHS

Exposure	Person-years	No. of Cases	Hazard Ratio (95% Confidence Interval)		
			Age-Adjusted	Covariate-Adjusted I[a]	Covariate-Adjusted II[b]
Smoking from menarche to before menopause, pack-years					
0	1 377 249	3871	1 [Reference]	1 [Reference]	1 [Reference]
1-10	568 388	1518	1.01 (0.95-1.07)	1.04 (0.98-1.11)	1.01 (0.95-1.08)
11-20	490 136	1412	1.08 (1.01-1.14)	1.16 (1.08-1.24)	1.14 (1.06-1.22)
21-30	365 237	1123	1.05 (0.99-1.13)	1.16 (1.08-1.26)	1.15 (1.07-1.24)
≥31	251 164	891	1.15 (1.07-1.24)	1.28 (1.18-1.39)	1.27 (1.16-1.38)
P for trend			<.001	<.001	<.001
Every increase of 20 pack-years			1.06 (1.03-1.09)	1.11 (1.07-1.15)	1.11 (1.07-1.15)
Smoking from menarche to before first birth, pack-years					
0	1 493 714	4174	1 [Reference]	1 [Reference]	1 [Reference]
1-5	1 014 089	2889	1.04 (0.99-1.09)	1.12 (1.05-1.21)	1.11 (1.04-1.20)
6-10	340 148	1060	1.14 (1.07-1.22)	1.22 (1.12-1.33)	1.19 (1.09-1.30)
11-15	103 217	335	1.18 (1.06-1.32)	1.25 (1.11-1.41)	1.21 (1.07-1.36)
≥16	101 006	357	1.22 (1.10-1.36)	1.30 (1.16-1.46)	1.25 (1.11-1.40)
P for trend			<.001	<.001	<.001
Every increase of 20 pack-years			1.20 (1.12-1.28)	1.22 (1.14-1.31)	1.18 (1.10-1.27)
Smoking after first birth to before menopause, pack-years					
0	1 641 615	4678	1 [Reference]	1 [Reference]	1 [Reference]
1-10	580 834	1563	1.02 (0.96-1.08)	0.95 (0.88-1.04)	0.94 (0.86-1.02)
11-20	448 257	1292	1.04 (0.98-1.11)	0.99 (0.90-1.09)	0.99 (0.90-1.09)
21-30	258 604	848	1.08 (1.00-1.16)	1.04 (0.94-1.16)	1.04 (0.94-1.16)
≥31	122 864	434	1.12 (1.01-1.23)	1.07 (0.94-1.21)	1.05 (0.92-1.19)
P for trend			.005	.05	.05
Every increase of 20 pack-years			1.05 (1.01-1.09)	1.04 (0.98-1.10)	1.04 (0.99-1.10)
Smoking after menopause, pack-years					
0	1 375 058	4946	1 [Reference]	1 [Reference]	1 [Reference]
1-5	256 793	839	1.00 (0.93-1.08)	0.91 (0.84-0.99)	0.94 (0.86-1.02)
6-10	142 069	465	0.94 (0.85-1.03)	0.83 (0.75-0.92)	0.89 (0.80-0.99)
11-15	87 360	313	0.96 (0.85-1.07)	0.83 (0.73-0.94)	0.90 (0.79-1.02)
≥16	111 990	424	0.94 (0.85-1.03)	0.81 (0.73-0.91)	0.88 (0.79-0.99)
P for trend			.08	<.001	.02
Every increase of 20 pack-years			0.96 (0.89-1.03)	0.87 (0.79-0.95)	0.93 (0.85-1.02)

Source: Xue *et al.* [28]

[a]In the assessment of smoking during a specific life period, smoking during the other life periods was adjusted for.

[b]Additionally adjusted for family history of breast cancer, history of benign breast disease, age at menarche, age at first birth, parity, oral contraceptive use, height, current body mass index, body mass index at age 18 years, physical activity, alcohol consumption, passive smoking status at home, and passive smoking status at work. Parity and age at first birth were not adjusted for in the analysis of smoking from menarche to before first or in the analysis of smoking after first birth to before menopause. Postmenopausal HT use was adjusted for in the analysis of smoking after menopause.

Smoking before First Birth and Breast Biology and Toxicology

Roo *et al.*[30] provide a succinct summary of the related breast biology and toxicology:

> "According to studies on breast development and cancer susceptibility, the relatively undifferentiated breast epithelial cells present before a first pregnancy may be particularly vulnerable to the carcinogenic effects of cigarette smoke.[31,32] Animal models have shown that cancer initiation can occur when chemical carcinogens come into

> contact with undifferentiated, highly proliferating mammary epithelium and is less likely after a full-term pregnancy, during which the mammary gland undergoes differentiation. In humans, the mammary gland is composed of developing lobules at menarche, and a first pregnancy and lactation trigger breast growth and differentiation.[32]" [30]

A more detailed and technical summary of the toxicology and breast biology as well as a list of the 20 tobacco smoke carcinogens suspected of being mammary carcinogens are provided in the Canadian Expert Report.[1]

Smoking Only before First Pregnancy Versus Only after First Pregnancy

A recent meta-analysis of active smoking before first pregnancy published by De Roo *et al.*[30] took a slightly different tack, focusing on women who smoked only before pregnancy compared to those who smoked only after first pregnancy. They reported a risk for only smoking before first pregnancy of 1.07 (95% CI 0.93-1.23) (5 studies) and a risk for smoking only after first pregnancy also of 1.07 (95% CI 0.99-1.15) (16 studies). The authors concluded that comparing ever smoking only before first pregnancy with ever smoking only after first pregnancy "provided no evidence that breast tissue is more susceptible to malignant transformation from smoking before the first pregnancy".[30] A closer examination, however, of the meta-analysis evidence and the Nurses' Cohort Study results (published after the meta-analysis), suggests that smoking before first pregnancy is likely the time of particular risk, consistent with current understanding of breast biology and the available toxicology:

1) Results in the meta-analysis differ by study design. The case-control studies suggest a smoking-only-before-first-pregnancy risk of about 1.04 (4 studies) and a smoking-only-after-first-pregnancy risk of about 1.12 (12 studies).[30] The one cohort study that isolated smoking only before first pregnancy found a 15% increase in risk and the four cohort studies looking at women smoking only after first pregnancy found no increase in risk (individual study risk estimates of 0.89,0.98, 1.01 and 1.03). [30] When the results of case-control and cohort studies of similar study quality differ, it is prudent to rely more on the cohort studies as some of the case-control studies may suffer from response or recall bias.
2) The meta-analysis was limited to a comparison of ever versus never smoking in the period before or after first pregnancy.[30] Thus one could be mixing into the same exposure category women who may have smoked a cigarette a day for four months as a 16 year old, with women who smoked 20 cigarettes a day for more than 20 years before a first pregnancy. In Table 2 of the article risk estimates by amount of smoking before first birth are included for the five cohort studies: the three studies with the high exposure category five or more years of smoking before a first pregnancy found risk estimates of 1.12,[20] 1.13 and 1.13,[22, 25], one with a high exposure category of greater than 10 years 1.39[26] and the fifth study, with categories of 1-4, 5-9, 10-14,15-19 and >=20 years found relative risks of 1.12, 1.19, 1.42 and 1.10.[21]
3) The extended analysis of the Harvard Nurses' Health Study,[28] published after the meta-analysis was completed, found statistically significant increases in risk, in

particular during the period from menarche though the first full term pregnancy, as discussed above.

The meta-analysis authors suggested that the small summary relative risks observed may simply be confounded by an unidentified factor – but that holds much less weight when a consistent dose-response relationship is observed across several studies. One must imagine an unidentified factor which causes a risk in the same dose-response pattern as smoking before first pregnancy and essentially restricted to that time period.

Given that there is biological plausibility for a smoking-before-pregnancy effect, [30]toxicological research that is consistent with the idea that the human breast is most vulnerable from menarche to before first full term pregnancy,[30] and a close evaluation of the current cohort evidence, confounding as an explanation of the observed risk seems quite unlikely.[18]

Active Smoking and *NAT2* Status

More than 50 epidemiological studies have been published evaluating a role for genetic polymorphisms related to carcinogen metabolism, modulation of oxidative damage, and DNA repair and the risk of breast cancer related to smoking.[11] Meta-analyses for specific gene/smoking interactions performed by Terry and Goodman generally resulted in inconsistent results complicated by small numbers of studies for any one polymorphism, small sample sizes for the individual studies and varying measures of smoking.[11] The one exception to this pattern was the interaction of smoking with *NAT2* polymorphisms. Three different meta-analyses including the one by Terry and Goodman found similar results with fairly consistent increases in breast cancer risk of 35 to 50% for long-term smokers with the *NAT2* slow acetylation status.[9,11,14] Ambrosone *et al.* performed both a meta-analysis and a pooled analysis of the *NAT2*-smoking interaction and breast cancer based on results from nine case-control and four case-control-within-a-cohort studies. Twenty or more pack-years of smoking was associated with statistically significant 41 to 49% increases in both pre- and postmenopausal breast cancer risk among *NAT2* slow acetylators in both the meta-analyses and the pooled analyses.[14] (See Table 1.3.) On the other hand, for *NAT2* fast aceytlators there was a non-significant increase in risk for premenopausal women with 20 or more years of smoking and no indication of increased risk for postmenopausal women. (Table 1.3.).

Active Smoking Conclusions

The weight of epidemiologic evidence for an active smoking-breast cancer link has clearly shifted and dramatically strengthened over the last decade. All nine large (>500 breast cancer cases) cohort studies reporting exposure metrics more detailed than ever/never and ex/current provide consistent evidence that smoking increases breast cancer risk. Eight of the nine studies are large high-quality North American cohort studies.

Table 1.3.

Summary of meta-analysis and pooled analysis of smoking pack-years, *NAT2* acetylators status, menopausal status and breast cancer risk

		***NAT2* slow acetylators**		***NAT2* rapid acetylators**	
Type of analysis	**Pack-years***	**Premenopausal RR (95% CI)**	**Postmenopausal RR (95% CI)**	**Premenopausal RR (95% CI)**	**Postmenopausal RR (95% CI)**
Meta-analysis	Never active	1.00	1.00	1.00	1.00
	<20	1.21 (1.00 to 1.45)	**1.28 (1.08 to 1.50)**	1.00 (0.80 to 1.24)	1.12 (0.93 to 1.36)
	≥20	**1.47 (1.08 to 2.01)**	**1.41 (1.15 to 1.72)**	1.34 (0.94 to 1.89)	0.98 (0.77 to 1.26)
Pooled analysis	Never active	1.00	1.00	1.00	1.00
	<20	1.05 (0.86 to 1.28)	**1.23 (1.03 to 1.46)**	0.91 (0.72 to 1.16)	1.10 (0.89 to 1.35)
	≥20	**1.49 (1.08 to 2.04)**	**1.42 (1.16 to 1.74)**	1.29 (0.89 to 1.86)	0.88 (0.69 to 1.13)

Source: Ambrosone *et al*.[14]

*Pack-year as a categorical variable were available from the following eight studies for meta-analysis: Ambrosone *et al*., 1996; Morabia *et al*.,2000; Chang-Claude *et al*.,2002; Egan *et al.*,2003; van dee Hel *et al*., 2003; Alberg *et al.*, 2004; Sillanpaa *et al.*, 2005; Lissowska *et al.*, 2006.Pack-year as a categorical variable were available from the following six studies of the pooled analysis: Ambrosone *et al*., 1996; Morabia *et al*., 2000; Chang-Claude *et al*., 2002; Egan *et al*., 2003; van dee Hel *et al*., 2003; Lissowska *et al*. 2006.

Bold type indicates statistically significant increases in summary risk.

Seven of the nine cohort studies were published since the last assessment of active smoking by the Surgeon General in 2004,[5] and for an additional one (the Nurses' Health Study) the new report extended follow-up by a decade resulting in an additional 5632 incident breast cancer cases to analyze.[5,28]

Based on the weight of evidence from epidemiologic studies, in particular eight large high-quality cohort studies, from toxicological studies and from understanding of biological mechanisms regarding the relationship between tobacco and breast cancer[1], in 2009 the Canadian Expert Panel on Tobacco and Breast Cancer Risk concluded that the relationship between active smoking and breast cancer was consistent with a causal interpretation. With the addition of the recent Nurses' Health Study cohort analysis, there is a strong case that the amount a woman smokes before the end of her first full-term pregnancy largely defines her active smoking-related breast cancer risk.

B. Secondhand Smoke and Breast Cancer Risk

The types and quality of exposure measures used in the studies of passive smoking and breast cancer have varied widely. At one end of the spectrum, the only question about adult exposure may be whether the woman's husband currently smokes (yes or no). At the other end are quantitative summaries of "total lifetime smoker-years" that are calculated based on combining exposure as a child, as an adult residentially and as an adult occupationally, based on lifetime residential and occupational histories.

In the evaluation of SHS and breast cancer risk, attention needs to be paid to 1) the quality of the SHS measures in the individual studies, 2) the resulting impact on exposure misclassification, and 3) the resulting bias in the analysis and interpretation of the passive smoking-breast cancer relationship when the quality of the exposure measures is inadequate and ignored.

Studies with inadequate measures of exposure may bias any underlying risks towards the null in a mathematically predictable way,[33] biasing the assessment of summary risks towards the null and thus biasing the judgment of the risk towards the null. Meta-analyses by Johnson,[10] the California Environmental Protection Agency[13,16] and the Surgeon General[17] have each demonstrated the impact of the comprehensiveness of the SHS measures on observed premenopausal risk estimates. (See Table 1.4.) Simply put, studies with comprehensive measures of SHS exposure reported increased premenopausal breast cancer risks and those without tended to show limited or no increase in risk.

A serious limitation of the published studies at the time of the California EPA's and US Surgeon General's meta-analyses was that respectively, only 5 of the 19 and 6 of the 21 case-control studies, and none of the 8 cohort studies had quantitative lifetime assessments of SHS. Many of the studies since 2006 — both case-control and cohort studies — continue to utilize SHS exposure measures that inadequately characterize a woman's lifetime exposure to SHS.[1]

When conducting meta-analyses of randomized controlled clinical trials, a set of study design criteria is critical to decide whether a study is of sufficient quality to be included in the analysis. In epidemiologic meta-analyses of observational research (case-control and cohort studies) all studies are often included that meet very basic quality criteria. This may be

suitable as a starting point, but the analyses need to separate the wheat from the chaff in terms of study quality to come up with meaningful risk estimates.

Table 1.4. Summary Risk Estimates for Breast Cancer Risk Associated with Ever Regular Secondhand Smoke Exposure in the 2005 California Environmental Protection Agency's and the 2006 US Surgeon General's Reports

	California EPA Report 2005		US Surgeon General's Report 2006	
Exposure	**n**	**RR (95% CI)**	**n**	**RR (95% CI)**
All studies	19	1.25 (1.08-1.44)	21	1.20 (1.08-1.35)
Premenopausal or Women < 50 (California EPA)	14	1.68 (1.31-2.15)	11	1.64 (1.25-2.14)
Premenopausal (Surgeon General)				
Premenopausal —Studies with lifetime exposure assessment	5	2.20 (1.69-2.87)	6	1.85 (1.19-2.87)
Postmenopausal	9	[a]	10	1.00 (0.88-1.12)

Source: Collishaw *et al*.[1]

[a] *The California EPA did not report a summary risk estimate for postmenopausal women but concluded that risk estimates from the nine studies with data on postmenopausal women 'cluster around a null association'.

Misclassification of SHS Exposure and Breast Cancer Risk

Careful estimation of exposure is central to accurately calculating risk in epidemiologic studies. The importance of this cannot be exaggerated.

Rothman and Greenland demonstrated how non-differential misclassification of exposure (similar levels of misclassification among cases and non-cases) can have a dramatic impact on observed risks when the exposure prevalence is high.[34] For example, in their hypothetical scenario, a true underlying relative risk of 5.0 for laryngeal cancer is reduced to 1.7 if half of the drinkers are (inaccurately) classified as non-drinkers.

Similarly, Repace and Lowrey (1985) showed that a risk ratio of 1.7 for passive smoking and lung cancer reduced to 1.2 if 38% of nonsmoking women with workplace exposure to secondhand smoke were classified as "unexposed" simply based on their spouses' non-smoking.[35] Table 1.5 summarizes the impact of exposure misclassification under 3 exposure scenarios: 10%, 80% and 90% of subjects exposed. When dealing with relatively rare exposures (for example, less than 10% actually exposed) the impact of substantial exposure misclassification (up to 50%) is likely to be small, resulting in minimally diluted relative risks (Scenario A). However, the situation is quite different where the exposure is extremely common, as demonstrated in scenarios B and C: 80 and 90% exposed. The problem arises from the fact that when exposure is very common, exposure misclassification can result in serious contamination of the referent group, that is, exposure misclassification can erroneously result in defining some fraction of unexposed persons as exposed. Scenarios B and C reflect the situation in many of the SHS studies done in developed countries, where a large majority of women have been exposed, but assessment of exposure has been limited.

Table 1.5. Exposure Misclassification and Relative Risk Dilution for Low and High Exposure Prevalence Situations

Actual percent exposed	Actual percent unexposed	Percent categorized as exposed	Percent categorized as unexposed	Percent of exposed subjects misclassified as unexposed	Percent contamination of the referent group with SHS exposed women	Dilution of risk estimate (%)	Dilution of an actual RR of 2.00	Dilution of an actual RR of 1.50	Dilution of an actual RR of 1.25
Scenario A: 10% of subjects actually exposed									
10	90	10	90	0	0%	0%	2.00	1.50	1.25
		9	91	10	1%	1%	1.99	1.49	1.24
		8	92	20	2%	2%	1.98	1.49	1.24
		7	93	30	3%	3%	1.97	1.48	1.24
		6	94	40	4%	4%	1.96	1.48	1.24
		5	95	50	6%	6%	1.94	1.47	1.23
Scenario B: 80% of subjects actually exposed									
80	20	80	20	0	0	0	2.00	1.50	1.25
		70	30	10	33%	33%	1.66	1.33	1.17
		60	40	20	50%	50%	1.50	1.25	1.12
		50	50	30	60%	60%	1.40	1.20	1.10
		40	60	40	66%	66%	1.32	1.16	1.08
Scenario C: 90% of subjects actually exposed									
90	10	90	10	0	0%	0%	2.00	1.50	1.25
		80	20	10	50%	50%	1.50	1.25	1.12
		70	30	20	66%	66%	1.33	1.12	1.06
		60	40	30	75%	75%	1.25	1.06	1.03
		50	50	40	80%	80%	1.20	1.03	1.01

In these situations, the ultimate impact of a major degree of exposure misclassification is to dramatically attenuate underlying risks in a mathematically predictable way, and thus introduce serious bias into the results. In these examples of SHS, we have considered only the consequences of misclassifying exposed persons as unexposed, a major issue in such investigations. In studies of some other types of environmental exposures, where exposures are uncommon, misclassifying unexposed persons as exposed can have equally dramatic effects in biasing estimates of risk toward the null.[36,37]

Studies with the more complete lifetime exposure assessment of SHS are those which have included quantitative measures covering the three major opportunities for exposure: childhood exposure from parents, adult residential exposure, and adult occupational exposure. The lifetime SHS exposure prevalence (ever regularly exposed to SHS) in these studies has generally been between 80 and 95%. Unfortunately, as noted, only six of the 24 case-control studies and none of the eight cohort studies published through 2008 had this level of comprehensive SHS exposure assessment.[1,18] Furthermore, some studies asked subjects whether or not they were "exposed to SHS" and to estimate how much they "were exposed". Questions formulated in this way require a highly subjective judgment by each subject, and different subjects may have widely differing perceptions as to what constitutes being exposed or the level of exposure, rendering analysis of the responses uncertain. A better form of questioning, used in a number of studies, asks about the settings and the time spent in the company of those who smoked in their presence. Questions of this sort can be much more objectively answered by respondents, be more easily validated, and provide higher quality information for analysis. The 2006 Surgeon General's report concluded that exposure models can be useful in estimating exposure to involuntary smoking. Kolb *et al.* (2010) observed that mathematical models can also be used to estimate retrospective exposure in studies of lung cancer and passive smoking for hospitality workers.[38]

The need for comprehensive exposure assessment may be less important for Asian studies where a woman's exposure may be limited to the home and primarily from the spouse, and indeed three of the first four cohort studies from Asia examining only spousal exposure suggested increased breast cancer risk with higher SHS exposure.[39-42]

Measures of adult exposure have been particularly limited in several recent studies. Here are three examples:

- In one large British cohort study, the assessment of adult SHS exposure was based on asking women age 53-69 if their spouse currently smoked. Only 11% of the women answered in the affirmative and this was used as the sole measure to assess adult SHS risk.[15] Other studies with similar populations and full assessment of lifetime exposure have generally reported 75 to 90 % of women exposed to SHS. With large numbers of men giving up smoking around their fifth decade and divorce rates of approaching 50% in Britain, SHS exposure from the current spouse simply failed to accurately categorize the women by their adult SHS exposure and thus provides a poor measure to assess SHS-breast cancer risk.
- Another recent large study in Britain of young women 36-45 evaluated only spousal exposure and found only 41% of never smokers exposed to SHS.[43] In contrast, a study of a similar British population with a comprehensive assessment of exposure found 93% of the never smoking women had experienced regular SHS exposure.[44]

- A third large study, in Canada, [45] asked participants "how many hours in a day the subjects were exposed to tobacco smoke of others as a child and approximately two years ago (the later for both working and non-working days)." Only the participants reporting more than two hours a day of SHS exposure on average were considered exposed, perhaps the equivalent of putting women who smoked less than five cigarettes a day into a non-smoker category. Only 54% of women were categorized as having been exposed. Another Canadian study, studying a similar population in the 1990's, had a more comprehensive SHS assessment based on a full residential and occupational history which included questions on living with people who smoked for each residence and working with people who smoked in the immediate work for each job held. That study found 89% of women who had never smoked had a history of SHS exposure overall, and 94% in younger women.[46]

Pirkle *et al.* (1996) found that although 88% of non-tobacco users had detectable serum cotinine, a marker for exposure to tobacco smoke, in the US NHANES III study, half denied receiving any SHS exposure at either home or at work.[47] Furthermore, many of those reporting "no SHS exposure" actually had higher cotinine concentrations than those who did report exposure. Thus, questionnaires alone may lead to substantial exposure misclassification and therefore underestimate the actual risk associated with SHS.

In summary, the three examples of inadequate SHS exposure assessment above are perhaps the most egregious, but not dissimilar to a number of the studies included in the meta-analyses. The exposure bias introduced by these inadequate exposure estimates — contamination of the "unexposed" referent group and the misclassification of exposure levels within the exposed — undermined the ability of these studies to evaluate SHS-breast cancer risk. Their results were predictably null.

The Differences in Estimated Risk from Isolated Ever Exposure Versus Comprehensive Higher Exposure Measures

Two recently published large American cohort studies,[27,48] the only two cohort studies to date with comprehensive exposure assessment, demonstrate the impact of estimating SHS risks based on partial measures in comparison to comprehensive measures of SHS exposure. The two analyses of the California Teachers Cohort study by Reynolds *et al.* demonstrate the impact that adequacy of the SHS measure can have on the observed relative risks. In the report from 2004, when only lifetime residential SHS exposure was available for analysis, results were largely null.[22,49] When a comprehensive measure of SHS, both residential and occupational, became available and a new analysis was published, results for age-specific exposures were also largely null: for any age <20 exposure, adjusted hazard ratio (HR) 1.06 (95% CI 0.94-1.19), any age ≥20 exposure HR 1.04 (95% CI 0.91-1.19), and for setting specific exposures, any home exposure HR 1.04 (95% CI 0.92-1.16), any work exposure HR 1.02 (95% CI 0.93-1.13) and any social exposure HR 1.00 (95% CI 0.90-1.10).[48](See Table 1.6.)

When cumulative lifetime exposure was analyzed in the California Teachers Cohort, however, hazard ratios for low, medium and high cumulative exposure were 1.17 (95% CI 0.91-1.49), 1.19 (95% CI 0.93-1.53) and 1.26 (95% CI 0.99-1.60) for postmenopausal

women.[48] Furthermore, Reynolds *et al.* reported a statistically significant dose-response relationship for those women with medium to high SHS exposure.[48]

The Women's Health Initiative Observational Study cohort,[27] also presented in Table 1.6, demonstrates a pattern of risk similar to the California cohort, with no indication of increased risk when measures of ever home or work exposure are analyzed in isolation. This is not surprising, given the huge range of exposure that would be encompassed by "any childhood", "any home" or "any occupational exposure", not to mention the huge variation in exposure any subject exposed in one setting might have in the other two. On the other hand, the highest cumulative exposure was associated with a RR of 1.32 (95% CI 1.04-1.67).[27]

In summary, there are now two large, high-quality cohort studies with adequate and comprehensive SHS measures. Both studies suggest little or no increased risk for any adult residential or any adult occupational exposure when these exposure scenarios are viewed in isolation, but revealed increases of 26% and 32% in postmenopausal risk among the women with the highest cumulative lifetime exposure.

Table 1.6. Relative Risks for Postmenopausal Breast Cancer from the Two Large Cohort Studies with Comprehensive SHS Exposure Assessment

SHS Exposure	California Teachers Cohort [48] Adjusted HR (95% CI)	Women's Health Initiative Cohort [27] Adjusted HR (95% CI)
No reported lifetime exposure	1.00	1.00
Any childhood exposure	1.06 (0.94-1.19)	1.19 (0.93-1.53)
Any adult home exposure	1.04 (0.92-1.16)	0.91 (0.70-1.19)
Any workplace exposure	1.02 (0.93-1.13)	1.01 (0.82-1.26)
Highest cumulative lifetime exposure (vs. no lifetime exposure from any source).	1.26 (0.99-1.60)	1.32(1.04-1.67)

Although the recent report from the Harvard Nurses' Health Study also reported on passive smoking[28] careful analysis of the questions asked and the cohort studied, demonstrates that it does not meet the criteria for a critical evaluation of breast cancer risks associated with SHS. As noted, the Nurses' Health Study provides some of the best recent data regarding direct smoking and breast cancer risk. In this instance, the researchers ascertained smoking behavior biennially following initial assessment of lifetime smoking behavior at intake in 1976. In contrast to the detailed, regularly updated information about active smoking, SHS exposure in the cohort was ascertained only once, in 1982. They asked three brief questions and assessment of occupational SHS exposure was limited to one question about current occupational exposure in 1982.[50-52] Thus, this study does not meet rigorous criteria for high quality data regarding SHS exposures justifying inclusion in meta-analyses of SHS. Results were null.

Reynolds *et al.*[48] point out that the "California Teachers Cohort study is not alone nor the only cohort to find passive smoking risk associations among postmenopausal or primarily postmenopausal women.[41,46,53-59] It is worth noting that these include three case-control studies with more complete exposure methods.[46,54,59]"

Three of five Asian cohort studies suggest increased risk for women with higher SHS exposure.[41,42,53] For example, the South Korean cohort study found an overall RR of 1.2

for wives of ex-smoking husbands, 1.3 for wives of current smokers and a risk of 1.7 (95% CI 1.0–2.8) for wives of current smokers who had lived with their husband's smoking for at least 30 years.[41]

Other Summarization of SHS Literature

A 2009 meta-analysis found no increase in breast cancer risk for ever passive exposure in eight cohorts [summary RR 0.99 (95% CI 0.93-1.05)].[15] The report, however, did not consider the quality of the SHS exposure measure or the level of exposure. In its brief special report in November 2009, the International Agency for Research on Cancer (IARC) summarized several hazardous exposures included an assessment of SHS using its three-level categorization system: evidence is sufficient, limited or suggests a lack of carcinogenicity. IARC classified breast cancer and SHS along with larynx, pharynx, liver pancreas and stomach cancer, as "tumour sites for which there is limited evidence" of human carcinogenicity.[60]

Secondhand Smoke Conclusions

Comprehensive measures of SHS, i.e. comprehensive, lifetime SHS exposure measures (quantitative measures of childhood, adult residential and adult occupational exposure) are needed to properly assess SHS exposure and breast cancer risk. Increased premenopausal breast cancer risk was consistently observed in three meta-analyses[10,16,17] which each highlighted the small subset of case-control studies with better-quality exposure measures. The first two cohort studies have recently been published which include comprehensive, quantitative, lifetime SHS exposure measures.[27,48] Both studies suggest that higher levels of total lifetime SHS exposure are associated with increased postmenopausal breast cancer risk as has previously been observed in three case-control studies with better SHS exposure assessment[46,59,60] as well as two Asian cohort studies.[41,53]

Overall Conclusions

Studies with higher precision in the measurement of active and passive smoking exposure provide strong and consistent epidemiologic evidence that both active and passive smoking exposure increase breast cancer risk. The concentration of the active smoking risk in the time before a woman's first birth, makes it all the more urgent to focus on finding ways to not have young women begin to smoke as teenagers. The WHO Framework Convention on Tobacco Control calls for more education, communication, and increasing of public awareness about the dangers of tobacco as part of comprehensive tobacco control. Communicating the new understanding of the breast cancer risks associated with active smoking and SHS, especially to girls and young women, should be an important component of that plan.

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Disclaimer

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Chapter 2

Cigarette Smoking and the Risk of Breast Cancer – A Systematic Review and Meta-Analysis

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Abstract

Cigarette smoking is one of the leading preventable risk factors for cancer in respiratory and non-respiratory sites. Carcinogens in tobacco smoke including polycyclic aromatic hydrocarbons, aromatic amines and N-nitrosamines may pass through the alveolar membrane and enter the blood stream, and be transported to mammary tissues through plasma lipoprotein. Numerous epidemiologic studies have been conducted to investigate the association between cigarette smoking and the risk of breast cancer and conflicting results have been generated. The inconsistency of these study results may be partly due to a postulated anti-estrogenic effect of smoking, which may potentially decrease the risk of breast cancer. In this chapter we performed a systematic review of the existing literature on the association between cigarette smoking and the risk of breast cancer and describe potential mechanisms underlying the associations. Study design and other methodological issues which may bias the smoking-breast cancer association were also discussed. Emerging evidences on the modification by carcinogen-metabolizing genes on the potential effect of smoking on the risk of breast cancer were also reviewed and summarized.

Introduction

The annual incidence of breast cancer ranges from 11.8 per 100,000 in Eastern China to 86.3 per 100,000 in North America [1]. Studies among migrants suggested that migrants tend

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to acquire the higher risk of breast cancer common to others in the host country [2]. These evidences suggest a major environmental component in the etiology of breast cancer. Tobacco smoking is one of the leading preventable risk factors of cancer in respiratory and nonrespiratory sites [3,4] and has been an established risk factor for at least 15 types of cancer [5]. Tobacco smoke contains potential human breast carcinogens including polycyclic aromatic hydrocarbons (PAHs), aromatic amines and N-nitrosamines [3,6-8]. Carcinogens in tobacco pass through the alveolar membrane and enter the blood stream [10], and are transported to mammary tissue through plasma lipoproteins [10,11]. Furthermore, because these breast carcinogens are lipophilic, they may be stored in breast adipose tissue and metabolized and activated by mammary epithelial cells [12,13]. The biological plausibility of a positive association between cigarette smoking and breast cancer risk has been supported by experimental studies which have shown a higher prevalence of smoking-specific DNA adducts and *p53* gene mutations found in the breast tissue of smokers compared to that in nonsmokers.[14,15].

Numerous epidemiologic studies have been conducted to investigate the association between cigarette smoking and breast cancer risk and inconsistent results have been generated from these studies, ranging from positive, null to negative association [12]. Previous studies have suggested that smoking may have antiestrogenic effect [16] as demonstrated by a positive association between cigarette smoking and increased risk of osteoporosis [17,18], an earlier age at natural menopause [19] and attenuated effects of hormone replacement therapy [16]. Because estrogen is an established risk factor for breast cancer [20], the antiestrogenic effect of cigarette smoking may lower the risk of breast cancer and thus the direction and magnitude of the overall association between cigarette smoking and breast cancer may differ according to the hormonal profile of the study population. Recent studies also have suggested that smoking may increase the risk of breast cancer among women with certain genotypes, such as N-acetyltransferase 2 (*NAT2*) [21] or may affect the risk of breast cancer differently according to various hormone receptor status [22,23].

On the other hand, because cigarette smoking is assessed mainly through self-report, various levels of comprehensiveness and accuracy in data collection may also affect the study results. Lifetime smoking exposure is comprised of many components, including active and passive smoking, as well as quantity, duration, initiation and cessation of smoking, which are difficult to assess or analyze comprehensively. In this chapter, we are going to conduct a systematic review and meta-analysis of epidemiologic studies on the association of various measurements of smoking with the risk of breast cancer. Since cigarette smoke is a well-known carcinogen, retrospective studies are subject to recall bias since breast cancer patients may report their exposure differently from non-cancer patients. Therefore, in this chapter, we focus primarily the review and analysis on prospective studies, except for studies involving genetic factors.

Methods

A systematic review of published prospective studies on the association between cigarette smoking and the risk of breast cancer was conducted. Studies with only qualitative assessment of smoking, such as “ever/never” and “never/past/current” were excluded, as

these measures do not adequately capture the relevant exposure and thus likely fail to detect the underlying association of breast cancer with cumulative exposure to smoking. Meta-analyses were conducted based on data from published studies on quantitative measures of active smoking including amount of smoking, duration of smoking, and age started smoking. Separate analyses were conducted for studies focused on premenopausal and postmenopausal women. For the meta-analysis, studies with overlapping study population were examined and only the study with larger population and/or longer follow-up were included. The software RevMan 5.1 was used to produce forest plots and summary effect estimates [24]. For the summary of each total or subtotal, the chi-square test statistic and P-value for heterogeneity across studies, the statistic I^2 measuring the inconsistency among results, and the test for overall effect (Z-statisticwith p-value) were calculated.

Results

Meta-Analysis on Various Measures of Active Smoking in Relation

Amount Of Smoking

Two nested case-control studies [25,26] and 15 cohort studies [22,27-40] have investigated the association between amount of smoking and the risk of breast cancer (Figure 2.1). The highest amount of cigarette smoking evaluated in these studies ranged from ≥10 [31,37] to ≥40 cigarettes per day [28, 32, 38]. Most of these studies identified a modest positive association and the summary effect estimate indicates a 16% (95% CI 9% - 23%) increased breast cancer risk associated with high amount of smoking compared to non-smoking, with a slightly higher magnitude from nested case-control studies (OR=1.30, 95% CI 0.96 – 1.76) than cohort studies (HR=1.15, 95% 1.09 – 1.23). Test for heterogeneity did not suggest the results across studies are significantly heterogeneous (P=0.45).

Duration of Smoking

The association of breast cancer risk and duration of smoking was evaluated in eight [22,32,35,36,38,40-42] (Figure 2.2) cohort studies. All these studies compared the incidence rate of breast cancer associated with smoking for a long duration to that associated with non-smoking. The longest duration of smoking evaluated in these studies ranged from ≥20 (35) to ≥50 years [40]. In all studies breast cancer risk was positively associated with longer duration of smoking [Summary RR (95% CI)=1.22 (1.15 - 1.30)], as compared with never smokers. The test for heterogeneity did not suggest the results across studies are significantly heterogeneous (P=0.17).

Age Started Smoking

The association of breast cancer risk and age started smoking was evaluated in 11 cohort studies [22,29,32,33,36-38,40-42] (Figure 2.3). The youngest age started smoking evaluated in these studies ranged from <15 [35,37,40] to <20 years [36]. In all studies breast cancer risk was modestly and positively associated with younger initiation of smoking [Summary RR (95% CI)=1.10 (1.06 - 1.15)], as compared with never smokers. The test for heterogeneity did not suggest the results across studies are significantly heterogeneous (P=0.10).

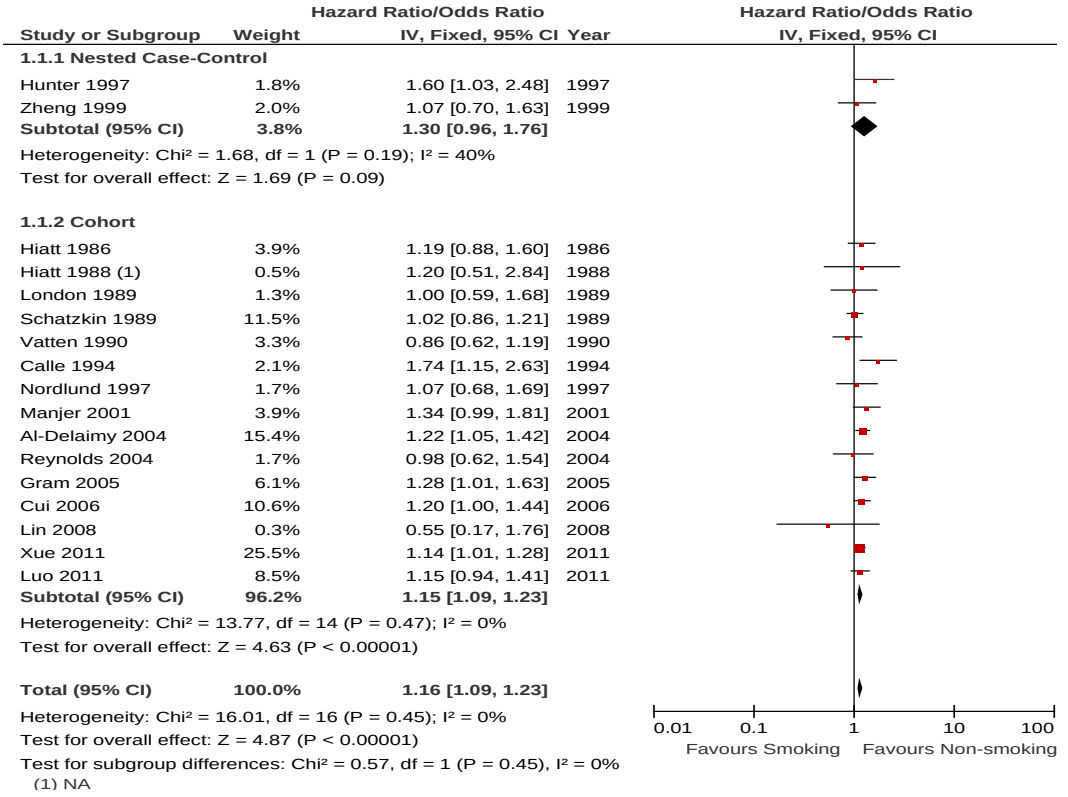

Figure 2.1. Meta-analysis on amount of smoking (cigarettes/day) and the risk of breast cancer.

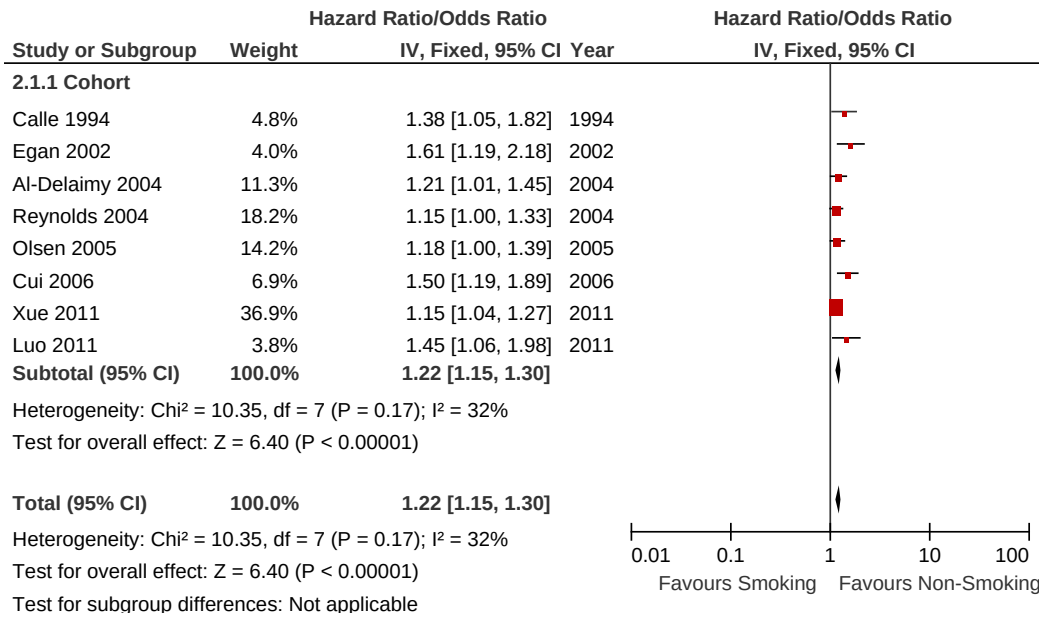

Figure 2.2. Meta-analysis on duration of smoking (years) and the risk of breast cancer.

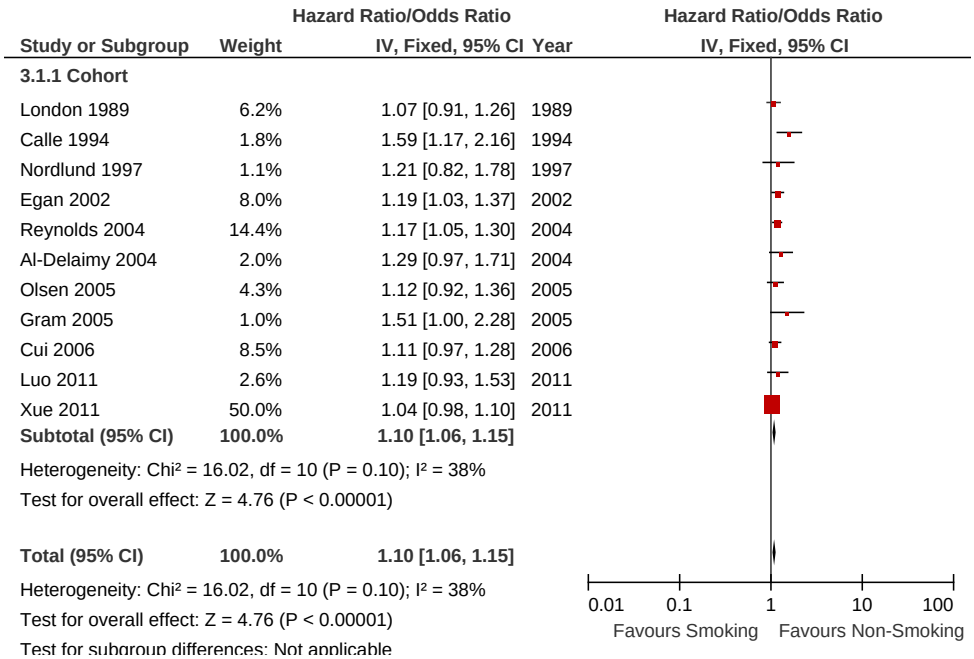

Figure 2.3. Meta-analysis on age started smoking (years) and the risk of breast cancer.

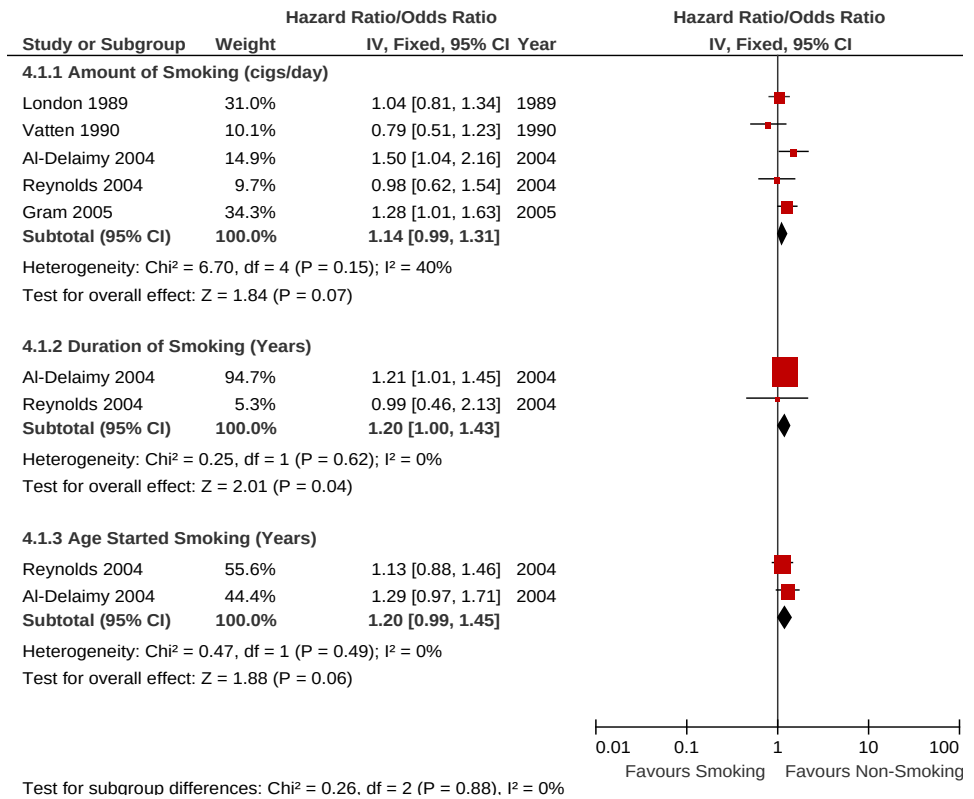

Figure 2.4. Meta-analysis on smoking measures and risk of premenopausal breast cancer.

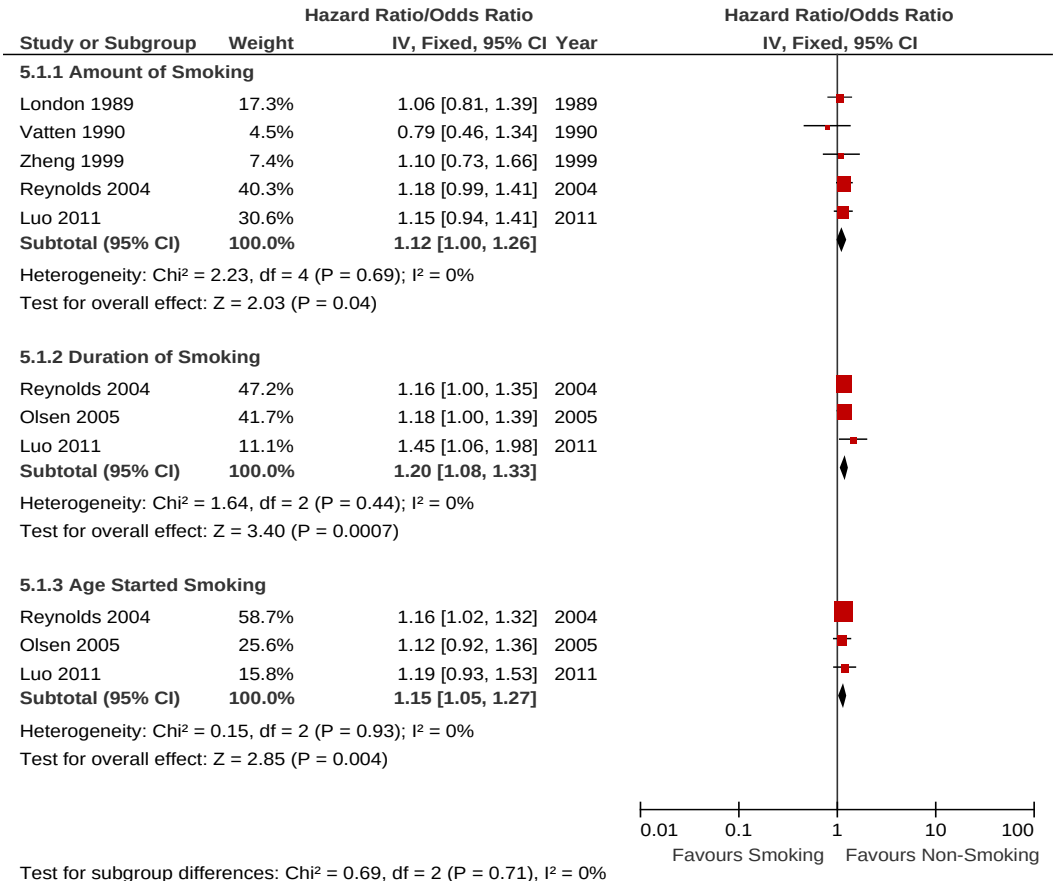

Figure 2.5. Meta-analysis on smoking measures and risk of postmenopausal breast cancer.

By Menopausal Status of Breast Cancer

There are evidences which suggest premenopausal breast cancer and postmenopausal breast cancer may have different underlying etiology. For instance, body mass index is associated with increased risk of postmenopausal breast cancer [43] but decreased risk of premenopausal breast cancer [44]. Similarly, size at birth was only found to be associated with premenopausal breast cancer but not postmenopausal breast cancer [45]. Few previous prospective studies have separately assessed cigarette smoking in relation to pre- and postmenopausal breast cancer (Figure 2.4 and Figure 2.5).

The summary effect estimates did not suggest that the association of premenopausal breast cancer with higher amount of smoking [HR (95% CI)=1.14 (0.99 - 1.31)], longer duration of smoking [HR (95% CI)=1.20 (1.00 - 1.43)] and younger age of smoking initiation [HR (95% CI)=1.20 (0.99 - 1.45) did not differ from that of postmenopausal breast cancer [HR (95% CI)=1.12 (1.00 - 1.26), 1.20 (1.08 - 1.33), 1.15 (1.05 - 1.27), respectively]. Test for heterogeneity did not suggest any significant heterogeneity for all the involved analysis.

By Smoking at Different Reproductive Period

A woman's hormonal profile and development of breast epithelium varies across different reproductive period. Therefore, smoking relative to major milestones of a women's reproductive life, such as menarche, first full-term childbirth and menopause, may have different impact on the risk of breast cancer.

Early age at the first birth has been associated with a decreased risk of breast cancer, possibly due to the terminal differentiation of breast cancer epithelium late in the last trimester of the pregnancy [46]. Therefore, the experience of a full-term pregnancy may be an indicator of the maturity and decreased susceptibility of breast cells to carcinogens. With regard to menopausal status, it was speculated that cigarette smoking may further reduce the low endogenous estrogen levels among postmenopausal women and thus convey protective effect against breast cancer, while any antiestrogenic effect of smoking may not be strong enough to materially reduce endogenous estrogen level among premenopausal women and thus leaving the dominant carcinogenic effect [22]. In a nationwide cohort study of female US radiologic technologists, smoking-related breast cancer risk was found to differ significantly by smoking during three reproductive periods [RR (95% CI)=1.06 (0.76 – 1.47), 1.03 (1.02 – 1.05), and 0.99 (0.98 – 1.00) for before menarche, from menarche to first childbirth and after first childbirth, respectively] [47]. Results based on 30 years of follow-up in the Nurses' Health Study suggested that every increase of 20 pack-years of smoking before menopause and especially before the first birth was associated with a higher incidence of breast cancer [HR (95% CI) =1.11 (1.07 – 1.15) and 1.18 (1.10 – 1.27), respectively] while smoking after menopause was associated with a non-significant decrease in the risk of breast cancer (HR (95% CI)=0.93 (0.85 – 1.02)] [22]. Another nested case-control study assessed the association between cigarette smoking during first pregnancy and breast cancer risk and the results did not suggest such association [48].

By Hormone Receptor Status of Breast Cancer

Both estrogen and progesterone mediate their functions through respective intracellular receptors, estrogen receptor (ER) and progesterone receptor (PR), which act as hormone-dependent transcriptional regulators [49,50]. The risk of breast cancer ER+/PR+ has been found to be preferentially associated with other hormone-related risk factors including endogenous sex steroid levels [51], BMI, and current use of postmenopausal hormones [52]. As the presence of significant amount of ER and PR in breast cancer cells at the time of diagnosis is generally taken as an important indicator of hormone dependence [50,53], theoretically cigarette smoking should assert greater protection from breast cancer with ER+ through its antiestrogenic effect. Nonetheless, there is evidence that cancer cells with ER+ and/or PR+ may also be more susceptible to DNA mutagenic effect of smoking. Benzo[a]pyrene (BaP), one of the most widely studied PAHs and endocrine-disrupting chemicals (EDCs) found in cigarette smoke, has been recognized to activate the aryl hydrocarbon receptor (AhR) and subsequently induce the conversion of BaP into Benzo[a]pyrene diolepoxide **(**BPDE), which forms DNA adducts [54]. In vitro studies have demonstrated that several steroid hormone receptors, including ER, PR and androgen receptor might interact with AhR in mediating cellular response [55,56]. Results from a recent in vitro

study indicated that BaP–DNA adduct formation and the DNA synthesis inhibition level were enhanced in a concentration-responsive manner in ER+ human breast cancer cell line, but there was no change in ER- cell line, suggesting that increased formation of BaP–DNA adducts may be mediated through ER expression [57].

Mixed results have been generated by studies which separately evaluated breast cancer according to ER and PR status. High amount of cigarette smoking has been found to be associated with ER+ breast cancer in some studies [22,23,29] but not others [34,35]. Similarly, a stronger association between heavy smoking and PR+ breast cancer was suggested by some studies [22] but not others [34]. Furthermore, when both ER and PR status were assessed simultaneously, no consistent pattern of a higher risk of breast cancer with ER+ and/or PR+ associated with smoking was observed [34].

Potential Effect Modification by Genotype

Several genetic factors including carcinogen-metabolizing genotypes, oxidative metabolism genotypes and DNA repair genotypes have been studied as potential effect modifier for the association between cigarette smoking and the risk of breast cancer [58]. Conflicting results have been generated from these studies.Such inconsistency may be related to inadequate sample size and lack of statistical power and precision, lack of uniform methods of smoking characterization, lack of consideration of gene expression in breast tissue or the frequency of variant alleles, and the differences among groups with known differences in disease incidence. Nonetheless, possible effect medication was suggested by some genotypes, such as *NAT2*, and glutathione S-transferase-M 1 (*GSTM1*). *NAT2* is a genotype involved in the metabolism of aromatic amines, a major class of tobacco smoke carcinogens. Variant alleles in *NAT2* result in slow clearance of aromatic amines. A pooled analysis and meta-analysis of 10 existing studies suggested a significant interaction between *NAT2* and smoking in influencing the risk of breast cancer, with higher pack-years of smoking significantly associated with breast cancer among women with *NAT2* slow genotype but not among rapid acetylators [21]. The GSTs are phase II enzymes that play key roles in detoxification of many potentially carcinogenic compounds, including PAHs, which are contained in tobacco smoke. A meta-analysis of previous studies suggested that the positive association between smoking and the risk of breast cancer tend to be stronger among women with *GSTM1*-null genotype [58]. Women with such genotype do not express the specific protein which has been shown to modulate cytogenetic damage in smokers [59,60].

Another challenge for the assessment of genotypes as potential effect modifier is to distinguish disease susceptibility related to haplotype or specific combination of variants in several genes. The mutagen sensitivity assay (MSA) is a phenotypic assay that accounts for the net results of several genetic pathways and the cumulative effects of low-risk genetic variants. It measures the frequency of chromosomal breaks induced by mutagens in short-term peripheral blood cultures and serves as a phenotypic marker of the combined effects of sensitivity to carcinogen exposure, and the individual's DNA damage response and repair capacity. In a study using bleomycin as the mutagen, ever smoking was found to be associated with the risk of breast cancer among women with hypersensitivity to bleomycin but not among bleomycin-hyposensitive women or bleomycin-sensitive women [61],

suggesting the effect of cigarette smoking on the risk of breast cancer may differ based on mutagen sensitivity status.

Passive Smoking

Extensive exposure to passive smoking has been suggested to induce breast cancer development since nitrosamines and other carcinogens found in tobacco smoke appear to be more concentrated in passive smoke than in mainstream smoke [62]. The role of passive smoking in the development of breast cancer has been assessed in numerous epidemiologic studies. A positive association between passive smoking and risk of breast cancer was found in several case-control studies [63-71], and in majority of these studies this association was statistically significant [63-69,71]. In contrast to the strong evidence from case-control studies, only two [40,72] out of eight cohort studies [22,36,37,39,41,72-74] identified a significantly increased risk of breast cancer among women who were expose to high intensity and long duration of passive smoking. These prospective cohort studies collectively suggested that passive smoking may not play an important role in the etiology of breast cancer.

Conclusion

The meta-analysis of various measures of active smoking in relation to the risk of breast cancer suggested that heavy smoking measured as high amount, long duration, and early age at initiation are associated with a modest increase in the risk of breast cancer. The association is likely slightly stronger for premenopausal breast cancer than postmenopausal breast cancer. Smoking relative to major milestones of a woman's reproductive life, especially before the first full-term childbirth and before menopause, was more strongly associated with the risk of breast cancer than smoking after menopause. Mixed results have been generated by studies which separated evaluated breast cancer according to ER and PR status. Similarly, conflicting results were generated from studies investigating potential effect modification by various genotypes related to carcinogen-metabolism, oxidative metabolism and DNA repair. Nonetheless, possible effect modification was suggested by some genotypes, such as *NAT2* and *GSTM1*. Results from prospective studies collectively suggest that passive smoking may not play an important role in the etiology of breast cancer. Though smoking in relation to the risk of breast cancer has been studied extensively in the past few decades, results remain controversial. Growing evidence suggests that carcinogen-metabolizing genes may modify the potential effect of smoking on the risk of breast cancer, but large studies with sufficient statistical power are needed to address the influence by haplotype or specific combination of variants in several genes.

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Chapter 3

Significance of Smoking in Cervical Carcinogenesis

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Abstract

The association between the infection by Human Papillomavirus (HPV) and the development of cervical intraepithelial neoplasia (CIN) and cervical carcinoma is well established nowadays. Discrepancies exist between the frequency of women infected by this virus and the number of women that develop the mentioned lesions. In the majority of cases, the infection caused by this virus is transitory.

It is believed that some cofactors, besides the presence of HPV, may be of great importance in its natural history. Among these factors are genetic, alimentary and environmental factors, use of hormonal contraceptives, smoking and immune status.

Many recent studies have verified a strong association between cigarette consumption and the development of cervical lesions. We hypothesize that there are several mechanisms involved in the genesis of this association. Although there are individual differences in the metabolism of the chemical substances in the cigarette smoke, besides individual genetic susceptibility, the harmful effect of smoking over the cervical tissue should be related to: high concentration of carcinogenic substances on cervical mucus causing direct DNA damage; modification of the vaginal flora, enhancing the risk of infection; increase of cellular proliferation index on the transformation zone; reduction of both cellular and humoral immune responses, causing difficulty in the recognition of HPV, as the persistence of its infection.

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Introduction

The world has a population of 2,337 million women ages 15 years and older who are at risk of smoking and also to suffer health risks related to it [1]. The trend of smoking in women is increasing nowadays and in some countries there are even more female smokers than male smokers. The prevalence of female smoking varies from <2,5% to 53,3% (Figure 3.1). Throughout most of Europe, where modern tobacco use began a century ago, rates of tobacco use by males and females have been converging for decades. Today, tobacco use rates are decreasing among European men while they are increasing among women, particularly in eastern, central and southern Europe and Latin America [1,2]. In most European Union countries, teenage girls are as likely to smoke as boys, if not more likely [3]. In the developing world, tobacco use rates for adult females remain relatively low, but could rise quickly among teenage females. In South-East Asia, the adult male smoking rate is ten times higher than the adult female rate [4]. Among 13–15-year-olds, however, the male smoking rate is only about two and a half times higher [3].

Several diseases are related to cigarette smoking, including vascular, heart and respiratory diseases, among others; 30% of all cancer-related deaths are associated with smoking. In fact, in addition to the well-known link between smoking and lung cancer, large epidemiological studies have shown an association of smoking with several other cancer sites: nose, oral cavity, oropharynx, hypopharynx, larynx, esophagus, pancreas, bladder, kidney, stomach, liver, colon, cervix and myeloid leukemia [5]. Cancer of the cervix uteri is the second most common cancer among women worldwide, with an estimated 529,409 new cases and 274,883 deaths in 2008. About 86% of the cases occur in developing countries, representing 13% of female cancers. The majority of cases are squamous cell carcinoma and adenocarcinomas are less common [1,6].

The etiological role of human papillomavirus (HPV) infection among women with cervical cancer is well-established, and HPV causes virtually 100% of cases of cervical cancer. There are more than 100 types of HPV, of which around 40 infect the genital area. The genital HPV types can be divided into two broad groups (low-risk and high-risk HPVs) depending upon their association (or lack of association) with cancers of the lower genital tract. Low-risk HPV types (6, 11, 42, 43, 44, 54, 61, 70, 72, and 81) are virtually never found in cancers. Therefore, they are also called non-carcinogenic HPV. High-risk (HR) HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) have been identified in cancers of the cervix, vagina, vulva, anus, and penis. Therefore, they are also called carcinogenic or oncogenic HPV [6,7]. Invasive squamous cell cervical cancers are preceded by a long phase of preinvasive disease, collectively referred to as cervical intraepithelial neoplasia (CIN), which is graded on a scale based on severity as CIN grade I (CIN1), CIN grade II (CIN2) and CIN grade III (CIN3 - used synonymously with carcinoma in situ of the cervix). The high-grade lesions (CIN 2 and 3) are considered to be true precursors of invasive cancer [8].

The majority of HPV infections are cleared spontaneously by the host's immune system over two years. Persistent infection by the oncogenic HPV types can at a low frequency (<10% of total infected women) undergo neoplastic progression to high-grade dysplasias. On average, it takes 12-15 years before a persistent HPV infection may ultimately lead to an overt cervical carcinoma. This argues that HPV-induced cervical carcinogenesis is multi-step

in nature and other cofactors farther than the presence of the virus are necessary for the development of these lesions [9,10].

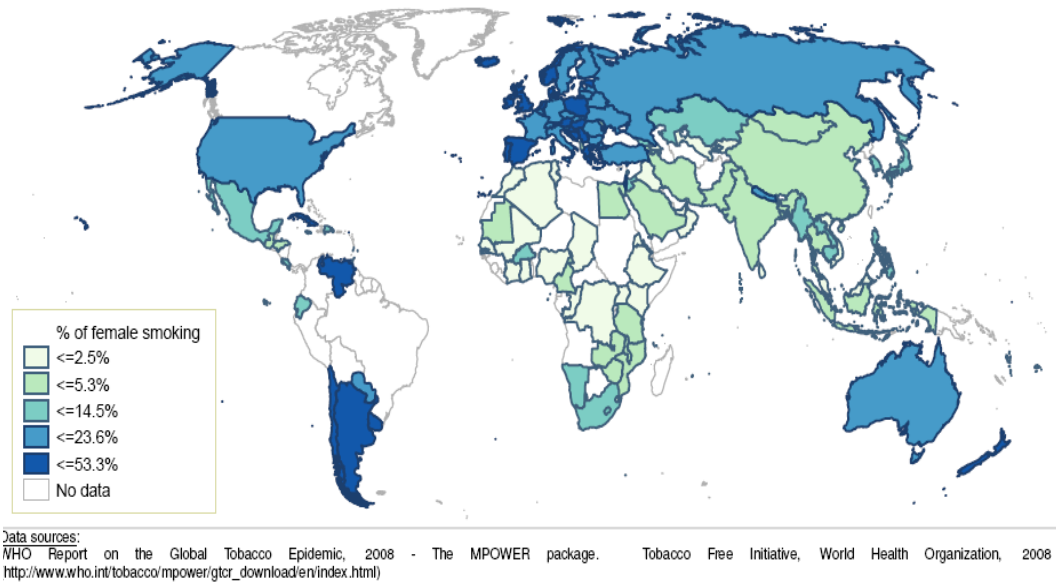

Figure 3.1. Global prevalence of female tobacco smoking.

Cofactors may be classified into three groups: (1) environmental or exogenous cofactors, including use of oral contraceptives (OCs), tobacco smoking, diet, cervical trauma, coinfection with human immunodeficiency virus (HIV) and other sexually transmitted agents; (2) viral cofactors, such as infection by specific types, coinfection with other types, HPV variants, viral load, and viral integration; (3) host cofactors, including endogenous hormones, genetic factors such as human leukocyte antigen and other host factors related to the host's immune response [11,12,13,14]. The mentioned cofactors interact in order to increase susceptibility of the host to HPV, thus favoring its infection, multiplication, and persistence in action. This chapter highlights the role of smoking as one of the main cofactors that leads to that effect [10,11]. Unhealthy life style, negatively rated life events, lack of social support, coping style, and distress, often associated with smoking, alcohol abuse, and illegal drugs addition, have been reported as risk factors for cervical cancer, especially among low-educated women [14].

1. Smoking and Cervical Lesions: Epidemiology

Winkelstein Jr. in 1977 [15] was the first to put the hypothesis that smoking is a risk factor for cervical cancer. Since then, the action of tobacco on cervical carcinogenesis has been a matter of scientific debate. Researchers subsequently began to explore the physiologic links between the two. Cigarette smoking has been linked to a two- to fivefold increase in cervical cancer risk [16,17,18]. Epidemiological studies have shown that twice as many cases of cervical intraepithelial neoplasia (CIN) advance to invasive disease in smokers as in

nonsmokers [19] and that the size of CIN lesions is reduced by 20% with cessation of cigarette smoking [20]. Such studies have demonstrated a clear relationship between smoking and the proliferation of tumorigenic neoplastic cells.

Plummer et al. reported the first multicentric case-control study in 2003 [21]. In analyzing eight studies on invasive cancer and two on carcinoma in situ, conducted by International Agency for Research on Cancer (IARC), between 1985 and 1997, the authors conclude that ever-smokers have an excess risk of cervical cancer that persists after controlling for the strong effect of HPV and for other potential cofactors of progression from infection to cancer, and they suggest that squamous cell carcinoma of the cervix should be added to the list of tobacco associated cancers, while for adenocarcinoma, further data should be warranted. In 2004, IARC revisited its previous conclusions and listed cervical cancer among those causally related to smoking [22]. Harris et al. in 2004, [23] that found among women with oncogenic HPV infection, smoking was associated with risk for both CIN1 and CIN2-3. Of the three smoking measures (smoking status, pack years of exposure, and number of cigarettes per day), number of cigarettes per day (>10 cigarettes) was the most strongly associated with risk for CIN1 and CIN2-3.

In 2006, The International Collaboration of Epidemiological Studies of Cervical Cancer [24] has brought together and combined individual data on 13,541 women with and 23,017 women without cervical carcinoma, from 23 epidemiological studies. After adjusting for potential confounders, current smokers were found to have a significantly increased risk of squamous-cell carcinoma (SCC) of the cervix compared to never smokers (RR = 1.60; 95% CI: 1.48–1.73). There was increased risk for past smokers also, though to a lesser extent (RR = 1.12 (1.01-1.25)), and there was no clear trend with time since stopping smoking (p-trend = 0.6). There was no association between smoking and adenocarcinoma of the cervix (RR = 0.89 (0.74-1.06) and 0.89 (0.72-1.10) for current and past smokers respectively), and the differences between the RRs for smoking and squamous cell and adenocarcinoma were statistically significant (current smoking $p<0.001$ and past smoking $p = 0.01$). In current smokers, the RR of squamous cell carcinoma increased with increasing number of cigarettes smoked per day and also with younger age at starting smoking ($p<0.001$ for each trend), but not with duration of smoking (p-trend = 0.3). Eight of the studies had tested women for cervical HPV-DNA, and in analyses restricted to women who tested positive, there was a significantly increased risk in current compared to never smokers for squamous cell carcinoma (RR = 1.95 (1.43-2.65)), but not for adenocarcinoma (RR = 1.06 (0.14-7.96)).

Syrjanen et al. [25] divided 3,187 women into groups comprising those who never smoked, those with a history of smoking and those who are current smokers. They found no increase in precancerous or CIN cytology among past or current smokers. Age and HPV status were the only independent predictors of CIN2. Using a multivariate model, however, the authors did find that smoking was an independent risk factor for HPV acquisition. Smoking may thus largely influence CIN by allowing HPV to proliferate in the cervical tissues.

Some other authors have shown that women with oncogenic HPV who smoke were more likely to be diagnosed with lesions ≥ CIN3 than nonsmokers. Smoking is also related to HPV persistence. In 2005, 5,060 women with minimally abnormal Papanicolaou smears were enrolled to assess associations between smoking behaviors and cases of cervical intraepithelial neoplasia grade 3 or cancer (> or =CIN3) identified throughout the study (n =

506) in women with oncogenic HPV (n = 3,133). It was concluded that women with oncogenic HPV and minimally abnormal Papanicolaou smears who smoke were up to three times more likely to be diagnosed with > or =CIN3 than nonsmokers [11]. Tolstrup et al. [26] used baseline information on tobacco exposures on 548 high-risk human papillomavirus positive women with normal cytology, comparing 94 women who developed high-grade squamous intraepithelial lesions with 454 women who remained cytologically normal. They concluded that smoking is associated with an increased risk of developing high-grade squamous intraepithelial lesions in women who are infected with oncogenic human papillomavirus.

Sarian et al. [27] performed a study which purpose was to assess the effect of smoking on the prevalence and incidence of high-risk human papillomavirus (HR-HPV) infection and cervical intraepithelial neoplasia in a large sample of Latin American women. The study examined baseline data on over 12,000 women included in the Latin American Screening Study (Brazil and Argentina), and over 1000 women followed-up for a period of 36 months. The authors concluded that smoking increases the risk of contracting HR -HPV infection and modifies the effect of a persistent hr-HPV infection by further increasing the risk of developing CIN2+. It seems that this effect modification persists over several years after smoking cessation.

To examine the effect of smoking on the incidence of low- and high-grade cervical intraepithelial neoplasia (CIN) in a subset of 150 women with a baseline Pap smear of atypical squamous cells (ASC) or a low-grade squamous intraepithelial lesion (LSIL), a prospective study in which a cohort of women with normal colposcopy and ASC/LSIL at baseline were followed at 6-month intervals of up to 36 months. The authors concluded that smoking contributes additional risk for developing high-grade CIN in women with ASC or LSIL cytology but normal colposcopy [28]. Xi et al. in 2009 [29] reported an analysis of 1,050 women HPV16 and/or HPV18 positives for viral DNA load. The authors concluded that higher HPV16 and HPV18 DNA load was associated with status of current, but not former, smoker. Among current smokers, the viral load did not appear to vary appreciably by the intensity and duration of cigarette smoking, in accordance, with previous study of Gunnell et al. [30] in 2006, that in testing for HPV16 DNA presence in first archival cervical smears from 375 cases of in situ cervical squamous carcinoma (CIS) and in 363 controls, it was found that current smokers with a high HPV16 viral load at time of first smear were at a particularly increased risk (27-fold) compared with current smokers without HPV-infection. A recent study [31], published in 2010, conducted on 2,011 women, 15–19 years old, recruited from 1988 to 1992 then regularly followed until 1997, concluded that there is no evidence to suggest that the risk of acquiring a HPV infection of any type, or a HPV16 or HPV18 infection, increases with either pack years of exposure to smoking or duration of current smoking episode, suggesting that smoking is not a important risk factor for HR-HPV infection.

Louie et al. [32] evaluated the potential impact of passive smoking of tobacco (PS) in the development of invasive cervical cancer (ICC). A pooled analysis of 1,919 couples enrolled in one of seven case-control studies involving cervical carcinoma in situ (CIS) or ICC was investigated. They concluded that PS could not be detected as an independent risk factor of ICC in the absence of active smoking. The combined effects of exposure to active and PS suggest its potential adverse role in cervical carcinogenesis.

2. Smoking and Cervical Epithelial Carcinogenesis

Tobacco smoke is the most widespread carcinogen in the world. More than 3,000 chemicals have been isolated from processed tobacco leaves. These are not only leaf constituents but also products derived from the soil, the atmosphere, the use of agricultural chemicals and from the process of curing, casings and flavoring of the leaves. When tobacco is burned during smoking, many other reaction products are formed, among which are >4,000 identified chemicals and an unknown number of unidentified chemicals. The products of mainstream smoke can be divided into particulate and gas phases. The particulate phase contains nicotine, nitrosamines [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone], N-nitrosonornicotine, metals (cadmium, nickel, zinc and polonium-210), polycyclic hydrocarbons and carcinogenic amines (4-aminobiphenyl). The vapor phase contains among the others carbon monoxide, carbon dioxide, benzene, ammonia, formaldehyde, hydrogen cyanide, N-nitrosodimethylamine and N-nitrosodiethylamine. Approximately 60 known carcinogens are present in tobacco smoke, the strongest are polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines and aromatic amines and the most prevalent present in the vapor phase are aldehydes, benzene and butadiene [5,33].

The amount of nicotine inhaled by a smoker depends not only on number of cigarettes smoked but also on the amount of nicotine per cigarette, the smoker's inhalation pattern, and the percentage of each cigarette smoked. (gather paragraphs)Among passive smokers the amount of nicotine inhaled depends on the smoking patterns of the other people sharing the same space and ventilation system and on the time spent in that space. . (gather paragraphs)Among both active and passive smokers there is great individual variation in the percentage of nicotine that is converted to cotinine and in the hepatic clearance of nicotine and cotinine, with some of this variation associated with gender and race [5,33].

Tobacco from both active and passive smoking has the main point of entry into the body via the airways; some constituents dissolve in saliva and are absorbed or swallowed. Alcoholic drinks act as solvents of the smoke constituents, thus facilitating their absorption. Virtually, all the organs and tissues are reached by the active products of smoking. Data from epidemiological studies confirm the widespread action of tobacco smoke on tissues and organs [5]. (gather paragraphs)The presence several compounds from cigarette smoke (nicotine and its major metabolite, cotinine) in the cervical mucus of smokers may indicate that inhaled tobacco- specific carcinogens could likewise become blood-borne and transported to the cervix, where they may damage cellular DNA. (gather paragraphs)Several studies have shown cigarette compounds and metabolites in cervical mucus. There may also be some variation related to the difference in amount and consistency of cervical mucus associated with such factors as time in the menstrual cycle and oral contraceptive use. . (gather paragraphs)

In women with sexual partners who smoke, it is unclear what proportion of the nicotine and cotinine levels found in cervical mucus is derived from cervical contact with semen, which has recently been shown to contain cotinine and what proportion from inhalation of environmental tobacco smoke [34,35,36].

2.1. Mechanisms of Action

The role of tobacco smoking in the multistage carcinogenesis at the cervix is not fully understood because of a paucity of prospective data. The exact mechanism of how smoking could lead to the induction of CIN and cervical cancer is still unknown and several hypotheses have been formulated, thus not making it possible to point out an isolated mechanism that would explain carcinogenesis related to cigarette smoking. The complexity of mixed carcinogens in cigarette smoke related to individual susceptibility could mean that different substances should cause different kinds of damages [37,38].

One of the main action mechanisms related to cervical carcinogenesis would be especially related to direct DNA exposition of cervical epithelial cells to high concentrations of such carcinogens, followed by the covalent change of such molecules, thus replacing nucleotides and potential mutagenic effects. Covalent alteration of DNA to form DNA adducts is considered an early step in chemical carcinogenesis and, therefore, detection of DNA adducts provides evidence of exposure of the cervix to carcinogens. Cellular repair systems can remove these DNA adducts and maintain a normal DNA structure. If DNA adducts persist unrepaired, they can cause miscoding during replication when DNA polymerase enzymes process them incorrectly. These mutations can cause the loss of normal cellular growth control functions, ultimately resulting in cellular proliferation and cancer [5,14,33,37,38]. The level of damage to cellular DNA would have individual variation, with the influence of genetic and environmental factors and the number and type of cigarettes consumed [33]. Prokopczyk et al. [35] reported no significant differences in smoking-related DNA damage (DNA adduct levels) between HPV-positive and HPV-negative smokers, suggesting that smoking DNA damage is not related with HPV infectivity.

To prove a causal link between an epigenetic change and an environmental or behavioral risk factor for a given disease, it is first necessary to show that the onset of exposure precedes the first detection of that epigenetic change in subjects who are still free of disease. Towards this end, a cohort of women aged 15-19 years, recruited soon after they first had sexual intercourse, were used to provide sequential observations on the relationship between cigarette smoking and the detection in cervical cytological samples of methylated forms of CDKN2A (p16) using nested methylation-specific polymerase chain reaction. The authors observed that among women who remained cytologically normal and who tested negative for human papillomavirus DNA in cervical smears during follow-up, those who first started to smoke during follow-up had an increased risk of acquiring CDKN2A methylation compared with never-smokers (odds ratio=3.67; 95% confidence interval 1.09-12.33; P=0.04). They conclude that smoking initiation is associated with the appearance of methylated forms of CDKN2A [39].

Genetic susceptibility to smoking is an important issue. Cervical cancer risk in smokers may be modified by genetic variants, as that described to interleukin 2 or to 8q24 chromosome polymorphisms [14]. In a recent study [40], the tumor suppressors p53, the fragile histidine triad and the interleukin-10 were under-expressed, and the cyclooxygenase-2 and the Ki-67 were over-expressed in smoking, compared with nonsmoking women with CIN.

PAHs, N-nitrosamines and aromatic amines are metabolized by a two phase process. Phase I involves the activation of the carcinogen by enzymes encoded by the CYP gene superfamily. Cytochrome p450 1A1 is responsible for the first step of PAH metabolism.

Other enzymes, such as CYP2C9, CYP1B1 and CYP2D6, are responsible for the activation of benzo-[a]-pyrene and nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone, whereas CYP2E1 metabolizes 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone. During the phase II process, carcinogens are transformed into hydrophilic elements to facilitate excretion. Glutathione S-transferases are mostly responsible for this process. This multigene superfamily detoxifies carcinogens from cigarette smoke as well as from other sources. Since phase I enzymes induce the formation of active carcinogens from procarcinogens, whereas phase II enzymes conjugate these compounds and make them suitable for excretion, it is reasonable to think that the overall carcinogenic effect of tobacco compounds should be measured as the final result of the combined action of the two categories of enzymes [5,38]. Differences in the processing of chemical substances in cigarette smoke related to specific genes which are responsible for the metabolism and detoxication of these enzymes, could also contribute to individual genetic susceptibility to carcinogens related to tobacco [38].

Most of the metabolic and DNA repair genes carry polymorphisms that are present in the general population at various frequencies. Some of these genetic variations alter the original gene function, thus increasing or decreasing the activity of the corresponding enzyme. For example, both GSTM1 and GSTT1 genes can be deleted and thus their conjugating activity can be absent. GSTM1 homozygous deletion is present at frequencies that vary from 30 to 50% in the general population, whereas the deletion of GSTT1 is around 20–30% in healthy subjects. Changes in enzymatic activity associated with polymorphisms in these genes may play a significant role in tobacco related cancer risk and genetic susceptibility. Variations in these genes involved in tobacco metabolism and/or DNA repair should produce a difference in local carcinogen levels; therefore, changes in levels of DNA damage should be observed as a consequence of the polymorphisms [5,38]. Most of the genetic polymorphisms described in the literature vary in frequency across ethnicity and geographic areas. This may be a further contributing factor to the observed variation in tobacco related cancer incidence among smokers with different ethnic background [5,38].

Smoking has also been associated with reduced occurrence of ectopy, perhaps, because smoking increases the rate at which columnar epithelium undergoes squamous metaplasia. This increased rate of squamous metaplasia might increase susceptibility of ectopic epithelium to malignant changes if there is exposure to certain pathogens such as human papillomavirus [41]. The in vivo effects of long-term nicotine exposure could affect persistent cellular proliferation, inhibition of apoptosis, and stimulation of vascular endothelial growth factor, with increased microvessel density [14].

There are several researches that correlate cigarette smoking and a higher cellular growth rate at different organs and tissues, both in human and animals. The effects of tobacco on pulmonary tissues are the subject of countless researches, due to its undoubtedly relation to lung cancer. Lee et al. [42], Hiroshima et al. [43], Miller et al. [44] and Lapperre et al. [45] found a significant increase in the number of proliferating cells within the bronchial epithelium of smokers in comparison to nonsmokers. Some other researchers have observed that smoking had an effect on the proliferation of cells in other tissues of the human body as oral gingival epithelium [46,47], cells of coronary walls [48] and also in some other types of epithelium of animals. This epithelial cell proliferation by increasing cell division could induce metaplasia, and this metaplasia is related/ precedes carcinomas. Cucina et al. [49] proposed that nicotine could lead to the increase of neointimal smooth muscle cells in vascular lesions by inducing the inhibition of physiological smooth muscle cell apoptosis and

the increase of smooth muscle cells proliferation. Apoptosis protects the organism by removing cells with DNA damage. The balance between mechanisms leading to apoptosis and those suppressing apoptosis has a major impact on tumors growth [38].

Waggoner and Wang [50] studied the effect of nicotine on cellular proliferation of human ectocervical, endocervical, malignant and human papillomavirus (HPV) 16 DNA-transformed cervical cell lines. Proliferation of nicotine-exposed cells was compared to unexposed cells with one-way analysis of variance. The authors observed that nicotine significantly stimulated epithelial cell growth in ectocervical and HPV DNA-transformed cell lines and did not significantly alter proliferation of endocervical cell lines. Their findings demonstrate that nicotine, in physiologically attainable concentrations, does not impair and occasionally enhances the proliferation of human cervical cells in vitro. The selective mitogenic effect noted among normal ectocervical and HPV-transformed ectocervical cells may relate to epidemiologic studies showing, among smokers, an increased risk of squamous cell carcinoma and not adenocarcinoma of the cervix. In 2004, Harris et al. [23] performed Ki-67 immunohistochemistry testing (marker for proliferation) on cervical transformation zone biopsy samples from 139 women with normal cervix in order to evaluate the effect of cigarette smoke on epithelial cell proliferation and metaplasia. The authors found that among women with oncogenic HPV DNA, the relation between number of cigarettes per day and intermediate to high level expression of Ki-67 exhibited a positive dose response relation. They suggest that the association between cervical lesions and smoking might be mediated through an effect of cigarette smoke on cell proliferation / metaplasia of cervical transformation zone.

Campaner et al. [51] evaluated the effect of smoking on cell proliferation in normal cervical epithelium. Among smoking women, there was no significant difference related to the number of cigarettes smoked per day or time of consumption and epithelial cell proliferation. However, the total amount of cigarettes smoked throughout presented significant association with Ki-67 staining ($p < 0.001$); the number of proliferating cells per mm^2 increased proportionally to the increase in consumption of cigarettes.

Smoking habits could also modify the vaginal environment, leading to increased local susceptibility to infectious agents and carcinogens. Alnaif and Drutz [52] and Ryckman et al. [53] observed that smoking independently affected vaginal flora, increasing the odds of developing bacterial vaginosis. Cherpes et al. [54] observed that cigarette smoking was among the independent predictors of herpes virus-2 infection. Porras et al. [55] described that smoking had an increase in *Chlamydia trachomatis* infection detection.

2.2. Smoking and Cervical Immunology

An impaired immunity of the cervix is of extreme importance and influences the natural history of cervical neoplasia. The quality of the immune response is a critical step in the defense against HPV infection, which may result or not in a more permissive environment for malignant transformation. Both innate and adaptive (cellular and humoral) immunity play a role in controlling HPV infection. However, the cornerstone of cervical immune surveillance directed to HPV infection is an intact cell-mediated immune system, which depends on the proper identification of antigens and their presentation to correct lymphocyte populations that leads to the destruction of the infected cells [56]. In untransformed HPV-infected

keratinocytes, the innate immunity is induced to eliminate the invading HPV pathogen through sensitization to HPV-related proteins by epithelial-residing Langerhans cells, macrophages, and other immune cells. Once the HPV infection escapes from initial patrolling by innate immunity, cellular immunity becomes in charge of killing the HPV-infected keratinocytes of the uterine cervix; It occurs through systemic immune response developing by dendritic cells (DCs) in the regional lymphoid organs or through local immune response developing by Langerhans cell (LCs) in the cervix. Thereby, DC/LC plays a critical role in eliciting innate and adaptive cellular immune responses against HPV infection [56]. Any factor capable of interfering in the human immune response, local or systemic, will be considered a cofactor, predisposing HPV infection, multiplication and persistence. As mentioned above, cigarette smoking is considered one of these co-factors. It has shown that it could induce diverse systemic and local changes in the immune system.

Some studies have tried to show the effects of the harmful substances absorbed by the human body during the act of smoking in the immunological defense of the organism. Smoking widely changes both cellular and humoral responses. Smoking individuals have decreased levels of circulating immunoglobulins (except by immunoglobulin E), decrease in the production of antibodies related to certain antigens, and decrease in releasing cytokines by immunocompetent cells. Changes in concentrations and functions of cytotoxic T lymphocytes also occur, as well as in the suppressor and natural killer cells; decrease in leukocyte migration and chemotaxis, as well as a decrease in phagocytes activity. Thus, the host could have difficulties in presenting an effective immune response against the various infectious aggressive agents, mainly HPV, allowing them to persist for a longer period of time [14,23].

Cigarette smoking may also exacerbate the carcinogenic potential of HPV, specifically via inhibition of interferon- γ and/or tumor necrosis factor- α, leading to a significant inhibition of apoptosis, which may promote tumor growth. The fact that some cigarette constituents have the ability to manipulate cytokine expression in a manner similar to that of HPV suggests that smoking may enhance the ability of HPV to evade the immune system [57].

Some researchers have observed a decrease in the concentration and function of cervical Langerhans cells and lymphocytes. Thus, changes in their density or/and function may profoundly influence the proper activation of the afferent and efferent arms of immune response in cases of HPV-related intraepithelial lesions; it could contribute to the development of CIN. Which constituent or metabolite of cigarette smoke is responsible for the change in these cells is unknown. After a broad literature review, we found just a few studies that evaluated the effect of smoking over Langerhans cells and lymphocytes in normal cervical epithelium, but they are not current data.

In 1988, Barton et al. [58] showed that current cigarette smoking was associated with a significant decrease in the Langerhans' cell population in normal cervical epithelium. Ex-smokers tended to have cell counts between those of smokers and non-smokers. There was a dose-response relation between number of cigarettes smoked daily and effect on cell counts. Poppe et al. [59] showed an association between smoking and reduction of the numerical densities of Langerhans cells and of helper/inducer T lymphocytes in the normal squamous epithelium of the transformation zone of the uterine cervix. They suggest a local impairment of cell-mediated immunity by smoking and emphasize that this immunosuppressive effect could support the concept that smoking is an independent risk factor for cervical neoplasia. A year later, Poppe et al [60] analysed cotinine levels in blood and cervical fluid of smokers and

non-smokers. The levels of this substance were not related to numerical cell densities of intraepithelial Langerhans cells or to macrophages in the stroma of the transformation zone of normal uterine cervices. However a decrease in the number of Langerhans cells was noted in smokers, especially in those using oral contraceptives. Macrophages were more numerous in the endocervical stroma of smokers, suggesting a local response to smoke constituents. The authors suggest a synergistic suppression of local cervical immunity by smoking and oral contraceptives.

On the other side, Szarewski et al. [61] showed that a reduction in smoking by 20 to 40 cigarettes per day was significantly associated with a reduction of between 6% and 16% in counts of Langerhans cells, CD8 and total lymphocytes. The objective of the study of Campaner et al. [62] was to evaluate the effect of smoking on intraepithelial Langerhans cells and T and B lymphocytes in normal cervical epithelium. They observed that the comparison of the number of intraepithelial Langerhans cells between smoking and nonsmoking women showed a significant difference (P=0.045), but it did not occur in relation to the number of T and B cells between the 2 groups. There was also no significant difference in relation to the number of cigarettes smoked per day, time of consumption, and total amount of cigarettes smoked throughout the lifetime.

The quantitative change in these antigen-presenting cells would promote events related to early cervical carcinogenesis due to an increase in the duration of oncogenic HPV infections, as well as decrease the likelihood of them disappearing. Smoking seems to affect negatively the early natural history of HPV infections. Smoking affecting clearance HPV infection remains a conflicting issue. For some authors, smoking has no influence in duration of HPV infection [63], for others tobacco delays the clearance of HPV infection [64,65].

To assess the relationship between smoking and spontaneous regression of cervical precursor lesions, a total of 516 women with low-grade squamous intraepithelial lesion (LSIL) were monitored by cytology and colposcopy every 4 months by Matsumoto et al. Probability of LSIL regression within 2 years was analyzed in relation to smoking behaviors. The study subjects included 258 never-smokers and 258 smokers (179 current and 79 former smokers). Probability of regression within 2 years was significantly lower in smokers than in never-smokers (55.0%vs 68.8%, P = 0.004). The risk of LSIL persistence increased with smoking intensity and duration and with younger age at starting smoking. Smokers had twice as high a risk of persistent HPV infection compared to never-smokers [66]. Burger et al. [67] evaluated 181 women with a report of cervical cytological abnormality in order to verify the prevalence of infection with oncogenic human papillomavirus and smoking habits. They observed that the prevalence of the virus increased in accordance with the number of cigarettes smoked. This relation remained after adjustment for age at first intercourse and lifetime number of sexual partners.

During the years following conservative treatment of cervical intraepithelial neoplasia (CIN), their risk of invasive cervical cancer is about 5 times greater than that of the general population. Acladious et al. [68] performed a nested case-control analysis, cases being defined as women who developed CIN within the 2 years of treatment and controls being sampled from those who did not experience treatment failure within 2 years. The cohort included 958 women of whom 77 (8%) experienced treatment failure (cases). The authors concluded that cigarette smoking is a factor, which, independently of HPV infection, influences the treatment outcome of CIN. Smokers and those who are HPV positive during follow-up appear to require longer, more intensive follow-up. Smoking also affects survival

among women diagnosed with cervical cancer. In 2009, Coker et al. [69], in analyzing 2661 women diagnosed with invasive cervical cancer from 1995–2005, found that, after adjustment for age and stage at diagnosis, cell type, rural residence, race, insurance coverage, and treatment-received, current smoker, were 35% more likely to die of any cause and 21% more likely to die of cervical cancer compared with known nonsmoking cases, in accordance with previous studies. Unfortunately, few smokers with cervical cancer quit or decreased consumption during treatment.

Conclusion

All meta-analyses and multi-institutional studies point out that smoking is an important cofactor for cervical squamous cancer and probably also for cervical adenocarcinoma. Acquisition of HR-HPV infection seems to be a smoking independent event; however, progression of the acquired infection is negatively affected by current smoking. Former smoking seems to be no so important.

From the above we can observe that, although there are individual differences in metabolism of chemicals in cigarette smoke, as well as individual genetic susceptibility, smoking women have a higher risk of developing cervical lesions. The deleterious effect of smoking on cervical tissue could be related to different mechanisms of action. Several hypotheses have been suggested and there is no single mechanism that could explain the smoking-related carcinogenesis.

Consequently we should encourage anti-smoking campaigns and guide the smokers carriers of HPV to abandon the habit of smoking in attempting to prevent viral persistence, local immunosuppression and subsequent progression to cervical lesions.

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Chapter 4

Association of Gene Polymorphisms with Lung and Colorectal Cancers in Relation to Smoking

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Abstract

Lung and colorectal cancer is a major cause of death and is influenced by genetic characteristics and environmental factors. Humans are exposed daily to a large variety of toxic and carcinogenic compounds due to habits such as tobacco smoking. Tobacco smoking produces major classes of carcinogenic compounds including polycyclic aromatic hydrocarbons, arylamines, and heterocyclic amines. Several of these compounds can produce bulky DNA adducts. The CYP enzymes are a critical importance for the metabolism of these carcinogens by *N*-oxidation. GSTs play an important role in the detoxification of carcinogens to reduced glutathione. NAT2 catalyze the metabolism of various aromatic amines and carcinogens, involving in detoxification by *N*-acetylation and activation by *O*-acetylation. In addition, DNA repair genes are increasingly being studied for cancer risk because of their critical role in maintaining genome integrity. The DNA repair pathways, including nucleotide excision repair (NER), base excision repair (BER) and double-strand break repair (DSBR) play an important role in repairing the DNA damage. In the NER pathway, XPD/ERCC2 protein is an evolutionarily conserved helicase. The BER pathway has a principal role in the repair of mutations caused by oxidized or reduced bases smoking-induced oxidative DNA base modifications and single-strand breaks are repaired by the BER pathway. OGG1 is a DNA glycosylase that removes 8-oxo-G and MUTYH is another DNA glycosylase that removes adenine paired with 8-oxo-G or 2-OH-A paired with guanine. APEX1 removes abasic sites formed in DNA cleavage by OGG1 and MUTYH and recruits DNA polymerase β and DNA ligase III. XRCC1 is a multidomain protein that interacts with poly-ADP-ribose polymerase, DNA ligase III and DNA polymerase β, and repairs DNA single-strand breaks by

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generating a single nucleotide repair patch. In the DSBR pathway, XRCC3 participates in DNA double-strand break/recombination repair. The single nucleotide polymorphism (SNPs) have been known useful markers of genetic susceptibility to cancers and required verification as predictive biomarkers. Recently, we have reported that these gene polymorphisms, *CYP1A1*, *CYP1A2*, *GSTM1*, *NAT2*, *XPD*, *OGG1*, *MUTYH*, *APEX1*, *XRCC1* and *XRCC3*, which play an interactive role in the risk for lung and colorectal cancers incidence in relation to smoking in Japanese population. In metabolism enzymes, our findings suggest that light smokers with intermediate-slow NAT2 activity are at highest risk for lung cancer and the gene-gene interaction based on intermediate-slow NAT2 activity and high CYP1A2 activity (*CYP1A2*1F A/A* genotype) would be increased a lung cancer risk among never smokers. These results also indicate that the *NAT2* in combination with *CYP1A1*2C*, *CYP1A2*1C*, or *GSTM1* genotypes may strongly confer susceptibility to colorectal cancer. In particular, the combination of *NAT2* plus *CYP1A1*2A*, *CYP1A1*2C*, or *CYP1A2*1F* genotypes, and that of *CYP1A2*1F* plus *CYP1A2*1C* genotype may define a group of persons who are genetically susceptible to colorectal cancer in never smokers. In DNA repair genes, the joint effect of tobacco exposure and *MUTYH* Gln324His and *APEX1* Asp148Glu show a significant association with lung cancer risk in smokers, and there is not significantly increased in non-smokers. We also report that *MUTYH* Gln324His and *APEX1* Asp148Glu constitute an increased risk of colorectal cancer, especially colon cancer. *MUTYH* Gln324His is strongly associated with colorectal cancer susceptibility in never smoking history, whereas *APEX1* Asp148Glu genotype constitutes an increased risk of colorectal cancer when accompanied by smoking exposure. These results indicate that these polymorphisms are associated with increased risk for lung and colorectal cancers in Japanese individuals in relation to smoking.

Introduction

Lung and colorectal cancer is a well-known cancer that is caused by a complex combination of genetic and environmental carcinogenic factors such as tobacco smoke. Tobacco smoke contains many chemical carcinogens and reactive oxygen species. DNA damage induced by these carcinogens or by endogenous metabolic processes can be manifested as gene mutations. Lung cancer is one of the most prevalent cancers worldwide and consists of three major histological subtypes, adenocarcinoma, squamous cell carcinoma, and small cell carcinoma. In recent years, adenocarcinoma, the most frequently encountered histological subtype, has accounted for the majority of lung cancers and is thought to be only minimally related to cigarette smoking [1, 2]. Squamous and small cell carcinomas, on the other hand, are strongly associated with smoking. In addition, the carcinogenic processes differ among the histological subtypes. Colorectal cancer (CRC) is also associated with genetic and environmental factors such as cooked meats and fish at high temperature [3, 4]. These factors result in the formation of carcinogenic compounds including polycyclic aromatic hydrocarbons (PAHs), arylamines, and heterocyclic amines (HCAs) [5].Several of these compounds can produce bulky DNA adducts [6]. The colorectal mucosa is exposed to these compounds through either the alimentary tract or the circulatory system. DNA adducts were detected in the colonic mucosa of smokers than in nonsmokers [7]. A previous study found that heavy smokers have a 2-3-fold elevated risk of colorectal adenoma [3].

SNPs are inherited genetic variants harbored by all the cells of the body. Their analysis can be easily done in blood tissue and is easier to adopt in the routine clinical setting [8].

Metabolic Enzymes

Carcinogens are metabolized by phase I and II enzymes (Table 4.1). The cytochrome P450 1A1(*CYP1A1*) gene belongs to the phase I enzymes and is involved in the activation step in the metabolism of PAHs, such as those found in tobacco smoke. Previous reports have shown that two *CYP1A1* gene polymorphisms, the *Msp*I polymorphism located in the 3'-flanking region of the gene (*CYP1A1*2A*: *Msp*I) and the Ile-Val polymorphism at amino acid residue 462 in the heme binding region of CYP1A1 protein (*CYP1A1*2C*: Ile462Val), are associated with susceptibility to several cancers (available at www.imm.ki.se/CYPalleles/cyp1a1.htm) [9, 10]. Another phase I enzyme, cytochrome P450 1A2 (*CYP1A2*) is also known to catalyze the *N*-oxidation of several amines such as HCAs formed when meat and fish are cooked well done or in tobacco smoke [11, 12]. Two polymorphisms of the *CYP1A2* gene, *CYP1A2*1C* (3858G→A) and *CYP1A2*1F* (164A→C), have been examined to associate with reduced enzyme activity (available at www.imm.ki.se/CYPalleles/cyp1a2.htm) [13, 14].

Table 4.1. Gene polymorphisms of metabolic enzymes and DNA repair

Gene	Region	Polymorphism (amino acid	Restriction enzyme
Metabolic enzymes			
*CYP1A1*2A*	3'-flanking region	T/C	*Msp* I
*CYP1A1*2C*	exon7 codon 462	A/G (Ile/Val)	*Bsr* DI
*CYP1A2*1F*	intron1 codon 164	A/C	*Apa* I
*CYP1A2*1C*	intron1 codon 3858	A/G	*Bst* I
GSTM1	exon4-5	deletion (null)	—
*NAT2*1* [a]	exon2 codon 64	G/A	*Kpn* I, *Bam* HI, *Taq* I
*NAT2*2*	exon2 codon 114	T/C	
*NAT2*3*	exon2 codon 197	C/T	
*NAT2*4*	exon2 codon 286	G/A	
DNA repair pathways			
XPD	exon23 codon 751	A/C (Lys/Gln)	*Mbo* II
OGG1	exon7 codon 326	C/G (Ser/Cys)	*Fnu* 4H1
MUTYH	exon12 codon 335	G/C (Gln/His)	*Hpy* CH4III
APEX1	exon5 codon 148	T/G (Asp/Glu)	*Bfa* I
XRCC1	exon10 codon 399	G/A (Arg/Gln)	*Msp* I
XRCC3	exon7 codon 241	C/T (Thr/Met)	*Nla* III

[a] Rapid: *1/*1; intermediate: *1/*2, *1/*3, *1/*4; slow: *2/*2, *2/*3, *2/*4, *3/*3, *3/*4, *4/*4.

Table 4.2. Genotype distribution in lung cancer and colorectal cancer

Gene	Lung cancer					Colorectal cancer				
	overall	adeno-carcinom	squamous cell	never-smokers	smokers	overall	colon	rectum	never-smokers	smokers
Metabolic enzymes										
*CYP1A1*2A*									*	
*CYP1A1*2C*										
*CYP1A2*1F*										
*CYP1A2*1C*										
GSTM1										
NAT2 rapid[d]									*	
NAT2 intermediate, slow					*[c]					
NAT2 intermediate, slow plus *CYP1A2*1F*				*[b]						
NAT2 rapid plus									*	
NAT2 rapid plus						*			*	
NAT2 rapid plus						*				
NAT2 rapid plus									*	
NAT2 rapid plus *GSTM1*						*				
*CYP1A2*1F* plus									*	
DNA repair pathways										
XPD										
OGG1										
MUTYH	*				*	*	*		*	
APEX1	*	*	*		*	*	*			*
XRCC1										
XRCC3										

[d]Rapid: *1/*1; intermediate: *1/*2, *1/*3, *1/*4; slow: *2/*2, *2/*3, *2/*4, *3/*3, *3/*4, *4/*4.
[b]Significant ($p < 0.05$).
[c]Significant for light smokers (0 < pack years < 30).

In contrast, the *CYP1A2*1C* G/G and *CYP1A2*1F* A/A genotypes caused a significant increase of CYP1A2 activity [13, 15, 16]. Glutathione S-transferases (GSTs) are enzymes involved in the phase II detoxification process by catalyzing the conjugation of reactive hydrophobic and electrophilic compounds to reduced gluthathione. GSTM1 null cannot effectuate the detoxification of activated PAHs [17]. *N*-acetyltransferase 2 (NAT2) is polymorphic and catalyzes both *N*-acetylation (deactivation) and *O*-acetylation (activation) of a variety of heterocyclic amine drugs and carcinogens [18]. *NAT2* polymorphisms are associated with leading to either slow or rapid acetylation for different cancers (http://louisville.edu/medschool/pharmacology/NAT2.html) [19]. These polymorphisms may increase lung or colorectal cancer risk in relation to smoking.

Gene Polymorphisms of Metabolic Enzymes and Lung Cancer Risk

Several metabolic enzymes have been investigated for their lung cancer susceptibility. Japanese studies have pointed to an increased risk of lung cancer in association with both the *CYP1A1*2A* and **2C* [9, 10]. The *GSTM1*-null genotype was found to be associated with a slight increase in the lung cancer risk [17]. The combination of *CYP1A1*2A* or **2C* variants and *GSTM1*-null genotype have been associated with a significantly increased risk of lung cancer in Japanese population [20, 21]. These studies indicated that *CYP1A1* variants play a major role in the activation of PAHs, and that *GSTM1*-null cannot effectuate the

detoxification of activated PAHs. Some studies have detected a positive association between the lung cancer risk with *NAT2* polymorphisms [22, 23].

We studied the *CYP1A1*2A*, *CYP1A1*2C*, *CYP1A2*1C*, *CYP1A2*1F*, *GSTM1* and *NAT2* polymorphisms involved in the metabolism of carcinogens associated with lung cancer (Table 4.2) [24]. We did not detect any association between the six genetic polymorphisms examined in this study for overall, lung adenocarcinoma or squamous cell carcinoma. These genotypes were no association with lung cancer among never smokers (pack-years = 0). For light smokers (0< pack-years ≤30), we observed a significant association for intermediate-slow genotypes of *NAT2* [the adjusted odds ratio (OR):10.9, 95% confidence intervals (95% CI): 1.75-67.5, p = 0.010], whereas the OR of that genotypes was not associated with lung cancer for heavy smokers (pack-years > 30). The intermediate-slow genotypes of *NAT2* was significantly associated with increased risk of lung cancer among light smokers. Previous studies reported that no overall association of *NAT2* acetylator genotypes to the lung cancer risk, but there was the increase risk with several factors, age, gender, or smoking dose [22, 25, 26]. In particularly, the *NAT2* slow acetylator genotype was associated with an increased risk for lung cancer in Japanese [25] or at lower pack-years [22]. Sørensen et al (2005) reported that *NAT2* fast acetylator genotype seemed to be protective against lung cancer for light smokers [26]. NAT2 enzyme is detoxification to many arylamines in tobacco smoke by *N*-acetylation [18]. The findings of our study indicate that the genetic susceptibility ascribable to NAT2 intermediate-slow acetylators may confer decreased the detoxification to tobacco mutagens, such as several arylamines, when tobacco expose is low.

Additionally, we detected that the joint association of *NAT2* intermediate-slow and *CYP1A2*1F* A/A polymorphisms for never smokers group was a significantly lung cancer risk compared with its association for ever smokers group (adjusted OR 4.95, 95% CI: 1.19-20.6, p= 0.028). HCAs or several arylamines, formed by cooking of meat or fish but little tobacco smoking, were activated by *N*-hydroxylation of CYP1A2 enzyme. The hydroxylated forms, can eventually covalent bound with DNA adduct-induced, are potent as proximate carcinogen. The *A/A* genotype of *CYP1A2*1F* represented a highly inducible genotype that was associated with an increased activity of CYP1A2 [15, 16]. Therefore, high CYP1A2 activity was possible to be increased a risk of lung cancer among never smokers. The hydroxylated forms may also be *O*-acetylated by NAT2 enzyme. The hydroxylated forms by *O*-acetylated are also can form DNA adduct-induced and are potent as ultimate carcinogen. *NAT2* slow acetylator genotype was associated with increased risk of lung cancer among non-smokers [27, 28]. Additionally, it is reported that when the joint effect of NAT2/CYP1A2 status, associated with slow genotypes of NAT2 and rapid CYP1A2 activity using caffeine metabolic ratio assay, was at highest risk for lung adenocarcinoma in nonsmoking Chinese women [29]. Our findings clearly show the promoting effect on the risk of lung cancer associated with combination of high CYP1A2 enzyme activity and NAT2 intermediate-slow acetylator activity. Therefore, high CYP1A2 activity and intermediate-slow NAT2 activity may be strongly increased the hydroxylated forms as proximate carcinogens, from HCAs and arylamines by *N*-hydroxylation, compared with the activation of the hydroxylated forms as ultimate carcinogens by *O*-acetylation.

Furthermore, we confirmed borderline significant association between *CYP1A2*1F* A/A and *CYP1A2*1C* G/G genotypes. The G/G genotype of *CYP1A2*1C* caused also a significant increase of CYP1A2 [13, 30], therefore, the G/G genotype of *CYP1A2*1C* genotypes

was supported to increase the CYP1A2 activity. This indicated that the joint association of *CYP1A2*1F* A/A and *CYP1A2*1C* G/G genotypes may lead to increase the CYP1A2 enzyme activity.

On the other hand, we found no association between the presence of *CYP1A1*2A*, *CYP1A1*2C* or *GSTM1* genes and lung cancer, although previous studies reported detecting genetic susceptibility to lung cancer in these genes [9, 10, 20, 21]. Also, our report indicated that the risk of the *GSTM1* -null plus *CYP1A1*2A* was not statistically significant in relation to smoking status.

We found that lung cancer risk was clearly associated with NAT2 intermediate-slow activity in individuals smoked low and significantly increased with the combination of NAT2 intermediate-slow activity and CYP1A2 high activity for never smokers.

Gene Polymorphisms of Metabolic Enzymes and Colorectal Cancer Risk

The CYP1A1 and CYP1A2 enzymes increased the activated PAHs, HCAs and several arylamines, formed by cooking of meats or fish but little tobacco smoking by *N*-hydroxylation. The hydroxylated forms, can eventually covalent bound with DNA adduct-induced, are potent as proximate carcinogen. *CYP1A2*1F* reported mainly the risk of colon and breast cancer [31, 32]. Among previous studies, *CYP1A1*2C* allele and *GSTM1* null has been associated with colorectal cancer risk [33, 34]. It is also reported that the highest colorectal cancer risk was associated with both high CYP1A2 activity and rapid NAT2 activity [35, 36].

We found in overall that the risk of colorectal cancer was not significant in these gene polymorphisms (Table 4.2) [37]**.** In never-smokers, the OR for *CYP1A1*2A* T/C genotype had a 3.06-fold increased risk of colorectal cancer **(**95% CI, 1.11−8.40; p = 0.030). The risk of NAT2 rapid genotype had a 5.38-fold increased risk of colorectal cancer (95% CI, 1.80−16.1; p = 0.003). We found no distribution of CYP1A1*2C, CYP1A2*1C, CYP1A2*1F or GSTM1 gene polymorphisms for colorectal cancer in never-smokers. In ever-smokers, the distribution of these genotypes was no association with colorectal cancer risk. Therefore, the association of *CYP1A1*2A* and *NAT2* polymorphisms for colorectal cancer risk was strongly increased compared with its in never-smokers. The *CYP1A1*2A* polymorphism was associated with a significantly increased risk of colorectal cancer in Japanese, although other studies were not detected [38, 39, 40]. Our findings support that the heterozygote for the rare *CYP1A1*2A* allele are expected to be at greater colorectal cancer risk without exposed to cigarette smoking in Japanese. *NAT2* rapid acetylator has a higher risk for colorectal cancer, explaining by the role of NAT2 in the *O*-acetylation to activation of *N*-hydroxy arylamines to potentially DNA-binding forms [41]. These results indicate that the NAT2 rapid acetylator seems to be at higher risk for colon cancers, in which *N*-acetylation is negligible and *O*-acetylation is an activation step such as a detoxification step such as HCAs [19]. We believe that the CYP1A1 activity and NAT2 rapid acetylator are increased in activating various carcinogens except tobacco mutagens in colon and rectum.

Additionally, we detected that the gene-gene interaction between *NAT2* and other polymorphisms was a significantly colorectal cancer risk in overall or never smokers, but not

ever-smokers. The risk of *NAT2* rapid plus combined *CYP1A1*2C* Ile/Val and Val/Val, *CYP1A2*1C* G/G or *GSTM1* null genotypes were significantly associated with colorectal cancer in overall (adjusted OR, 3.12; 95%CI, 1.15−8.51; p = 0.026 for *CYP1A1*2C*, adjusted OR, 3.25; 95%CI, 1.09−9.74; p = 0.035 for *CYP1A2*1C*, adjusted OR, 4.20; 95%CI, 1.09−16.1; p = 0.037 for *GSTM1*, respectively). In never-smokers, the risk of *NAT2* rapid plus combined *CYP1A1*2A* T/C and C/C, *CYP1A1*2C* or *CYP1A2*1F* A/A genotypes were specifically increased for colorectal cancer (adjusted OR, 15.9; 95%CI, 1.87−135.8; p = 0.011 for *CYP1A1*2A*; adjusted OR, 5.71; 95%CI, 1.49−21.9; p = 0.011 for *CYP1A1*2C*; adjusted OR, 9.14; 95%CI, 2.05−40.7; p = 0.004 for *CYP1A2*1F* , respectively). Therefore, the rapid NAT2 activity in combination with high CYP1A1 activity, high CYP1A2 activity, or low GSTM1 activity, may be strongly increased the final hydroxylated forms as ultimate carcinogens in colon and rectum.

Further, we observed a significant association with *CYP1A2*1F* A/A plus *CYP1A2*1C* G/G genotypes in never-smokers (adjusted OR, 6.16; 95%CI, 1.26−30.1; p = 0.025). This finding indicate that the joint association of *CYP1A2*1F* and *CYP1A2*1C* strongly lead to increase the CYP1A2 enzyme activity [13, 15, 16, 24]. The joint effects among *CYP1A1*2A*, *CYP1A1*2C*, *CYP1A2*1C* and *GSTM1* were also no association with colorectal cancer risk.

These results show that the combination of *NAT2* rapid plus *CYP1A1*2C*, *CYP1A2*1C*, or *GSTM1* genotypes is associated with the susceptibility to colorectal cancer. In particular, the combination of *NAT2* rapid plus other genotypes or *CYP1A2*1F* plus *CYP1A2*1C* seems to be remarkably increased association with colorectal cancer susceptibility in never-smokers.

DNA Repair Pathways

The DNA repair pathways, including nucleotide excision repair (NER), base excision repair (BER) and double-strand break repair (DSBR), play an important role in repairing the DNA damage resulting from chemical alterations of a single base, such as methylated, oxidized, or reduced bases (Table 4.1) [43, 44].

In the NER pathway, the xeroderma pigmentosum group D/ excision repair cross-complementing group 2 (XPD/ERCC2) protein is an evolutionarily conserved helicase, a subunit of transcription factor II H [44]. The BER pathway plays an important role in repairing the DNA damage resulting from chemical alterations of a single base, such as methylated, oxidized, or reduced bases [43]. The most stable product of oxidative DNA damage, 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxoG), causes G:C→T:A transversions, because 8-oxoG pairs with adenine as well as cytosine [45]. In human cells, the proteins that repair these mutations are 8-oxo-guanine glycosylase-1 (OGG1), which is involved in direct repair by 8-oxoG DNA glycosylase, and mutY homolog (MUTYH), which is involved in repair of adenine to 8-oxoG mismatch or that of guanine to 1,2-dihydro-2-oxoadenine (2-OH-A) mismatch due to its glycosylase activity [46, 47]. The 2-OH-A level is increased by exposure to reactive oxygen species [48]. The most stable product of oxidative DNA, Apurinic/apyrimidinic endonuclease-1(APEX1/APE1) exhibits 3′-phosphodiesterase activity that removes the abasic sites from cleaved DNA by OGG1 and MUTYH proteins [49]. X-ray cross-complementing group 1(XRCC1) acts as a scaffold for other proteins, such as DNA polymerase β, ligase III, and ADP-ribose polymerase, in the gap-filling step [50]. In addition,

smoking-induced oxidative DNA base modifications and single-strand breaks are repaired by the BER pathway.

In the DSBR pathway, X-ray repair cross-complementing groups 3 (XRCC3) participates in DNA double-strand break/recombination repair and likely participates [51]. Therefore, gene polymorphisms of DNA repair pathways may increase the risk of lung or colorectal cancer with respect to exposure to tobacco smoke.

Gene Polymorphisms of DNA Repair Pathways and Lung Cancer Risk

Genetic variation in DNA repair genes are thought to modulate DNA repair capacity and are suggested to be related to cancer risk [52].The variant alleles Asp312Asn and Lys751Gln in *XPD* have been associated with relatively high risks of lung cancer in Caucasian population [53, 54], but a recent study concluded that the *XPD* Lys751Gln are associated with a statistically significant lung cancer risk than Asp312Asn in the Chinese population [55]. In some patient- control studies, OGG1 Ser326Cys appeared to be associated with an increased risk for lung cancer [56-58], whereas the findings of this association study have been inconsistent [59]. The association between APEX1 Asp148Glu or XRCC1 Arg399Gln polymorphisms and lung cancer risk has been evaluated in a number of epidemiological studies [52, 60, 61]. A recent meta-analysis showed that the *XRCC1* 399Gln/Gln genotype was associated with an increased risk of lung cancer among Asians but not among Caucasians [59]. There are several reports that *XRCC3* Thr241Met polymorphism and lung cancer risk was associated in Caucasian population [62, 63].

We attempted to analyze the association among and between XPD Lys751Gln, OGG1 Ser326Cys, MUTYH Gln324His, APEX1 Asp148Glu, XRCC1 Arg399Gln and XRCC3 Thr241Met gene polymorphisms (Table 4.2) [64, 65]. The MUTYH His/His genotype was significantly associated with increased risk of lung cancer (adjusted OR 3.03, 95%CI 1.31−7.00, p = 0.010). In different histological types of lung cancer, the MUTYH His/His genotype was a significantly borderline association for both adenocarcinoma and squamous cell carcinoma, that suggested a potential interaction between this polymorphism and lung cancer risk regardless these subtypes. Moreover, a joint effect between tobacco smoking and the MUTYH His/His genotype for the risk of lung cancer was statistically increased in smokers (adjusted OR 3.82, 95%CI 1.22−12.00, p = 0.022), whereas that was not in non-smokers. This finding suggested that the effect of *MUTYH* Gln324His for lung cancer risk is not different between smoking habits. Previous study has shown that the identified variants of the *MUTYH* gene, containing Gln324His, were unlikely to predispose significantly to the risk for lung cancer in Caucasians [66]. The discrepancy between this study and ours might reflect the differences in genetic background, carcinogen exposure in different populations or sample sizes. Recent study has reported that the MUTYH enzyme activity in Gln324His polymorphism was only 66 % active from the substrates compared with the wild type [67]. It was reported that the 2-OH-A level compared to repair of adenine opposite 8-oxo-G was increased in human cancerous tissues compared to normal tissues [68]. Therefore, it is also possible that the MUTYH enzyme having 324His variation may have partially a reduced activity in repair of 2-OH-A opposite guanine. This suggested that *MUTYH* Gln324His might

also be associated with risk for lung cancer, related to the decreased MUTYH enzyme activity.

APEX1 Asp/Glu and Glu/Glu genotypes showed a increased risk for development of lung cancer **(**adjusted OR 2.78, 95% CI 1.58−4.90, p = 0.0004). The *APEX1* Asp/Glu and Glu/Glu genotypes was statistically significant for both adenocarcinoma (adjusted OR 2.24, 95% CI 1.18−4.25, p = 0.014) and squamous cell carcinoma (adjusted OR 4.75, 95% CI 1.79−12.60, p = 0.002). Moreover, the *APEX1* Asp/Glu and Glu/Glu genotypes was significantly increased (adjusted OR 3.61, 95%CI 1.74–7.50, p = 0.001), whereas that not in non-smokers. We found a strong statistically significant interaction between *APEX1* Asp148Glu and smoking. This polymorphism was located within the endonuclease domain of the protein [69], but it did not reduce endonuclease activity [70]. Instead it may lead to a reduced ability to communicate with other BER proteins, in turn leading to reduced repair efficiency, and a possibility that the Glu allele may have higher sensitivity to ionizing radiation [71]. A recent study reported an association between the *APEX1* 148Glu allele and increased risk in the development of lung cancer among light, current Japanese smokers [60]. Our findings are consistent with these previous studies and suggest that APEX1 variation may also play a role in predisposition to lung cancer.

XRCC1 Arg399Gln showed no statistically significant risk for lung cancer. The XRCC1 Arg399Gln was a borderline significant for adenocarcinoma, whereas that not for squamous cell carcinoma. The XRCC1 Arg399Gln were not statistically significant in relate to smoking status. For XRCC1 Arg399Gln variants, we found a tendency to increase on lung adenocarcinoma cancer risk. The XRCC1 Arg399Gln has associated with higher mutagen sensitivity and higher levels of DNA adducts [72]. It has previously reported to have an important genetic determinant of squamous cell carcinoma of the lung [73] or adenocarcinoma [74]. It was also reported that XRCC1 Arg399Gln might be prognostic factors in non-smoking female patients with lung adenocarcinoma [75]. It may be attributable to differences in the carcinogenesis pathways among the histological types of lung cancer.

We found that no significant effect was apparent between *XPD Lys751Gln***,** *OGG1* Ser326Cys, **or *XRCC3* Thr241Met** and lung cancer risk, in combination to smoking status. The *XPD* **Lys751Gln** have been observed a lower DNA repair capacity for UV-induced DNA damage in *XPD* 751Gln alleles [76]. The recent meta-analysis revealed an association between lung cancer and the *XPD* 751Gln alleles [77].

Our results didn't confirm an association between these polymorphisms and the risk of lung cancer. It has been reported that the *OGG1* Cys allele in Japanese patients is associated with an increased risk for lung cancer [57, 58]. The variant OGG1 is deficient in its catalytic activity, was not stimulated by the AP endonuclease [78]. A recent report has suggested that *OGG1* Ser326Cys is not associated with lung cancer by meta-analysis [60]. The *XRCC*3 241Met allele has previously been associated with less efficient DNA repair and eliminated aberrant cells with mitotic defects [72, 79]. However, several studies have also been shown to explain the lack of association between *XRCC3* Thr241Met and lung cancer risk in Caucasian population [80, 81]. Therefore, our finding in a Japanese population is consistent with the results from these studies.These results suggest that the *MUTYH* Gln324His and *APEX1* Asp148Glu **gene** polymorphisms appear to play an important role in modifying the risk for lung cancer in the Japanese population.

Gene Polymorphisms of DNA Repair Pathways and Colorectal Cancer Risk

DNA repair genes are increasingly being studied for cancer risk because of their critical role in maintaining genome integrity. *XPD* Lys751Gln, *OGG1* Ser326Cys, *APEX1Asp148Gln, XRCC1* Arg399Gln *and XRCC3* Thr241Met have also been linked to a risk of colorectal cancer [82-86]. In *MUTYH* gene, it was shown that the inherited variants Tyr165Cys and Gly382Asp have been associated with colorectal tumors in Caucasians, not in East Asians including Japanese [87-89]. Recent studies reported that MUTYH Gln324His mutation was the most frequent mutation in Japanese patients with adenomatous polyposis, and the gene polymorphisms was associated with the risk of proximal colon cancer in the Japanese population [90, 91]. To our knowledge, few previous studies have examined the effect of these polymorphisms on the association between smoking and colorectal cancer [92, 93]. The *XPD Lys751Gln, OGG1* Ser326Cys, *MUTYH Gln324His, APEX1 Asp148Glu, XRCC1 Arg399Gln and XRCC3 Thr241Met* gene polymorphisms were analyzed to evaluate genetic susceptibility to colorectal cancer and the possible modification effect on the relationship between smoking and colorectal cancer risk (Table 4.2) [94].

The *MUTYH* Gln/His and His/His genotypes, and *APEX1* Asp/Glu and Glu/Glu genotypes carry a significant risk for carcinogenesis of colorectal cancer *(*adjusted OR3.53, *95%CI* 1.44−8.70, p = 0.006 for *MUTYH;* adjusted OR 2.33, *95%CI* 1.21−4.48, p = 0.011 for *APEX1*, respectively). For subsites, these genotypes were statistically significant for colon cancer (adjusted OR 3.95, 95%CI 1.28−12.20, p = 0.017 for *MUTYH*; adjusted OR 3.04, 95%CI 1.38−6.71, p = 0.006 for *APEX1*, respectively), but not for rectal cancer. Therefore, the cancer subsite-specific study indicated that the *MUTYH Gln324His* and *APEX1 Asp148Glu* have a colon cancer-specific risk. Tao *et al*. reported *MUTYH* Gln324His in Japanese was statistically significantly associated with increased risk of proximal colon, but not distal colon or rectal cancer [91]. Therefore, their results are consistent with our study. Moreover, a recent study found that the activity of *MUTYH* Gln324His *is 34% less active than that of wild type [67]*. 8-oxo-G is generated by direct oxidation of DNA by a hydroxyl radical, whereas 2-OH-A is exclusively generated by oxidation of dATP in the nucleotide pool [46, 47]. The 2-OH-A level is increased in human cancerous tissues compared to normal tissues [95]. Thus, for colorectal cancer, it is also possible that the enzyme of *MUTYH* Gln324His may have partially impaired in repair of 2-OH-A opposite guanine, compared to repair of adenine opposite 8-oxo-G, because of the difference in the origin of each oxidized base. Furthermore, the *APEX1* Asp148Glu genotype has a specifically association with colon cancer risk. A previous study reported that this genotype was especially an increased risk of colon cancer risk [96].

Moreover, a joint effect between tobacco smoking and the *MUTYH Gln324His* for the risk of colorectal cancer showed a significant association with colorectal cancer risk in non-smokers (adjusted OR 4.08, 95%CI 1.22−13.58, p = 0.022), but not in smokers. These results show that the *MUTYH Gln324His* are associated with colorectal cancer susceptibility with never smoking history. The *APEX1 Asp148Glu* in smokers was significantly increased (OR 5.02, 95%CI 1.80−13.99, p = 0.002), whereas that in non-smokers did not show a significant. Smokers with the *APEX1 Asp148Glu* showed an increased risk of colorectal cancer. A previous study didn't found about the effect of smoking habit on association between the

APEX1 Asp148Glu genotype and colorectal cancer risk [96]. This polymorphism is located within the endonuclease domain of the protein [73], but it does not reduce endonuclease activity [97]. The 148Glu allele has also been associated with increased mitotic delay after exposure to ionizing radiation [71].

In contrast, the *XPD Lys751Gln*, *OGG1* Ser326Cys, *XRCC1 Arg399Gln and XRCC3 Thr241Met* were not statistically significant for overall, colon cancer, rectal cancer, or in relation to smoking status. Previous reports have suggested that *OGG1* Ser326Cys is associated with colorectal cancer in Caucasians [83, 98], but not among Koreans [99]. The *XRCC1* 399Gln allele has been linked with a reduced risk of colorectal adenomas [84, 85], and XRCC1 has also been associated with improved progress in patients who underwent chemotherapy, but not in those who received surgery alone [83]. The smoking has an effect on colon adenoma risks among carriers of *XRCC1* codon 399 Arg alleles [92, 93]. Recent report suggested that the Thr241Met polymorphism of the *XRCC3* gene can modify the risk of colorectal cancer [86]. However, our finding in a Japanese population is not consistent with these results. While, the recent study suggested that *XPD Lys751Gln may not be associated with colorectal cancer development in meta-analysis [100]*.

The *MUTYH* Gln324His and *APEX1* Asp148Glu polymorphisms are important risk factors for colorectal cancer, especially colon cancer, in the Japanese population. In particular, the *MUTYH* Gln324His is associated with colorectal cancer susceptibility in never smoking history, whereas the *APEX1* Asp148Glu constitutes an increased risk of colorectal cancer in combination with smoking exposure.

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Chapter 5

The Effect of Cigarette Smoking on Progression in Different Laryngeal Lesions

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Abstract

The association between cigarette smoking and an increased risk of laryngeal carcinoma has been definitely demonstrated in numerous studies. The aim of the present study was to assess the prevalence of smoking habit in patients with different laryngeal pathologies. The prevalence of cigarette smoking was compared between patients with laryngeal tumors and those with nonmalignant laryngeal lesions. Data on all patients with indications for direct microlaryngoscopy at ENT Department, Split University Hospital Center, during a five-year period were analyzed. The study included 562 patients with various laryngeal pathologies, divided into three groups as follows: group 1, benign lesions; group 2, precancerous lesions; and group 3, tumors. The majority of patients (82.92%) had a long history of smoking. The proportion of smokers was lowest in benign lesion group (72.13%), higher in precancerous lesion group (81.48%) and highest in malignant lesion group (97.14%). There was a statistically significant difference in the prevalence of cigarette smoking between patients with laryngeal tumors and those with benign or precancerous lesions (χ^2=68.5; P=0.00). The mean number of cigarettes per day was 20.54±14.80, and was lowest in benign lesion group (15.67±13.41) and highest in malign lesion group (26.33±12.70). The mean length of smoking habit was 26.44±16.92 years, ranging from 19.57±16.03 years in benign lesion group to 35.20±12.12 years in malign lesion group. Collectively, ozr results clearly pointed to the increased prevalence of laryngeal diseases in smokers, with a statistically significant difference between patients with benign laryngeal lesions and those with laryngeal tumors. A great part of

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these lesions are reversible in the initial stage with smoking cessation. Therefore, there is only one and obvious advice: quit smoking now and forever.

Keywords: Cigarette smoking, precancerous lesion, laryngeal tumor

Introduction

Smoking is inhalation of smoke produced by burning tobacco leaves in a cigarette, cigar or pipe. North America is considered the original habitat of tobacco. Archeological finds excavated in the area once populated by the Amerindian Mayan tribe show priests smoking pipes during religious ceremonies.

The Amerindian tribes used to smoke the pipe of peace at various rituals, especially on terminating warfare and making peace [Figure 1]. The history of tobacco and various smoking related events and consequences for the humankind dates back to 1492, when Christopher Columbus discovered the North American continent. Columbus' sailors and followers were the first European smokers [1]. The Spaniards started planting tobacco in San Domingo in 1550. The term "tobacco" was coined after the Tobago Island, wherefrom the first international traders imported tobacco. In 1560, Jean Nicot, French ambassador in Portugal, brought tobacco seeds to the Portugal royal court and presented it to the queen Catherine de Medici as a medicinal and miraculous plant. The Latin name of tobacco was then coined after his name as *Nicotiana*. They transferred the plant to Europe, first to Spain and Portugal, wherefrom it was disseminated all over the European countries and other continents.

Figure 1. Indians smoke the pipe.

In the 18th century, snuff held sway; the 19th century was the age of the cigar; the 20th century saw the rise of the manufactured cigarette and with it a greatly increased number of smokers. Although the worldwide use of tobacco has steadily increased since the 16th century, early public statements showed its disapproval as stated by James I of England in his Counterblast to Tobacco in 1604: "Smoking is a custom loathsome to the eye, hateful to the nose, harmful to the brain, dangerous to the lungs, and in the black, stinking fume thereof nearest resembling the horrible Stygian smoke of the pit that is bottomless." Thus, even though its health risks have been acknowledged for centuries, tobacco use throughout the world continues to increase.

The five centuries of tobacco smoking can be divided into two periods according to opinion about the beneficial and detrimental effects of this phenomenon. The first period that lasted for more than four centuries was the era of empiricism, based on the good and bad experiences of smokers and nonsmokers.

However, there were no strong arguments or evidence on either side. At that time, the attitude toward smoking ranged from attributing medicinal properties to tobacco through placing a ban on smoking, with offenders being severely punished including corporal punishment, even death penalty (Turkey), and confiscation of property. Tobacco was long attributed medicinal properties, e.g., tobacco juice was recommended in the treatment of "French disease", i.e. syphilis, tobacco leaves were applied over lesions to facilitate healing or were used to relieve inflammation and headache. Tobacco enema was used in the management of constipation. Until 1812, British military physicians had devices for medical use of tobacco smoke always available. Tobacco snuffing was a transitional form between tobacco medical use and use for pleasure. Initially, tobacco snuff was used to relieve headache and ocular pain, but then over decades it turned to a trendy phenomenon characterized by some specific rituals and ceremonies.

At the beginning of the 20th century, the first researches of detrimental effects of tobacco on human health, economy and state as a whole were launched, thus opening the second (scientific) period in terms of attitudes toward tobacco smoking. Nicotine, the pure major tobacco alkaloid, was isolated around 1828. In 1856, after the Crimean War, industrial cigarettes spread rapidly all over Europe, followed by large-scale cigarette smoking peaking between the two world wars. In 1936, results of the first studies in mice were published, demonstrating that tobacco smoke inhalation caused airway cancer in mice.

The association between tobacco and lung cancer was initially demonstrated by Doll and Hill in the 1950s in the UK [2]. Since then, additional case-control studies [3] and prospective cohort studies [4] have all affirmed the association between tobacco and the development of lung cancer. Indeed, lung cancer was rare in the early decades of the 20*th* century, but with the increase in smoking tobacco, it has become an alarming epidemic.

Carcinogenicity of tobacco tar substances was demonstrated in 1953. In 1960, a decree was passed in the USA on each cigarette package to carry a label explicitly stating the health risk of cigarette smoking. In 1964, a report published by the American health service (developed by Luther Terry, director of the American health service, and 150 scientists from various fields) pointed to cigarette smoke as the main culprit causing numerous diseases and death. This report was widely known as "Terry's bomb" because it came as surprise condemning tobacco smoking and proclaiming tobacco smoke as the cause of diseases and premature death [5]. The First World Conference on Smoking and Health was held in 1967,

and in 1971, the World Health Organization (WHO) decided on systematic struggle against tobacco smoking and defined it as a type of dependence.

According to current WHO estimate, the rate of tobacco smoking is 41% of men and 21% of women in industrialized countries, while the respective percentage in developing countries is 48% and 8%, yielding one 1 billion and 200 million people worldwide, with about 5 million smoking related deaths *per* year [6].

Tobacco is the second most common cause of death in the world. Half of current smokers, i.e. around 650 million people, will probably die from adverse tobacco effects. Data on hundred thousands of people having never smoked who will die from diseases caused by inhaling environmental tobacco smoke are as disturbing indeed.

In 2003, the mean rate of cigarette smoking in the European Union (EU) was 28.4% in old member countries and 30.3% in new member countries. In Europe, the highest rate of cigarette smoking was recorded in Albania (39%), followed by Bosnia and Herzegovina (37.6%), Serbia, Montenegro and Macedonia (36% each) and Russian Federation (35.8%), whereas the lowest rate was found in Sweden (17.5%), Belgium (20%), Finland (23%), Slovenia (23.7%) and Croatia (27.4%).

More than 4000 chemicals have been identified in tobacco smoke, and some 60 are known or suspected carcinogens [7]. Each cigarette brings approximately 10 mg of soot, tar, ash, phenols, benzpyrene, hydrogen cyanide, formaldehyde, and radioactive polonium 210 into the lungs of the smokers.

Nicotine is one of the most detrimental substances in tobacco smoke. Following initial excitation of the central nervous system (respiratory center, vasomotor center, vomiting center), additional dose increase leads first to tremor and seizures, and further increase to lethal dose results in paralysis and death. The action of nicotine upon adrenal gland leads to the release of epinephrine and norepinephrine, which in turn results in heart rate increase, microvascular constriction and blood pressure elevation. In addition to these effects, tobacco smoke components inhaled to the lungs pass to the circulation and cause lesions to the vascular endothelial cells.

Carbon monoxide (CO), binding to hemoglobin 200 times faster than oxygen, is one of the harmful tobacco smoke compounds. In smokers, 10%-15% of hemoglobin may be bound to CO, thus considerably reducing the body oxygen supply, which is a highly adverse effect in individuals with cardiac diseases, angina pectoris in particular. It also increases vascular wall permeability for cholesterol and favors atherosclerotic plaque formation.

In young male smokers (aged 35-54), the rate of sudden cardiac death is 2- to 4-fold that in age-matched nonsmokers [8]. In pregnant smokers, fetal oxygen supply is reduced by CO, thus posing a risk for fetal development. Therefore, pregnant women frequently give birth to low birth weight neonates, while sudden infant death is also more common in infants exposed to environmental tobacco smoke.

Tobacco smoking is currently considered the main risk factor for bronchial, pulmonary, oral oral, laryngeal, nasal and nasal sinus, pharyngeal, esophageal, pancreatic, renal and bladder carcinoma, while squamous cell carcinoma of the cervix, gastric cancer and myeloid leukemia are more common in smokers.

Some conditions are significantly more frequently found in smokers, e.g., coronary heart disease, cerebrovascular disease, atherosclerotic aortic aneurysm and atherosclerotic peripheral vascular disease. Along with hyperlipidemia (increased blood lipids) and arterial hypertension (elevated blood pressure), smoking is a major risk factor for vascular disease.

Smoking is the main cause of numerous lung diseases. Tobacco smoke contains a number of irritants that stimulate mucus formation and lead to ciliary epithelium dysfunction and bronchiole narrowing, and eventually to chronic obstructive pulmonary disease (COPD). In smokers, COPD mortality is 6-fold that in nonsmokers. In addition, smoking is a predisposing factor for respiratory infection and asthma exacerbation.

Tobacco smoking influences reproductive health. The women smoking more than 20 cigarettes a day are at a higher risk of primary tubal factor infertility and ectopic pregnancy. The women smoking in pregnancy have a higher risk of giving birth to low birth weight neonates, fetal death and premature delivery. Smoking women more frequently suffer from menstrual impairments (painful, irregular menstruation) and earlier menopause (by 2-3 years), thus earlier cessation of the estrogen protective action against osteoporosis and cardiovascular diseases.

Environmental tobacco smoke exerts its detrimental effect on nonsmokers found in premises filled with smoke [9]. Nonsmokers living with smokers are at a 20%-30% higher risk of bronchial and lung carcinoma, and 25%-30% higher risk of coronary disease. In infants and small children, inhaling tobacco smoke frequently results in the development of bronchitis, pneumonia, asthma, reduced pulmonary function, acute and chronic otitis media. Recent studies indicate that exposure to environmental tobacco smoke doubles the risk of macular degeneration in the elderly as the main cause of vision loss in EU.

Because of the demonstrated highly adverse effects of tobacco smoke on nonsmokers, legal acts restricting and forbidding tobacco smoking in many public premises have been adopted in most countries [10].

The majority of adult smokers had their first cigarette lit before having completed their high school education. Among other decrees, it is forbidden to sell tobacco products to individuals younger than 18 and by cigarette machines. Important adult smokers, such as family members, movie stars, athletes and others idolized and impersonated by children and adolescents in particular have great impact on them. Tobacco smoking is not just a health risk by itself, as research results show that adolescent smokers are at a higher risk of alcohol and psychoactive drug abuse than their peer nonsmokers are.

The Centers for Disease Control and Prevention (Atlanta, USA) and WHO have launched the Global Youth Tobacco Survey (GYTS) with the aim to perceive the issue of tobacco use in young people from various standpoints. In Croatia, GYTS was conducted in 2002 and 2006, including children aged 13 to 15. Survey results revealed 67.1% and 59.9% of study children to have tried smoking or experimented with cigarettes in 2002 and 2006, respectively, while every fourth study subject (24.8%) reported current cigarette smoking in 2006 (16.6% in 2002). Although still very young, almost half of current smokers (41.7%) reported their wish to quit smoking [11].

Initial experimentation with smoking arises from curiosity or due to peer group influence, later it turns to pleasure and habit, along with development of the self-medication phenomenon.

A smoker tends to maintain a certain level of substance concentration in the blood; when it falls below that level, he/she will light another cigarette, and if not, then he/she feels a strong need of cigarette accompanied by withdrawal symptoms (i.e. irritability, concentration difficulties, tiredness and depressive mood, increased appetite and weight gain).

There is a clear dose-response relationship between cancer risk and tobacco use. A lifetime smoker is at a 20- to 30-fold risk of a nonsmoker [12]. Worldwide, cancer is

responsible for 1 of 8 deaths (more than HIV/AIDS, tuberculosis and malaria combined, and tobacco use is responsible for one-third of all cancer-related deaths. The International Agency for Research on Cancer (IARC) estimated that there were approximately 12.7 million new cases of cancer diagnosed in the world in 2008, and 7.6 million deaths attributed to it [13].

Furthermore, tobacco is responsible for 87% of all deaths attributable to lung cancer, now the single most common cancer in the world. Currently ranking ninth, it is estimated that by 2030, lung cancer will be the sixth most common cause of death in the world [10, 14].

Tobacco smoking-attributable illness extends beyond cancer and includes stroke, heart attack, and COPD. Indeed, total tobacco-attributable deaths are projected to rise from 5.4 million in 2005 to 6.4 million in 2015 and to 8.3 million in 2030, with estimated 600,000 deaths attributable to second-hand smoke [14]. These projections are based on models that show a three- to four-decade lag between the rise in smoking prevalence and the increase in smoking-attributable mortality that results from it.

Worldwide, cigarette consumption is increasing at a rate of about 3% annually [6]. In Asia, Southern and Eastern Europe, and developing countries, tobacco use is increasing at about 8% *per* year. Yet, in some industrialized countries, smoking rates are decreasing, while the global burden of lung cancer has shifted significantly from approximately 31% to up to 55% of cases occurring in developing countries [15]. Although the number of adult smokers in the USA has declined appreciably, from 42.4% in 1965 to 20.6% in 2008, the persistently large burden of tobacco use is distributed unequally across different classes, races, ethnicities, and geographies [Figure 5,2]. The estimated numbers of lung cancer cases worldwide has increased by 51% since 1985 (+44% in men and +76% in women). In men, this increase is due solely to population growth and aging; in fact, there has been a small (3.3%) decrease in the actual age-standardized incidence (risk). However, the ASRs have increased by 22% in women.

This overall upward trend disguises considerable difference between countries. This makes the widening disparities in cancer-related mortality between developed and developing countries even more tragic. Indeed, the WHO estimates that 40% of all cancers diagnosed today could have been prevented, partly by maintaining healthy diet, promoting physical activity, and preventing infections that may cause cancer, but largely through tobacco control [16,17].

China is the biggest tobacco market, based on total cigarettes consumed. Some 350 million smokers in China consume around 2200 billion cigarettes a year, or about 41% of the global total [18]. However, the industry in China is state owned. Outside of China, the four largest publicly listed international tobacco companies account for 46% of the global market. High-tar cigarettes, banned in developed countries, continue to be sold in the developing world. For example, nicotine contents for Indonesian *kreteks* or clove cigarettes are between 1.7 and 2.5 mg *per* stick compared with <0.05 and 1.4 mg per stick for cigarettes sold in the USA [19].

Yet, in some industrialized countries, smoking rates are decreasing at about 1% a year, largely due to the implementation of significant anti-tobacco programs. In China and many other developing countries, the rate of tobacco-related deaths is rising rapidly. Indeed, lung cancer rates in China have already been increasing by about 4.5% a year.

The Family Smoking Prevention and Tobacco Control Act, a United States federal law that gives the Food and Drug Administration (FDA) the power to regulate tobacco industry, was signed into law on June 22, 2009 by President Barack Obama.

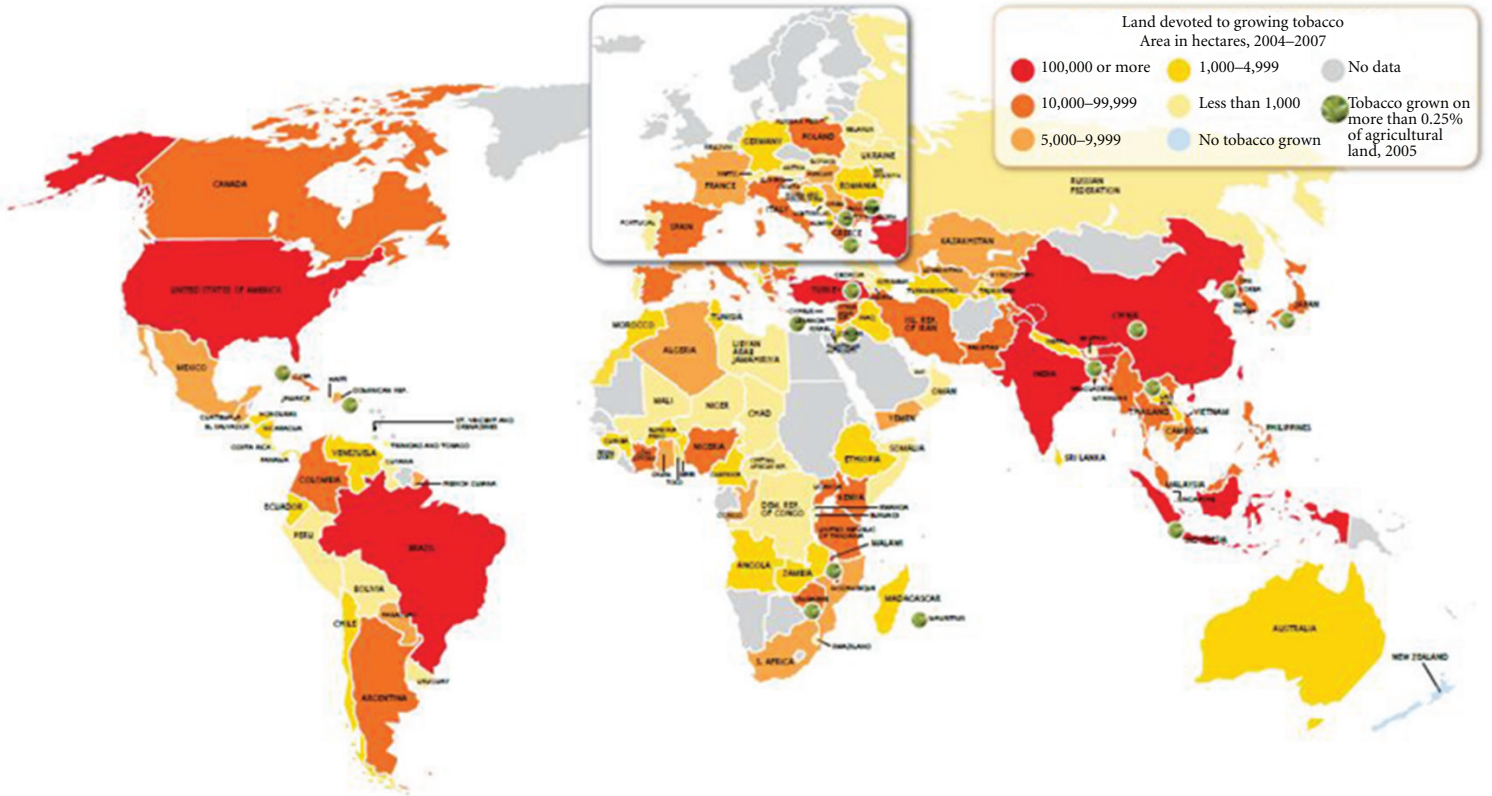

Figure 2. World land devoted to growing tobacco. *The Tobacco Atlas, 3rd Edition.* American Cancer Society 2009, http://www.cancer.org/. All rights reserved."

The Tobacco Control Act requires that cigarette packages and advertisements have larger and more visible graphic health warnings (including nine new textual warning statements and color graphics depicting the negative health consequences of smoking) and prohibition on the manufacture of products that use the terms “light,” “low,” “mild”, and similar descriptors [20].

In Croatia, the most common seat of cancer in men were bronchi and lungs (20%), while laryngeal carcinoma ranked sixth (4%) in 2007 [21]. In the 40-49 age group, laryngeal carcinoma was the second most common carcinoma, following only bronchial and lung carcinoma. In women, lung carcinoma ranked fourth (6%), while laryngeal carcinoma was by far less common (0.9%). In EU countries and in Croatia, the incidence of laryngeal carcinoma was 12.3/100,000 and 15.3/100,000, respectively. In men, the incidence rate was 25-fold that in women.

Cigarette smoking is a major factor of laryngeal carcinogenesis. In smokers, the relative risk of laryngeal tumor development is 5- to 30-fold that recorded in nonsmokers, it increases with early onset of smoking habit and depends on the length of smoking habit and association with other risk factors. Maier *et al*. report on 96.5% of patients with squamous cells carcinoma of the larynx to be smokers, with a 5.6 relative risk of carcinoma in smokers [22]. DeStefani *et al*. found 97.2% of smokers and a relative risk of 14.7 [23]. Wunder *et al*. report on a 13.5 and 34.4 relative risk of tumor development in subjects smoking up to 20 and more than 20 cigarettes daily, respectively [24]. In the study by Falk *et al*., the relative risk of squamous cell carcinoma was 6.0 in subjects smoking up to 30 cigarettes and 19.2 in those smoking more than 30 cigarettes daily [25]. The risk tended to decrease in former smokers with at least 5-year history of nonsmoking, to reach the level observed in nonsmokers after 15 years [26,27].

In Croatia, 27.4% of the population over 18 years of age smoke daily. The high prevalence of smoking habit among young individuals aged 18-29 is a cause for concern, with special reference to east Croatia where the prevalence rises to as high as 46% [11]. Statistical data show a male predominance of smoking (34% of male *vs*. 22% of female). Polls taken in Istria revealed adolescents in Istria County to start smoking as early as age 12-13 (32.09%); the habit of cigarette smoking was more regularly practiced by female adolescents (35.72%).

The association between cigarette smoking and an increased risk of laryngeal carcinoma has been definitely demonstrated in numerous studies; however, tobacco smoke causes laryngeal mucosa lesions, thus certainly favoring the development of other laryngeal diseases as well. Therefore, the aim of the present study was to assess the prevalence of smoking habit in patients with different laryngeal pathologies. The prevalence of cigarette smoking was compared between patients with laryngeal tumors and those with nonmalignant laryngeal lesions.

Patients and Methods

Data on all patients (N=562) undergoing direct microlaryngoscopy at ENT Department, Split University Hospital Center, Split, Croatia, over a five-year period were collected and analyzed. Results were processed by standard statistical methods and are presented in tables.

Results

The study included 562 patients with different laryngeal pathologies, divided into three groups according to histopathologic diagnosis as follows: 244 (43.4%) patients with benign laryngeal lesions (e.g., polyps, cysts, hemangiomas, etc.) including patients with normal histopathology findings; 108 (19.2%) patients with precancerous laryngeal lesions, including laryngeal papilloma; and 210 (37.4%) patients with malignant lesions of the larynx [Table 1].

Whereas the benign lesion group showed an equal sex distribution, male patients predominated in the precancerous lesion and malignant lesion groups (χ^2=34.8; *P*=0.00). Overall, there were 408 (72.6%) male and 154 (27.4%) female patients [Table 2].

Patient distribution according to age (age range 19-81; mean age 53.53±14.21 years) is shown in Table 2. Duncan test following analysis of variance at the level of significance revealed the malignant lesion group to be statistically significantly older than the benign and precancerous lesion groups (F=15; *P*=0.00).

Table 1. Age distribution of study patients

	Histopathologic diagnosis			
Age (yrs)	Benign lesion group	Precancerous lesion group	Malign lesion group	Total
Mean ± SD	48.14±14.09	53.13±14.81	61.41±10.08	53.53±14.21
Total	244 (43.4%)	108 (19.2%)	210 (37.4%)	562 (100%)

Table 2. Sex distribution of study patients

	Histopathologic diagnosis			
Sex	Benign lesion group	Precancerous lesion group	Malign lesion group	Total
Male, n (%)	116 (20.6)	94 (16.7)	198 (35.3)	408 (72.6)
Female, n (%)	128 (22.8)	14 (2.5)	12 (2.1)	154 (27.4)
Total, N (%)	244 (43.4)	108 (19.2)	210 (37.4)	562 (100)

Table 3. Patient distribution according to cigarette smoking, number of cigarettes *per* day and length of smoking habit

	Histopathologic diagnosis			
	Benign lesion group	Precancerous lesion group	Malign lesion group	Total
Smokers n (%)	176/244 (72.13)	88/108 (81.48)	204/210 (97.14)	466/562 (82.92)
Cigarettes *per* day mean ± SD	15.67±13.41	23.04±18.20	26.33±12.70	20.54±14.80
Length of smoking (yrs), mean ±SD	19.57±16.03	28.70±19.61	35.20±12.12	26.44±16.93

Most study patients (82.92%) had a long history of cigarette smoking [Table 3]. The proportion of smokers was lowest in the benign lesion group (72.13%) and highest in the malignant lesion group (97.14%).

There was a statistically significant difference in the prevalence of smoking habit between patients with laryngeal tumors and those with benign or precancerous laryngeal lesions (χ^2=68.5; *P*=0.00).

The mean number of cigarettes daily was 20.54±14.80; it was lowest in the benign lesion group (15.67±13.41 cigarettes) and highest in the malignant lesion group (26.33±12.70 cigarettes). The mean length of smoking was 26.44±16.93 years; it was also shortest in the benign lesion group (19.57±16.03 years) and longest in the malignant lesion group (35.20±12.12 years).

Discussion

In the group of patients with malignant laryngeal tumors, the prevalence of cigarette smoking was 97.14%, which is consistent with literature data. In Croatia, the prevalence of smoking among women has been on an increase in recent years. The mean rate of smoking habit in women worldwide is 12%, whereas in Croatia it reaches 22% or even more in particular areas. In Istria County, 35.72% of female high school students smoke regularly *vs.* 34.26% of their male counterparts. However, the marked increase in the rate of cigarette smoking in female population does not appear to be associated with an increased prevalence of laryngeal tumors in this population group. In the present study, a total of 12/210 (5%) malignant tumors were diagnosed in female patients, of which only one was nonsmoker. Other factors (e.g., hormonal, lower number of cigarettes daily, etc.) must also be involved in carcinogenesis in women [28].

In the benign lesion group, there were 72.13% of smokers, yielding a statistically significant difference from the malignant lesion group with 97.14% of smokers (χ^2=68.5; *P*=0.00). Polls taken at the national level show the mean rate of smoking habit in Croatia to be 27.4%; accordingly, the number of smokers in the benign, precancerous and malignant lesion groups was 2.6-fold, 2.9-fold and 3.5-fold mean rate recorded in Croatia.

Comparison of the benign and malignant lesion groups revealed the latter to be characterized by a significantly older age (48.14 *vs.* 61.41 years), greater number of cigarettes daily (15.67 *vs.* 26.33 cigarettes), and longer history of smoking (15.67 *vs.* 35.20 years).

A number of factors have been implicated in the increased prevalence of tumors in smokers. Some fifty compounds with known carcinogenic effects have been isolated from tobacco smoke [27]. These mostly include tar substances (e.g., polycyclic aromatic hydrocarbons) and many other carcinogens such as toluidine, urethane, polonium, naphthylamine, vinyl chloride, etc. One of the adverse tobacco smoke compounds is CO, which binds to hemoglobin 200 times faster than oxygen. In smokers, 10% to 15% of hemoglobin can be bound to CO, thus considerably reducing the body oxygen supply, which may pose great risk in individuals with heart diseases, angina pectoris in particular. In addition, the vascular wall permeability for cholesterol increases, thus favoring the atherosclerotic plaque formation.

The increase in the number of smokers correlates directly with the increase in the prevalence of lung carcinoma. In China and many other developing countries, the rate of tobacco-related deaths is rising rapidly. Indeed, the rate of lung cancer in China has already been increasing by about 4.5% a year.

A significant increase in lung carcinoma in women recorded in the past 20 years, which correlates directly with the increase of cigarette smoking in women, should also be noted. On the other hand, the rate of laryngeal carcinoma in women showed no increase in spite of the increasing smoking habit recorded in this population group. The significantly higher prevalence of lung carcinoma than laryngeal carcinoma in smokers is definitely associated with some anatomic determinants. The carcinogenic effect of tobacco smoke has been clearly demonstrated, thus a higher prevalence of carcinoma is expected to be proportional to the level of tissue exposure to tobacco smoke. On smoking, tobacco smoke passes over laryngeal mucosa and ends in pulmonary alveoli, where it stays until expiration. Therefore, pulmonary alveolar cells are exposed to the adverse action of tobacco smoke for a considerably longer time, while smoke in part remains there in the form of residual lung volume, thus protracting the harmful effects of nicotine and other carcinogenic tobacco smoke compounds. In addition, some other factors such as poor dietary habits, stress, alcohol, etc. intensify the action of these carcinogenic compounds. The lower prevalence of laryngeal carcinoma in female smokers is related to hormonal changes. Glottal disease (Reinke's edema) occurs exclusively in middle-aged female smokers; on the other hand, in women the prevalence of laryngeal carcinoma is 25 times lower as compared with men [29]. Laryngeal tumors usually occur in the elderly, with 70% of patients aged 50-70, only 1% aged <30 and 0.1% younger than 15, mostly children treated with radiotherapy for benign laryngeal lesions, juvenile papilloma in most cases [30,31]. Only 54 squamous cell carcinoma cases, 32 in male and 19 in female children, were documented in 1980 [32]. Seven tumors were recorded in children aged 1-5, 14 tumors in children aged 6-10, 35 tumors in children aged 11-15, and two tumors in children of unknown age.

Certain malignant tumors originate from one malignantly altered cell, so-called monoclonal malignant cell, whereas others develop by progression from precancerous lesions [33]. Lung carcinoma generally develops by malignant mutation of one or more cells due to their exposure to prolonged and intensive action of tobacco smoke. Laryngeal mucosa epithelium is less exposed to the action of tobacco smoke than cells of pulmonary alveoli, and there also are considerable anatomic differences between these two types of epithelium. While bronchioles are lined with ciliated stratified columnar epithelium, thinner bronchioles are covered by ciliated simple columnar epithelium, and alveoli are lined with thin epithelium composed of thin anuclear cells and small nucleated cells. Laryngeal mucosa is composed of stratified squamous epithelium and ciliated stratified columnar epithelium. The glottis, lingual aspect of the epiglottis and inner aspect of arytenoid cartilage are lined with stratified squamous epithelium. It consists of three layers*: stratum basale* (basal layer) lies on the basal membrane and consists of one row of columnar cells; *stratum spinosum* (malpighian layer) composed of several cell rows with well pronounced intercellular bridges, so that cells do not adhere to each other, with mitotic figures seen in this layer; and *stratum superficiale* (superficial layer) composed of several rows of flattened squamous cells that undergo desquamation but usually not keratinization.

The rest of laryngeal mucosa is lined with ciliated stratified columnar epithelium that contains a number of mucus secreting goblet cells. It also consists of three layers: basal layer

consists of one row of cuboid cells; intermediate layer is composed of columnar or cuneiform cells, most of them with numerous endings; and superficial layer composed of columnar ciliated cells and goblet cells. The lower parts of these cells reach up to the basal membrane.

Almost 99% of malignant laryngeal tumors arise from squamous epithelium, although it covers only a minor part of laryngeal mucosa. Some of these tumors arise from a single malignantly altered monoclonal cell. However, inherited genetic instability of malignant cells, and environmental and micro-environmental effects rapidly lead to biological variations, i.e. phenotypic and genotypic changes of malignant cells and their metastases (malignant cell heterogeneity) [34].

This means that some tumors show signs of malignancy from the very onset, whereas others pass through certain stages. Therefore, some patients reported hoarseness for only a few months, whereas in others hoarseness had persisted for years before they were diagnosed with laryngeal carcinoma. Such observations are additionally supported by frequent finding of dysplastic lesions of various stages along tumor edges. Dysplastic lesions always arise from the stratified squamous epithelium cells. Epithelium thickness is gradually reduced with aging, so the basal layer of stratified squamous epithelium cells come closer to the surface and the action of tobacco compounds. In young individuals, epithelial thickness is around 50 microns, whereas in the elderly it is only 29.9 microns. Thus, the cells of the stratified squamous epithelium basal layer are more exposed to the action of tobacco smoke. In addition, in the elderly the index of cell division is twofold that in children (1.72 *vs*. 0.88).

On the other hand, the area of stratified squamous epithelium is expanding to the area of stratified columnar epithelium with aging, in smokers in particular, which can be excellently visualized by contact endoscopy [35] [Figure 3]. That is why laryngeal tumors are rare before age 30 and then they are generally associated with radiotherapy for laryngeal papilloma in childhood. In smokers, stratified squamous epithelium expands to the area of stratified columnar epithelium, i.e. the fine columnar epithelium is replaced by the more resistant squamous epithelium, thus reducing mucus secretion, which results in dry throat and cough.

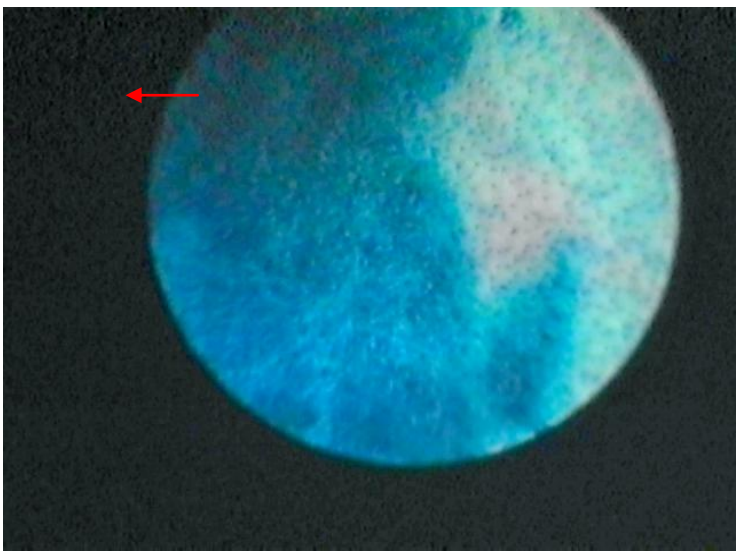

Figure 3. Spreading of stratified squamous epithelium to the area of stratified columnar epithelium, contact endoscopy, 60x.

The expansion of stratified squamous epithelium influences mucociliary transport of the mucosa, thus slowing down the removal of nicotine compounds from mucosal surface, i.e. protracting and cumulating their action.

Stratified squamous epithelium develops only after birth, and with aging it also spreads to other parts of the larynx, especially after age 40, and in chronic irritation of laryngeal mucosa.

The stratified columnar epithelium cilia are responsible for the mucociliary transport of discharge from the trachea toward higher parts, which occurs at a rate of 4 to 21 mm/min (15-17) [36,37]. This means that laryngeal mucosa is transporting discharge actively from lower to higher parts, and it is supported by coughing if the system of mucociliary transport is impaired. The mucociliary transport from trachea is continued to subglottis mucosa, crossing over vocal cords, and then directing it to the posterior commissure and hypopharynx. Ciliary movements result in shifting the mucoserous layer to the surface. Serous layer is in direct contact with the cilia and it contains a lot of water, whereas mucous layer is in contact with laryngeal lumen. This mucoserous layer protects laryngeal mucosa from external agents (tobacco smoke, dust, etc.) and from dehydration due to airflow through laryngeal lumen. Tobacco smoke causes damage to cell cilia, thus impairing mucociliary transport, which results in discharge retention and cough [38].

The entire mechanism of laryngeal mucosa defense and alteration in smokers can be observed by microscopic analysis of the cells of laryngeal epithelial mucosa. In the initial stage, the cilia of the stratified columnar epithelium are lost, followed by the stratified columnar epithelium metaplasia to stratified squamous epithelium. The very expansion of the stratified squamous epithelium to the area of stratified columnar epithelium points to the intensity of smoking and to laryngeal mucosa sensitivity to exogenous factors. Reduction of the ciliated stratified columnar epithelium area results in lower laryngeal mucosa humidity and impairs mucociliary transport, which causes dry throat, discharge retention and cough, all this favoring further mucosal lesions. These changes are reversible if irritative factors are eliminated. Upon elimination of irritative factors, the cilia can regenerate in 5-20 days [38]. These changes can be very well visualized *in vivo* by contact endoscopy [35,39]. As tumor cells derive from the basal layer of mucosal epithelium, hypertrophy, i.e. epithelial thickening is the first measure of cell defense, reducing the basal layer cell contact with carcinogenic substances from tobacco smoke. Clinical examination of the patient shows thickened vocal cords of slightly uneven surface and harsh voice. If the action of the agent continues, hyperkeratosis, i.e. a layer of dead cells on epithelial surface, may occur. On examination, these patients have whitish layers on their vocal cords or other parts of laryngeal mucosa, which is described as leukoplakia. The next stage is gradual changing of the epithelial basal layer cells, described as dysplasia levis.

The cells undergo gradual changing, with an increasing number of mitoses and cell nucleus hyperchromatism, and such altered cells involve ever more epithelial layers; involvement of two-thirds of the epithelium is called dysplasia gravis. During a 10-year follow up, the progression of leukoplakia without dysplasia and dysplasia levis to carcinoma was recorded in only 3% and progression of dysplasia gravis to carcinoma in up to 30% of cases [40,41,42]. In this stage, only a few superficial epithelial layers remain unchanged. When these lesions involve full thickness of the epithelium, then carcinoma in situ is diagnosed. Microscopic picture shows very little difference between dysplasia gravis and carcinoma in situ, and according to some classifications these two lesions are classified in one

group. When tumor cells penetrate basal membrane, the finding is described as invasive carcinoma.

This theory is supported by the frequent finding of dysplasia, dysplasia gravis in particular, along with tumor lesions [42]. This theory also explains the finding of tumor multicentricity, which occurs with gradual progression of dysplastic lesions in particular parts of the mucosa. It should be noted that the initial changes are reversible, even in the stage of dysplasia levis, if the action of irritative factors, i.e. tobacco smoke, is discontinued. Results of a study conducted at ENT Department, Split University Hospital Center in Split, Croatia, supported this theory [39]. Laryngeal mucosa epithelium was analyzed by *in vivo* contact endoscopy in patients operated at our department for some other diagnoses; patients younger than 20 were excluded. In our group of 150 patients free from any clinical signs of laryngeal disease, dysplasia levis was diagnosed in six and dysplasia gravis in two patients, all of them long-term smokers; dysplastic lesions were not diagnosed in any nonsmoker.

This paper clearly shows the major role of smoking in the development of laryngeal carcinoma as well as in the occurrence of benign changes, precancerous lesions in particular. Some diseases such as Reinke's edema occur exclusively in middle-aged female smokers; these lesions are reversible by smoking cessation in the early stage of edema formation, but in later stage when the edema turns gelatinous these lesions are permanent and require operative therapy.

Placing a ban on smoking in public premises, cigarette advertising, selling cigarettes to those aged <18 and good education can lead to considerable decrease in the number of smokers, as seen in the USA and west European countries, where the number of smokers has greatly decreased in recent years, in contrast to east European countries, South America and Asia, where the number of smokers continually rises, especially in females [43,44,45].

Conclusion

Study results pointed to the increased prevalence of laryngeal diseases in smokers and a statistically significant difference in the prevalence of smoking habit between patients with benign laryngeal lesions and those with laryngeal tumors. The greater number of cigarettes daily and longer history of smoking contributed significantly to the increased prevalence of laryngeal carcinoma. A great part of these lesions are reversible in the initial stage with smoking cessation. Therefore, there is only one and obvious advice: quit smoking now and forever.

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Chapter 6

Impact of Smoking on Oral Mucosa and Reproduction: Effects on Humans and Experimental Models

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Abstract

Nowadays, cigarette smoking remains the single most important avoidable cause of death in the developed world. Particularly smoking is the most significant exogenous risk factor for diseases, specially the oral cavity and reproductive system. The aim of this chapter is to summarize data generated from smoking on oral mucosal cells and reproduction,. In particular, we focused on the role of DNA damage, mutagenesis, proliferation status, apoptosis dysregulation, tumor suppressor genes, xenobiotics metabolizing enzymes, on oral mucosa and periodontal tissues as well as fertility in humans and experimental models. Taken together, these data have demonstrated relevant biomarkers for understanding the noxious activities exerted by smoking on oral mucosa cells and reproduction.

Keywords: Oral squamous cell carcinoma, p53; bcl-2; bax; rats; humans

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Introduction

There are at least 10.000 years ago, the central american indians made use tobacco as cigarette smoke (CS) in religious rituals. This began to be industrialized since 1840 and it is composed of dried leaves of the plant known as tobacco (nicotine and *nicotine rusticum tabacum*) and other illicit substances.

The tobacco plant is native to the Western hemisphere, and the use of tobacco in smokelles forms (placed in the mouth or inhaled as a powder through the nose) predates the arrival and exploration of the West by Europeans.

It is known that CS contains a toxic collection of more than 4000 chemicals including nicotine, which combined give rise to addictive stimulant and euphoriant properties [1]. There are some main clinical consequences of prolonged exposure to CS. First, it causes several chronic respiratory ailments, including chronic bronchitis, emphysema and lung fibrosis and it is associated with an increased in respiratory infections. Second, it is associated with an incidence of a variety of cancers including lung, oral, oesophagus, pancreas and colon [2], and third, CS increases the risk of atherothrombotic clinical events such as myocardial infarction and stroke [3].

Use of tobacco has a devastating effect on the health and well-being of the public. About 500 million people alive today will eventually be killed by tobacco use. By 2030, tobacco is expected to be the single biggest cause of death worldwide, accounting for about 10 million deaths per year. One-half of these deaths will occur among people 35 to 69 years of age, losing an average of 20 to 25 years of life. The effects of tobacco use on the public's oral health also are alarming. All forms of tobacco including cigarettes, cigars, pipes and smokeless tobacco have been established as causal for oral and pharyngeal cancer [4].The gaseous components of cigarette smoke as carbon monoxide (CO) and carbon dioxide (CO2), are responsible for the reduction of oxygen to the organs of smokers. Nicotine fulfills all the criteria of an addictive, including psychoactive effects, drug-reinforced behavior, compulsive use, relapse after abstinence, physical dependence, and tolerance. Nicotine stimulates specialized receptors in the brain which produce both euphoric and sedative effects. It has been known for many years that nicotine shares many features of drug dependence with opioids, alcohol and cocaine. This includes similar disappointing patterns of relapse. It is for this reason that most attempts at smoking cessation are not successful, despite the fact that the majority of smokers are aware that smoking is harmful to their health, and so would like to quit. Nowadays, cigarette smoking remains the single most important avoidable cause of death in the developed world. The WHO reports that smoking is responsible for 4.9 million deaths worldwide annually, which amounts to over 10.000 deaths per day. If current trends of expansion of consumption are maintained, these numbers increase to 10 million deaths annually by the year 2030, half of which individuals of working age (between 35 and 69 years) [4].

The aim of this chapter is to show data generated from smoking on oral mucosa, focusing studies conducted by our research group. In particular, we reviewed studies demonstrating the role of DNA damage, mutagenesis, proliferation status, apoptosis dysregulation, tumor suppressor genes, xenobiotics metabolizing enzymes taking into consideration recent evidences in humans and experimental models.

The Noxious Effects of CS on Oral Tissues

The lack of reports of oral cancer studies with mainstream cigarette smoke in experimental animals is a continuing problem for researches trying to design potentially reduced risk products for those smokers who are either unwilling or unable to quit smoking. Although a quite extensive literature covers the genotoxic and carcinogenic properties of individual CS components in experimental tests systems, less information is available on genotoxicity and carcinogenicity of CS as a complex mixture and it is difficult to reproduce these effects in animal models [5]. The major assays is about inhalation with cigarette smoking, and even these studies produce only small percentages of animals with pulmonary tumors (e.g adenomas with the occasional adenocarcinoma) as opposed the highly invasive carcinomas (e.g small cell and squamous cell) [6].

It is known that tobacco smoke plays a major role in the pathogenesis of lung cancer, cancer at the other sites and a variety of chronic degenerative diseases [7]. In spite of the dominant role of cigarette smoke (CS) in cancer epidemiology, all studies performed during the past 60 years have shown that this complex mixture is either negative or weakly tumorigenic in experimental animals [8]. Laboratory animals have extensively been used for evaluating the carcinogenicity of typical CS components, such as benzo(a)pyrene [B(a)P], as a prototype of polycyclic aromatic hydrocarbons and 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone, as a prototype of tobacco-specific nitrosamines. Previous reviews of inhalation studies with mainstream cigarette smoke in experimental animals have concluded that the results do not agree with the epidemiologic evidence that smoking causes lung cancer and other human diseases [9,10].

The most convincing medium-term bioassay for CS tumorigenicity has been developed during the last decade [11]. This bio assay involves the whole-body exposure of A/J mice or other mouse strains to ECS for 5 months, followed by recovery in filtered air for an additional 4 months. Studies performed by Balansky et al 2007 and Witschi et al 2005, showed statistically significant increases in the yield of surface lung tumors in the majority of the experiments realized [11,12].

The effects of CS are almost exclusively due to the Environmental Cigarette Smoking (ECS) gas phase and mainly due to 1-3 butadiene [13].This suggests that tumorigenicity in the A/J mouse model is because of some as yet unidentified carcinogen(s) present in the ECS gas phase or because of free radical-mediated oxidative stress of the lung [12], however, the increase of ECS-related tumor multiplicity is low [13]. Izzotti et al (2010), provided evidence that the whole-body exposure of rodents to ECS, during the first 4 weeks of life, dysregulates miRNA expression in the apparently healthy lung tissue, by analyzing the expression of 484 miRNAs in the lungs of Sprague-Dawley rats [14]. It was shown that ECS cause an extensive dysregulation of several miRNAS, which is correlated with the formation of bulky DNA adducts and with overexpression of a number of genes and proteins in the same tissues [14]. D'Agostini et al (2001) founded no lung tumor in SKH-1 hairless mice exposed whole-body to environmental cigarette smoking (ESC) for 6 months [15]. However, after 28 days of exposure, ESC produced significant alterations in the respiratory tract of SKH-1 mice, including the formation of micronucleated cells and polynucleated pulmonary alveolar macrophages, induction of proliferation and apoptosis in the bronchial epithelium and enhancement of oxidative DNA damage and bulky DNA adducts in the lung [16].

p53 protein may be highly related to carcinogenesis, the progression of disease and that is controlled by a p53-dependent pathway [16]. One of the mechanisms that hampers the formation of lung tumors in rodent exposed to CS may be represented by removal of damaged cells via apoptosis. D'Agostini showed that apoptosis was induced strongly in bronchial/bronchiolar epithelium and in pulmonary alveolar macrophages of Sprague-Dawley rats exposed whole-body to ECS and mainstream cigarette smoke [15] . The results provide evidence that p53 mutant A/J mice differ significantly from their wt littermate controls in certain background characteristics, as well as in susceptibility to molecular alterations and induction of lung tumors after exposure to ECS. This finding indicates that the loss of p53 contributes to genomic instability by permitting inappropriate survival of cells that would normally undergo apoptosis in response to DNA damage. In addition, the higher background proliferation rate of the bronchial epithelium and its lower sensitivity to ECS-induced apoptosis in mutant mice are in line with the known mechanisms of p53 [15,17].

Our group studied the effects of cigarette smoking in rat tongue mucosa through the Bcl-2 gene family. The Bcl-2 and bax are two important effectors genes during intrinsic apoptotic pathway. The bcl-2 pro-oncogene was originally discovered by analisys of the t [14,18] chromosomal translocation associated with human follicular B-cell lymphoma [18,19]. Our results demonstrated no histopathological changes in epithelial cells of tongue mucosa in the negative control group. In the same way, no remarkable differences were noticed in the experimental group. The Bcl-2 gene encodes a membrane protein localized to the nuclear membrane, the inner surface of mitochondria and the endoplasmatic reticulum [20]. Bax, another member of the Bcl-2 family, is considered to be a major effector of apoptosis [21].

Thus, the bcl-2/bax ratio controls the relative susceptibility of cells to stimuli, which induce apoptotic cell death [22]. We founded an over expression of Bcl-2 in the rat tongue keratinocytes after CS exposure. This is consistent with published data reporting that tobacco products are able to exert a suppressive effect on the signalizing of the death pathway contributing to tumor growth [23]. Other authors have argued, however, that bcl-2 expression was not significantly different between smokers and those nerve smokers [24]. Taken together, our results support the notion that CS was able to induce mutations in Bcl-2 oncogene leading to its overexpression as far as to inhibit the apoptotic regulation appears to play a pathogenic role in malignancies. There seems to be evidence that up regulation of Bcl-2 induced by CS may be associated with a risk factor in the progression of oral cancer.

Glutatione S- transferases (GSTs) are a family of enzymes involved in detoxification of xenobiotics. GSTs exist as homo-or-hetero-dimers and have been grouped into at least seven distinct classes [25]. The main function of GSTs is to catalyze the conjugation of glutathione to an electrophilic site of a broad range of potentially toxic and carcinogenic compounds, thereby making such compounds less biologically active and enabling excretion [26]. Expression of placental glutathione S-transferase in rat tongue mucosa exposed to CS was also one of the studies performed by our group that revealed that under controlled experimental conditions used herein, histological normal tissue harbors genetic altered cells able to express GST-P. The induction of GST-P in the tongue mucosa of these animals may facilitate cell proliferation and inhibit apoptosis, hence allowing the clonal expansion of a population of initiated keratinocytes leading to oral carcinogenesis [27]. GST-P expression may reflect the carcinogenic effect of CS in rat oral mucosa and the genetic susceptibility of animals in relation to continuous carcinogens exposure.

There is an inherent need for research using experimental models in studies linking cigarette smoking with oral cancer. Animal models of CS-induced cancer are important for several reasons. We would like to emphasize the significance for exploring the mechanisms involved in CS-related cancer, and specially, oral cancer, for studying the interactions between CS and cells from experimental models and perhaps provide understanding of the potential extrapolations to smokers.

On the other hand, an increase in plaque accumulation, a higher incidence of gingivitis and periodontitis, a higher rate of tooth loss , and an increased resorption of the alveolar rigde have been found among smokers, there was various factores predispose this, but smoking was the most significant factor [28].

Although periodontal diseases are infections caused by dental plaque, risk factors could modify the periodontal response to microbial aggression. Tobacco smoking is considered one of these factors was strongly associated with both attachment and bone loss. Smokers are more susceptible than nonsmokers to advanced and aggressive forms of periodontitis. In smokers, there seems to be a relationship between periodontal attachment loss, number of cigarettes smoked daily, and number of years of tobacco consumption. Probing depth and gingival recession are greater in smokers than in non-smokers mostly at buccal surfaces. Smokers have less inflammatory response and bleeding on probing than non-smokers, at the same plaque level. Moreover, the effects of cigarette smoking on periodontal status are independent of the plaque index and oral hygiene of the patient, due to the direct influence of tobacco on periodontal tissues. In an interesting study, 240 dental patients were selected according to previously defined criteria and they were divided into two groups according to their periodontal status. Patients with established periodontitis constituted the case group. The remaining patients constituted the control group. Smoking status, probing depth, gingival recession, clinical attachment level, tooth mobility, periodontal bleeding index and plaque index were determined for each participant. Smoking was considered a risk factor strongly associated with periodontitis. The effects of smoking on periodontal tissues were dependent on the number of cigarettes. The effect of tobacco on periodontal periodontium; tobacco tissues seems to be more pronounced in men than in women [29].

Smoking is, also a major cofactor for periodontitis. Smokers are approximately three times more likely to develop periodontitis and respond less favorably to periodontal therapy. The risk for developing periodontitis correlates with the number of cigarettes smoked. The exact nature of the relationship between periodontitis and smoking remains unclear. Recent studies indicated that smoking impairs the immune response to periodontal pathogens because of a decreased chemotaxis, a decreased phagocytic capacity of polymorphonuclear leukocytes and because of decreased levels of IgG and IgA. The conflictions reports the effects of smoking on the oral microbial flora, but other studies do not. Such bacterial adherence is known to be a first important step in the pathogenesis of infections (*Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Tannerella forsythensis, Escherichia coli,* and *Candida albicans*) [29,30].

Nicotine is a key constituent of cigarette smoke, causing adverse health effects. Its concentration as well as that of its primary metabolite, nicotinine, is known to be elevated in smokers, albeit there is some discussion regarding the concentration of cotinine and nicotine in the gingival crevice. In a study using bacterial colonization of the epithelial cells refer that the susceptibility of epithelial cells to become colonized by either *A. actinomycetemcomitans* or *P. gingivalis* could be altered by nicotine, cotinine, or cigarette smoke extract in a time-

dependent, species-specific manner. These findings support the hypothesis of an increased patient susceptibility for bacterial adhesion to epithelial cells in smokers [30].

Therefore, smoking has direct effects on homeostatic mechanisms in the periodontium, as well as any possible influence on the periodontal microflora, changes periodontal tissue vascularity, altered fibroblast attachment and function, suppression of osteoblast proliferation, stimulation of osteoclasts, increased gingival crevicular fluid flow, altered polymorphonuclear neutrophil function , decreased production of IgA or IgG, and decreased lymphocyte proliferation. The polymorphonuclear neutrophil is the first line defense against bacteria in the gingival sulcus. In periodontal diseases is reduced the number of PMN, and smoking also many significant negative effects on PMN function: phagocytosis, super oxide and hydrogen peroxide generation, integrin expression and protease inhibidor production, that result in several periodontitis. [31].

Ina longitudinal study (4 years), some researchers investigated the risk of periodontal disease and tooth loss, associated smoking and drinking habits [32]. The authors suggested that cigarette smoking was found to be an independent risk factor for diseases periodontal and tooth loss. Alcohol consumption was a limited factor for loss tooth but was unrelated to periodontal disease.

The majority of evidence in the literature is inconclusive on the effect of smoking on the microflora, but some data suggests that the main effect of smoking is on the immune and inflammatory response [33-35], which frequently reduces the clinical signs of gingival inflammation such as redness and bleeding.

Tobacco smoking has been found to be a major environmental factor associated with generalized forms of severe periodontitis. The epidemiologic studies on the relationship between tobacco use and periodontal diseases consistently reported that cigarette smokers are five times more likely to develop severe periodontitis than nonsmokers [36].

The evaluation of serum imunoglobulin (Ig)G levels in smokers with periodontities and its potential role as a risk indicator of the disease process has been postulated. Serum immunoglobulin (Ig) G,IgA, and IgM levels were estimated with immunoturbidimetricassay. The IgG subclass (IgG1, IgG2, IgG3, and IgG4) levels were performed using single radial immunodiffusion assay. Levels of serum IgG and IgA were significantly lower in smokers compared to non-smokers and healthy controls. Although IgM levels were low in smokers, it was not significant. Of the four subclasses of IgG studied, the IgG2 was found to be significantly lower among smokers with periodontitis. This indicates that cigarette smoking may be associated with the suppression of B-cell function and immunoglobulin production. The alteration of antibody levels further explains the potential mechanism by which smoking exacerbates periodontal disease [37].

Cigarette smoking has also been suggested as a risk factor for periodontitis. Thousands of components are present in cigarette smoke, including nicotine, which may play an important role in the observed effects of smoking on cell metabolism. However, the mechanisms underlying these effects are unclear. Using DNA microarrays, some researchers groups have monitored differentially expressed genes, responsive to nicotine, in a macrophage-like human cell line. Among these were genes related to inflammation and other immune responses, such as phospholipase A2 and interferon. Consistent with the array findings, the authors found similar changes in mRNA expression after analysis using the real-time polymerase chain reaction. That suggests that nicotine causes excess inflammation and disturbs host defense mechanisms against pathogens [38].

In fact, clinical evidence shows that cigarette smoking is one of the most significant risk factors for periodontal diseases, in the radiographic studies of alveolar bone to evaluate the effect of smoking on alveolar bone. It was observed that smoking produces an adverse effect on clinical periodontal variables and alveolar bone height and density, acting as a potential risk factor for alveolar bone loss, even at an early age with low tobacco consumption. It is very important to inform young smokers about the risk of this habit in relation to periodontal health [39].

Periodontitis is a bacteria-induced, irreversible chronic inflammatory mucosal disease characterized by the destruction of the soft and hard supporting structures of the teeth. Tobacco smokers are more susceptible than non-smokers to infections with periodontal pathogens [40], are more likely to develop severe periodontitis and to prove refractory to treatment [41]. Paradoxically, smokers show reduced clinical signs of inflammation in response to dental plaque than non-smokers, particularly the key diagnostic index of gingival bleeding on probing and edema [41,42]. Again, the mechanisms underlying this phenomenon are poorly characterized, *Porphyromonas gingivalis*, a Gram negative, *asaccharolytic* anaerobe, is a key periodontal pathogen whose numbers are increased in tobacco smokers.

Tobacco smokers are more susceptible to periodontitis than non-smokers but exhibit reduced signs of clinical inflammation. The underlying mechanisms are unknown. It has been established that cigarette smoke extract (CSE) represents an environmental stress to which *P. gingivalis* adapts by altering the expression of several virulence factors – including major and minor fimbrial antigens (FimA and Mfa1, respectively) and capsule – concomitant with a reduced proinflammatory potential of intact *P. gingivalis* [43].

The impact of tar and nicotine contents of cigarettes on chromosomal damage in oral mucosa cells of smokers, monitored the effect of smoking different cigarette types, on induction of nuclear anomalies including micronuclei (MN), broken eggs (BE), binucleates (BN), condensed chromatin (CC), karyorrhexis (KR), karyolysis (KL) and pyknosis (P) in exfoliated buccal cells has been revealed by some studies. The frequencies of KR, CC, KL, BE and BN were increased significantly only in smokers of medium (MF) and non-filtered (NF) while MN levels were only elevated in the group that smoked NF cigarettes. These findings also suggest that nicotine potentially protects cells against DNA reactive carcinogens contained in tobacco smoke [44].

Smoking and Reproduction

Effects of Smoking on Male Reproductive Function

Male reproductive function may be disturbed by a variety of conditions ranging from environmental contaminants [45] to pathologies such as varicocele, cancer and diabetes [46-48]. According to Sharpe (2010), several life-style related (e.g. obesity, smoking) and environmental factors appear to negatively affect both perinatal and adult testes [49].

Tobacco smoking, a widely recognized health hazard, has been shown to adversely affect male reproductive health [50]. Evidence suggests that certain components in cigarette smoking (alkaloids, nitrosamine, nicotine, cotinine and hydroxycotinine) interact with the

gamete cells affecting their function and viability, through mechanisms involving free radicals production [50,51].

Besides human-based investigations, animal models exposed to cigarette smoking or its isolated substances have been used to elucidate the major mechanisms of tobacco reproductive toxicity. Taken together such studies have shown as smoking effects: altered Leydig and Sertoli cells physiology, with disruption in testosterone production, decreased gonadal weight, altered germinal cell kinetics, increased sperm head abnormalities, increased testicular lipid peroxidation, decreased sperm counts and motility, higher sperm DNA fragmentation, disrupted testicular apoptotic index, meiotic disturbance during oogenesis and spermatogenesis, gonadal histologic alterations due to hypoxemia and altered sexual maturity to individuals prenatally exposed [50,52-57]. Concerning the tobacco influence in the hormonal status, Yamamotto et al. (1998) assumed that smoking may influence the reproductive ability by causing impaired spermatogenesis secondary to various hormonal alterations [58]. According to such authors, the lower levels of testosterone after hCG stimulation in cigarette smoke-exposed rats reflects a detrimental effect of exposure on Leydig cells secretory function. The authors also indicated the dysfunction in the Leydig and Sertoli cells as responsible for the lower values for caudal epididymal sperm count and motility, through a disturbance in spermatogenesis or epididymal sperm maturation which are testosterone-dependent processes. Also working with smoke-exposed rats, Audi et al. (2006) discussed that nicotine inhibits release of gonatropins, FSH and LH from pituitary, acting through hypothalamus and blocking the neural stimulus to GnRH. Such decrease in gonadotropins is refected in the atrophy of gonads leading to a reduction in the gonadal weight and functional properties [55].

Jana et al. (2010) demonstrated, in Wistar rats, a significant reduction in testicular key androgenic enzyme activities with lowering in plasma and intratesticular testosterone concentration after nicotine treatment. Such authors revealed, through Western blot and reverse transcriptase-PCR analysis, that nicotine induced a marked decrease in the expression of testicular steroidogenic acute regulatory protein (StAR) [53]. Lowering in sperm counts in animal model, as described by Yamamotto et al. (1998), is a direct consequence of hormonal disruption and has also been described to humans to which increased incidence of oligo/azoospermia has been found [58;59].

Reduction in sperm production may be also attributed to other events possibly mediated by cigarette substances. An increase in germinal cell apoptosis in rat testis was observed by Rajpurkar et al. (2002) in a protocol of 45 days of cigarette-smoke exposition. The authors argued that the cigarette substances are responsible to induce testicular apoptosis probably by an imbalance in oxidant-antioxidant mechanism within the testis, generating a large amount of reactive-oxygen species (ROS) [52].

Besides to the possible apoptosis stimulation, smoke-induced reactive oxygen species are also responsible to induce alteration in sperm plasma membrane and high degree of DNA fragmentation [50, 54]. Sepaniak et al. (2006) concluded that sperm DNA fragmentation can be considered as an independent parameter with diagnostic, prognostic and strategic value in the treatment of infertility. According to Zenzes et al. (1999) and Zenzes (2000), cigarette smoke constituents and/or their DNA-reactive metabolic intermediates reacts directly with spermatozoa causing a oxidative DNA lesion of guanine (8-hydroxydexyguanosine or 8-OHdG), a major damage found in lung cells of smokers, but also occurring in spermatozoa in association with smoking and seminal plasma cotinine [50,60]. Smoking-related adducts of in

spermatozoa arise from oxidative damage since oxidant radicals in cigarette tar are assumed to bind to DNA producing nicks. As the repair capacity of ejaculated is minimal, these genetic damages are transmissible [50,60]. Agarwal and Said (2005) attributed the increase in seminal leukocyte concentrations as responsible, in part, for the increased levels of seminal ROS frequently observed in infertile smoker men [61].

In addition to DNA damage, sperm morphology has also been found to be altered due to tobacco consumption in both rodents and humans [62,63]. Mak et al. (2000) suggested that smoking is associated with the impaired disposal of residual sperm cytoplasm by the testis and⁄or epididymis in men. Such retention of residual cytoplasm correlated with semen ROS levels and decreased sperm fertilizing capacity [63]. According to Agarwal and Said (2005), retained residual cytoplasm promotes spermatozoa to generate endogeneous ROS via mechanisms involving the cytosolic enzyme glucose-6-phosphate dehydrogenase, leading to peroxidative DNA damage [61]. Excess of cytoplasm were also found in the ultrastructural studies by Aydos et al. (2001) in spermatids from nicotine-exposed rats, accompanied by lipid droplets accumulation and irregular shaped acrossome [64].

Another detrimental effect of smoking is related to the meiotic division mechanics. Zenzes (2000) discussed that alkaloids from cigarette bind to tubulin leading to disturbances in microtubule polymerization, which affects chromosome segregation and increase the rate of dissomic spermatozoa [50]. Also found as a consequence of cigarette substances are the gonadal histoarchitecture disturbances. In rats, Audi et al. (2006) found testes with disruption of the normal orderly progression of spermatogonia, with tubules containing only one layer of these cells [55]. Erpek et al. (2004) described a gradual decrease in seminiferous tubule diameter in albino mice submitted to passive smoking, alcohol or passive smoking + alcohol, showing a possible synergistic effect of such substances. Degenerated germinal epithelium and decrease in Leydig cell population were also more severe in the combination of cigarette and alcohol [65]. Nicotine was also responsible for the decrease in Leydig cell number and hypospermatogenesis described to albino mice by Gawish et al. (2010) [66]. Others cigarette substances such as benzo(a)pyrene and cadmium also have been found to affect the gonadal structure. Benzo(a)pyrene has long been found to cause germ aplasia and increase in interstitial testis tissue in mice [67] and cadmium has been shown to exert high degrees of testis degenerative changes in a dose-dependent manner in rats [68].

An increasing number of investigations has been shown substances which can protect the organism against the detrimental effects of cigarette smoking chemicals. Russo et al. (2006) have described the action of propolis in protecting human sperm DNA from damage induced by benzo(a)peryne [69]. Gawish et al. (2010) have demonstrated that green tea was able to prevents the decrease in Leydig cells number in mice testis during nicotine treatment [66], whereas Jana et al. (2010) have obtained good results using the taurine – a sulfur-containing aminoacid with antioxidant properties – which prevented the degeneration of germ cells to some extent, restored the spermatogenesis moderately (with increase in sperm counts) and decreased the sperm head abnormalities in nicotine-treated albino rats [53].

Effects of Smoking on Female Fertility

Smoking is a differential between men and women in cancer mortality patterns attributable to lifestyle. In most countries, being born male is the greatest predictor for

tobacco use, with overall prevalence of 48% in this population and 12% in women globally [70]. However, the use of tobacco between young girls and women is increasing, mainly because the tobacco industry is stimulating specially this population, without forget to continue targeting the men [71]. Moreover, girls aged 12-17 are more vulnerable to initiate smoke than males and they take less time to become tobacco dependence [72]. This event is relation to social and familiar aspects, depression occurrence, desire of weight control and physiology effects, mainly the influence of the levels gonadal hormones [71,72]. It is very important to underline that men or women are starting to smoke tobacco younger than in the past decades, sometimes before 15 years old, influencing the sexual maturity in puberty and reducing the fertility in the reproductive age [71].

The effects of cigarette smoke on human fertility are dose-dependent and are influenced by time and type of exposure [73]. Generally, women who smokes cigarettes have an increased risk for infertility and they take longer to get pregnant than women who do not smoke [74,75]. The changes induced by tobacco smoke and some metabolites like benzo[a]pyrene, nicotine and cadmium are present in steps as: folliculogenesis, steroidogenesis, preimplantation embryo development, embryo implantation, uterine flow velocity and myometrial activity [73].

Female infertility in smoking patients is related to significant reduction in number of oocytes and increased rate of oocyte destruction with advancing age than non-smokers [50]. Experimental researches demonstrated follicle loss by apoptosis involving Bax pathway or increased rate of follicle recruitment [76,77] and inhibition of ovarian follicles growth by induction granulosa cell apoptosis [78,79]. Moreover, cotinine (a metabolite of nicotine) compromises the developmental potential of follicles when it is incorporated into ovarian granulosa-lutein cells, and it can inhibit apoptosis in different cell lines, contributing to the pathogenesis of ovarian tobacco-related cancer [50]. Cadmium can interfere with cell-cell junctions and the adherence of cells, leading changes in granulosa cell morphology [80].

Additionally, nicotine can blocks the meiotic metaphase I or disturb the homologue segregation at anaphase I with premature centromere separation and premature anaphase [81,82]. It has been described degenerative changes in chromatin after at resumption of meiosis I into metaphase II, mainly by change the meiotic spindle [50]. According to Zenzes *et al.* (1995) smokers has an increased frequency of oocyte diploidy probably resulting from prevention of first polar body extrusion, indicating meiotic immaturity [83]. Also, changes in DNA can occurs by the action of Benzo[a]pyrene (BaP), a member of the policyclic aromatic hydrocarbons (PAH) found in the follicular fluid of women smokers, mainly leading the formation of DNA adducts [79,84].

Event well known in smokers, oxidative stress lead cellular apoptosis and aneuploidy, manly to produce cytoskeletal alterations and cellular fragmentation [50,73]. Cadmium (Cd) is a major inducer of oxidative stress.

In a study, granulose cells with cadmium exposure showed a maximum increase in lipid peroxides and catalase activity, along with decreased glutathione status and superoxide dismutase activities [85]. This event can lead damage lipids, proteins, nucleic acids, DNA and RNA and affect the fertilizing ability of the gametes [47]. Additionally, oxidative stress induces granulose cell death followed by destruction of follicular walls [86].

The production of a viable oocyte is modulated by a complex interaction of endocrine, paracrine and autocrine factors leading to follicular maturation, granulosa cell maturation, ovulation and luteinization [61]. Tobacco smoke can lead to decrease ovulation by inhibition

of estradiol production due nicotine and other compounds exposition [87]. It has been observed lower levels of estriol, estradiol, and estrone during the luteal phase of menstrual cycles and during the follicular phase in female smokers [88]. Nicotine exposure is relation to decrease of uterine weight, myometrium and endomerium and increase of ovarian cholesterol levels due reduction of granulosa cell aromatase activity, responsible to convert androstenedione to estradiol [89,90].

Steroidogenesis is regulated by hypotalamic-pituitary-gonadal axis and occurs in a cooperative fashion between granulosa and theca interna cells. While the theca interna converts cholesterol enzymatically to progesterone, which in turn is converted to androstenedione, the granulosa cells sensitize testosterone from that and this is aromatized to from estradiol [80; 88]. Cadmium decrease luteinizing hormone levels in blood and progesterone synthesis [88]. This mechanism also is changed by nicotine, modulating both its steroidogenic activity of theca interna and its vascularization [88,91].

Whereby progesterone controls endometrial response, it is critical for early pregnancy maintenance, decreased this hormone has been implicated as a cause of infertility and fetal loss [92]. There is an increase in spontaneous abortion occurrence among pregnant smokers [50]. There was reported that nicotine have been identified in the endometrium and uterine fluid, suggesting a toxic environment for embryo development [73]. Furthermore, benzo[a]pyrene has inhibitory effect on endometrial cell proliferation and cell adhesion molecules loss which can affect trophoblast implantation [93]. Smoking is associated with decreased trophoblastic migration due to induce a generalized dysfunction of both villous and cell columns of trophoblast and to impair mitotic and proliferative ability of cytotrophoblast [73; 93].

Cigarette compounds cause abnormal placental morphology and pregnant smokers has showed reduces human chorionic gonadotropin (hCG) levels, a important hormone in trophoblastic differentiation [73,94,95]. Secondhand smoke exposure lead greater risk for preterm birth and their newborn are more likely to have respiratory distress syndrome, neonatal intensive care unit admissions and immediate newborn complication. Nicotine and cotinine can be found in fetal hair, meconium, placental tissue and cord blood, demonstrating directly action of compounds tobacco smoke on fetus [96]. In systematic review and meta-analysis, studies shown pregnant women who are exposed to secondhand smoke are more likely experience stillbirth and birth to a child with a congenital malformation, including neural tube defects [97].

Thus, the damage caused by smoking is evident in female fertility in all stages cycle from meiotic stages of oocytes to the embryonic and fetal development for both smokers and passive smokers. Importantly, fetal exposure to toxic agents in cigarette smoke can lead to various physiological, biochemical and metabolic changes affecting child development and adult life.

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Chapter 7

Effect of Chronic Smoke in Human Oral Mucosa: The Morphological Point of View

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Abstract

The relationship between tobacco smoking and diseases of the respiratory system is worldwide demonstrated and the human oral mucosa represents the first barrier exposed to both physical (heat) and chemical stresses derived from cigarette smoke. The knowledge about oral epithelial homeostasis after exposure to a chronic exogenous stimulus such as smoke is crucial in basic oral biology, but many issues have to be still elucidated.

The integrity of the physiological barrier offered by the oral mucosa is maintained thanks to desmosomes, tight junctions (TJs), and adherens junctions (AJs). After the exposure of the whole human oral mucosa to smoke, the molecular composition of the epithelial junctions can be affected, but only few studies reported experimental data on the consequences of smoke on intercellular adhesion and keratinocyte terminal differentiation.

We present a review of the recent literature and original data about the outcome of smoke on TJ and AJ molecular composition in keratinized human oral mucosa explants of young healthy smoking women, together with a quantitative analysis of keratinocyte proliferation, to further understand the epithelial response to smoke.

The pattern distribution of occludin, E-cadherin, and β-catenin was comparable in control and smoker groups. The quantitative analysis of cell proliferation demonstrated that no differences existed between the two groups (BrdU/mm^2 mean value ± 1SD: 161 ± 42.02 for controls; 113.5 ± 64.7 for smokers). TJs and AJs molecular composition was thus maintained after chronic exposure to cigarette smoke and also epithelial proliferation resulted unaffected.

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The lack of evidence for a modification in TJs and AJs in the healthy mucosa of chronic smokers, actually, can not exclude an initial modification by cigarette compounds as proposed by previous studies, an event which may occur earlier than in desmosomes. Subsequently, the expression pattern of AJs and TJs is possibly reverted to the physiological pattern observed in the present study, whereas desmosomes modify their molecular composition as we recently demonstrated in biopsies obtained from healthy young chronic smoker women.

Thus, when the molecular composition of desmosomes is affected by smoke, AJs and TJs can represent good candidates for maintaining the intercellular adhesion and, consequently, the physiological barrier as "compensatory mechanism".

Introduction

The relationship between tobacco smoking and cancer of the respiratory system is widely demonstrated, but the smoke role in increasing the risk of death for other cancers and cardiovascular diseases was also reported. For this reason the World Health Organization (WHO) indicated cigarette smoke as one of the five leading global risk for mortality in the world [1]. Nonetheless, this habit has an even more increasing popularity in Western countries, particularly in many low- and middle- income countries, with about one billion males and 250 million females smoking in the world [2]. The commercially manufactured cigarettes are made up of a mixture of tobacco, paper, and often, but not always, a filter is present. When the combustion starts, this filter is crossed by the mainstream smoke, directly entering the smoker's oral cavity, while the sidestream smoke is released in the environment from the farthest point of the cigarette [3].

Smoke Chemical Composition

In a cigarette, a huge number of substances is present and, during each puff, more than 4700 chemical are generated as the products of high temperature combustion, reaching up to 950°C and thus making smoke a potent physico-chemical stress for the respiratory system [2, 3]. In particular, a group of chemical compounds known as Hoffman analytes has a high carcinogenic potential due to the presence of volatile aldehydes, metals, aromatic amines, and N-nitrosamines, among which the tobacco-specific- nitrosamines were demonstrated to be of particular significance [4]. The life-threatening action of smoke is also linked to the presence of radioactive compounds, the biologically harmful reactive oxygen species (ROS), and organic radicals [5, 6]. On the other hand, nicotine, the most abundant component of cigarette, is the natural alkaloid responsible for the tobacco dependence, despite its rapid degradation and removal from the body. These pharmacokinetic and pharmacodynamic properties represent the major obstacle in realizing the full benefits of nicotine which was initially used in the 15th and 16th Centuries as a treatment for toothache, migraine, and other ailments [7]. Probably for its properties, nicotine was the cigarette component most investigated up to now in oral keratinocytes both in vivo [8] and in vitro experimental studies [9, 10]. Other works focussed on other single components of the tobacco smoke, in particular

aldehydes [11] and comprehensive analysis of the smoke effects was carried out only in cell cultures or on the salivary antioxidant content in smokers [12].

Smoke and Oral Mucosa

Only recently, the viability of cultured human gingival keratinocytes and engineered oral mucosa after exposure to whole smoke was investigated [13]. Actually, it is noteworthy to consider that, among the different organs of the respiratory system, the human oral mucosa represents the first barrier exposed to both physical and chemical stresses exerted by cigarette smoke in toto. This is the reason why a large spectrum of adverse effects occurs in the mouth of chronic smokers. The first oral changes associated with tobacco use are represented by xerostomia, a reduced vascularisation, salivation, and wound healing, accompanied by an increased tartar production.

Table 1. Summary of the main published studies focussing on the effects of cigarette smoke and its constituents on experimental models of oral mucosa

Stressing Agent	Experimental Model	Reported effect	References
Nicotine	Rat oral mucosa	Hypertrophy and reduction of the epithelial thickness	Caldeira et al; *Arch. Oral Biol.* 52, 83 (2007)
	Reconstructed human oral mucosa	Cell cycle arrest	Kwon et al; *Skin Pharmacol. Appl. Skin Physiol.* 12, 227 (1999)
	Immortalized and malignant oral keratinocytes	Cell cycle alterations and differentiation hint	Lee et al; *J. Oral Pathol. Med.* 34, 436 (2005)
Smokeless tobacco	Co-cultures of human immortalized keratinocytes and fibroblasts	Alterations of adherens and tight junctions and of cornified envelopes	Coppe et al; *Mol. Cancer Res.* 6, 1085 (2008)
Acetaldehyde	Normal and immortalized oral keratinocytes	DNA adducts formation	Vaca et al; *Chem. Biol. Interact.* 108, 197 (1998)
Cigarette smoke condensate	Oral squamous cell carcinoma cells	Increased mobility	Allam et al; *Arch. Oral Biol.* 56, 1154 (2011)
Whole cigarette smoke	Normal human oral mucosa from smokers	Increased expression of genes involved in inflammation and xenobiotic degradation processes Dose-dependent increase in genetic aberrations Modifications in desmoglein 3 and keratin 10 expression	Shani et al; *Oral Oncology* 46, 96 (2010) Boyle et al ; *Cancer Prev. Res.* 3, 266 (2010) Donetti et al; *Arch. Oral Biol.* 55, 815 (2010)
	Normal human oral keratinocytes and engineered oral mucosa	Increased apoptosis	Semlali et al; *J. Periodont. Res.* 46, 533 (2011)

Progressively, nicotinic stomatitis can be evident on smoker's hard palate, most commonly in men over the age of 45. Similarly, in these subjects melanosis due to hyperpigmentation of the maxillary and mandibular alveolar mucosa and periodontal disease take place, with the latter worsened by continued smoking during therapy [14-17]. A worsening towards both pre-cancerous lesions and, in the end, squamous cell carcinomas, represents about 90% of oral malignancies. Only a small number of oral cancers is represented by sarcomas [18].

On the basis of these premises, the knowledge of oral epithelial homeostasis after exposure to an exogenous stimulus such as smoke is crucial in basic oral biology. As mentioned above, most studies have been carried out in primary or immortalized keratinocyte cell cultures [11] or in a three-dimensional reconstructed oral mucosa [9, 10] and considered only single cigarette components, leaving thus many issues to be still elucidated.

Two recent works reported a cytogenetic [19] and transcriptome [20] analysis, but the direct effect of chronic cigarette smoke as a whole on the morphological features of human oral mucosa has not yet been evaluated, in particular regarding the maintenance of the physiological barrier.

Human Oral Mucosa

The three dimensional arrangement of the human oral mucosa grants an efficient barrier against several physico-, chemical-, and bacterial agents. Underneath, the connective compartment represents a structural support aimed at sustaining from both a mechanical and a nutritional point of view the above placed oral epithelium facing the cavity of the mouth. In this epithelium, oral keratinocytes are arranged in several layers with different orientations with respect to the surface (Figure 1).

Starting from the most profound layer upwards, the basal layer is constituted by a monolayer of cylindrical cells able to replicate and lying on a basal membrane interposed between the connective tissue and the basal cells.

When keratinocytes exit from the basal layer, they lose their proliferative ability and undergo a complex process of maturation and differentiation, known as terminal differentiation (TD). Immediately over the basal layer, keratinocytes constitute a multilayered compartment, the spinous layer, in which abundant intercellular junctions are present, accompanied by a well developed cytoskeletal apparatus made up mainly by intermediate filaments of cytokeratins.

Keratinocytes gradually change their orientation until becoming flattened and aligned, with their major axis parallel to the epithelial surface in the granular layer, where their cytoplasm is filled with keratohyalin granules and keratin filaments. In those areas of the oral mucosa particularly subjected to mechanical stress at the epithelial surface, oral keratinocytes progressively lose their nucleus and almost all intracellular organelles, forming the stratum corneum or horny layer, which represents the first compartment facing the oral cavity. In some areas of the mouth, oral keratinocytes are still nucleated also in the horny layer.

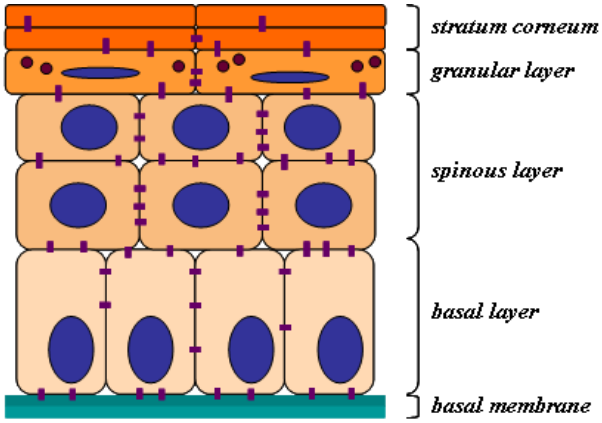

Figure 1. Schematic representation of the three-dimensional structure of keratinized human oral mucosa.

Human Oral Mucosa and Intercellular Adhesion

Along with the epithelial arrangement, the three-dimensional physiological barrier offered by the oral mucosa is maintained by desmosomes, adherens junctions (AJs), and tight junctions (TJs). In all these junctions, a similar design is shared, with a i) hydrophobic transmembrane domain crossing the cell membrane, ii) a hydrophylic extracellular part interacting with the corresponding extracellular portion of the adjacent cell, and iii) a hydrophilic intracellular tail linking the cytoskeletal filaments. However, from a molecular point of view, each junction has unique components. Both desmosomes and AJs characterize the junctional apparatus throughout the whole living oral epithelium and their distribution is strictly linked with the TD process. Conversely, TJs are confined to the uppermost granular layer and are specific of the late stages of keratinocyte TD. For this reason, the constitutive adhesive proteins in the different intercellular junctions are considered as TD biomarkers. Desmosomes are strong intercellular junctions in which desmosomal cadherins, i.e. desmocollins (Dscs) and desmogleins (Dsgs), are linked to the keratin intermediate filaments by an intracellular bridge constituted by the plaque proteins plakoglobin and plakophylin [21]. Thanks to their structure, desmosomes are most abundant in tissues and organs subjected to mechanical stress, as epidermis, oral epithelium, and myocardium, but the different Dsc and Dsg isoforms are specifically expressed in the various tissues. For example, in the oral mucosa, throughout the whole epithelium, the most distributed isoform is Dsg3, while the “skin type” desmosomal cadherins are Dsc1 and Dsg1. In the last two decades, desmosomes were clearly reported to have additional roles besides adhesion, as in cell signalling during development, tissue morphogenesis and wound healing. [21-26]. A pivotal role during epithelial differentiation is played by E-cadherin, a transmembrane protein present in AJs with an extracellular domain interacting with the homologous domain of the adjacent keratinocyte. The intracellular tail is linked to actin microfilaments thanks to cytoplasmic proteins as β-catenin [27]. The altered expression of the E-cadherin/β-catenin complex is considered an important biomarker for the epithelial-to-mesenchymal transition (EMT), the

process responsible for the cellular neoplastic transformation as it reveals an enhanced motility of cells [28, 29]. In TJs plasma membranes of adjacent cells are fused in the characteristic "kissing points" [30]. Within this junctions several proteins were identified, among which occludin represents the transmembrane protein and zona occludens 1 (ZO-1) together with claudins are responsible for the maintenance of the adhesive properties. This type of intercellular junction is crucial for preventing the paracellular way to substances and microbes entering or exiting the epithelial compartment [27]. A significant correlation between the adhesion strength and the TD process is established by calcium concentration which is relevant both to determine the differentiation degree of keratinocytes and to stabilize the reciprocal bonds among the different proteins constituting all the above cited intercellular junctions.

Smoke and Intercellular Adhesion

The direct effect of an exogenous stress as smoke on the different junctions was reported in two studies analyzing the AJs and TJs molecular composition in cultured cells. First, both E-cadherin and ZO-1 expressions were reduced in co-cultures of pretreated fibroblasts and immortalized keratinocytes by smokeless tobacco [31]. Additionally, ZO-1 disassembly was observed in bronchial cultured epithelial cells following cigarette smoke exposure [32]. However, from a morphological point of view, scattered evidences are available on the whole human oral mucosa exposed to the smoke in toto. We recently demonstrated that in biopsies obtained from healthy young chronic smoker women the expression of two epithelial differentiation markers as Dsg3 and keratin 10 was affected, suggesting that the overall process of keratinocyte terminal differentiation was altered, without damages to the three-dimensional structure of the oral mucosa [33]. On the basis of i) these observations and ii) of the studies reporting that TJs and AJs are a smoke target [31, 32], we decided to investigate the smoke outcome in keratinized human oral mucosa on the molecular composition of these junctions to further understand the molecular mechanisms underlying the epithelial response to smoke.

The present study stands in continuation with the previous one and was carried out on biopsies of keratinized human oral mucosa. Human biopsies were obtained from healthy young chronic smoking women (n=5) compared with a parallel group of non-smoker healthy volunteers (n=5), as the smoking habit among women is ever more spreading.

The expressions of intercellular adhesion biomarkers for TJs (occludin) and AJs (E-cadherin and β-catenin) were investigated by indirect immunofluorescence. In particular, these last two proteins were considered as markers for epithelial-mesenchymal transition.

Furthermore, keratinocyte proliferation was quantitatively evaluated after incubation and incorporation of 5-bromo-2'-deoxyuridine and results were expressed as BrdU-positive cells/mm^2 of viable epithelium measured with an image analysis system. These results were then compared with the control group.

The three dimensional architecture of oral mucosa was well preserved in both control and smoker groups (Figure 2). By the tetrachromic histochemical Dane and Herman's method specific for prekeratins and keratins, we observed a homogenous orange staining in controls,

but an abrupt reduction of colour intensity in uppermost layers of the oral epithelium of smokers (Figure 3, black arrows).

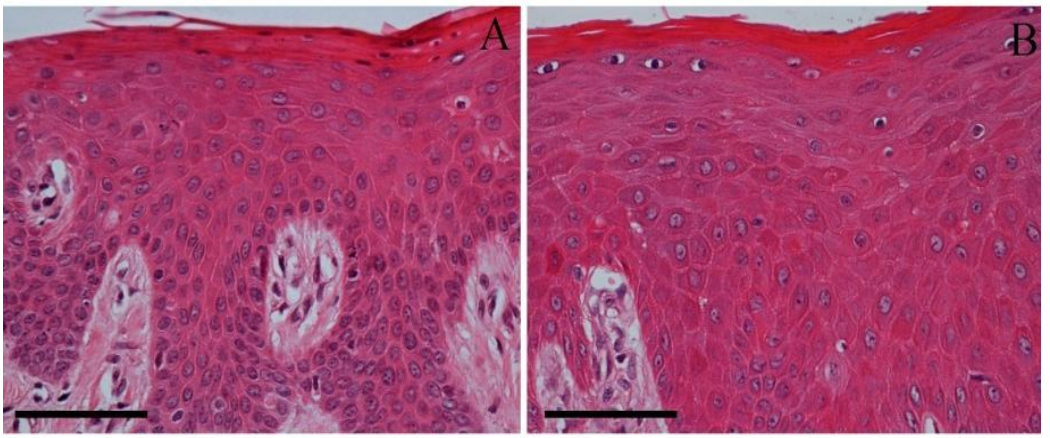

Figure 2. Representative micrographs of human oral mucosa sections stained with haematoxylin and eosin. (A) control and (B) smoker. Bars = 50 μm.

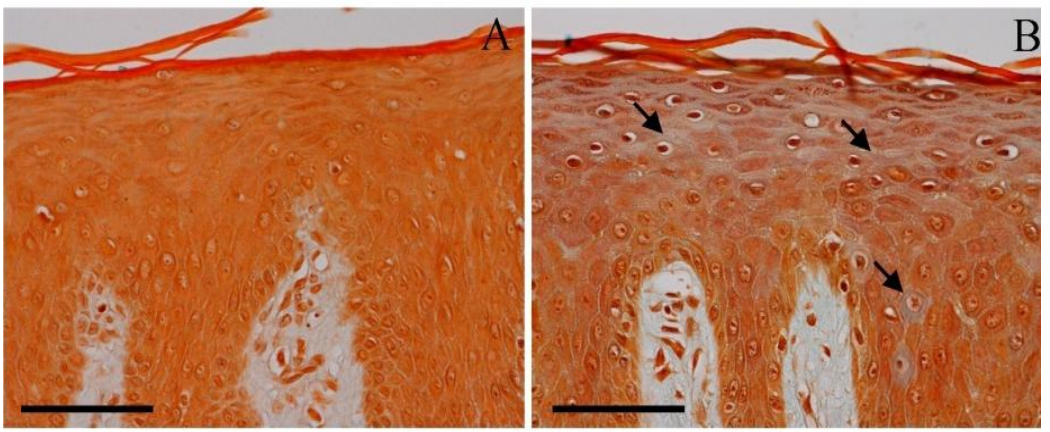

Figure 3. Representative micrographs of human oral mucosa sections stained with Dane-Herman tetrachromic method specific for prekeratins and keratins. (A) control and (B) smoker. Black arrows in B indicate cells with pale cytoplasm in smokers. Bars = 50 μm.

By immunofluorescence, we never found differences between the two experimental groups regarding the pattern distribution of occludin, E-cadherin, and β-catenin (Figure 4).

The analysis of cell proliferation demonstrated that proliferating cells were always located in the basal layer of the oral epithelium (Figure 5) and no differences existed between the two groups from a quantitative point of view (Figure 5).

We can thus conclude tha TJs and AJs molecular composition was maintained after chronic exposure to cigarette smoke and also epithelial proliferation resulted unaffected.

Interestingly, on the other hand, we previously demonstrated that desmosomes, despite their strength in maintaining intercellular adhesion, were the only junctions affected in their molecular composition after a prolonged exposure to smoke.

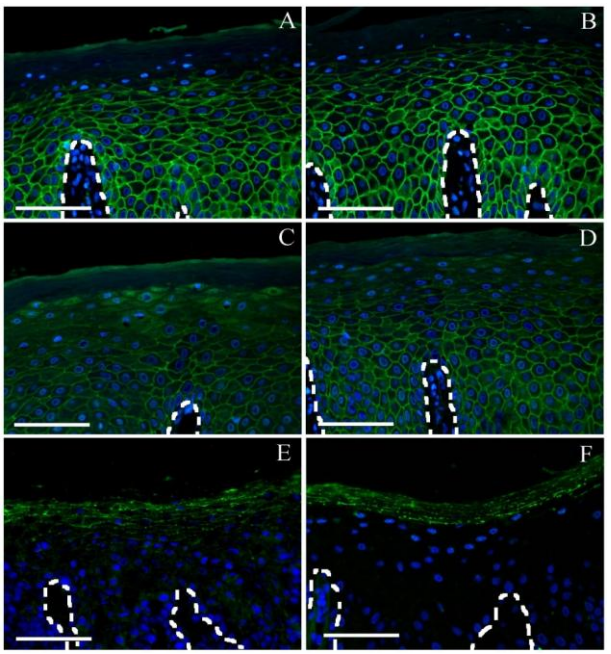

Figure 4. Representative micrographs of human oral mucosa sections after immunofluorescence evaluation of the epithelial distribution of E-cadherin (A and B), β-catenin (C and D), and occludin (E and F). (A, C, and E) control and (B, D, and F) smoker. White dot lines indicate the basal membrane. Bars = 50 μm.

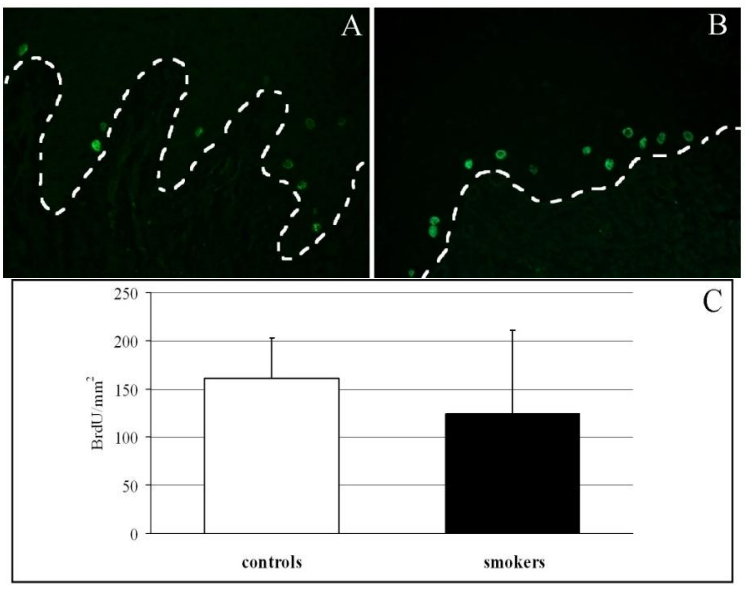

Figure 5. Nuclei of proliferating keratinocytes in human oral mucosa sections after immunofluorescence evaluation of BrdU incorporating cells. (A) control and (B) smoker. In (C) the quantitative results are reported. The mean value of BrdU/mm^2 obtained in controls (n=5; white column) and smokers (n=5; black column) + 1 SD is shown. BrdU: 5-bromo-2'-deoxyuridin. Bars = 50 μm.

Conclusion

The effect of cigarette smoke on the intercellular junctions remains controversial. To explain the discrepancy of our data with those obtained in previous experimental studies we must consider the different experimental settings used and the different smoke components analyzed. Moreover, we can hypothesize that the microenvironment and the three-dimensional arrangement of the oral mucosa play a crucial role in the keratinocyte response to smoke, thus allowing the maintenance of the physiological barrier with the preservation of the molecular composition of both AJs and TJs.

However, the lack of evidence for a modification in these junctions in our experimental conditions can not exclude that they might be influenced by cigarette compounds in an earlier phase than desmosomes and that the molecular composition of adherens and tight junctions could progressively be reverted to the physiological pattern also in smokers.

Thus, when the molecular composition of desmosomes is affected after a prolonged exposure to smoke, AJs and TJs can represent good candidates for maintaining the intercellular adhesion and, consequently, the physiological barrier thanks to a "compensatory mechanism". Future studies will be aimed at investigating the acute effects of smoke in a three dimensional model of organotypic culture of human oral mucosa to elucidate the first early steps in the epithelial response.

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Chapter 8

Cigarette Smoking and Risk Factors of Cardiovascular Disease

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Abstract

Cigarette smoking continues to be a major health hazard, and it contributes significantly to cardiovascular morbidity and mortality. Both active and passive cigarette smoke exposures predispose to cardiovascular events. Although, the underlying mechanisms remain unclear, cigarette smoke increases inflammation, thrombosis, and oxidation of cLDL. Recent experimental and clinical data support the hypothesis that cigarette smoke exposure increases oxidative stress as a potential mechanism for initiating cardiovascular dysfunction. This study aimed to investigate the effects of cigarette smoking on cardiovascular risk indicators: homocysteine, criteria of metabolic syndrome especially lipid profile, and paraoxonase activity, and to determine the correlation between these factors and two biological tobacco markers: plasma thiocyanate (SCN^-) and cotininuria. The initial study was conducted on 300 voluntary subjects: 138 non-smokers and 162 smokers aged respectively 38.47 ± 21.91 and 35.55±16.03 years. Folate, vitamin B12 and homocysteine (tHcys) were measured by immunoassay. Total cholesterol (TC), triglycerides (TG), cholesterol HDL (cHDL) and cholesterol LDL (cLDL) were determined by enzymatic colorimetric methods. ApoA1, ApoB and Lp(a) were analyzed by immunoturbidimetry. Paraoxonase activity was measured by kinetic method. Cotinine was measured using an immuno-enzymatic method and SCN^- by a

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selective electrode. In smokers, we found a significant increase in tHcys and decrease in folate and vitamin B12 levels compared to non-smokers. tHcys was strongly correlated with the consumption duration and the number of cigarettes consumed. Folate and vitamin B12 were significantly reduced in subjects smoking for more than 20 years compared to those who smoked less than 5 years. Among smokers, we noted a positive correlation between tHcys and both SCN^- and cotininuria, and a negative correlation between cotininuria and plasma folate. Smokers showed significant higher levels of TC, TG, cLDL, Lp(a) and ApoB/ApoA1 ratio and lower levels of cHDL than non-smokers. In addition, TG values were significantly higher in subjects smoking more than 30 cigarettes/day compared to those smoking 5-10 cigarettes/day. Additionally, we noted a significant decrease of paraoxonase activity in smokers compared to nonsmokers, with regression of paraoxonase activity according to number of cigarettes. The prevalence of metabolic syndrome was significantly higher in smokers compared to nonsmokers (OR=2.9;95% IC: 1.61- 5.31). In smokers, 94.5% met the criteria for hypertriglyceridemia, 92.5% for low cHDL, 73.5% for hypertension, 37.7% for high fasting glucose and 34% for obesity. In conclusion, cigarette smoking lowers the levels of anti-risk factors but raises the risk factors. This funding confirms the high risk of cardiovascular diseases in smokers.

Introduction

Cardiovascular disease is among the leading causes of death in developed countries. Cigarette smoking is a serious health problem and most important avoidable causes of death in world [1]. Smoking has been strongly implicated as a risk factor for chronic obstructive pulmonary disease, cancer and atherosclerosis, etc. [2, 3]. The leading causes of death from smoking are cardiovascular diseases (1.69 million deaths), chronic obstructive pulmonary disease (0.97 million deaths) and lung cancer (0.85 million deaths) [4]. Cigarette smoking is one of the major risk factors for cardiovascular disease for both males and females [5]. Many reports find that cardiovascular disease is the most important cause of smoking related premature death [6]. Up to 50% of avoidable deaths in the industrialized world have been attributed to smoking, half of which show cardiovascular disease. Investigators have attempted to elucidate the mechanisms of the pathogenesis associated with cigarette smoking, but the conclusions were not consistent. A basic hypothesis is that free radicals cause oxidative damage to macromolecules such as lipids, proteins and DNA; therefore, free radicals are believed to be instrumental in the pathogenesis of diseases [4]. Endothelial injury is considered to be a key initiating event in the pathogenesis of atherosclerosis [7]. It seems reasonable to hypothesize that cigarette smoking may exert its effects through endothelial damage, as proposed by Pittilo [8]. Cigarette smoking is also associated with increased monocyte-endothelial cell adhesion when endothelial cells are exposed to plasma from healthy young smokers [1]. In addition, results from the European Concerted Action Project case control study showed that plasma total homocysteine was increased by cigarette smoking [1]. Homocysteine is an intermediary metabolite of the essential amino acid methionine. The association between increased circulating homocysteine concentrations and premature vascular thrombotic events in individuals with hereditary homocystinuria is well established [1]. This process may include platelet activation, smooth muscle cell proliferation, and enhanced leukocyte binding to the endothelium [1]. In recent years, a relationship between milder degrees of hyperhomocysteinaemia and vascular disease has emerged, and this has

been the subject of intense research. Hyperhomocysteinemia can be caused by a wide range of disorders, the most important of which are genetic defects of the enzymes involved in homocysteine metabolism and/or deficiencies of their co-factors: folate (former vitamin B9), vitamin B12 and vitamin B6 [1].

Among other effects, cigarette smoking causes lipid peroxidation, and it has been suggested that lipid peroxidation contributes to the increased risk of premature atherosclerosis and coronary heart disease among smokers. Metabolic activity of high-density lipoprotein can prevent the oxidation of LDL, which might be mediated by certain enzymes. Paraoxonase (PON) is one of the enzymes located on HDL particle, and it has been shown in vitro that PON reduces the accumulation of lipid peroxidation products on LDL. Furthermore, it has been demonstrated that PON hydrolyzes lipid peroxides also in human atherosclerotic lesions, strengthening the hypothesis that PON is also anti-atherogenic in vivo. Smokers are at greater risk of developing insulin resistance and subsequently diabetes than non-smokers [9, 10]. Metabolic syndrome has been documented to increase the risk of cardiovascular disease [11]. Although, the pathophysiology of metabolic syndrome is unclear. Thus, smoking is theoretically an important risk factor for metabolic syndrome. Smoking is an escalating health problem especially in developing countries such as India. Cigarette smoking is a known risk factor for peripheral, coronary and cerebral atherosclerotic vascular diseases. Cigarette smoking leads to the uptake of many hazardous compounds and their metabolites extracted from burning tobacco. These substances may be electrophilic and react with biological molecules, and give rise to oxidative stress through the formation of reactive species or the initiation of lipid peroxidation chain reactions in the membranes [1]. Plasma lipoprotein abnormalities are major risk factor for the occurrence of atherosclerotic vascular disease [2]. Cigarette smoking has been found to alter the lipoprotein levels [8]. Previously published reports suggest that oxidatively modified low density lipoprotein is taken up by macrophages to form foam cells in culture and aggravate the process of atherosclerosis [4]. Also, the effects of elevated lipid levels and changes in lipoprotein among cigarette smokers were demonstrated earlier. The effects of cigarette smoking on serum apolipoprotein A1 and apolipoprotein B in smokers without other risk factors of atherosclerotic vascular disease and dose response relationship were studied. The correlation of Apo A1 with cHDL and Apo B with cLDL as coronary risk factors was also examined along with the effects of smoking on cHDL/Apo A1 and cLDL/Apo B. The aim of this study was to investigate the effects of cigarette smoking on cardiovascular risk indicators: plasma homocysteine levels, criteria of metabolic syndrome especially lipid profile, and paraoxonase activity, and to determine the correlation between these factors and two biological tobacco markers: plasma thiocyanate (SCN^-) and urinary cotinine.

Materials and Methods

Study Design

Population

The study was performed on 300 voluntary subjects: 138 non-smokers (62 men and 76 women) aged 38.47 ± 21.91 years and 162 current smokers (145 men and 17 women) aged

35.55 ± 16.03 years. Subjects with peripheral vascular disease, diabetes mellitus, renal disease, hepatic disease and hyperlipidemia or hypertension or receiving any medication were also excluded. None of these subjects was vegetarian or vegan or used vitamin supplements.

Written informed consent was obtained from all voluntary adult participants and from the parents of minors.

Samples

After a 12 h overnight fasting, venous blood from each subject was drawn in tubes containing lithium heparinate for classical biochemical parameters, and in tubes containing EDTA/K_3 for plasma homocysteine, folates, vitamine B12 and thiocyanates. Blood samples were immediately centrifuged at +4°C and stored at –80°C until analyses.

Urine samples were obtained from the smokers and nonsmokers. These samples were either used the same day or frozen at -20°C until required for analysis. All the samples were analysed for urine cotinine.

Methods

Smoking Questionnaire

Smoking habits were investigated by standard questionnaire: questions covered both previous and current smoking habits, including the duration of smoking (age at start, years of smoking) and number of cigarettes smoked per day. Participants were classified into: (1) never smokers: those who had never smoked cigarettes; (2) current smokers: those who smoked regularly at least 1 cigarette/day during the previous year. The majority of subjects were able to provide information on the number of cigarettes they smoked and the duration of smoking. All subjects were questioned about their socio-demographic characteristics including age, gender, education and employment.

Biochemical Assays

Lipid Profile Assay

Total cholesterol (TC), HDL cholesterol (cHDL) and triglycerides (TG) were determined by enzymatic methods, and apolipoprotein (ApoA1, ApoB) and lipoprotein (Lp(a)) levels were determined by immunoturbidimetric techniques using Konelab 30™ equipment.

Homocysteine and Vitamin Profile

Plasma homocysteine concentrations were determined by an immunometric assay using the AxSYM® (Abbott Laboratories, Abbott Park, IL 60064, Barcelaneta, Puerto Rico). Folic acid and vitamin B12 were determined using an immunoenzymatic method (Elecsys 2010™ Roche Diagnostics, Indianapolis, IN, USA).

Paraoxonase Activity

Paraoxonase activity was determined using paraoxon (1.2 mmol/L) as the substrate in 0.1M Tris/ HCl buffer at pH= 8.0, containing 2mM $CaCl_2$ (0.5ml final volume). The sample to be tested was added (5µL) to start the reaction and the increase in the absorbance at 405nm

was recorded (Araoud et al, 2011). One international unit (IU) of paraoxonase activity is defined as 1 µmol of p-nitrophenol formed per minute, and activity was expressed as IU/L of plasma.

Tobacco Biomarkers

Cotinine levels were determined using homogenous enzymes immunoassay method (Konelab 30™, Thermo Electron Corporation, Finland) and expressed as micrograms per micromol of creatinine in urine. Plasma thiocyanates levels were determined using selective electrodes (Ionometer Seven Multi S80, Mettler Toledo, Switzerland) and expressed as milligrams per liter in plasma.

Clinical Evaluation

BMI was calculated as weight (kg) divided by height (m2).

Criteria for Metabolic Syndrome

The metabolic syndrome was defined according to NCEP ATP III criteria by presence of three or more of the following:

1. Abdominal obesity: waist circumference >102 cm in men and > 88 cm in women;
2. Hypertriglyceridemia: ≥150 mg/dL (1.69 mmol/L);
3. Low c HDL: < 40 mg/dL (1.04 mmol/L) in men and <50 mg/dL (1.29 mmol/L) in women;
4. High blood pressure: ≥130/85 mm Hg;
5. High fasting glucose: ≥110 mg/dL (≥ 6.1 mmol/L).

These five parameters were designated metabolic syndrome risk factor components' in the current study, and metabolic syndrome was diagnosed when three or more metabolic syndrome risk factor components were present.

Statistical Analysis

The statistical analyses were performed using the SPSS 17.0. Quantitative variables were presented as mean ± SD and comparisons were performed using the Student's t test. Qualitative variable comparisons were performed using the χ^2 test. Odds ratios (ORs) and their 95% confidence interval (CI) were calculated. Adjustment for potential confounder factors was determined by binary logistic regression. A comparison between smokers and non-smokers in paraoxonase activity was performed using analysis of variance (ANOVA) after adjustment for potential confounder factors (gender, age, BMI and lipid profile). Breslow- low of tarone: χ^2 interaction test was used to study the combined effect of two parameters. The statistical significance level was set at $p < 0.05$. All variables with a p value < 0.25 between the two studied groups (smokers and non-smokers) were considered as confounding factors for further OR adjustment.

Results

Risk Factors of Cardiovascular Disease in Smokers

Effect of Cigarette Smoking on Plasma Homocysteine Concentrations

As shown in Table 8.1, plasma homocysteine concentrations were significantly higher in smokers than in non-smokers. Also, there was no significant difference in plasma folic acid concentrations between the two groups. However, plasma vitamin B12 concentrations were significantly lower in smokers. After adjustment of plasma homocysteine concentrations for potential confounders, we noted a significant difference between smokers and non-smokers (p= 0.0001).

Table 8.1. Variation of homocysteine, folic acid, vitamin B12, thiocyanate and cotininuria levels according smoking status

Parameters	Smokers n = 162	Non smokers n = 138	p
Homocysteine, µmol/L	18.46 ± 9.64	12.75 ± 3.94	0.04
Folic acid, nmol/L	4.30 ± 1.56	4.45 ± 2.45	0.56
Vitamin B12, pmol/L	363.94±140.96	407.45±192.97	$< 10^{-7}$

Table 8.2. Odds ratio of lower paraoxonase activity associated with smoking status

	OR	CI 95%	*p*-value	OR adjusted	CI 95%	*p*-value
Hyperhomocysteinemia > 15µmol/L	3.2	1.9-5.4	$< 10^{-4}$	2.8	1.29- 6.12	0.009

Homocysteine: 15µmol/L a median, OR adjusted for Age, gender, folates and vitamin B12.

Table 8.3. Variation of homocysteine, folic acid, vitamin B12 levels according consumption duration and number of cigarettes smoked/day

Parameters		Homocysteine µmol/L	Folic acid nmol/L	Vitamin B12 pmol/L
Consumption duration (Years)	[1-5[(n= 15)	18.06 ± 9.76*	4.18 ± 47*	418.84± 27.49*
	[5-5[(n=50)	18.31 ± 8.22	4.17 ± .50	385.12 ± 44.31
	[15-0](n=22)	18.43 ± 11.59	4.12 ± .59	352.09 ± 38.24
	> 20 (n= 26)	20.22 ± 8.05*	3.89 ± 17*	339.08± 41.28*
Cigarettes (smoked/day)	[5-20](n=82)	18.22 ± 11.23	4.32 ± .35	364.58 ± 45.78
	[21-30] (n=15)	18.85 ± 11.82	4.33 ± .57	359.43 ± 61.39
	> 30 (n=16)	23.33 ± 14.10	4.04 ± .50	344.05 ± 106.33

* $p < 0.05$.

We calculated the odds ratio of hyperhomocysteinma (> 15 µmol/L) before and after adjustement for confounder factors (age, gender, folates and vitamin B12) associated with smoking status, we noted a significant association between smoking status and hyperhomocysteinemia in the two situations (table 8.2) (OR= 3.2; $p < 10^{-4}$ before and 2.8; p = 0.009 after).

Table 8.3 shows that folic acid and vitamin B12 were significantly lower in subjects smoking more than 20 years compared with those smoking less than 5 years. We found an important correlation between homocysteine concentration and duration of smoking, between folic acid and duration of smoking (r= 0.989, p= 0.01), and between vitamin B12 level and duration of smoking (r = 0.989, p = 0.007). We found an important correlation between homocysteine concentrations and number of cigarettes smoked/day (r = 0.839), but without any difference between groups. However, for folic acid and vitamin B12, we found no significant correlation with number of cigarettes smoked/day, but we noted a slight difference between groups.

Table 8.4. Variations of lipid profile and paraoxonase activity according to smoking status

	Smokers (n = 162)	**Nonsmokers (n = 138)**	***p* - value**
Age (years)	35.6 ± 16.0	38.5 ± 21.9	0.172
Sex ratio	2.87	0.69	< 0.001
BMI (kg/m²)	24.24± 3.17	25.63 ± 4.36	0.003
TC (mmol/L)	4.13 ± 1.18	3.70 ± 1.04	0.005
cHDL (mmol/L)	0.94 ± 0.25	1.07 ± 0.27	0.001
cLDL (mmol/L)	1.35 ± 0.56	1.16 ± 0.61	0.01
TG (mmol/L)	1.79 ± 1.03	1.40 ± 1.24	< 0.0001
Apo B/Apo A1	0.83 ± 0.52	0.52 ± 0.15	0.03
Lp (a) (g/L)	0.23 ± 0.23	0.18 ± 0.19	0.04
PON 1 activity(IU/L)	94 ± 104	158 ± 133	0.001

Table 8.5. Odds ratio of lower paraoxonase activity associated with smoking status

	OR	**CI 95%**	**p-value**	**OR adjusted**	**CI 95%**	***p*- value**
PON1 activity < 90 IU/L	3.21	1.7 – 5.8	< 10-4	3.03	1.5 – 5.9	0.001

PON1 activity: 90 IU/L a median OR adjusted for Age, gender, BMI and lipid profile.

Paraoxonase 1 (PON1) Activity and Lipid Parameters in Smokers

As shown in table 8.4, PON1 activity and the concentration of cHDL were significantly lower in smokers than in non-smokers. Also, smokers had significantly higher levels of TC, TG, cLDL, Lp(a) and ApoB/$ApoA_1$ ratio than non-smokers. In addition, TG values were significantly higher in subjects smoking more than 20 cigarettes/day as compared to those smoking less than 10 cigarettes/day. We noted a significant decrease of PON1 activity in smokers compared to non smokers (94 ± 104 *Vs* 158 ± 133 IU/L; p = 0.001), with regression of PON1 activity according number of cigarettes/day. After adjustment of PON1 activity levels for potential confounders (lipid profile, BMI, gender and age), we noted a significant

difference between smokers and non-smokers (p = 0.002). To evaluate the adjusted association between smoking status and lower paraoxonase activity, we calculated odds ratio of lower paraoxonase activity (< 90 IU/L) associated with smoking status and adjusted for confounder factors (age, gender, BMI and lipid profile). We noted a significant association between smoking status and lower paraoxonase activity in the two situation (OR = 3.21; $p < 10^{-4}$ before, OR = 3.03; p = 0.001 after) (table 8.5).

In smokers, we found a significant positive correlation between paraoxonase activity and cHDL (r = 0. 4447; p < 0. 0001) (figure 8.1).

Table 8.6 shows the relationship between alcohol intake and the plasma lipids. Alcohol consumption was associated with increases in the plasma concentrations of both c HDL, and TG. There was a significant positive association between alcohol intake and Lp (a). There was a tendency for TC to increase with alcohol intake, although levels were only significantly raised in drinkers. Alcohol consumption had no significant effect at apolipoproteins in smokers; however we found a significant increase of Apo B in drinker's smokers.

Prevalence of Metabolic Syndrome in Smokers

The metabolic syndrome was more prevalent in smokers than non-smokers (OR=2.9; 95% CI: [1.61-5.31]). This was exclusively due to a higher prevalence of dyslipidemia (i.e. high triglycerides and/or low cHDL; p< 0.001), whereas abdominal obesity were less frequent in smokers (table 8.7).

In smokers, the positive association between metabolic syndrome and tobacco status parameters including both number of cigarettes smoked/day (OR= 1.8; CI 95% [0.6 - 2.9]) and consumption duration (OR= 2.4; CI 95% [1.2 - 5.4]) was found. Risk of metabolic syndrome increased when cigarettes smoked/day exceeds 20 and when the consumption duration of smoking was ≥10 years (table 8.8).

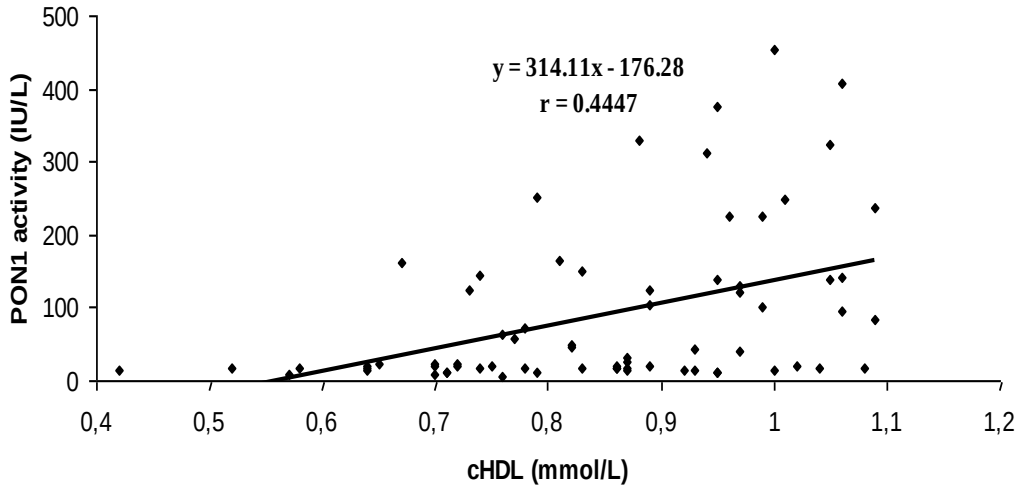

Figure 8.1. Correlation between PON1 activity and cHDL concentration in smokers.

Table 8.6. The effect of alcohol consumption on plasma lipids concentration in smokers

	Consumers (n= 126)	No Consumers (n=36)	p
TG (mmol /L)	1.82 ± 1.08	1.54 ± 0.79	0.05
TC (mmol /L)	4.20 ± 1.17	3.86 ± 1.16	0.11
cHDL (mmol /L)	0.97± 0.26	0.90 ±0.19	0.05
cLDL (mmol /L)	1.34 ±0.52	1.38 ± 0.70	0.82
ApoB/ApoA1	0.85 ± 0.56	0.75 ± 0.33	0.16
Lp (a) (g/L)	0.22 ± 0.19	0.29 ± 0.31	0.05

Table 8.7. Prevalence of metabolic syndrome risk factor components according to smoking status

	Smokers	Non-smokers	OR 95%	*p*-value
Hypertriglyceridemia (%)	45.2	10	3.39	$< 10^{-3}$
Low cHDL (%)	75.3	15	2.21	0.001
Hypertension (%)	11	9	1.2	0.4
Hyperglycemia (%)	13	10	1.2	0. 3
Obesity(%)	15	30	0.4	0. 003
Metabolic syndrome (%)	27	10.3	2.9	0.01

Table 8.8. Odds ratio of metabolic syndrome associated with smoking status parameters

	OR; CI 95%	*p*-value
Cigarettes smoked/day	1.8; [0.6-2.9]	0.02
Consumption duration/years	2.4; [1.2-5.4]	0.01

Table 8.9. Variation of urinary cotinine, urinary and plasma SCN- levels according smoking status, gender and alcoholic beverage

		Urinary cotinine (µg/µmol Cr)	p	Plasma SCN- (µmol/L)	P
Smoking status	Smokers	231.43 ± 205.22	**$< 10^{-7}$**	100.25 ±1.36	**$5\ 10^{-4}$**
	Non-smokers	73.22 ± 73.71		99.60 ± 0.91	
Gender	Men	222.88 ± 195.76		100.15 ± 1.39	
	Women	310.67 ± 277.40	**0.03**	100.70 ± 0.34	**10^{-4}**
Alcoholic beverage	Yes	222.19 ± 191.35	**NS**	100.37±1.5	**0.03**
	No	235.40 ± 211.96		99.80±0.51	

Cr: creatinine.

Table 8.10. Correlation between urinary cotinine, urinary and plasma SCN^- levels and consumption duration, cigarettes smoked/day and BMI

		Urinary cotinine (µg/µmol Cr)	Plasma SCN^- (µmol/L)
Consumption duration (Years)	**[1-5[**	158.61 ± 230.05	100.19±1.48*
	[5-15[	222.23 ± 187.40	100.21 ± 1.57
	[15-20]	252.34 ± 195.97*	100.42±0.15*
	> 20	272.88 ± 228.75*	100.43±1.07*
Cigarettes smoked/day)	**[5-10]**	133.89 ± 149.04*	99.47 ± 0.41*
	[11-20]	217.07 ± 204.90	100.31 ± 1.48
	[21-30]	309.09 ± 194.44*	100.89±1.55*
	> 30	341.38 ± 220.29*	102.07±2.95*
BMI (kg/m^2)	**< 25**	251.86 ± 216.65	100.24 ± 1.44
	[25- 27[	196.00 ± 111.96	100.00 ± 0.69
	[27- 30]	135.64 ± 137.59	100.62 ± 1.63
	> 30	50.26 ± 65.46	100.93 ± 2.04

* $p < 0.05$.

Correlation between these Factors and Two Biological Tobacco Markers

Urinary cotinine and plasma SCN^- levels were both significantly higher in smokers than in nonsmokers and correlated well with the number of cigarettes smoked per day. Urinary cotinine was significantly correlated with duration of consumption ($F_{3\text{-}109} = 3.43$; $p = 0.019$; $r = 0.9961$), and there was a negative correlation between body mass index and urinary cotinine ($r = 0.9989$; $p < 0.05$) (tables 8.9 and 8.10).

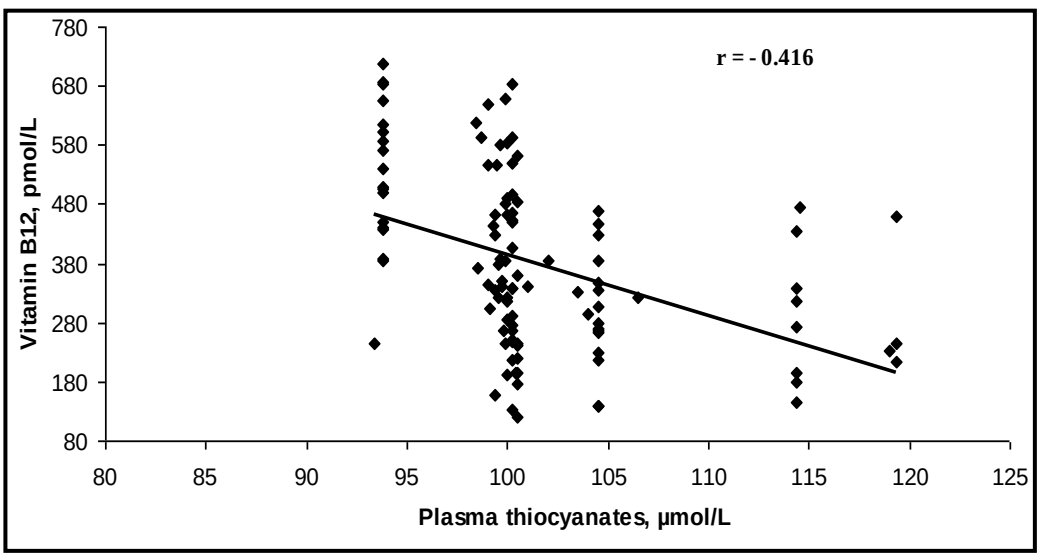

Figure 8.2. Correlation between Vitamin B12 and plasma thiocyanate.

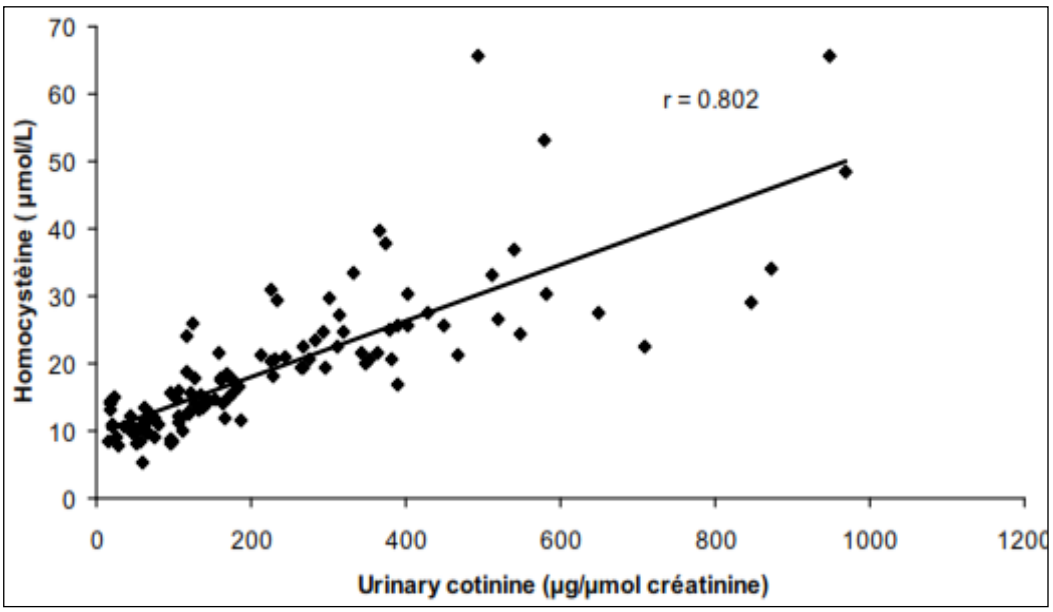

Figure 8.3. Correlation between urinary cotinine and plasma homocysteine concentration in smokers.

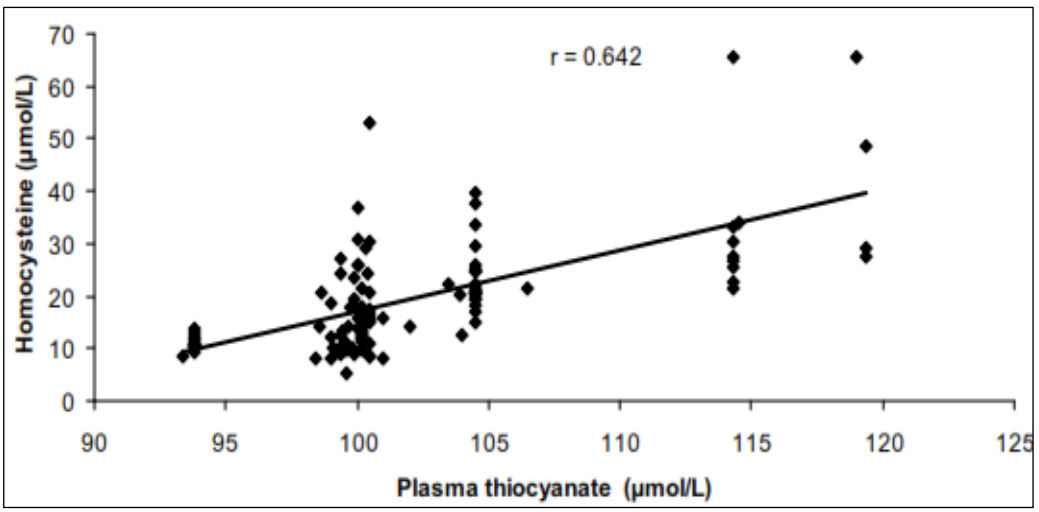

Figure 8.4. Correlation between plasma thiocyanate and plasma homocysteine concentration in smokers.

Figure 8.2 shows the negative correlation between plasma thiocyanates and plasma vitamin B12 (r = 0.416). Figures 8.3 and 8.4 show that the correlation between urine cotinine and plasma homocysteine concentrations (r = 0.802) is greater than that between plasma thiocyanates and homocysteine concentrations (r = 0.642). However, the significant correlation (r= 0.825) was found only in individuals who smoked more than 20 cigarettes/day. We found significant dissimilarity between smokers and non-smokers with respect to urine cotinine ($p < 10^{-7}$) and plasma thiocyanates concentrations ($p = 5.10^{-4}$). Paraoxonase activity

had significant negative correlation with urine cotinine (r= - 0.271; p = 0.03) and at the limit of the statistical significance with plasma thiocyanates (r= - 0.188; p = 0.06).

Discussion

Our data showed that plasma vitamin B12 was significantly lower and plasma homocysteine significantly higher in smokers than in non-smokers. It is known that hyperhomocysteinemia is linked to inadequate intake of vitamins, particularly B-group vitamins, and therefore may be amenable to nutritional intervention. The study by Bostom and Lathrop [13] is the only one in which concentrations of all three vitamins known to influence hyperhomocystinaemia were determined. It has been recognised that smoking affects the nutritional status of folic acid, vitamin B12 and vitamin B6, each of which regulates homocysteine metabolism, and/or because cigarette smokers have poorer diets than non-smokers; smokers are more likely to choose white bread, sugar, meat, butter, whole milk and eggs, and less likely to consume whole-wheat bread, high-fibre breakfast cereals, fruits and vegetables, than non-smokers. The usual dietary sources of vitamin B12 are meat and meat products (including shellfish, fish, poultry and eggs). The results obtained in our study are in accordance with the results of a study by Pagan et al. [14], who found plasma vitamin B12 concentrations to be significantly lower in smokers than in non smokers. Univariate analysis of local and systemic vitamin B12 concentrations showed significantly lower buccal mucosa vitamin B12 concentrations in current smokers. Although, there is wide documentation of the adverse effects of cigarette smoking on a variety of diseases and disturbances, the direct effects of smoking on nutrient concentrations are less well studied. Detrimental effects of cigarette smoke on systemic concentrations of vitamin B12 have been known for decades. Many of these published studies, however, have not considered other factors, including dietary intake, which might explain the differences in vitamin B12 status among smokers and non-smokers.

Several mechanisms might explain the increased risk in smokers with raised plasma homocysteine. Nicotine and carbon monoxide separately produce tachycardia, hypertension and vasoconstriction and both produce direct endothelial damage. Hyperhomocysteinemia has been associated with impaired endothelial function and abnormal flow mediated vasodilatation has been demonstrated with mild hyperhomocysteinemia [15]. Smoking may also damage the vascular tree via platelet activation, lipid peroxidation, enhanced tissue factor activation, increased fibrinogen levels and smooth muscle proliferation [15]. The fact that both of these risk factors can exert similar effects would suggest strong potential for interaction between them to produce vascular damage. While both smoking and homocysteine may damage the vascular tree independently, they are also related. Plasma homocysteine level was significantly affected by several B vitamins. Plasma folic acid was a significant factor affecting plasma homocysteine concentration in the smoker group. Our results were similar to those reported recently; O'Callaghan et al. [15] found that current smokers had higher plasma homocysteine levels and lower folic acid levels than those who never smoked. These data suggest that different factors contribute to the high plasma homocysteine concentrations in study subjects. Other factors such as genotype may also play an important role in the modulation of plasma homocysteine levels [1].

However, Plasma concentrations folic acid of the smokers was lower than those of the non-smokers, but not significantly different. It is important to mention that plasma folic acid is related to recent consumption, while red blood cell folic acid is an indicator of folic acid stores. People who smoke cigarettes are known to differ from persons who have never smoked with respect to several lifestyle behaviours, including eating less healthful diets. The causes for this deficiency are presently unclear; although a number of mechanisms have been proposed, including diminished dietary intake, poor absorption of polyglutamyl folic acids, decreased hepatic uptake and retention, increased urinary excretion of folic acid, impaired formation or hydrolysis of polyglutamates, and increased folic acid catabolism [16]. Several of the hundreds of chemical components of tobacco smoke have been shown to interact with folic acid coenzymes, transforming them into biologically inactive compounds. These chemical interactions may have physiological significance, which is supported by reports of lowering circulating folic acid levels in smokers. Reactive oxygen species can be produced by cigarette smoke-induced phagocytic cells and cause oxidative damage to DNA, proteins and lipids, which may be closely related to cardiovascular disease [16]. Chemical components found in tobacco smoke interact with the above and transform them into inactive compounds reducing their active concentration in biological fluids and possibly alter the ability of the cell to store and metabolise folate [10]. The lower plasma folate levels found in our study most likely follow the mentioned mechanism, and other studies have confirmed the finding [17].

We found a significant relation between raised homocysteine levels and number of cigarettes smoked per day in smokers but without difference between groups. A similar study also reports significantly higher basal homocysteine levels in smokers as compared to non-smokers, which is also related to the number of cigarettes smoked per day [18]. The Hordaland studies [18] found that the plasma homocysteine concentration increase markedly with the daily number of cigarettes smoked. In the multivariate analysis, sex, age, folate intake, cigarette smoking, and coffee consumption were found to be the most important determinants of plasma homocysteine concentration [17]. In addition, we found an important correlation between homocysteine level and duration of smoking; we noted that plasma homocysteine level increase when duration of consumption exceeds 5 years. We found an important correlation between folic acid level and duration of smoking, and between vitamin B12 and duration of smoking. However, for folic acid and vitamin B12 levels, we did not found significant correlation with number of cigarettes per day, but we noted a slight difference between groups. We showed that folic acid and vitamin B12 were significantly lower in subjects smoking more than 10 years compared with those smoking less than 5 years.

Among gender, the current study demonstrates that men smokers had high levels of homocysteine and a low level of folate and vitamin B12, but this difference was not significant, this lack association may result from a small size of women smokers' subject in this study.

Previous studies have demonstrated a fall in PON1 activity and cHDL concentration and a rise in TC, TG, cLDL, Lp(a) and Apo B/ApoA_1 ratio in smokers. It is known that smoking is associated with coronary artery disease and other vascular disorders. For the occurrence of cardiovascular disease among smokers alteration in plasma lipid profile was implicated. In this context, the mechanisms for the altered lipid profile among smokers were recalled [19]. First, nicotine stimulates the release of adrenaline from the adrenal cortex leading to increase

serum concentration of free fatty acids which further stimulates hepatic synthesis and secretion of cholesterol as well as hepatic secretion of very low density lipoprotein and hence increased TG [19]. Second, smoking decreases oestrogen levels and further leads to decreased cHDL concentration [19]. Also, cHDL concentration was inversely related to VLDL concentration in serum. Finally, smoking increases insulin resistance and thus, causes hyperinsulinemia. LDL and TG are elevated in hyperinsulinemic conditions due to decreased activity of lipoprotein lipase [19]. Also, human serum paraoxonase is a polymorph enzyme which has been shown to play an important role in lipid metabolism. PON1 significantly decreases lipid peroxidase generation during LDL oxidation in the presence of HDL modification by lipid peroxidase [20, 21]. Smoking impairs PON1 activity and thereby compromises anti-oxidant defense mechanism [22]. Moreover, a decrease in PON1 activity in smokers can be explained by the effects of several of the hundreds of chemical components of tobacco smoke have been shown to be responsible for inhibition of PON1 activity are various reactive aldehydes (acetaldehyde, formaldehyde and α,β-unsaturated aldehydes, such as acrolein and acrotonaldehyde), as well as aromatic hydrocarbons [23]. In addition, urinary cotinine and plasma thiocyanates concentrations were both significantly higher in smokers than in nonsmokers. Although, urine cotinine and plasma thiocyanates are influenced by the diet and the industrial pollution, it remains a reliable indicator of the smoking status [24]. We noted a significant association between smoking status and lower paraoxonase activity before and after adjustments for confounder factors. Cigarette smoke has a high content of oxidants that promote a pro-oxidant effect in blood plasma and tissues, which probably contributes to the increased incidence of cardiovascular disease present in smokers. The information available on the molecular mechanisms of action of cigarette smoke is limited. However, recent observations suggest that the pro-oxidant effect of smoking is, in part, related to PON1 activity inhibition caused by cigarette smoke [22].

We showed a significant positive correlation between paraoxonase activity and cHDL values. Paraoxonase is a calcium-dependant esterase closely associated with the high density lipoprotein subfraction that contains apolipoprtein A1 in human serum. Previous studies have suggested that HDL can prevent oxidation of LDL and that some oxidised LDL phospholipids are physiological substrates for serum PON1 [22].

This study showed that current smokers have a significantly higher risk for development of metabolic syndrome that was associated with abnormality in triglyceride level and cHDL level but not significantly related to the presence of high blood pressure, abnormal fasting glucose concentrations, or increased waist circumference. Among this group, there was a dose-dependent association among smoking amount and development of metabolic syndrome, high TG, and low cHDL levels. Metabolic syndrome and its individual components, high triglyceride level and low cHDL levels, were significantly higher in current smokers with a smoking amount ≥20 cigarettes/day. Our study supports the findings of previous studies [23-24] that metabolic syndrome is more frequent in current smokers than in those who have never smoked. Ishizaka et al [25] demonstrated that exposure to environmental tobacco smoke had a dose-response relationship with metabolic syndrome. Our study also revealed that cigarette smoking had a dose-dependent association with metabolic syndrome. This finding was consistent with previous studies [26-27]. After analysis of the components of metabolic syndrome, we found that there is also a dose dependent association among smoking, high TG levels, and low cHDL levels, a finding that was also reported by other studies [27, 28]. Why is smoking, even many years after cessation, associated with increased

prevalence of metabolic syndrome? Although we cannot determine the underlying mechanism in this type of cross-sectional study, there are several possible explanations for the association between smoking and metabolic syndrome in smokers. First, mechanisms for these long lasting effects of smoking on insulin resistance may include vascular changes that lead to decreased glucose uptake by skeletal muscle [29]. Second, we have demonstrated that smoking increased the circulating white blood cell count, a marker of inflammation, in subjects undergoing general health screening [30]. It is possible that proinflammatory cytokines, such as tumor necrosis factor may explain the association between smoking, increased white blood cell count, and metabolic syndrome [31, 32]. Third, Miyazaki et al. have reported that both current and former smoking is negatively associated with plasma levels of adiponectin [33]. Decreased adiponectin is thought to play a major role in the development of insulin resistance [34]; thus, it may be an underlying mechanism of metabolic syndrome in smokers. Fourth, nicotine stimulates the release of adrenaline by the adrenal cortex, causing a mobilization of free fatty acids from adipocytes thus stimulating [35] hepatic triglyceride synthesis and VLDL secretion, and in turn increasing triglycerides concentrations. Moreover, smokers have lower levels of lecithin-cholesterol acyl-transferase [36, 37]; the enzyme involved in the conversion of cholesterol to cholesterol ester, necessary for HDL mediated removal of cholesterol from peripheral tissues.

Urinary cotinine and plasma SCN^- concentration were both significantly higher in self smokers than in nonsmokers and correlated well with the number of cigarettes smoked per day and with the duration of consumption. Cotinine in body fluids is the most frequently used biomarker of tobacco smoke exposure [38, 39]. Cotinine has been shown to be the most specific and most sensitive marker; however, the urinary cotinine concentration is regarded as the best biomarker available for detection of exposure to tobacco smoke and for discriminating active smokers from nonsmokers. A mean of 70 - 80% of nicotine is converted to cotinine, which has a half- life of about 17 hours [38]. We noted that the urinary cotinine level was significantly correlated with the number of cigarettes smoked per day. However, cotinine is no longer considered the major metabolite of nicotine; which probably explains why the urinary cotinine level is only roughly related to daily cigarette consumption, because the correlation of urinary cotinine with the number of cigarettes smoked per day is related to that observed in serum or plasma specimens [40].

In this study, we found a significant correlation between mean urinary cotinine levels and duration of consumption. This correlation can be explained by the long half-life of cotinine, which is eliminated from the body after a few days and is mainly excreted in the urine. Moreover, smoking induces changes in nicotine disposition: the rate of cotinine disappearance from the urine is significantly slower in smokers than in nonsmokers [40]. Therefore the determination of this marker in urine is a good alternative to discriminate smokers from nonsmokers.

We found a significant correlation between plasma homocysteine level and urinary cotinine concentration in the whole group of active smokers, the important correlation found between urinary cotinine and plasma homocysteine in smokers was not surprising, because urinary cotinine levels were determined as a marker of tobacco smoke exposure [24]. There was a negative correlation between plasma thiocyanate and plasma vitamin B12 concentration. In smokers alone, this latter correlation was more pronounced, and the tendency for high plasma thiocyanate levels to be associated with relatively low plasma vitamin B12 concentrations was striking. Summarized, the results show that urine excretion

of B12 is raised in smokers, and that a high of plasma thiocyanate tends to be associated with an increase in vitamin B12 excretion and a relatively low plasma vitamin B12 concentration. The association between high plasma thiocyanate levels and low plasma B12 concentration, which is especially marked in smokers, recalls that between high plasma cyanide concentration and low plasma vitamin B12 [41]; these two observations are probably related. More than one hypothesis might be put forward to explain these results. Thus it could be postulated that subjects with relatively low plasma vitamin B12 concentrations have a reduced ability to detoxicate cyanide by pathways involving this vitamin, so that detoxication by the thiocyanate pathway is increased. This would not, however, readily explain the association between high plasma thiocyanate and high excretion of vitamin B12. Alternatively, the low plasma vitamin B12 concentration might reflect vitamin B12 depletion, possibly resulting from conversion of tissue cobalamins to cyanocobalamin, a form relatively readily excreted by the kidney [42]. However, the increment in vitamin B12 excretion associated with smoking is so small in relation to the amount probably absorbed daily and to the liver stores that it would seem unlikely that appreciable depletion could be caused in healthy people by this means. This consideration, together with the very poor correlation between plasma vitamin B12 and urine B12 excretion, suggests that some factors, other than increased renal excretion of vitamin B12, must operate to produce the relation between high plasma thiocyanate and low plasma vitamin B12 concentration. It is possible that high plasma cyanide concentrations disturb the equilibrium between plasma and urine vitamin B12. At the moment the main significance of this work is that it shows further definite, if unexplained, interrelationships between smoking, cyanide metabolism, and bodily handling of vitamin B12, and gives further support to the idea that high loads of cyanide might produce derangements of vitamin B12 metabolism. The effects of smoking are slight in healthy subjects, but in patients already in marginal vitamin B12 balance they might become significant.

Conclusion

Cigarette smoking lowers the levels of anti-risk factors and raises those of risk factors. This funding confirms the high risk of cardiovascular diseases in smokers. The present study suggests that chronic cigarette smoking can exert a deleterious impact on plasma homocysteine levels, so the smokers with high plasma homocysteine are at greatly increased risk of cardiovascular disease. They also have reduced levels of those B-vitamins that modulate homocysteine metabolism. Compared to people who never smoked, smokers had an increased prevalence of metabolic syndrome. As metabolic syndrome was an independent risk factor for cardiovascular disease, associations between smoking and cardiovascular disease may in part be mediated by this metabolic syndrome. Although, cessation of smoking is the ideal objective, it is not always attainable, and therefore any strategy to prevent the detrimental effects of smoking is desirable and should therefore be offered intensive advice to help them cease smoking.Chapter

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Chapter 9

Potential Role of Cigarette Smoking in Two Emerging Endemic Diseases: Chronic Kidney Disease and Diabetes Mellitus

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Abstract

The fight against cigarette smoking is a global challenge. Smoke is harmful in both active and passive smokers and it has a role in the initiation and progression of certain chronic kidney diseases (CKD), in the initiation of type 2 diabetes mellitus and in the development and progression of diabetic nephropathy and cardiovascular complications of diabetes mellitus (DM). Chronic kidney failure per se raises the risk of cardiovascular morbidity and mortality. Therefore tobacco use can be considered as a starter and/or confounder process that diminishes life quality or even shortens life expectancy. Cigarette smoking is a risk factor for chronic kidney disease and a predictor for the risk of end-stage kidney failure in the general population both in women and men. Smoking is the most significant factor associated with albuminuria in hypertensive patients, moreover, GFR-decline is generally faster in smokers versus non-smokers. Cigarette consumption seems to have a cumulative effect in the development of atherosclerotic renal artery stenosis.

In IgA nephropathy, which is the most frequent primary glomerulonephritis, smoking habit is clearly related to worse disease progression. The role of tobacco in the course of autosomal dominant polycystic kidney disease is yet controversial. However, in lupus nephritis smoking is associated both with faster progression and poorer outcomes after transplantation. Smokers on renal replacement therapy have generally a higher risk for mortality.

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The pathomechanism is probably multi-causative and somewhat different in the specific kidney diseases, but common „steps" are also present as shown in histology results. Smoking causes insulin resistance - thus increases the risk of developing metabolic syndrome and type 2 diabetes. Both in type 1 and type 2 diabetes, smoking increases the risk of development and progression of nephropathy and nearly doubles the rate of progression to end-stage renal failure. Cigarette smoking causes not only a deterioration in renal function, but also elevates microalbuminuria and proteinuria in diabetes mellitus. The pathomechanism of diabetic nephropathy is also complex, the rise in the urinary podocyte excretion is probably an early sign. In the future, having tools for the detection of the smoking-induced damage by biomarkers or ultrasound of the kidney would be a great help in the understanding of the pathomechanism. Chronic kidney diseases and diabetes mellitus should be prevented, therefore the intervention in the smoking habit - as a modifiable risk factor - should begin very early!

List of Abbreviations

ACE	angiotensin converting enzyme
ACR	albumin-to-creatinine ratio
ADPKD	autosomal dominant polycystic kidney disease
app	approximately
ARB	angiotensin receptor blocker
CAPD	continuous ambulantory peritoneal dialysis
CI	confidence interval
cyclic GMP	cyclic guanosine monophosphate
DM	diabetes mellitus
DNP	diabetic nephropathy
dsDNA	double-stranded deoxyribonucleic acid
eGFR	estimated glomerular filtration rate
ESRD	end-stage renal disease
ESRF	end-stage renal failure
FSGS	focal segmental glomerulosclerosis
HR	hazard ratio
MDRD	modification of diet in renal disease
NKF-KDOQI	National Kidney Foundation – Kidney Disease Outcomes Quality Initiative
NO	nitric oxide
OR	odds ratio
RR	relative risk
SH group	sulph-hydril group
SLE	systemic lupus erythematosus
$TGF_{\beta 1}$,	tissue growth factor β1

Introduction

The fight against cigarette smoking is a global challenge. Worldwide 1.2 billion people smoked in 2000 [1], a number that is projected to increase to 1.6 billion by 2030 [2]. Tobacco currently causes an estimated 5 million deaths annually and if the actual trends continue, the number of deaths will be doubled by 2030 [1]. Chronic cigarette consume is harmful in both active and passive smokers and it has a role in the initiation and progression of certain chronic kidney diseases (CKD), type 2 diabetes mellitus and in the progression of diabetic nephropathy and cardiovascular complications of diabetes mellitus (DM). It is also evident that chronic kidney failure raises the risk of cardiovascular morbidity and mortality, thus tobacco use can be considered as a starter and/or confounder of processes that diminish life quality or even shorten life expectancy. Noteworthy is the „memory for smoking" of the organism, namely the deleterious effects of tobacco consumption do not last only until the cessation of cigarette smoking but even years longer.

Tobacco consume is involved in the initiation and progression of the most common causes of CKD [3] e.g. diabetic nephropathy, ischemic nephropathy, nephrosclerosis, IgA nephropathy - the most frequent primary glomerulonephritis - or autosomal dominant polycystic kidney disease - the most common cystic kidney disease [4, 5] and some less frequent diseases like lupus nephritis [6]. Smoking causes insulin resistance – thus increases the risk of metabolic syndrome [7-9] and type 2 diabetes [10, 11]. Both in type 1 and type 2 diabetes smoking increases the risk of the initiation and progression of nephropathy [8]. The pathomechanisms of these diseases are complex and yet not fully understood, therefore each step can be crucial for the outcome of the disease for each individual. In this chapter we would like to underline the facts and theories that support the fight against an addiction that can destroy one's and the „surrounders" life.

Definition and Complications of Chronic Kidney Disease

The intended redefinition of chronic kidney disease (CKD) includes both kidney function and albuminuria [12]. On the basis of analyses of 45 cohorts including 1,555,332 subjects from general, high-risk and kidney disease populations the attendees of a Controversies Conference organized by „Kidney Disease: Improving Global Outcomes" (KDIGO) in 2009 agreed that both glomerular filtration rate and stages of albuminuria should determine the classification of chronic kidney disease for a better estimation of the prognosis of the kidney patients [12].

The decline in glomerular filtration rate and/or presence of proteinuria increase the risk of cardiovascular diseases [13, 14]. According to the NKF-KDOQI Guidelines cardiovascular disease is the leading cause of death in non-diabetic patients with chronic kidney disease, moreover the cardiovascular disease mortality is more likely than development of kidney failure in non-diabetic patients with chronic kidney disease [14], which means that these patients die due to fatal cardiovascular complications before they reach the end-stage renal failure. A prospective population-based cohort performed in Iceland with 16,958 people aged

33-81 years with a median follow-up of 24 years showed that even the earliest stage of chronic kidney disease is associated with higher risk of coronary heart disease [13].

It has been shown that albuminuria in a population with chronic stable coronary disease, including only partly diabetics, is an independent predictor of cardiovascular and all-cause mortality [15]. Albuminuria is not only a cardiovascular risk factor but it is also associated with cancer incidence [16]. A 10.3 year follow-up of 5,425 subjects without diabetes or previous cancer in the Tromsø Study has showed that the albumin-to-creatinine ratio (ACR) at baseline significantly correlated with the incidence of cancer, even after adjustment for age, gender, body mass index, physical activity, and smoking ($P < 0.001$). Participants with ACR in the highest quintile were 8.3- and 2.4-fold more likely to receive bladder cancer and lung cancer, respectively, compared with those with ACR in the lowest quintile after similar adjustments [16].

Smoking as a Risk Factor for Initiation and Progression of CKD in the General Population

There are more and more evidences accumulating in the field of the connection between cigarette smoking and CKD. Cigarette smoking is a risk factor for chronic kidney disease [17] and a predictor for the risk of end-stage kidney failure in the general population both in women and men [18]. A community-based, prospective observational study of 20 years duration in 23,534 men and women in Washington County, Maryland revealed a significant association between current cigarette smoking and the risk of CKD in both women and men (in women hazard ratio [HR] 2.9; 95% confidence interval [CI]: 1.7 - 5.0; and in men HR 2.4; 95% CI: 1.5 - 4.0) [17]. A prospective open cohort study using general practitioners databases with the goal of developing risk algorythms for estimating the individual 5-year risk of moderate-severe CKD and end-stage kidney failure in a primary care population (775,091 women and 799,658 men aged 35-74 years, contribution of 4,068,643 and 4,121,926 person-years of observation respectively) involved cigarette smoking as one of the main factors in the models [18]. A comprehensive review showed an overall evidence for current smoking as a risk factor for incident chronic kidney disease [19]. An increased risk of developing chronic kidney disease among smokers was significantly associated with male gender (relative risk [RR] 2.4, 95% CI: 1.2-4.5), >20 cigarettes smoked per day (odds ratio [OR] 1.51, 95% CI: 1.06-2.15, and relative risk 2.3, 95% CI 1.2-4.3), and smoking >40 years (OR 1.45, 95% CI: 1.00-2.09) [19]. In the Chronic Renal Insufficiency Cohort (CRIC) Study (3,612 participants, 46% women, 47% diabetics) among others, previous cigarette exposure was associated with lower eGFR [20]. Results from the Italian Longitudinal study of Aging (ILSA) revealed that during a 3.6 years follow-up of 2,981 subjects, aged 65-84 years, heavy current smoking (> 20 cigarettes/day) showed to be a risk factor for pathological loss of renal function (OR 2.3, 95% CI: 1.0-5.3) in a multiple logistic regression analysis model [21]. The third National Health and Nutration Examination Survey – a cross-sectional analysis of 15,719 adults - in the US revealed an association between cigarette smoking and albuminuria [22]. Current smoking was more common in persons with albuminuria (26%) compared to normal albumin-to-creatinine ratio (21%), and after adjusting for other risk factors, among hypertensives,

current smokers were 1.85 (95% CI: 1.29 - 2.64) times more likely to have albuminuria than never smokers [22], moreover current smokers with more than 40 pack-years were at highest risk for albuminuria. The role of passive smoking was also highlighted, among non-smoking hypertensives, those exposed to passive smoke (highest vs. lowest quartile of serum cotinine) were 1.41 (95% CI: 1.04 - 1.90) times more likely to have albuminuria, and surprisingly the association between tobacco use and albuminuria disappeared in former smokers among hypertensives if they stopped smoking for at least 1 year [22]. An age-associated decline in renal function is more marked in patients with co-existent cardiovascular risk factors, among these smoking seems to have an important role in the detrimental effect on renal function also in individuals without co-presence of other cardiovascular risk factors or renal diseases [23]. In the cross sectional PREVEND (Prevention of REnal and Vascular ENd stage Disease) study with 7,476 participants, compared to non-smokers, current smokers had higher median albumin excretion, and were more likely to have microalbuminuria and high-normal albuminuria with either elevated or decreased GFR, all differences showed a dose-dependent manner (above or below than 20cigarettes/day) [24]. In an analysis of 12,866 randomly assigned men of the The Multiple Risk Factor Intervention Trial (MRFIT) current smoking had an adjusted hazard ratio of 1.84 (95%CI: 1.35 - 2.51) for end-stage renal disease after 25 years [25]. A 10-year follow-up study with 123,764 (male: 41,012, female: 82,752) adults aged 40 years and over showed that smoking was a predictor of CKD in both genders (RR for CKD stages 3 and 4 1.13 and 1.16, respectively) [26]. In a Norwegian population-based cross-sectional study involving 30,485 men and 34,708 women a significant, dose-dependent elevation in risk for CKD (GFR<45ml/min per 1.73 m^2) was found above a cumulative lifetime exposure of 25 pack-years (adjusted RR 1.42 for 25 to 49 pack-years and 2.05 for >50 pack-years, respectively) [27]. The results of these studies - summerized in ***Table 9.1*** – also suggest a great benefit when a healthy subject or a kidney patient quits smoking.

Role of Tobacco in Autosomal Dominant Polycystic Kidney Disease

Tobacco consume plays also a role in the progression of certain specific kidney diseases. The facts concerning the influence of smoking in autosomal dominant polycystic kidney disease (ADPKD) are somewhat controversial. ADPKD accounts for 5-10% of patients with end-stage renal disease [28]. A Turkish epidemiological study [28] included 1,139 patients with ADPKD, where 20.3% were current smokers and 15% were ex-smokers, which underlines the importance of the topic. In a study performed on 270 ADPKD patients, 32 subjects with established proteinuria had a significant larger pack-year smoking history, higher mean arterial pressure, larger renal volumes, lower creatinine clearances than did their non-proteinuric counterparts, moreover, smoking history was the only significant independent variable determing the level of proteinuria [29]. In a retrospective multicenter, matched case-control study including patients with primary renal disease (IgA nephropathy and ADPKD) a significant dose-dependent increase of the risk to progress to end-stage renal failure (ESRF) was found in male smokers compared with non-smokers or moderate smokers [30]. A small sample size and modest average tobacco consumption caused the subgroup of women to be excluded from analysis [31]. Nevertheless, after adjustment to the ACE-inhibitor treatment,

the risk of ESRF was not increased in heavy smokers, which suggests a pivotal role of the renin-angiotensin system in the pathomechanism of the smoking-induced kidney damage. A study of 554 patients with type 1 ADPKD did not find, however, that cigarette smoking influences the disease course [32].

Smoking and IgA Glomerulonephritis

Cigarette smoking promoted in a dose-dependent manner the risk of progression to end-stage renal failure in male patients with IgA nephropathy in a retrospective case-control study [5, 30]. The pathomechanism – similar to that in ADPKD – probably involves the renin-angiotensin system because the history of ACE inhibitor treatment abolished the significance of the tobacco effect [5, 30].

Another clinical trial included 295 primer glomerulonephritis cases, 116 IgA nephropathy, 80 membranous nephropathy and 99 nephrotic syndrome with either minimal change nephropathy or focal segmental hyalinosis and 242 matched hospital controls [33]. In men the percentage of ever-smokers was significantly higher among cases with chronic renal failure than those without. Moreover, a dose-effect relationship was observed with both the daily and the cumulative dose of tobacco consumption, wich suggests a causative role of smoking [33].

Smoking was significantly related to chronic renal failure among cases who were older than 40 years and/or hypertensive and the results did not differ among the three histologic types mentioned above [33]. A single center retrospective study performed on 223 patients – both women and men – failed to confirm the influence of smoking on the progression of the disease [34], but the smoking habits were not well defined, women were included in a relevant number - of note the difference between the tobacco effects among the sexes is well defined [35] -, and past smokers seemed to have a poorer outcome of IgA nephropathy compared to current smokers [36]. A recent study including patients suffering from IgA nephropathy, FSGS or membranous glomerulonephritis also failed to confirm the role of smoking in the progression to ESRD independent of the baseline estimated GFR [37]. The study design and the fact that minimum 75% of the patients received ACE inhibitor or ARB treatment may explain that finding. In a most recent retrospective cohort study including 971 IgA nephropathy patients in 3 major nephrology centers in Japan during a 5.8 years observational period, 117 participants progressed to a 50% increase in serum creatinine level and 47 advanced to ESRD [38].

Current smokers and number of cigarettes smoked in the period of kidney biopsy were significant predictors of outcomes and the association of current smoking with adverse outcomes was stronger in those with lower compared with higher estimated glomerular filtration rates which confirms that smoking is - in a dose-dependent manner - a key prognostic factor in IgA nephropathy [38]. Also, in a study performed in a Japanese population – 485 patients with stage 1 and stage 2 chronic kidney disease (IgA nephropathy, lupus nephritis, minimal change GN, FSGS etc.) - smoking habit was clearly related to accelerated disease progression [39].

Cigarette Smoking – Importance in Hypertensive Nephropathy

Smoking is a classical risk factor for the initiation of ischemic nephropathy and for the progression of hypertensive nephropathy. At the beginning of the 20th century it was known that cigarette smoking elicits an acute transient rise in blood pressure [40]. Although in the first analysis of a large epidemiological cross-sectional study (IRSA) lower systemic blood pressure was described among current smokers compared with non-smokers [41], a subsequent analysis in men revealed a higher risk of hypertension in smokers [42]. The examination of the albumin excretion in the morning urine of 631 hypertensive subjects showed by multivariate analysis that smoking was the most significant factor associated with albuminuria [43]. In a prospective, 7-year-long study, performed on 225 hypertensive patients, GFR declined generally faster in smokers versus non-smokers, independent of urine albumin/creatinine ratio. However, the risk of GFR-decline increased robustly in subjects in whom the albumin/creatinine ratio was higher than 200 mg/g [44]. In patients with primary hypertension the prevalence of microalbuminuria is almost double in smoking than non-smoking lean patients and it has been shown that smoking is the strongest predictor for albuminuria [31]. The LIFE study has found that hypertensive and heavy smokers (> 20 cigarettes/day) with left ventricular hypertrophy had a 1.6-fold higher prevalence of microalbuminuria and a 3.7-fold higher prevalence of macroalbuminuria than never-smokers [31].

Smoking: A Common Origin of Ischemic Nephropathy

Ischemic nephropathy or atherosclerotic renal artery stenosis is an important cause of end-stage renal failure in patients older than 65 years [35]. In a retrospective study of 218 subjects who underwent angiography to investigate peripheral vascular disease – which is common in smokers - the incidence of atherosclerotic renal artery disease was significantly higher in those patients with femoral artery atherosclerosis than in those without femoral lesion [45]. Smokers have also, not surprisingly, a higher risk of critical atherosclerotic renal artery stenosis [46]. In an arteriography study performed on 67 patients older than 50 years the percentage of smokers was 80.5% in the group with significant atheromatous stenoses of the renal artery and 44.4% in the group of smokers without significant stenoses [47]. The fact that the presence of atheromatous stenoses of renal arteries was connected to the number of cigarettes and the exposure time, and not to the current compartment of patients towards smoking suggested a cumulative effect of smoking [47]. In an observational multicenter Spanish study 69.8% of the elderly patients with bilateral renal artery stenosis and chronic renal failure were smokers [48]. Both in unilateral and bilateral atherosclerotic renal artery stenosis the prevalence of smokers is higher compared to patients without stenosis [35]. Smoking is also a strong risk factor for cholesterol embolism, which could also contribute to the decline in renal function in patients with ischemic nephropathy [35]. In an Italian study of elderly patients with peripheral atherosclerosis multiple regression analysis showed that

smoking and LDL cholesterol were associated with the decrease of renal plasma flow, the degree of the latter was parallel with the severity of the peripheral atherosclerotic lesion [49].

Tobacco Use in Lupus Nephritis

The effect of smoking on the course of lupus nephritis is an underexamined field. In a meta-analysis performed by Costenbader et al. [50] current smokers compared with non-smokers had a significantly elevated odds ratio (OR) for development of SLE (OR: 1.50, 95% CI, 1.09-2.08). Moreover, a study performed with black women supported an increased risk of SLE among smokers [51]. There is no evidence for the link between the initiation of lupus and smoking, but the latter has a great impact on the progression of lupus nephritis. In a retrospective cohort study of 160 adults with lupus nephritis the median time to ESRD among smokers was 145 months and among non-smokers it was greater than 273 months even in a multivariable analysis adjusting for differences in age, gender, socioeconomic status, renal histology, and immunosuppressive treatment [6]. The significant independent association between current smoking and dsDNA seropositivity (OR=3.5, 95% CI 1.2 to 10.5) in a multivariate cohort which included 410 white SLE patients [52] gives an indirect proof of the connection between lupus nephritis and smoking, given the well established association of dsDNA autoantibodies with lupus nephritis. At the same time it can serve as one potential plausible explanation for the pathomechanism of smoking-induced kidney damage in lupus, e.g. the formation of DNA adducts with resultant autoantibodies to the damaged DNA. A study including 97 patients with lupus nephritis requiring renal transplantation and matched non-lupus controls revealed that subjects with lupus nephritis had inferior transplantation outcomes, with more than twice the risk of allograft loss compared with the control kidney transplant patients, moreover smoking status was associated with allograft loss in a multivariate model [53]. Because the life expectancy of lupus patients has improved from an approximately 4-year survival rate of 50% in the 1950s to a 15-year survival rate of 80% today, the bimodal pattern of mortality in lupus e.g. lupus or infection as main causes of death in the first period and myocardial infarction and stroke in the long term period [54] suggests that cigarette smoking due to direct macrovascular damage and indirectly via accelerating the progression of CKD in lupus nephritis can contribute to the mortality in this special autoimmune disease.

Patients on Renal Replacement Therapy (End-stage Renal Failure; Hemodialysis; CAPD; Transplantation) – Effect of Smoking

In the Chronic Renal Impairment in Birmingham (CRIB) prospective cohort study of 382 stage 3-5 CKD patients with a mean follow-up of 4.1 years for ESRD and 6.0 years for death, smoking showed an association with ESRD independently of age and sex, moreover, current smoking was an independent predictor of death [55]. In hemodialysis patients active smoking causes a lower serum albumin level compared to non-smokers, of note, low albumin level is

predictor of increased mortality in this CKD population [35]. Tobacco use increases cardiovascular mortality (myocardial infarction, carotid artery stenosis, peripheral vascular disease) and death both in non-diabetic and diabetic hemodialysis subjects [35]. In peritoneal dialysis patients smoking is a significant survival risk factor [35].

In a retrospective study of 1,334 transplant patients after adjustment for multiple predictors of graft failure, smoking more than 25 pack-years at transplantation was associated with a 30% higher risk of graft failure compared to those who smoked less intensively or did not smoke at all [56]. The increase in graft failure was due to an increase in deaths (adjusted RR 1.42; 95% CI: 1.08 - 1.87, P = 0.012) [56]. However, having quit smoking more than 5 years before transplantation reduced the relative risk of graft failure by 34% [56]. In a cohort study of 645 adult renal allograft recipients pretransplant smoking caused a reduced overall graft and death-censored graft survival [57]. Pretransplant smokers had kidney survival of 84%, 65%, and 48% at 1, 5, and 10 years, respectively; in contrast, non-smokers had an increased graft survival, namely 88%, 78%, and 62% (P = 0.007) [57].

Conceptions for the Pathomechanisms of Smoking-induced Kidney Damage in CKD Patients

The pathomechanism is probably multi-causative [58], but oxidative stress, alteration in cell membrane processes and signal transduction pathways likely play a pivotal role (Table 9.2).

Hyperfiltration

Our human study showed that both nicotinic and nicotine-free cigarette smoke transiently reduces the resistance index of renal arteries, which is indicative of increased vasodilation of the examined vessels due to a component of smoke other than nicotine (Figure 9.1.). In our animal experiments water-soluble components of cigarette smoke – probably via hydrogene peroxide - elicited dose-dependent acute relaxation of renal arteries [59] (Figure 9.2.), which, together with the elevated mean arterial pressure during smoking [40] and endothelial dysfunction [60-62] could elicit hyperfiltration and increased albuminuria. Parallel with the stimulation of the sympathetic nervous system, smoking causes a significant but transient (app. 30 minutes) increase of blood pressure, an effect which was observed among healthy subjects, hypertensive patients, type 1 and type 2 diabetics and also in patients with primary renal disease [31]. Also in chronic renal diseases hyperfiltration and glomerular hypertension accelerate the progression [63] according to Brenner's theory [64], which can be aggravated by cigarette smoking. In a study performed in an apparently "healthy" population subclinical inflammation (serum C-reactive protein level) was associated with cigarette smoking-induced hyperfiltration and proteinuria [65]. In IgA glomerulonephritis patients an increase in the urinary albumin/creatinine ratio was described, which could be also developed due to higher glomerular capillary pressure [8].

Nicotine

Nicotine has been „accused" as a link between cigarette smoking and the progression of renal injury by its mitogenetic effects and by inducing the production of extracellular matrix in human mesangial cells via reactive oxygen species [4, 66]. Nicotine also increases sympathetic activity via both stimulation of cathecolamine release from peripheral nerve endings and the adrenal medulla and via direct stimulation of postganglionic sympathetic nerve endings [8]. In a human study, the administration of nicotine gum to non-smokers was associated with increased mean arterial pressure and heart rate and renal vasoconstriction, the latter possibly through inhibition of a cyclic-GMP-dependent vasoactive mechanism [67]. However, in chronic smokers a tolerance to this renal effect of nicotine was observed despite the maintenance of the systemic response to nicotine [67]. The antidiuretic effect of the nicotine content of tobacco due to increased vasopressin secretion, and a possible increase in single-nephron GFR could also contribute to the deleterious consequences of tobacco use [8, 68]. Nicotinic acetylcholine receptors, which mediate cell proliferation, are expressed on human mesangial cells [66, 69]. Exposition of mesangial cells to cigarette smoke induced an increase in TGF-β_1, which is a major factor in the development of renal fibrosis [69]. In smokers, due to nicotine, the plasma concentration of another substance, endothelin-1, a strong vasoconstrictor and at the same time a growth promoter of endothelial cells, vascular smooth muscle cells and mesangial cells [70] is increased [71, 72]. The consecutive glomerulomegaly, nephromegaly, and enlarged kidney size – the latter also observed in middle-aged smokers [73] - are established risk factors for kidney disease progression.

Oxidative Stress

Parallel to the deterioration of a chronic kidney disease the concentration of markers of oxidative stress (malondialdehyde, and hydrogen peroxide) rises, whereas protein SH groups (as important antioxidants) and activity of antioxidant enzymes (glutathione peroxidase, catalase and superoxide dismutase) decrease [74], hereby CKD patients are more susceptible to the oxidative stress either caused directly- or induced by cigarette smoke. Water-soluble constituents of cigarette smoke induce vascular reactive oxygen- and nitrogen species production, enhance inflammatory gene expression, and lead to endothelial dysfunction [75]. Nitric oxide (NO) bioavailability is reduced in smokers [76] and patients with chronic kidney disease have a reduced whole body NO production [77], which can play an important role in increasing renal vascular tone and perhaps also in mesangial cell proliferation [8].

Tubulointerstitial Injury

Both in diabetic and non-diabetic smokers a proximal tubular cell dysfunction and a tubular cell damage was observed [78], which is important in tubulointerstitial injury and thus in the progression of CKD. The excretion of N-acetyl-β-hexosaminidase, as a marker of proximal tubular cell damage, was dose-dependently elevated in smokers [78]. Cadmium – one of the app. 4,000 components of cigarette smoke – has also a toxic effect on proximal tubular cells in vitro [79], an observation confirmed by a population-based study, where

diabetics were more susceptible to the toxic effects of cadmium [80]. Smoking 20 cigarettes daily for a longer period leads to 45 to 70% higher accumulation dosages of cadmium in the renal cortex [69].

Gene Modification

Cadmium and strontium modify the expression of several genes in the endothelial cells, which can play a role in the pathogenesis of accelerated atherosclerosis induced by cigarette smoking [4]. In ADPKD patients a potential smoking-induced second PKD allele mutation in the unaffected parent could promote cyst formation, because loss of heterozygocity and intragenic mutations in the PKD-1 gene were already described, suggesting a two-hit model of cystogenesis due to inactivation of both copies of the gene [8].

Reversibility

The „point of no return" of smoking-induced kidney damage is not established yet, but there are some data about the reversibility of the process. A representative study performed in the general population revealed that cessation of cigarette smoking led to normalisation of urinary albumin excretion only in non-heavy smokers, namely in subjects who smoked less than 20 cigarettes per day [81].

Lifetable analyses were used to estimate gains in life expectancy in non-diabetics and diabetics of the Multiple Risk Factor Intervention Trial (MRFIT), and it was concluded that cessation of smoking would prolong life by a mean of around four years in a 45-year-old non-diabetic man and three years in a diabetic man, whereas aspirin and antihypertensive treatment would provide approximately one year of additional life expectancy in both categories [82]. This means that reversibility has its borders and probably both intensity, duration, form of tobacco consume and associated diseases determine it.

Histological Alterations of the Kidney due to Tobacco Consume

Renal Vessels

Smoking-induced structural renal artery damage has been shown already in the 1980s. An autopsy study described an intima thickening in arterioles of smokers [83], whereas in another investigation the thickening of the renal arterioles was attributed to increase in collagen in smooth muscle [84]. Smoking promotes not only atherosclerosis of the vessels but it is also a risk factor for cholesterol microembolism [85].

Glomerular and Tubulointerstitial Alterations

It can probably also be learnt from histology results about the pathomechanism of cigarette-induced kidney damage. In case reports the histopathologic changes in the kidneys of non-diabetic smokers include focal segmental or focal global glomerulosclerosis, ischemic glomeruli, interstitial fibrosis, tubular atrophy, arterial sclerosis and arteriolar hyalinosis [86]. Electron microscopy showed glomerular capillary wall thickening caused by subendothelial expansion by cellular elements and new basement formation resulting in segments of double contours [86].

Role of Cigarette Smoking in the Development of Insulin Resistance, Impaired Fasting Glucose, Type 2 Diabetes Mellitus and Metabolic Syndrome

The topic of diabetes and diabetic nephropathy is more established so it deserves a separate discussion. Smoking causes insulin resistance - thus increases the risk of developing type 2 diabetes [10,11] - and elevates the risk of the initiation of diabetes mellitus and metabolic syndrome [7-9]. In 1992 it has been already shown that in otherwise healthy volunteers chronic smokers were insulin resistant, hyperinsulinaemic, and dyslipidaemic compared with a matched group of non-smokers [87]. The quantification of insulin resistance by euglycemic clamp technique in middle-aged male patients confirmed that smoking habits were independently related to the degree of insulin resistance [88]. The smoking-induced rise in insulin reistance can lead to worsening of glycemic control, the latter has a pivotal role in the progression of diabetic nephropathy [89]. A prospective cohort study (CARDIA) including 5,115 participants with a 15-year follow-up ascertained that a strong association exists between both active and passive tobacco smoke exposure and subsequent development of impaired fasting glucose or diabetes [90]. During the follow-up period 16.7% of participants (black and white men and women aged 18-30 years with no glucose intolerance at baseline) developed glucose intolerance [90]. The 15-year incidence of glucose intolerance was highest among smokers (21.8%), followed by never smokers with passive smoke exposure (17.2%), and then previous smokers (14.4%); it was the lowest for never smokers with no passive smoke exposure (11.5%) [90]. Current smokers (hazard ratio 1.65, 95% confidence interval 1.27 - 2.13) and never smokers with passive smoke exposure (1.35, 1.06 - 1.71) remained at higher risk than never smokers without passive smoke exposure after adjustment for multiple baseline sociodemographic, biological, and behavioural factors [90]. Among smokers, total pack-years smoked was associated with increasing risk of incident diabetes, moreover the association of tobacco exposure with diabetes was greatest among white women and men [90]. Another prospective study with a 6 year follow-up including 41,810 male health professionals aged 40-75 years revealed that after controlling for known risk factors men who smoked 25 or more cigarettes daily had a relative risk of developing type 2 diabetes of 1.94 (95% CI 1.25 - 3.03) compared with non-smokers [91]. The same research group has found that among women (114,247 nurses) during a 12-year-long observation period the relative risk of diabetes, adjusted for obesity and other risk factors, was

1.42 among subjects who smoked 25 or more cigarettes per day compared with non-smokers [92]. Smoking is an important factor in the initiation of diabetes, which was confirmed in the Nurses' Health Study (n=84,941, 16 years of follow up), where 91 percent of the cases of diabetes were found in women with obesity, lack of exercise, a poor diet, and smoking, suggesting that many cases of diabetes could be prevented with a healthier lifestyle [93]. In the multiple risk factor intervention trial (MRFIT) in subjects with normal glucose tolerance at baseline (n = 11,827) an intervention program (diet guidance: reduced saturated fat, cholesterol, and calorie intake; increase of physical activity, intensive control of blood pressure, and cessation of smoking) was associated with a lower risk of type 2 diabetes in the non-smokers (HR 0.82, 95% CI 0.68 - 0.98), but not in the smokers [94].

The study of 21,068 male physicians aged 40 to 84 years in the Physicians' Health Study detected also that smokers had a dose-dependent increased risk of developing type 2 diabetes mellitus compared with never smokers [95]. The age-adjusted relative risk was 2.1 (95% confidence interval [CI]: 1.7 - 2.6) for current smokers of > or = 20 cigarettes per day, 1.4 (95% CI: 1.0 - 2.0) for current smokers of <20 cigarettes per day, and 1.2 (95% CI: 1.0 - 1.4) for past smokers.

After multivariate adjustment for body mass index, physical activity, and other risk factors, the relative risks were 1.7 (95% CI: 1.3 - 2.3) for current smokers of > or = 20 cigarettes per day, 1.5 (95% CI: 1.0 - 2.2) for current smokers of <20 cigarettes per day, and 1.1 (95% CI: 1.0 - 1.4) for past smokers [95]. Total pack-years of cigarette smoking was also associated with the risk of type 2 diabetes mellitus (P for trend <0.001) [95]. Estimates from the Physicians' Health Study suggest that in the United States, where approximately 25 percent of people smoked at this time, approximately 10% of the incidence of type 2 diabetes may be attributable to cigarette smoking. In 2007 a systematic review of 25 prospective cohort studies (N=1.2 million participants with 45,844 incident cases of diabetes during a follow-up period ranging from 5 to 30 years), 24 reported adjusted relative risks (RR) greater than 1 (range for all studies, 0.82-3.74) [96].

The pooled adjusted RR was 1.44 (95% CI: 1.31 - 1.8), the risk of developing diabetes was higher for heavy smokers (≥20 cigarettes/day; RR, 1.61; 95% CI: 1.43 - 1.80) than for lighter smokers (RR,1.29; 95% CI: 1.13 - 1.48) and lower for former smokers (RR, 1.23; 95% CI: 1.14 - 1.33) compared with active smokers, which is consistent with a dose-response phenomenon [96]. The organism „remembers for a long time" the detrimental effects of smoking: in a prospective study of 7,735 men the benefit of giving up smoking regarding the risk of new-onset diabetes mellitus was only apparent after 5 years of smoking cessation, and risk reverted to that of never-smokers only after 20 years [97].

Evaluation of data from 2,273 subjects from the National Health and Nutrition Examination Survey III showed that among adolescents, 5.6% met the criteria for metabolic syndrome and the prevalence increased with tobacco exposure: 1.2% for non-exposed, 5.4% for those exposed to environmental tobacco smoke, and 8.7% for active smokers; moreover, at overweight adolescents a similar relationship could be detected (5.6%, 19.6%, and 23.6% respectively) [98]. In a multivariable logistic regression model environmental tobacco smoke exposure was independently associated with the metabolic syndrome among adolescents (environmental tobacco smoke: OR, 4.7, 95%CI, 1.7 - 12.9; active smoking: OR, 6.1; 95% CI, 2.8 - 13.4) [98].

Cross sectional data of 5,033 Japanese adults revealed that in this population too - both former and current - smoking was significantly associated with an increased incidence of

metabolic syndrome (OR, 1.77, 95% CI 1.42 - 2.22), the latter was found to be an independent risk factor for carotid plaque formation (OR, 1.72, 95% CI 1.43 – 2.08) [99]. It has been also noticed that smoking causes a body fat distribution typical for the metabolic sndrome [100]. For a better overview, the studies are presented also in summary in **Table 9.3.**

Cigarette Smoking Promotes the Commencement of Diabetic Nephropathy in a Mixed Population of Type 1 and Type 2 Diabetic Patients

Although there is a great body of evidence of the harmful effect of smoking on diabetic nephropathy, in the 2011 ADA recommandations for physicians this topic is perhaps not accentuated enough [101]. Both in type 1 and type 2 diabetes smoking increases the risk of development of nephropathy and almost doubles the rate of progression to end-stage renal failure [8]. Cigarette smoking causes not only a deterioration in renal function [102], but also elevates microalbuminuria and proteinuria in diabetes mellitus [8]. In a follow-up study of 185 – either type 1 or type 2 – diabetics (44 smokers and 141 non-smokers) without signs of overt renal disease, the GFR estimated with the MDRD formula remained constant during the minimum 3 years of follow-up in non-smokers (from 107±33ml/min baseline to 106±31ml/min), whereas GFR decreased significantly (from 95±26ml/min baseline to 83±22ml/min) in smokers [102]. This relationship persisted when adjusted for retinopathy, glycaemic control, age, body constitution, ACE-inhibitor treatment, blood pressure control or severity of proteinuria [102].

Development of Diabetic Nephropathy in Type 1 Diabetes Mellitus

A study including 668 type 1 diabetic patients confirmed that tobbaco consume is a risk factor for the development of nephropathy and revealed that the prevalence of nephropathy was higher among heavy smokers than among non-heavy smokers, 19.2% versus 12.1%, respectively. An increasing frequency of nephropathy was found with increasing cigarette consumption [103]. A study group in Denver investigated 359 young patients with type 1 diabetes mellitus and they found that smoking was a significant risk factor of increased albumin excretion also in a logistic regression model controlled for duration of diabetes, glycohaemoglobin level, blood pressure, age and gender [104]. Also in a four year follow-up of a cohort of 137 insulin-dependent diabetics smoking was one of the significant determinants of persistent microalbuminuria [105]. In an ESRF retrospective study it was shown that tobbacco consumption in a dose-dependent manner shortens the time period between the onset of diabetes and the commencement of albuminuria or proteinuria in type 1 diabetic patients [106].

Cigarette Smoking – Effect on the Progression of Diabetic Nephropathy of Type 1 Diabetics

Among type 1 diabetics with microvascular complications, albuminuria and retinopathy were found to progress more in smokers and the former improved significantly when subjects ceased smoking [104]. A case control study involved 192 cigarette-smoking patients with type 1 diabetes mellitus, who were compared with non-smoking controls pair-matched for sex, duration of diabetes and age [107].

Although the glycosylated haemoglobin values and the prevalence of hypertension were similar between the two groups, macroproteinuria was found significantly more often, in 19.3% of the smoking and in 8.3% of the non-smoking patients [107]. Moreover, proliferative retinopathy was present in 12.5% of the smoking and in 6.8% of the non-smoking patients [107].

Thus cigarette consumption seems to be a risk factor both for overt proteinuria and proliferative retinopathy in type 1 diabetics. In a retrospective study data of type 1 diabetic patients with end-stage diabetic nephropathy and a control group matched for sex, age and duration of diabetes were analysed [108]. Smokers – especially those with a large daily consumption – had an earlier onset of proteinuria than non-smokers; moreover, tobacco use was proposed as a trigger for progression of incipiente to overt nephropathy [108]. Tobacco consume is also an independent variable associated with the rate of decrease of creatinine clearance in the predialysis phase both in type 1 and type 2 diabetic patients [109]. The rate of loss of glomerular filtration rate was 1.24±0.29ml/min/month in smokers versus 0.86±0.31ml/min/month in non-smoker type 1 diabetics; the respective values for subjects with type 2 diabetes mellitus were 1.21±0.34ml/min/month and 0.73±0.38ml/min/month [10, 109].

In a prospective, follow-up study over one year with treated hypertensive type 1 diabetics the progression of nephropathy – defined as an increase in proteinuria or serum creatinine or decrease in creatinine clearance – was more common in smokers (53%) and ex-smokers (33%) than in non-smokers (11%) [110].

In a prevalence survey of 3,250 men and women aged 15-60 years with type 1 diabetes mellitus from 31 diabetes centers in Europe 35% of the men and 29% of the women were smokers [111]. Current smokers had a higher prevalence of microalbuminuria and total retinopathy than did those who never smoked; moreover, ex-smokers had a higher prevalence of macroalbuminuria and proliferative retinopathy than did those who never smoked, but both had a similar prevalence of microalbuminuria [111], which can be perhaps interpreted that if someone quits smoking in time, the progression of diabetic nephropathy could be reduced to the level of that of a non-smoker.

An observational extension of the randomized prospective Diabetes Control and Complications trial revealed that among 1,105 type 1 diabetics who had normal urine albumin excretion at baseline, a 4.3-fold greater rate of GFR decline could be observed in active versus non-active smokers (-0.77 versus -0.18 ml/min per 1.73m^2/year) [112].

Initiation of Nephropathy in Type 2 Diabetes Mellitus Due to Chronic Cigarette Smoking

In a cross-sectional study of 1,203 type 2 diabetic patients the prevalence of smokers was higher in patients with microalbuminuria [113], which was confirmed by another smaller study performed in Germany, where current smoking was significantly correlated with an increased risk of microalbuminuria [114]. A prospective study documented that smoking is also an independent predictor of the de novo development of microalbuminuria in type 2 diabetes [115]. In a population-based cohort of 1,574 type 2 diabetics cigarette smoking was an independent variable related to micro- and macroalbuminuria [116]; the latter was confirmed also by other authors [117].

Tobacco as a Progression Promoter of DNP in Type 2 Diabetes

A population-based prospective study in southern Wisconsin of individuals with type 2 diabetes showed that during a four-year follow-up the relative risk of developing gross proteinuria was 2- to 2.5-fold higher in heavy smokers compared to non-smokers [118]. In 933 type 2 diabetic patients using a multivariate logistic regression analysis controlling for diabetes duration, glycosylated hemoglobin, gender and race, one of the most significant predictors of microalbuminuria and macroalbuminuria was smoking pack-year [119]. In a prospective follow-up study of type 2 diabetic patients with normal renal function at the beginning, smokers had significantly faster decline of the creatinine-clearance (1.24±0.34ml/min/month) than non-smoking patients (0.99±0.35 ml/min/month), while systolic and diastolic blood pressure as well as serum cholesterol, triglycerides and HbA_{1c} were not significantly different in the two patient groups [120]. Another prospective study including type 2 diabetics with normal initial renal function, manifest nephropathy and with well-controlled blood pressure - partly due to ACE inhibitor treatment -, the increase in serum creatinine was more pronounced in smokers as compared with non-smokers, i.e., from 93±7 µmol/L to 157±18µmol/L versus from 95±3 µmol/L to 117±4µmol/L [121]. Regression analysis (follow-up time, mean blood pressure and initial plasma creatinine) revealed that cigarette smoking was the only factor that significantly predicted the decline in GFR [121]. In a more recent prospective study involving 227 white patients with type 2 diabetes mellitus and nephropathy a faster rate of GFR decline was independently associated with heavy smoking during a follow-up of 6.5 years [122]. In a cross-sectional study involving 32,208 type 2 diabetic patients without known albuminuria smoking was an independent risk factor for increased urine albumin excretion [123]. A follow-up of 185 subjects with type 1 and type 2 diabetes with and without nephropathy showed that smoking was independently associated with a decrease in estimated GFR; moreover, the relation was independent of proteinuria [102].

Diabetic Smokers with End-stage Renal Failure and Renal Replacement Therapy

In diabetic patients with end-stage renal failure, smoking decreases survival on commencement of dialysis [124]. The 1- and 5-year survival rates in diabetic patients with tobacco consumption were 68 and 9%, respectively, while in non-smoking patients these rates were 80 and 37%, respectively ($P<0.05$) [125]. As a potential explanation, hemodialyzed diabetic cigarette smokers showed higher fibrinogen and systemic blood pressure values and a higher incidence of myocardial infarctions when compared with non-smoker diabetic patients on hemodialysis [125].

Smoking as a Risk Factor for All-cause Mortality in Type 1 and Type 2 Diabetic Patients with CKD

The relationship between CKD and all-cause mortality in type 1 diabetes was underlined in the Finnish Diabetic Nephropathy Study, which was a multicenter prospective study including 4,201 adult diabetics with a mean follow-up of 7 years [126]. The presence of microalbuminuria, macroalbuminuria, and end-stage kidney disease was associated with 2.8, 9.2, and 18.3 times higher standardized mortality ratio, respectively, compared to the general population [126]. In addition, the glomerular filtration rate was independently associated with mortality: both individuals with impaired kidney function and those demonstrating hyperfiltration had an increased risk of death [126].

The Casale Monferrato Study, a population-based cohort (n=1,538 type 2 diabetics; median age 68.9 years, 11 years follow-up) has found that chronic kidney disease (lower eGFR) conferred an increased risk of all-cause mortality of 23% and of cardiovascular mortality of 18% independently of both cardiovascular risk factors and albumin excretion rate. However, in an analysis stratified by albumin excretion rate categories, a significant increasing trend in risk with decreasing eGFR was evident only in people with macroalbuminuria [127]. Since cigarette smoking is a risk factor for the development of both diabetes and chronic kidney disease and the main complications of diabetes and CKD are the cardiovascular ones - the latter triggered also directly by tobacco use -, it is evident that chronic cigarette smoking is one of the major modifiable elements in the formation of potentially fatal illnesses.

Possible Contribution of Smoking to Diabetic Nephropathy

The potential pathomechanisms involved in the development of smoking-induced diabetic nephropathy are summerized in **Table 9.4.**

Podocyte Damage

Cigarette smoking also rises the urinary podocyte excretion [128]. First it may occur in patients with early diabetic nephropathy [129], and second, it can predict long-term urinary albumin excretion in type 2 diabetes and microalbuminuria [130]. Smoking increases urinary albumin level even at albumin concentrations below that of microalbuminuria [31]. In a prospective study including 80 type 2 diabetic patients and 30 healthy controls, urinary podocytes were detected by immunofluorescense microscopy in 35 diabetic subjects with microalbuminuria (27 smokers and 8 non-smokers, 1.4±0.7 cells/ml) but were not detected in the remaining 45 patients (23 smokers and 22 non-smokers) or the 30 healthy subjects [128]. More podocytes were excreted in the urine in smokers (27 of 50 patients) with microalbuminuria than in non-smokers (8 of 30 patients) with microalbuminuria ($P = 0.017$, χ^2 test). Interestingly, the urinary podocytes disappeared after 3 years in 77% of patients who had stopped smoking, whereas urinary podocytes increased in all patients who continued to smoke (from 1.1±0.8 to 1.7±0.4 cells/ml, $P<0.01$) [128]. These data suggest first that smoking may be associated with podocyte injuries in patients with early diabetic nephropathy [128], and second that podocyte excretion is probably an early and potentially reversible sign of diabetic nephropathy.

Hyperfiltration and Limited or Abolished Glomerular Autoregulation

In insulin-treated diabetics a higher prevalence of hyperfiltration was found in smokers than in non-smokers, moreover, the glomerular filtration rate was directly dependent on the intensity of smoking [131]. The fact that in the same study no correlation could be shown in users of oral snuff suggested that another component of tobacco apart from nicotine was responsible for the hyperfiltration [131]. It supports our theory [59] that not only nicotine but other components of cigarette smoke are also crucial in the hyperfiltration process, namely hydrogen peroxyde reduces the vasomotor tone of renal arteries, which could lead to hyperperfusion of kidneys also in diabetics. The elevated mean arterial pressure due to cigarette smoke can harm the glomeruli of patients with diabetic nephropathy in a greater extent than non diabetics, because autoregulation of GFR is impaired or abolished both in type 1 [132] and type 2 [133] diabetic patients with diabetic nephropathy.

Nicotine

Animal experiments and human studies indicated that nicotine exposure could induce a reduction of insulin release, and negatively affect insulin action, suggesting that this substance of cigarette could be a cause of insulin resistance [11]. Animal and human studies suggest that either acute or chronic nicotine exposures could negatively affect insulin action both in smokers preceding type 2 diabetes mellitus and in type 2 diabetic patients, which means that nicotine can contribute to type 2 diabetes development and aggravation of the disease through enhancing insulin resistance [11]. It has been already shown that functional nicotinic receptors are present in pancreatic islets and beta cells, so nicotine could, at least in part, negatively affect beta-cell function [11]. Moreover, nicotine increases apoptosis of islet

β-cells [11]. Mitochondrial dysfunction, oxidative stress, and inflammation are involved as underlying mechanisms of nicotine-induced pancreatic β-cell loss [11].

Genetic Predisposition

A cross-sectional analysis in 1,209 normo-albuminuric type 2 diabetics has shown a genetic predisposition to develop albuminuria in smokers, who carried the DD-genotype of the ACE gene [134].

Heavy Metals

In diabetic patients low-level cadmium exposure has been also associated with early onset of diabetic nephropathy [69].

Impaired Vasodilation

Smoking also impairs the responsiveness of intrarenal arteries to vasodilators, which is one of the potential mechanisms behind the progression of diabetic nephropathy [135].

Elevated Resting Energy Expenditure

Smoking is an independent risk factor for elevated resting energy expenditure in patients with diabetic nephropathy and since resting energy expenditure is not attributable to heightened oxidative stress and inflammation, it provides an additional mechanism by which smoke may lead to poor outcomes in subjects with diabetic nephropathy [136].

Reversibility

In type 1 diabetics with nephropathy and with adequate control of blood pressure and glycemia, the progression of nephropathy slowed down among diabetic subjects, who had stopped smoking [104]. Cigarette smoking-induced increased TGF-β_1 excretion (the promoter of renal fibrosis) was reduced after smoking cessation, which can underline the beneficial consequences of quitting smoking [69].

Histology in Diabetic Nephropathy and Metabolic Syndrome

Histology may also add some information to the understanding of the pathomechanism in diabetic nephropathy. Chronic cigarette smoking could contribute to the development of

„idiopathic" nodular glomerulosclerosis [137], and noteworthy, diabetic nodular glomerulosclerosis is a separate entity in the histopathologic classification of diabetic nephropathy [138].

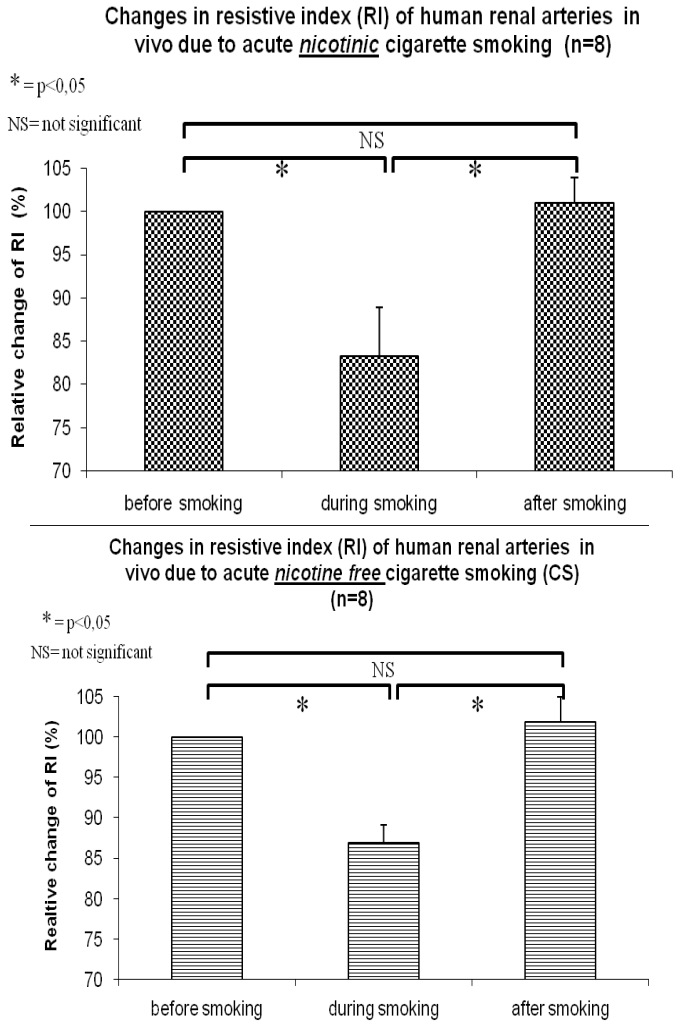


Figure 9.1. Reduction in the resistance index of the segmental renal arteries due to either nicotinic or nicotine-free cigarette smoke.

In a small sample study with metabolic syndrome patients 2 out of 3 individuals with histology-proven nodular glomerulosclerosis were smokers [139]. Histopathologic features in smoking-induced renal damage in 18 type 1 diabetic patients involved a more pronounced matrix volume and greater ratio in mesangial - to - glomerular volume and greater basement membrane thickness in smokers than in non-smokers [69]. The increase in basement membrane thickness in 96 type 2 diabetics was found to be a dose-dependent alteration (non-smokers: 398.0±92.5 nm, moderate smokers: 438.6±80.9 nm, heavy smokers: 471.0±113.3 nm) [140]. In a Skandinavian study, where changes of kidney function, microalbuminuria and kidney biopsy-proven structural glomerular parameters, namely glomerular volume,

matrix/glomerular volume fraction, mesangial/glomerular volume fraction and the basement membrane thickness were analysed in type 1 diabetics in an 8-year period, the smoking group had a definitely higher rise in albumin ecretion rate and a tendency to larger decline in GFR than the non-smokers. Moreover, smoking was an independent risk factor for decline in GFR in a multivariate analysis [141]. We have also data about the reversal of established lesions of diabetic nephropathy after pancreas transplantation [142], which can give hope for the patients who decide to quit smoking, so that the smoking-induced alterations could be also reversible until a certain degree of damage.

Future Perspectives

In the future having tools to help the understanding of the pathomechanism and the detection of the stage of the kidney damage caused by cigarette smoking would be of great help. Specifically, for the detection of the probably initial step, e.g. hyperfiltration, not only measuring the glomerular filtration rate via creatinine clearance would be important, but perhaps pointing out of potential specific signs of the damage by ultrasound or specific biomarkers would be of great importance. The risk of cancer as a recently highlighted „non-cardiovascular" long-term complication of diabetes mellitus rises with tobacco consume too, so the eventually triggering role of one or another harm factors can be in the forefront of research [143]. It is worth to quit smoking in time, because in some cases, like idiopathic nodular glomerulosclerosis or in the renal transplant it is proven that quitting smoking reduces the rate of progression of renal failure [135], moreover, cessation of smoking alone may reduce the risk of progression in the decline of GFR by 30% also in patients with type-2 diabetes [9]. However, the role of smoking both in the development and progression of membranous nephropathy, FSGS or minimal change glomerulonephritis and the „point of no return" in other renal diseases are not established yet. Age-related chronic kidney diseases and diabetes mellitus should be prevented, therefore intervention in the smoking habit - as a modifiable risk factor - should begin very early!

Table 9.1. Smoking as a risk factor for initiation and progression of chronic kidney disease in the general population

Author	number of patients	study design	follow-up (years)	primary end-point	hazard ratio/ relative risk/odds ratio (95%CI)	conclusion
Haroun et al.	23,534 women and men	po*	20	kidney disease/ end-stage renal disease	HR 2.9 in women (CI:1.7-5)HR 2.4 in men (CI:1.5-4)	association between current smoking and risk of CKD
Hippisley-Cox et al.	777,091 women 799,658 men	poc*	5	moderate/ severe CKD, ESRF	-	smoking is a main risk factor for CKD
Jones-Burton et al.	74-157,377	cr*	2-18.5	incident CKD	OR 1.51[a] (CI:1.06-2.15 RR 2.3[a] (CI: 1.2-4.3) OR 1.45[b] (CI:1.0-2.09)	>20 cigarettes smoked/day[a] &>40 years duration[b] are main risks for incident CKD
Lash et al.	3,612	pc*	8	progression of CKD	-	lower eGFR associated with previous smoking

Table 9.1. (Continued)

Author	number of patients	study design	follow-up (years)	primary end-point	hazard ratio/ relative risk/odds ratio (95%CI)	conclusion
Baggio et al.	2,981	l*	3.6	pathological increase of renal function >26.5 µmol/l of SCr	OR 2.3 (CI:1.0-5.3)	current smokers >20 cigarettes smoked per day have pathological rise in GFR
Hogan et al.	15,719rp*	cs*	-	risk of albuminuria in hypertensives & non-hyper-tensives	OR 1.85 (CI:1.29-2.64) OR 1.41 (CI:1.04-1.90)	current smokers have increased risk for albuminuria passive smoking increased risk for albuminuria
Pinto-Sietsma et al.	7,476	cs*	-	association between smoking and albuminuria and abnormal renal function -high normal albuminuria microalbuminuria -elevated GFR -decreased GFR	 RR 1.33 *vs* (CI:1.1-1.61) RR 1.98 (CI:1.49-2.64) RR 1.92 *vs* (CI:1.54-2.39) RR 2.15 (CI:1.52-3.03) RR 1.82 *vs* (CI:1.31-2.53) RR 1.84 (CI:1.12-3.02) RR 1.53 *vs* (CI:1.04-2.24) RR 1.83 (CI:1.05-3.20)	smokers *vs* non-smokers assoc. between smoking albuminuria and altered GFR <20cigarettes/day >20cigarettes/day <20cigarettes/day >20cigarettes/day <20cigarettes/day >20cigarettes/day
Ishani et al.	12,866 men	it*	25	ESRD	HR 1.84 (CI:1.35-2.51)	elevated risk for ESRD in current smokers *vs* non-smokers
Yamagata et al.	123,764 gp*	fu*	10	development of CKD	HR 1.4[c] (CI:1.16-1.69) HR 1.26[d] (CI:1.14-1.41) HR 1.13[e] (CI:1.05-1.22) HR 1.16[f] (CI:1.06-1.26)	current smoking rised risk for CKD stage I/II *in womec*[d] and *in men*[d] & elevated risk for CKD st. III/IV *in women*[e] and *in men*[f]
Hallan et al	30,485 men 34,708 women	pbcs*	-	risk for CKD	RR 1.52 (CI:1.13-2.06)	elevation in risk of CKD lifetime exposure of >25 pack-years

* Abbreviations: assoc, association; cr, comprehensive review; cs, cross-sectional; f, follow-up; gp, general population; it, intervention trial; l, longitudinal; pc, prospective cohort; po, prospective observational; poc, prospective open cohort; pbcs, population-based cross-sectional; rp, representative population.

Table 9.2. Patomechanisms of smoking-induced kidney damage in the general population and in patients suffering from chronic kidney disease

- Hyperfiltration due to smoking
  - repetitive acute hyperperfusion + chronic endothelial dysfunction → hyperfiltration
- Nicotine
  - mitogenetic effects; reactive oxygen species → extracellular matrix overproduction
    mesangial cell proliferation → TGF-β_1* overproduction → renal fibrosis
  - sympathetic activity
    a/ rise in mean arterial pressure and heart rate
    b/ renal vasoconstriction → vasopressin secretion → antidiuresis
  - rise in single nephron GFR*
  - endothelin-1 →vasoconstriction and overgrowth of endothelial cells + vascular smooth muscle cells + mesangial cells → glomerulomegaly + nephromegaly → kidney disease progression
- Oxidative stress
  - parallel to CKD* development: rise in malondialdehyde + hydrogen peroxide
  - decrease in glutathione peroxidase + catalase + superoxide dismutase
  - water-soluble constituents of cigarette smoke
    → vascular reactive oxygen- and nitrogen species production
    → inflammatory gene expression → endothelial dysfunction
  - smokers: decrease in NO* bioavailability + CKD* patients: lower whole body NO production → elevated renal vascular tone + possible mesangial cell proliferation
- Tubulointerstitial injury
  - in smokers dose-dependent elevation in the excretion of N-acetyl-β-hexosaminidase = proximal tubular cell damage marker
  - cadmium – toxic effect on proximal tubular cells
- Gene modification
  - cadmium + strontium → modification of the expression of genes in endothelial cells

  → accelerated atherosclerosis
  - in ADPKD* potential smoking-induced second PKD* allele mutation → cyst formation
- Reversibility
  - unknown „point of no return" of smoking-induced kidney damage
  - in general population cessation of smoking → normalisation of urinary albumin excretion only in non-heavy smokers
  - cessation of smoking → prolongation of life by approximately 4 years in a 45-year- old non-diabetic man and 3 years in a diabetic man

* Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; CKD, chronic kidney disease; GFR, glomerular filtration rate; NO nitric oxyde; PKD, polycystik kidney disease; TGF-β1, tissue growth factor β1.

Table 9.3. Role of cigarette smoking in the development of insulin resistance, impaired fasting glucose, type 2 diabetes mellitus and metabolic syndrome

Author	number of patients	study design	follow-up (years)	primary end-point	hazard ratio/ relative risk/odds ratio (95%CI)	conclusion
Facchini et al.	40	es*	-	rise in plasma insulin concentration after oral glucose challenge	p<0.001	chronic smokers are more insulin resistant than non-smokers
Houston et al.	3,459 white and black men and women	pm*	15	incidence of glucose intolerance among young adults	HR 1.65[a] (CI:1.27-2.13) HR 1.35[b] (CI:1.06-1.71)	higher incidence of glucose intolerance in current smokers[a] *or* passive smoke exposure [b] *vs* non-smokers
Rimm et al.	41,810 male	cq*	6	incidence of type 2 diabetes mellitus	RR 1.94 (CI:1.25-3.03)	higher incidence of type 2 diabetes in >25 cigarettes smoked/day *vs* non-smokers
Hu et al.	84,941 women	co*	16	risk of type 2 diabetes	RR 1.15[c] (CI:1.07-1.25) RR 1.20[d] (CI:1.03-1.41) RR 1.34[e] (CI:1.2-1.5)	elevated risk for type 2 diabetes in former smokers[c] or current smokers with 1-14cig/day[d] or >15cig/day[e] *vs* never smokers
Manson et al.	21,068 men	rdpt*	13	risk of developing type 2 DM	RR 2.1[f] (CI:1.7-2.6) RR 1.4[g] (CI:1.0-2.0) RR 1.2[h] (CI:1.0-1.4)	elevated risk for type 2 diabetes in current smokers with >20 cig/day[f] or <20 cig/day[g] or past smokers[h] *vs* never smokers
Willi et al.	1,2 mio	mpc*	5-30	association between smoking and incidence of type 2 DM	pooled RR 1.44 (CI:1.31-1.58) RR 1.61[i] (CI:1.43-1.80) RR 1.29[j] (CI:1.13-1.48) RR 1.23[k] (CI:1.14-1.33)	dose-dependent increased risk for type 2 DM smokers >20 cig/d[i] or <20 cig/d[j] or former smokers[k] *vs* non-smokers

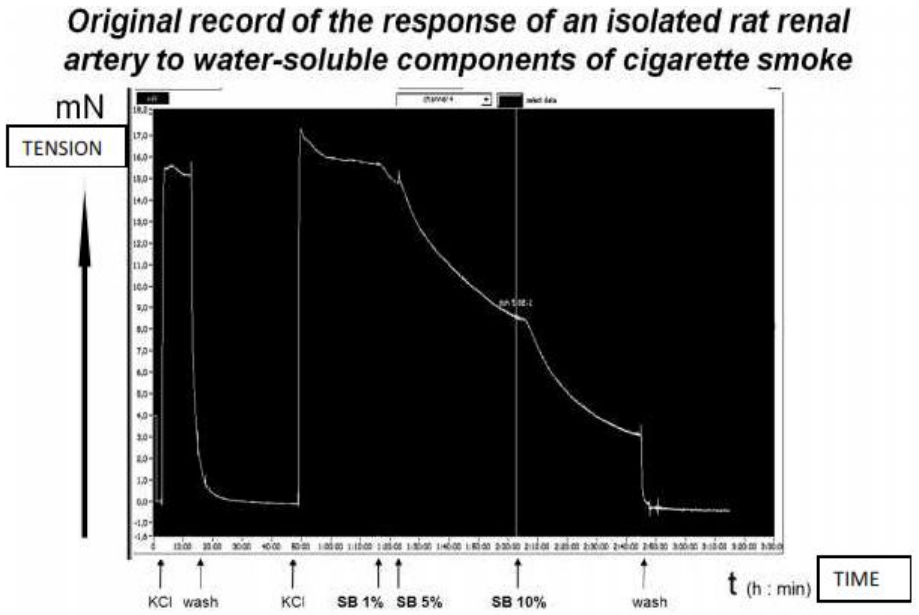
Author	number of patients	study design	follow-up (years)	primary end-point	hazard ratio/ relative risk/odds ratio (95%CI)	conclusion
Wannamethee et al.	7,735 men	pr*	16.8	risk of type 2 DM due to smoking *vs* quitting of smoking	RR 1.74[l] (CI:1.24-2.43) RR 1.79[m] (CI:1.20-2.68) RR 1.71[n] (CI:1.19-2.45) RR 1.18[o] (CI:0.5-2.77) RR 1.79[p] (CI:1.2-2.68) RR 1.33[q] (CI:0.92-1.90) RR 1.42[r] (CI:0.87-2.31) RR 0.95[s] (CI:0.54-1.67)	decreased risk of type 2 DM only after >20ys cessation of tobacco in all current smokers[l] light smokers (<20 cig/d)[m] heavy smokers (>20 cig/d)[n] primary pipe/cigar smokers[o] secondary pipe/cigar[p] ex-smokers[q] 11-19years since quitting[r] >20years since quitting[s] *vs* never smokers
Davey et al.	12866 men	it*	6-7	influence of comprehensive intervention on the risk of developing type 2 diabetes in smokers *vs* non-smokers	HR 1.26[t] (CI: 1.1-1.45) HR 0.82[u] (CI: 0.68 0.98)	no benefit of lifestyle intervention in prevention of diabetes among smokers[t] *vs* non-smokers[u]
Weitzman et al.	2,273 adoles-cent subjects	cs*	-	prevalence of metabolic syndrome	OR 4.7[v] (CI:1.7-12.9) OR 6.1[x] (CI:2.8-13.4)	independent association of environmental tobbaco smoke exposure[v] *or* active smoking[x] with the metabolic syndrome
Ishizaka et al.	5,033 subjects	cs*	-	incidence of metabolic syndrome	OR 1.77[y] (CI:1.42-2.22) OR 2.38[z] (CI:1.95-2.91)	higher incidence of metabolic sy in former smoking[y] & current smoking[z]

* Abbreviations: co, cohort; cq, cohort questionnaire; cs, cross-sectional; es, experimental study; it, intervention trial; mpc, meta-analysis of 25 prospective cohorts; pr, prospective; pm, prospective multicenter; rcmt, randomized controlled multicenter trial; rdpt, randomized, double-blind, placebo-controlled trial.

Table 9.4. Possible contribution of smoking to diabetic nephropathy

- Podocyte damage
  - cigarette smoking → rise in urinary podocyte excretion (early sign of diabetic nephropathy; prediction of long-term urinary albumin excretion in type 2 DM* and microalbuminuria;
  - possible reversible stage
- Hyperfiltration and limited or abolished glomerular autoregulation
  - among insulin-treated diabetics higher prevalence of hyperfiltration in smokers than in non-smokers + GFR* directly dependent on the intensity of smoking
  - cause: non-nicotinic component of smoke (no correlation in users of oral snuff)
  - impaired or abolished autoregulation of GFR* both in type 1 and type 2 diabetics with diabetic nephropathy → harm of glomeruli due to elevated mean arterial pressure caused by smoking
- Nicotine
  - higher insulin resistance → development and aggravation of type 2 diabetes
  - presence of functional nicotinic receptors in pancreatic islets and beta cells → negative impact on beta-cell function (mitochondrial dysfunction, oxidative stress, and inflammation) + apoptosis of islet β-cells
- Genetic predisposition
  - carriers of the DD-genotype of the ACE* gene in normo-albuminuric type 2 diabetic smokers → predisposition to develop albuminuria
- Heavy metals
  - low-level cadmium exposure - association with early onset of diabetic nephropathy
- Impaired vasodilation
  - smoking → impaired responsiveness of intrarenal arteries to vasodilators
- Elevated resting energy expenditure
  - smoking: independent risk factor for elevated resting energy expenditure in patients with diabetic nephropathy → poorer outcome of diabetic nephropathy
- Reversibility
  - in type 1 diabetics with nephropathy and with adequate control of blood pressure and glycemia after stopping smoking → slow down of the progression of nephropathy
  - smoking cessation → reduction in cigarette smoking-induced increased TGF-β_1* excretion → possible reduced renal fibrosis

* Abbreviations: ACE, angiotensin converting enzyme; DM, diabetes mellitus; GFR, glomerular filtration rate; TGF-β1, tissue growth factor β1.



Abbreviations: SB, smoke buffer; KCl, potassium chloride; ACh, acetylcholine; mN, milliNewton.

Figure 9.2. Water-soluble components of nicotinic cigarette smoke elicit an acute and dose-dependent relaxation of renal arteries. This original record shows the change in the isometric tension of one rat renal artery due to cigarette smoke in a Danish Multimyograph.

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Chapter 10

Effects of Cigarette Smoking on Oxidative Stress Biomarkers

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Abstract

Cigarette smoking is known to contribute to many diseases, including cancer, chronic obstructive pulmonary disease, stroke, cardiovascular disease and peptic ulcers. Investigators have attempted to elucidate the mechanisms of the pathogenesis associated with cigarette smoking, but the conclusions are inconsistent. A basic hypothesis is that free radicals cause oxidative damage to macromolecules such as lipids, proteins and DNA. However, there is still limited information regarding the relationship between cigarette smoking and plasma antioxidant concentrations in disease-free individuals. Therefore, the aim of this study was to determine the effects of cigarette smoking on oxidative stress risk indicators such as malondialdehyde (MDA), uric acid, bilirubin, albumin, homocysteine and paraoxonase (PON1) activity and to determine the correlation between these factors and two biological tobacco markers: plasma thiocyanate (SCN^-) and urinary cotinine. The initial study was conducted on 138 nonsmokers aged 38.47 ± 21.91 years and 162 smokers aged 35.55 ± 16.03 years. MDA, bilirubin, albumin and uric acid were measured by colorimetric method, homocysteine (tHcys) by immunoassay, PON1 activity by kinetic method, cotinine by immuno-enzymatic method and SCN^- by a selective electrode. In smokers, we found a significant increase in MDA, tHcys and a significant decrease in bilirubin, albumin, uric acid and paraoxonase activity compared to non-smokers. A statistical significant negative correlation was noted between the

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smoking status parameters, including both the number of cigarettes smoked/day and the plasma uric acid, as well as between the duration of smoking and the plasma uric acid. Among smokers, we noted a negative correlation between uric acid and both plasma thiocyanates, and cotininuria. We found a positive significant correlation between MDA and number of cigarettes smoked/day and a negative significant correlation between albumin and number of cigarettes smoked/day, tHcys was strongly correlated with the consumption duration and the number of cigarettes consumed, and we observed a positive correlation between tHcys and both SCN^- and cotininuria. Additionally, we noted a significant regression of paraoxonase activity in smokers when the number of cigarettes smoked/day exceeds 20. We noted a significant association between smoking status and oxidative stress biomarkers perturbation mainly with lower PON1 activity and hyperhomocysteinema. Moreover, both cigarettes smoked/day and consumption duration were significantly associated with these perturbations particularly with higher MDA levels. In conclusion, we have shown that smokers have higher levels of MDA and tHcys than nonsmokers in spite of balanced antioxidant profiles. Additionally, we have found that smoking decreases plasma antioxidant concentrations.

Introduction

Cigarette smoking is a major risk factor for atherosclerosis. Although the relative importance of potential mechanisms of smoking induced vascular injury are unknown, direct delivery of oxidants and the subsequent promotion of platelet and neutrophil activation suggest the importance of oxidative stress in the pathogenesis of smoking-induced tissue injury.

Oxidative stress (OS) is a disturbance in the redox state of an organism or a disturbance in the balance between production of reactive oxygen species (ROS) and endogenous antioxidant defenses, leading to oxidation of lipids, proteins, and DNA in ways that impair cellular function. A free radical is any molecule with one or more unpaired electrons in its outer electron shell and ROS are any chemically reactive molecules that contain oxygen. Although often used interchangeably, not all free radicals are ROS, and not all ROS are free radicals. Nitric oxide (NO) is itself a ROS. Other biologically important ROS include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), which is a ROS, but not a free radical, and peroxynitrite (ONOO).

Cigarette smoking is a serious health problem and most important avoidable causes of several diseases in world. Smoking has been strongly implicated as a risk factor for chronic obstructive pulmonary disease, cancer, and atherosclerosis. The leading causes of death from smoking are cardiovascular diseases (1.69 million deaths), chronic obstructive pulmonary disease (0.97 million) and lung cancer (0.85 million deaths) [1].

Cigarette smoke is a complex mixture of chemicals containing more than 4000 different constituents. In the last 30-40 years, a large body of knowledge has accumulated identifying the exact chemical composition of cigarette smoke both qualitatively and quantitatively. Some of the compounds identified include different pyridine alkaloids such as nicotine, ammonia, acrolein, phenols, acetaldehyde, N-nitrosamine; polycyclic aromatic hydrocarbons such as benzopyrine; combustion gases such as carbon monoxide, nitrogen oxides, hydrogen cyanide; trace metals, α-emitter radioactive elements such as polonium, radium, and thorium Two major phases were identified in cigarette smoke: a tar phase and a gas phase, both are

rich in oxygen-centered, carbon-centered and nitrogen-centered free radicals as well as non-radical oxidants [2].

From the analysis of each phase, it was estimated that a single cigarette puff contains approximatively. These include various compounds, which are capable of causing an increase in the generation of various ROS like superoxide ($O_2^{\bullet -}$) hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals. These reactive oxygen species in turn are capable of initiating and promoting oxidative damage in the form of lipid peroxidation [3]. Evidence suggests that ROS may play important roles in the pathogenesis in myocardial infarction. Following ischemia, ROS are produced during reperfusion phase. ROS are capable of reacting with unsaturated lipids and of initiating the self-perpetuating chain reactions of lipid peroxidation in the membranes [2]. Numerous reports have demonstrated the increased risk of coronary problems in smokers [3, 4]. Smoking is thought to have an influence on the prevalence of myocardial infarction by means of several mechanisms, including atherosclerotic injury, increase in platelet aggregation, increase in the levels of adhesion molecules and fibrinogen and vasoconstriction. Cigarette smoking leads to the uptake of many hazardous compounds. Such compounds or their metabolites may be electrophilic and thereby able to react with biological macromolecules, or they may give rise to oxidative stress by formation of reactive species or the initiation of radical chain reactions [2]. Smoking is the most important cause of chronic obstructive pulmonary disease and may enhance oxidative stress not only through increasing oxidants but also by weakening the antioxidant defence mechanisms. Several studies have demonstrated increased susceptibility of LDL to oxidation and higher levels of oxidized LDL in smokers. This would provide an important causal mechanism that links smoking with vascular disease given the numerous pathological effects of oxidized LDL. Smoking may enhance oxidative stress not only through the production of reactive oxygen radicals in smoke but also through weakening of the antioxidant defence mechanisms. In this context, a recent study showed that cigarette smoke inhibited the enzymatic activity of paraoxonase (PON). Given its hypothesized, antioxidant role, this could also contribute to the increased oxidation of LDL in smokers [5].

PON (EC 3.1.8.1) is a calcium-dependent esterase that circulates in plasma associated with HDL and contributes to the protective effect of this lipoprotein on LDL oxidation [6]. Some authors have extended this suggested antioxidant role of PON to a general prevention of per-oxidative damage to cell membranes.

Studies by several groups have shown diverse markers of oxidative stress to be increased in smokers compared with nonsmokers.

Thiobarbituric acid reactive substances (TBARS) and malonyldialdehyde (MDA) are the most commonly assayed substances. Other markers of lipid peroxidation include hydroxynonenanal and isoprostanes. MDA, the end product of lipid peroxidation, is used as a marker of oxidative stress [6].

Homocysteine (Hcy), a sulfur-containing amino acid, is not found in our daily diet. It is primarily formed from the demethylation of methionine during DNA/RNA methylation. L-Hcy is the primary active form in a variety of tissues or cells, and it has been suggested that increased levels of plasma Hcy may play a role in the pathogenesis of various diseases, particularly at the cardiovascular level. The cellular and molecular mechanisms underlying the adverse effect of hyperhomocysteinemia have not been fully elucidated [7]. Al-Obaidi et al [8] suggested that Hcy potentiates the production of thrombin in endothelial cells. Thrombin is a potent activator of a unique group of protease-activated receptors (PARs) that

belong to the G protein-coupled receptor family. Activation of PARs induces generation of ROS, up-regulates NADPH oxidase, and down regulates thioredoxin [9] in endothelial cells. A hyperhomocysteinemia increase oxidative stress and is closely related to accumulation of asymmetric dimethyl-arginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor that inhibits the activity of endothelial NOS (eNOS) and inducible NOS (iNOS). The inhibitory effect of ADMA on NO synthesis is removed by dimethylarginine-dimethylaminohydrolase (DDAH), which catalyzes the conversion of ADMA to L-arginine, citrulline, and dimethylamine. Elevation of ADMA concentrations attenuated acetylcholine-induced coronary vasodilatation, indicating impairment of endothelial NO signaling. NO is an important mediator of many physiological phenomena [10].

Uric acid is mainly synthesized from adenine and guanine-based purines. Because uricase is lacking in man, uric acid appears to be an end-product of the purine pathway. It is thus traditionally considered as a metabolically inert and waste compound without any physiological significance. However, uric acid can be oxidized following a non-enzymatic degradation and has thus proved to be a selective antioxidant, capable especially of reaction with hydroxyl radicals and hypochlorous acid. Uric acid may be found in all tissue compartments with the exception of the lipid phase. In plasma, uric acid, albumin and ascorbic acid, account for more than 85% of total antioxidant capacity. Thus, measuring levels of specific antioxidant molecules, such as plasma uric acid can yield valuable information and low levels of such antioxidants may provide suggestive evidence of oxidative stress [10].

Un-conjugated bilirubin is a pigment resulting from heme catabolism. The oxidative cleavage of heme, catalyzed by heme oxygenase, results in the formation of carbon monoxide, iron and biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase. Whether bilirubin is merely a waste product or has a physiological role is still a matter of debate. In fact, despite the number of studies pointing to its antioxidant capacity, the role of bilirubin as a scavenger of ROS is still controversial. Interaction of bilirubin with human erythrocytes was shown to induce morphological alterations, as well as membrane structure disturbance, with loss of phospholipid asymmetry. In addition, disruption of lipid fluidity, protein order and redox status was observed in rat mitochondrial membranes and whole nerve cells, events that were accompanied by mitochondrial swelling, increased permeability and cytochrome c release. bilirubin also impairs the release and uptake of the neurotransmitter glutamate, suggesting the involvement of excitotoxicity in the mechanisms of Bb-induced neuronal death [11].

Albumin, the most abundant circulating protein in the plasma, exerts important antioxidant activities. The molecule acts through its multiple-binding sites and free radical-trapping properties (Figure 10. 1). In physiological or pathological conditions, function associated with changes in the redox status, the albumin structure, and its beneficial antioxidant properties can be altered. In general, albumin constitutes the major plasma protein target of oxidant stress. An indirect antioxidant activity of albumin comes from its ability to transport bilirubin, which binds with high affinity to the molecule at Lys 240. Such albumin-bound bilirubin was shown to act as an inhibitor of lipid peroxidation, bilirubin bound to albumin in the primary site, was shown to protect a-tocopherol from damage mediated by peroxyl radicals and to prolong the survival of human ventricular myocytes against in situ-generated oxidative stress. Another aspect of antioxidant activity of albumin may come from its capacity to bind homocysteine. Elevated plasma homocysteine is a well-known risk factor

for atherosclerosis and may act through oxidation of LDL [12]. A direct protective effect of albumin is indicated from many epidemiological studies [13]. We review the evidence that cigarette smoking is pro-oxidant in vivo and discuss particularly the implications of these findings for the mechanism of smoking-associated cardiovascular disease. In addition, we briefly discuss methods to measure oxidant stress in smokers and their relevance to potential antioxidant strategies in smokers.

The aim of this study was to study the effects of cigarette smoking on six oxidative stress risk indicators: malondialdehyde (MDA), uric acid, bilirubin, albumin, homocysteine and paraoxonase (PON1) activity levels and to determine the correlation between these factors and two biological tobacco markers: plasma thiocyanate (SCN-) and urinary cotinine.

Materials and Methods

Study Design

Population

The study was performed on 300 voluntary subjects: 138 non-smokers aged 38.47 ± 21.91 years and 162 current smokers aged 35.55 ± 16.03 years. All subjects were questioned about their age, gender, cigarette and alcohol consumption habits. The biological and socio-demographic characteristics are shown in Table 10.1. Differences between smokers and non smokers for gender, body mass index (BMI) alcoholic beverage consumption and lipid profile are noted. Therefore, these variables were considered as potential confounder factors for this analysis. Subjects with peripheral vascular disease, diabetes mellitus, renal disease, hepatic disease and hyperlipidemia or hypertension or receiving any medication were also excluded. None of these subjects was vegetarian or vegan or used vitamin supplements.

Written informed consent was obtained from all voluntary adult participants and from the parents of minors.

Samples

After a 12 h overnight fasting, venous blood from each subject was drawn in tubes containing lithium heparinate for classical biochemical parameters, and in tubes containing EDTA/K_3 for plasma homocysteine and thiocyanates. Blood samples were immediately centrifuged at +4°C and stored at –80°C until analyses.

Urine samples were obtained from the smokers and nonsmokers. These samples were either used the same day or frozen at -20°C until required for analysis. All the samples were analysed for urine cotinine.

Methods

Smoking Questionnaire

Smoking habits were investigated by standard questionnaire: questions covered both previous and current smoking habits, including the duration of smoking (age at start, years of smoking) and number of cigarettes smoked per day. Participants were classified into: (1)

never smokers: those who had never smoked cigarettes; (2) current smokers: those who smoked regularly at least 1 cigarette/day during the previous year. The majority of subjects were able to provide information on the number of cigarettes they smoked and the duration of smoking. All subjects were questioned about their socio-demographic characteristics including age, gender, education and employment.

Biochemical Assays

MDA, bilirubin, albumin and uric acid were measured by colorimetric method, on the Konelab 30™. Plasma homocysteine concentrations were determined by an immunometric assay using the AxSYM® (Abbott Laboratories, Abbott Park, IL 60064, Barcelaneta, Puerto Rico). Folic acid and vitamin B12 were determined using an immunoenzymatic method (Elecsys 2010™ Roche Diagnostics, Indianapolis, IN, USA).

Total cholesterol (TC), HDL cholesterol (cHDL) and triglycerides (TG) were determined by enzymatic methods, and apolipoprotein (ApoA1, ApoB) and lipoprotein (Lp(a)) levels were determined by immunoturbidimetric techniques using Konelab 30™ equipment.

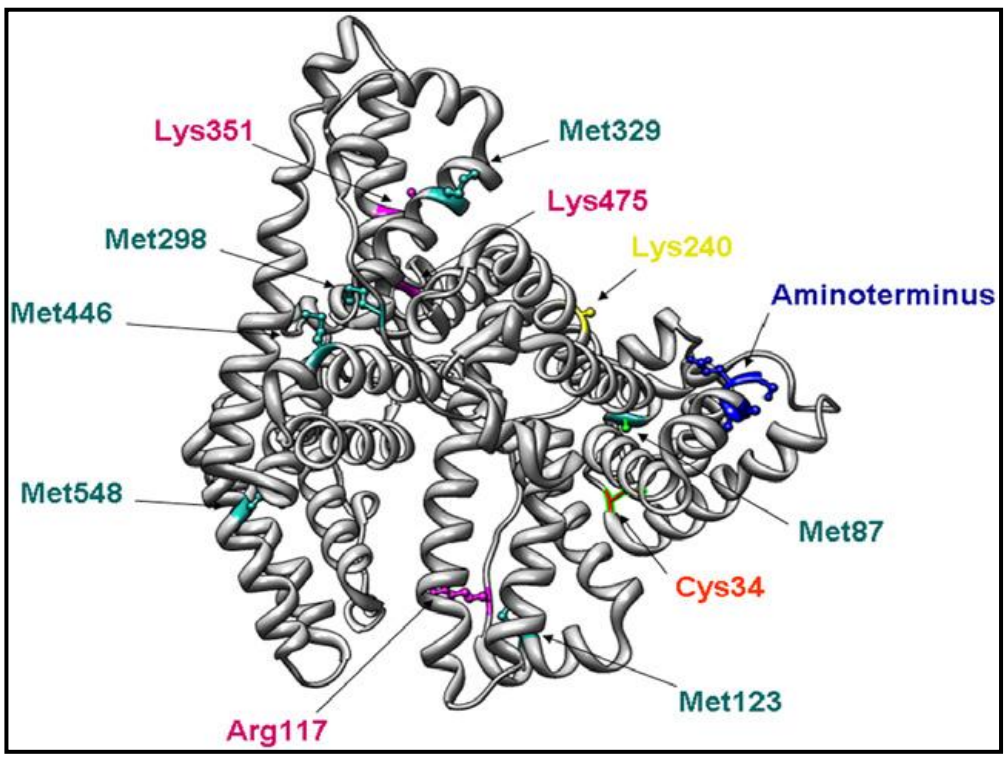


Figure 10.1. Main sites in albumin involved in its antioxidant activity. Lateral carbon chain of residues involved in albumin antioxidant properties are developed and colored. Amino terminus four amino acids of the protein are shown in blue. This sequence is involved in the metal binding of the protein. The sole free cysteine in the protein (Cys34) is shown in red. The free main sites of ligation of PUFA in albumin are shown in purple. Lys240 in albumin (yellow) is involved in bilirubin ligation. The six methionine residues are depicted in green [12].

Table 10.1. Socio-demographic, clinical and biological characteristics of study population

	Smokers (n=162)	**Non smokers (n=138)**	**p-value**
***Age* (years)**	35.55 ± 16.03	38.47 ± 21.91	0.172
***BMI* (kg/m²)**	24.25 ± 3.17	25.63 ± 4.36	**0.003**
Sex ratio	8.64	0.81	**< 0.001**
Alcoholic consumption			
Yes	37	16	**< 0.001**
No	125	122	
Lipid profile			
CT (mmol/L)	4.13 ± 1.18	3.70 ± 1.04	**0.005**
HDLc (mmol/L)	0.94 ± 0.25	1.07 ± 0.27	**0.001**
LDLc (mmol/L)	1.35 ± 0.56	1.16 ± 0.61	**0.01**
TG (mmol/L)	1.79 ± 1.03	1.40 ± 1.24	**< 0.0001**
ApoB/ApoA1	0.83 ± 0.52	0.52 ± 0.15	**0.03**
Lp (a) g/L	0.23 ± 0.23	0.18 ± 0.19	**0.04**
Vitamins profile			
Folates (nmol/L)	4.30 ± 1.56	4.45 ± 2.45	0.56
Vitamin B12 (pmol/L)	363.94± 140.96	407.45 ± 192.97	**$< 10^{-7}$**

Paraoxonase Activity

Paraoxonase activity was determined using paraoxon (1.2 mmol/L) as the substrate in 0.1M Tris/ HCl buffer at pH= 8.0, containing 2mM $CaCl_2$ (0.5ml final volume). The sample to be tested was added (5µL) to start the reaction and the increase in the absorbance at 405nm was recorded. One international unit (IU) of paraoxonase activity is defined as 1 µmol of p-nitrophenol formed per minute, and activity was expressed as IU/L of plasma.

Tobacco Biomarkers

Cotinine levels were determined using homogenous enzymes immunoassay method (Konelab 30™, Thermo Electron Corporation, Finland) and expressed as micrograms per micromol of creatinine in urine. Plasma thiocyanates levels were determined using selective electrodes (Ionometer Seven Multi S80, Mettler Toledo, Switzerland) and expressed as milligrams per liter in plasma.

Clinical Evaluation

BMI was calculated as weight (kg) divided by squared height (m^2).

Statistical Analysis

The statistical analyses were performed using the SPSS 17.0. Quantitative variables were presented as mean ± SD and comparisons were performed using the Student's t test. Qualitative variable comparisons were performed using the χ^2 test. Odds ratios (ORs) and

their 95% confidence interval (CI) were calculated. Adjustment for potential confounder factors was determined by binary logistic regression. Differences between groups were evaluated with ANOVA, followed by the Tukey post -hoc tests after adjustment for potential confounder factors (gender, age, BMI, and lipid profile). The statistical significance level was set at $p < 0.05$. All variables with a p value < 0.25 between the two studied groups (smokers and non-smokers) were considered as confounding factors for further OR adjustment.

Results

Risk Factors of Oxidative Stress in Smokers

Effect of Cigarette Smoking on Plasma Total Homocysteine Concentrations

As shown in Table 10.2, plasma total homocysteine concentrations were significantly higher in smokers than in non-smokers after and before adjustment.

Table 10.2. Variation of oxidative stress parameters of study population

Parameters	Smokers n= 162	Non smokers n = 138	p	*p
thcys(µmol/L)	18.52 ± 9.71	12.82 ± 3.96	$< 10^{-4}$	0.02
PON1 activity (IU/L)	95 ± 104	154 ± 133	0.001	< 0.001
Albumin (g/L)	44.37 ± 7.99	53.38 ± 6.32	$< 10^{-4}$	< 0.001
Bilirubin (µmol/L)	11.53 ± 6.51	13.89 ± 11.97	0.03	0.01
Uric acid (µmol/L)	199 ± 97	250 ±131	$< 10^{-4}$	$< 10^{-4}$
MDA (nmol/L)	8.22 ± 2.52	6.60 ± 3.77	$< 10^{-4}$	$< 10^{-4}$

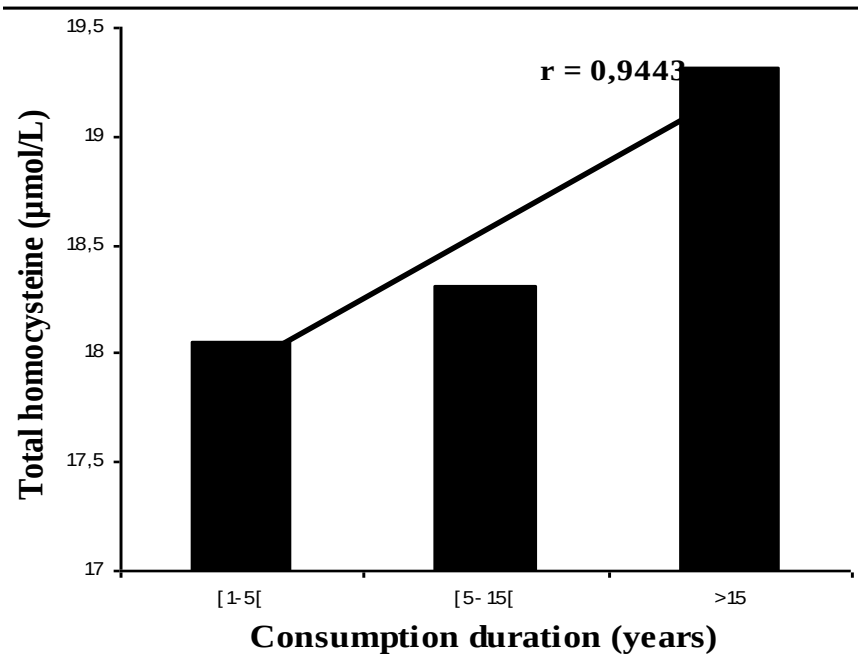
* p: adjusted for confounder factors: thcys (Age, gender, BMI, folates and vitamin B12), PON1(Age, gender, BMI and lipid profile), Albumin (Age, gender, BMI, alcohol consumption and lipid profile), Bilirubin (Age, gender, BMI and lipid profile),MDA(Age, gender, BMI and lipid profile), Uric acid (Age, gender, BMI and lipid profile).

We calculated the odds ratio of hyperhomocysteinma (> 15 µmol/L) after and before adjustment for confounder factors (age, gender, folates and vitamin B12) associated with smoking status, we noted a significant association between smoking status and hyperhomocysteinemia in the two situations (table 10.3) (OR= 3.2; $p < 10^{-4}$ before and 2.8; p = 0.009 after). We found an important correlation between total homocysteine concentration and duration of smoking (0.9443) and number of cigarettes smoked/day (r = 0.9170), but without any difference between groups (Figure 10.2).

Paraoxonase 1 (PON1) Activity in Smokers

We noted a significant decrease of PON1 activity in smokers compared to non smokers (94 ± 104 *Vs* 158 ± 133 IU/L; p = 0.001), with regression of PON1 activity according number of cigarettes/day. After adjustment of PON1 activity levels for potential confounders (lipid

profile, BMI, gender and age), we noted a significant difference between smokers and non-smokers ($p < 0.001$) (table 10.2).



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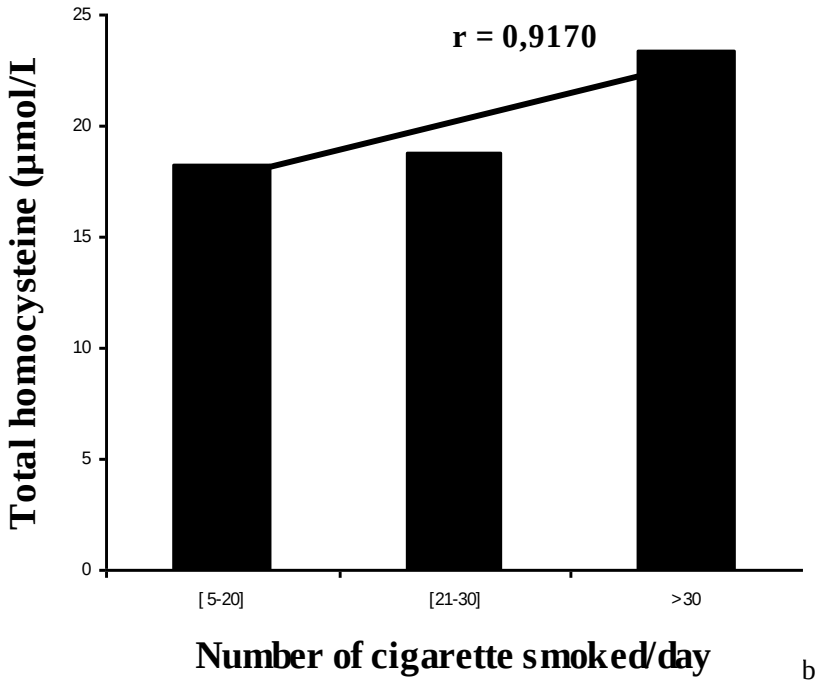


b

Figure 10.2. Correlation between tHcys concentration and smoking status (number of cigarettes smoked and consumption duration). a: Homocysteine and consumption duration b: Homocysteine and number of cigarettes smoked.

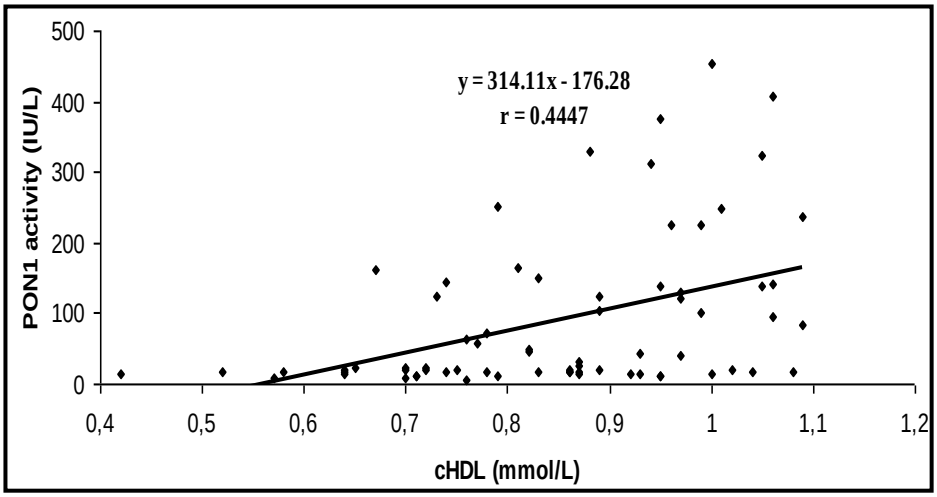


Figure 10.3. Correlation between PON1 activity and HDLc concentration in smokers.

To evaluate the adjusted association between smoking status and lower paraoxonase activity, we calculated odds ratio of lower paraoxonase activity (< 90 IU/L) associated with smoking status and adjusted for confounder factors (age, gender, BMI and lipid profile). We noted a significant association between smoking status and lower paraoxonase activity in the two situations (OR = 3.21; $p < 10^{-4}$ before, OR = 3.03; p = 0.001 after) (table 10.3). In smokers, we found a significant positive correlation between paraoxonase activity and cHDL (r = 0. 4447; p < 0. 0001) (Figure 10.3).

Table 10.3. OR of different stress oxidant parameters according to smoking status

	OR CI 95%	**p**	*** OR CI 95%**	***p**
PON1 activity < 90 IU/L	3.21 [1.7 -5.8]	**$< 10^{-4}$**	3.03 [1.5 5.9]	**0.01**
thcys > 14 µmol/L	3.21 [1.9-5.4]	**$< 10^{-4}$**	2.81 [1.296.12]	**0.009**
Hypoalbuminemia < 47.8 g/L	4.36 [2.15-8.8]	**$< 10^{-4}$**	2.51 [3.22-19.6]	**$< 10^{-4}$**
Bilirubin < 10.4 µmol/L	1.30 [0.8-2.07]	0.16	2.47 [1.18-5.18]	**0.01**
Hypouricemia M:< 200 µmol/L;W < 150 µmol/L)	1.21 [0.7-2.09]	0.49	1.27. [0.372.24]	0.37
MDA > 7.2 nmol/L	3.75[2.306.13]	**$< 10^{-4}$**	6.7. [3.08-14.8]	**$< 10^{-4}$**

M: Men; W: Women; Thcys: 14µmol/L a median, PON1 activity: 90 IU/L a median Albumin: < 47.8 g/L a median, Bilirubin: < 10.4 µmol/L a median, MDA: > 7,2nmol/L a median.

*p: adjusted for confounder factors: thcys (Age, gender, BMI, folates and vitamin B12), PON1(Age, gender, BMI and lipid profile), Albumin (Age, gender, BMI, alcohol consumption and lipid profile), Bilirubin (Age, gender, BMI and lipid profile),MDA(Age, gender, BMI and lipid profile) and Uric acid (Age, gender, BMI and lipid profile).

Effect of Cigarette Smoking on Uric Acid Levels

We noted that plasma uric acid was significantly lower in smokers than in nonsmokers before and after adjustment (Table 10.2). It was also significantly lower in smoking men than

non-smoking ones (203 ± 100 *Vs* 337 ± 100; $p < 10^{-7}$). The same result was found for women (172 ± 72 *Vs* 223 ± 130; p = 0.02) (Figure 10.4).

We found an association between smoking status and hypouricemia before and after adjustment to potential confounders factors (1.21[0.7-2.09], p = 0.49; 1.27 [0.37-2.24], p= 0.37; respectively) (table 3). In this study, we found a significant difference between subjects smoking more than 20 cigarettes/day and those smoking less than 20 cigarettes/day (χ^2 = 22.4; $p < 10^{-7}$). We noted a significant decrease of uric acid concentration when the smoking duration exceeds 5 years ($\chi^2 = 3.89$; p = 0.04) (Table 10.4).

The number of cigarettes smoked/day was significantly higher in subjects having uric acid values below 200 µmol/L than in others (24 ± 9.58 *Vs* 19 ± 9.55 cigarette/day; p = 0.01) (table 10.4).

A statistical significant negative correlation was noted between the smoking status parameters, including both the number of cigarettes smoked/day ($F_{3\text{-}161} = 12.063$; r = - 0.9968; p < 0.05; Figure 10.5a) and the duration of smoking ($F_{3\text{-}161} = 1.305$; r = - 0.9406; p = 0.274, Figure 10. 5b) and serum uric acid.

Table 10.4. Variation of uric acid according to the number of cigarettes smoked and consumption duration

	Cigarettes smoked/days		***P***	**Consumption duration (years)**		***P***
	< 20	**≥ 20**		**< 5**	**≥ 5**	
Uric acid < 200 µmol/L (n =72)	147 ± 24	98 ± 67	$5\ 10^{-4}$	125 ± 62	99 ± 65	**0.02**
Uric acid ≥ 200 µmol/L (n = 90)	270 ± 44	264 ± 9	0.58	267 ± 46	267 ± 49	0.95
Chi², p	22.4**; p < 10^{-7}**			3.89; **p = 0.04**		

Effect of Cigarette Smoking on Bilirubin Levels

In smokers, we found a significant decrease in bilirubin levels (11.53 ± 6.51 *Vs* 13.89 ± 11.97; p= 0.03) compared to non-smokers (table 10.2). We calculated the odds ratio of hypobilirubinemia (< 10.4 µmol/L) before and after adjustment for confounder potentials factors (age, gender, lipid profile) associated with smoking status, we noted a significant association between smoking status and hypobilirubinemia in the two situations (1.30 [0.8-2.07], p = 0.16 before; 2.47 [1.18-5.18], p = 0.01 after) (table 10.3).

Effect of Cigarette Smoking on Albumin Levels

In smokers, we found a significant decrease in albumin (44.37 ± 7.99 *Vs* 53.38 ± 6.32 g/L; $p<10^{-4}$) compared to non-smokers and a negative significant correlation between albumin and number of cigarettes smoked/day (r = - 0.449; $p < 10^{-4}$) (table 10.2). After adjustment for potentials confounder factors such as lipid profile, BMI, age and gender, we noted a

significant association between smoking status and lower albumin levels (4.36 [2.15-8.8], $p < 10^{-4}$ before; 2.51 [3.22-19.6], $p < 10^{-4}$ after) (table 10.3).

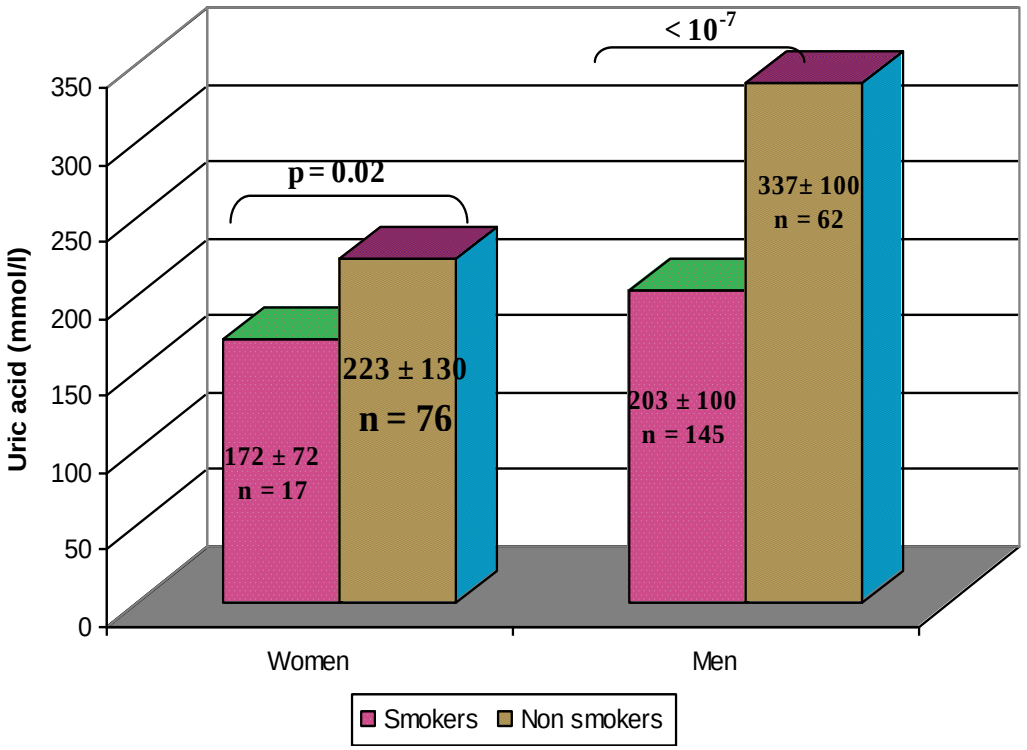
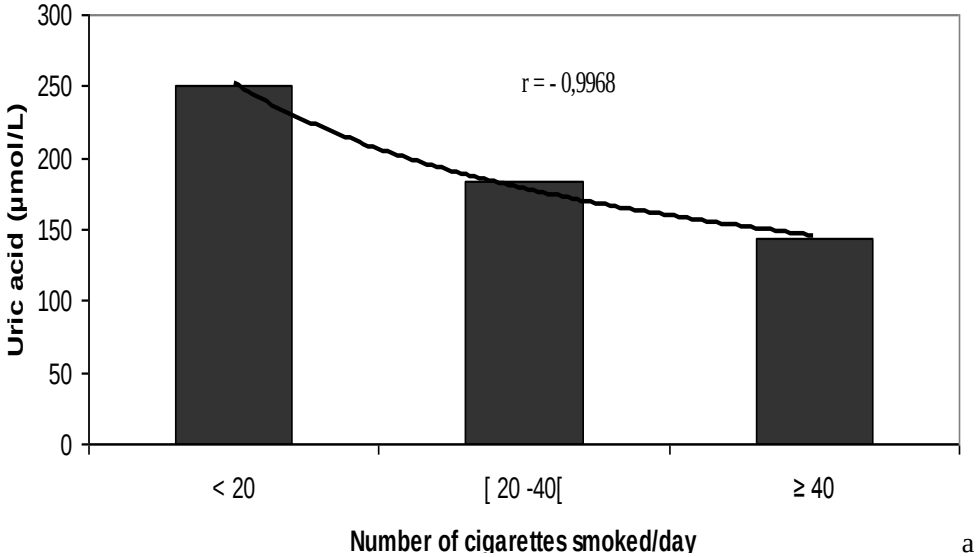


Figure 10.4. Variation of uric acid in men and women according to smoking status.

Effect of Cigarette Smoking on MDA Levels

We found significant increase in MDA (8.22 ± 2.52 *Vs* 6.60 ± 3.77; $p < 10^{-4}$) in smokers compared to non smokers before and after adjustment (table 10.2). After adjustment for potentials confounder factors such as lipid profile, BMI, age and gender, we noted a significant association between smoking status and higher levels of MDA levels (OR = 3.75;CI 95% [2.30-6.13], $p < 10^{-4}$ before; OR= 6.70 CI 95% [3.08-14.80], $p < 10^{-4}$ after) (table 10.3). In smokers, we found a positive significant correlation between MDA and number of cigarette smoked/day (r= 0.535; $p < 10^{-4}$). MDA levels were elevated in subjects who were smoking for more than 10 years. Moreover, both cigarettes smoked/day and consumption duration were significantly associated with these perturbations particularly with higher MDA levels (OR= 4.6; CI 95% 0.6-3.2; OR= 1.9; CI 95% [0.8-3.6]; respectively).

In smokers, MDA levels were significantly correlated PON1 activity (r =0.792; p = 0.01), and with tHcys concentration (r =0,600; p = 0.04), albumin (r = -0.781; p = 0.02) and bilirubin levels (r = -0.697; p= 0.03) (table 10.5).



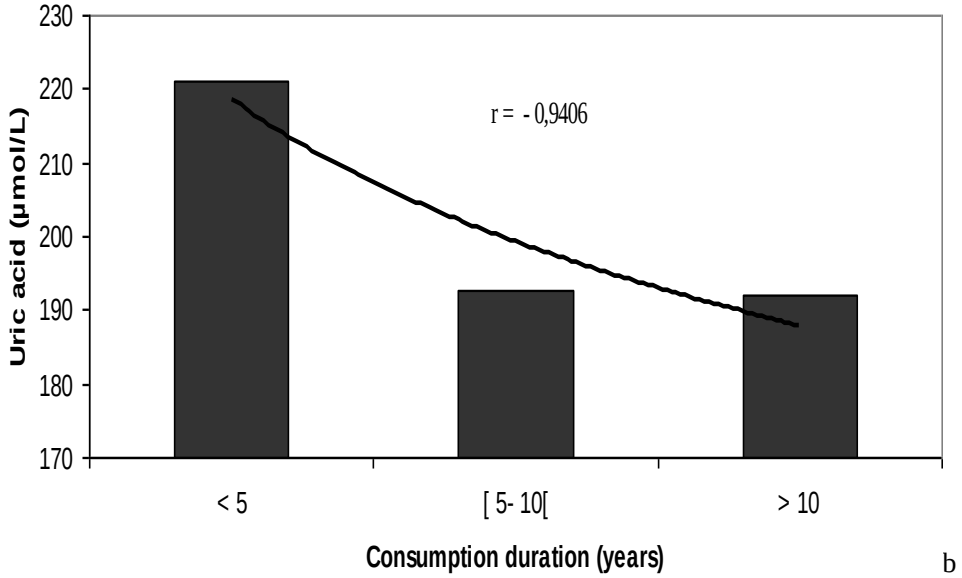


Figure 10.5. Correlation between uric acid concentration and smoking status (cigarettes smoked/day and consumption duration); a. Uric acid levels and cigarette smoked/day; b: Uric acid levels and consumption duration.

Correlation between the Studied Oxidative Stress Factors and Two Biological Tobacco Markers

Urinary cotinine and plasma SCN^- levels were both significantly higher in smokers than in nonsmokers and well correlated with the number of cigarettes smoked per day. Urinary cotinine was significantly correlated with duration of consumption ($F_{3\text{-}109} = 3.43$; $p = 0.019$; $r = 0.9961$), and there was a negative correlation between body mass index and urinary cotinine ($r = 0.9989$; $p < 0.05$) (tables 10.6 and 10.7).

Table 10.5. Correlation between MDA levels and oxidative stress factors

	MDA levels	
	Pearson r coefficient	p
PON1 activity	0.792	0.01
Albumin	-0.781	0.02
Bilirubin	-0.697	0.03
thcys	0.600	0.04
Uric acid	0.207	0.179

Figures 10.6 and 10.7 show that the correlation between urine cotinine and plasma total homocysteine concentrations (r = 0.802) is greater than that between plasma thiocyanates and homocysteine concentrations (r = 0.642). However, a significant correlation (r = 0.825) was found only in individuals who smoked more than 20 cigarettes/day. Paraoxonase activity had significant negative correlation with urine cotinine (r = - 0.271; p = 0.03) and at the limit of the statistical significance with plasma thiocyanates (r= - 0.188; p = 0.06).

Figures 10.8 and 10.9 show a negative correlation between urinary cotinine and plasma uric acid levels (r = - 0.580), and between plasma SCN^- and uric acid concentrations (r = - 0.437).

Discussion

Our data showed that plasma total homocysteine is significantly higher in smokers than in non-smokers. It is known that hyperhomocysteinemia is linked to inadequate intake of vitamins, particularly B-group vitamins, and therefore may be amenable to nutritional intervention. The study by Bostom and Lathrop [14] is the only one in which concentrations of all three vitamins known to influence hyperhomocystinemia were determined. It has been recognised that smoking affects the nutritional status of folic acid, vitamin B12 and vitamin B6, each of which regulates homocysteine metabolism, and/or because cigarette smokers have poorer diets than non-smokers; smokers are more likely to choose white bread, sugar, meat, butter, whole milk and eggs, and less likely to consume whole-wheat bread, high-fibre breakfast cereals, fruits and vegetables, than non-smokers. The usual dietary sources of vitamin B12 are meat and meat products (including shellfish, fish, poultry and eggs). The results obtained in our study are in accordance with the results of Pagan et al [15]. Several mechanisms might explain the increased risk in smokers with raised plasma homocysteine. Nicotine and carbon monoxide separately produce tachycardia, hypertension and vasoconstriction and both produce direct endothelial damage. Hyperhomocysteinemia has been associated with impaired endothelial function and abnormal flow mediated vasodilatation has been demonstrated with mild hyperhomocysteinemia. Smoking may also damage the vascular tree via platelet activation, lipid peroxidation, enhanced tissue factor activation, increased fibrinogen levels and smooth muscle proliferation [16]. The fact that both of these risk factors can exert similar effects would suggest strong potential for interaction between them to produce vascular damage. While both smoking and homocysteine may damage the vascular tree independently, they are also related. Plasma homocysteine level was significantly affected by several B vitamins. Plasma folic acid was a significant factor

affecting plasma homocysteine concentration in the smoker group. Our results were similar to those reported recently; O'Callaghan et al [16] found that current smokers had higher plasma homocysteine levels and lower folic acid levels than those who never smoked. These data suggest that different factors contribute to the high plasma homocysteine concentrations in study subjects. Other factors such as genotype may also play an important role in the modulation of plasma homocysteine levels [17].

Table 10.6. Variation of urinary cotinine and plasma SCN^- levels according smoking status, gender and alcoholic beverage

		Urinary cotinine (µg/µmol Cr)	p	PlasmaSCN⁻ (µmol/L)	P
Smoking status	Smokers	231.43 ± 205.22	$< 10^{-7}$	100.25 ±1.36	$5\ 10^{-4}$
	Non-smokers	73.22 ± 73.71		99.60 ± 0.91	
Gender	Men	222.88 ± 195.76		100.15 ± 1.39	
	Women	310.67 ± 277.40	**0.03**	100.70 ± 0.34	10^{-4}
Alcoholic beverage	Yes	222.19 ± 191.35		100.37 ± 1.5	
	No	235.40 ± 211.96	**NS**	99.80 ± 0.51	**0.03**

Cr: creatinine.

However, plasma folic acid concentrations of the smokers were lower than those of the non-smokers, but not significantly different. It is important to mention that plasma folic acid is related to recent consumption, while red blood cells folic acid is an indicator of folic acid stores. People who smoke cigarettes are known to differ from persons who have never smoked with respect to several lifestyle behaviours, including eating less healthful diets. The causes for this deficiency are presently unclear; although a number of mechanisms have been proposed, including diminished dietary intake, poor absorption of polyglutamyl folic acids, decreased hepatic uptake and retention, increased urinary excretion of folic acid, impaired formation or hydrolysis of polyglutamates, and increased folic acid catabolism [18]. Several of the hundreds of chemical components of tobacco smoke have been shown to interact with folic acid coenzymes, transforming them into biologically inactive compounds. These chemical interactions may have physiological significance, which is supported by reports of lowering circulating folic acid levels in smokers. Reactive oxygen species can be produced by cigarette smoke-induced phagocytic cells and cause oxidative damage to DNA, proteins and lipids, which may be closely related to cardiovascular disease [18].

Chemical components found in tobacco smoke interact with the above and transform them into inactive compounds reducing their active concentration in biological fluids and possibly alter the ability of the cell to store and metabolise folate [19]. The lower plasma folate levels found in our study most likely follow the mentioned mechanism, and other studies have confirmed the finding [20].

Table 10.7. Variations of urinary cotinine and plasma SCN^- levels according to consumption duration, number of cigarettes smoked/day and BMI

Parameters		Urinary cotinine (µg/µmol Cr)	Plasma SCN (µmol/L)
Consumption duration (Years)	**[1-5[**	158.61 ± 230.05	100.19 ± 1.48*
	[5-15[	222.23 ± 187.40	100.21 ± 1.57
	[15-20]	252.34 ± 195.97*	100.42 ± 0.15*
	> 20	272.88 ± 228.75*	100.43 ± 1.07*
Cigarettes smoked/day)	**[5-10]**	133.89 ± 149.04*	99.47 ± 0.41*
	[11-20]	217.07 ± 204.90	100.31 ± 1.48
	[21-30]	309.09 ± 194.44*	100.89 ± 1.55*
	> 30	341.38 ± 220.29*	102.07 ± 2.95*
BMI (kg/m^2)	**< 25**	251.86 ± 216.65	100.24 ± 1.44
	[25- 27[	196.00 ± 111.96	100.00 ± 0.69
	[27- 30]	135.64 ± 137.59	100.62 ± 1.63
	> 30	50.26 ± 65.46	100.93 ± 2.04

* $p < 0.05$.

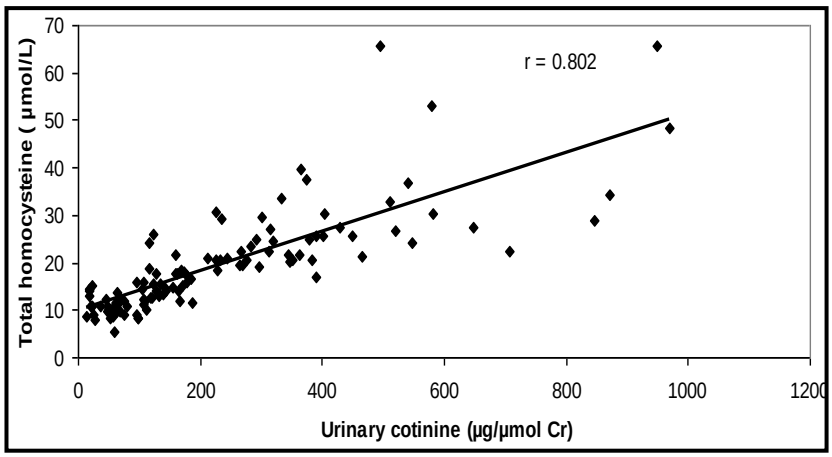


Figure 10.6. Correlation between urinary cotinine and plasma homocysteine concentration in smokers.

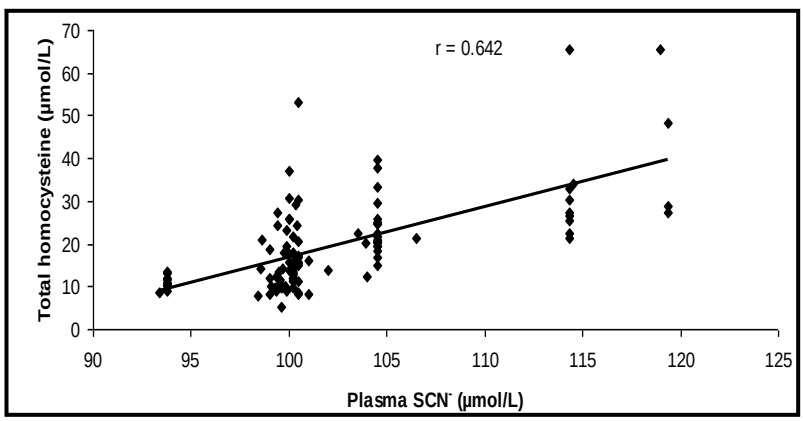


Figure 10.7. Correlation between plasma SCN^- and plasma homocysteine concentration in smokers.

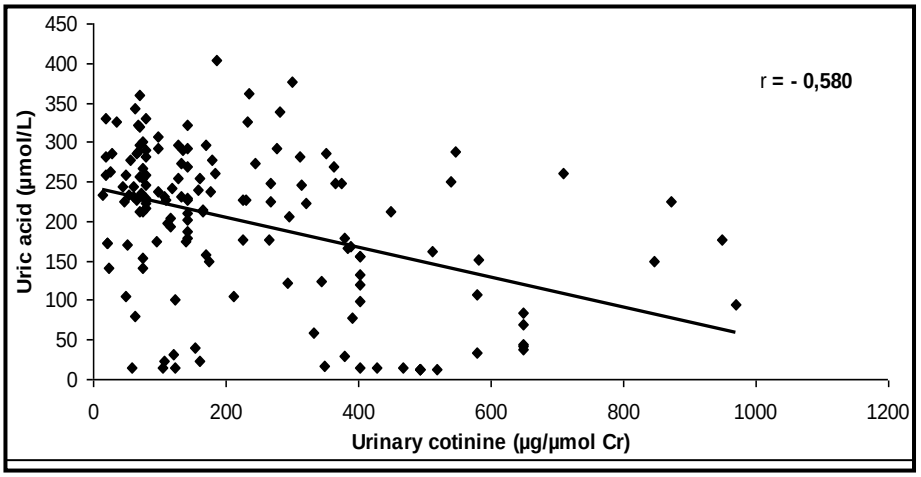


Figure 10.8. Correlation between uric acid concentration and urinary cotinine levels.

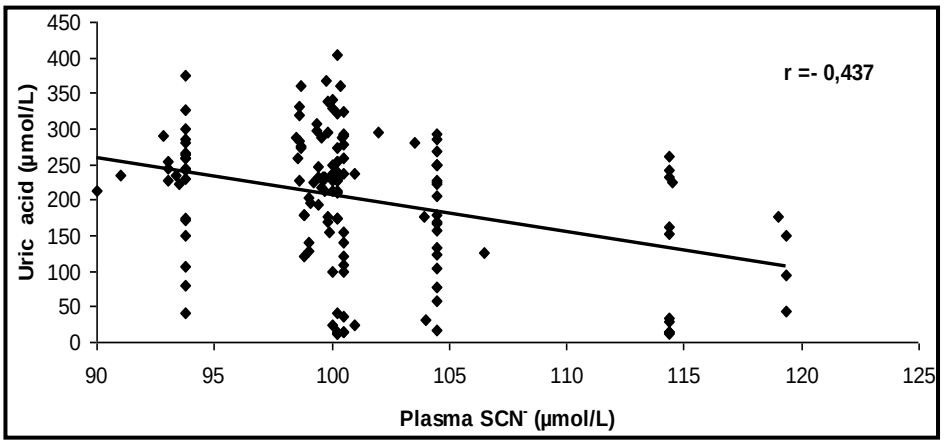


Figure 10.9. Correlation between uric acid concentration and plasma SCN^- levels.

We found a significant relation between raised homocysteine levels and number of cigarettes smoked per day in smokers but without difference between groups. The mechanism for the increase in the plasma homocysteine concentration by cigarette smoking is not clear. A similar study also reports significantly higher basal homocysteine levels in smokers as compared to non-smokers, which is also related to the number of cigarettes smoked per day [21]. The Hordaland studies [21] found that the plasma homocysteine concentration increase markedly with the daily number of cigarettes smoked. In the multivariate analysis, sex, age, folate intake, cigarette smoking, and coffee consumption were found to be the most important determinants of plasma homocysteine concentration [20]. In addition, we found an important correlation between homocysteine level and duration of smoking; we noted that plasma homocysteine level increase when duration of consumption exceeds 5 years. Among gender, the current study demonstrates that men smokers had high levels of homocysteine, but this difference was not significant, this lack association may result from a small size of women smokers'subject in this study.

Previous studies have demonstrated a fall in PON1 activity and cHDL concentration. It is known that smoking is associated with coronary artery disease and other vascular disorders. For the occurrence of cardiovascular disease among smokers alteration in plasma lipid profile was implicated. In this context, the mechanisms for the altered lipid profile among smokers were recalled [22]. First, nicotine stimulates the release of adrenaline from the adrenal cortex leading to increase serum concentration of free fatty acids which further stimulates hepatic synthesis and secretion of cholesterol as well as hepatic secretion of very low density lipoprotein and hence increased TG [22]. Second, smoking decreases oestrogen levels and further leads to decreased cHDL concentration [22]. Also, cHDL concentration was inversely related to VLDL concentration in serum. Finally, smoking increases insulin resistance and thus, causes hyperinsulinemia. Also, human serum paraoxonase is a polymorph enzyme which has been shown to play an important role in lipid metabolism. PON1 significantly decreases lipid peroxidise generation during LDL oxidation in the presence of HDL modification by lipid peroxidise [23, 24]. Smoking impairs PON1 activity and thereby compromises anti-oxidant defense mechanism [25]. Moreover, a decrease in PON1 activity in smokers can be explained by the effects of several of the hundreds of chemical components of tobacco smoke have been shown to be responsible for inhibition of PON1 activity are various reactive aldehydes (acetaldehyde, formaldehyde and α,β-unsaturated aldehydes, such as acrolein and acrotonaldehyde), as well as aromatic hydrocarbons [26]. In addition, urinary cotinine and plasma thiocyanates concentrations were both significantly higher in smokers than in nonsmokers. Although, urine cotinine and plasma thiocyanates are influenced by the diet and the industrial pollution, it remains a reliable indicator of the smoking status [27]. We noted a significant association between smoking status and lower paraoxonase activity before and after adjustments for confounder factors. Cigarette smoke has a high content of oxidants that promote a pro-oxidant effect in blood plasma and tissues, which probably contributes to the increased incidence of cardiovascular disease present in smokers. The information available on the molecular mechanisms of action of cigarette smoke is limited. However, recent observations suggest that the pro-oxidant effect of smoking is, in part, related to PON1 activity inhibition caused by cigarette smoke [25].

We showed a significant positive correlation between paraoxonase activity and cHDL values. Paraoxonase is a calcium-dependant esterase closely associated with the high density lipoprotein subfraction that contains apolipoprtein A1 in human serum. Previous studies have suggested that HDL can prevent LDL oxidation and that some oxidised LDL phospholipids are physiological substrates for serum PON1 [27].

Many, but not all epidemiological studies, have suggested that high plasma uric acid is a risk factor for cardiovascular diseases, and they aimed at evaluating its prognosis implications and potential utility in the therapy monitoring [28, 29]. This raised level of plasma uric acid, parallel to an increased risk of cardiovascular diseases, could be either primary or secondary to the underlying causes of the cardiovascular diseases [30]. However, the specific role of plasma uric acid in this constellation remains uncertain although it may be involved in the platelet adhesiveness, aggregation or inflammation, and it may be implicated in the genesis of hypertension [31]. In contrast, there is some evidence that the increase of plasma uric acid is protective against the cardiovascular diseases since uric acid acts as an endogenous antioxidant (31, 32); and the higher plasma uric acid levels found in cardiovascular diseases patients suggest that any protective antioxidant effect of uric acid is hidden by other negative effects in these pathogeneses.

In this study, plasma uric acid level in smokers was significantly lower than in nonsmokers ($p = 0.0003$), both in men ($p < 10^{-7}$) and women ($p = 0.02$). This could confirm the effect of cigarette smoking on uric acid levels independently of the gender. In addition, we noted a significant negative correlation with the smoking status including the average number of cigarettes smoked/day and the smoking duration. Moreover, we noted that the uric acid levels decrease when the smoking duration exceeds 5 years. This finding is in agreement with other studies showing a low plasma uric acid in regular smokers [33], and a reduction of antioxidants including uric acid in smokers, indicating that oxidative stress increases each time a cigarette is smoked [34, 35]. Other studies proved that even nonsmokers exposed to cigarette smoke have a significantly lower plasma antioxidant status than unexposed nonsmokers, independently of the differences in the dietary antioxidant intake [35]. Others studies proved that the administration of uric acid increases the circulating antioxidant defences and allows the restoration of endothelium-dependent vasodilatation [36]. A decrease of uric acid in smokers can be explained by the inactivation of xanthine oxidase by cyanide which is eliminated as thiocyanate [37]. Therefore, high plasma uric acid concentrations might be protective in situations characterized by an increase of cardiovascular risk, and oxidative stress such as smoking [33], and a reduction of its level which increases susceptibility to oxidative damage and accounts for the excessive free radical production [35]. Therefore, the possibility that uric acid confers protection against the development of atherosclerosis, in view of its antioxidant properties, has been recognized [35].

In this study, we found a significant decrease of serum creatinine levels in smokers compared to nonsmokers although these values are not pathological. This can confirm that all the subjects studied are without any renal failure, since the determination of creatinine has been reported to be useful in evaluating renal handling of uric acid and as concentrations of this parameter are highly dependent on endogenous production as well as on renal excretion [38]. Therefore, low plasma uric acid level in smokers is attributed to a reduction of endogenous production.

This finding is in agreement with other studies that proved that the reduction of antioxidants including uric acid in smokers is due to both the chronic exposure to cigarette smoke that is a significant source of oxidative stress and to the low intake of dietary antioxidants [39].

Some of the relationships between tobacco and urea or uric acid are very significant; however, they are all very weak. If these relationships have the same origin, a hypothetical renal mechanism must first be considered. In fact, the blood urea is a product of the catabolism of proteins and their amino acids whereas uric acid originates from the oxidation of purines. Moreover, the two molecules, while circulating in the blood, remain unlinked, either directly or by a common carrier. On the contrary, they are both excreted by the kidney, and in the disease processes, they generally vary in the same way: a rise in blood uric acid is well-known as an early sign of renal failure. An increase in renal excretion of urea and uric acid under the influence of tobacco is therefore a reasonable hypothesis, and it is supported by the known action of nicotine on the metabolism of cathecholamines and the effect of these substances on renal function [40].

Urinary cotinine and plasma SCN^- concentrations were both significantly higher in smokers than in nonsmokers and they were well correlated with the number of cigarettes smoked per day and with the duration of consumption. Although, cotinine and plasma SCN^- are influenced by the diet and the industrial pollution, it remains a reliable indicator of the

smoking status [41]. We found a negative correlation between the plasma uric acid level and both urinary cotinine concentration ($r = - 0.408$) and plasma SCN^- concentration ($r = - 0.337$) in active smokers. The important correlation found between urinary cotinine and plasma uric acid in smokers was not surprising because the urinary cotinine and plasma SCN^- levels were determined as a marker of tobacco smoke exposure [27].

In our study, plasma antioxidant levels were closely, but inversely related to the levels of plasma nicotine metabolites. It can be explained that more regular cigarette smoking will markedly affect plasma nicotine metabolites, and thus decrease plasma antioxidant levels. Furthermore, our finding suggests that plasma nicotine metabolites are appropriate as biomarkers for smoking consumption. These biomarkers should be applied use in future studies on cigarette smoking.

Analysis of TBARS in plasma is a widely used method for the evaluation of lipid peroxidation. The simplest and most frequently used assay for lipid oxidation is the thiobarbituric acid or TBA assay. Usually under strong acidic condition and heating, biological samples are reacted with TBA leading to the formation of pink- colored products which can be measured by colorimetric or fluorometric methods [42]. A frequent misconception among researchers unfamiliar with the field is that what is actually measured by the TBA assay is in fact exclusively MDA derived from lipid oxidation. However, it is well known that several TBA-derivatized substances that are unrelated to lipid oxidation may be formed during sample preparation and contributes to the overall absorbance or fluorescence.

Moreover, genuine MDA and MDA-like substances may also be formed during the assay which is performed in boiling sulphuric acid [42]. Consequently, measured absorbance or fluorescence does therefore not correspond to the concentration of MDA in vivo but rather to a range of products appropriately termed thiobarbituric acid reactive substances or TBARS. One major problem with the TBA assay is that it is performed in numerous variations making comparison of results between different laboratories extremely difficult.

We found significant increase in MDA in smokers compared to non smokers. In smokers, we found a positive significant correlation between MDA and number of cigarette smoked/day. MDA levels were elevated in subjects who were smoking for more than 10 years. Moreover, both cigarettes smoked/day and consumption duration were significantly associated with these perturbations particularly with higher MDA levels. Cigarette smoke may be expected to induce peroxidation of cellular membrane lipids. Cigarette smoke contains numerous precursors in the tar and gas phases, which were converted to electrophilic compounds during burning, and/or during biotransformation in the body. These reactive electrophiles cause lipid peroxidation by abstracting a proton from the methylene bridge adjacent to double bonds of fatty acids. After a series of reactions, MDA is formed as a reactive aldehyde among other degradation products [42]. These findings, although supporting that MDA is a weak biomarker for individual exposure, may also indicate that the recorded number of cigarettes smoked by an individual may be a poor estimate for the actual exposure to the smoke toxins. Smokers are often exposed for longer periods to cigarette smoke from other smokers than are nonsmokers. Also, some smokers do not inhale the smoke from their own cigarettes. These factors may affect the relation between MDA and the exposure indicators. On a group basis, however, our finding of a significantly increased MDA in smokers is supported by the findings of Kalra et al [43]. Cigarette smoke is known to

increase production of oxygen free radicals by polymorphonuclear leukocytes, and to decrease activities of some free radical scavengers. Nicotine, a major toxic component of cigarette smoke, is a well established procarcinogen [44]. However, it has been reported that nicotine disrupts the mitochondrial respiratory chain leading to an increased generation of superoxide anion and hydrogen peroxide [44]. This may lead to oxidative damaged macromolecules including lipid, DNA, RNA, antioxidant enzyme in subsequent cells through disruption of cellular functions and integrity. Some studies have reported that smokers have poorer dietary habits and consume significantly less ascorbic acid than non-smokers [45] which may be the reason for an increased risk of cancer and cardiovascular diseases. The significant differences in food intake reported here between smokers and non-smokers have also been shown in other communities, where smokers have a higher intake of non-vegetarian food items and a lower intake of fruits, vegetables and milk products [45]. According to the hypothesis proposed by Whichelow et al [46] and Grunberg et al [47], smokers may find sweet foods, such as fruit juice, less palatable than fried foods which were found to be the case in the present study. This may be due to the effect of smoking (via nicotine) on their sense of taste [48]. In our study, the extent of lipid peroxidation was found to be higher in smokers than in non-smokers, as shown by the significantly higher levels of MDA. Circulating erythrocytes are particularly susceptible to oxidative damage as they are exposed to a high partial pressure of oxygen, have membranes rich in polyunsaturated fatty acids and contain large amounts of iron that can potentiate a free radical reaction.

In smokers, MDA levels were significantly correlated PON1 activity, and with tHcys concentration, albumin and bilirubin levels. During the auto oxidation of homocysteine in plasma, reactive oxygen species are generated. The latter initiates lipid peroxidation in cell membranes (potentially responsible for endothelial dysfunction) and in circulating lipoprotein, oxidized LDLC may trigger platelet activation as well as some of the homeostatic abnormalities reported in such patients. Thus, the oxidative stress induced by homocysteine may be a key process in the pathogenesis of thrombosis in hyperhomocysteinemia. These results confirm the correlation between homocysteinemia and MDA levels in smokers. In a smutch as increased MDA level, a well-known lipid peroxidation marker, and reduced serum PON1 activity reflect increased oxidative damage in smokers [49].

In this study, we have shown that plasma total bilirubin concentrations are inversely related to cigarette smoking. This inverse association was found to occur in individuals without cardiovascular disease as well as in individuals with minimal cardiovascular disease and severe cardiovascular disease. These findings have been confirmed in studies of subjects with early familial coronary artery disease [50].

Even though the antioxidant role of bilirubin has been known for over a century, its in vitro antioxidant properties have been more identified since 1987 [51]. A number of recent in vitro studies have shown that bilirubin is more efficient than α-tocopherol at inhibiting LDL oxidation and is a more efficient protector of human ventricular monocytes than either vitamin C or vitamin E [52]. Considerable interest has recently been focused on LDL oxidation since it is believed to be an early event in atherogenesis and because cigarette smoking appears to cause an increase in LDL oxidation [53]. Studies need to be performed to determine if low serum bilirubin concentrations are associated with increases in oxidized LDL and if antioxidants can prevent or minimize LDL oxidation caused by cigarette smoking. Simple and reliable indices of oxidant injury, including those produced by smoking, need to

be developed and validated. A number of methods are currently being used and are based on measurement of malondialdehyde, conjugated dienes, prostanes, or lipid peroxides [54]. Other methods involve measurement of vitamin C and E by high performance liquid chromatography or gas chromatography. Reagents and equipment for measuring plasma bilirubin concentrations, by contrast, are available in most clinics and hospitals worldwide and the costs per test are very low compared to most of the above mentioned tests. Also, reagent and laboratory standardization procedures have been established to insure accurate quantification of plasma bilirubin concentrations.

In this study, the effects of smoking on plasma bilirubin concentrations were studied and plasma bilirubin was found to be lower in individuals who smoke than in those who do not. We noted a significant association between smoking status and hypobilirubinemia in the two situations. These studies confirm the in vitro studies showing that cigarette smoke decreases serum bilirubin concentrations [53]. Further studies will have to be performed to determine if other antioxidants are also decreased with cigarette smoking, if serum bilirubin is an effective measure of oxidative stress and if it can be used to monitor antioxidant therapy. Chronic smoking may lead to a deficiency of both bilirubin and other antioxidants and these deficiencies could lead to oxidant injury, higher concentrations of oxidized LDL, increased plaque formation and increases in DNA oxidation productions. We have shown that serum bilirubin, which is an endogenous antioxidant, is decreased in individuals with coronary artery disease. Even though cigarette smoke has been shown to cause decreases in serum bilirubin concentrations in vitro [55], the association between cigarette smoking and serum bilirubin concentrations has been limited to studies of serum bilirubin as a risk factor for coronary artery disease [50]. In both of those studies, the low serum bilirubin concentrations were primarily examined to determine if it was independent of smoking as a risk factor for coronary artery disease.

In smokers, we found a significant decrease in albumin compared to non-smokers and a negative significant correlation between albumin and number of cigarette smoked/day. After adjustment for potentials confounder factors such as lipid profile, BMI, age and gender, we noted a significant association between smoking status and lower albumin levels.

The observation that the smokers in this study had lower plasma albumin concentrations than non-smokers also suggests the induction of an acute-phase response to smoking, since albumin is a negative acute-phase protein. Cigarette smoking is strongly associated with low serum albumin levels in this and other studies. Lower plasma albumin concentrations in smokers agrees with certain [51], but not all [52], epidemiological findings, and in the present study, does not appear to arise from an alteration in the rate of albumin synthesis. It is probable that the mechanism responsible is an accelerated trans-capillary loss of albumin into the extra-vascular space [53]. Moreover, there may be dietary effects on albumin synthesis, since smokers have been shown to consume less protein and more energy than non-smokers [54]. A wide range of biologic mechanisms have been put forward to account for the association between serum albumin and cardiovascular disease and all-cause mortality. Serum albumin is a negative acute-phase reactant synthesized in the liver, and in inflammatory states, hepatic synthesis is switched from serum albumin to other acute phase proteins [55]. The chronic inflammatory nature of atherosclerosis is well established [55], and it has been suggested that the reduced serum albumin level may be an indicator of this vascular response. As smoking aggravates atherosclerosis, it is hypothesized that smokers with the lowest levels of serum albumin are those whose inflammatory vascular response is greatest [55]. The

observation that lowered serum albumin is associated with cardiovascular disease and all-cause mortality in smokers and ex-smokers but not in never smokers, suggests that the association might merely be an indicator of the inflammatory process (atherosclerosis). In smokers, you found a negative correlation between tHcy and albumin concentrations ($r = -0.3957$). This correlation can be explained by another aspect of antioxidant activity of albumin from its capacity to bind homocysteine. Elevated plasma homocysteine is a well-known risk factor for atherosclerosis and may act through oxidation of LDL [12].

Urinary cotinine and plasma SCN^- concentration were both significantly higher in self smokers than in nonsmokers and correlated well with the number of cigarettes smoked per day and with the duration of consumption. Cotinine in body fluids is the most frequently used biomarker of tobacco smoke exposure [56, 57]. Cotinine has been shown to be the most specific and most sensitive marker; however, the urinary cotinine concentration is regarded as the best biomarker available for detection of exposure to tobacco smoke and for discriminating active smokers from nonsmokers. A mean of 70 – 80% of nicotine is converted to cotinine, which has a half- life of about 17 hours [56]. We noted that the urinary cotinine level was significantly correlated with the number of cigarettes smoked per day. However, cotinine is no longer considered the major metabolite of nicotine; which probably explains why the urinary cotinine level is only roughly related to daily cigarette consumption, because the correlation of urinary cotinine with the number of cigarettes smoked per day is related to that observed in serum or plasma specimens [41]. In this study, we found a significant correlation between mean urinary cotinine levels and duration of consumption. This correlation can be explained by the long half-life of cotinine, which is eliminated from the body after a few days and is mainly excreted in the urine. Moreover, smoking induces changes in nicotine disposition: the rate of cotinine disappearance from the urine is significantly slower in smokers than in nonsmokers [41]. Therefore the determination of this marker in urine is a good alternative to discriminate smokers from nonsmokers. We found a significant correlation between plasma total homocysteine level and urinary cotinine concentration in the whole group of active smokers, this important was not surprising, because urinary cotinine levels were determined as a marker of tobacco smoke exposure [27]. There was a negative correlation between plasma thiocyanate and plasma vitamin B12 concentration. In smokers alone, this latter correlation was more pronounced, and the tendency for high plasma thiocyanate levels to be associated with relatively low plasma vitamin B12 concentrations was striking. Summarized, the results show that urine excretion of B12 is raised in smokers, and that a high of plasma thiocyanate tends to be associated with an increase in vitamin B12 excretion and a relatively low plasma vitamin B12 concentration. The association between high plasma thiocyanate levels and low plasma B12 concentration, which is especially marked in smokers, recalls that between high plasma cyanide concentration and low plasma vitamin B12 [20]; these two observations are probably related. More than one hypothesis might be put forward to explain these results. Thus, it could be postulated that subjects with relatively low plasma vitamin B12 concentrations have a reduced ability to detoxicate cyanide by vitamin B12 pathway, so that detoxication by the thiocyanate pathway is increased. This would not, however, readily explain the association between high plasma thiocyanate and high excretion of vitamin B12. Alternatively, the low plasma vitamin B12 concentration might reflect vitamin B12 depletion, possibly resulting from conversion of tissue cobalamins to cyanocobalamin, a form relatively readily excreted by the kidney [58]. However, the increment in vitamin B12 excretion associated with

smoking is so small in relation to the amount probably absorbed daily and to the liver stores that it would seem unlikely that appreciable depletion could be caused in healthy people by this means. This consideration, together with the very poor correlation between plasma vitamin B12 and urine B12 excretion, suggests that some factors, other than increased renal excretion of vitamin B12, must operate to produce the relation between high plasma thiocyanate and low plasma vitamin B12 concentration. It is possible that high plasma cyanide concentrations disturb the equilibrium between plasma and urine vitamin B12. At the moment, the main significance of this work is that it shows further definite, if unexplained, interrelationships between smoking, cyanide metabolism, and bodily handling of vitamin B12, and gives further support to the idea that high loads of cyanide might produce derangements of vitamin B12 metabolism. The effects of smoking are slight in healthy subjects, but in patients already in marginal vitamin B12 balance they might become significant.

Conclusion

Cigarette smoking is one of the most important exogenous factors, which cause 3-fold higher incidence of oxidative stress in smokers. Free radical-mediated oxidative stress appears to play a central role in cigarette smoking-mediated atherothrombotic diseases. The results of the present study clearly show that cigarette smoking induces an oxidative stress in smoking by augmenting lipid peroxidation and diminishing both enzymatic and non enzymatic antioxidant status. The above findings also support the hypothesis that the atherogenic effects of smoking are mediated in part by free radical damage to lipids. The low antioxidant status of smokers may predispose them to oxidant and cytokine inflicted tissue damage and disease, which may manifest itself as coronary heart disease, atherosclerosis and cancer.

In light of these findings it is concluded that smokers are more susceptible to oxidant stress as a consequence of insufficient antioxidant potential and greater oxidative burden. The consumption of antioxidant foods should be recommended to smokers in order to compensate for higher oxidant load. Additionally, they must be encouraged to stop smoking. In particular, young smokers should quit promptly before health problems arise, so as to have the optimal benefits of cessation.

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Chapter 11

Causality of Cigarette Smoking among Young Men in Taiwan

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Abstract

A cross-sectional survey was conducted among young adult military conscripts in the southern and eastern regions of Taiwan from August 1 to December 31, 2001. A total of 3,617 young adult conscripts (19–25 years old) who had served more than 1 month were included in this study. Forty-eight subjects with incomplete or missing data were excluded from the final analysis. Informed consent was obtained from the participants before survey. From this study, we found that education level, betel-nut chewing, alcohol intake, smoking of peers, and the attitudes of parents and peers toward smoking are all associated with the risk of a young adult conscript becoming a habitual cigarette smoker. Subjects with more education may have more cultural, intellectual, socioeconomic, and psychosocial resources to help them face adverse. The cross-sectional survey design limits exploration of the causal relationship between lifestyle factors, attitude of peers and adverse behaviors among young adults.

Introduction

Substantial evidence has shown that cigarette smoking is a leading cause of morbidity and mortality in chronic diseases such as cardiovascular disease, cancer and chronic

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obstructive pulmonary disease [1, 2]. In our previous paper, we reported that the prevalence of cigarette smoking is slightly elevated during military service and is even higher among young conscripts when compared with the general population [3]. In Taiwan, almost all young male adults are required to serve in the armed forces. Therefore, preventing smoking among young adult conscripts is an important national health issue.

Many general characteristics such as age, education level, area of residence, time served in the military, and parental education level are potential risk factors associated with cigarette smoking in adolescents and young adults. In a military study, age difference was a contributing factor in explaining cigarette smoking among young conscripts [4]. Furthermore, in Europe, national health data showed that smoking was more prevalent among less educated people than among more educated people [5, 6]. This agrees with many studies that show the trends in cigarette smoking may be associated with education level [7, 8]. In addition, a subject's area of residence, service period, and parents' education levels are also associated with cigarette smoking [9-13].

The use of alcohol, betel-nut chewing, and cigarettes contribute to substantial health risks and are often used concurrently among adults. Ko et al [14] found that concurrent smoking and drinking habits were closely related to betel-nut chewing preferences. Similarly, Wen et al [15] demonstrated a relationship between betel-quid chewing and cigarette smoking, and the 2 were associated with a combined effect that may lead to serious health consequences. Recently, Weitzman and Chen [16] found that over 98% of current smokers also drink alcohol, and smoking and drinking are powerfully interrelated. Many studies have reported the concurrence of smoking and drinking among adults [17].

The prevalence of smoking was found to be higher in subjects who were raised in environments in which there were many smokers, particularly when parents and peers were smokers [18-22]. In addition, young adults were more likely to smoke when their parents or peers expressed positive attitudes toward smoking [23].

There is growing concern that during military service, young conscripts might develop a habit of cigarette smoking. Adequate understanding of the risk factors associated with smoking will not only lead to understanding the total burden to society, but is also useful in the development of effective prevention strategies. The purpose of this study was to identify the most important risk factors that influence smoking among military conscripts in Taiwan.

Methods

Study Sample and Study Design

A cross-sectional survey was conducted among military conscripts in the southern and eastern regions of Taiwan from August 1 to December 31, 2001. A total of 3,617 young adult conscripts who had served more than 1 month were included in this study. Forty-eight subjects with incomplete or missing data were excluded from the final analysis. Informed consent was obtained from the participants before survey.

Table 11.1. General characteristics and cigarette smoking status of 3,569 young military conscripts in Taiwan*

	Nonsmoker (n = 1,715)	Past smoker (n = 24)	Current smoker (n = 1,830)	χ^2 test
Age (yr)				211.3†
≤ 20	406 (37.0)	8 (0.7)	685 (62.3)	
21	566 (43.0)	10 (0.8)	739 (56.2)	
22	259 (56.8)	2 (0.4)	195 (42.8)	
23	199 (65.2)	2 (0.7)	104 (34.1)	
≥ 24	285 (72.3)	2 (0.5)	107 (27.2)	
Education level				501.7†
≤ Junior high school	124 (19.1)	4 (0.6)	523 (80.3)	
Senior high school	898 (45.2)	16 (0.8)	1,072 (54.0)	
College	355 (68.7)	2 (0.4)	160 (30.9)	
≥ University	338 (81.4)	2 (0.5)	75 (18.1)	
Region of residence in Taiwan				12.9†
Northern	304 (54.5)	4 (0.7)	250 (44.8)	
Middle	204 (47.7)	3 (0.7)	221 (51.6)	
Southern	1,130 (47.0)	15 (0.6)	1,258 (52.4)	
Eastern	77 (42.8)	2 (1.1)	101 (56.1)	
Time served in military (mo)				24.1†
1–6	264 (43.2)	6 (1.0)	341 (55.8)	
7–12	599 (49.2)	7 (0.6)	612 (50.2)	
13–18	534 (53.1)	5 (0.5)	467 (46.4)	
> 18	318 (43.3)	6 (0.8)	410 (55.9)	
Betel-nut chewing status				690.1†
No	1,688 (58.9)	20 (0.7)	1,159 (40.4)	
Yes	27 (3.8)	4 (0.6)	671 (95.6)	
Alcohol drinking status				272.6†
No	1,643 (53.6)	21 (0.7)	1,399 (45.7)	
Yes	72 (14.2)	3 (0.6)	431 (85.2)	

* Data presented as n (%); † $p < 0.05$.

Data Collection and Measurement

All participants completed a structured questionnaire concerning sociodemographics, lifestyle, and the attitudes and behavior of family members and peers. The complete list of questions is shown in Tables 1 and 2. The questionnaire used in this study had been tested by 68 military conscripts before survey. The validity and consistency of the questionnaire were acceptable to measure the habit of smoking among these subjects. The content validity of our questionnaire about the attitude of smoking was 0.96, split-half reliability was 0.75, and Cronbach's alpha was 0.8.

With regard to sociodemographics, we divided the education level of the military conscripts into "junior high school or below", "senior high school", "college", and "university or above" on the questionnaire. For parents, due to the likelihood of a lower education level

for that generation, we added "elementary school". We defined smoking based on a modification of the World Health Organization questionnaire [24]. A current smoker was defined as a subject who smoked ≥ 1 cigarette/day during the past 30 days and had smoked > 100 cigarettes in their lifetime, or who considered himself a current habitual smoker. A past smoker was defined as a subject who had not smoked cigarettes during the past 30 days, but had smoked > 100 cigarettes in their lifetime, or who did not consider himself a current habitual smoker. A nonsmoker was defined as a subject who had not smoked cigarettes during the past 30 days and had not smoked > 100 cigarettes in his lifetime, or who considered himself a nonsmoker.

We defined drinking based on drinking frequency, alcohol concentration, or a history of habitual drinking. A current drinker was defined as a subject who consumed ≥ 2 drinks/week of liquor (or equal alcohol concentration/week) in their lifetime or who was a habitual drinker before or during military service [25].

We also defined habitual betel-nut chewing based on chewing frequency and history. A current betel-nut chewer was defined as a subject who had chewed ≥ 1 betel nut during the past 30 days, had chewed ≥ 1 betel nut/week, and had chewed > 50 betel nuts in their lifetime before or during military service [25].

Statistical Analysis

We conducted χ^2 tests for each characteristic (e.g. age, education level, etc.) to evaluate the impact of each factor on cigarette smoking status (i.e. nonsmoker, past smoker, current smoker). We used multivariate logistic regression analyses to assess which factors could best predict cigarette smoking behavior among young adults in Taiwan. A 2-tailed p value < 0.05 was considered statistically significant. All statistical analyses were conducted using the SAS statistical package (SAS Institute Inc., Cary, NC, USA).

Results

The general characteristics and cigarette smoking status of the 3,569 subjects are presented in Table 11.1. All subjects were male, with a mean age of 22 ± 2 years. Overall, the prevalence of current cigarette smokers was 51.3% among young adults in Taiwan. Smoking was significantly associated with age, education level, region of residence, and time served in the military (all $p < 0.05$). The highest prevalence of cigarette smoking was observed among the youngest subjects with the lowest educational levels. The prevalence of current cigarette smokers went from 62.3% to 27.2% as age increased from ≤ 20 to ≥ 24 years old. More dramatically, the prevalence of current smokers was 80.3% among subjects with an education level ≤ junior high school, while it was only 18.1% among subjects with a university degree. A somewhat higher prevalence of cigarette smokers was observed among residents of eastern Taiwan compared to residents in other regions. The prevalence of current smokers was reduced among subjects who had served 18 months compared to those who had served ≤ 6 months in the military. However, among subjects who had served > 18 months, smoking prevalence was similar to that observed among subjects who had served ≤ 6 months. The

adverse behavior of cigarette smoking was significantly correlated with alcohol drinking and betel-nut chewing (all $p < 0.05$).

Table 11.2. Factors associated with cigarette smoking among 3,569 young military conscripts in Taiwan*

	Nonsmoker (n = 1,715)	Past smoker (n = 24)	Current smoker (n = 1,830)	χ^2 test
Father's education level				35.9†
≤ Elementary school	692 (44.5)	13 (0.8)	851 (54.7)	
Junior high school	393 (46.0)	4 (0.5)	457 (53.5)	
Senior high school	427 (51.9)	5 (0.6)	391 (47.5)	
≥ College or above	203 (60.4)	2 (0.6)	131 (39.0)	
Mother's education level				8.2
≤ Elementary school	882 (47.0)	12 (0.7)	981 (52.3)	
Junior high school	412 (46.7)	7 (0.8)	463 (52.5)	
Senior high school	340 (50.7)	4 (0.6)	326 (48.6)	
≥ College or above	81 (57.0)	1 (0.7)	60 (42.3)	
Father's smoking status				60.4†
No	784 (56.0)	5 (0.4)	610 (43.6)	
Yes	931 (42.9)	19 (0.9)	1,220 (56.2)	
Mother's smoking status				12.4†
No	1,624 (48.8)	23 (0.7)	1,679 (50.5)	
Yes	91 (37.5)	1 (0.4)	151 (62.1)	
Father's attitude towards son's smoking				360.2†
Does not approve	1,133 (62.9)	10 (0.6)	657 (36.5)	
Approve	32 (14.1)	3 (1.3)	192 (84.6)	
No comment	550 (35.7)	11 (0.7)	981 (63.6)	
Mother's attitude towards son's smoking				246.7†
Does not approve	1,393 (56.2)	17 (0.7)	1,067 (43.1)	
Approve	10 (7.7)	1 (0.8)	118 (91.5)	
No comment	312 (32.4)	6 (0.6)	645 (67.0)	
Percentage of peers who smoke				317.4†
Less than half	427 (79.6)	3 (0.6)	106 (19.8)	
About half	470 (53.8)	7 (0.8)	397 (45.4)	
More than half	818 (37.9)	14 (0.6)	1,327 (61.5)	
Peer attitudes toward subjects' smoking				252.4†
Do not approve	556 (71.3)	8 (1.0)	216 (27.7)	
Approve	103 (28.2)	2 (0.6)	260 (71.2)	
No comment	1,056 (43.6)	14 (0.6)	1,354 (55.8)	

* Data presented as n (%); † $p < 0.05$.

Table 11.3. Factors associated with cigarette smoking among 3,569 young military conscripts in Taiwan

Independent variables	OR	95% CI
Education level		
≤ Junior high school	5.36	3.773–7.69
Senior high school	2.66	2.002–3.58
College	1.63	1.177–2.30
≥ University	1.00	
Betel-nut chewing status		
No	1.00	
Yes	16.81	11.355–25.91
Alcohol drinking status		
No	1.00	
Yes	2.11	1.548–2.90
Father's attitude towards son's smoking		
Does not approve	1.00	
Approve	3.28	2.022–5.43
No comment	1.96	1.594–2.41
Mother's attitude towards son's smoking		
Does not approve	1.00	
Approve	3.11	1.477–7.12
No comment	0.99	0.799–1.24
Percentage of peers who smoke		
Less than half	1.00	
About half	2.43	1.822–3.26
More than half	3.16	2.422–4.15
Peer attitudes toward subjects' smoking		
Does not approve	1.00	
Approve	2.27	1.600–3.22
No comment	1.94	1.558–2.42

OR = odds ratio; CI = confidence interval.

The characteristics of the subjects' parents and peers are presented in Table 11.2. Smoking was significantly associated with only the father's education level, the smoking habits of the parents and peers, and the attitudes of parents and peers toward smoking (all $p < 0.05$). Smoking prevalence was highest among subjects whose parents and peers approved of smoking. Prevalence of 84.6%, 91.5%, and 71.2% were observed among subjects whose fathers, mothers, and peers approved of smoking, respectively. Prevalence of smoking was also high among subjects whose parents and peers were current smokers. Prevalence of 56.2%, 62.1%, and 61.5% were observed among subjects whose fathers, mothers, and > 50% of peers smoked, respectively. Smoking prevalence was influenced by the relative education levels of the father and mother to a similar extent. Smoking prevalence was 54.7% among subjects whose fathers had the least education, and only 39.0% among subjects whose fathers had the most education.

Multivariate logistic regression indicated that the factors most significantly associated with smoking behavior of young adults were: education level, betel-nut chewing, alcohol

drinking, parents' attitude toward smoking, proportion of peers who currently smoked, and peer attitude toward smoking (Table 11.3). Based on the odds ratios (OR), subjects who chewed betel nuts had the highest probability of cigarette smoking (OR, 16.81; 95% confidence interval [CI], 11.35– 25.91). Subjects with an education level ≤ junior high school had the second highest probability of cigarette smoking (OR, 5.36; 95% CI, 3.77–7.69). Subjects whose parents approved of smoking had the next highest probability of smoking (father's approval—OR, 3.28 and 95% CI, 2.02–5.43; mother's approval—OR, 3.11 and 95% CI, 1.47–7.12), and a similar probability was observed when > 50% of the subjects' peers were current smokers (OR, 3.16; 95% CI, 2.42–4.15). Finally, subjects with a drinking habit were as likely to smoke as subjects whose peers approved of smoking (drinking—OR, 2.11 and 95% CI, 1.54–2.90; peer approval—OR, 2.27 and 95% CI, 1.60–3.22).

Discussion

In this cross-sectional study, we found that the prevalence of smoking among young military adults was significantly associated with education level, betel-nut chewing, alcohol drinking, parental and peer attitudes toward smoking, and the proportion of peers who smoke. Further, after adjusting for potential confounding factors, we found that age, region of residence, period of service in the military, and parents' education levels were not significantly associated with cigarette smoking in this population.

We found that education was a strong predictor of habitual cigarette smoking among young adult conscripts. A person's education level may reflect their capacity to take in new information and to act on it [6, 13, 26, 27]. In addition, subjects with more education may have more cultural, intellectual, socioeconomic, and psychosocial resources to help them face adverse personal circumstances in a healthy way compared to those with less education. We found that lifestyle habits such as alcohol drinking and betel-nut chewing were also associated with cigarette smoking even after controlling for potential confounding factors. The betel-nut is popular in certain Asian countries and it is predominantly used by men.28 Males chew betel nut to project a "macho" image, and it is often used on social occasions [14, 29]. Our results are consistent with previous reports that betel-nut chewers were more likely to have habits like cigarette smoking or drinking of alcoholic beverages [14],and that alcohol drinking, betel-nut chewing, and cigarette smoking are likely to cluster together in adult subjects [16, 17, 30].

Our results suggest that parents who approve of smoking are more likely to have children who smoke as young adults. This is consistent with the results of Shakib et al, [18] who also identified parental approval of smoking as one of the most important determinants of adolescent smoking. In the Chinese culture, children are taught to take heed of their parents and elders and to act according to their guidance without objection [31]. Therefore, parents' attitudes toward smoking may have direct effects on a subject's smoking habits, and may be a good target in a smoking prevention program. Further, our models suggest no significant relationship between the parents' and subject's smoking habits. This is in contrast to some studies which found that young adults with parents who smoke are more likely to become smokers [18, 19]. Our results suggest that the smoking status of adult military conscripts might be related to their peers' smoking status and attitudes toward subjects' smoking,

because they live in the military base most of their time, not with the family, which could explain these findings.

Our findings show that young adults are more likely to smoke if their friends smoke or express approval of smoking. This result agrees with those of Unger et al, [32] who showed that both perceived access and peer influences are significant risk factors for habitual smoking. Other studies also identified peer influence as one of the determinants of smoking in young adults [20-22]. Peers often mimic and act as reference groups in support of opinions, attitudes, and practices of adverse behaviors. Young adults commonly start smoking in order to identify with friends. Flay et al [20] clearly showed that friends who smoke have both direct and indirect influences on initiation of smoking. This evidence suggests that peers can be used as an important resource to help young adults in a smoking cessation program.

Our study has several limitations that should be noted. First, the information we collected on smoking habits was based on a self-report structured questionnaire, and misclassifications may have occurred if underreporting of smoking was systematic; for example, underreporting may be linked to socio-characteristic status. However, underreporting associated with socio-characteristic status has previously been shown to have little or no effect [33, 34]. Thus, we assume that any misclassifications are likely to be minimal and random and would only attenuate our results. Second, this study examined the relationship between subjects' reported smoking status and their perceptions of smoking among parents and friends. However, we did not actually collect data from their parents and friends. The perception of smoking among friends may be more closely related to a subject's own smoking habits than to the actual number of friends who smoke [35]. Finally, the cross sectional survey design limits exploration of the causal relationship between lifestyle factors, attitude of peers and adverse behaviors among young adults. Previous evidence has indicated that affiliation with friends who smoke leads to smoking behavior, but studies have also shown that adolescents who smoke tend to seek out friends who are also smoking [21]. Further studies are necessary to examine peer influence more closely.

In conclusion, this study has identified the most effective ways to approach individuals at high risk for cigarette smoking and to develop population-based multifactorial interventions to help young adult conscripts control or quit smoking in the future. We should also propose more anti-smoking programs and a cigarette smoking-free environment to the Department of Defense.

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Chapter 12

Review **– Bacteria, Mold and Microbial Toxinsof Cigarettes**

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Abstract

Prior assessments of the harmful effects of cigarette smoke have focused on the chemicals in mainstream and sidestream smoke that arise from the burning of the tobacco. The criterion for harm has been derived primarily from registries of carcinogens including those of the US National Toxicology Program and the International Agency for Research on Cancer. Overlooked in these assessments has been the propensity for harm from microbes and microbial-derived biological elements in tobacco. Various microorganisms and microbial-derived toxins are known to exist in: (i) different types of fermented tobacco (Virginia bright, burley and Oriental tobacco); (ii) tobacco that has been processed using different methods (air- or flue-cured); (iii) tobacco imported from different countries, some of which have few or no laws regulating the use of anti-microbial agents; and (iv) tobacco used currently in smoking and smokeless tobacco products that are being currently marketed. Examples of the referenced microbial elements include: (i) different microbes (bacteria, fungi and spores); (ii) microbial toxins (endotoxins, exotoxins and mycotoxins); and (iii) a large and heterogeneous group of bacterial and fungal molecules that induce inflammation. Harm may arise from the (a) microbe-mediated formation of nicotine-derived carcinogens (tobacco-specific N-nitrosamines; TSNA), (b) elaboration of inflammation-inducing factors (lipopolysaccharide, LPS); (c) colonization in the lungs of long-term smokers with impaired immunity (chronic obstructive pulmonary disease; COPD); and (d) induction of microbial antigen-evoked immune responses. Also discussed herein are US patents that have been awarded to inventors who have discovered novel schemes to prevent or reduce

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the propensity for harm associated with microorganisms in cigarettes and oral tobacco products.

Abbreviations

AFL-B1	aflatoxin-B1
AP-1	activator protein 1
ATSDR	Agency for Toxic Substances Disease Registry
CA EPA	California Environmental Protection Agency
COPD	chronic obstructive pulmonary disease
DC	dendritic cell
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
HPHC	harmful and potentially harmful components
IARC	International Agency for Research on Cancer
IL-1β	interleukin-1 beta
IκB	kinase of the IκK complex
IκK	signal transduction protein that regulates inflammation
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MD-2	protein associated with TLR4
MNR	microbial nitrate reductase
Mon	monocyte
MyD88	myeloid differentiation primary response gene 88
MΦ	macrophage
NF-κB	nuclear factor-kappa B
NNK	nitrosamines; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butone
NTP	National Toxicology Program
OSM	oncostatin M
PAH	polycyclic aromatic hydrocarbons
PMN	polymorphonuclear leukocyte
PREPS	potential reduced exposure products
ROS/RNS	reactive oxygen species/reactive nitrogen species
TCA	Tobacco Control Act
TLR	Toll-like receptors
TNFα	tumor necrosis factor-alpha
TPSAC	Tobacco Products Scientific Advisory Committee, of the FDA
TRAF6	TNF receptor associated (protein) factor
TRAM	adapter protein, related to TRIF
TRIF	adapter protein
TSNA	tobacco specific nitrosamines
WTPM	Wet total particulate matter

1. Introduction

FDA List of Harmful Constituents of Tobacco Smoke

The most recent listing of "harmful and potentially harmful" constituents (HPHC) in tobacco products and tobacco smoke is one that was published on 12 August 2011 in the *Federal Registry* by The Food and Drug Administration (FDA) [1]; updated on 06/01/2012. The FDA listing of HPHC originates from the Tobacco Control Act (TCA) [2]. The 2009 TCA "requires the FDA to establish and periodically revise as appropriate, a list of harmful and potentially harmful constituents (HPHC) including smoke constituents, to health in each tobacco product by brand and by quantity in each brand and subbrand" [1, 2].

Agents in the FDA's HPHC list were assembled from different registries. The organizations that were identified by the FDA included the U.S. Environmental Protection Agency (EPA); International Agency for Research on Cancer (IARC); US National Toxicology Program (NTP); the Agency for Toxic Substances Disease Registry (ATSDR); and the California Environmental Protection Agency (CA EPA). The FDA has also reviewed lists of HPHC established by various organizations, and from other countries [1].

The FDA HPHC list tabulates, in alphabetical order, 97 chemical constituents. Each of the HPHC entities is identified as a carcinogen, respiratory toxicant, cardiovascular toxicant, reproductive or developmental toxicant, and/or addictive substance [1]. Of these chemicals, 84.5% (N=82/97) are known carcinogens. The largest group of chemicals is that of polycyclic aromatic hydrocarbons (PAH) [N=16/97; 16.5%]; all of the listed PAH are carcinogens. The second group of chemicals is that of nitrosamines (NNK) [N = 9/97; 9.3%]; all of the listed NNK are carcinogens. Several metals/metalloids are present, including arsenic, cadmium, cobalt, lead, mercury, and nickel; all of the listed metals are carcinogens [1].

Fifteen of the 97 agents (15.5%) are non-carcinogens. Toxicants in this non-carcinogen class included carbon monoxide, hydrogen cyanide, methyl ethyl ketone, nicotine, phenol, and toluene [1].

The FDA acknowledges that in preparing the HPHC list they have focused on five disease outcomes: (1) cancer, (2) cardiovascular disease, (3) respiratory effects, (4) developmental or reproductive effects and (5) addiction [1].

Certain shortcomings have also been acknowledged by the FDA. The criteria that the FDA has "tentatively selected are limited to those that relate to carcinogens, toxicants, and addictive chemicals or chemical compounds in tobacco products and tobacco smoke." Thus, the Year 2011 listing may not include all substances that are harmful or potentially harmful. Likewise, as additional information is obtained, other criteria may be incorporated [1]. In this context, it is notable is that the FDA did not address tobacco-associated chronic inflammation. There is a consensus of opinion that that chronic inflammation is commonly associated with the use of smoking tobacco and smokeless tobacco. The prevailing wisdom of the medical and scientific communities is that chronic inflammation is a primary contributor to the cellular and molecular mechanisms that proceed, and subsequently, culminate in tobacco-associated malignant and non-neoplastic diseases of the lung and airways, as well as diverse cardiovascular maladies.

It is to be emphasized that the FDA list is not novel. The FDA list has been preceded by other tabulations and writings of harmful chemicals that have been identified in mainstream

and sidestream smoke of cigarettes. One such list is the widely cited listing known as the "*Hoffmann Analytes*" [1-6]. Other guides have been published, and updated periodically during the last two decades [3, 4, 5, 7-11]. A historical review of this topic was published in 2009 [4].

A comprehensive listing of harmful or potentially harmful tobacco and/or tobacco smoke components has been issued by the FDA's Constituent Subcommittee of the Tobacco Products Scientific Advisory Committee (TPSAC) [3]. A draft list that has been prepared by TPSAC lists 106 components. Of the 106 components, all were defined as "biologically adverse at one time or another over the pervious years by one or more investigators" [3].

In 2011 Alan Rodgman, a chemist who worked for R. J. Reynolds Tobacco Company for many years, published an 18-page critique of the FDA listing. Dr. Rodgman concluded that the TPSAC should "amend the list to reduce the number of problems and anomalies in it…and also convey such amendments to the Food and Drug Administration" [3].

A docket folder has been established by the FDA, and a request for public comments was announced [12]. Today, this docket contains the written communications from different individuals and groups, including representatives of the manufacturers of smoking and smokeless tobacco products.

One challenge to the current FDA initiative has been to define the phrase: "harmful and potentially harmful constituent." The final guidance of the FDA is: "to include any chemical or chemical compound that is or potentially is inhaled, ingested, or absorbed into the body, and causes or has the potential to cause direct or indirect harm to users or non-users of tobacco products" [1].

Also published in 2011 was a list of hazardous tobacco smoke components prepared by a group assembled from four different European countries. A structured search of the literature resulted in a database of 2,256 different tobacco smoke components. From this data base, 98 components were identified, and documentation has been presented for their cancer and non-cancer inhalation risk values [8]. The authors recommend that this listing be used in preference to the Hoffmann analytes for regulatory purposes [8].

Structured Studies of Tobacco Smoke Chemical Toxicants

Structured studies to identify chemical components in cigarette smoke began in the 1950s. By way of example, a paper by Kosak in 1954 presented a listing of chemicals found in tobacco smoke that consisted of fewer than one hundred components [13]. Today, a review published in 2011 [5], and detailed in a book published in 2009 [4], has identified tobacco smoke as having 5,685 different chemicals [4, 5]. It is anticipated that additional components will be identified using new technologies [4, 5].

The chemicals in mainstream and sidestream smoke have been assigned to different classes; a partial listing includes: aldehydes, alcohols and phytosterols, aldehydes and ketones, carbolic acids, esters, lactones, aldehydes, carbohydrates and their derivatives, phenols and quinones, ethers, nitriles, acyclic amines, amides, imides, N-nitrosamines, nitroalkanes, nitroarenes, and nitrophenols, nitrogen heterocyclic compounds, and miscellaneous components [4, 5, 7, 9-11].

To facilitate the analysis of the complex aerosol, the cigarette smoke that emerges from a filtered or non-filtered cigarette (mainstream smoke) is classified into two phases. The two

phases are established by passing whole smoke through a glass-fiber Cambridge filter. The Cambridge filter retains more than 99.9% of the microparticulates (mean particle diameter, ~ 0.3 to 0.5 μm) [4, 7, 9]. Thus, the material captured onto the filter is the submicron particulate ('tar') phase. When the smoke is inhaled, the smoke particles are deposited in the lung, including the most terminal portion – the "deep" lung. The numerous (~ 1.0×10^9 particles/35 cc puff of cigarette smoke) [4, 7, 9] particles are responsible in part for the black appearance of the lung, as viewed in cadavers or as observed in fresh surgically-excised lung tissue.

The portion of the cigarette smoke that passes through the Cambridge filter is the vapor (gas) phase. The major portion of the gas phase is due to water, and components of air (e.g., nitrogen and oxygen) that are drawn into and through the cigarette during smoking [1, 2, 4, 5, 7, 9-11].

The carcinogenic compounds in cigarette smoke may be divided into four types. The first type of carcinogens is the NNK. There is a consensus of opinion that the NNK are most probably the most carcinogenic, and deadly, agents in tobacco smoke. Most probably, they are also the most thoroughly studied tobacco smoke carcinogens. The second type of carcinogen is aldehydes; these are produced by the burning of sugars and cellulose in tobacco. The third type are polycylic aromatic hydrocarbons (PAH's), which form in the cigarette behind the fire line of the burning tobacco. Heavy metals and metalloids in tobacco smoke arise primarily from tobacco plant fertilizers. These form the fourth type of tobacco smoke carcinogens [14; also see 4, 5, 7, 9, 10]. The molecular mechanism by which tobacco smoke causes diseases at different organ sites has been reviewed, and these papers have been published in a NOVA book [15].

Attempts to craft cigarettes that deliver a flavorful and satisfying smoke without these carcinogens have proven difficult. Most efforts have focused on engineering novel filters having absorbents, such as charcoal, to eliminate some of the mainstream smoke components. The complexity and heterogeneity of the numerous toxicants in cigarette smoke has prompted investigators to design potential reduced risk products (PREPs). Some of the PREPS have the appearance of a cigarette; however, they contain little or no natural tobacco, and deliver a nicotine-containing "artificial smoke." Whether some of these devices are less hazardous than a conventional cigarette remains unknown [reviewed in 14, 16].

Aflatoxin B1 – First Listing of a Microbe-derived Carcinogen of Tobacco

The recent HPHC listing of the 97 compounds by the FDA includes aflatoxin B1 (AFL-B_1). The FDA is the first, and only, national or international regulatory agency or authority to list a microbial product as a harmful or potentially harmful constituent of tobacco.

Aflatoxin-producing mold and the chemical identification of AFL-B1 in different foods and livestock feed has been is well established. AFL-B_1 is but one of several aflatoxins that are produced by many species of *Aspergillus* (e.g., *A. flavus*). Moreover, aflatoxins are among the most carcinogenic substances known (NTP and IARC) [1, 4, 5, 7, 9-11].

AFL-B1 producing *Aspergillus* is known to colonize and contaminate tobacco and other crops, (e.g., corn, wheat, rice, peanuts and spices) [reviewed in 4, 11, 17, 18]. Accordingly, the US FDA has established action levels for aflatoxin present in food [17, 18]. Aflatoxins are present also in moldy livestock feed [17, 18]. Thus, the presence of AFL-B1 in moldy tobacco

is not unexpected. In addition to its carcinogenicity, aflatoxins have been associated with a variety of general adverse health effects [reviewed in 17].

AFL-B_1 has been identified in cured tobacco as well as in smoking and smokeless tobacco products that are being marketed [4, 19-22]. The FDA, however, has not established action levels for aflatoxin, or other mycotoxins, present in US or imported tobacco. Various methods, including inventions by the tobacco companies, have been described in awarded US Patents to prevent AFL-B1 in tobacco [21, 22].

Tobacco Microbes and Microbial Toxins – Propensity for Harm

Presented herein is a synopsis that highlights some of the identified tobacco-associated microbial components and their propensity for harm. A number of investigators have reported the presence of microbes and microbial toxins in tobacco products [reviewed in 23]. Some researchers have theorized that microbial constituents may, in and of themselves, be hazardous, produce hazardous toxins, or produce substances that interact with the known chemical constituents of tobacco smoke to augment the potential for harm.

The intent of this Chapter is to identify gaps in our understanding of the harm of cigarette smoke, and to document the need to achieve a scientifically based objective understanding of the potential health risks of currently marketed cigarettes that have microbes or microbial elements. Also, we will address tobacco product stewardship, and the responsibility of the tobacco industry to reduce harmful constituents of cigarette smoke using available, efficient and cheap technologies; US Patents have been awarded for some of these inventions.

Tobacco Microbes, Cigarette Smoke and Carcinogenic Nitrosamines

Tobacco-specific nitrosamines (TSNA) are a group of carcinogens that are present in (a) cured tobacco, (b) smokeless tobacco products, (c) tobacco of smoking products (cigarettes, cigars and pipes), (d) mainstream cigarette smoke, and (e) sidestream cigarette smoke. The products include American- and foreign-made articles [1, 2, 5, 7, 9-11, 24].

TSNA are among the most thoroughly studied of all tobacco-associated carcinogens [10]. Two of the most carcinogenic TSNA are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butone (NNK) and N'nitrosonor-nicotine (NNK) [4, 7, 10, 15, 24, 25]. The formation from nicotine *in vitro* and during tobacco curing and carcinogenicity was described in 1978 [25], more than 35 years ago. Freshly harvested tobacco leaves, often referred to as "green" tobacco, regardless of the tobacco type, is of no value – it is not consumed by humans or livestock, and it is not used in tobacco products [19, 20, 21]. Green tobacco contains no carcinogens. Moreover, at the time of harvest, the green tobacco leaf has relatively few microbes. Of the microbes that are present, most are on the surface of the leaf.

TSNA are not present at trace levels in freshly harvested tobacco. During processing curing and storage, there is a marked increase in the number of fungi and bacteria, and TSNA. Many factors and parameters contribute to the formation of TSNA [4, 7, 9, 10, 16, 20, 21, 24, 25].

A partial listing of factors that affects the growth of microbes includes the country in which the tobacco was grown, tobacco growing conditions in the field, particularly the use of

nitrogen-rich fertilizers. Other factors include the type of tobacco (Virginia flue-cured bright tobacco, burley tobacco or Oriental tobacco). Also known to be important is the tobacco leaf stalk position, tobacco leaf (lamina with and without stems). The growth of bacteria and mold are affected significantly by the harvesting conditions, curing methods (air-cured versus different flue-cured procedures), fermentation practices and storage conditions (temperature, ventilation, humidity, and light) [4, 16, 19, 20].

It is known that the formation of TSNA is dependent primarily on the growth of microbes during tobacco processing, curing, fermentation and storage. Operationally defined, the color-change of tobacco from green to yellow is attributed to drying; this is the initial phase of tobacco curing. Production of an enzyme, microbial nitrate reductase (MNR), from bacteria normally present in tobacco, mediates or facilitates TSNA formation. Green leaf tobacco contains no nitrites. However, MNR converts naturally occurring nitrate to nitrite under anaerobic conditions. It is perceived that during drying, the tobacco cell wall breaks down. This results in the release of nutrients ("sap"-like substance) that promote the rapid growth of microorganisms. The nitrosamines of tobacco are thought to be formed upon the reaction of NO_2, N_2O and N_2O_4, under anaerobic conditions [24, 25].

The nitrosation of nicotine produces the carcinogenic nitrosamines NNN and NNK. The degradation of nicotine and the formation of TSNA have been described [24-27]. Recently, the molecular basis for induction of human cancer by TSNA has been described recently [28].

US Patents have been awarded during the last decade to inventors for technologies to reduce or prevent the growth of tobacco leaf microbes and, thereby, hinder the formation of tobacco-specific carcinogenic nitrosamines in smoking and smokeless tobacco products. By way of example, a partial listing of patents awarded is referenced [29-32]. Lists of prior patents, US and Foreign, and papers in scientific journals are cited in these patents that detail this subject. Collectively, the patents define the current state of knowledge, provide a historical overview of NNK formation, detail the propensity for human risk, and describe innovative, technically simple and inexpensive schemes for preventing and/or eliminating NNK from cigarette tobacco.

For example, schemes described in these patents to reduce TSNA include: (a) maintenance of an aerobic, versus an oxygen-poor anaerobic environment, to hinder the growth of microbes that produce MNR; (b) increased ventilation and air circulation between tobacco leaves; (c) irradiation of harvested green tobacco with microwave, ultraviolet light, and gamma radiation; (d) washing or soaking green tobacco leaves with different chemical solutions (bleach) that are known to kill microbes on tobacco – some of these methods are used for processing vegetables and other foods; (e) treatment of tobacco leaves with antibacterial and antifungal agents (antibiotics); and (f) exposure of the tobacco to different gases [29-32].

In addition to these writings that detail the formation of TSNA in flue-cured tobacco, other papers have described the factors that influence the formation of TSNA in air-cured tobacco [33].

One approach to achieve fermentation of tobacco without producing TSNA is to destroy all of the natural-occurring microbes on the tobacco leaf. Curing and fermentation is then achieved by "inoculating" the tobacco with selected microbes that lack MNR. The role of bacteria in fermenting tobacco for cigarettes was known as early as 1899 [34] – more than 110 years ago. Also remarkable is that a similar scheme to sterilize tobacco, and then

"inoculate" the sterilized tobacco with selected microbes, grown in a laboratory, was described in a patent seven years later [35]. The inventors concluded that:

> "Our invention is particularly applied to the treatment of tobacco comprising, first, sterilizing (with hot air) the tobacco under such conditions and sufficiently long-continued as to destroy the original bacteria and spores existing thereon, then adding to the sterilized tobacco bacteria or cultures, thereof to produce a new and characteristic flavor, and subsequently subjecting the tobacco to fermentation." [35]

Variation of the aforementioned method and other technologies have been described in US Patents to the formation of NNN and NNK in tobacco to be used for cigarettes [20, 29, 36-41]. The control of microbes in cigarette tobacco has been studied by RJ Reynolds [27], Philip Morris [42] and other tobacco companies.

The procedures mentioned above are intended to treat tobacco leaves before they are processed and incorporated into the cigarette or other products.

Another approach to reduce the potential for harm of microorganisms is to destroy the microbes in the finished tobacco products. A US Patent awarded in 2008 notes that:

> "One commonality of all tobacco products, however, is that they contain viable microorganisms." So as to safeguard against tobacco associated microorganisms from causing human disease, the inventor proposed different methods to sterilize cigarettes and cigars so that "they are essentially free of pathogens." [43]

It was noted also in this US Patent that: "Some tobacco products may contain pathogens or potential pathogens which may contribute to, or be causative agents of, human disease, animal disease, or plant disease." In addition, "While some tobacco products may, from time-to-time, be naturally free of such unwanted pathogens, comprehensive testing to determine the presence or absence of all unwanted species in tobacco is presently problematic, especially as many pathogens have probably not yet been identified." By way of example, sterilizing procedures included ionizing irradiation from a ^{60}Cobalt source, chemical treatment and combinations thereof [43].

The inventor postulates that: "The treated products were expected to be equally acceptable to consumers and, perhaps more acceptable because of the knowledge that the treated products contained a reduced content of potential pathogens" [43].

In addition to eliminating NNN and NNK from smoking tobacco products, US Patents have been awarded for eliminating or abating TSNA in oral tobacco products (snus and snuff) [reviewed in 44].

Summarily, these writings have been selected from a large number of US Patents that document that NNN and NNK in tobacco and tobacco smoke can be reduced significantly. Many of the technologies have been crafted to be efficient, inexpensive, and readily incorporated into currently used large-scale tobacco processing methods.

Tobacco companies have been criticized as establishing relatively simple and cheap technologies to reduce carcinogen levels, but have not applied their knowledge to smoking and smokeless tobacco products. The question arises as to why the application of this technology by the tobacco industry has been neglected? This question has been the focus of a paper for leading scientists in the field of TSNA research [45].

2. Tobacco Microflora, Curing and Fermentation

The Microbiology of Tobacco

It has been known for more than 100 years that tobacco contains microbes, and that the microbes play a pivotal role in the fermentation of tobacco [35]. The microbes present on the surface of tobacco in the field or freshly harvested green tobacco are a heterogeneous population that includes diverse bacteria, fungi and spores. Curing procedures in which the tobacco is hanged in a barn, as is practiced for fermenting Virginia bright tobacco, exposes the tobacco leaves to conditions that foster the growth of the microbes [29-33].

Other writings have detailed the identification and growth of diverse microbial populations, and the influence of different parameters, a partial listing of those that are most important includes temperature, ventilation, humidity and duration. For many years, the fermentation of tobacco has been regarded primarily as an art, based upon the experience and practices of the tobacco farmer, rather than science. Today, standardized protocols and prescribed methodologies are not used. Thus, considerable variation in the growth of the tobacco-associated microbes is to be expected.

The microbiology of tobacco has been the focus of many investigations. It is not surprising to learn that most all of the major tobacco companies have studied this issue for many years. Listed below are different topics addressing bacteria, mold and mycotoxins in tobacco, and references. Most of the reports were from tobacco industry documents, and retrieved from the Legacy database. Isolation of viable fungi from snuff [46] and the microbiology of cigarettes, pipes, cigars and snuff [47-56].

The tobacco microflora of the community has been studied [57-63]. Quantitative studies have been performed of the microflora on green tobacco, at different stages of processing, including curing, fermenting and storage [64-66]. Moreover, different groups have established a data base of tobacco microbes [67-69].

Chemical and microbiological changes during curing: [70-83]. Also studied was the growth of mold and fungi on tobacco during storage [78-83]. Comparative examinations have been made of cigarettes from mold-damaged and non-damaged tobacco [84, 85]

Notable is the documentation of the growth of *Aspergillus* from tobacco and tobacco products [86-88].

Biological and Chemical Components in Tobacco – A New Perspective

Presented in Table 12.1 is a listing of chemical and biological components of cigarette smoke. Comment This listing has been compiled by the authors to emphasize the harm and potential for harm that may arise from “biological” components. This is the first registry of cigarette smoke components that includes biological components. This listing includes diverse microbes and microbial toxins present in tobacco smoke and are reasonably anticipated to be harmful or potentially harmful to the smoker, particularly with respect to the elicitation of an innate and/or cognitive immune response. One should consider the additive or synergistic response that may be provoked by an admixture of chemical and biological toxicants.

Table 12.1. Chemical component categories of cigarette smoke, with an emphasis of microbes and microbe-derived factors of tobacco flakes and microparticles

1. Total Particulate Matter
Water
Nicotine
'Tar'
2. Vapor Phase Elements
Water
Nitrogen
Oxygen
Other
3. Miscellaneous agents
Metals (Cd & Pb)
Pesticides & growth regulators
Isotopes (^{210} Polonium)
Free radicals (ROS/RNS)
Additives (flavorings & burn accelerators)
4. Microbes and microbial category; tobacco flakes and microparticulates
Bacteria and spores (living and dead)
Gram-positive bacteria (diacetyl lipopeptide)
Gram-negative bacteria (triacyl lipopeptides)
Bacterial toxins (endotoxin, LPS)
Cellular components
Bacterial antigens (allergens)
Immunostimulatory activity (antigens)
Inflammation-inducing agents (lipopeptides)
Perforins (hemolysin)
Bacterial cell lysate (CpG motif)
Mold, Yeast and Fungi
Mycotoxins (aflatoxin-B1)
Immunostimulatory elements (β1-glucans)

The first category consists of wet total particulate matter (WTPM) and consists primarily, as defined by weight, of water, nicotine and 'tar'. The second group of vapor phase elements (water, nitrogen, oxygen and other). The third group in this conventional listing of chemical components consists of miscellaneous agents, and these includes, metals, pesticides, isotopes, free radicals, enzymes, and cigarette additives. These three groups are no different than the chemicals listed by the FDA in the HPHC list.

The biological components include, but are not limited to, microbes and microbe-derived elements. The biological components are to include elements that are released from the cigarette column and into mainstream smoke. Incorporated into another grouping of biological components are tobacco flakes and tobacco microparticles that lie freely on the cut surface of the cigarette filter. These are sucked into mainstream smoke, and inhaled.

A partial listing of relevant tobacco-associated microbial elements include bacteria (Gram-positive and Gram negative). Also included are bacterial toxins, such as endotoxins (LPS). A large and diverse population of immunostimulants is to be included. These include:

(a) bacterial antigens (allergens); (b) imunostimulatory activity of spores (Bacillus spores), and inflammation inducing agents (LPS). Also included are factors that cause the destruction of human red blood cells (hemolysin) and diverse enzymes.

Mold, yeast and fungi are also included. This classification includes mycotoxins that are known human carcinogens (mycotoxins; aflatoxins, AFL type B1. For completeness, β1-glucan is also included.

We have also listed bacteria, spores and toxins that are known immunostimulants. Bacteria, Gram-positive and –negative, living or dead, are known immunostimulants. By way of example, *bacillus* spores are known to stimulate the immune system [89]. Thus, microbes and cell wall components elicit immune responses. Likewise, toxins produced by the bacteria also activate the immune system. Other bacterial products may also prove harmful, including perforins, such as those that cause hemolysis, and various enzymes.

3. Chronic Inflammation and Tobacco Smoke

Tobacco Microbes and Lung Leukocyte Toll Receptors

There exists a consensus of opinion that chronic inflammation plays an important role in the etiology of tobacco-associated malignant and non-malignant diseases. These include pathologies of the lung, heart and mouth. Chemicals derived from smoking (cigarettes, cigars and pipes), and non-smoking (snuff, snus, and long-cut) tobacco products that are currently being marketed are known to contain diverse toxicants that are either known or which are suspect of inducing chronic inflammation.

As noted above, the current FDA HPHC list includes but one microbe-derived agent, namely aflatoxin B1. Today, it is widely recognized that cigarettes and other tobacco products contain bacteria, mold/yeast/fungi, spores, and microbial toxins. For example, endotoxin (lipopolysaccharide, LPS), a potent inflammation-inducing agent, derived from Gram-negative bacteria, exists in cigarette tobacco. LPS has been identified in the tobacco of cigarettes and chewing tobacco. Notable is that LPS been identified in mainstream smoke and environmental cigarette smoke.

Cigarette Smoke, Chronic Inflammation, and Impaired Immunity

Chronic inflammation is associated with malignant transformation, tumor growth, and possibly, tumor metastasis; reviewed in: [90-94]. Examples of the association of cancer with chronic inflammation include (a) lung cancer, and cigarette smoke (aerosol); (b) malignant mesothelioma, and asbestos (fibers); (c) stomach cancer, and *H. pylori* (bacteria); (d) malignant melanoma, and ultraviolet sun light (irradiation); (e) liver cancer, and aflatoxin (mycotoxin), and (f) cancer of the uterine cervix, and human papilloma virus. Thus, malignancy at diverse body sites, and of different tissues, is associated with chronic inflammation provoked by assorted items that include smoke, bacteria, fibers, irradiation, toxin and viruses.

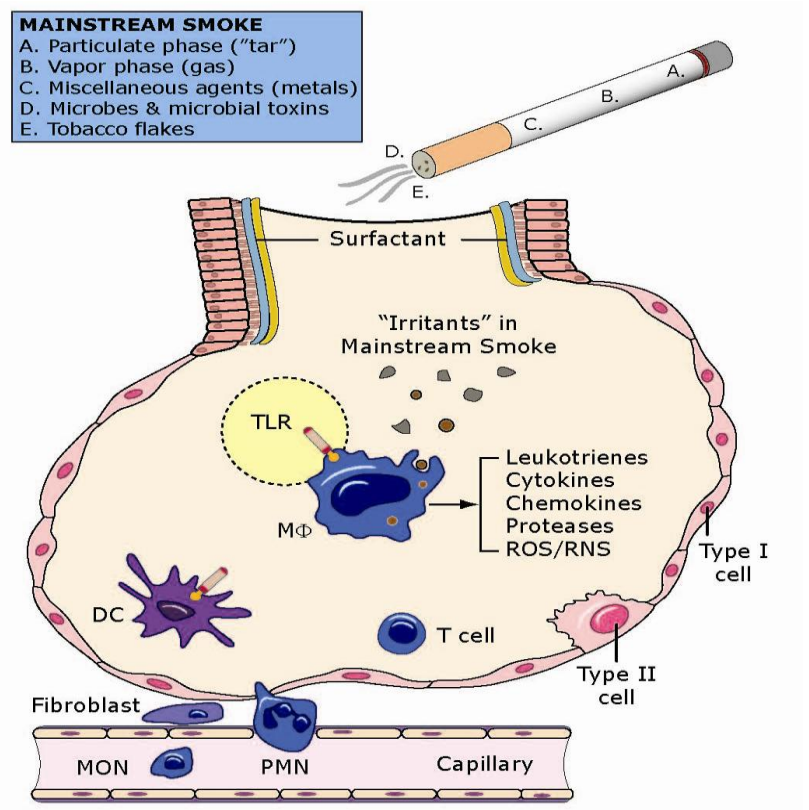

Figure 12.1. Illustration of the multiple components of mainstream cigarette smoke and the induction by these "irritants" of a pro-inflammatory response. Irritants in mainstream smoke activate lung MΦ to produce proinflammatory mediators. The mediators are diverse factors which, by way of example, are known to include leukotrienes, cytokines, chemokines, proteases and reactive oxygen and reactive nitrogen species. Most probably, some of the chemicals that arise from the burning of tobacco induce chronic inflammation of the lung of long-term smokers. In addition, different microbes and microbial components may also be present in mainstream smoke, and these have the propensity to induce chronic inflammation. These biological components are recognized by surface membrane Toll receptors that are expressed on lung macrophages (MΦ). The chemical and biological irritants of cigarette smoke may also activate other leukocytes, including dendritic cells (DC), T cells, monocytes (MON) and polymorphonuclear leukocytes (PMN). The irritants may also affect Type I and Type II lung epithelial cells and fibroblasts.

Cigarette smoke is known to induce chronic inflammation of the lung [95-102]. More recently, a substantial body of information has been obtained to suggest that long-term cigarette smoking may not only have an adverse affect on systemic immunity but also skews both innate and adaptive immune responses [103-107].

Inhaled Microbes and the Alveolus

Shown in Figure 12.1 is a proposed scheme of the induction of chronic inflammation of the lung by diverse components in mainstream cigarette smoke.

Two large and heterogeneous classes of chemicals are defined as the particulate phase ('tar") and the vapor phase (gas) [box insert in illustration]. Other chemicals are defined as miscellaneous components (e.g., metals). Biological components also exist in mainstream

smoke. They include microbes (bacteria, mold and spores) and different microbial toxins. The microbial toxins may include bacteria-derived endotoxins (lipopolysaccharide; LPS) and fungus-produced mycotoxins (aflatoxin-B; AFL-B1).

Also present in mainstream cigarette smoke are tobacco microparticles that are sucked from the cut surface of the filter. Studies have shown that bacteria grow from a single flake of tobacco placed on a blood agar dish.

The irritants are distributed into the mouth and airway, and some may be transported to the terminus of the lung – the alveolus. The inhaled chemical and biological "irritants" are perceived as inducing tobacco-associated chronic lung inflammation. A structured review of the literature addressing the inflammatory response, as measured using diverse *ex vivo* assays, of human and animal lung MΦ and epithelial cells to tobacco smoke has recently been published [108].

Chronic inflammation is mediated by the interaction of the irritants with different leukocyte subsets, including macrophages (MΦ). The MΦ are derived from monocytes (MON) of the blood. Polymorphonuclear (PMN) leukocytes, some of which are signaled by chemokines, are recruited from blood circulating in capillaries adjacent to the alveolus. Other leukocyte subsets that are known to mediate chronic inflammation include antigen-processing and -presenting dendritic cells (DC). T cells, including helper and suppressor T cell subsets also participate.

Highly conserved Toll-like receptors (TLR) recognize different microbes and microbial-derived elements, and play a pivotal role in chronic inflammation and cancer; reviewed in [109-111]. Activation of the TLR initiates transmembrane signaling. As shown in Figure 12.1, TLR-mediated activation of MΦ results in the production of diverse factors. A partial listing of pro-inflammatory factors includes leukotrienes, cytokines, chemokines, proteases and reactive oxygen species/reactive nitrogen species (ROS/RNS).

Numerous highly fluorescent MΦ, designated "smoker cells," exist in the lung of smokers and subjects who have quit smoking within five years. The highly fluorescent MΦ, however, are not present in the lung of never smokers. The fluorescence of these cells is associated with tobacco tar in tobacco smoke, and particularly to polycyclic aromatic hydrocarbons. These cells are most probably the source of the pro-inflammatory mediators induced by tobacco smoke [reviewed in 112].

These factors interact with different leukocyte subsets, including those that are mentioned herein, as well as type I and type II lung epithelial cells. It is theorized that chronic inflammation is sustained from each cigarette that is smoked, and continues for as many years as the subject smokes.

Toll Receptor Mediated Pro-inflammatory Response

Chronic inflammation of the lung is mediated by (spores, not illustrated), fungi and bacteria. Moreover, activation is also induced by other microorganisms (e.g., mycoplasma, viruses and parasitic protozoa). A schematic diagram of Toll-mediated transmembrane signals, cytosol regulatory elements, and nuclear activation by fungi and bacteria is presented in Figure 12.2; also see [109-111].

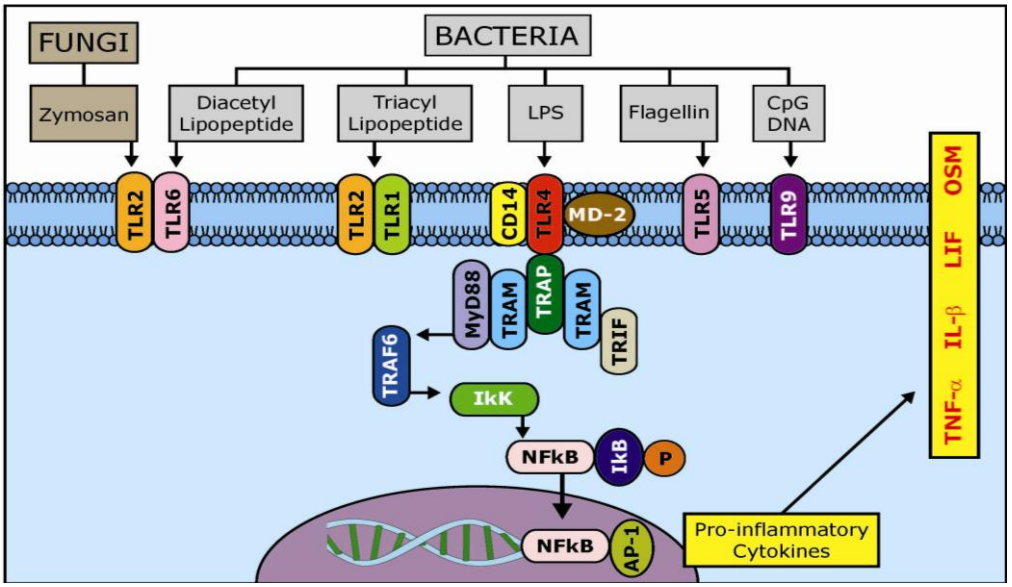

Figure 12.2. A schematic diagram of Toll-mediated transmembrane signals, cytosol regulatory elements, and nuclear activation by fungi and bacteria. Activation of lung MΦ and other leukocytes generates diverse pro-inflammatory cytokines which, if persistent, is thought to induce chronic inflammation. Fungi and bacteria, either whole or cell fragments (e.g., LPS and flagellin), engage ligand-specific Toll receptors expressed on the surface membrane of MΦ. Mediation of the transmembrane signaling by different adaptor molecules (MyD88) and other elements (enzymes) culminates the activation of genes for the production of various pro-inflammatory substances (see Fig. 12.1). Illustrated here, by way of example, is the production of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), leukemia inhibitory factor (LIF) and oncostatin M (OSM).

Notable is that chronic inflammation is induced by living or dead microorganisms, whole or membrane fragments (e.g., flagellin). Moreover, chronic inflammation is induced by diverse bacteria that include Gram-positive (e.g., diacetyl lipopeptide) or Gram-negative (e.g., triacetyl lipopeptide) bacteria. Chronic inflammation is also induced by diverse microbial toxins, some of which are among the most potent inflammatory agents (e.g., endotoxin, LPS).

Immunostimulation is induced by *Bacillus. Bacillus* is commonly found in cigarette tobacco and on the surface of the filter. Inflammation may be induced by cell wall components of fungi (1→3 β-D glucans, not illustrated, and zymosan) and bacteria (e.g., flagellin). Thus, an intact microorganism is not required to illicit an inflammatory reaction. LPS, an endotoxin derived from Gram-negative bacteria is a potent inducer of inflammation. In addition, other elements that are unique to certain bacteria are inflammatory.

These irritants are recognized by different TLRs that are present on the cell surface membrane of different leukocyte subjects.

A partial listing of TLR that signal inflammation includes TLR1, TLR2 and TLR4. An irritant interacts with a TLR, either alone or in combination with another TLR, and this initiates transmembrane signaling by different cytosol components, including various adaptor molecules (MD-2, TRAM, TRIF, TRAF6 and NFκB).

One pathway, truncated for illustrative purpose, is that which is activated by LPS. This signaling pathway is mediated by the binding of LPS to TLR4 and MD-2 complex. Mediated

by different adaptor proteins (e.g., MyD88) and under the regulation of I kappa Kinase (IκK), nuclear factor kappa B (NFκB) enters the nucleus and binds to the activator protein-1 (AP-1). AP-1 transcription factor regulates the gene expression to a variety of stimuli. The activation of certain MΦ genes results in the secretion of numerous and diverse pro-inflammatory cytokines, a partial listing of which includes Tumor Necrosis Factor-alpha (TNF-α), interleukin-1β (IL-1β), Leukemia Inhibitory Factor (LIF) and Oncostatin M (OSM).

LPS in Tobacco Smoke

In 1999, Hasday and his colleagues reported the identification of bacterial endotoxin as an active component in cigarette tobacco and cigarette smoke [113]. The authors showed that the dose of LPS delivered from smoking one pack of cigarettes was comparable to that of the LPS that had been previously shown to be associated with adverse health effects in cotton textile workers. With the knowledge that LPS is one of the most potent inflammation-inducing agents, the work by Hasday attracted considerable attention; reviewed in [114].

In 2004, Larsson reported that they were able to demonstrate unequivocally that high levels of LPS are inhaled during active cigarette smoking, and more importantly, that environmental tobacco smoke may involve inhalation of amounts of endotoxin that are dramatically greater than those existing in indoor environments free from tobacco smoke [115].

In 2006, these findings were confirmed and extended [116].

Particularly notable is that studies of Larsson and colleagues used a novel state-of-the-art mass-spectrometry-based assay that circumvents the problems that are often associated with the biologically-based LPS assay.

4. COPD and Chronic Inflammation

Bacterial Colonization of the Lung

Chronic obstructive pulmonary disease (COPD) is often observed for long-term smokers. COPD is characterized by non-reversible airflow limitations. The lung of a healthy person is sterile. In contrast, the lung of a subject with COPD is often colonized by bacteria, and is often associated with persistent inflammation. Structural changes in the lung include marked changes in the architecture of the airways and alveolar spaces [reviewed in: 117-122].

Two questions arise: (a) Is COPD in some subjects attributed in part to chronic inflammation that arises solely from the chemical components in cigarette smoke, or is it attributed in part to inhaled smoke containing microbes and microbial elements?, and (b) Do some of the bacteria that colonize the lung of subjects with COPD arise from viable bacteria in mainstream smoke?

Prevailing wisdom would suggest that long-term smoking would damage the airway, reduce pulmonary host-defense mechanisms, activate microbial-antigen associated cognitive immune response, sustain chronic inflammation that is fueled by toxicants in smoke as well as irritants produced by colonized bacteria, and provide a favorable microenvironment for

bacterial growth – all of which may augment malignant transformation and lung cancer promotion.

5. Transfer of Tobacco Flakes into Mainstream Smoke

Tobacco Bacteria Escape Pyrolysis

The filter of a cigarette is often contaminated with loose tobacco flakes. The presence of the flakes can, for some cigarettes, be seen readily with the naked eye. Microparticles of raw tobacco are also present, and these can be seen easily with a stereo zoom microscope. Moreover, the flakes are observed lying loosely on the cut surface of the filter which, for most all cigarettes, is a longitudinal milk-white plug of plastic-like cellulose acetate fibers.

In one examination, the filters of 11 different brands of cigarettes were examined in freshly opened packs. For all brands, cigarettes were observed with tobacco flakes on the filter. Examination of the filters with the naked eye showed that 127 of 208 (61.1%) of the filters had tobacco particles [123].

The release of tobacco flakes into mainstream smoke was also described in 1958 [124]. These pioneering investigations sought to understand the deposition of the flakes in the respiratory tract.

The release of flakes from the filter of cigarettes can be illustrated by using a stereo zoom microscope to craft a map of the distribution of tobacco flakes on the cut surface of the filter. The filter is re-examined and the map is reviewed after the first puff of smoking.

These and related studies illustrate that tobacco flakes having microbes and microbial components escape destruction by burning.

A tobacco flake may be perceived as a small matrix, which can readily be inhaled, for transporting bacterial and fungal agents into mainstream tobacco smoke. Thus, the burning of the tobacco during cigarette smoking does not exclude the smoker to the harm and potential for harm that is attributed to the inhalation of tobacco-associated microbes, some of which are viable, and microbial toxins.

The tobacco flakes that contaminate the filter arise from tobacco that escapes from the non-filter, sometimes called the distal end, of the cigarette. Most probably, the flakes are jarred loose during manufacturing, shipment, and daily transportation, especially in a pack in which more than one-half of the cigarettes have been used [125, 126].

6. Immune Response to the Inhalation of Tobacco-associated Bacillus

Cigarettes, Tobacco, Bacteria and Spores

Flue-cured tobacco contains numerous bacteria, and these play a critical role in the curing and fermentation of the leaf that is required for a tobacco product [89, 127, 128]. One of the most commonly found bacteria are *Bacillus.* The *Bacillus* species have been considered a

nonpathogenic, spore-forming, soil microorganism. Spore-forming *Bacillus* species are inhabitants of the gastrointestinal tract of humans. *B. subtilis* is itself immunogenic. Moreover, *B. subtilis* can stimulate the expression of the toll-like receptor genes for TLR2 and TLR4 [89].

Smoking (cigarettes, cigars) and non-smoking (snus and snuff) have tobacco with different *Bacillus* species; examples of which include *B. subtilis, B. lichenformis, and B. pumilus* [89, 126, 127].

Some bacteria grow in unique microenvironments, and some are difficult to grow using traditional broth- and agar-based methods. This technical difficulty may also apply to growing bacteria that have adapted to growing in unique conditions that develop during the curing and fermentation of tobacco. Accordingly, it is anticipated that conventional methods may not accurately define the microflora of diverse tobacco products [129]. Consequently, there may be an incomplete understanding of the bacterial diversity in the tobacco of cigarettes, and the impact that these microbes and microbial toxins may impose on the smoker [128].

Recently the bacterial metagenome of cigarettes were characterized using a 16S rRNA-based taxonomic microarray as well as traditional cloning and sequencing methods [128]. The brands included Camel, Marlboro, Kool, and Lucky Strike. The results of this study showed that the number of microorganisms in cigarettes may be as vast as the number of chemicals in these products. Fifteen different classes of bacteria were identified. Particularly noteworthy was the detection and identification of a broad range of potentially pathogenic microorganisms. More than 90% of the tobacco samples from the cigarettes contained *Actinetobacter*, *Bacillus*, *Burkholderia*, *Closteridium*, *Klebsiella*, *Pseudomonas aerogenosa*, and *Serratia*. Other bacteria that are known to be potentially pathogenic to humans and which were detected using the metagenomic technology were *Campylobacter*, *Enterococcus*, *Proteus*, and *Staphylococcus* [128].

Also reported in 2010 were the results of an investigation of the diversities of unaged and flue-cured tobacco leaves using a 16S rRNA sequence analysis scheme [130].

Others have reported the identification of potentially pathogenic bacteria in commercial cigarettes. One study was undertaken to assess the bacterial diversity of cigarettes that were thought to be linked to severe pneumonitis in US Military personnel deployed in Operation Iraqi Freedom [131]. Eight species of *Bacillus*, including five new species, and one new species of *Kurthia* were isolated from the cigarettes. Some of these species have been identified elsewhere to cause hypersensitivity pneumonitis and other respiratory syndromes [130]. This study was of particular interest to many because the cigarettes were made in Iraq and had not been manufactured by a major tobacco company.

In undertaking this investigation, the military asked to whether the cigarettes that had been purchased by soldiers from street vendors had been intentionally altered by adding pathogenic bacteria and/or mold. This scenario was rejected. However, it raises the issues as to whether cigarettes can be used to deliver pulmonary pathogens.

The authors reported previously the establishment of a novel bioassay which showed that bacteria were grown routinely from a single flake of tobacco that had been placed on the surface of a sheep blood agar plate [123]. Of eight different popular brands of cigarettes, bacteria grew from most all (>90%) of the flakes. Similarly, bacteria were grown from a single flake, and also with a high frequency, from tobacco that had been retrieved from cigar filler and from smokeless tobacco (snus, snuff and long-cut).

Some bacteria from a single flake of cigarette tobacco induced hemolysis of the sheep blood in the agar dishes. The destruction of the red blood cells was readily visible as a yellow zone surrounding a single tobacco flake. Expanding studies documented the hemolysis of human blood in agar or nutrient broth cultures. Thus, as will be discussed later, bacteria could be carried deep into the respiratory tract by a single tobacco flake that had been sucked from the cut surface of a cigarette filter, and transported into the bolus of smoke that is inhaled deep into the lung. Thus, a single flake of tobacco may be a matrix for delivering a microbial pathogen into the respiratory tract of an immunologically compromised long-term smoker.

Cigarettes, Tobacco, and Mold

Mold has been identified in the tobacco of popular-brand cigarettes, and concern has been raised as to the propensity of these microbes as a health risk to the smoker. Presented herein is a partial listing of papers that have identified mold in cigarettes [82,132-136] and in marijuana [135].

Forgacs observed that the tobacco of all cigarettes contained fungal mycelia and spores. In part, the origin of his health concern is based upon the knowledge of the: (a) widespread fungal contamination of tobacco products; (b) heat stability of the mycotoxins; (c) known animal toxicity; (d) reasonable assumption that some of the fungi are carcinogenic; and (e) potency at low doses [also see 137].

As early as 1971, Papavassiliou and co-workers concluded that: "[C]igarettes are contaminated with various fungi." They studied cigarettes that had been manufactured in the US, Canada, England, France, Belgium, Germany, Jordan and Egypt. Hundreds of strains of fungi were isolated. The Greek scientists discovered that the most prominent fungi were *Aspergillus* (28 strains from Greek cigarettes; and 35 strains from other countries). Their findings raised the question as to whether there was an association of the fungi with allergies [133].

In 1983, Kurup and colleagues reported the identification of allergenic fungi in smoking materials and discussed the health implications of their findings [134]. Concern has been expressed as to the health risks associated with mold in cigarettes.

Writing in the Journal of the American Medical Association, Verweij and co-workers addressed the propensity of health risks associated with fungal contaminates of tobacco and marijuana [135]. They concluded that: "[A]ll cigarette brands tested (N=14 brands) had some degree of fungal contamination, although not every cigarette was found to have a positive culture."

Another study was conducted by a group of investigators in Sweden who characterized the microbiological composition of tobacco products using culture and chemical analysis. Gas chromatography-tandem mass spectrometry was used for determining LPS (bacteria biomarker) and peptidoglycan (fungal biomarker) [132]. Significant differences were observed in the measured microbial components in cigarettes produced in different countries of Europe versus Asia. The authors note that tobacco smoke is a bioaerosol, and that this may explain the respiratory disorders among smokers, and non-smokers who inhale second hand smoke [132].

7. Chewing Tobacco and Microbes

Potential Pathogens and Aflatoxin

Studies have been conducted by investigators of the tobacco industry and health community to identify and characterize harmful and potentially harmful microbial components in chewing ("oral") tobacco [partial listing, 79, 138-140].

It is notable that as early as 1951 a study published in the New England Journal of Medicine raised the question as to whether there was an association between the bacteria identified in snuff used by the patient, bacteria in the patient's sputum and his COPD. *Pseudomonas aeruginosa*, often colonized in COPD patients, and a few colonies of *Staphylococcus aureus* were identified in bacteriological examinations of the subject's sputum [138]. The patient used snuff, and it was theorized that the snuff may have been the source of the pathogens.

A study was then undertaken of 22 samples of previously unopened packs of snuff. The following microorganisms were grown from more than 50% of the snuff samples: *Bacillus rubitilles*, *Staphylococcus aureus* (coagulase positive), *Staphylococcus albus* (coagulase positive), *Pseudomonas aeruginosa*, *Staphylococcus aureus* (coagulase negative) and *Staphylococcus albus* (coagulase negative) [138].

Nine different species of *Aspergillus* in stored leaves of chewing tobacco were reported in a study published in 1991 [139] approximately 18 of the *Aspergilli* were found to be mycotoxigenic. All aflatoxigenic strains of *A. flavus* produced aflatoxin B1 (AFLB1) is a constituent listed on the HPHC FDA list of tobacco and tobacco smoke [1] The mycotoxins patulin and ochratoxin were produced by *A. ochraceus*. Sterigmatocystin was produced by three different strains [139].

The microbiological quality of chewable tobacco mixes ("Gutka") used in India has been investigated by Warke [20] Of the 15 samples studied, all contained aflatoxin B1, listed as a known carcinogen by the FDA [1] and aflatoxins B2 and G2. Samples exposed to radiation ^{60}Co displayed a marked reduction of viable CFU. In 1992, Rubenstein reported the identification of large number (> 10^6 CFU) of a *Bacillu*s species in chewing tobacco sold in the US [140].

Summary

The authors have identified writings by many investigators who have studied the microbial flora of tobacco from the field, during curing and fermentation, prolonged storage, and in diverse smoking and smokeless products. The FDA, NTP, IRAC and other regulatory organizations have failed to address the harm or potential for harm that may be attributed to microbes and microbial components that are present in both smoking and smokeless products.

There is a gap in our understanding of the harm that may be associated with microbial components in tobacco. Specific recommendations include.

a. Technically simple, inexpensive and readily available methodologies that have been defined in US Patents and other writings should be implemented for eliminating or reducing NNK from all tobacco products.
b. Chronic inflammation should be incorporated by regulatory authorities in defining adverse health outcomes for those who use smoking or smokeless tobacco products. This assessment should include different body sites (mouth, nasopharynx and lung). The assessment should include chemical agents in mainstream tobacco smoke, some of which arise from the pyrolysis of tobacco, and biological agents, some of which arise for diverse microorganisms, whole or part, living or dead, including bacteria and fungi, and toxins produced by these organisms.
c. Harm and propensity for harm associated with microbiological agents of smoking and smokeless tobacco have been identified by investigators, and a structured investigation should be undertaken to define final guidance of these risks.

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